# Development of potential *Trichoderma* based formulation for management of collar rot disease of tomato caused by *Sclerotium rolfsii*

काशी हिन्दू विश्वविद्यालय
BANARAS HINDU UNIVERSITY
AN INSTITUTION OF NATIONAL IMPORTANCE ESTABLISHED BY AN ACT OF PARLIAMENT

**THESIS**

Submitted in partial fulfilment of the requirements for the degree of

**Doctor of Philosophy**
**in**
**Mycology and Plant Pathology**


**Submitted by**
*Rahul Singh Rajput*

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**I.D. No. PM-15045** **2019** **Enrolment No. 381285**

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I, **Rahul Singh Rajput**, research scholar under the supervision of **Dr. H.B. Singh**, Professor, Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, give undertaking that the thesis entitled **"Development of potential *Trichoderma* based formulation for management of collar rot disease of tomato caused by *Sclerotium rolfsii*"** submitted by me for the degree of Doctor of Philosophy is a record of first hand research work done by me during the period of study.

I avail myself to responsibility such as an act will be taken on behalf of me, mistakes, errors of fact and misinterpretation are of course entirely my own.

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**Place**: B.H.U., Varanasi **(Rahul Singh Rajput)**

**Annexure-E**
**(See clause XIII.2 (b) (iii))**

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I, **Rahul Singh Rajput**, **I. D. No**: PM-15045, **Enrolment No**.: 381285, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out by me under the supervision of **Dr. H.B. Singh**, Professor, Department of Mycology and Plant Pathology, for the period from September 2015 to August 2019 at Institute of Agricultural Sciences, Banaras Hindu University. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

**Dr. H.B. Singh**
(Supervisor's Signature)

**(Signature of the Head with seal)**

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# Development of potential *Trichoderma* based formulation for management of collar rot disease of tomato caused by *Sclerotium rolfsii*


**THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF**

**DOCTOR OF PHILOSOPHY (AGRICULTURE)**
**in**
**MYCOLOGY AND PLANT PATHOLOGY**

**Institute of Agricultural Sciences**
**Banaras Hindu University**
**Varanasi-221005-India**

**by**
*Rahul Singh Rajput*

**APPROVED BY RESEARCH PROGRAMME COMMITTEE (RPC)**

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**EXTERNAL EXAMINER:**

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*An aim is the eternal condition of success which is attainable at perfection in everything by the bearer with the association of their predecessors, teachers, family members, friends and colleagues. No words would adequately express my feelings for them yet these lines are not exaggeration but feelings, which come straight from my heart.*

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***Date:*** ***(Rahul Singh Rajput)***

***Place:*** *B.H.U., Varanasi*

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| < | Less than |
| % | Per cent |
| ˚C | Degree Celsius |
| > | Greater than |
| ± | Plus Minus |
| ≥ | Greater than equal to |
| µl | Micro litre |
| µm | Micro metre |
| BOD | Biological Oxygen Demand |
| CFU | Colony forming unit |
| cm | Centimetre |
| CTAB | Cetyl trimethyl ammonium bromide |
| dapi | Days After Pathogen Inoculation |
| DAS | Days After Sowing |
| DAT | Days After Transplanting |
| DMRT | Duncan's multiple Range test |
| EDTA | Ethylene diamine tetraacetic acid |
| *et al.* | And co workers |
| etc. | (et cetera) and the others |
| Fig. | Figure |
| FW | Fresh weight |
| g | Gram |
| h | Hour |
| ha | Hectare |
| HCl | Hydrochloric acid |
| NaOCl | Sodium Hypochlorite |
| hrs | Hours |
| i.e | (ed est) that is |
| m² | Square metre |
| mg | Milligram |

| | |
|---|---|
| $min^{-1}$ | Per minute |
| ml | Millilitre |
| ml $l^{-1}$ | Millilitre per Litre |
| $ml^{-1}$ | Per millilitres |
| mm | Millimetre |
| mM | Millimole |
| mt | Million Tonnes |
| NaCl | Sodium Chloride |
| NaOH | Sodium Hydroxide |
| NB | Nutrient Broth |
| NBT | Nitroblue tetrazolium chloride |
| No. | Number |
| O.D | Optical Density |
| PAL | Phenylalanine ammonia lyase |
| PO | Peroxidase |
| PPO | Polyphenol oxidase |
| PDA | Potato Dextrose Agar |
| PDB | Potato Dextrose Broth |
| p.s.i. | Pound per square inch |
| R | Replication |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| Sr. No | Serial number |
| T | Treatment |
| TPC | Total Phenol content |
| UV | Ultra violet |
| viz. | (videlicet) namely |
| WP | Wettable powder |
| w/v | Weight/Volume |
| w/w | Weight/weight |

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# PREFACE

Since this thesis is written as the final document for Doctorate degree in Mycology and Plant Pathology and primarily aimed to isolate high temperature tolerant *Trichoderma* spp. having the potential to mitigate high temperature stress and collar rot in tomato plants. The work will be of great intent for development of bioformulations which have the ability to mitigate high temperature stress to a certain extent. Also, the study will be helpful to minimize the crop losses and in turn provide food safety and food security in India.

The work described in this thesis was carried out between 2015-19 at the Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, B.H.U., Varanasi, under the supervision of Dr. H.B. Singh, Professor, Department of Mycology and Plant Pathology.

*Chapter I* - The introduction part which provides much of the general background. This chapter justifies the reason for choosing this research topic, importance of study in reference & lastly objectives of the research work.

*Chapter II* - The review of literature deals with the findings related to role of beneficial micro-organisms in mitigating abiotic stress and also collar rot of tomato caused by *S. rolfsii*. Attempts made by various scientists in managing collar rot have also been described.

*Chapter III* - The materials and methods deals with the methodology employed for carrying the research work and methods for analysis.

*Chapter IV* - The results and discussion which explains the results obtained with appropriate reasons and support.

*Chapter V* - The summary and conclusion gives a brief description of the results of the investigation and outcomes of the research work concluded.

In the last, references which have been consulted during the course of investigation have been cited.

# CHAPTER I

# INTRODUCTION

*Chapter-I*

# INTRODUCTION

The demand for food is increasing day by day in order to feed the growing population. However the food production mainly depends upon the agronomic operation and plant protection measures adopted. The losses in production due to abiotic and biotic stresses have been estimated to be 25-30% (Lesk *et al*., 2016; Hsiang *et al*., 2017)

Abiotic and biotic stresses are the major threat in global food security hampering the overall production and productivity. Abiotic stresses refer to environmental factors such as temperature, rainfall, wind, soil nutrient content and water availability while biotic stresses include all pests related issues such as phytopathogens, insects and weeds. Crops are affected drastically with these stresses under field conditions. Abiotic stresses not only affect the production and productivity of crops but also promote the incidence of plant diseases. The projections for increasing food demand at 2050 shows that the crop production will needed to be doubled for completing this target. Therefore, an urgent need is required to reduce the crop losses by both abiotic and biotic stresses. The conventional practices for protecting crops from various plant diseases by using chemical pesticides is an effective method. Presently, chemical pesticides cover more than 95% of world pesticide market shares and prevent approximately 50% loss in crop yield (Jeschke, 2016). To reduce the adverse effect of biotic and abiotic stresses, a wide range of chemicals are being used in agriculture particularly in vegetable production. In the last few decades, chemical pesticides do not show a significant reduction in crop loss rate instead a rising

global concern about the hazardous effect of synthetic agrochemicals on the environment and human health (Bedi *et al*., 2013; Lewis *et al*., 2016).

Vegetables play an important role in human dietary and provide different type of essential nutrients, vitamins, amino acids and carbohydrates (Saini *et al*., 2016; Henning *et al*., 2017). After China, India holds second rank and share approximately 15% of vegetables production of world (Ahmad *et al*., 2019). These agrochemicals definitely help growers to improve vegetable production but these hazardous chemicals get accumulated in the plant system and during the course of consumption, may create health problems. These chemicals also disturb the soil health, microbial diversity and ultimately contaminate the soil. Continuously use of these chemicals is raising global concern to the environment and human health that provoked an urgent need to be shifted on a safer alternative way for plant disease management.

**1. 1 Tomato plant**

Tomato (*Solanum lycopersicum* L; Synonym: *Lycopersicum esculentum* Mill.) is one of the most widely cultivated vegetable crop all over the globe. Tomato belongs to the family Solanaceae which includes about more than 3500 species, found in the diverse range of habitats (Knapp, 2004). The native place of tomato crop is tropical Central and South America particularly in Peru, Ecuador, Bolivia and Andes (Salunkhe *et al*., 1987; Peralta *et al*., 2008). The plant is adapted to wide variety of climate and cultivated in almost every part of the world. It is world's most highly consumed vegetable due to its status as a basic ingredient in a large variety of raw, cooked or processed foods (Naika *et al*., 2005; Bergougnoux, 2014). Over the last century, tomato has attained tremendous popularity as an important vegetable crop. It is 7$^{th}$ most important crop after maize, rice,

wheat, potatoes, soybeans and cassava and 2nd vegetable crops in the world after potato with 160 million tons production during 2017 (Renna *et al*., 2018). It is not only traded in the fresh market but is also used in the processing industry in soups, paste, concentrate, juice, and ketchup (Lenucci *et al*., 2006). It is an incredible source of important nutrients such as lycopene, β-carotene and vitamin C, which all have the positive impacts on human health.

**1. 2 Economic importance and production statistics**

Tomato plant is very versatile and can be utilized both in fresh and processed forms. In both cases, world production and consumption has grown quite rapidly over the last 25 years. With their culinary role in the daily diet, tomatoes also represent a low energy food with very unique constituents that have positive effects on human health. Among vegetables, raw and processed tomato foods are the richest source of lycopene. About 85% of lycopene in daily diet is obtained from fresh and processed products (Soares *et al*., 2017).

The worldwide production of tomatoes in year 2018 was estimated around 170.8 million tons. China, the leading producer of tomatoes, accounted for 31% of the total tomato production (Guan *et al*., 2017; Bisen and Singh, 2019; Bhargava and Srivastava, 2019). India and United States are second and third highest production of tomato in the world respectively. In India, tomato stands third in production share with 11.5 % of total production of vegetable crops in the year 2015-16 after potato and onion (nhb.gov.in/area-pro/NHB_Database_2017-18). Total cultivated area under tomato in year 2017-18 was 20,782 (‘000 ha) and total tomato production was 797 (‘000 MT) with productivity of 25 MT/ha (Figure 1.1). In India leading tomato producing states are Madhya Pradesh,

followed by Karnataka, Andhra Pradesh, Telangana, Gujrat, Odisha and West Bengal (Figure 1.2).

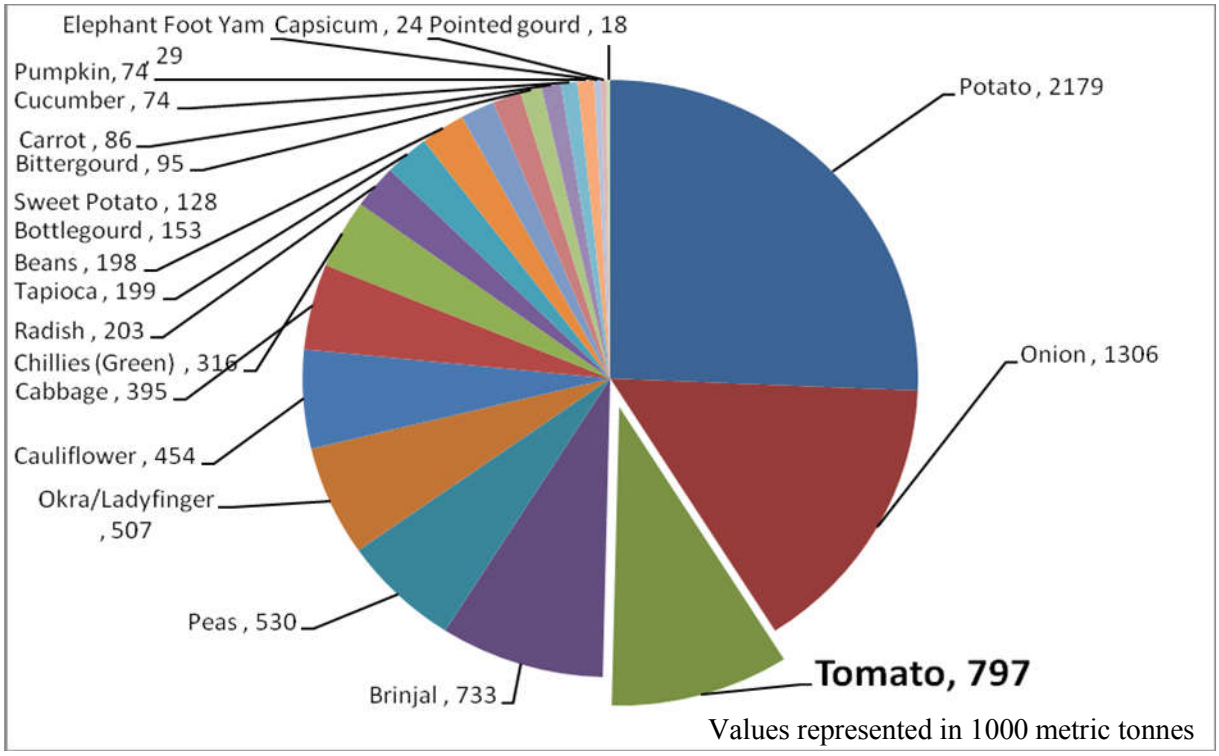



**Figure 1.1** Production share of tomato [Horticultural Statistics at Glance-2018 (http://agricoop.nic.in/statistics/publication-reports)

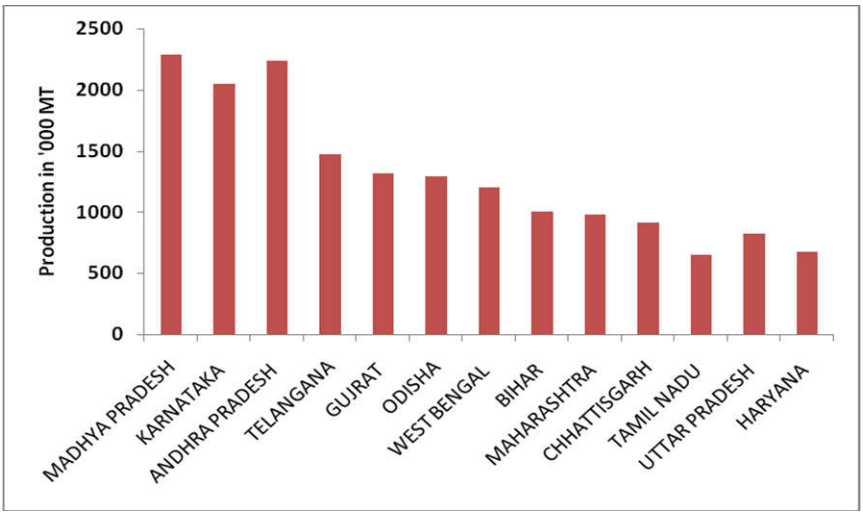



**Figure 1.2** Top ten tomato producing states in India (2015-16) [Horticultural Statistics at Glance-2018 (http://agricoop.nic.in/statistics/publication-reports)]

## 1. 3 Nutritional significance of tomato

Tomato is a key constituent of the alleged "Mediterranean diet", which is linked with high intake of vegetables, less protein consumption and thought to reduce risk of chronic degenerative diseases (Agarwa and Rao, 2000; Soares *et al*., 2017; Hurtado-Barroso *et al*., 2019). The most common phytochemicals present in raw tomato are the carotenoids including lycopene (60-40%), phytoene (10-12%), neurosporene (7-9%) and carotenes (10-15%) [Clinton *et al*., 1998; Gerster, 1997; Rao *et al*., 1998; Coyago-Cruz *et al*., 2019]. Tomato is a treasure of riches when it comes to antioxidants and contributes largely to the daily consumption of a major amount of these molecules (Table 1.1). Various studies have shown that intake of raw tomato and tomato based products reduces risk of heart diseases and cancer (Clinton, 1998; Giovannucci *et al*., 2002; Hoffman and Gerber, 2015). This protective potential of tomatoes has been mainly credited to bioactive components with antioxidant properties (Rao *et al*., 1998; Borguini and Torres, 2009). Major antioxidants of tomatoes include carotenoids such as β-carotene (a precursor of vitamin A) and mainly lycopene which is largely responsible for the red color of the fruit. Other vitamins such as ascorbic acid and tocopherols, and phenolic compounds such as flavonoids (naringenin, chalconaringenin, rutin, kaempferol and quercetin) and hydroxycinnamic acid are also present in tomato (Borguini and Torres, 2009; Kotikova *et al*., 2011). These compounds play an important role in reducing the high level of reactive oxygen species (ROS) responsible for many important diseases, through free-radical scavenging, metal chelation, inhibition of cellular proliferation, and modulation of enzymatic activity and signal transduction pathways (Clinton, 1998; Crozier *et al*., 2009)

**Table 1.1 Major nutritional constituents of tomato (per 100.00 g)**

| Nutrient | Amount |
|---|---|
| Lycopene | 2573 µg |
| L-ascorbic acid (Vitamin C) | 24.66 mg |
| Vitamin A | 74.97 mcg |
| Vitamin K | 7.9 µg |
| Pyridoxine (Vitamin B6) | 0.008 mg |
| Tocopherol (Vitamin E) | 0.54 mg |
| Vitamin B1 (Thiamine) | 0.037 mg |
| Protein | 0.9 g |
| Carbohydrate | 3.9 g |
| Fat | 0.2 g |
| Dietary fiber | 1.2 g |
| Potassium | 237 mg |
| Manganese | 0.114 mg |
| Phosphorus | 24.20 mg |
| Magnesium | 11.00 mg |

**Source:** https://fdc.nal.usda.gov/fdc-app.html#/?query=ndbNumber:11529

## 1. 4 Constraints in tomato production

Low yield of tomato production has been attributed to the various biotic and abiotic stresses (Figure 1.3). Temperature, relative humidity, and soil characteristics are the major abiotic factors that can directly or indirectly influence productivity. In addition to the direct impact on growth, these abiotic factors can also have an impact on the proliferation and damage caused by pests and diseases. The major insect pests that attack on tomato are fruit borers, thrips, aphids, leaf miners, and caterpillars. Tomato is attacked by a number of destructive diseases caused by fungi, bacteria, virus, nematode and mycoplasma which lead to considerable reduction in quality and quantity (Adhikari *et al*., 2017; Singh *et al*., 2017). The important diseases attacking tomato are damping off (*Pythium aphanidermatum*), early blight (*Alternaria solani*), buck eye rot (*Phytophthora parasitica*), late blight (*Phytophthora infestans*), fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*), collar rot (*Sclerotium rolfsii*), white mold (*Sclerotinia sclerotiorum*), powdery mildew (*Levellula taurica)*, bacterial leaf spot (*Xanthomonas campestris* pv. *vesicatoria*), bacterial canker (*Clavibacter michiganensis* pv. *michiganensis*), tomato mosaic (Tomato Mosaic Virus, TMV), tomato yellow leaf curl (Tomato yellow leaf curl virus) and tomato big bud (MLO). The diseases which are most devastating and causes huge economic losses are collar rot, stem rot, damping-off, black collar rot, early and late leaf spot, fungal and bacterial wilt (Middleton *et al.*, 1994; Podile and Kishore, 2002).

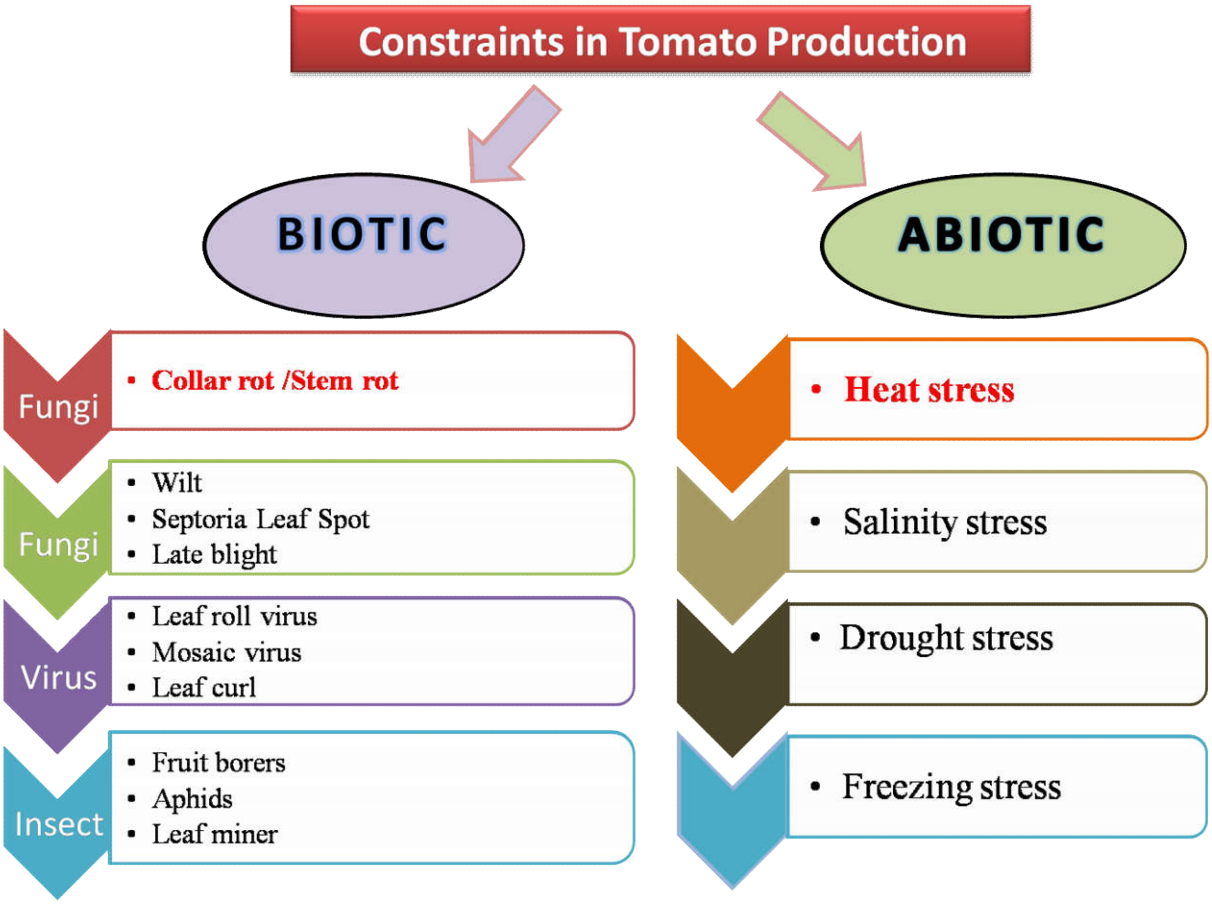



**Figure 1.3** Various biotic and abiotic constraints tomato of production

### 1.4.1 Biotic stress: Collar rot disease

*Sclerotium rolfsii* is a cosmopolitan pathogen which is a serious threat to large number crops. *S. rolfsii* is the most devastating polyphagous soilborne pathogen having a broad ecological distribution. The fungus infects over 500 species of plants worldwide including important crops and numerous weed species (Punja, 1985). Most of the plants susceptible to the necrotrophic pathogen are dicotyledonous and belong to Solanaceae, Cruciferac, Umbelliferac, Compositac Chenopodiaceae and Leguminosae, few are monocotylenous plant such as onion and tulip (Boland and Hall, 1994). Diseases caused by *S. rolfsii* are known by different names such as damping off of seedlings, stem canker,

crown blight, root, crown, bulb, tuber and fruit rots in many crops and most common is collar rot (Aycock, 1966; Farr *et al*., 1989; Singh *et al*., 2013). Soilborne inoculum (sclerotia) is more important in causing infection and disease development. The collar rot disease occurs primarily in the warm temperate, subtropics and tropics regions of the world, especially where high temperatures and high moisture conditions are available (Aycock, 1996). The disease is found to be present in almost every part of tomato cultivating regions of India including Uttar Pradesh with substantial losses in yield. As improved varieties and new cultivars having resistance to diseases are being developed, it will become easier to grow the crop in more marginal condition. However the sclerotia forming soil borne pathogen like *S. rolfsii* is major threat to production of tomato because resistant variety against soilborne pathogen are rare and not available in India. In India tomato is grown throughout year and root rot in tomato plant is commonly observed. Symptoms are characterized by water-soaked spots on stems, fruits or petioles which usually have an irregular shape. These spots enlarge and a cottony mycelium covers the affected area, the fungus spreads and the plant becomes a soft, slimy, water-soaked mass. *S. rolfsii* produces hyaline, septate, branched and multinucleate hyphae and mycelium may appear white to tan in culture and in plants. During the infection process, the pathogen produces a large amount of oxalic acid killing host epidermal cells well before penetration (Dakwa, 1965). The enzyme secreted by *S. rolfsii* enhances the host cell permeability so that leaking of electrolytes are more benefited to the invading pathogen and helpful for their further invasion into host cells (Kator *et al*., 2015). Figure 1.4 shows the diagrammatic representation of different stages of infection of *Sclerotium rolfsii.*

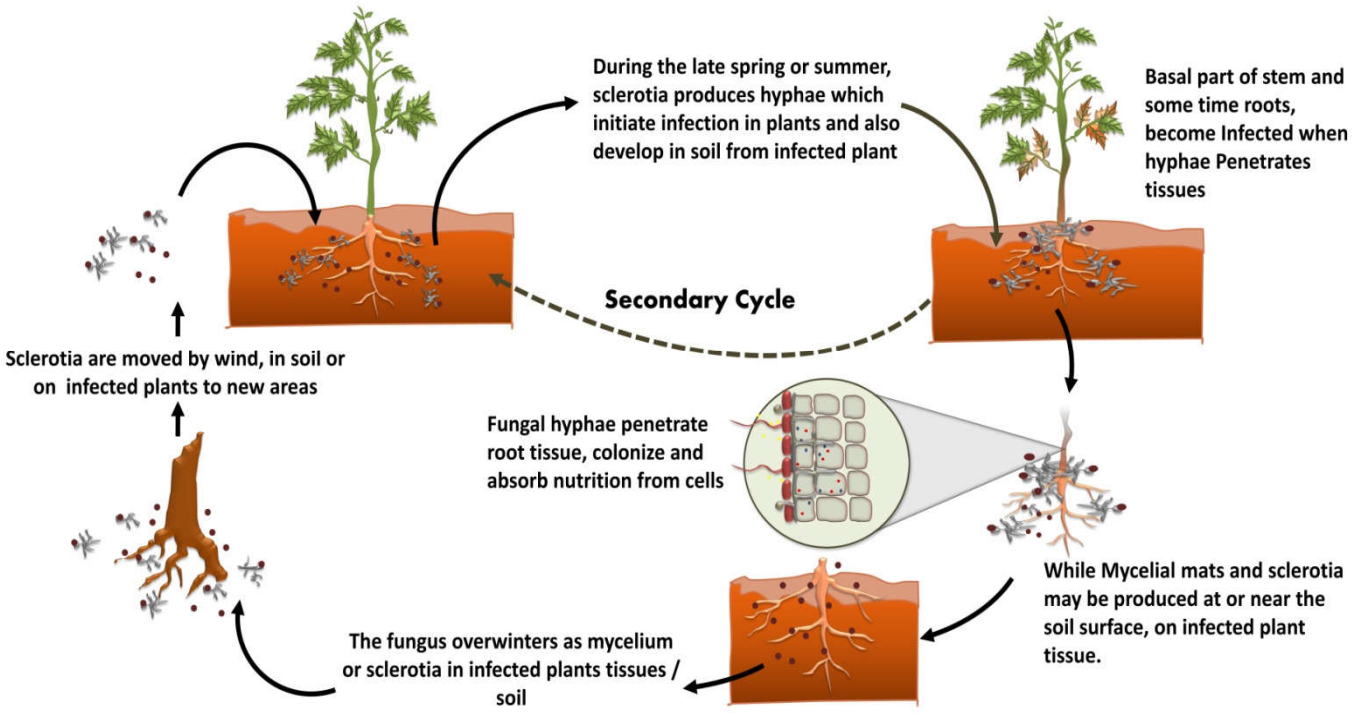



**Figure 1.4** Disease cycle of collar rot disease of tomato (*Solanum lycopersicum* L.) caused by *Sclerotium rolfsii*

The cottony mycelium usually produces numerous sclerotia. Pathogen produces no asexual conidia. Long-term survival of pathogen is mediated through the sclerotium; a pigmented multi-hyphal structure that can remain viable over long periods of time (8 years) under unfavorable conditions for growth (Chet, 1975, Willets and Wong, 1980; Amule *et al*., 2014). Sclerotia can germinate to produce mycelia or apothecia depending on environmental conditions. Apothecia produce ascospores, which are the primary means of infection host plants. These ascospores are forcibly discharged from apothecial surface and are critical for survival and spread of disease in the field (Steadman, 1983). The combined effects of light, temperature, moisture are considered the important factors for germination of sclerotia and subsequent apothetical development. Optimum temperature for pathogen life cycle and infection is 15-20°C Infections of above ground plant parts

occur when sclerotia germinate to produce apothecia which in turn release airborne ascospores. At the soil line, infection may result from either ascospore or sclerotial infection, while below ground infection result from mycelium produced from sclerotin. Pathogen has ability to infect plants at any stage of growth, makes white mold very serious disease. The fungus can survive on infected tissues, in the soil, and on living plants. The pathogens have been reported to cause heavy loss in various crops such as lettuce, celery, potato, tomato, cabbage, soybean and sunflower. Foot and root rot disease by *S. rolfsii* had caused approximately 30-35% loss in eggplant (Siddique *et al*., 2018). Wide host range, long term survival of *S. rolfsii* narrows down the opportunity for disease management using either crop rotation or resistant varieties. It is difficult to breed for resistance to *S. rolfsii* since resistance is governed by multiple genes. Also, source of genetic resistance is limited which has hampered the development of resistant genotypes against the pathogen (Bera *et al*., 2016). Management of the pathogen using chemicals/ fumigants like methyl bromide (Apablaza, 2000) has been reported with limited success since the chemicals are known for their toxicity and detrimental effect on the ozone layer (Duniway, 2002). Managing disease by indiscriminate use of pesticide of pesticide cause many negative consequences mainly resistance, resurgence and residue aspects. Also due to the present day concern about the environment, human health and development of resistance among the plant pathogens as a result of use of chemicals, biological control has emerged as an attractive for the management of plant diseases.

### 1.4.2 Abiotic stress: Heat stress

Abiotic stress is defined as any aspect exerted by the environment on the optimal functioning of an organism. Abiotic stresses like heat, cold, freezing, drought salinity,

flooding or oxidizing agents usually cause protein dysfunction (Wang *et al*., 2004). Plants are frequently exposed to a plethora of unfavorable or even adverse environmental conditions, thereby, pose serious threats to the sustainability of crop yield (Bhatnagar-Mathur *et al*., 2008). The complex nature of the environment, along with its erratic conditions and global climate change, are rising gradually, which is creating a more adverse situation (Mittler and Blumwald, 2010). Abiotic stresses alter plant metabolism leading to harmful effects on growth, development and productivity. These stresses initiate the production of harmful chemical compounds known as reactive oxygen species (ROS) which includes superoxide radical, hydroxyl radical, hydrogen peroxide etc. They can cause cellular damages by inactivation of enzymes, degradation of protein and altering the gene response (Choudhary *et al*., 2013). Due to global warming, a gradual increase in the overall temperature of the earth's atmosphere noticed. Inter-governmental panel on climatic change has reported that global mean temperature will increase 0.3°C per decade, reaching to 1°C above the current value by the year 2100 (IPCC, 2007) and it will play a negative role in the field of agriculture by decreasing crop yields. This decrease in crop yield is because of the shortened life cycle and hastened senescence due to high-temperature stress in different agro-climatic zones (Porter, 2005). High day and night temperatures as well as high soil and air temperatures hamper plant growth and cause considerable pre- and post-harvest losses (Hall, 2001).

Heat injuries are both short and long term. Severe heat stress for short-term can cause cellular damage and ultimately cell death. In the same way, high-temperature stress over a long period of time results in the calamitous collapse of cellular organization and death (Schoeffl *et al*., 1998). High-temperature stress results in altered gene expression,

inactivation of enzymes in chloroplast and mitochondria, production of reactive oxygen species, inhibited protein synthesis and protein degradation. High temperature increases the rate of reproductive development of plant and as a result, the time for photosynthesis is reduced, which ultimately reduces the total fruit or grain yield. Plants can be damaged in different ways by heat stress, and different species and cultivars differ in their sensitivity to high temperatures (Hall, 2001). Tomato production is limited by high daytime temperature and especially by high night temperature (Moore and Thomas, 1952). High temperature can also cause impairment of pollen and anther development by the elevated temperature that contributes to decreased fruit set in tomato (Peet *et al*., 1998; Sato *et al*., 2000). Temperature affects not only the time of fruit ripening but also the rate of fruit growth. Elevating the temperature often increases the fruit growth rate, but it has a greater effect in hastening maturity and, as a result, the final mean weight of tomato fruit is reduced (Hurd and Graves, 1985; Sawhney and Polowick, 1985). The process that are reported to be adversely affected by high-temperature stress includes meiosis in pollen and ovule mother cell, stigma position, number of pollen grain retained by stigma, ovule viability, pollen tube growth and fertilized embryo (Kinet and Peet, 1997). High-temperature stress impaired mitochondrial functions and resulted in induced oxidative damage (Mittler, 2002; Suzuki and Mittler, 2006). Under high temperature, RuBisCO can lead to the production of $H_2O_2$ as a result of its oxygenase reactions (Kim and Portis, 2004). The main effects of ROS include autocatalytic peroxidation of membrane lipids and pigments, modification of membrane permeability and functions (Xu *et al*., 2006).

## 1.5 Role of plant growth promoting microbes (PGMs) in biotic and abiotic stress management

The biological management of collar rot disease has been attempted by many researchers in the last few decades. According to the overall research done till date, it was found that the disease can be managed to a significant extent by the use of fungicides, host plant resistance, biological means combined with other management strategies (Suryawanshi, 2009; Singh, 2013). Earlier reports reveal that biocontrol agents like *T.viride* and *T. harzianum* effectively colonize and reduce the germination of sclerotia of *Sclerotium rolfsii* and found to be a potent tool against this fungus (Ray and Mukerjee, 2002; Naik *et al*., 2009; Kotasthane *et al*., 2015). Other bioagents such as *Pseudomonas fluorescens*, *B. subtilis Alcaligenes faecalis* and certain botanicals have also been found to have a profound effect in the management of disease caused by *S. rolfsii* in several crops (Srivastava *et al*., 2001; Ray *et al*., 2016a, 2016b). These beneficial microbes are also involved in the mitigation of various abiotic stress include salinity (*Azospirillum* sp., *Pseudomonas syringae*, *P. fluorescens*, *Bacillus* spp.), drought (*Azospirillum* spp., *P. putida*, *Bacillus* spp.) and nutrient deficiency (*Bacillus polymyxa*, *Pseudomonas alcaligenes*). In addition to plant growth promoting rhizobacteria, drought and salinity tolerant isolates of *Trichoderma* spp. and the effect of other strains of *Trichoderma* in amelioration of such abiotic stresses has also been reported. Mitigation of abiotic stresses in the crops could be done by increasing nutrient uptake (phosphorus, magnesium, nitrogen and calcium), biochemical (accumulation of betaines, proline, polyamines, carbohydrates and antioxidants), physiological and molecular changes.

### 1.6 Microbial pesticide and their existing formulations

The commercial development and market success of microbial pesticide depend upon the broad spectrum activity of formulated biological control agents and its ease of application technology. However, market penetration of microbial pesticide products in pest control management has increased significantly in recent years (Glare and O'Callaghan, 2017). The main reason behind such increasing demand is awareness in public on the benefits of biopesticide use and also the adverse effect of chemical pesticides on the environment (Lamichhane *et al*., 2016). The growth of the global biopesticides market is expected to reach US$ 6.6 billion by 2020 with estimated CAGR (compound annual growth rate) of 18.8 % from 2015 to 2020 (http://www.marketsandmarkets.com/Market-Reports/biopesticides267.html). While in India, the demand for biopesticides both in terms of volume and value is expected to show the growth with compounded annual rates of 18.3% and 19% over the 2015-2020 period (http://www.businesswire.com/news/home/20160217/Indian-BiopesticidesMarketGrowth-Trends-Forecast). Increasing demand of safe food is the key driver in enhancing the biopesticide market growth in sustainable agricultural practices. In worldwide biopesticide market, bacterial products holds 60% market share followed by fungal, viral and other products holding 27, 10 and 3%, respectively (Singh *et al*., 2016). Indian biopesticide market has much more opportunities to excel in the growth of this sector in the near future. The major commercialized products which have high market demand in Indian biopesticide market is *Trichoderma* based products which holds almost major 60% of total fungal BCAs market share (Rajput *et al*., 2018). Currently 355 *Trichoderma* spp. based

products are available in market for managing different soil and seedborne diseases, registered with CIBRC (Figure 1.5)

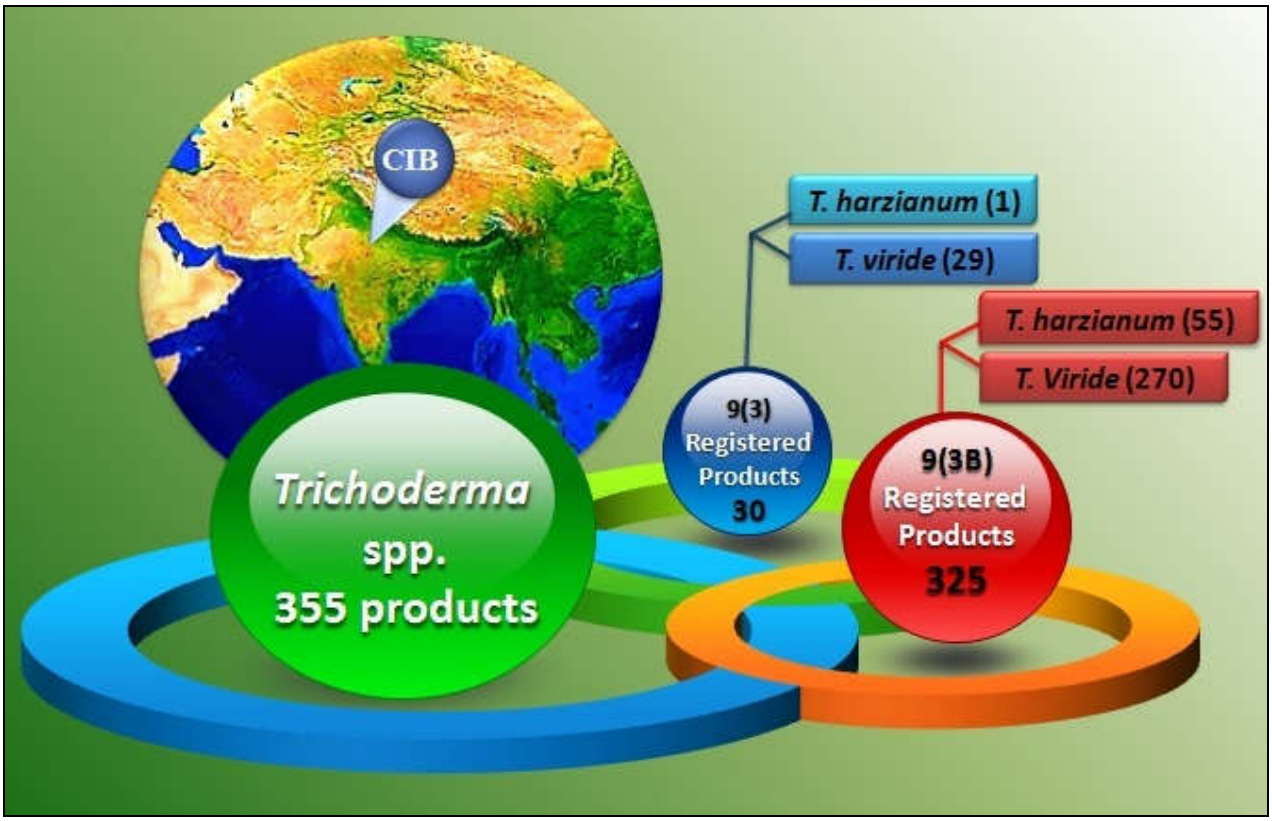



**Figure 1.5** Commercially registered *Trichoderma* based products registered under section 9(3) & 9(3B) with CIBRC [http://cibrc.nic.in/bpr.doc]

**1.7 Need of novel formulation**

Although many commercial products of *Trichoderma* spp. are available for the management of soilborne plant diseases (Howell, 2003; Harman and Custis, 2004; Bisen *et al*., 2016), but none of them are related to thermotolerant *Trichoderma* strains (Singh *et al*., 2014b; Woo *et al*., 2014; Keswani *et al.*, 2016). Among the different propagules like conidia, chlamydospores and vegetative mycelium, the conidia have been most widely employed in preparing biocontrol formulations (Elad *et al*., 1993; Mukherjee and Raghu, 1997; Ramanujam *et al*., 2010; Mukherjee *et al*., 2014). Active

ingredients of *Trichoderma* based products are conidia which are sensitive to different environmental stresses, such as summer temperature and UV radiation (Rangel *et al*., 2004, 2005; Ying and Feng, 2004). The high temperature in the soil during summer season also reduces the biocontrol efficacy of *Trichoderma* against phytopathogens (Mukherjee and Raghu, 1997; Kredics *et al*., 2003). High temperatures stress disturbs the membrane of fungal cells and damage macromolecules (Crisan, 1973; Nicholson *et al*., 2000). Thus, the persistency and performance of a fungal formulation in the field depend, to a large extent, upon conidial tolerance to stress factors. Of those, thermo tolerance is an important trait for the fungal candidates to possess (Kredics *et al*., 2003) For instance; the upper thermal limits of *T. harzianum* are around 37°C for conidial germination (Samuels, 1996; Sanogo *et al*., 2002) and 37–39°C for hyphal growth (Fargues *et al*., 1996, 1997; Hallsworth and Magan 1999). These are often close to or below the upper thermal limits of summer (Zaid season), e.g. > 45°C. To date, research has mostly been focused on the identification of biocontrol and plant growth promoting (PGP) strains of *Trichoderma* and very few reports are available on high-temperature tolerant *Trichoderma* that are effective at temperature up to 35°C (Begoude *et al*., 2007; Moustafa and Abdel-Azeem 2008). Therefore, there is an urgent need to explore thermotolerant *Trichoderma* strains that can retain their biocontrol potential at elevated temperature (>35°C) and develop their formulation that can be used even at high temperature conditions in the field.

**Aim of study**

Most of the rhizospheric microbes including *Trichoderma* strains have low osmotic tolerance level, hence fail to perform as biocontrol agents under high-temperature

condition in the field (Bérard *et al*., 2015). Therefore, the choice of bioagents should be fine-tuned with soil hydrological parameters. The main aim of the present study was to explore thermotolerant *Trichoderma* strains having biocontrol potential against *S. rolfsii* and develop their formulations based on eco-friendly and cost effective approaches. The secondary aim was to elucidate the defense mechanism during *Trichoderma* interaction with tomato plants upon *S. rolfsii* challenged inoculation. To fulfill these aims following objectives were designed in the present study.

**Objectives**

1. To isolate, characterize and identifying of abiotic stress tolerant *Trichoderma* species from various agro ecosystems
2. To develop of commercially viable and cost effective formulation using potential strains of *Trichoderma* species
3. To study the shelf life of *Trichoderma* based formulation at room temperature
4. To study the impact of *Trichoderma* based formulation on plant growth promotion and biological control potential (*viz*. SOD, POx, MDA, PAL, TPC) against *Sclerotium rolfsii* in tomato under green house conditions

# CHAPTER II

# REVIEW OF LITERATURE

# Chapter-II

# REVIEW OF LITERATURE

The literature pertinent to the present study entitled **"Development of potential *Trichoderma* based formulation for management of collar rot disease of tomato caused by *Sclerotium rolfsii.*"** is included in this chapter.

## 2.1 Host Plant: Tomato

Tomato (*Solanum lycopersicum* L; Synonym: *Lycopersicum esculentum* Mill.) is the world's most highly used and dominating vegetable, as it has a position of basic ingredient among a large variety of either raw, cooked or convenience foods (Bergougnoux, 2014; Naika *et al*., 2005). It is known by various names such as golden apple (Italin), love-apple, pomme d'amour (French), tomate (Spanish), jitomate (Spanish) etc. In India it is popularly known as "Tamatar or Paatal". Tomato is mainly grown owing to its high nutritional value. It is rich in nutrients, proteins, anti-oxidants, vitamins and a good source of lycopene (Borguini and Torres, 2009; Kotikova *et al*., 2011). It constitutes about 94.5 g of water in 100g of fresh weight of fruit which in very high in comparison to other vegetables (Ripoll *et al*., 2016; Lahoz *et al*., 2016).

### 2.1.1 History

Tomato is one of the vegetable crop which is most widely cultivated all over the globe. Tomato exists in the family Solanaceae which comprises of more than 3500 species, found within a diverse range of habitats (Knapp, 2004). The native place from where the tomato crop originated was Central and South America especially in Peru,

Bolivia, Ecuador and Andes (Salunkhe *et al*., 1987; Peralta *et al*., 2008). Although tomato plant is cultivated in nearly every chunk of the world, it is acclimatized to a wide variety of climate.

### 2.1.2 Botany

Tomato is a herbaceous annual plant in the family Solanaceae grown for its edible fruit. Plant can be erect with short stem height (determinate type) or vine-like with long, spreading stems (indeterminate type), ranging from three to six feet. The stem of tomato plant is covered with minute hairs and the leaves are arranged spirally. Leaves are more or less hairy, strongly odorous, pinnately compound, and up to 45 cm (18 inches) long. Pollination in tomato occurs through self pollination with flowers borne at the tip of auxiliary and side branches of plant. The five petaled flowers are yellow, 2 cm (0.8 inch) across, pendant, and clustered (Figure 2.1). Each flower has male and female parts, the stamens and pistil, wind and the vibration of bees' wings help move the pollen to the female part of the blossom. After fertilization, the petals and stamens drop off, and the new tomato develops. An unpollinated flower dies and drops off the plant. The tiny green tomatoes develop slowly from the first two to three weeks.

Fruits are berries that vary in diameter from 1.5 -7.5 cm (0.6 - 3 inches) or more. They are usually red, scarlet, or yellow, though green and purple varieties do exist, and they vary in shape from almost spherical to oval and elongate to pear-shaped. Each fruit contains at least two cells of small seeds surrounded by jelly like pulp. In market, tomato is classified as “Hybrid-type” or “desi-type,” based primarily on fruit size, shape, and colour. In “Hybrid-type”, fruits are about twice the size of desi-type fruit.

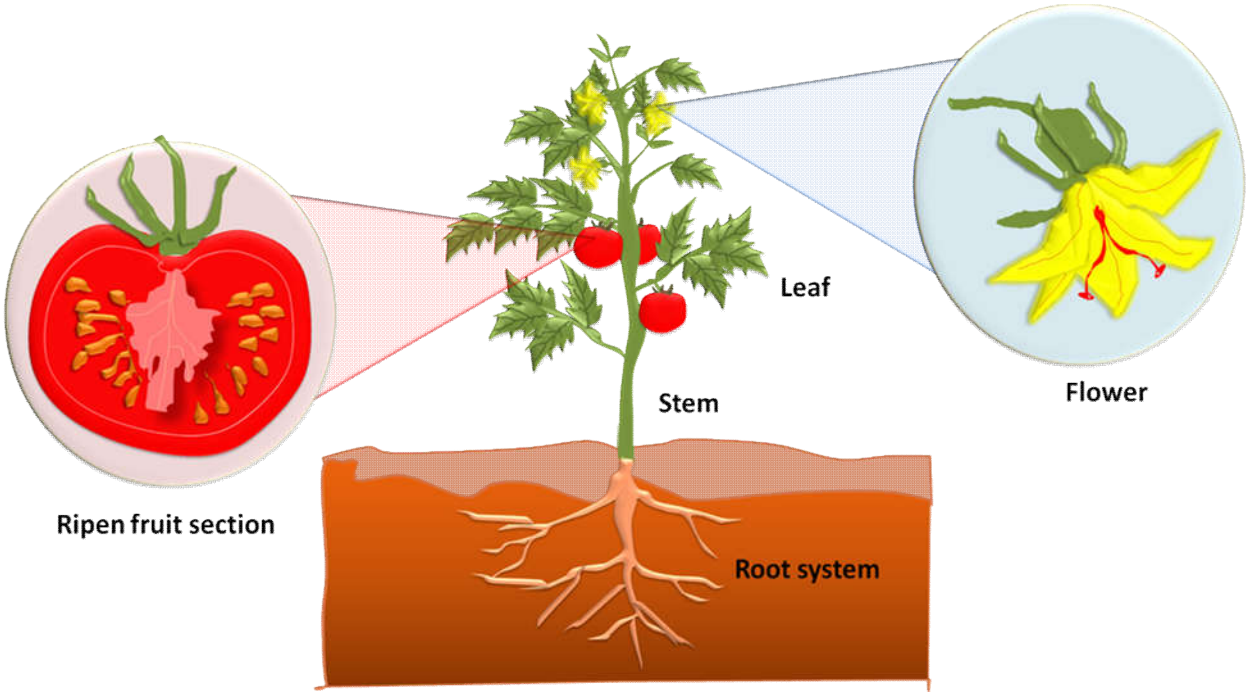



**Figure 2.1** Diagrammatic representation of tomato plant

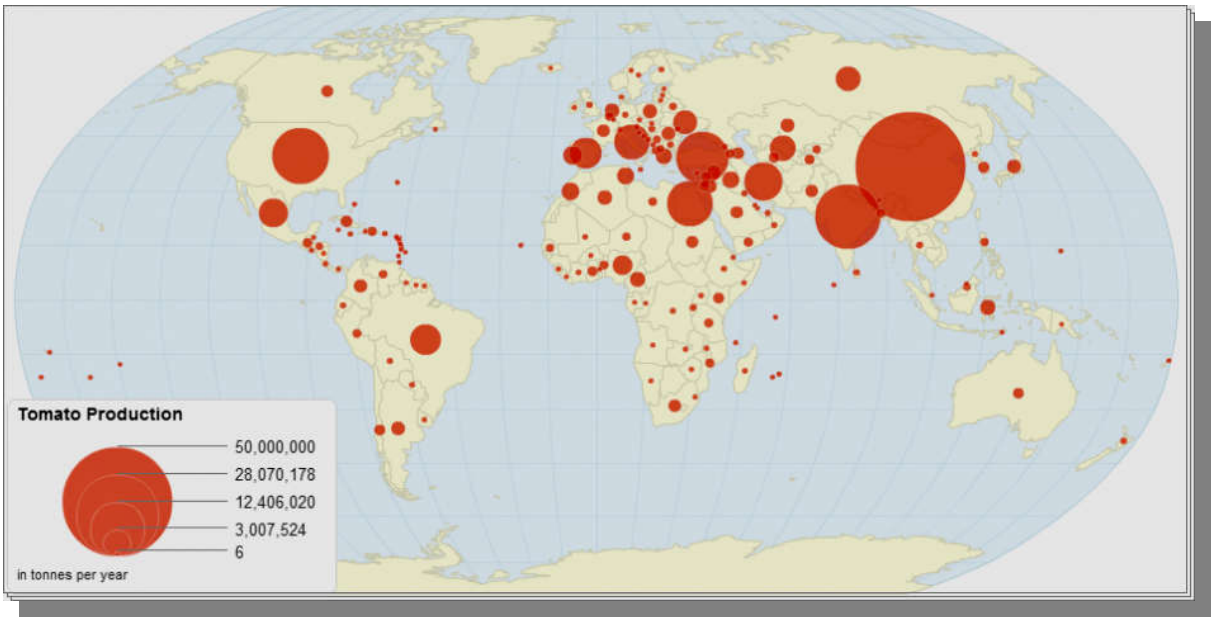
### 2.1.3 Climatic conditions

Tomato grows well under good moisture conditions with ideal temperature ranging from 24 ºC - 30 ºC (Paull, 1999; Saadi *et al.,* 2015). The water use efficiency of tomato plant depends upon various factors such as climatic conditions, soil type, and length of growing season (Hsiao, 1973; Egamberdieva *et al*., 2017; Rodríguez-Ortega *et al*., 2019). The plant performs well under annual rainfall between 400-600 mm; however its productivity may be enhanced through genotype selection and manipulation of planting density. Owing to its deep taproot, tomato is moderately drought tolerant as it is able to extract moisture from deep layers of soil profile, but its productivity is reduced by the recurrence of the terminal droughts. The plant requires fertile, sandy-loam soil with good internal drainage and they do not tolerate water-logged conditions (Bitterlich *et al.*, 2019; Ximenez-Embun *et al*., 2018). Tomato grows well in soils having a pH range of

6.0-7.0. It prefers soil with good residual soil moisture content or storage. The time of fruiting depends on type and variety and ranges from 65-90 days (Baloch *et al.*, 2017; Nazi *et al*., 2019)

### 2.1.4 Global distribution of tomato

Over the last century, tomato has attained a tremendous popularity as an important vegetable crop in the world (Figure 2.2). In year 2018, the total production of tomatoes was evaluated to be around 170.8 million tons worldwide which made tomato the seventh most crucial crop species after maize, wheat, rice, soybeans, potatoes and cassava. Also tomato is the 2$^{nd}$ vegetable crop predominant in the world after potato (Renna *et al*., 2018). China is producing 31% of the total global production making them the leading producer of tomatoes, while India and United States are the second and third highest producer of tomato in the world.




**Source:** http://chartsbin.com/view/32687

**Figure 2.2**: Tomato growing region throughout the world

In India tomato is mainly grown in central and southern regions. Six states mainly Madhya Pradesh, Karnataka, Andhra Pradesh, Teleangana, Gujrat and Odisha are the leading producers which altogether contribute 91% of the produce and 90% of the area (http://agricoop.nic.in/statistics/publication-reports).

### 2.1.5 Economic importance of tomato

Tomato being sold in markets for cooking is also used in processed food industry. In processing industry tomato are used to produce soups, concentrate, paste, juice and ketchup on a large scale. It is a phenomenal source of many bioactive components with antioxidant properties (Rao *et al*., 1998; Borguini and Torres 2009) such as lycopene, vitamins including tocopherols and ascorbic acid, and phenolic compounds such as flavonoids and hydroxycinnamic acid derivatives and β-carotene, precursor of vitamin A. All these nutrients are reported to have positive effects on human health (Borguini and Torres, 2009; Clinton, 1998; Kotikova *et al*., 2009; Kotikova *et al*., 2011). All these bioactive compounds may have an important role in restraining the ROS causing many important diseases, via free-radical hunting, metal chelation, obstruction of cellular proliferation, and regulation of enzymatic activity and signal transduction pathways (Clinton, 1998; Crozier *et al*., 2009). Nearly 85% of lycopene in daily diet is obtained from fresh tomato products, so raw and processed tomato contains the highest source of lycopene (Soares *et al*., 2017). It has been reported that intake of either raw tomato or tomato based products can decrease the risk of heart related diseases and also cancer (Hoffman and Gerber 2015; Clinton 1998; Giovannucci *et al*., 2002). As tomato is necessary for maintaining human health (Chapagain and Wiesman, 2004), hence strategies are needed for increasing its production and quality, which is foremost

important for the producers (Gruda, 2005; Flores *et al*., 2010). Also it was reported that mineral composition in soil has a significant effect on the antioxidant properties of tomato fruit with an increase in overall tomato fruit quality (Toor *et al*., 2006; Dorais *et al*., 2008).

### 2.1.6 Effect of abiotic stress on plant

Abiotic stress is defined as any aspect exerted by the environment on the optimal functioning of an organism. Protein dysfunction is usually caused due to abiotic stresses like heat, cold, freezing, drought salinity, flooding or oxidizing agents (Wang *et al.,* 2004). Plants are frequently exposed to a plethora of unfavourable and adverse environmental conditions, thereby, posing many serious threats to the sustainability of crop yield (Bhatnagar *et al*., 2008). Due to continuously changing environment, plants are constantly challenged by various abiotic stresses such as temperature extremes, drought salinity, heavy metal toxicity, UV-B radiation, etc. which results to considerable losses in the yield and quality of a crop (Hasanuzzaman *et al*., 2012). A more adverse situation is being created due to the complex nature of the environment, along with its erratic conditions and global climate changes (Mittler and Blumwald, 2010)

A number of abnormal environmental parameters are collectively termed abiotic stress. Abiotic stresses alter plant metabolism leading to harmful effects on growth, development and productivity. If the stress becomes very high and continues for an extended period it may lead to an unbearable metabolic load on cells, reducing growth, and in severe cases, result in plant death. However, plant stress may vary depending on the types of factor and on the prevailing period. These stresses are associated with production of certain deleterious chemical entities called ROS which includes hydrogen

peroxide ($H_2O_2$), superoxide radical ($O_2^-$), hydroxyl radical ($OH^-$), etc. ROS are capable of inducing cellular damage by degradation of proteins, inactivation of enzymes, alterations in the gene (Choudhary *et al*., 2013).

**2.1.6.1 Emerging global threat to tomato production: Heat stress**

Abiotic stresses are considered as greatest constraint to crop production worldwide. It has been estimated that more than 50% of yield reduction is the direct result of abiotic stresses (Rodriguez *et al.,* 2005; Acquaah, 2007). Due to the global warming, gradual increase in the overall temperature of earth's atmosphere has been noticed. Inter-governmental panel on climatic change has reported that global mean temperature will increase 0.3°C per decade, reaching to 1°C above the current value by the year 2100 (IPCC, 2007) and will play a negative role in the field of agriculture by decreasing crop yields. This decrease in crop yield is because of shortened life cycle and hastened senescence due to high temperature stress in different agro-climatic zones (Porter, 2005). High day and night temperatures as well as elevated soil and air temperatures hamper plant growth and cause considerable pre- and post-harvest losses (Hall, 2001).

Heat stress can be said as a stage when the temperature perceived by the plant is high enough to cause irreversible damage to the plants. Heat injuries are of both short and long term. Severe heat stress for short-term can cause cellular damage and ultimately cell death. Similarly, high temperature stress over a long period of time results in calamitous collapse of cellular organization and death (Schoeffl *et al*., 1998). High temperature stress results in altered gene expression, inactivation of enzymes in chloroplast and mitochondria, production of ROS, inhibited protein synthesis and protein degradation.

Long-term effects may be reduction in the size of tissues and organs and hampered morphological development (Wahid *et al*., 2007). It can also be cause a transitory damage when high temperature increases the rate of reproductive development and as a result, the time for photosynthesis is reduced, which ultimately reduces the total fruit or grain yield. Plants can be damaged in different ways by heat stress, and different species and cultivar differ in their sensitivity to high temperatures (Hall, 2001).

Tomato production is limited by high daytime temperature and especially by high night temperature (Moore and Thomas, 1952). Peet *et al*. (1997) demonstrated that daily mean temperature is more critical then night temperature. At daily mean temperature of 29ºC; fruit number, fruit weight per plant and seed number per fruit were markedly decreased compared with those at 25ºC. High temperature also causes impairment of pollen and anther development leading to decreased fruit set in tomato (Peet *et al*., 1998; Sato *et al*., 2000). Temperature affects not only the time of fruit ripening but also the rate of fruit growth. Pearce *et al.* (1993) found that in the short term the expansion of tomato fruits was closely related to temperature and did not appear to be limited by assimilate supply. The growth rates of fruit were found to be positively related to fruit temperature between 10 and 30ºC, with an increase in fruit diameter of 5 µm/h/ ºC (Pearce *et al*., 1993). Elevated temperature often increases the fruit growth rate, but it has a greater effect in hastening maturity and, as a result, the final mean weight of tomato fruit is reduced (Hurd and Graves, 1985; Sawhney and Polowick, 1985). Marcelis and Baan Hofman-Eijer (1993) reported that the consequence of temperature on the growth of cucumber was dependent on assimilate availability. Process that are reported to be adversely effected by high temperature stress include, meiosis in pollen and ovule mother

cell, stigma position, number of pollen grain retained by stigma, ovule viability, pollen tube growth and fertilized embryo (Kinet and Peet, 1997). High temperature stress impaires mitochondrial functions and results in induced oxidative damage (Suzuki and Mittler, 2006). Extreme temperature stress accelerates the generation and reactions of ROS including $O_2$, $O_2^-$, $H_2O_2$ and $OH^-$, thereby inducing oxidative stress (Mittler, 2002). Under high temperature, Rubisco can lead to production of $H_2O_2$ as a result of its oxygenase reactions (Kim and Portis, 2004). The main effects of ROS include autocatalytic peroxidation of membrane lipids and pigments leading to modification of membrane permeability and functions (Xu *et al*., 2006).

## 2.2 Collar rot disease of tomato caused by *Sclerotium rolfsii*

### 2.2.1 Host range, Distribution and Economic Importance

*Sclerotium rolfsii* is a polyphagous, soilborne fungal pathogen which causes disease on a wide range of agricultural and horticultural crops. Although no worldwide compilation of host genera has been published but more than 500 hundred plant species have been reported separately by many researches all over the world and this pathogen can cause huge economic losses to crops (Punja, 1985; Sennoi *et al*., 2010; Osemwegie *et al*., 2010; Majumder *et al*., 2016). It also causes damping off of seedlings, crown blight, root, crown, bulb, tuber, stem canker and fruit rots in several other crops (Aycock, 1966; Farr *et al*., 1989; Singh *et al*., 2014; Singh and Gaur, 2017; Acabal *et al*., 2019). Soilborne inoculum (sclerotia) is more important in causing infection and disease development (Mukherjee *et al*., 1993; Pacheco *et al*., 2016; Ray *et al*., 2017; Shrestha *et al*., 2018). This disease occurs primarily in warm temperate, subtropical and tropical regions of the world, especially in the regions of high temperatures and high moistures

(Aycock, 1996; Lehner and Mizubuti, 2017). As *S. rolfsii* causes collar rot disease which is one of the major constraints in tomato (*Solanum lycopersicon* L.) all over the globe (Dixit *et al*., 2016; Parvin *et al*., 2016; Mahato *et al*., 2017). The first report of collar rot in tomato dates back to 1892 with Peter Henry Rolfs' discovery of this fungus in association with tomato blight in Florida (Aycock 1966). The incidence of *S. rolfsii* have been reported in many countries across the globe including Europe, Belgium, Bulgaria, Spain, Sweden, Asia, China, Japan, Pakistan and India (Figure 2.3). In India, it has been reported from Arunachal Pradesh, Assam, Bihar, Delhi, Haryana, Jammu & Kashmir, Madhya Pradesh, Uttar Pradesh, West Bengal, Manipur, Meghalaya (Mahato *et al*., 2017; Sajad and Jamaluddin, 2017; Dinesh *et al*., 2018; Sahu *et al*., 2019).

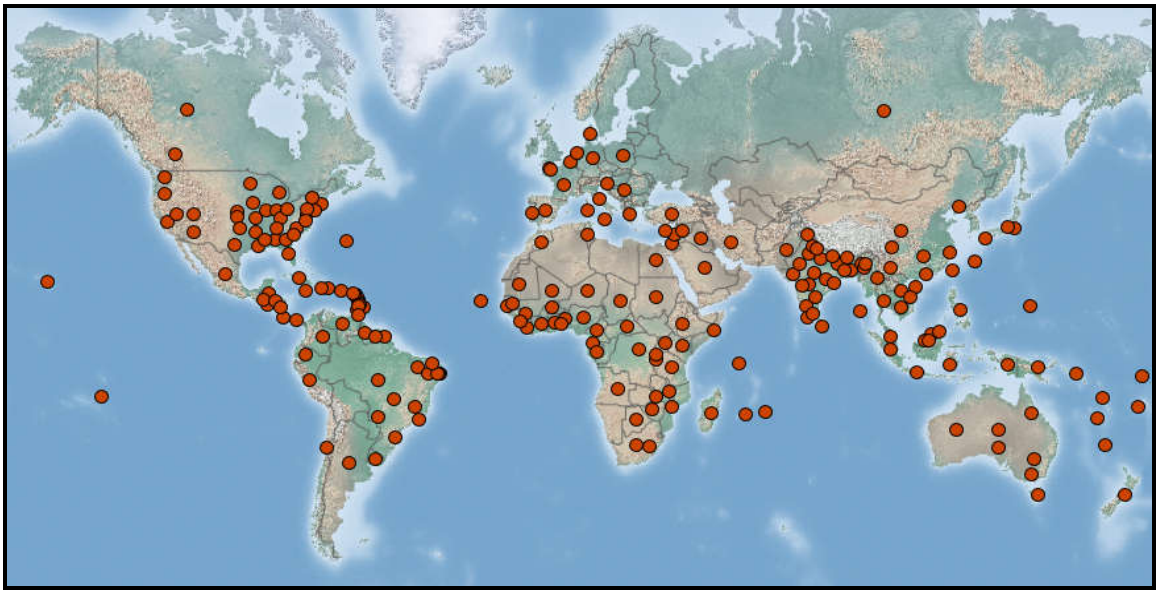


**Figure 2.3** Global distributions of *Sclerotium rolfsii* pathogen causing collar rot disease in tomato crop (https://www.plantwise.org/KnowledgeBank/datasheet)

*S. rolfsii* is causal organism of collar rot disease and can affect their hosts at any stage of growth, including seedlings, mature plants, and harvested products (Sahu *et al*.,

2019; Siddique *et al*., 2018). This disease can causes approximately 30% crop loss in both field and greenhouse conditions and under severe condition were it may reach up to 95% (Suriyagamon *et al*., 2018). In tomato, severely infected plants show reduction in weight (84.3 %) and size of fruit (62%). Nearly total loss is observed when plants are infected from early to mid-bloom as compared to plant infected near harvest (Amin and Jampala, 2018).

The loss caused by pathogen varies with their host. It has been reported that the pathogen caused 25% seedling mortality in groundnut (Rakholiya *et al*., 2012; Kumar and Thirumalaisamy, 2018) and overall 25% yield loss (Manjula *et al*., 2014), 25-35% crop loss on Tomato (Thiribhubanamala *et al*., 1999), 40-55% yield loss of cowpea (Fery and Dukes, 2002; Adandonon *et al*., 2017 ), 40-50% plant mortality of Crossandra (Harinath Naidu, 2000; Bhosale *et al*., 2018) and 5-20% crop loss of peppermint (Singh and Singh, 2004; Shaikh *et al*., 2018).

**2.2.2 Symptomatology**

Symptoms appear on tomato after extended periods of warm and rainy weather (Mullen, 2001; Billah, 2017). The pathogen *S. rolfsii* inflicts severe damage right from germination till harvest of the crop in all seasons. The disease symptoms can usually be found on tissues with high water content and in close proximity to the soil. This fungus can spread quickly in the field from plant to plant. Infection is usually restricted to plant parts that are in direct contact with the soil (Ray *et al*., 2016; Siddique *et al*., 2018). Limited symptom occurs on the adjoining region of stem and the root at point of injury. The lesions develop around the stem eventually forming a "collar zone" which disrupting the translocation of nutrients from the top to the root zone (Sanoubar and Barbanti, 2017;

Hasan and Meah, 2019). The fungus forms a typical white, fan-shaped mycelium which develops on the infected tissue and surrounding soil along with sclerotia (Punja and Grogan, 1982; Mukherjee and Raghu, 1997; Xu *et al*., 2008; Bholanath and Papiya, 2017). Sclerotia are spherical in shape with 1.0-3.5 mm in size (diameter); they are white when immature, turning tan and then reddish brown when mature and act as resting structure (Sarma *et al*., 2002; Mahato and Biswas, 2017). The pathogen overwinters in soil and plant debris as sclerotia which germinates under favourable environmental conditions and initiate the infection in plant and start the colonization in the root and shoot tissues (Coley-Smith and Cooke, 1971; Brewster *et al*., 2001; Salvi *et al*., 2017). After infection, the basal part of the stem turn pale brown and soft but not watery. The whole plant can collapses or die either at the seedling stage or at mature stage. In the seedling stage, the entire rows or large patches of dead seedlings can be supplemented by gap filling but in the case of mature plants, this is not possible. As a consequence, collar rot becomes a very destructive disease leading to huge economic losses. Fruit may occasionally become infected and then it rots and drops off without becoming spotted.

### 2.2.3 Biology of *Sclerotium rolfsii*

#### 2.2.3.1 Taxonomy

The first report of fungus was done by Rolfs (1892) as a causal agent of tomato blight from Florida region in United State of America. Saccardo (1911) has named the fungus as *S. rolfsii* and Cooper (1961) has reported *S. rolfsii* as an omnipathogenic fungus, due to its ability to attack a large number of monocot and dicot plant. At beginning, researchers assigned *Sclerotium* genus into the Fungi Imperfecti class which possess a diverse range of species and these species were grouped as a single genus

because they shared some similar phenotypic characters. These fungi generally produces small, tan to dark-brown/black, spherical sclerotia, that function as survival structures. As later on with knowledge of their sexual states, some fungi were reclassified into other genera, either in the Basidiomycota or Ascomycota, and sometimes renamed according to their teleomorph (sexual stage).

Cruzi (1931) first reported the perfect stage of *S. rolfsii* as *Corticium rolfsii* (Tu and Kimbrough, 1978). They proposed to classify the pathogen in the genus *Athelia* and the binominal name, *Athelia rolfsii* (Cruzi) Tu and Kimbrough, has been used since. Some *Sclerotium* species still do not have any known sexual state, but by exploring molecular identification methods each species can now be assigned to the right genus. For example, based on sequence analysis of the rDNA large subunit (LSU) and internal transcribed spacer (ITS) regions, some *Sclerotium* spp. were renamed and moved from Ascomycota to Basidiomycota, whereas others, including *S. denigrans* and *S. perniciosum,* were moved from the Basidiomycota to Ascomycota (Xu *et al*., 2010)

The genetic diversity of *S. rolfsii* has been studied by a variety of techniques, including mycelial compatibility, restriction fragment length polymorphism (RFLP) analysis of ITS-rDNA, and by ITS-rDNA or LSU sequencing (Harlton *et al.*, 1995; Okabe *et al.*, 2000; Okabe *et al.*, 2001; Punja and Sun, 2001; Okabe and Matsumoto, 2003; Xu *et al.*, 2010). Harlton *et al.*, (1995) found 49 mycelial compatibility groups (MCGs) and 12 RFLP-ITS groups in a worldwide collection of isolates, but could not establish correlations between MCGs and pathogenicity. However, Xu *et al.* (2010) reported a close relationship between *S. rolfsii*, *S. rolfsii* var. *delphinii* and *S. coffeicola* by LSU sequence analysis. The phylogeny of *S. rolfsii*, *S. delphinii* and *S. coffeicola*

based on ITSrDNA shows two clades with most *S. rolfsii* strains. One clade contains most of the *S. delphinii* strains, and one clade contains strains of both *S. rolfsii* and *S. delphinii,* suggesting a close relationship between the latter two species.

Colonies of *S. rolfsii* can be readily distinguished on infected plant material or artificial media by gross morphological characteristics. Rapidly growing, silky-white hyphae tend to aggregate into rhizomorphic cords (Aycock, 1966; Harlton *et al.*, 1995). In culture, agar media are rapidly (2-3 days) covered with mycelium, including aerial hyphae. The optimum temperature for hyphal growth and sclerotial formation is 27-30ºC (Aycock, 1966; Mathur and Sinha, 1970; Punja, 1985; Punja and Rahe, 1993; Domingues *et al*., 2016). Sclerotia (0.3-3.0 mm diameter) begin to develop after 4-7 days of growth (Punja and Rahe, 1993) when hyphae cluster together as a compact mass. After an initial white appearance, the sclerotia quickly turn dark brown (Aycock, 1966). Sclerotia are made up of three different layers; Cortex cells, rind and medulla (Figure 2.4).

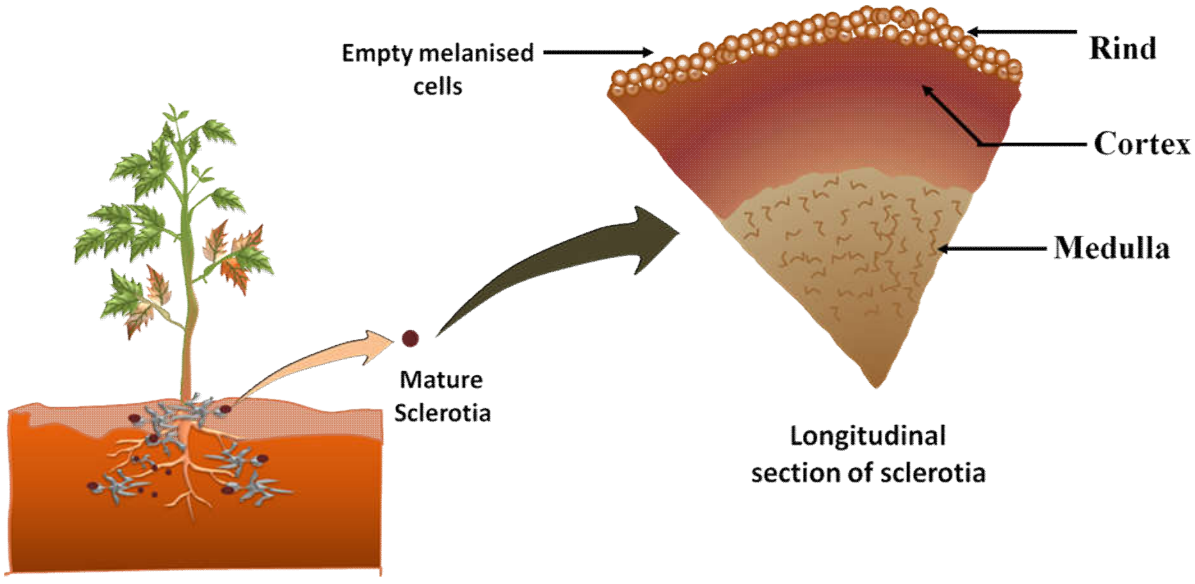



**Figure 2.4** Pictorial representation of different layer present in mature sclerotia

Cortex cells contained vesicles and rind have empty melanised cells which may helps in resistance of sclerotia (Chet *et al.*, 1967; Chet, 1975). Willets (1971) reported that melanin pigment might act as an inhibitor of both chitinases and glucanases.

**2.2.3.2 Infection mechanism**

Sclerotia contain viable hyphae and serve as the primary inoculum source in the disease and infection in plant. Sclerotia of *S. rolfsii* are easily spread in soil and get attached to shoes, hand tools, vehicle tires, machinery, or in splashing water while long-distance movement occurs by means of sclerotia in plant material or soil during shipment (Mullen, 2001). During favourable weather conditions, sclerotia resume their activity by either eruptive or hyphal germination (Punja and Grogan, 1981). Eruptive germination means that aggregates of white mycelium burst out of the sclerotial rind. An external food source is not required for this type of germination. Sclerotia can germinate eruptively only after being induced by dry conditions or volatile compounds (Punja and Grogan 1981). Growth of individual hyphae from sclerotia is in response to availability of exogenous nutrients (Punja and Damiani, 1996).

During infection, *S. rolfsii* secretes oxalic acid and tissue cell wall degrading (CWD) enzymes (Aycock, 1966; Punja and Damiani, 1996). Oxalic acid plays an important role in the virulence of *S. rolfsii* (Kritzman *et al.*, 1977; Punja, 1985; Ansari, and Agnihotri, 2000). By producing oxalic acid as well as pectinolytic and cellulolytic enzymes, the pathogen kills and disintegrates host tissues before it penetrates (Prasad and Naik, 2008). Oxalic acid acts as corrosive agent to tissues of hundreds of genera of plants (Ghaffar, 1976). Mahadevan and Sridhar (1986) reported that the production of oxalic acid by *S. rolfsii* enabled the pathogenesis by promoting the activity of poly

galacturnonase (PG) by creating an acidic environment in plant tissues, which inactivated the prohibitins and phytoalexins and thus reducing host resistance.

Presence of calcium in plant tissue reduces the activity of hydrolytic enzymes i.e. PG and polymethyl galacturonase (PMG) because of its association with pectic acid that forms calcium pectate which is not hydrolysed by PG and PMG. *S. rolfsii* produces oxalic acid that combines with calcium of plant tissues and form calcium oxalate instead of calcium pectate (Figure 2.5). Now, this calcium oxalate can easily hydrolyse by PG and PMG enzymes (Deacon, 2006). Massive mycelial growth on plant tissues produces large quantities of oxalic acid that facilitate penetration of hyphae into tissue (Aycock, 1966; Godoy *et al*., 1990). Oxalic acid and tissue-degrading enzymes work together to break down cell walls, resulting in tissue maceration and the pathogen then absorb nutrients from the macerated tissue (Aycock, 1966). Maceration interrupts transport of water and nutrients in plant tissues, thereby causing wilting, yellowing, and necrosis (Bateman and Beer, 1965).

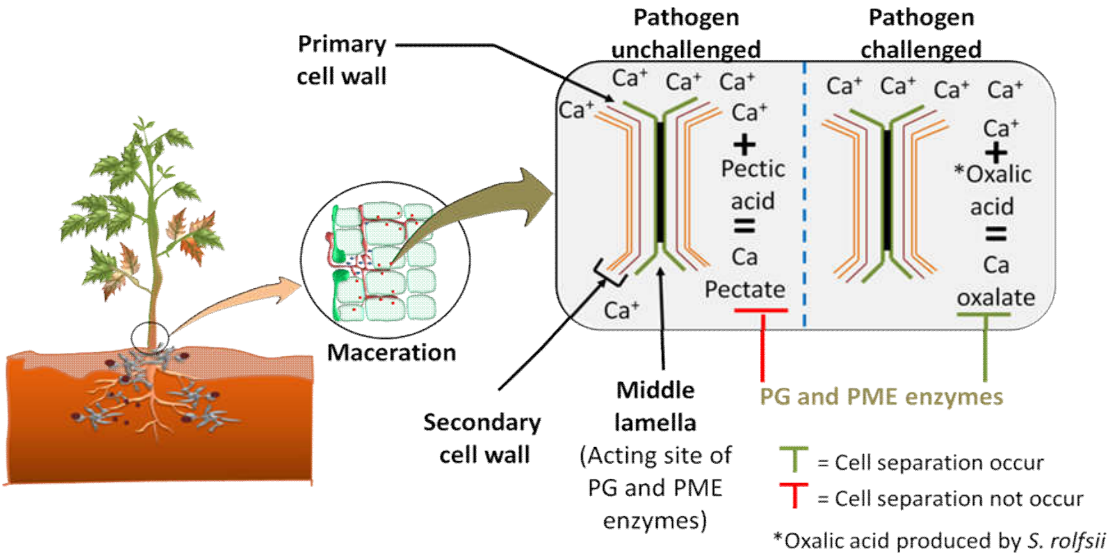



**Figure 2.5** Maceration (cell separation) process of plant tissue by *Sclerotium rolfsii*

### 2.2.3.3 Survival of sclerotia

Sclerotia are the primary structures that ensure the survival of *S. rolfsii* pathogen in unfavourable conditions. Sclerotia are formed from masses of hyphal strands. Mature sclerotia include an outer layer (rind), an underlying cortical layer, and a central medullary region comprised of loosely interwoven hyphae (Chet, 1967; Deacon, 2006). Sclerotia of *S. rolfsii* survived from 2 months to 7 years in field soil depending on environmental conditions (Aycock, 1966).

**1. Effect of temperature and moisture on sclerotia survival**

Temperature and moisture may interact in influencing survival of *S. rolfsii* under field conditions (Beute and Rodriguez-Kabana, 1981). To isolate the impact of specific environmental variables, survival of sclerotia has also been studied under controlled conditions. Cycles of drying and wetting, as well as cycles of freezing and thawing, may decrease survival of sclerotia of *S. rolfsii* (Punja and Jenkins, 1984). Stimulation of eruptive germination during drying following wetting was associated with rind cracking and leakage of nutrients, permitting activity of lytic microorganisms (Smith, 1972). Therefore, Smith (1972) suggested attempting biological control of *S. rolfsii* through alternate wetting and drying of soil. Matti (1988) reported little difference in the proportion of viable sclerotia of *S. rolfsii* recovered under a range of controlled temperature regimes (0-40 °C) or under moderate to low soil water holding capacity. In saturated soil, however, sclerotia lysis and mortality increased significantly, and survival was < 2 weeks. Similarly, in moist soil (0.033 MPa) survival of sclerotia was significantly lower when temperature was >20 °C than ≤20 °C, whereas no significant difference between these temperatures was observed in drier soil (-0.5 MPa). Matti

(1988) found that only 11% of sclerotia survived on the soil surface, whereas 94% survived at 10 cm soil depth, after alternating 7 day cycles of wetting and drying over 8 weeks. The relative absence of soil drying at 10 cm depth might account for greater survival than at the soil surface. Unlike sclerotia of *S. cepivorum*, *Botrytis cinerea* and *B. tulipae*, sclerotia of *S. rolfsii* tend to leak large quantities of nutrients into soil after a succession of wetting and drying periods, resulting in increased microbial Colonization (Coley-Smith, 1974)

**2. Effect of soil characteristics on sclerotia survival**

Survival of sclerotia can be affected by their depth of burial in soil. The survival of *S. rolfsii* decreased when depth of burial was greater than 2.5 cm, and that survival decreased in proportion to depth of burial (Smith *et al*., 1989). Punja and Jenkins (1984) attributed this trend in part to increasing gravitational pressure at greater depths, which may enhance substrate leakage from sclerotia. Soil texture and pH may also affect survival of sclerotia. Mitchell *et al*. (1990) and Alexander and Stewart (1994) showed more rapid sclerotial degradation and reduced survival in soil with higher clay content and relatively low pH (~6), and lower survival in clay loam than in sandy loam for *S. sclerotiorum*. Alexander and Stewart (1994) attributed lower survival in clay loam to greater water holding capacity, which affected drying and wetting of soil, resulting in greater microbial activity. Factors such as drying, wetting, and heating that increase activity of soil microorganisms near sclerotia and predispose sclerotia to antagonism may accelerate their mortality rate (Alexander and Stewart, 1994). Ubiquitous soil microorganisms such as *Trichoderma* spp., *Fusarium* spp., *Penicillium* spp. and

*Aspergillus* spp. can penetrate the rind and destroy the inner sclerotial tissues (Coley-Smith, 1974; Elad *et al*., 1984). For *Trichoderma*, this process is facilitated by production of the enzymes β-1, 3 glucanase and chitinase (Elad *et al*., 1982).

**3. Impact of sclerotia size on survival**

Alexander and Stewart (1994) repaoted that the relatively large sclerotia of *S. rolfsii* and *S. sclerotiorum* survived longer than the smaller sclerotia of *S. minor* and *S. cepivorum.* They related greater durability to the fact that larger sclerotia may contain more nutrients in the cortex and thus be more resistant to prolonged periods of unfavorable conditions (Alexander and Stewart, 1994; Chet, 1975). Smolinska and Horbowicz (1999) documented much higher resistance to plant-derived toxic volatiles from plants by *S. sclerotiorum* than *S. cepivorum* sclerotia, concluding that larger size of *S. sclerotiorum* sclerotia may impede penetration of volatiles into the cortex. Similarly, *Botrytis elliptica,* which has larger sclerotia than *B. cinerea* or *B. tulipae*, appeared more resistant to fungicides than sclerotia of the latter two species (Hsiang and Chastagner, 1992). Larger sclerotia size confers smaller surface to volume ratio, which means that sclerotia are less exposed to the environment, and may provide a protective effect by shielding the cortex from temperature extremes and microbial attack (Schmidt-Nielson, 1984).

**2.3 Effect of increasing global temperature on disease**

The agricultural sector has gained an interesting impact in the economic development by contributing significantly to food security, national income and employment. However, despite this importance, agriculture continuously faces different challenges principally the climate change. A drastic change in climate i.e. changes in

temperatures, rainfall imbalance with also changes in the frequency and the intensity of exceptional weather events negatively affects the crop and also the livestock systems (OECD, 2015). As the changing environment remarkably influences the plant health, pathogen activity and their antagonists, the changes in environmental conditions are strongly related with the intensity of loss induced by the disease, and also it was found that changes in environmental conditions are also provoking the arrival of new diseases (Anderson *et al*., 2004). Hence threatening and emerging with re-emerging plant diseases were reported that were causing remarkable yield loss in several crops (Bandyopadhya and Frederiksen, 1999; Hibakr *et al*., 2007; Rhouma *et al*., 2007; Gaaliche *et al*., 2018). Collar rot disease caused by *S. rolfsii* re-emerged and became a major factor limiting yield, which ultimately became a challenge to both farmers and scientists. *S. rolfsii* occurs worldwide but is more predominant through the tropics and warmer regions of the temperate zones (Punja, 1985; Dasgupta and Mandal, 1989). Hence environmental conditions may have an effect on plant infection and furthermore on the levels of the induced losses. It was reported previously that optimal growth, germination and sclerotial production occurred at 25°C-30°C (Abeygunawardena and Wood, 1957; Mahen *et al*., 1995; Tripathi and Khare, 2006; Lin *et al*., 2009). Also temperature variations may affect the growth patterns of the sclerotia (Punja, 1985). This pathogen was found to be most active at relatively warm temperatures (27°C to 32°C) in California (Browne *et al*., 2002); while the optimum growth of this pathogen occurred at 30°C-35°C in Tunisia (Daami-Remadi *et al*., 2010). The temperature requirements indicate that its distribution and occurrence is more towards warmer regions of the world. Hence future changes in environmental conditions like changes in the ambient temperature may directly or

indirectly impact plants, plant pathogens, and ultimately plant diseases (Li *et al*., 2013; Suzuki *et al*., 2014; Ashoub *et al*., 2015).

### 2.4 Management practices adapted for collar rot disease

Owing to of its wide host range, fast growth rate, and the production of large numbers of persistent sclerotia, Collar rot disease caused by *S. rolfsii* is very difficult to control (Punja, 1985; Lakpale *et al.*, 2007, Singh *et al*., 2013; Siddique *et al*., 2018; Sahu *et al*., 2019). Current control measures comprises the use of various cultural, physical, chemical, and biological control strategies for the effective management of disease (Punja and Rahe, 1993; Mehan and Hong, 1994; Mehan *et al.*, 1995; Mukherjee and Raghu, 1997; Le, 2004; Nguyen *et al*., 2004; dos Santos *et al.*, 2005; Vargas Gil *et al.*, 2008; Sahni *et al*., 2008).

#### 2.4.1 Cultural and physical practices

Cultural and physical management techniques found effective against soil-borne *S. rolfsii* are deep ploughing, solar heating, adequate use of chemical fertilizers and crop rotation (Mullen, 2001; Katan, 2002). Summer deep ploughing was found effective in reducing the viability of sclerotia or to kill hyphae of the pathogen in the fields by burying sclerotia or disease tissues in the depth 6-20 centimetres of soil (Elad *et al.*, 1980; Porter and Merriman, 1983; Mihail and Alcorn, 1984). In warm and humid regions, soil solarization has been found effective in the control of the pathogen (Hagan 2004). For solar heating, soils mulched with transparent polyethylene for 6 weeks in July-August followed by sown with groundnuts in spring, showed significant decrease in the percentage of diseased plants and rotten pods (Grinstein *et al.*, 1979).

Rotation with non-host crops not only improves the soil nutritional status, but also may adversely affect pathogen inoculum densities. For *S. rolfsii,* however, this strategy is not very effective due to its broad host range. Nevertheless, Taylor and Rodriguez Kabana (1999) reported that stem rot of groundnut can be suppressed by rotation with cotton. Also paddy rice was recommended as a rotation crop with groundnut in order to reduce stem rot in Vietnam (Le, 1977).

Use of incorporating organic amendments in soil such as compost, oat or corn straw, vermicompost and farm yard manure (FYM) reduce the incidence of collar rot disease and enhances populations of beneficial soil microbes (Bulluck and Ristaino, 2002). Singh *et al*. (1980) reported the effect of neem (*Azadirachta indica*) oil and aqueous extracts of leaf, trunk bark, fruit pulp on four soil-borne pathogens i.e. *F. oxysporum* f.sp. *ciceri, R. solani, S. rolfsii*, and *S. sclerotiorum*, which incite wilt and rot in gram (*Cicer arietinum*). Growth of the four pathogens in liquid medium was inhibited by extracts and oil. Neem oil showed maximum inhibitory effect while fruit pulp suppressed formation of Sclerotium of *R. solani* at all the concentrations. Neem oil and pine bark extracts or pine bark powder also has detrimental effect on growth of *S. rolfsii* (Kokalis-Burelle and Rodriguez-Kabana, 1994).

Singh and Handique (1997) reported that the essential oil from the leaves of American mint (*Hyptis suaveolens* L.), a herbaceous annual weed, has been showed significant antifungal activity against the soil-borne sclerotial forming fungi, *R. solani, S. rolfsii* and *S. sclerotiorum.* Treatment with the oil drastically reduced the ascospore germination of *S. sclerotiorum* and when oil used with combination of antagonistic fungus *T. harzianum* control wilt as well as rot diseases of knol-khol *(Brassica caulorapa*

Pasq.) caused by *S. sclerotiorum.* The oil appears to inhibit the fungal growth, but does not destroy the viability of these sclerotial fungi.

Ammonium compounds were shown to inhibit germination of sclerotia and promoted colonization of sclerotia by soil microorganisms (Prasad and Naik, 2008). Other cultural practices that suppressed *S. rolfsii* growth include adjusting the soil pH to about 6.5 by adding lime (Bulluck and Ristaino, 2002) and aerification of the soil (Mullen, 2001). Some cultural practices, such as soil replacement and solarization, are labour-intensive and expensive, and can be impractical in many commercial scale production systems (Edmunds *et al*., 2003).

**2.4.2 Chemical control**

*S. rolfsii* can be controlled to a considerable extent by used of fungicides such as Vitavax-200 (combination of Thiram 38.7% and Carboxin 38.7%), Ziram, tebuconazol and flutolanil (Kaur and Mukhopadhyay, 1992; Mehan *et al*., 1994; Yaqub and Shahzad, 2006. However, tolerance to tebuconazole has been reported for *S. rolfsii* populations in USA (Wadsworth and Melouk, 1984; Franke *et al.*, 1998; Shim *et al.*, 1998). Other fungicides that are used to control collar rot disease include difenoconazole, carbendazim, flusilazole and chlorothalonil (Cilliers *et al*., 2003). When difenoconazole was tested in combination with *T. harzianum*, a biocontrol agent of *S. rolfsii,* no reduction of the effect of *T. hazianum* was observed (Cilliers *et al.*, 2003). Although fungicides can protect tomato plants from infection by *S. rolfsii* but their harmful effect on ecosystem can gradually minimize use of these harmful chemicals. Therefore integration of several different control measures is proposed to provide sustainable management of *S. rolfsii* and other diseases of tomato. In this context, biological control can be an alternative or

supplement to current management practices for *S. rolfsii* (Singh *et al.* 2003; Singh and Singh, 2004; Dey *et al*., 2004; Tonelli *et al*., 2010)

### 2.4.3 Biological control approach

Biocontrol of plant diseases has been promoted as a tool to achieve sustainable and improved crop production systems that are less dependent on agrochemicals. Successful biological control practices usually utilize naturally occurring, antagonistic microorganisms that are able to reduce the activities of plant pathogens. Such antagonists can compete with pathogens for nutrients, inhibit pathogen growth by secreting antibiotics, or reduce pathogen populations through parasitism. In addition, some of these microorganisms induce resistance in host plants, which enhances the plant's ability to defend itself from pathogen attack (Ram *et al*., 2018). The most widely studied microorganisms with antagonistic activity against plant pathogens and with beneficial effects on plant growth, belong to the bacterial genera *Bacillus*, *Pseudomonas, Rhizobium,* or the fungal genus *Trichoderma* (Mukherjee *et al.* 1995; Rangeshwaran *et al.* 2000; Anahosur, 2001; Lorito *et al.* 2010; Singh *et al*., 2013; Singh *et al*., 2014; ).

Application of these beneficial microorganisms can be done for soil, seeds or planting materials treatment and it has been used as a sustainable greener approach to control plant diseases (Cook and Baker, 1989). Fungal biocontrol agents may directly or indirectly kill sclerotia or mycelium of *S. rolfsii*. Lectins produced by *S. rolfsii* were proposed to serve as recognition factors for fungal biocontrol agents (Prasad and Naik, 2008). Among the fungal biocontrol agents, *Trichoderma* species are the most widely studied (Table 2.1).

**Table 2.1** Fungal biocontrol agent *Trichoderma* used for the management of *Sclerotium rolfsii* in different crops

| BCAs | Crop/ Study type | References |
|---|---|---|
| *T. lignorum* | *In vitro* study | Weindling, 1934 |
| *T. harzianum* | Ryegrass | Wells *et al*., 1972 |
| *T. harzianum* | Groundnut | Backman and Rodriguez-Kabana, 1975 |
| *T. harzianum* | Bean | Elad *et al*., 1980 |
| *T. harzianum* | Sugar Beet | Upadhyay *et al*., 1986 |
| *T. harzianum* | Snapbean | Papavizas and Lewis, 1989 |
| *T. viride* | Tomato | Wokocha, 1990 |
| *T. harzianum* | Chickpea | Kaur and Mukhopadhyay, 1992 |
| *T. koningii* | Tomato | Latunde-Dada, 1993 |
| *T. harzianum* | Beet, Carrot, Elephant Foot Yam and Bitter Gourd | Mukherjee and Raghu, 1993 |
| *T. harzianum* | Sugar beat | Abada, 1994 |
| *T. harzianum, T. virens* | Chili | Jinantana and Sariah, 1998 |
| *T. parceramosum, T. pseudokoningii* | Rice | Cuevas *et al*., 2001 |
| *T. harzianum* | Mung bean | Mishra *et al*., 2000 |
| *T. harzianum* | Tomato | Dutta and Das, 2002 |
| *T. koningii* | Tomato | Tsahouridou and Thanassoulopoulos, 2002 |
| *T. viride* | Groundnut | Manjula *et al*., 2004 |
| *T. harzianum, T. virens* | Mint | Singh and Singh, 2004 |
| *T. hazianum* | Groundnut | Karthikeyan *et al*., 2006 |
| *T. harzianum* | Chickpea | Maurya *et al*., 2008 |
| *T. harzianum* | Sunflower, Mung bean | Yaqub and Shahzad, 2008 |
| *T. harzianum* (ITCC-4572) | Groundnut | Ganesan *et al*., 2007 |

| | | |
|---|---|---|
| *T. harzianum,*<br>*T. viride* | Eternity plant *(Zamioculcas zamiifolia)* | Jegathambigai *et al*., 2010 |
| *T. harzianum* | Faba bean | Abdel-Kader *et al*., 2011 |
| *Trichoderma spp.* | Groundnut | Bagwan *et al*., 2011 |
| *Trichoderma* spp. | Chilli | Joshi *et al*., 2011 |
| *T. hazianum* | Tomato | Singh *et al*., 2013 |
| *T. harzianum* | Artichoke<br>(*Helianthus tuberosus* L.) | Sennoi *et al*., 2013 |
| *T.* spp. | Cucumber, Bottle gourd<br>and Bitter gourd | Kotasthane *et al*., 2015 |
| *T. harzianum*, *T. viride*<br>*T. virens* | Lentil | Kushwaha *et al*., 2018 |
| *T. harzianum* | Tomato | Suriyagamon *et al*., 2018 |
| *Trichoderm*a sp. T76<br>12/2 | Snake fruit (*Salacca zalacca*)<br>and Lettuce | Wonglom *et al*., 2019 |
| *T. harzianum* | Barley | Faruk, 2019 |

In a direct interaction, hyphae of *Trichoderma* penetrate the rind and the cortex of sclerotia and lyse the medullar tissue (Desai and Schlosser, 1999). Degraded sclerotia become dark, soft and disintegrate under slight pressure and it was shown that chitinase and β-1, 3-glucanase play a role in the interaction between *T. harzianum* and *S. rolfsii* (Prasad and Naik, 2008). Next to *Trichoderma,* several other fungal genera have been tested for their ability to control diseases caused by *S. rolfsii* on bean, carrot, chilli, ginger, wheat, lentil, sesame, soybean, sugar beet, sunflower, tomato, or groundnut. These antagonistic fungi include *Gliocladium virens, Gliocladium roseum, Glomus fascicatum, Penicillium pinophilum* and *Gigaspora margarita.* In addition to fungal, several bacterial genera and species have been studied for biocontrol of *S. rolfsii*. Most of them belong to the genera *Pseudomonas* and *Bacillus* (Table 2.2).

**Table 2.2** Bacterial biocontrol agents (BCAs) used for the management of *Sclerotium rolfsii* in different crops

| BCAs | Mode of action | Crop | References |
|---|---|---|---|
| ***Pseudomonas* spp.** | | | |
| *P. fluorescens* | Unknown | Groundnut | Ganesan and Gnanamanickam, 1987 |
| *P. chlororaphis* | Siderophore production | Maize | Sharma and Johri, 2003 |
| *P. fluorescens* | Unknown | Betelvine | Singh *et al*., 2003 |
| *P. fluorescens* | Unknown | Groundnut | Manjula *et al*., 2004 |
| *P. fluorescens* | Unknown | Potato, Maize, Groundnut | Bhatia *et al*., 2005 |
| *P. fluorescens* | Interference with CWDE & ISR | Groundnut | Kishore *et al*., 2005 |
| *P. syringae* | Unknown | Tomato | Sahni *et al*., 2008 |
| *P. fluorescens* | Unknown | Pepper | Abeysinghe, 2009 |
| *P. putida* | Unknown | Pepper | Abeysinghe, 2009 |
| *P. aeruginosa* | Interference with CWDE & ISR | Chili, Pepper | Siddiqui and Meon, 2009 |
| *Pseudomonas* sp. | Unknown | Tomato | De Curtis *et al*., 2010 |
| *Pseudomonas* sp. | Antibiosis | Chickpea | Hameeda *et al*., 2010 |
| *Pseudomonas* sp. | ISR | Tomato | Pastor *et al*., 2010 |
| *P. fluorescens* | ISR | Groundnut | Senthilraja *et al*., 2010 |
| *P. monteilii* | Antibiosis | Groundnut | Rakh *et al*., 2011 |

| | | | |
|---|---|---|---|
| *Pseudomonas* sp. | ISR | Groundnut | Tonelli *et al*., 2011 |
| *Pseudomonas* sp. | PHZ production | Groundnut | Le *et al*., 2012 |
| *Pseudomonas* sp. | Unknown | Tomato | Singh *et al*., 2013 |
| *P. fluorescens* P-60 | Unknown | Dragon Fruit | Hamarawati *et al*., 2018 |
| ***Bacillus* spp.** | | | |
| *B. subtilis* | Unknown | Groundnut | Abd-Alla, 2003 |
| *B. subtilis* | ISR | Groundnut | Abd-alla, 2005; Abd-Allah and El-Didamony, 2007 |
| *B. subtilis* | Antibiosis | Pepper | Abeysinghe, 2009 |
| *B. subtilis* | Unknown | Tomato | De Curtis *et al*., 2010 |
| *Bacillus* spp. | Antibiosis | Chickpea | Hameeda *et al*., 2010 |
| *Bacillus* sp. | ISR | Groundnut | Tonelli *et al*., 2011 |
| *B. amyloliquefaciens* | ISR | Groundnut | Rajyaguru *et al*., 2017 |
| **Other bacterial BCAs** | | | |
| *Streptomyces* sp. | Antibiosis | Sugar Beet | Errakhi *et al*., 2007 |
| *Burkholderia cepacia* | ISR | Chili Pepper | Siddiqui and Meon, 2009 |
| *B. cepacia* | Unknown | Tomato | De Curtis *et al*., 2010 |
| *S. aureofaciens* | Antibiosis | Chili Pepper | Boukaew *et al*., 2011 |
| *Agrobacterium* | Competition | Tomato | Pelzer *et al*., 2011 |
| *Streptomyces* sp. RP1A-12 | Antibiosis | Groundnut | Jacob *et al*., 20018 |

*Pseudomonas* strains can restrict *in vitro* hyphal growth or reduce germination of sclerotia of *S. rolfsii* (Ganesan and Gnanamanickam, 1987; Kishore *et al.*, 2005; Ganesan *et al.*, 2007; de Curtis *et al.*, 2010; Pastor *et al.*, 2010; Tonelli *et al.*, 2010). Although pseudomonads are well known for the production of a diverse array of antimicrobial compounds, including 2,4 diacetylphloroglucinol, pyrrolnitrin, pyoluteorin, rhizoxins, phenazines and lipopeptides (Raaijmakers *et al*., 2002; Haas and Defago, 2005; Raaijmakers *et al*., 2009; Daes *et al*., 2010; Raaijmakers *et al*., 2010); the role of these or other bioactive compounds in biocontrol of *S. rolfsii* has not been studied in detail. Also for most of the tested *Pseudomonas* strains, there is a lack of knowledge on the genes involved in their activity against *S. rolfsii*. Next to *Pseudomonas*, several *Bacillus* species and strains have been studied for their efficacy to control stem rot disease of groundnut. Pre treatment of groundnut seeds with *B. subtilis* protected groundnut seeds against *S. rolfsii* and significantly increased the number of pods (Abd-Allah 2005). Also for the *Bacillus* strains and other bacterial genera tested till date, little or no knowledge is available on the fundamental mechanisms involved in their activity against *S. rolfsii*. Moreover, most of these studies were conducted under controlled conditions and only few studies were performed under field conditions.

**2.5 Biological control using *Trichoderma* spp.**

*Trichoderma* spp. are free living fungi which are commonly present in soil and root ecosystems and provide a protection for plant against various plant pathogenic fungi like *Rhizoctonia* spp., *Botrytis cinerea*, *Pythium* spp. and *Fusarium* spp. (Howell, 2003; Rajput, 2014; Singh *et al*., 2016). The discovery of this *Trichoderma* genus was done by

Persoon (1794) over two hundred years ago in Germany and the potential role of for the biological suppression of plant pathogenic fungi was first reported by Weindling in 1934. *Trichoderma* spp. have been scrutinized as a biocontrol agent for over 75 years ago but they have become achieved more commercial attention and popularity in recent years; after that it has been extensively used as biocontrol agent (BCA) all over the world for last few decades and it is one of the most acceptable biocontrol agent used for effective management of different soil and seed-borne fungal diseases in several crop plants (Harman, 2004; Singh, 2014; Rai *et al*., 2016)

### 2.5.1 Identification of *Trichoderma* spp.

The proper and accurate identification of isolated *Trichoderma* spp. is most required and necessary before applying in field as biocontrol agent. Samules *et al*. (2002) reported that *Trichoderma* spp. can be responsible for the outbreak of green mold disease in commercially grown *Agaricus bisporus.* The fungus *Trichoderma longibrachiatum* observed as an opportunistic fungal pathogen to immune-compromised patients (Keswani *et al*., 2014). The taxonomy and species identification were vague until around 1969, and explicit after the report of Rifai (1969). As it is seems difficult and cumbersome to identify microbes solely on the basis of morphology, hence advanced tools like genealogical concordance phylogenetic species recognition (GCPSR) and DNA-barcode system are used which gives the proper identification of *Trichoderma* spp. based on sequence analysis (Druzhinina *et al*., 2006**;** Verma *et al*., 2007).

### 2.5.2 Action mechanisms

The antagonistic behaviour and broad-spectrum activities exhibited by *Trichoderma* fungi against many soilborne phytopathogens are well known. *Trichoderma* spp. are able to manage plant pathogenic fungi through the mycoparasitism, rhizosphere competence, nutrient competition and enzyme, induced defence responses (Biswas and Sen, 2000; Howell, 2003; Singh *et al*., 2014; Ram and Singh, 2017) [Table 2.3].Out of which some are more extensively studied and reported, one is mycoparasitism which involves the release of various enzymes like chitinases, glucanases, cellulases etc. that degrades the cell wall of fungal pathogens (Kubicek and Harman, 1998; Howell, 2003; Vinal, 2008). Second is antibiosis which is release of secondary metabolites antimicrobial in function and further competition for nutrients (Vinal 2008; Shi *et al*., 2012; Gajera *et al*., 2013; Pacheco *et al*., 2016). A graphical representation of action mechanism of *Trichoderma* spp. against phytopathogenic fungi has been shown (Figure 2.6). It has been also found that it increases induce systemic resistance (ISR) in plants and promote plant growth (Samuels, 2006). It has been proven in plentiful study reports that *Trichoderma* spp. are more prominent biocontrol agents for managing plant disease and currently various commercial products of *Trichoderma* in solid and liquid formulation are available in the market as biofungicide or as enhancers for plant growth (Papavizas, 1985; Harman, 2004).

#### 2.5.2.1 Mycoparasitism

This is a kind of hyphal interaction with parasitism which involves complex consecutive processes, where *Trichoderma* spp. shows extending growth towards other

pathogenic fungi (Chet *et al*., 1981), while the expanding *Trichoderma* keep secreting extracellular exo-chitinase in minute quantities which diffuses and hydrolyzes the cell wall of fungal pathogen. The pathogen hence releases some oligomers as a result of cell disruption which in turn conceived by *Trichoderma* originates huge production of endo-chitinases which are toxic to the pathogen (Brunner *et al*., 2003).

All this intensify the action of diffusion by *Trichoderma* against the pathogenic fungi (Brunner *et al*., 2003). After the binding of *Trichoderma* with the host it further releases various cell wall degrading enzymes and peptaibol antibiotics (Harman *et al*., 2004). These synergistic actions cause the degradation of host fungus by creating many ruptures through which fungal hyphae of *Trichoderma* penetrates, and ultimately leads to the cytoplasmic leakage of host cell bodies (Desai and Schlosser, 1999; Harman *et al*., 2004).

**2.5.2.2 Antibiosis**

*Trichoderma* generates a large number of metabolites with anti-microbial properties and these diverse type of metabolites have biocontrol potential against a wide range of phytopathogens. The hyphal penetration was not seen in co-culturing of *Trichoderma* spp. and *F. moniliforme*/*Aspergillus flavus,* showing that the inhibitory effect on pathogen was not solely due to mycoparasitism (Calistru *et al*., 1997). Also the inhibition by *T. harzianum* (T-12) and *T. koningii* (T-8) on the peas of *Pythium* species was not mainly due to mycoparasitism or competition but due to the production of toxic factors in spermosphere (Lifshitz *et al*., 1986). These observations concluded that the metabolites produced by *Trichoderma* spp. might play a major role in antibiosis. Also it

was found that the refined secondary metabolites of *Trichoderma* spp. in field conditions perform more rapidly in constraining bacterial infections than the application of whole organism (Fravel, 1988).

**2.5.2.3 Competition**

As mycoparasitism and antibiosis, another factor, competition is necessary for regulating the existence of pathogen. It is a key mechanism in which the biocontrol microbial strain competes for food and nutrients against the pathogenic microbial strain, this in some way or the other effects the pathogen's viability (Waghunde *et al*., 2016). Carbon and iron are the two crucial elements needed for the persistence and viability of many filamentous fungi. Competition for carbon can be seen not only in *Trichoderma* but also many other fungi *F. oxysporum* (Alabouvette *et al*., 2009). At the iron starving conditions, the fungi start producing minute ferric-iron specific chelators for mobilization of iron from the surrounding environment. As reported in the case of *T. harzianum* T35 which competes against *F. oxysporum* for both nutrients and rhizosphere colonization (Tjamos *et al*., 1922). Chet and Inbar (1994) reported that the siderophore produced by the *Trichoderma* isolates can also inhibit the growth of other fungi via producing effective chelators for iron. Hence, *Trichoderma* spp. exceeds against phytopathogens for accessible iron in soil and effectively inhibits its growth.

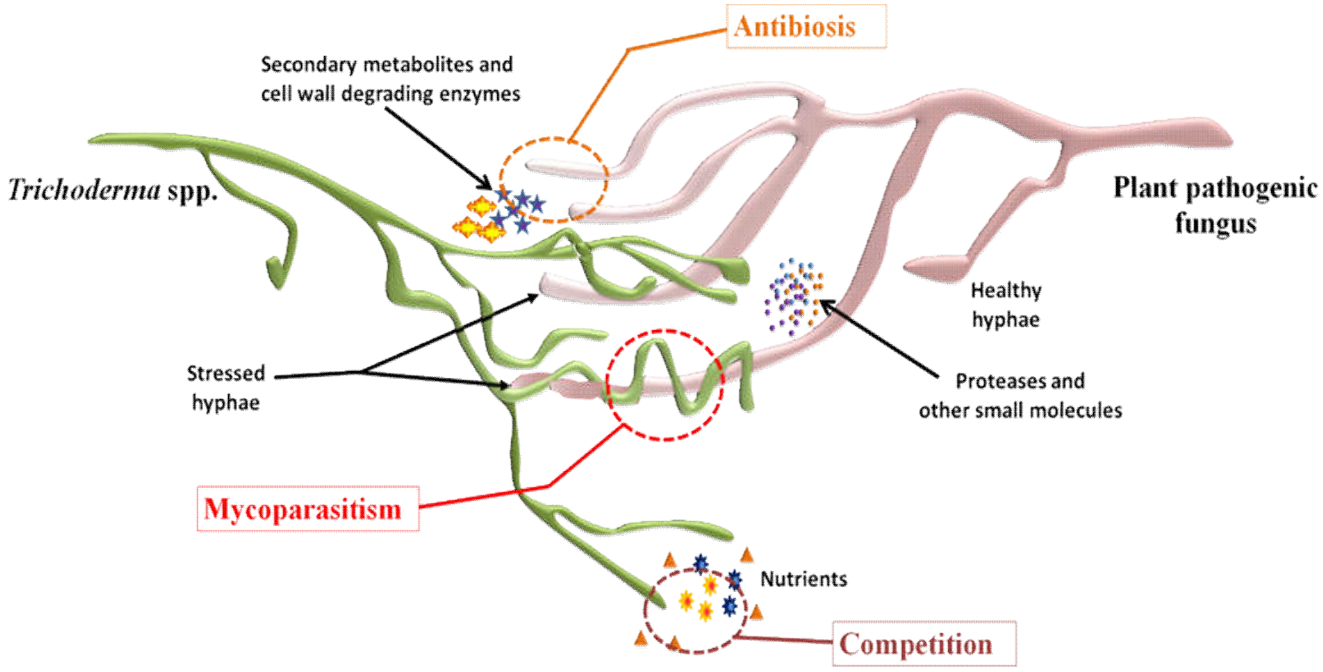



**Figure 2.6** Graphical representations of mechanism of action *Trichoderma* spp. action against different phytopathogenic fungi

**Table 2.3** Mode of action of *Trichoderma* spp. action used against *Sclerotium rolfsii*

| BCAs | Mode of action | References |
|---|---|---|
| *T. lignorum* | Mycoparasitism | Weindling, 1934 |
| *T. harzianum* | Mycoparasitism | Wells *et al*., 1972 |
| *T. harzianum* | Mycoparasitism | Backman and Rodriguez-Kabana, 1975 |
| *T. viride* | Proteolytic activity | Rodriguez-Kabana *et al*., 1978 |
| *Trichoderma* sp. | Mycoparasitism | Bell *et al*., 1982 |
| *T. harzianum* | Volatile and nonvolatile metabolites | Upadhyay and Mukhopadhyay, 1983 |
| *T. harzianum* | Mycoparasitism | Elad *et al*., 1980; Elad *et al*., 1984 |
| *T. harzianum* | Mycoparasitism | Henis *et al*., 1984 |
| *T. harzianum* | Mycoparasitism | Upadhyay *et al*., 1986 |
| *T. harzianum* | Mycoparasitism and antibiosis | Mukherjee *et al*., 1986 |

| | | |
|---|---|---|
| *T. harzianum* | Antibiosis | Debao, 1993 |
| *T. harzianum* | Mycoparasitism | Mukherjee *et al*., 1995 |
| *T. harzianum* | Mycoparasitism | Benhamou and Chet, 1996 |
| *T. harzianum* | Chitinase enzyme production | Lima *et al*., 1997 |
| *T. harzianum* | Mycoparasitism | Desai and Schlosser, 1999 |
| *T. viride* | Antibiosis | Viterbo *et al*., 2002 |
| *T. viride* | Mycoparasitism and antibiosis | Küçük and Kivanc, 2004 |
| *T. harzianum* | Mycoparasitism and ISR | Prasad and Naik, 2008 |
| *T. harzianum* | Chitinase and an endo-β-1, 3-glucanase enzyme production | El-Katatny *et al*., 2001 |
| *Trichoderma* sp. | Volatile metabolites | Amin *et al*., 2010 |
| *Trichoderma* sp. | Mycoparasitism | Joshi *et al*., 2010 |
| *Trichoderma* sp. | Mycoparasitism | Kotasthane *et al*., 2015 |

*Trichoderma* has also the capability to acquire ATP from the various types of sugars, such as those mostly available within the environment of fungus: glucan, chitin and cellulose, and others, all of them can be turned into glucose (Chet *et al*., 1997). The *Ceratocystis paradoxa* causing pineapple disease in sugarcane fields can also be controlled by the filtrates of *Trichoderma* strains containing antifungal properties (Rahman *et al*., 2009). *Trichoderma* produces various proteins which play an important role in root colonization competition with various other root colonizers (Saloheimo *et al*., 2002; Viterbo *et al*., 2004; Brotman *et al*., 2008) and some help to maintain a symbiotic relationship with other host plants (Smolskr *et al*., 2012).

**2.6 *Trichoderma* as bioinoculant and biostimulant**

Bioinoculants have a crucial involvement in maintaining soil environment abundant in micro-nutrients and macro-nutrients through phosphate and potassium

solubilisation, nitrogen fixation, the release of different plant growth regulating substances, exhibition of antibiotics and biodegradation of surplus organic matter present in soil (Bhardwaj *et al*., 2014). *Trichoderma* strains simply colonize around plant roots that intensify root growth and development, consumption and nutrient usage, crop productivity, and protection against abiotic stresses (Benítez *et al*., 2004). It was found that the use of *Trichoderma* spores on seeds amplify crop yield (Chet *et al*., 1997; Bisen *et al*., 2015) whereas same amplification of crop was obtained when seeds and *Trichoderma* both were separated by cellophane membrane. Reports suggested that biopesticides are capable of enhancing soil micronutrients such as Zn, Fe, Cu, Mn etc. and protect crop from various phytopathogens (Kaya *et al*., 2009; Jain *et al*., 2014; Singh *et al*., 2016). This stipulates that *Trichoderma* makes some growth factors as a consequence of which enhanced rate of seed germination and yield can be seen (Haque *et al*., 2012; Khan *et al*., 2017; Almammory and Matloob, 2019). Production of cytokinin-like compounds (zeatin) and gibberellins GA3 or GA3- related compounds by *Trichoderma* spp. are the agents responsible for the biofertilizer activity by the *Trichoderma* (Osiewacz, 2002). *Trichoderma* strains also produce gluconic, citric or fumaric acid by the metabolism of different carbon sources that causes acidification in their vicinity (Benítez *et al*., 2004). In turn, these organic acids solubilize micronutrients, phosphates, and mineral cations including iron, magnesium and manganese (Harman *et al*., 2004). Therefore, the usage of *Trichoderma* as biofertilizer in the soil can result in solubilisation of the cations and production of phytohormones that ultimately enhances the crop yield (Swain *et al*., 2018; Wang *et al*., 2018).

## 2.7 Different formulations *Trichoderma* spp.

Generally the bioformulations are classified according to their physical state i.e., dry or liquid formulations. Under the dry formation wettable powder (WP), powders for seed dressing (DS), dusts (DP), microgranules (MG), granules (GR) and water dispersible granules (WG) comes while liquid state comprises oil dispersions (OD), suspension concentrates (SC), capsule suspensions (CS), suspo-emulsions (SE), emulsions and ultra low volume formulations (Singh *et al*., 2014; Singh *et al*., 2016.).

A total of 611 fungal based biopesticide are available in the Indian biopesticide market and in that 354 *Trichoderma* spp. based product now currently available for managing different soil and seedborne diseases. A review of the status of biopesticides showed that India has the capacity to produce 1850 MT of *Trichoderma* formulations per annum while the requirement is 22038 MT with a market value of Rs 260 crores (Sriram *et al.*, 2013) which indicated that there are requirement of more big *Trichoderma* formulations producing industrial giants. There are 86 firms who have received *T. viride* mother culture from Tamil Nadu Agricultural University (TNAU), Coimbatore which registered with CIB (Central Insecticide Board), India for commercial production. In addition, there are 32 integrated pest management (IPM) centres and 35 commercial companies established for biopesticide production under the necessary guidelines and assistance of the Ministry of Agriculture and Farmers Welfare. To accelerate the production of potentially screened selected biocontrol agents, the State Departments of Agriculture and Horticulture of Tamil Nadu, Gujarat, Andhra Pradesh, Uttar Pradesh, Kerala, Madhya Pradesh, Chhattisgarh and Karnataka have installed numerous advance

biocontrol laboratories. Indian Council of Agricultural Research (ICAR) institutions along with States Agricultural Universities (SAUs) are take initiatives for the research and production for the microbial biopesticides (Kumar *et al*., 2016). The number of biocontrol research laboratories and production units is continuously increasing day by day (Chaturvedi, 2005; Wahab, 2009; Bharti *et al*., 2017).

**2.8 Need for new bioformulation with prolonged shelf life**

The shelf life of the biopesticides is a principal point that determines the success in commercialization of its products. Short lived shelf life of *Trichoderma* spp. in any formulation can create a vital issue for development of commercial formulations. Further research has to be done in this field to enhance the shelf life and existence of *Trichoderma* spp. in any formulation. So far several types of formulation have been developed and different authors have explained different criteria for the stability of their formulation. According to Cumagun and Ilag (1997) formulations incorporating dried conidial pellets of *T. harzianum* are much more effective in reducing the sclerotia germination of *Rhizoctonia solani* as compared to the liquid formulations. Usually formulations containing *Trichoderma* spp. which are based on liquid fermentation are more susceptible to desiccation than the solid state fermentation based formulations.

Th-10 isolate from *T. harzianum* was successful in controlling *Fusarium* wilt of banana. It was found that the dried banana leaf was the best carrier material for growth with high density of propagules of *T. harzianum,* but with addition of jaggery to dried banana leaves increased its multiplication which increased to a shelf-life of more than six months as the stored substrate (Thangavelu *et al*., 2003). Survivability of *Trichoderma*

spp. conidia can increase in alginate pellets formulation when further supplemented with 10% cellulose (Shaban and El-Komy, 2001). Kolombet *et al*. (2008) studied the effect of different modifications in formulating the *T. asperellum,* which includes addition of starch as a food base, reduction of pH and addition of minute amounts of copper to deduct the metabolic activity. This gave the shelf life of six month from the developed formulation. Formulation of bentonite-vermiculite although maintained the colony-forming unit of *T. harzianum* for 8 weeks but also enhanced the shoot weight of melon plants and gave resistance against *Fusarium* wilt disease (Medina *et al*., 2009).

The addition of chitin in production media and also talc formulation of *T. harzianum,* can increase the shelf-life of formulation further by two months (Sriram *et al*., 2010). Al-Taweil *et al*. (2010) suggested that shelf-life of *Trichoderma* can be increased by using alginate and paraffin oil formulation. Addition of glycerol in culturing media as osmoprotectant can also increase the shelf-life of *T. harzianum* formulation. In an experiment it was found that the shelf life of formulation was increased to 7-12 months by addition of 3%-6% glycerol in the culture media, in comparison to formulation without glycerol having shelf-life of about 4-5 months (Sriram *et al*., 2011).

Further, immobilization of microorganism is an effective method to improve and enhance shelf life with field efficacy. Microencapsulation, an important immobilization technology increases shelf life of microorganism in comparison to other type of formulations with control delivery of microbes and also enhances its field application (John *et al*., 2011). *Trichoderma* conidia spray dried and microencapsulated with various sugars such as molasses, sucrose or glycerol, can extensively increase the survival

percentage of conidia even after drying (Jin and Custin, 2011). *T. harzianum* SQRT037 conidia formulated with organic fertilizers exhibited increase in controlling *Fusarium* wilt of cucumber as compared to the treatment including formulation made of only conidial suspension (Yant *et al*., 2011). Muñoz-Celaya *et al*. (2012) also reported that microencapsulation of *T. harzianum* conidia in 1:1 ratio blend of malto dextrin–gum Arabic polymeric matrix can give 11 fold higher conidia survival in comparison with non-encapsulated conidia after spray-drying.

The increase in atmospheric temperature is imposing a serious threat to agricultural production all over the globe (Ge *et al*., 2019; Wiebe *et al.*, 2019). Soil microbial population dynamics also gets interfered by high-temperature stress due to changes in soil microclimate and rapid loss of soil moisture. A large number of *Trichoderma* spp. based commercial products are available in the global biopesticide market (Singh *et al.*, 2014; Woo *et al.*, 2014; Keswani *et al.*, 2016) but none of them contain heat stress tolerant strain to counter the negative effect of increasing atmospheric temperature. During summer (April-June), the elevated atmospheric temperature hampers the efficacy of *Trichoderma* during their application on field. High temperatures stress can disturb the membrane of fungal cells (Crisan, 1973) and may perhaps cause damage *via* protein denaturation (Setlow and Setlow, 1998) and DNA damage through base loss leading to depurination (Nicholson *et al.*, 2000). Thus, a fungal candidate with greater tolerance to the upper thermal limits of summer is of greater potential for commercial development, as long as it has acceptable virulence to phytopathogens.

Therefore, there is an urgent need to explore thermotolerant *Trichoderma* strains and develop their formulation that can be used even at elevated temperature conditions in the field. Application of such thermotolerant *Trichoderma* based formulation will definitely aid in effectively management of both the biotic and abiotic stresses and minimize the crop losses.

# CHAPTER III

# MATERIALS AND METHODS

*Chapter-III*

# MATERIALS AND METHODS

The present investigation entitled **"Development of potential *Trichoderma* based formulation for management of collar rot disease of tomato caused by *Sclerotium rolfsii*"** was undertaken during the year 2016 to 2018. The details of the materials and methods used during the course of investigation have been described as under.

## 3.1 Experimental site

The *in vitro* experiment was conducted in the Plant Health Clinic and Biocontrol Laboratory of Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India. The *in vivo* experiment was carried out in the polyhouse and field of the same department, where tomato crop was raised in earthen pots (18 x 10 cm) and all physical precautions were kept in view in order to protect the crop from the external damage. The site of the experiment was unaltered during experimentation period. The university is located in the south- eastern part of Varanasi city which is at 25°18' N latitude, 83°3' E longitude and at an altitude of 75.7 m above mean sea level.

## 3.2 Collection of diseased samples from different districts of Eastern Uttar Pradesh

### 3.2.1 Survey for incidence of collar rot from different zones of Eastern Uttar Pradesh and collection of diseased samples

Random roving method of survey was carried out to record the severity of collar rot in tomato. The survey was conducted during *Rabi* season 2016 - 17 in five

districts of Eastern Uttar Pradesh i.e. Azamgarh, Jaunpur, Mau, Mirzapur and Varanasi, (Figure 3.1). The disease severity was estimated on the basis of individual plants scoring on a 0-5 visual scale (Table 3.1) of increasing severity (Latunde-Dada, 1993) while the mean disease rating (MDR) was calculated according to Pal *et al*. (2001).

$$MDR = \frac{a \times 0 + b \times 1 + c \times 2 + d \times 3 + e \times 4 + f \times 5}{a + b + c + d + e + f}$$

Where; a, b, c, d, e and f are the number of plants with the disease rating of 0, 1, 2, 3, 4 and 5 respectively.

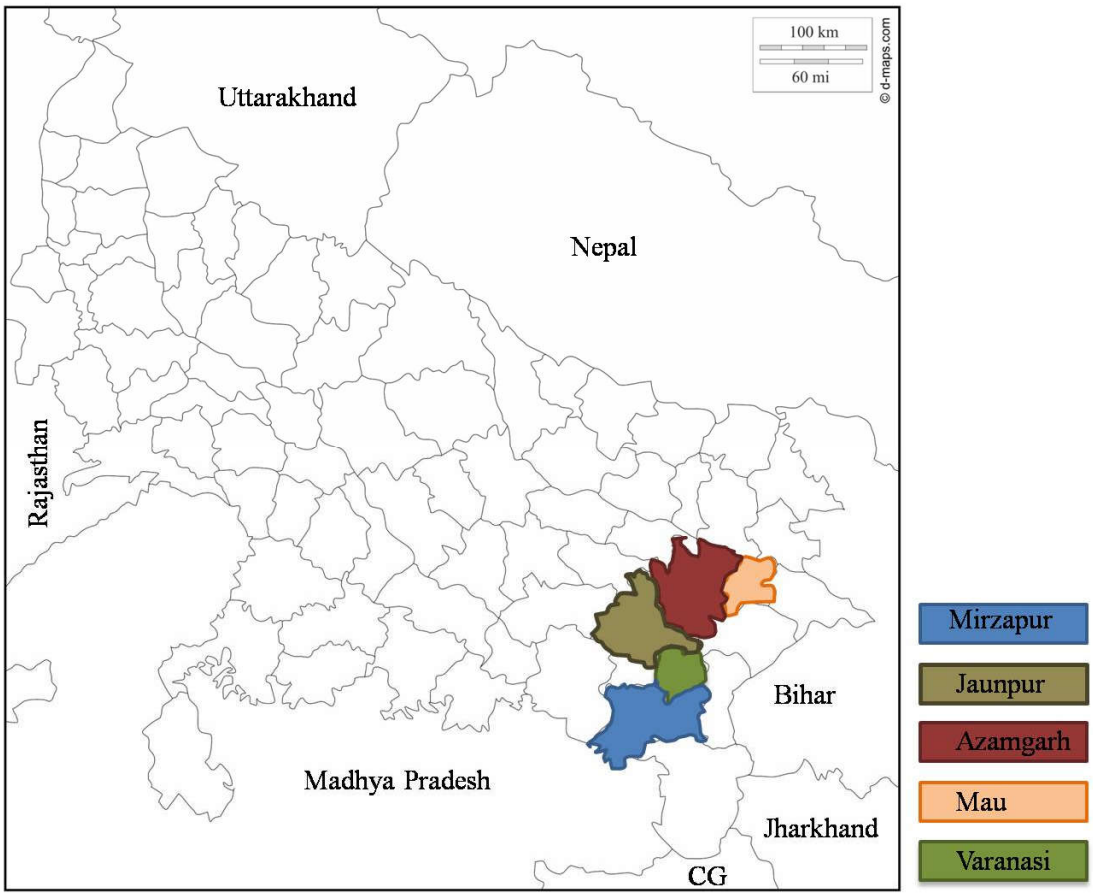



**Figure 3.1** Survey of different districts of Eastern Uttar Pradesh for collection of *Sclerotium rolfsii* isolates.

Tomato plants showing typical collar rot symptoms were collected separately in paper bags and brought to the laboratory for isolation of associated pathogen and further investigations. The diseased samples were collected from areas having maximum soil moisture content in the locality as the pathogen aggravates in high soil moisture conditions.

**Table 3.1** Rating scale used to record disease severity of collar rot in Tomato (Latunde-Dada, 1993)

| Rating | Observation |
|---|---|
| **0** | No symptoms |
| **1** | Wilting symptoms in few leaves |
| **2** | Slight infection; mycelium mat on soil surface only |
| **3** | Moderate infection; wilting and blight symptoms, presence of mycelium mat on stem base region |
| **4** | Severe infection; advance wilting and presence of abundant sclerotia at stem base region |
| **5** | Plant died |

### 3.2.2 Isolation of the pathogen

The pathogen *Sclerotium rolfsii* was isolated on PDA medium from infected root of tomato plant. A small portion of diseased tissue along with a portion of adjacent healthy tissue were cut into small pieces (3 to 5 mm in length) and then surface sterilized with 0.1% $HgCl_2$ for 30 seconds. The pieces were then rinsed thrice with sterilized distilled water. Sterilized and rinsed pieces were inoculated aseptically on sterilized Petriplates containing Potato dextrose agar (PDA) medium. The inoculated Petriplates were incubated at 20- 25 $^0$C for five to six days. When the fungal colony developed, a small cut was made on mycelium with cork borer and was transferred on another petriplate containing PDA medium to obtain pure culture. The mycelial bit was also transferred to fresh PDA slants in order to store it for future use.

### 3.2.3 Maintenance and storing of the pathogen

The pure culture of the pathogen *S. rolfsii* was maintained on PDA slants throughout the period of investigation by periodic sub culturing on fresh media and stored in a refrigerator at 4 $^0$C.

### 3.2.4 Pathogenicity test

The tomato cultivar **"Kashi Amrit"** was used for testing the pathogenicity of the pathogen. Tomato seeds were surface sterilized by using 1% sodium hypochlorite for 1 min and was rinsed twice with sterilized distilled water and then air-dried. Soil mixture containing sandy loam soil, vermicompost, and farmyard manure (2:1:1) was autoclaved for 30 min at 15 lbs pressure for three consecutive days. Half amount of soil was also mixed with crushed mycelial powder of *S. rolfsii*. The seeds were sown in bottom hole transparent plastic cups (10×5 cm$^2$) under greenhouse conditions. Untreated seeds sown in pathogen infected and pathogen uninfected soil served as positive and negative controls, respectively. The studies on pathogenicity test was done 25 DAG (days after seed germination) as the symptoms are not produced at the initial stages of crop growth.

## 3.3 Collection of samples from different agro-ecosystems for isolation of thermotolerant *Trichoderma* spp.

Samples were collected from the different locations of agriculture fields where mean temperature of April to June, 2016 was recorded high (Figure 3.2). In addition different composting sites were also targeted to explore thermotolerant *Trichoderma* spp., as these sites always maintained a high temperature in depth of the pit. The soil samples were collected from the rhizospheric regions of various crops and composting samples were collected from a depth of 7-10cm from surface.

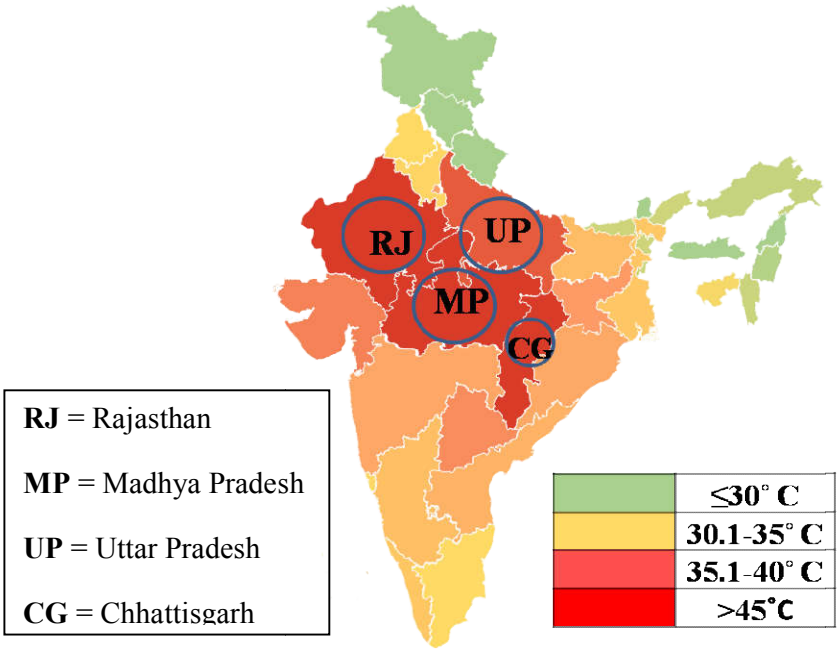



**Figure 3.2** Survey of different high temperature sates of India for collection of high temperature tolerant *Trichoderma* spp. isolates. (https://data.gov.in/catalog/all-india-seasonal-and-annual-minmax-temperature-series)

Both soil and composting site samples were collected randomly and kept in plastic bags for further use. The locations for the collection of soil samples are very common agricultural fields. It was also confirmed that these fields did not involve any endangered or protected species. We used sampling procedures that did not harm the plant diversity of the locations.

### 3.4 Isolation of *Trichoderma* spp.

*Trichoderma* spp. in the collected soil and composting samples were isolated by using serial dilution method on *Trichoderma* selective media (TSM) (Elad and Chet, 1983). One gram of sample was suspended in 10 ml of sterilized distilled water (SDW). Thereafter, samples were serially diluted as $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$. For each soil sample 100µl of soil suspension from $10^{-4}$ and $10^{-5}$ was inoculated on TSM plate. Plates were incubated at 28±2℃ for 4 to 5 days (Figure 3.3). *Trichoderma* spp. were identified based on their colony colour, size, shape and appearance on TSM

plate. Then it was sub-cultured on potato dextrose agar (PDA) medium for further identification.

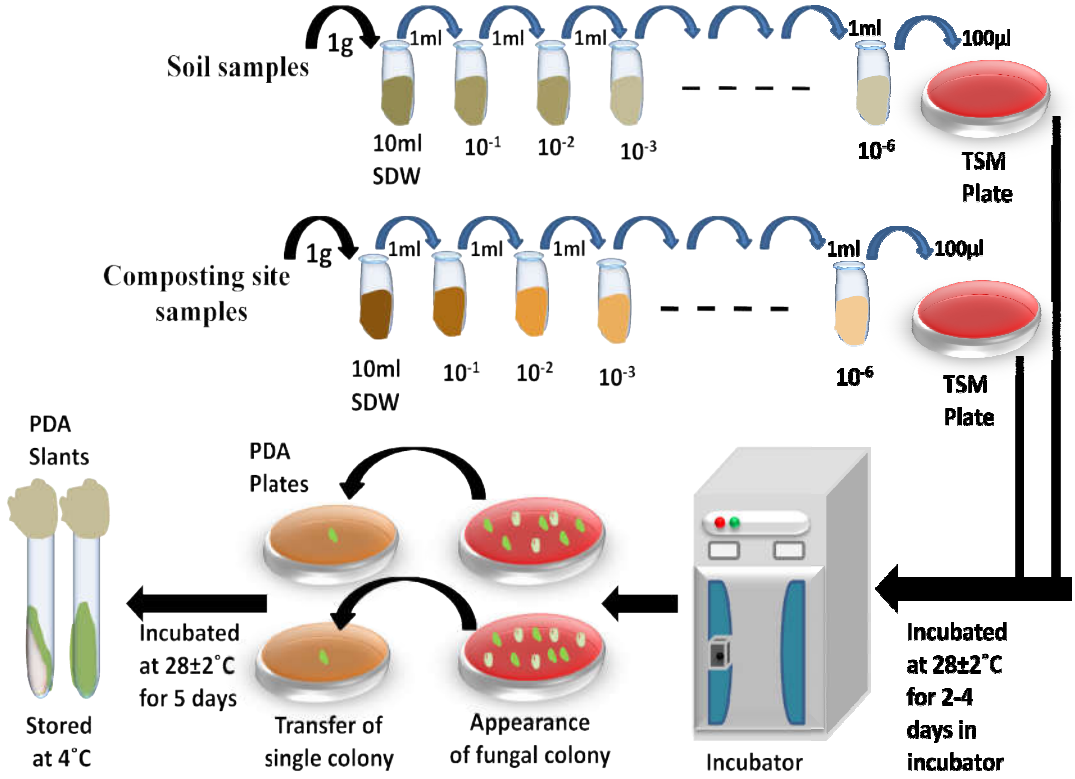



**Figure 3.3** Isolation of *Trichoderma* spp. from soil samples collected from various sites through serial dilution method on TSM plate.

### 3.4 Screening of potential *Trichoderma* isolates

#### 3.4.1 Assessment of conidial thermo tolerance and survivability of *Trichoderma* isolates

Each *Trichoderma* isolate was grown separately on Potato dextrose agar (PDA) for one week at 27 ± 2℃. Thereafter, 5 mm diameter piece was taken from each fully sporulated isolate, and spores were suspended in 1 ml of 0.02% Tween-80. The complete suspension was placed upright in water-bath at 47℃. During the wet stress, 100µl samples were taken out at 60 min intervals and serially diluted upto $10^{-4}$ dilution. The diluted samples was inoculated on rose bengal added PDA plates. Plates

were placed in incubator at 28±2˚C and colonies were counted after sporulation (Li and Feng, 2009) (Figure 3.4).

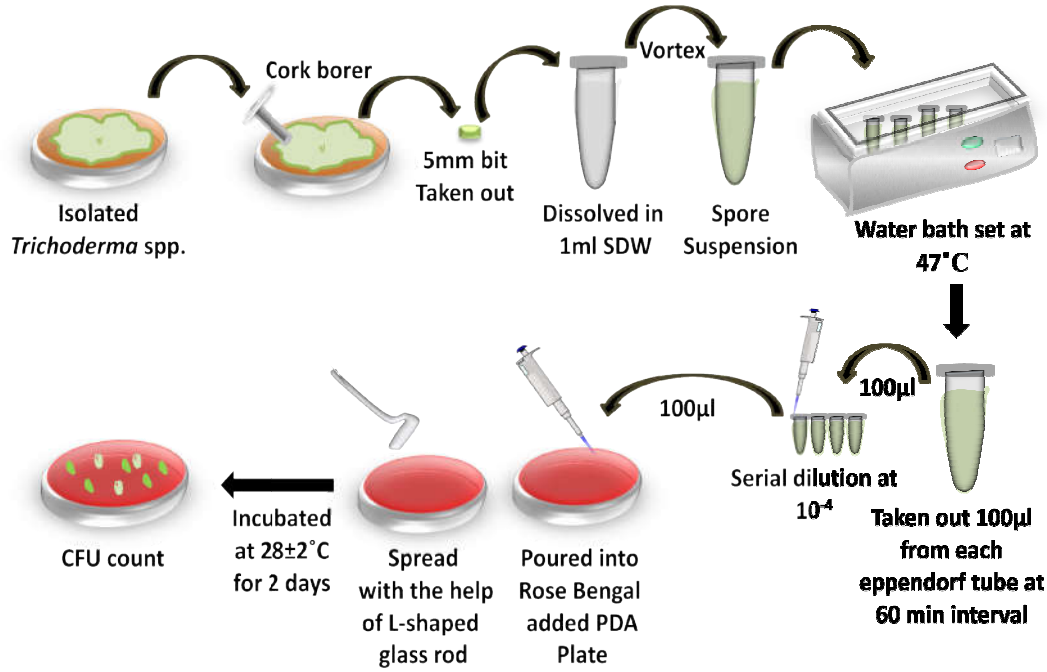



**Figure 3.4** Procedure for assessment of conidial thermo tolerance and survivability of *Trichoderma* isolates

### 3.4.2 Assessment of mycelial growth of the selected *Trichoderma* isolates under different temperature regime

On the basis of spore survivability at 47°C, *Trichoderma* isolates that retained the maximum number of colonies after wet temperature stress were further selected to examine their mycelial growth at different temperature. The selected *Trichoderma* isolates were subjected to 20, 25, 30, 35, 40 and 45˚C by inoculating 5mm of the mycelial disc of each isolate separately on PDA plates. Three replicates for each isolate were maintained and colony growth at each temperature was assessed regularly by measuring the diameters of the colonies (Li and Feng, 2009; Poosapati *et al*., 2014). The average linear growth rate (ALG) was calculated by using the formula:

$$\text{ALG (mm/day)} = \frac{(C5 - C1)}{4}$$

Where; ALG = Average linear mycelia growth; C5 = colony diameter in mm after 5 days of inoculation; C1 = colony diameter in mm after 1 day of inoculation

**3.4.3 *In vitro* antagonism of selected *Trichoderma* isolates against *Sclerotium rolfsii***

The selected thermotolerant *Trichoderma* isolates were further screened for their antagonistic activity against *S. rolfsii* by dual culture assay. Five mm disc of *Trichoderma* isolate and *S. rolfsii* were placed opposite to each other at a distance of 2 cm from the edge of plate. The inoculated plates were kept at 28 ± 2℃ and the radial growth of pathogen after six days of incubation was observed for antagonistic effect. The percent inhibition of pathogen was calculated by using the following formula:

$$I = \frac{(C-T) \times 100}{C}$$

Where;

I = percent (%) inhibition in mycelia growth; C = growth of pathogen in control plates; T = growth of the pathogen in dual culture plates.

**3.5 *In vitro* assessment of selected *Trichoderma* strains for plant growth promoting traits (PGP) and hydrolytic enzyme production**

**3.5.1. Indole acetic acid (IAA) production:** IAA was quantitatively analysed using the method of Loper and Scroth (1986) using different concentrations of tryptophan (0, 150, and 300 mg $ml^{-1}$). 5 mm mycelium disc of selected *Trichoderma* isolates were taken from seven days old culture plates and inoculated into 10 ml of tryptophan broth media which stored into 30 ml glass vials. After inoculation, glass vials were incubated at room temperature (30 ± 2˚C), 40˚C and 45˚C for 4 to 5 days. Thereafter,

cultures were centrifuged at 15000 rpm for 10 min and the supernatant (2 ml) thus obtained was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink colour indicated IAA production. Absorbance of the pink colour developed was recorded at 530 nm and IAA produced was quantified using standard curve of IAA.

**3.5.2. Phosphate solubilization:** Selected isolates were screened for phosphate solubilization on NBRI-BPB broth as described by Mehta and Nautiyal (2001). Solubilization of tricalcium phosphate was quantified in liquid medium as described by King (1932). Five mm disc of selected *Trichoderma* isolates were inoculated in 25 ml of NBRI-BPB broth and were incubated at room temperature (30 ± 2˚C), 40˚C and 45˚C for 4 to 5 days. Cultures were centrifuged at 15,000 rpm for 10 min. 1 ml of supernatant was mixed with chloromolibidic acid (10 ml) and cholorostannous acid (0.25 ml) and the volume was made up to 50 ml with distilled water. Absorbance of the blue colour developed was recorded at 600 nm. The amount of soluble phosphorus was quantified from the standard curve of $KH_2PO_4$.

**3.5.3. Siderophore assay:** Selected isolates were screened for siderophore production assay according to Schwyn and Neilands (1987). 5 mm bit was taken out from seven days culture plates and placed on Chromo azurol Sulphonate (CAS) medium agar plates. Thereafter, plates were incubated for 96 hr at 28 ± 2˚C. The discolouration of medium around culture showed the positive result.

**3.5.4. Amylolytic activity:** Amylase activity (Hankin and Anagnostakis, 1975) was assessed by growing the *Trichoderma* strains on starch agar medium (Starch 20.00g,

Beef extract 3.00g, Peptone 5.00g, Agar 16.00g, Distilled water 1000 ml). The medium was aseptically transferred to petri dishes and inoculated with a 5 mm agar disc takeout from 5 day old culture plates of each strain separately and incubated at room temperature (30 ± 2˚C), 40˚C and 45˚C for 4 to 5 days in darkness. The plates were flooded with solution of 1% iodine in 2% potassium iodide. The clear zone formed surrounding the colony was considered positive for amylase activity

**3.5.5. Proteolytic activity:** For protease test (Vijayaraghavan and Samuel, 2013), the selected *Trichoderma* strains were grown on casein agar medium (Peptic digest of animal tissue 5.00g, Beef extract 1.50g, Yeast extract 1.50g, Sodium chloride 5.00g, Agar 15.00g, Casein 10.00g, and Distilled water 1000ml).The medium was aseptically transferred to petriplates and inoculated with a 5 mm agar disc takeout from 5 day old culture plates of each strain separately and incubated at 26± 2°C in darkness for 3 to 5 days. The plates were flooded with Bromo Cresol Green dye. The clear distinct zone indicates proteolytic activity. A distinct zone surrounded by greenish-blue colour is pH dependent (8.0 ± 0.2). The proteolytic activity appears as a colourless zone, while the rest of the plates as greenish-blue in colour.

**3.5.6. Chitinase activity:** The Chitinase Detection Medium (Agrawal and Kotasthane, 2012) consisted of a basal medium comprising (4.5 g of Colloidal chitin, 0.30g of $MgSO_4.7H_2O$, 3.00g of $NH_4SO_4$, 2.00g of $KH_2PO_4$, 1.00 g of Citric acid monohydrate, 15.00g of Agar, 0.15g of Bromo cresol purple and 0.20 ml of Tween-80) per liter, pH was adjusted to 4.7 and then autoclaved at 121˚C for 15 min. Colloidal chitin was prepared from commercial chitin (HiMedia) and was amended in the chitinase assay medium as a sole carbon source. Colloidal chitin (Murthy and Bleakley, 2012) was prepared and stored at 4˚C until further use. After cooling, the

medium was poured in to petri plates and allowed to solidify. The actively growing *Trichoderma* culture plugs of the isolates to be tested for chitinase activity was inoculated into the medium and incubated at 26 ± 2˚C for 3-5 days and observed for the colored zone formation. Chitinase activity was identified by the formation purple colored zone. Bromo cresol green reagent sharply increases the colour intensity of the plate, as it binds on unhydrolyzed protein in the plate. Colour intensity and diameter of the purple coloured zone were taken as the criteria to determine the chitinase activity.

**3.6 Molecular identification of potential *Trichoderma* strains**

*Trichoderma* strain BHU R2 showed better performance in all aspects (heat tolerance, biocontrol potential, plant growth promoting and enzymatic activity), was selected for molecular identification. Identification of *Trichoderma* strain was carried out by sequence analysis of internal transcribed spacer (ITS)-1 region of rRNA. Amplification of this region was done by using universal primers, ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-R (5'-TCCTCCGCTTATTGATAT-3'). The PCR reaction was performed in a thermocycler (Techne, UK) with following conditions; initial denaturation at 94˚C for 1 min followed by 35 cycles of 4 min, annealing at 56˚C for 1 min, elongation period at 72˚C for 50 s and a final extension step of 7 min at 72˚C. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis with ethidiumbromide (0.5ug/ml) and subjected to sequencing. The sequence was subjected to BLAST in NCBI and was also analyzed by using Trichokey and Trichoblast tools to obtain the identification of isolate. After confirmation, the sequence was submitted to NCBI Genbank for generating accession number. The obtained nucleotide sequences were also used for phylogenetic and molecular evolutionary analysis, conducted by using MEGA version 6 (Tamura *et al*.,

2007). Phylogenetic tree construction was done by neighbor- joining method (Kimura, 1980; Tamura and Nei, 1993).

### 3.7 Development of commercially viable and cost effective formulation using potential strain of *Trichoderma*

#### 3.7.1. Preparation of mass inoculum of *Trichoderma* BHU R2

For preparation of mass inoculum of *Trichoderma* strain BHU R2, plastic trays were surface sterilized with 100% ethanol and air dried under aseptic condition. Thereafter, 250 ml of sterilized potato dextrose broth (PDB) poured into in tray and then inoculated with 5mm disc (3-4 in numbers) of selected *Trichoderma* BHU R2 strain which obtained from seven day old culture plate. Inoculated trays were incubated in B.O.D. at 28±2˚C for six days. Full grown *Trichoderma* BHU R2 trays were removed outside and mat of *Trichoderma* was aseptically placed on sterilized blotting paper for removal of excess moisture. Thereafter, whole blotting paper was placed under laminar air flow for drying and after drying the mat was removed carefully for encapsulation process (Figure 3.5).

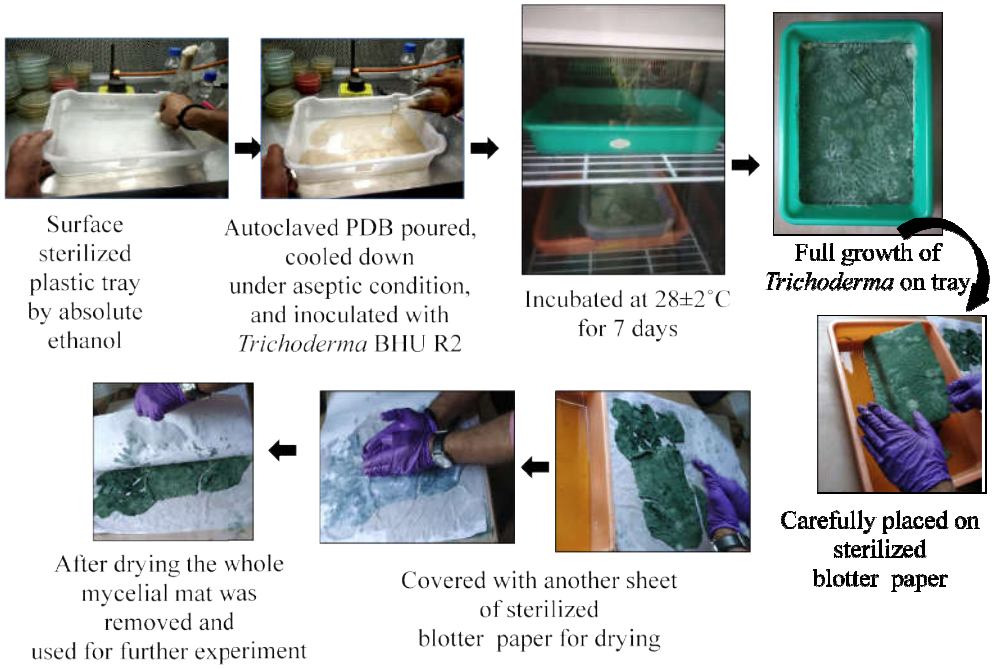



**Figure 3.5** Procedure for mass inoculum production of *Trichoderma* strain BHU R2 to encapsulate in hard gelatin capsules

### 3.7.2. Encapsulation of inoculum in the gelatin capsules

For encapsulation, hard gelatin capsules were used because it provides protection to microbial entity from harsh environmental conditions during storage, reduces the amount of biopesticide for application purpose and gives an easy storage as well as transport option. Other than this it can also be used for mass production of biocontrol agents. Three different type of mixing combinations were made for preparation of 10% w/w formulation which was used further as carrier/ filling material.

1. **Mix A** = Rice bran (84.9 g) + Okra gum powder (5 g) + Rutin (0.1g)
2. **Mix B** = Coal powder ( 84.9 g) + Okra gum powder (5 g) + Rutin (0.1g)
3. **Mix C** = Rice bran (39.95 g) + Coal powder (39.95 g) + Okra gum powder (10 g) + Rutin (0.1 g)

10 g of prepared *Trichoderma* inoculum was added in each combination to make final 10% w/w formulation which was used for encapsulation (Figure 3.6). Survivability of *Trichoderma* BHU R2 spores was checked after the storage of capsules at room temperature upto 6 month at 1 month interval. Unencapsulated *Trichoderma* BHU R2 inoculum was used as control.

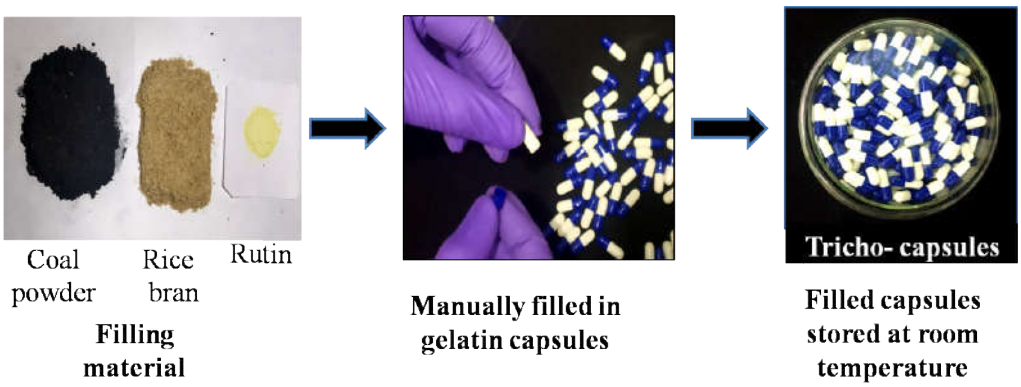



**Figure 3.6** Procedure for encapsulation of *Trichoderma* strain BHU R2 in hard gelatin capsules

**3.7. 3. Seed Bio-priming with *Trichoderma* strain BHU R2 and encapsulation in the gelatin capsules**

Tomato seeds were surface sterilized with 1% sodium hypochlorite (NaOCl) for 1 min and rinsed thrice with sterile distilled water and dried under a sterile air stream on autoclave blotting paper. After that seeds were soaked in prepared spore suspension of *Trichoderma* BHU R2 in 1% okra gum solution and seeds treated with SDW only served as control. The excess suspension was drained off and the seeds were dried for 2 h under sterile air stream. Okra gum solution acts as adhesive agent and helped in coating of microbes on seed surface. For priming, the treated seeds were kept as a heap in the moist chamber at 28–30 °C, maintaining 98% relative humidity for 24 h. The coated microbial cells were supposed to grow on the seed surface under this condition and form a protective layer over the seed coat. After incubation, two

seeds were kept into a single gelatin capsule with filling material (Figure 3.7) while unencapsulated bioprimed seed used as control. Survivability of *Trichoderma* BHU R2 spores was checked after storage of capsules at room temperature upto 6 month in 1 month interval.

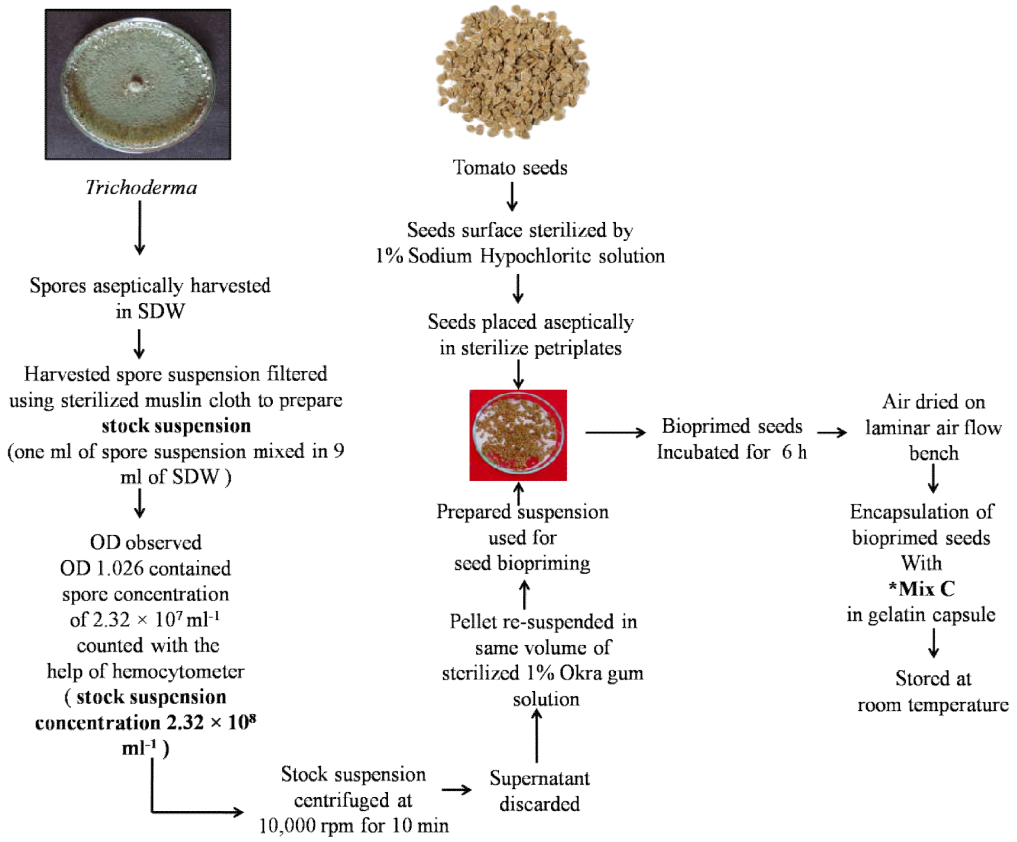



**Figure 3.7** Procedure for encapsulation of *Trichoderma* strain BHU R2 primed seed in hard gelatin capsule

### 3.8 Evaluation of *Trichoderma* BHU R2 shelf life in encapsulating formulations

Shelf life of *Trichoderma* BHU R2 was observed by serial dilution technique and CFU counting method at 1 month interval upto 6 month. Single capsule was taken out from both the formulations and dissolve in 10 ml of SDW containing glass vial under aseptic condition. Thereafter, glass vials were safely vortexed for obtaining the homogenize solution and serially diluted upto $10^{-6}$ dilution. 100 µl of this diluted

solution was taken and poured on rose bengal added PDA plates. Plates were incubated at 28±2˚C for 2 days and CFU was counted (Figure 3.8). The CFU was calculated by using formula:

$$\text{CFU} = \frac{\text{CFU per plate } \times \text{Dilution factor}}{\text{Weight of substrate (gm)} \times \text{Amount plated (ml)}}$$

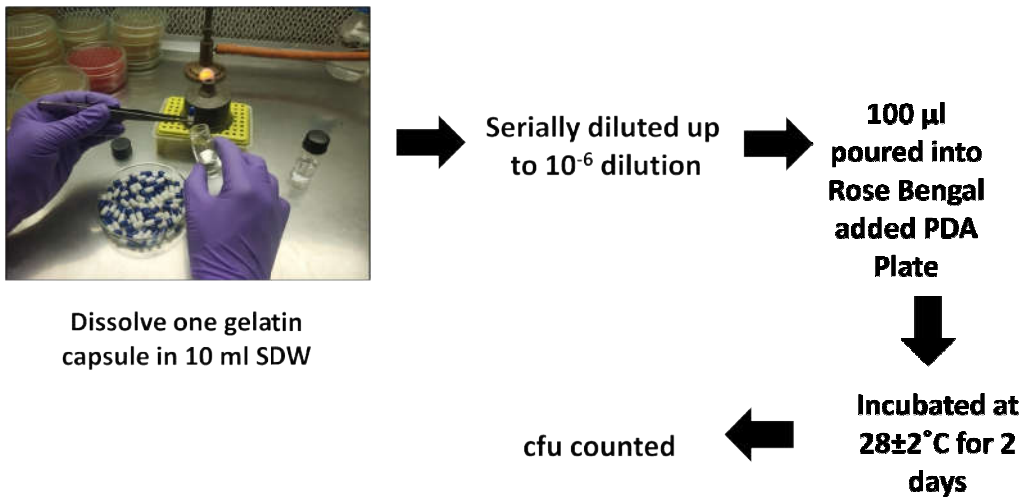



**Figure 3.8** Procedure for evaluating the shelf life of *Trichoderma* encapsulated formulations

**3.9 Evaluate the impact of encapsulated formulations on plant growth promotion and biocontrol potential of collar rot of tomato caused by *Sclerotium rolfsii***

**3.9.1. Effect of Tricho-capsule treatments on plant growth promotion and biocontrol potential**

**3.9.1.1. Preparation of treatments through Tricho-capsule**

Single Tricho-capsule was taken out and dissolved in 100 ml of SDW. This spore suspension was further used for seed bioprimg as well as root dip treatment of tomato seedlings (Figure 3.9). Tomato seeds (cv. Kashi amrit) were surface sterilized by 1% NaOCl solution for 1 min, followed by rinsing with SDW thrice and dried under a sterile air stream on pre-sterilize blotting paper. Sterilized seeds were divided into two parts; one part of sterilized seeds were primed with the prepared spore

suspensions of BHU R2 while second part remain as unprimed and then both was sowed separately in sterilized soil. After 15 days of seed sowing (DAS), seedlings were uprooted for transplanted into field. BHU R2 spore suspension primed seedlings were directly transplanted into field while unprimed seed seedlings were dipped into spore suspension of BHU R2 for root dip treatment and then transferred into the field for transplanting. After 15 days of transplanting (DAT), plants were challenged with the pathogen *S. rolfsii*.

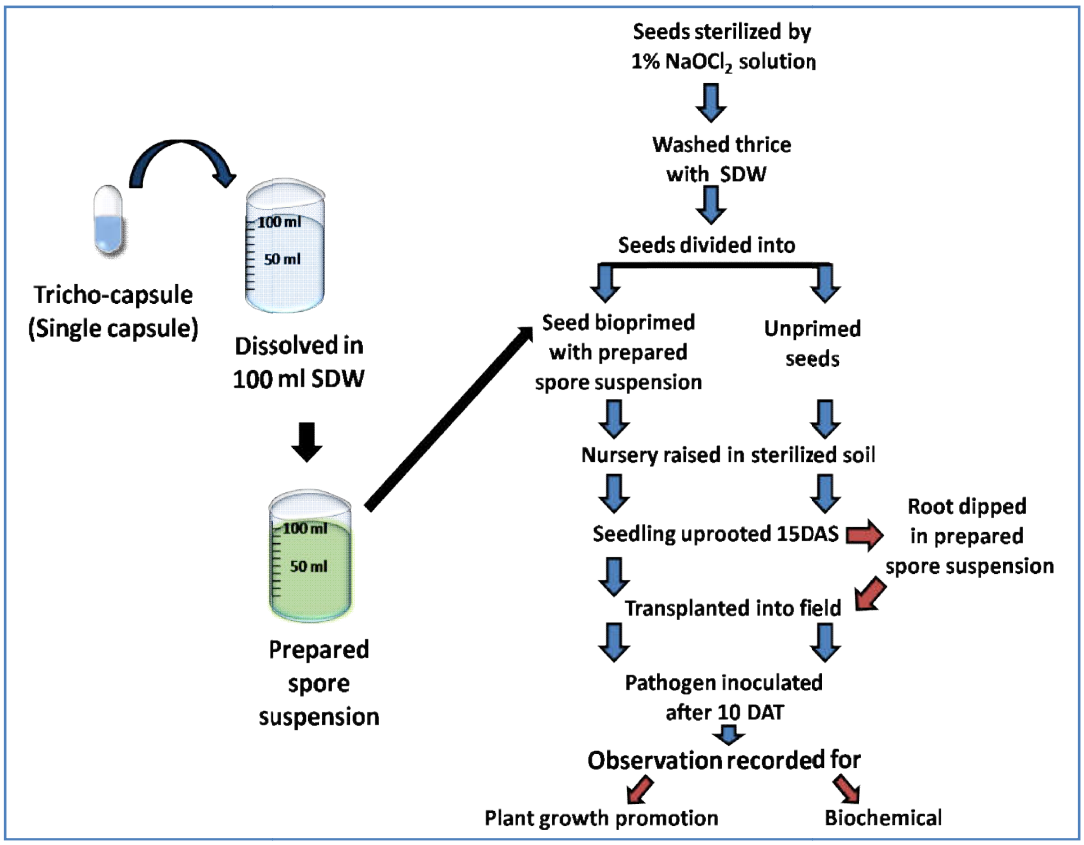



**Figure 3.9** Procedure for preparation of treatment through Tricho-capsule.

The pathogen *S. rolfsii* was mass multiplied on corn meal-sand media (Corn seed 250g, washed sand 750g, distilled water 250ml) at 26±2˚C for 15 days. The colonized culture of the pathogen was blended for homogenization of inoculum before application in field. Prepared inoculum (active fungal mycelium and sclerotia)

was inoculated at the rate of 10g per plant on the top soil of the plants and covered with sterilized potting mixture. Irrigation was done manually in every alternate day to maintain adequate moisture level in the field. Pathogen infected and pathogen uninfected untreated seedlings served as positive and negative controls, respectively (Table 3.2). Sampling for plant growth promotion was done after 30 days of transplanting (DAT). Percent mortality was reordered in only pathogen challenged treatments after 15 days of pathogen inoculation (DAI) by using following formula (Erkilic *et al*., 2006) given as below

$$\%DS = \frac{\Sigma(n \times v)}{N \times V} \; 100$$

Where; n= score of infection according to scale, v= number of seedlings per category, N= total number of seedlings screened, V= highest score for infection

The experiment was conducted with three replications and the data were pooled for analysis.

**Table 3.2** Combination of treatments used for conducting experiment

| Treatment | Details |
|---|---|
| **T 1** | Control |
| **T 2** | Pathogen (*S. rolfsii*) challenged only |
| **T 3** | Biopriming only |
| **T 4** | Root dipping only |
| **T 5** | Biopriming + Pathogen (*S. rolfsii*) challenged |
| **T6** | Root dipping + Pathogen (*S. rolfsii*) challenged |

**Details of the layout plan**

Experimental design: Randomized block design (RBD)

Number of treatment: 6

Number of replication: 3

### 3.9.1.2. Observations recorded

Random sampling technique was adopted for recording the observations of various plant growth promoting traits after 30 days of transplanting (DAT) while yield attributing traits were recorded after 60 days of transplanting (DAT). Three plants of each treatment from each replication were selected to record the data on various characters. Mean data of treatment was used for statistical analysis. Recommended package of practices were applied to raise a healthy crop.

**Plant growth promoting attributes**

- **Shoot length (cm)**

It was measured in centimeter from the ground level (base of the plant) to the tip of the main axis of the plant after stretching the main shoot of plant at the time span of 30 days after sowing with the help of meter scale.

- **Root length (cm)**

It was measured in centimeter from the ground level (base of the plant) to the tip of the main root of the plant at the time span of 30 days after sowing with the help of meter scale.

- **Number of lateral roots**

The numbers of lateral roots were counted from one randomly selected plant per replication.

- **Determinations of fresh and dry weight (g)**

After washing the plants in the tap water and softly wiped with using blotting paper, fresh weight was determined by using an electronic balance (Sartorius BT-224S) and the values were expressed in grams. After taking fresh weight, the plants were placed to 100°C pre-heated hot air oven for one hour. Then they were placed in

an oven, maintained at 60±2°C for drying purpose. The weight was measured regularly till to get constant value, expressed in grams.

**Yield attributing traits**

- **Number of fruits / plant**

The fruits from one randomly selected plant were taken from each replication and their numbers were counted.

- **Average fruit yield (kg/ plant)**

The fruits from one randomly selected plant were taken from each replication and their weight was measured.

**3.9.1.3. Biochemical estimation of anti-oxidative enzyme activities and phenol content in plant**

The pathogen *Sclerotium rolfsii* was inoculated at near of ground portion of stem and a small scar made to initiate early infection in tomato plants. Observations were recorded at 24, 48, 72 and 96 hours after pathogen inoculation.

1. Superoxide dismutase (Fridovich *et al.,* 1974)
2. Peroxidase (POx) activity (Hammerschmidt *et al*., 1982)
3. Malondialdehyde (MDA) content Ohkawa *et al*., (1979)
4. Phenylalanine ammonia lyase (PAL) (Brueske, 1980)
5. Total phenol content (TPC) (Zheng and Shetty, 2000)

**Sample collection for biochemical analysis**

Leaves of tomato plants from all the treatments were collected randomly at 24, 48, 72 and 96 h after pathogen inoculation. Leaves were washed separately by placing them on sieve (1mm diameter) under running tap water and used for further studies.

**Assessment of anti-oxidative enzyme activities in plant**

**1. Superoxide dismutase (SOD) activity**

Superoxide dismutase activity was estimated by using Fridovich method (1974). 0.1g of leaf sample was homogenized in liquid nitrogen and suspended in 2 ml of extraction buffer (0.1M phosphate buffer containing 0.5 mM EDTA at pH 7.5). The homogenate was centrifuged at 15,000 rpm for 20 min at 4˚C and the supernatant was collected. The enzyme reaction mixture consisted of 200 mM methionine 2.25 mM nitrobluetetrzolium chloride (NBT), 3 mM EDTA, 100 mM phosphate buffer (pH 7.8), 200 µl of enzyme extract and the final volume was maintained to 3ml. 2 µM riboflavin (0.4 ml) was added for the initiation of reaction and the tubes were placed under the light of two 18W fluorescent light lamp for 15 min. After 10 min, termination of the reaction was carried by switching off the light and the tubes were kept in dark. For control, a complete reaction mixture was taken without enzyme extract. The optical density (O.D.) was recorded at 560 nm on a Thermo Scientific UV1 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). One unit of the SOD activity defined the amount of enzyme reducing the absorbance to 50% in comparison to control devoid of enzyme.

**2. Peroxidase (POx) activity**

Peroxidase activity was estimated spectrophotometrically with slight modification in Hammerschmidt method (Hammerschmidt *et al*., 1982**).** 0.1 g of leaf tissue was homogenized in liquid nitrogen and suspended in 5 ml of 0.1 M phosphate buffer (pH 7.0) at 4˚C. The complete homogenate was centrifuged at 16,000 rpm at 4˚C for 15 min and the supernatant was collected. The enzyme reaction mixture contained 100mM phosphate buffer, 0.05 M pyrogallol (1.5 ml), enzyme extract (50

µl) and 1% $H_2O_2$ (0.5 ml). The absorbance was recorded at 30 s interval for 3 min at 420 nm. The enzymatic activity was expressed in U / min / g fresh weight.

**3. Malondialdehyde (MDA) content**

The Malondialdehyde amount was estimated by the thiobarbituric acid (TBA) reaction. The reaction assay was carried by the method of Ohkawa *et al*., (1979). 0.1g of leaf sample was homogenized with 4.0 ml of 20% trichloroacetic acid (TCA, w/v) containing 1% TBA (w/v) for 30 min at 95˚C. The reaction was stopped by cooling on ice for 10 min and the product was centrifuged at 10,000×g for 15 min. The reaction product was measured at 532 nm and the concentration of MDA was expressed as nmole / g fresh weight.

- **Assessment of phenol content in plant**

**4. Phenylalanine ammonia lyase (PAL) activity in plant**

The activity of phenylalanine ammonia lyase (PAL) was assessed by using the method of Brueske (1980). 0.1 g of leaf sample was homogenized in liquid nitrogen and suspended in 5 ml of sodium borate buffer (pH 7.0) contained 1.4 mM mercaptoethanol. The complete homogenate was centrifuged at 16,000 rpm at 4˚C for 15 min and supernatant was collected. In enzyme reaction mixture, 0.5 ml of borate buffer (pH 8.7), 1.3 ml of distilled water and crude enzyme extract (200 µl) was added. To initiate the reaction, 0.5 ml of l-phenylalanine (0.1mM, pH 8.7) was added in each tube and incubated at 32˚C for 30 min. Reaction was terminated by adding 0.5 ml of trichloroacetic acid (1 M). The Absorbance was recorded at 290 nm and PAL activity was measured in terms of t-cinnamic acid (t-CA) formed and it was expressed as µM TCA/ g fresh weigh.

**5. Total phenol content (TPC) in plant**

Assessment of total phenol content was performed by the method of Zheng and Shetty (2000). Leaf sample (0.1 g) was taken in glass vials (10 ml size) containing 5ml of 95% ethanol. For maximum extraction vial was stored at 0˚C for 48 hours. Thereafter, sample was homogenized and centrifuged at 15,000 rpm for 10 min and the supernatant was collected. 1 ml of the supernatant was mixed with1 ml of 95% ethanol, 5 ml of SDW, 0.5 ml of 1 N Folin-Ciocalteau reagent was added and after 5 min, 1 ml of 5% $Na_2CO_3$was added and the complete mixture was incubated at room temperature for 60 min. The absorbance was recorded at 725 nm and the absorbance values were converted to mM Gallic acid equivalent (GAE) g /fresh weight.

**3.9.1.4. Estimation of nutritional content of tomato fruits**

The ascorbic acid content was estimated by the method of Klein and Perry (1982) and the lycopene content was determined by using the method of Fish et al. (2002). The total carbohydrate content in tomato fruits was determined by Anthrone method (Hedge and Hofreiter 1962) and protein determination was done by using Lowry method (Lowry et al. 1951).

**3.9.1.5. Estimation of mineral content in tomato fruits**

Randomly five fruit samples were collected from all the treatments to analyze the mineral content. The Fruit samples were oven dried at 65˚C until the constant weight was obtained. Thereafter the dried fruit samples were grinded and sieved through a 0.5 mm mess sieve for analysis of mineral components. For analysis of total Nitrogen content, Micro-Kjeldahl procedure as described by IITA (1982) was used. Total phosphorus content was determined by Vanadomolybdate method by using

spectrophotometer, and flame photometry was used for the analysis of K and Ca. Concentrations of nutrients were expressed as µg/g FW of fruit material.

### 3.9.2. Effect of encapsulated bioprimed seeds on nutrient use efficiency and plant growth promotion

In the greenhouse trial, the experiment was conducted in earthen pots (3 litre) and laid out as Completely Randomized Block Design (CRD) having four treatments with three replication of each treatment (Table 3.3). Potting mixture consisted of sand and soil (1:1 ratio) was filled in the autoclavable polypropylene bags which was sterilized for three consecutive days at 15lb pressure for 30 min in an autoclave and filled in earthen pots. Ten bioprimed seed capsules were sown in each pot at an approximate depth of 1.5 cm. For assessment of plant growth parameters, five random plant samples were uprooted from pot after 30 days of seed germination (DAG). After uprooting, the plants were washed thoroughly by placing them on sieve (1mm diameter) under running tap water to remove adhering soil particles from roots. Thereafter, plants were subjected for drying by placing them on two layers of blotting papers. Fresh weight (FW) of shoot and root were taken and then oven dried at 80˚C for determining dry weight (DW). Shoot length (SL), root length (RL), number of lateral roots (LR) were also estimated.

**Table 3.3** Combination of treatments used for conducting experiment

| Treatment | Details |
|---|---|
| **T 1** | Control (No fertilizer) |
| **T 2** | 75% RDF+EBS** |
| **T 3** | RDF* + Unprimed seed |
| **T 4** | Only EBS |

**RDF* (Recommended dose of fertilizer) = 75:100:50 (N: P: K) kg/ha**
**EBS** = Encapsulated bioprimed seed**

### 3.10 Statistical analysis

The data in the tables were analyzed by using one way ANOVA. The data are expressed as the mean of three independent replications ± standard deviation. Means were compared by DMRT ($P \leq 0.05$), using SPSS version 16.0. Different letters superscript in the column data indicates significant difference between the variants across the treatments and vertical bars indicate standard deviations of the means.

# CHAPTER IV

# EXPERIMENTAL FINDINGS

Chapter-1V

# EXPERIMENTAL FINDINGS

The results of the experiments conducted on different objectives of the thesis are presented in this chapter.

## 4.1 Collection of diseased samples from different districts of Eastern Uttar Pradesh, isolation and purification of *Sclerotium rolfsii*, test of pathogenicity and study of percent disease incidence of selected *S. rolfsii* isolates on tomato plants in pots

### 4.1.1 Survey for collar rot disease incidence at different zones of Eastern Uttar Pradesh and collection of diseased samples

A roving survey was conducted during March to April, 2016 in five districts of Eastern Uttar Pradesh to analyze the status of collar rot incidence and to collect diseased samples infected by *Sclerotium rolfsii* under field condition. The areas having maximum root rot incidence were selected and infected samples were collected from those areas. The data pertaining to observations on the crop stages of tomato showing the typical collar rot symptoms, the mean disease rating (MDR) of the surveyed plots and the name of the isolates of *S. rolfsii* were presented in Table 4.1. The mean disease rating of the infected plants was recorded on a rating scale of 0-5. The disease severity of plants varies with area and different crop stages like seedling, flowering and fruiting stages. Forty samples were collected from different districts of Eastern Uttar Pradesh from which twenty different isolates of *S. rolfsii* were isolated in laboratory in aseptic condition on potato dextrose agar (PDA) plates.

**Table 4.1** Collection of disease plant sample from different Tomato growing districts of Eastern Uttar Pradesh

| S. No. | District | Isolate name | Location | Latitude/ Longitude | Mean disease rating |
|---|---|---|---|---|---|
| 1 | Azamgarh | Sr 1 | Ainhia | 26°06'90" N 83°20'66" E | 4.3 |
| | | Sr 2 | Baddopur | 26°06'16" N 83°21'14" E | 4.1 |
| | | Sr 3 | Narauli | 26°04'91" N 83°17'63" E | 3.2 |
| | | Sr 4 | Paranapur | 26°05'92" N 83°16'79" E | 2.6 |
| 2 | Jaunpur | Sr 5 | Chitarsari | 25°76'13" N 82°68'67" E | 1.6 |
| | | Sr 6 | Mainipur | 25°76'21" N 82°67'37" E | 3.8 |
| | | Sr 7 | Tajapur | 25°75'34" N 82°68'91" E | 4.2 |
| | | Sr 8 | Tamarashapur | 25°75'94" N 82°67'11" E | 3.7 |
| 3 | Mau | Sr 9 | Barpur | 25°93'22" N 83°57'99" E | 4.4 |
| | | Sr 10 | Indarpur | 25°92'28" N 83°58'7" E | 3.9 |
| | | Sr 11 | Malibpura | 25°93'83" N 83°58'29" E | 2.7 |
| | | Sr 12 | Rastipur | 25°93'37" N 83°58'63" E | 2.9 |
| 4 | Mirzapur | Sr 13 | Bashi | 25°12'79" N 82°54'79" E | 3.3 |
| | | Sr 14 | Chintamanpur | 25°13'47" N 82°53'62" E | 3.7 |
| | | Sr 15 | Ranibari | 25°13'32" N 82°52'79" E | 3.9 |
| | | Sr 16 | Rajapur | 25°12'46" N 82°52'16" E | 4.1 |
| 5 | Varanasi | Sr 17 | BHU | 25°26'41" N 82°99'39" E | 4.4 |
| | | Sr 18 | Ganeshpuram | 25°27'36" N 82°97'31" E | 4.1 |

| | | | | | |
|---|---|---|---|---|---|
| | | Sr 19 | Kandwa | 25°26’94” N 82°95’14” E | 3.4 |
| | | Sr 20 | Nasirpur | 25°25’76” N 82°97’31” E | 3.8 |

### 4.1.2 Isolation, purification, maintenance of *S. rolfsii* isolates

The isolates were purified by single sclerotial isolation technique and were identified as *S. rolfsii* based on morphological and cultural characters using the descriptions given by C.M.I (1970). The isolates were designated serially from Sr 1 to Sr 20. The purified isolates were maintained in PDA slants and stored at 4°C for further use.

### 4.1.3 Test of pathogenicity of *S. rolfsii* isolates

` After isolation and purification of *S. rolfsii* from the collected samples, they were subjected to the pathogenicity tests on susceptible genotype of chickpea cultivar ‘Kashi amrit” through soil inoculation method. Out of 20 isolates of *S. rolfsii* tested for pathogenicity, all isolates showed typical collar rot symptoms like formation of white mycelium mat on basal portion of stem and wilting. The adjoining region of the plant near to soil got almost rottened. Thus, all 20 isolates showed positive result for Koch’s postulate which indicating their pathogenicity to tomato and selected as representative samples for further study. The colony characteristics of these isolates were studied .These selected isolates were also further studied for per cent disease severity (% DS) through ‘soil inoculation’ method. The results of pathogenicity were presented in Table 4.2.

**Table 4.2** Pathogenicity test using *S. rolfsii* isolates collected from different districts of Eastern Uttar Pradesh

| S. No. | Isolate name | Collar rot symptoms | Koch’s postulates |
|---|---|---|---|
| 1 | Sr 1 | +ve | +ve |
| 2 | Sr 2 | +ve | +ve |
| 3 | Sr 3 | +ve | +ve |

| | | | |
|---|---|---|---|
| 4 | Sr 4 | +ve | +ve |
| 5 | Sr 5 | +ve | +ve |
| 6 | Sr 6 | +ve | +ve |
| 7 | Sr 7 | +ve | +ve |
| 8 | Sr 8 | +ve | +ve |
| 9 | Sr 9 | +ve | +ve |
| 10 | Sr 10 | +ve | +ve |
| 11 | Sr 11 | +ve | +ve |
| 12 | Sr 12 | +ve | +ve |
| 13 | Sr 13 | +ve | +ve |
| 14 | Sr 14 | +ve | +ve |
| 15 | Sr 15 | +ve | +ve |
| 16 | Sr 16 | +ve | +ve |
| 17 | Sr 17 | +ve | +ve |
| 18 | Sr 18 | +ve | +ve |
| 19 | Sr 19 | +ve | +ve |
| 20 | Sr 20 | +ve | +ve |

### 4.1.4 Studies on cultural variability in the selected *S. rolfsii* isolates

Variability in the cultural and morphological characters of eleven isolates of *S. rolfsii* was studied by growing on PDA medium. The variations were recorded on the basis of colony diameter, colony colour, colony texture and sclerotial characteristics (Table 4.3).

**Colony diameter:** The data on colony diameter presented in the table 4.3 revealed that there was a significant difference in the colony growth recorded among the eleven isolates of *S. rolfsii* which varied from 39.2 to 81.3 mm. Isolate Sr 1, Sr 2, Sr 7, Sr 9 and Sr 17 showed significantly highest colony growth ≥ 60 mm while the least colony diameter was observed with the isolate Sr 16 (39.2 mm).

**Colony colour**: Based on visual observation on colony colour, the isolates were divided into three groups (Table 4.4). Pure white colour colony was observed in 7 isolates i.e Sr 1, Sr 2, Sr 4, Sr 7, Sr 9, Sr 18 and Sr 17 while Sr 10, Sr 11, Sr 12, Sr 15 and Sr 19 showed creamy white. Remaining 7 isolates i.e Sr 3, Sr 5, Sr 6, Sr 8, Sr 13, Sr 14 and Sr 20 showed light brown type of colony.

**Colony texture:** The isolates were categorized into two groups on the basis of colony appearance (Table 4.5). 12 isolates produced fluffy colony while rest of 8 isolates had produced compact type growth.

**Sclerotia:** All 20 isolates of *S. rolfsii* produced sclerotia, but the initiation of sclerotial development and arrangement was found to differ (Table 4.6). Only two i.e. Sr 9 and Sr 17 showed early sclerotial development on 6$^{th}$ day in PDA plates while majority of the isolates showed delayed sclerotial development. Sclerotia were found dark brown to light brown in colour with proper round to globoid shape about 2-3 mm size.

**Table 4.3** Variability in cultural characteristics of selected isolates of *S. rolfsii*

| S. No. | Isolate name | Radial growth (mm) | Colony texture | Colony colour | Sclerotial development | Sclerotia colour |
|---|---|---|---|---|---|---|
| 1 | Sr1 | 75.2 | Fluffy | Pure white | 8$^{th}$ day | Dark brown |
| 2 | Sr 2 | 73.3 | Fluffy | Pure white | 10$^{th}$ day | Brown |
| 3 | Sr 3 | 47.7 | Compact | Light brown | 15$^{th}$ day | Light brown |
| 4 | Sr 4 | 51.2 | Fluffy | Pure white | 10$^{th}$ day | Brown |
| 5 | Sr 5 | 37.1 | Compact | Light brown | 16$^{th}$ day | Light brown |
| 6 | Sr 6 | 48.4 | Compact | Light brown | 15$^{th}$ day | Light brown |
| 7 | Sr 7 | 74.8 | Fluffy | Pure white | 10$^{th}$ day | Brown |
| 8 | Sr 8 | 41.8 | Fluffy | Light brown | 14$^{th}$ day | Light brown |
| 9 | Sr 9 | 77.2 | Fluffy | Pure white | 6$^{th}$ day | Dark brown |
| 10 | Sr 10 | 52.4 | Fluffy | Creamy white | 11$^{th}$ day | Brown |
| 11 | Sr 11 | 31.3 | Compact | Creamy white | 16$^{th}$ day | Light brown |
| 12 | Sr 12 | 35.8 | Compact | Creamy white | 15$^{th}$ day | Light brown |
| 13 | Sr 13 | 39.9 | Compact | Light brown | 15$^{th}$ day | Light brown |
| 14 | Sr 14 | 52.3 | Compact | Light brown | 12$^{th}$ day | Light brown |
| 15 | Sr 15 | 69.2 | Fluffy | Creamy white | 10$^{th}$ day | Brown |
| 16 | Sr 16 | 49.7 | Compact | Creamy white | 17$^{th}$ day | Light brown |
| 17 | Sr 17 | 81.3 | Fluffy | Pure white | 6$^{th}$ day | Dark brown |
| 18 | Sr 18 | 68.7 | Fluffy | Pure white | 10$^{th}$ day | Brown |
| 19 | Sr 19 | 51.2 | Fluffy | Creamy white | 10$^{th}$ day | Brown |
| 20 | Sr 20 | 50.3 | Fluffy | Light brown | 12$^{th}$ day | Light brown |

**Table 4.4** Variation in colony colour of *Sclerotium rolfsii* isolates

| Colony colour | Colony in the PDA Petri plate | Isolate detail |
|---|---|---|
| **Pure white** |  | **Sr 1, Sr 2, Sr 4, Sr 7, Sr 9, Sr 18 and Sr 17** |
| **Creamy white** |  | **Sr 10, Sr 11, Sr 12, Sr 15 and Sr 19** |
| **Light brown** |  | **Sr 3, Sr 5, Sr 6, Sr 8, Sr 13, Sr 14 and Sr 20** |

**Table 4.5** Variation in colony type of *Sclerotium rolfsii* isolates

| Colony type | Colony in the Petri plate | Isolate detail |
|---|---|---|
| **Fluffy** |  | **Sr 1, Sr 2, Sr 4, Sr 7, Sr 8, Sr 9, Sr 10, Sr 15, Sr 17, Sr 18, Sr 19, Sr 20,** |
| **Compact** |  | **Sr 3, Sr 5, Sr 6, Sr 11, Sr 12, Sr 13, Sr 14, Sr 16,** |

**Table 4.6** Variation in sclerotial arrangement of *Sclerotium rolfsii* isolates

| Type of sclerotial arrangement | Arrangement in the Petri plate | Isolate detail |
|---|---|---|
| **Only center** |  | **Sr 5, Sr 6, Sr 11, Sr 12 and Sr 13** |
| **Only periphery** |  | **Sr 1, Sr 4, Sr 9 and Sr 17** |
| **Periphery and center** |  | **Sr 2, Sr 3, Sr 7, Sr 8, Sr 10, Sr 14, Sr 15, Sr 16, Sr 18, Sr 19 and Sr 20** |

### 4.1.4 Study of the effect of selected isolates of *S. rolfsii* on tomato plants in pots

The studies of per cent disease severity (% DS) of 20 isolates of *S. rolfsii* were studied by soil inoculation methods in pots under greenhouse conditions. The data were recorded from 15 DAI to 60 DAI (Table 4.7). It is evident from Table 4.4, only Sr1 (19.57%), Sr 9 (17.47%) Sr 17(21.68%) and Sr 18(16.21%) isolates showed % DS at 15 DAI while none of other isolates showed % DS up to 30 DAI. At 45 DAI, highest % DS recorded in treatment with Sr 17 (53.85%) followed by Sr 9 (49.72%), Sr 1 (48.98%), Sr 18 (44.62%) and Sr 7 (38.56%) respectively. All 20 isolates showed different levels of % DS at 60 DAI. Maximum % DS recorded at 60 DAI, in treatment with Sr 17 (89.63 %) followed by Sr 9 (80.26%), Sr 1 (78.34%), Sr 18 (74.21 %), Sr 7 (69.30 %) and similarly the values of % DS decreased with other isolates. Sr 17 showed the highest % DS amongst all isolate from 15-60 DAI while Sr 9 recorded the second highest position from 15-60 DAI. Other isolates i.e. Sr 1, Sr 2, Sr 7, Sr 10, Sr 15 and Sr 18 also showed % DS more than 60.00% while the remaining ones i.e. Sr 3, Sr 4, Sr 5, Sr 6, Sr 8, Sr 11, Sr 12, Sr 13, Sr 14, Sr 16, Sr 19 and Sr 20 showed % DS less than 60.00%.

From the observations of Table 4.4, Sr 17 isolated from Varanasi, was found to be the most aggressive amongst all the *S. rolfsii* isolates and thus Sr 17 was selected as test pathogen for carrying out further experiments.

**Table 4.7** Effect of selected *S. rolfsii* isolates on disease severity of tomato through soil inoculation method**.** Results are expressed as mean of triplicates ± S.D.

| **Name of the isolate** | **Per cent disease severity (% DS)** | | | |
|---|---|---|---|---|
| | **Days after inoculation (DAI)** | | | |
| | **15** | **30** | **45** | **60** |
| Control | 0 ±0 | 0 ±0 | 0 ±0 | 0 ±0 |
| Sr1 | 19.57 ±6.54 | 29.94 ±6.23 | 48.98 ±8.33 | 78.34 ±7.33 |

| | | | | |
|---|---|---|---|---|
| Sr 2 | 0 ±0 | 23.34 ±6.38 | 35.84 ±6.38 | 64.15 ±8.25 |
| Sr 3 | 0 ±0 | 0 ±0 | 21.62 ±7.25 | 35.85 ±6.33 |
| Sr 4 | 0 ±0 | 0 ±0 | 36.85±7.25 | 39.45±7.33 |
| Sr 5 | 0 ±0 | 0 ±0 | 29.23±5.82 | 34.28±5.75 |
| Sr 6 | 0 ±0 | 0 ±0 | 24.13 ±8.33 | 42.37±9.33 |
| Sr 7 | 0 ±0 | 24.83 ±5.45 | 38.56 ±6.25 | 69.30 ±7.37 |
| Sr 8 | 0 ±0 | 0 ±0 | 34.12±4.88 | 43.22±7.25 |
| Sr 9 | 17.47 ±4.17 | 27.31 ±6.25 | 49.72 ±8.33 | 80.26 ±9.25 |
| Sr 10 | 0 ±0 | 0 ±0 | 23.63 ±7.33 | 61.70 ±8.36 |
| Sr 11 | 0 ±0 | 0 ±0 | 18.39±6.33 | 22.38±7.58 |
| Sr 12 | 0 ±0 | 0 ±0 | 20.51±5.63 | 25.43±6.88 |
| Sr 13 | 0 ±0 | 0 ±0 | 25.41±6.15 | 33.36±8.33 |
| Sr 14 | 0 ±0 | 18.39 ±5.87 | 25.36 ±8.33 | 48.51±6.33 |
| Sr 15 | 0 ±0 | 20.76 ±6.33 | 33.52 ±8.25 | 68.46 ±9.25 |
| Sr 16 | 0 ±0 | 0 ±0 | 20.87 ±9.33 | 32.36 ±10.33 |
| Sr 17 | 21.68 ±5.25 | 37.63 ±6.45 | 53.85 ±8.37 | 89.63 ±9.33 |
| Sr 18 | 16.21±6.65 | 25.36±7.25 | 44.62 ±7.33 | 74.21±8.43 |
| Sr 19 | 0 ±0 | 0 ±0 | 25.31±5.63 | 38.47±6.88 |
| Sr 20 | 0 ±0 | 0 ±0 | 23.81±6.15 | 35.86±8.33 |

## 4.2 Sample collection, isolation, characterization and identification of abiotic stress tolerant *Trichoderma* species from various agro ecosystems.

### 4.2.1 Survey for collecting samples from different agro-ecosystem

A roving survey was conducted during April-June, 2016 in 10 districts of different state to collect soil and composting samples for isolation of abiotic (high temperature) stress tolerant *Trichoderma.* Total eighty samples were collected from Allahabad, Varanasi and Mirzapur from Uttar Pradesh; Damoh and Hoshangabad from Madhya Pradesh; Raipur and Bilaspur from Chhattisgarh; Jodhpur and Shri Ganganagar from Rajasthan (Table 4.8).

**Table 4.8** Collection site details for isolation of thermotolerant *Trichoderma* isolates

| S. No. | District | Latitude/ Longitude | Location |
|---|---|---|---|
| **1** | Varanasi, UP | 25°29’25” N / 83°03’35” E | Ramnagar, |
| | | 25°15'51" N 82°59'42" E | BHU |
| **2.** | Allahabad, UP | 25°54’92” N / 82°08’65” E | Phulpur |
| | | 25°37’02” N / 81°85’08” E | Dhanuha |
| **3** | Mirzapur, UP | 25°20’67” N / 82°49’17” E | Vindhyachal |
| | | 25°12’51” N / 82°57’36” E | Sarauli |
| **4.** | Damoh, MP | 23°83’66” N / 79°42’80” E | Tidori |
| | | 23°84’34” N / 79°42’49” E | Rastoriya |
| **5.** | Maihar, MP | 24°27’86” N / 80°75’62” E | Manpur |
| | | 24°25’40” N / 80°77’68” E | Harnampur |
| **6.** | Hoshangabad, MP | 23°22’40” N / 80°00’47” E | Mohaniya |
| | | 23°19’86” N / 80°08’95” E | Amakhoh |
| **7.** | Raipur, CG | 21°30’66” N / 81°65’69” E | Urkura |
| | | 21°28’85” N / 81°60’41” E | Gondwara |
| **8.** | Bilaspur, CG | 22°76’98” N / 81°89’46” E | Belghna |
| | | 22°07’64” N / 82°12’75” E | Tifra |
| **9.** | Jodhpur, RJ | 25°91’85” N / 73°31’45” E | Pali |
| | | 25°82’72” N / 73°06’09” E | Gadhwara |
| **10.** | Shri Ganganagar, RJ | 29°89’49” N /78°87’04” | Sector 4 C |
| | | 29°92’42” N /73°84’26” | 9Z |

### 4.2.2 Isolation, purification and maintenance of *Trichoderma* isolates

One hundred fifty two isolates were recovered from TSM agar plates after subjecting samples to serial dilution. On the basis of their colony colour, appearance and morphology the fungal isolates were categorized which were further characterized as *Trichoderma* according to the historic species concept established by Rifai (1969). These isolates were again transferred aseptically to Potato dextrose agar (PDA) plates and pure isolates were also maintained on PDA slants at 4˚C for further studies (Plate 1).

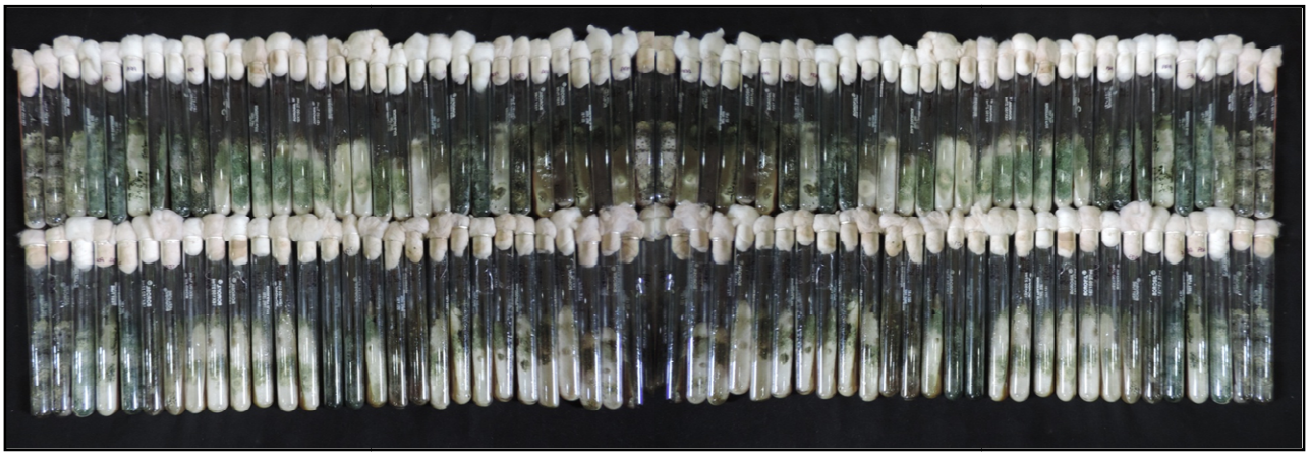


**Plate 1** Culture tubes showing *Trichoderma* isolates collected from different locations

### 4.1.3 Assessment of conidial thermotolerance and survivability of *Trichoderma* isolate

All the *Trichoderma* isolates showed variations in spore viability on thermal stress at 47˚C. Most of the spores of *Trichoderma* isolates lost their survivability which declined to zero or nearby zero within 60 min, while some isolates retained their viability up to 300 min. Sixteen *Trichoderma* isolates were selected from this study which retained their spore viability for more than 90 min of heat stress (Figure 4.1).

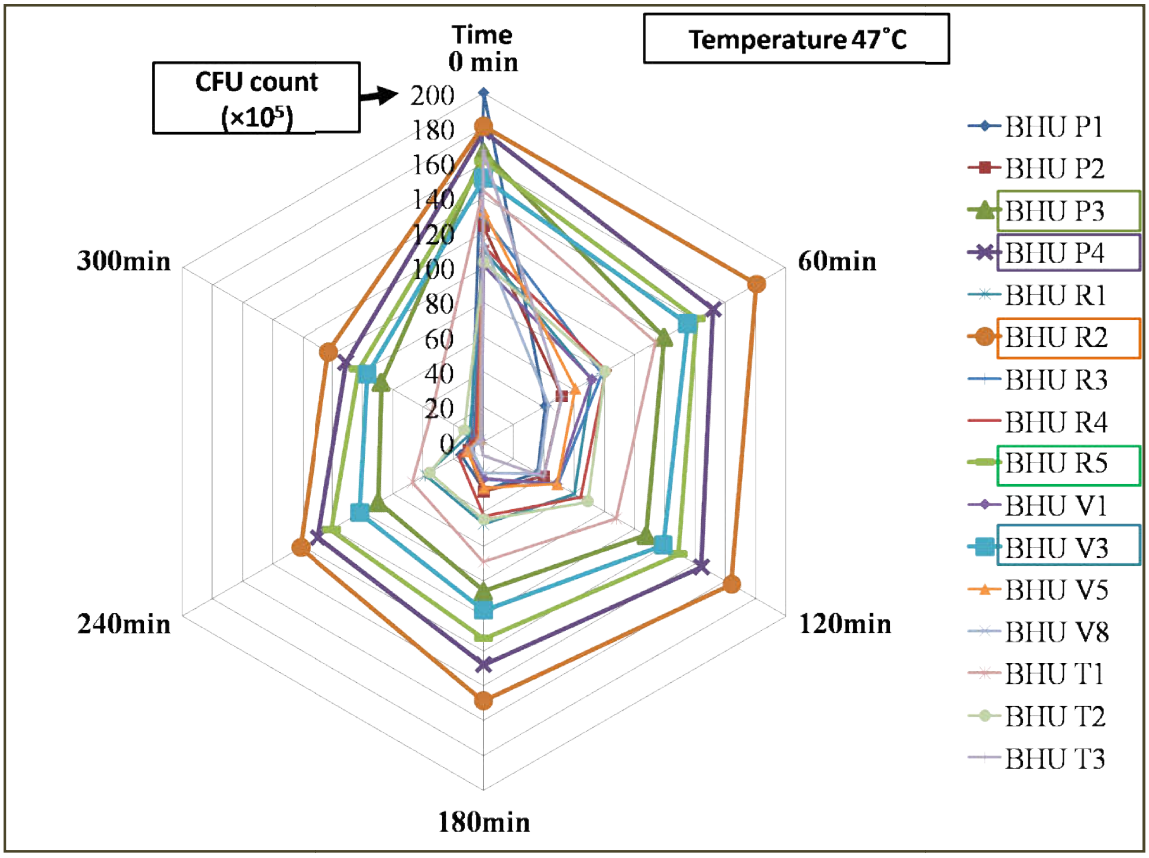



**Figure 4.1** Conidial thermo tolerance and survivability of *Trichoderma* isolates at 47˚C temperature

### 4.1.4 Assessment of mycelial growth of the selected *Trichoderma* isolates under different temperature

Selected *Trichoderma* isolates showed distinct variations in colony morphology and sporulation pattern at different temperatures. The growth pattern of mycelia varied with temperature (Table 4.9). The isolate BHU R2 showed the maximum temperature tolerance with mycelial growth up to 45˚C while BHU R1 showed least temperature tolerance with mycelial growth up to 20˚C. The isolates namely BHU V3 (18.49±0.71) and BHU R2 (19.07±1.01) showed highest average linear growth rate (mm/day) at 20˚C and 25˚C respectively while lowest average linear growth rate (mm/day) observed in

BHU V5 (9.50±0.75) BHU T1 (13.54±0.80) and at 20˚C and 25˚C. BHU R2 showed highest average linear growth rate at 30˚C (19.50±0.75), 35˚C (18.40±0.89), 40˚C (15.58±0.86) and 45˚C (12.24±0.66). The highest reduction in growth of mycelium at 45˚C was observed in BHU R1 (1.45±0.41).

**Table 4.9** Average linear growth (ALG) of selected thermo tolerant *Trichoderma* isolates in terms of mycelium growth (mm/day) against different temperature range. Results are expressed as the mean of three replicates, Mean± SD.

| **Thermo-tolerant *Trichoderma* isolates** | **Temperature range** | | | | | |
|---|---|---|---|---|---|---|
| | **20˚C** | **25˚C** | **30˚C** | **35˚C** | **40˚C** | **45˚C** |
| **BHU P1** | 16.33±0.72 | 17.75±0.75 | 17.21±0.80 | 12.00±0.83 | 4.00±0.98 | 3.25±0.75 |
| **BHU P2** | 14.75±1.15 | 15.75±0.65 | 12.83±1.01 | 8.90±0.87 | 6.75±0.43 | 2.83±081 |
| **BHU P3** | 15.75±0.43 | 16.17±0.63 | 13.50±1.14 | 11.89±0.69 | 7.16±0.59 | 5.08±0.71 |
| **BHU P4** | 17.42±1.44 | 17.58±0.38 | 17.41±0.75 | 15.00±1.02 | 10.33±0.84 | 7.25±0.64 |
| **BHU R1** | 15.61±0.87 | 15.00±0.63 | 16.83±0.62 | 11.08±0.63 | 2.83±0.69 | 1.18±0.98 |
| **BHU R2** | 19.07±1.01 | 18.33±0.66 | 19.50±0.75 | 18.40±0.89 | 15.58±0.86 | 12.24±0.66 |
| **BHU R3** | 16.92±0.63 | 16.58±1.13 | 16.66±1.10 | 8.08±1.10 | 5.08±0.67 | 2.58±0.67 |
| **BHU R4** | 11.33±1.04 | 15.33±0.80 | 14.50±1.12 | 11.66±0.87 | 4.33±1.10 | 2.61±0.69 |
| **BHU R5** | 18.79±0.81 | 16.12±0.43 | 17.35±1.19 | 13.91±1.14 | 10.08±0.78 | 6.89±0.65 |
| **BHU V1** | 15.14±0.95 | 15.50±0.87 | 15.41±0.96 | 7.50±0.61 | 4.83±0.87 | 2.08±0.89 |
| **BHU V3** | 18.49±0.71 | 18.65±0.66 | 17.00±0.56 | 12.12±1.03 | 8.41±1.12 | 6.12±0.67 |
| **BHU V5** | 9.50±0.75 | 14.75±0.43 | 12.83±1.10 | 10.12±0.67 | 3.41±0.89 | 2.41±0.74 |
| **BHU V8** | 13.42±1.26 | 16.83±0.38 | 12.58±0.86 | 9.47±0.88 | 5.41±0.68 | 2.10±0.68 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **BHU T1** | 15.00±1.30 | 13.54±0.80 | 15.58±1.09 | 11.56±1.14 | 7.08±0.62 | 2.09±0.58 |
| **BHU T2** | 14.33±1.15 | 15.42±1.04 | 14.91±0.68 | 10.23±0.68 | 3.91±1.02 | 1.45±0.41 |
| **BHU T3** | 15.33±1.12 | 15.12±0.98 | 16.10±0.59 | 8.90±1.09 | 4.75±0.75 | 2.03±0.52 |
| **CD (0.05%)** | **1.66** | **1.34** | **2.14** | **2.76** | **1.89** | **2.38** |

### 4.1.5 *In vitro* antagonism of selected *Trichoderma* isolates against *Sclerotium rolfsii*

Five high temperature tolerant *Trichoderma* isolates coded as BHU P3, BHU P4, BHU R2, BHU R5 and BHU V3 were selected to check the antagonistic activity against *S. rolfsii* under *in vitro* condition. All the selected isolates showed antagonistic activity against *S. rolfsii* (Plate 2). The highest percent inhibition in mycelial growth of pathogen was observed in *Trichoderma* strain BHU R2 (93.63±1.56) followed by BHU P4 (81.05±1.86), BHU R5 (78.44±1.71). The minimum inhibition was recorded by BHU P3 (64.97±2.35) followed by BHUV3 (68.92±1.49) (Figure 4.2).

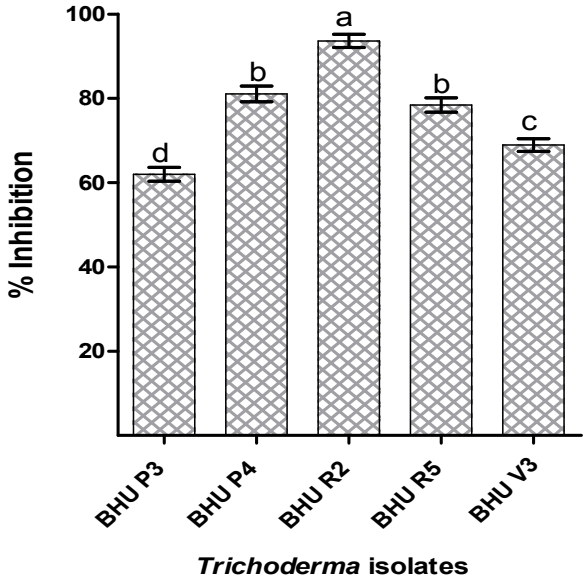



**Figure 4.2** Percent inhibition of *S. rolfsii* mycelium growth by selected high temperature tolerant *Trichoderma* isolates. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$.

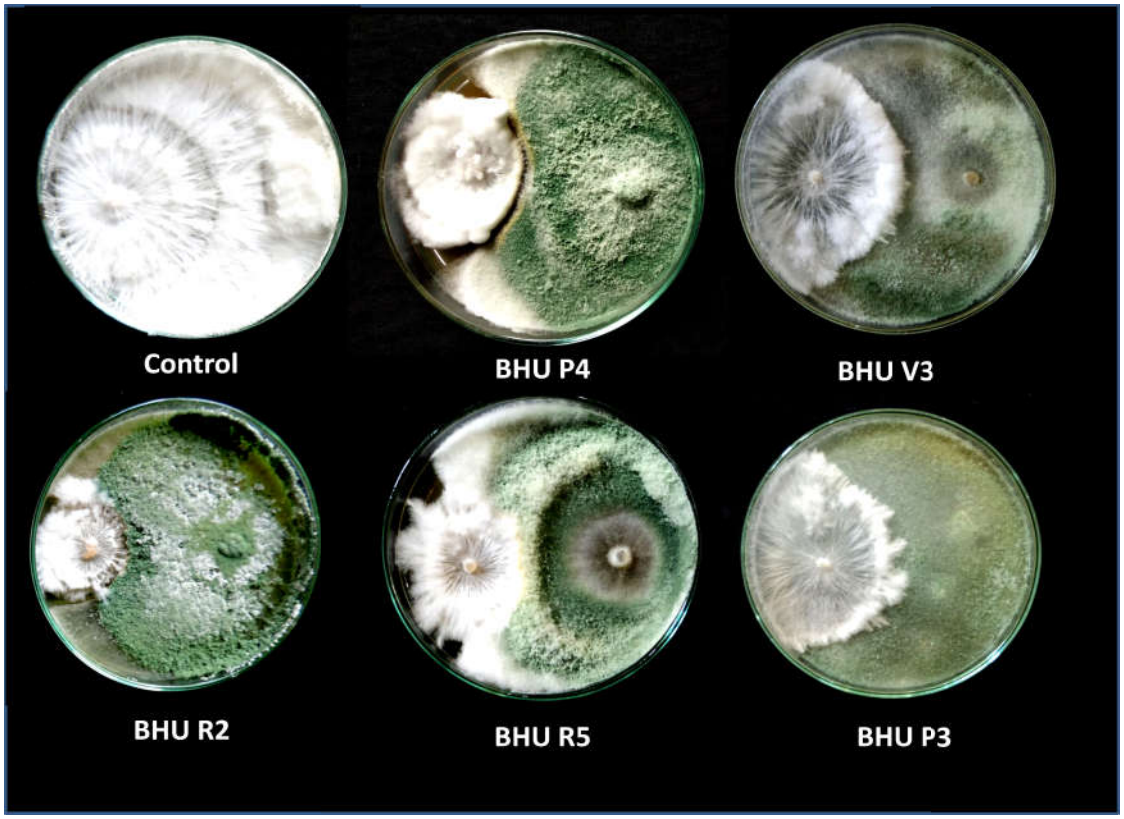



**Plate 2** Dual culture plate assay of selected high temperature tolerant *Trichoderma* isolates against *S. rolfsii*

### 4.1.6 *In vitro* effect of selected *Trichoderma* strains on plant growth promoting traits, siderophores and hydrolytic enzymes production

The potential antagonist with high temperature tolerant *Trichoderma* isolates namely BHU R2, BHU P4, BHU R5 and BHU V3 were selected to analyze different plant growth promoting traits and hydrolytic enzyme production activity at 40˚C, 45˚C and ambient room temperature (30±2˚C) conditions. IAA production was observed highest in BHU R2 strain at room temperature condition (57.73±1.10) followed by BHU P4 (48.21±1.03), BHU R5 (45.73±0.99) and BHU V3 (41.77±0.87). At 40˚C and 45˚C, the higher activity was observed in BHU R2 strain (47.76±0.90; 35.62±1.1) followed by BHU P4 (39.74±0.97; 30.32±0.08), BHU R5 (35.25±1.05; 26.62±1.02) and BHU V3 (31.30±1.01; 24.62±1.00).

Similarly, phosphate solubilization activity was found increased in the isolate *Trichoderma* BHU R2 strain at room temperature (165.47±2.24) followed by BHU P4 (150.87±1.94), BHU R5 (135.24±1.98) and BHU V3 125.10±1.19). At 40˚C and 45˚C, highest phosphate solubilization was observed in BHU R2 strain (119.68±2.23; 82.23±2.01) followed by BHU P4 (97.50±2.19; 63.37±1.95), BHU R5 (90.17±2.00; 51.33±1.79) and BHU V3 (79.35±2.27; 44.30±1.9) [Figure 4.3 (a), (b)]. Siderophore production, amylolytic, proteolytic and chitinase activity were found higher in BHU R2 as compared to other isolated strains at 40˚C, 45˚C (Table 4.10).

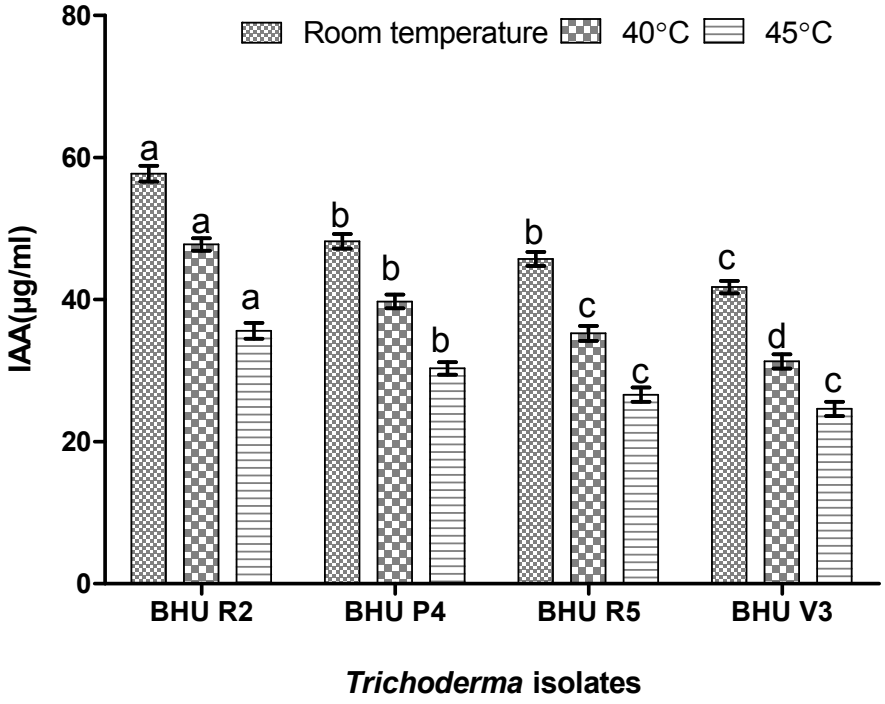



**Figure 4.3 (a)** Production of IAA (µg/ml) by selected *Trichoderma* isolates at different temperatures. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$

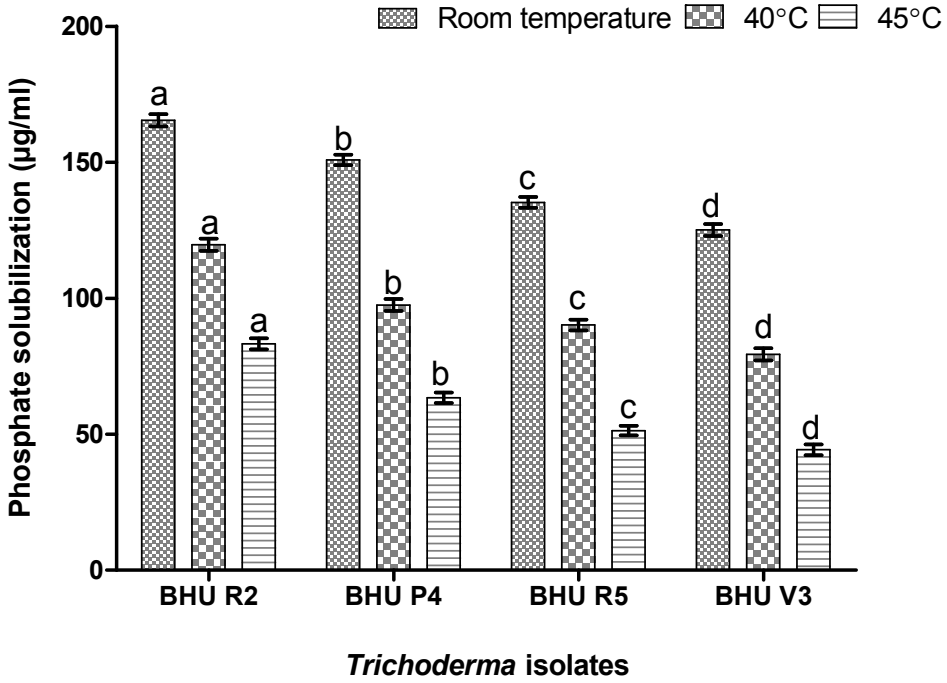



**Figure 4.3 (b)** Phosphate solubilization (µg/ml) by selected *Trichoderma* isolates at different temperatures. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$

**Table 4.10** Siderophore production and enzymatic activity of selected *Trichoderma* isolates

| Parameters | Room temperature (30±2˚C) | | | | 40˚C | | | | 45˚C | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **BHU R2** | **BHU P4** | **BHU R5** | **BHU V3** | **BHU R2** | **BHU P4** | **BHU R5** | **BHU V3** | **BHU R2** | **BHU P4** | **BHU R5** | **BHU V3** |
| Siderophore production | +++ | ++ | +++ | ++ | ++ | + | + | - | ++ | - | - | - |
| Amylolytic activity | ++ | ++ | ++ | ++ | ++ | - | + | - | ++ | - | - | - |
| Proteolytic activity | +++ | +++ | +++ | ++ | +++ | ++ | ++ | + | ++ | + | + | + |
| Chitinase activity | +++ | +++ | +++ | +++ | ++ | + | + | + | ++ | + | - | - |

Where,

+++ = High

++ = Medium

+ = Low

- = Absence

### 4.1.7. Molecular identification of potential *Trichoderma* strains

Most prominent thermotolerant *Trichoderma* strain BHU R2 was identified at molecular level (Plate 3). Sequence of ITS-1 region showed nearest phylogenetic link with *Trichoderma pseudokoningii* with 99.67% homology (Figure 4.4). After submission of sequence to GenBank database, the accession number MH729058 was generated for *Trichoderma* strain BHUR2.

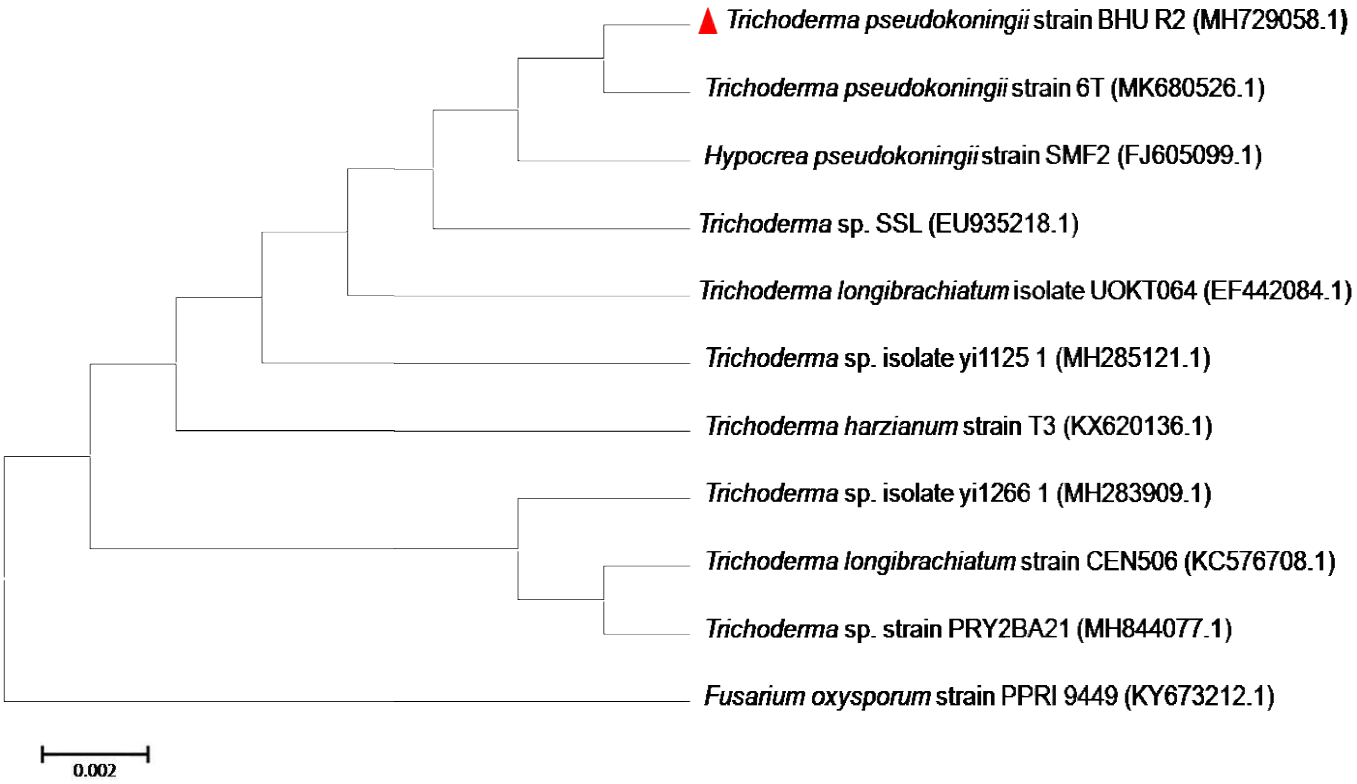
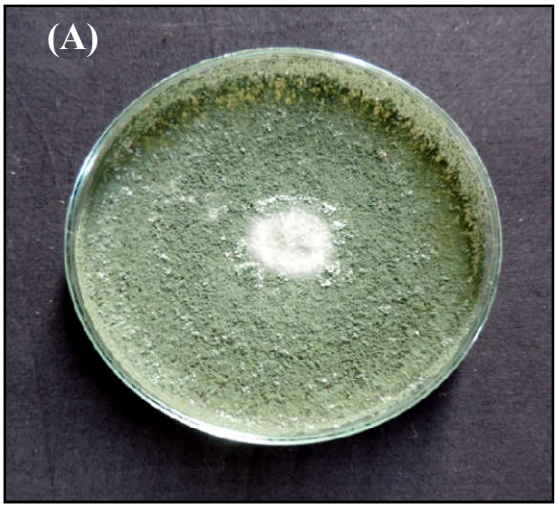
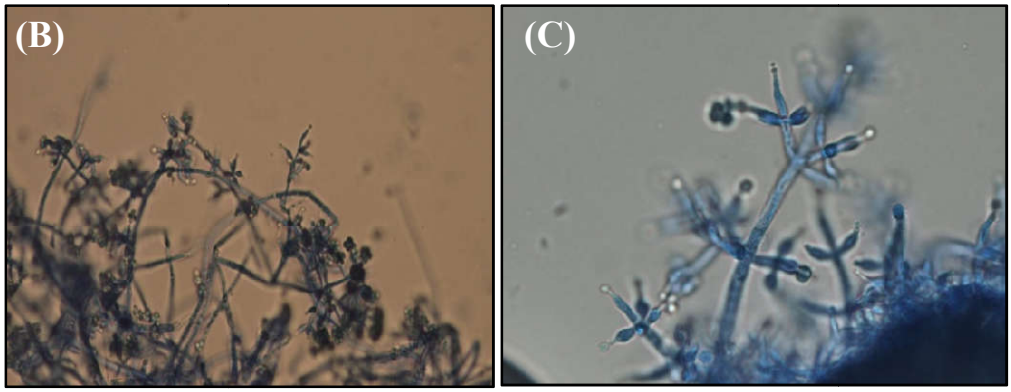



**Figure 4.4** Phylogenetic tree of *T. pseudokoningii* strain BHU R2 based on ITS sequence. Bootstrap values are based on 1000 replications. The isolate whose sequences have been submitted in NCBI Gen Bank are indicated through asterisk with their respective Accession number




**a)** Seven days old culture of *T. pseudokoningii* strain BHU R2 plate view




**b)** Microscopic view of *T. pseudokoningii* strain BHU R2

**Plate 3** *T. pseudokoningii* strain BHU R2 (a) Seven days old culture on PDA plate; (b) slide showing conidophore and conidia of *T. psudeokonii 4*0X magnification and (c) same as 100X magnification microscopic view

### 4.2 Evaluate the shelf life of encapsulating formulations

#### 4.2.1 Effect of gelatin encapsulation with different filling material on shelf life of *T. pseudokoningii* BHU R2

Three different mixing ratio; Mix A, Mix B and Mix C with *Trichoderma pseudokoningii* BHU R2 spores were filled in gelatin capsules separately. Capsules (Tricho-capsule) were stored at room temperature and the shelf life of the formulation was assessed through CFU count method at one month interval for six months. At initial stage, CFU count ($79.33 \times 10^7$ / g) was recorded to be similar in all the capsule formulations. After 30 days of storage, CFU count was found stable in capsule formulations in comparison to control. At 60 days after storage, CFU count was significantly decreased in the control (45±2.16) followed by Mix A (67.00±2.00), Mix B (69.33±1.83) and Mix C (78.67±2.08). There was significant decrease in *T. pseudokoningii* BHUR2 shelf life after 90 days of storage in control (unencapsulated). After 90 days of storage, significantly higher CFU count was recorded in Mix C with respective of other mixture and control. In Mix C *T. pseudokoningii* BHUR2 showed higher shelf life at 120 days (78.33±2.08), 150 days (77.00±2.12) and 180 days (76.17±2.21) in comparison to unencapsulated control (Figure 4.5).

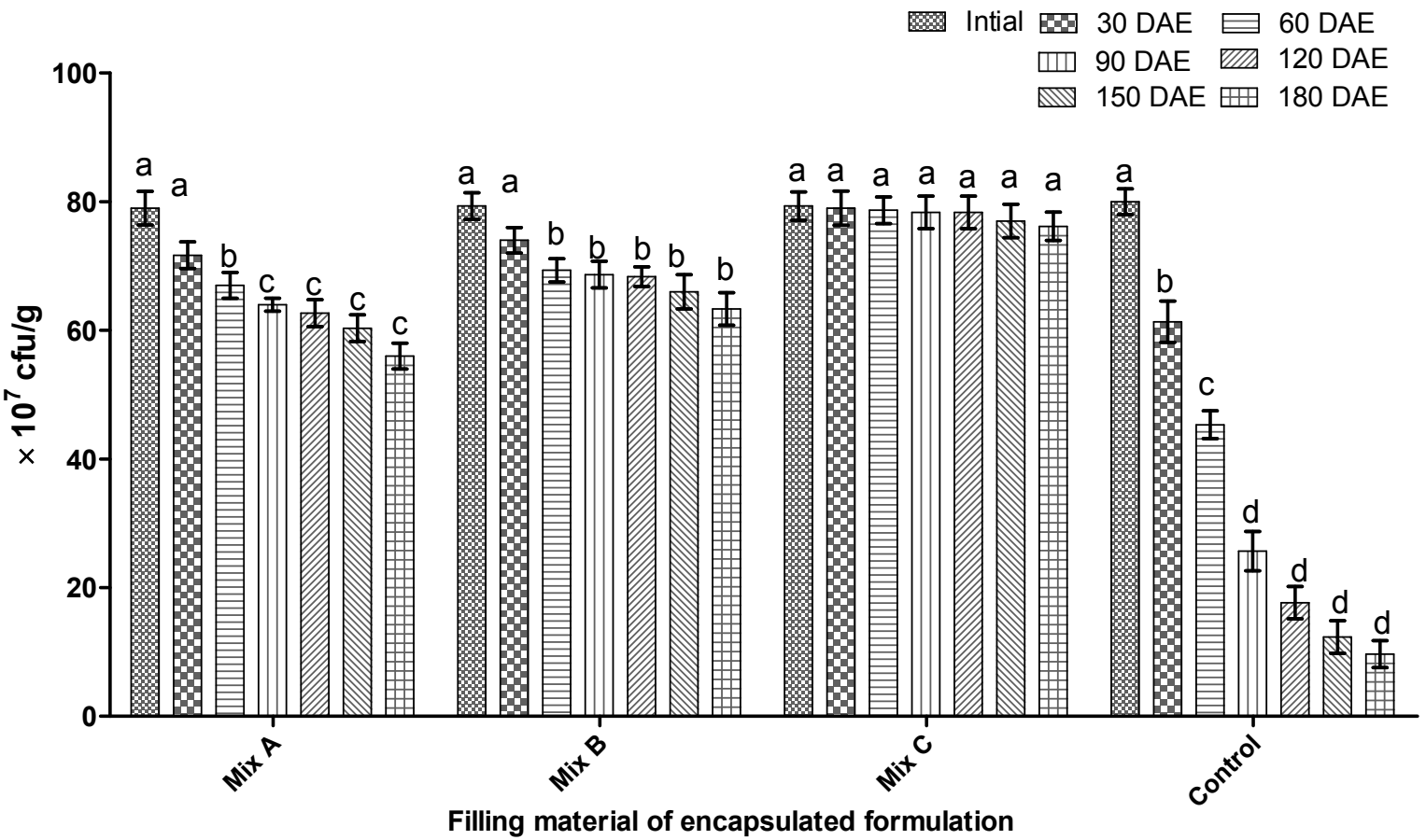



**Figure 4.5** Effect of gelatin encapsulation with different filling material on shelf life of *Trichoderma*. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at p 0.05

**Mix A** = Rice bran (84.9 g) + Okra Gum powder (5 g) + Rutin (0.1g) + *Trichoderma* (10g) filled in gelatin capsules

**Mix B** = Coal Powder (84.9 g) + Okra Gum powder (5 g) + Rutin (0.1g) + *Trichoderma* (10g) filled in gelatin capsules

**Mix C** = Rice bran (39.95 g) + Coal Powder (39.95 g) + Okra Gum powder (10 g) + Rutin (0.1 g) + *Trichoderma* (10g) filled in gelatin capsules

**Control** = Only *Trichoderma*, not filled in gelatin capsules

### 4.2.1 Effect of gelatin encapsulation of bioprimed seeds on shelf life of *T. pseudokoningii* BHU R2 spores

*T. pseudokoningii* BHU R2 spore suspension primed tomato seeds were encapsulated in hard gelatin capsules while unencapsulated primed seeds were used as control and stored at room temperature. The shelf life of *T. pseudokoningii* BHU R2 spores on primed seed surface was estimated by serial dilution and CFU count method at 1 month interval up to 6 months. Initial spore load on primed seeds surface was recorded $2.37\times10^7\pm$ 0.5 CFU per seed. After 30 days storage of encapsulated bioprimed seeds (EBS) and unencapsulated bioprimed seeds, CFU count was found significantly higher in EBS ($2.30\times10^7\pm0.5$) when compared with control. Significant decrease in CFU count was observed in unencapsulated primed seeds in comparison of EBS (Table 4.11). After 60 days of storage, highest CFU count was estimated in EBS ($2.30\times10^7\pm0.17$) when compared of unencapsulated bioprimed seeds ($1.07\times10^6\pm0.15$). Gradual decrease in CFU count of EBS was observed after 90 days of storage. After 180 days of storage, EBS recorded $1.57\times10^7\pm0.21$ CFU count while $0.77\times10^6\pm0.25$ CFU count was recorded in unencapsulated seeds.

**Table 4.11** Effect of bioprimed seed encapsulation on *T. pseudokoningii* BHU R2 spore shelf life different time intervals.

| Treatment | CFU count | | | | | | |
|---|---|---|---|---|---|---|---|
| | Initial CFU count | Days after Storage at room temperature | | | | | |
| | | 30 | 60 | 90 | 120 | 150 | 180 |
| *EBS | $2.37\times10^{7}\pm0.5$ | $2.30\times10^{7}\pm0.10$ | $2.30\times10^{7}\pm0.17$ | $2.23\times10^{7}\pm0.15$ | $1.97\times10^{7}\pm0.21$ | $1.77\times10^{7}\pm0.15$ | $1.57\times10^{7}\pm0.21$ |
| **Control | $2.37\times10^{7}\pm0.5$ | $1.10\times10^{7}\pm0.10$ | $1.07\times10^{6}\pm0.15$ | $0.93\times10^{6}\pm0.15$ | $0.90\times10^{6}\pm0.26$ | $0.83\times10^{5}\pm0.12$ | $0.77\times10^{6}\pm0.25$ |
| CD (5%) | - | 0.226 | 0.370 | 0.471 | 0.539 | 0.306 | 0.523 |

Results are expressed as means of three replicates and ± indicate standard deviations of the means.

***EBS = Encapsulated bioprimed seed**

****Control = Unencapsulated bioprimed seed**

## 4.3 Impact of encapsulated formulations on plant growth promotion and biological control potential against *S. rolfsii* in tomato.

### 4.3.1 Effect of Tricho-capsule treatments on plant growth promotion and biological control of *S. rolfsii*

#### 4.3.1.1 Effect of treatments on mortality of tomato plants

The field experiment was conducted to assess the effect of *T. pseudokoningii* BHU R2 on diseased tomato plants, caused by pathogen *S. rolfsii*. Seeds primed with *T. pseudokoningii* BHU R2 spore suspension showed significant reduction in mortality of tomato plants (Plate 4). The least mortality was observed in treatment T5 (16±0.89) followed by treatment T6 (22±0.84) in comparison to pathogen challenged control plant (82±0.84) [Figure 4.6].

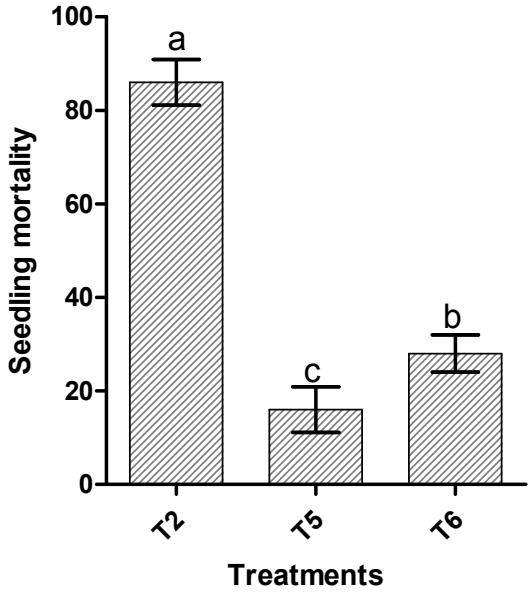



**Figure 4.6** Effect of treatments on the seedling mortality of tomato plants. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T2 = Pathogen challenged only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

**(A)**

**(B)**

**(C)**

**Plate 4 (A)** Tomato plant infected with *S. rolfsii*; **(B)** Basal region of stem completely colonized by the sclerotia and **(C)** Healthy tomato plant treated with *T. pseudokoningii* BHU R2

#### 4.3.1.2 Effect of different *Trichoderma* application methods on plant growth promoting and yield attributing traits

Shoot length, root length, lateral root numbers, fresh and dry weight of shoot & root were recorded after 30 days of transplanting while numbers of fruits per plant and average fruit yield per plant was recorded after 60 days of transplanting.

**Plant growth promoting traits**

- **Shoot length (cm)**

  Increase in shoot length was recorded in *T. pseudokoningii* BHU R2 treated treatments during pathogen challenged condition. Shoot length was observed significantly higher in treatment T3 (34.78±1.47), followed by T5 (27.75±1.55), T4 (22.77±1.72), T6 (18.85±1.588) in comparison to T1 (16.04±1.41) and T2 (12.09±1.41) [Figure 4.7(a); Plate 5(A)].

- **Root length (cm)**

  Root length was also recorded higher in *T. pseudokoningii* BHU R2 treated treatments as compared to other treatment. Root length was observed significantly higher in treatment T3 (28.63±1.25), followed by T5 (21.77±1.34), T4 (19.33±1.19), T6 (16.76±1.18) in comparison to T1 (13.45±1.40) and T2 (8.80±1.23) [Figure 4.7(b); Plate 5(B)].

- **Lateral root numbers**

  Increase in lateral root numbers was recorded in *T. pseudokoningii* BHU R2 treated treatments. Number of lateral root were observed significantly higher in treatment T3 (32.29±1.24), followed by T5 (27.35±1.50), T4 (23.69±1.44), T6 (16.95±1.57), T1 (15.95±1.57) and T2 (13.68±1.40) [Figure 4.8].

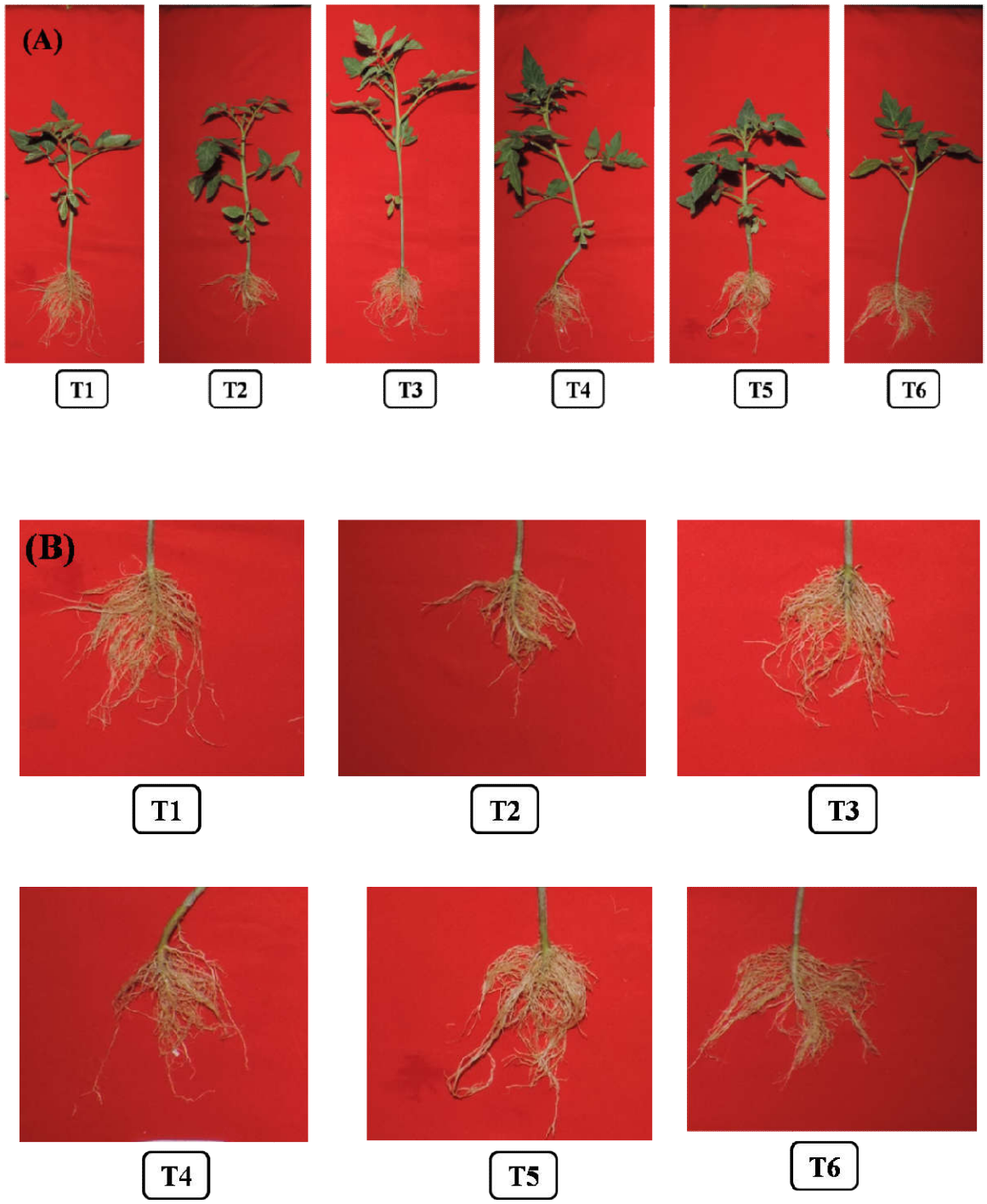



**Plate 5** Effect of different application methods of *Trichoderma* on shoot (A) and root (B) length. T1 = Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

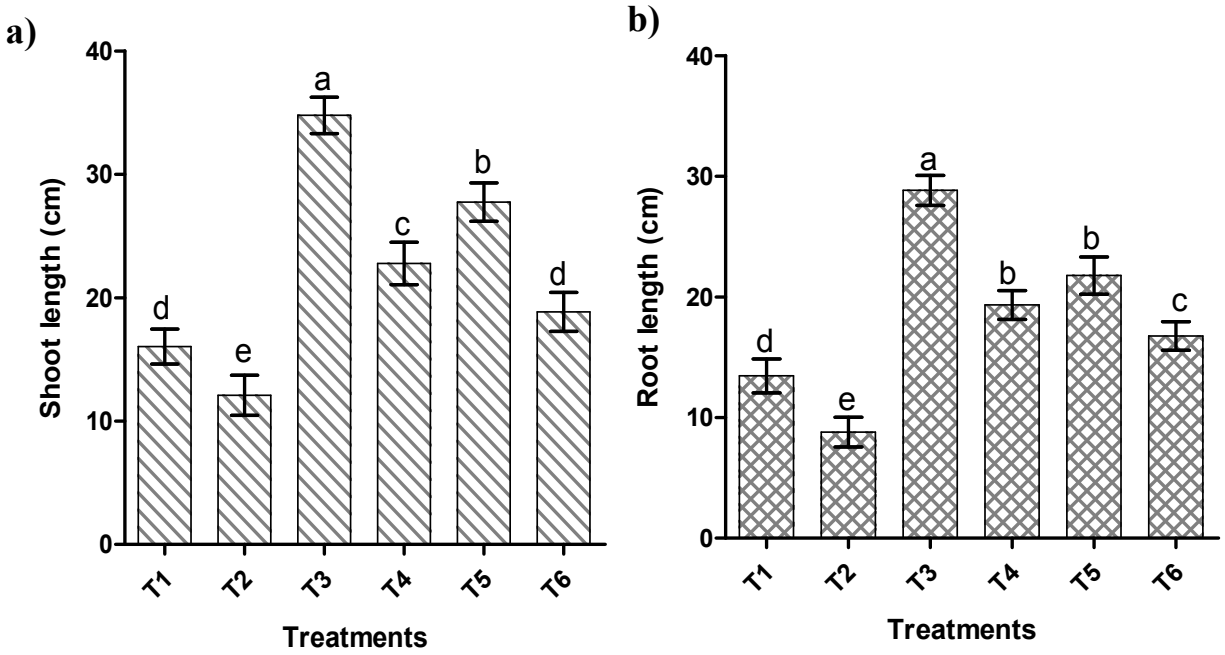



**Figure 4.7** Effect of different application methods of *Trichoderma* on shoot (a) and root (b) length. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 = Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

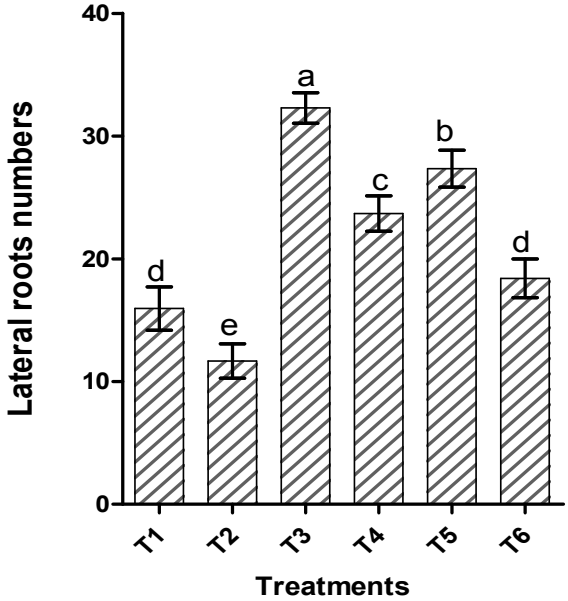



**Figure 4.8** Effect of different application methods of *Trichoderma* on lateral root numbers. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Ducan's multiple range test at $p \leq 0.05$; T1 = Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged

- **Determinations of fresh and dry weight (g) of shoot**

The increase in shoot fresh and dry weight was observed in *T. pseudokoningii* BHUR2 treatments in comparison to other treatments (Figure 4.9). Shoot fresh weight was recorded significantly higher in treatment T3 (32.29±1.24), followed by T5 (3.91±0.21), T4 (3.63±0.22), T6 (2.87±0.19) in comparison to T1 (2.05±0.25) and T2 (1.14±0.27). Similarly, shoot dry weight was recorded significantly higher in treatment T3 (0.82±0.06), followed by T5 (0.57±0.03), T4 (0.48±0.04), T6 (0.36±0.04) in comparison to T1 (0.24±0.05) and T2 (0.14±0.03).

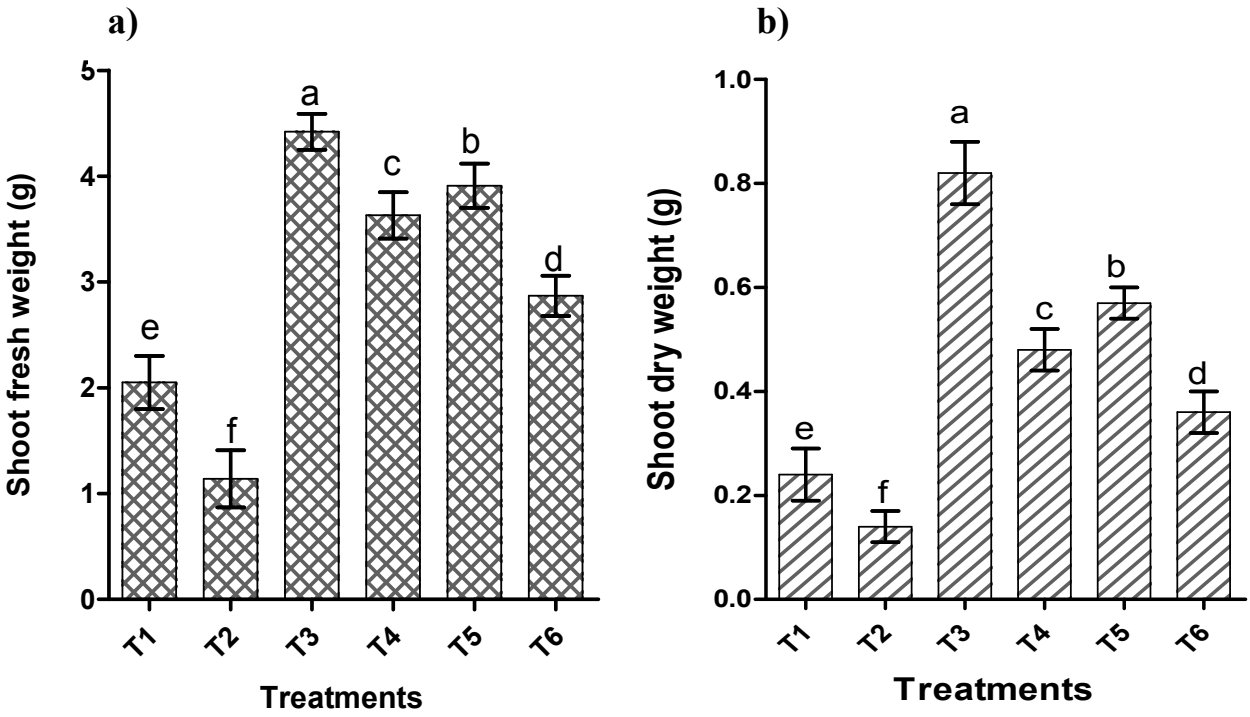



**Figure 4.9** Effect of different application methods of *Trichoderma* on shoot fresh (a) and dry (b) weight. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 = Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged

- **Determinations of fresh and dry weight (g) of Root**

The increased Root fresh and dry weight was observed in *T. pseudokoningii* BHUR2 treatments (Figure 4.10). Root fresh weight was recorded significantly highest in treatment T3 (2.77±0.12), followed by T5 (2.23±0.14), T4 (1.73±0.16), T6 (1.39±0.12), T1 (1.04±0.15) and T2 (0.68±0.13). Similarly, root dry weight was recorded significantly highest in treatment T3 (0.41±0.02), followed by T5 (0.34±0.03), T4 (0.25±0.02), T6 (0.18±0.03), T1 (0.10±0.03) and T2 (0.03±0.01).

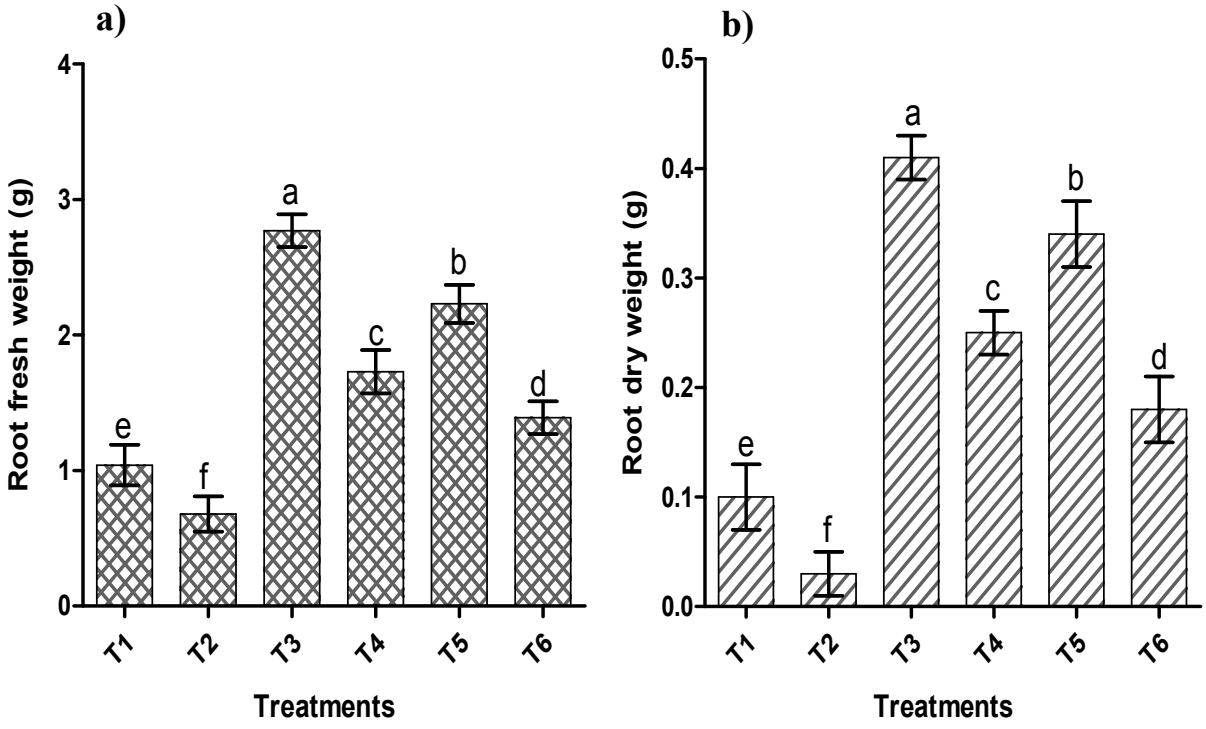



**Figure 4.10** Effect of different application methods of *Trichoderma* on root fresh (a) and dry (b) weight. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 = Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

**Yield attributing traits**

- **Numbers of fruits per plant**

An increased number of fruits per plant as recorded in *T. pseudokoningii* BHUR2 treated plants (Figure 4.11). Number of fruits was observed significantly higher in treatment T3 (26.01±1.44), followed by T5 (21.81±1.36), T4 (18.03±1.14), T6 (14.03±1.27) in comparison to T1 (12.68±1.34) and T2 (9.24±1.21).

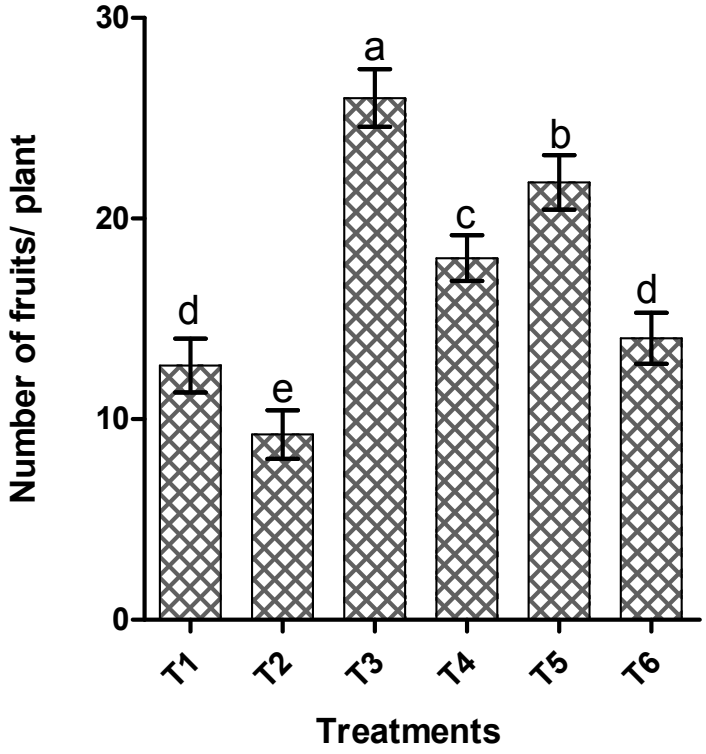



**Figure 4.11** Effect of different application methods of *Trichoderma* on number of fruits per plant. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 = Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

- **Average fruit yield (kg/plant)**

An increased in average fruit yield (kg/ plant) was recorded in *T. pseudokoningii* BHUR2 treatments (Figure 4.12). Number of fruits was observed significantly higher in treatment T3 (26.01±1.44), followed by T5 (21.81±1.36), T4 (18.03±1.14), T6 (14.03±1.27) in comparison to T1 (12.68±1.34) and T2 (9.24±1.21).

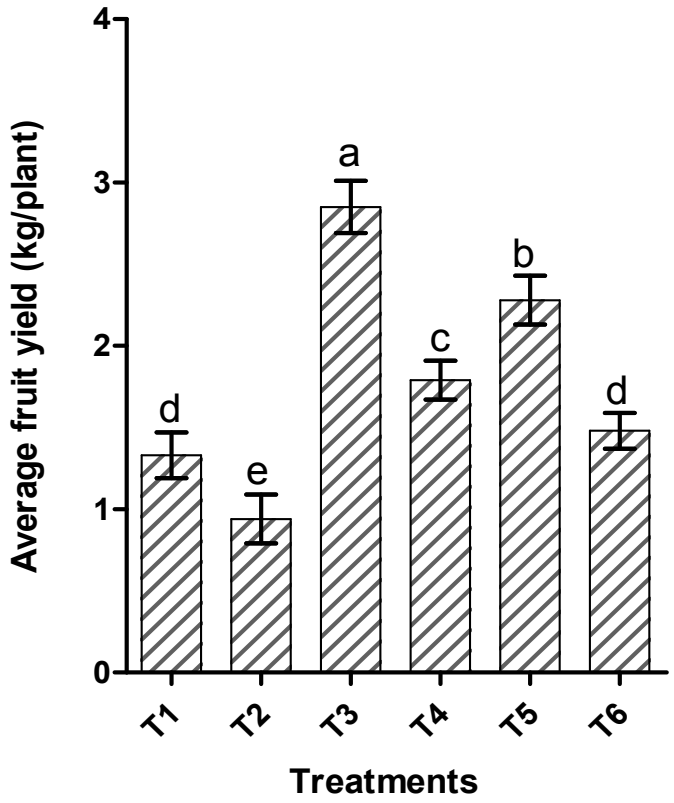



**Figure 4.12** Effect of different application methods of *Trichoderma* on average fruit yield (kg/ plant). Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 = Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged

#### 4.3.1.3 Effect of treatments on anti-oxidative enzyme activities and phenol content in tomato plant

- **Anti-oxidative enzyme activities in tomato plant**

The effect of treatments on SOD & POx activity and MDA content were estimated over a time spawn of 4 days at 24 h intervals after the pathogen inoculation.

1. **SOD activity**

The SOD activity was increased consistently and attained their maximum levels at 72h followed by 48h after inoculation. The SOD activity was recorded significantly higher in all the *Trichoderma* inoculated treatments in comparison to pathogen inoculated and un-inoculated control, while the highest activity was estimated in the seeds primed by high temperature tolerant *T. pseudokoningii* BHUR2 spore suspension (Figure 4.13). The SOD activity was recorded 1.52 and 1.34 folds increased in T5 and T6 respectively, as compared to pathogen inoculated control (T2) at 72 h.

2. **POx activity**

The POx activity was increased consistently and attained their maximum levels at 48 h followed by 24 h after inoculation. The POx activity was recorded significantly enhanced in all the *Trichoderma* inoculated treatments in comparison to pathogen inoculated and un-inoculated control, while the highest activity was estimated in the seeds primed by high temperature tolerant *T. pseudokoningii* BHUR2 spore suspension. The POx activity was recorded 1.31 and 1.12 folds increased in T5 and T6 respectively, as compared to pathogen inoculated control (T2) at 48 h (Figure 4.14).

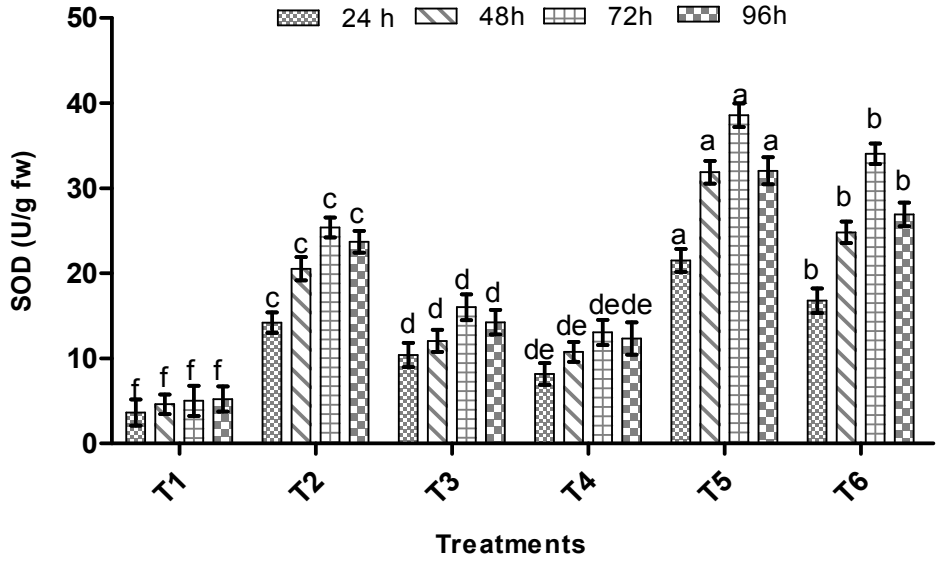



**Figure 4.13** Effect of different application methods of *Trichoderma* on Superoxide dismutase activity (SOD) at different time intervals. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

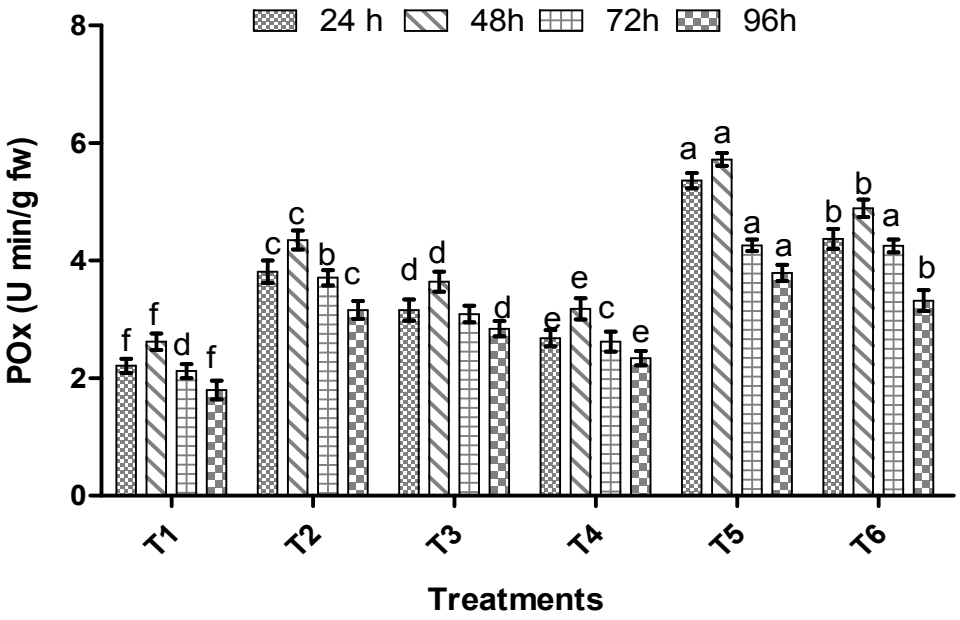



**Figure 4.14** Effect of different application methods of *Trichoderma* on peroxidase activity (POx) at different time intervals. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 = Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged

### 3. MDA content

The MDA content was increased consistently and attained their maximum levels at 96 h followed by 24 h after inoculation in pathogen inoculated control. The MDA content was significantly reduced in all the *Trichoderma* inoculated treatments in comparison to pathogen inoculated control, while the highest reduction in MDA content was estimated in the seeds primed by high temperature tolerant *T. pseudokoningii* BHUR2 spore suspension. The MDA content was recorded 3.79 and 2.64 folds reduced in T5 and T6 respectively, as compare to pathogen inoculated control (T2) at 96 h (Figure 4.15).

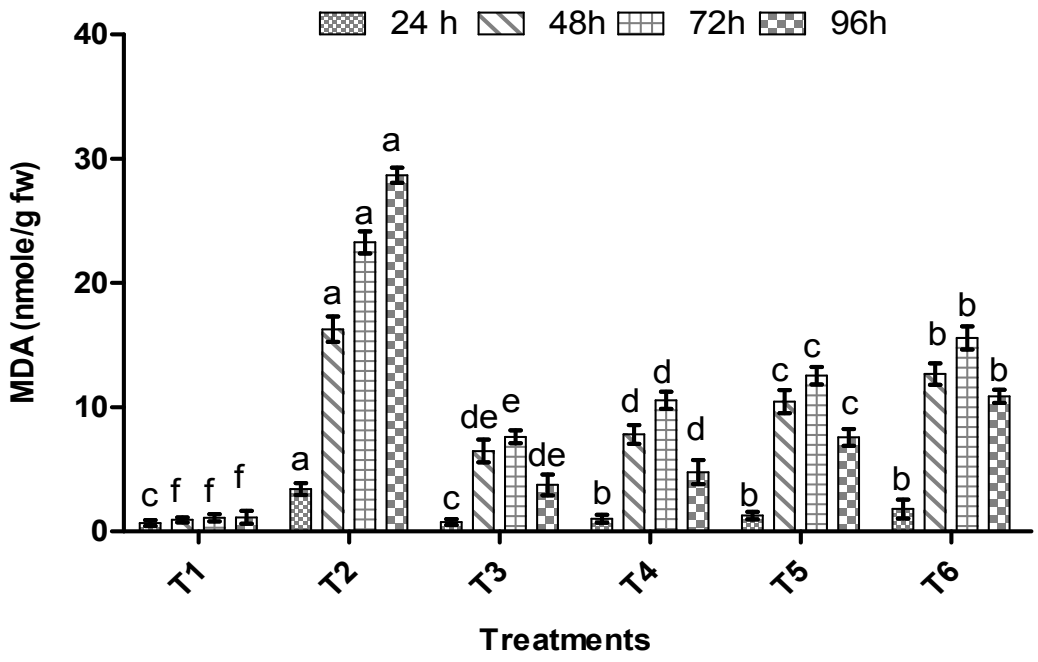



**Figure 4.15** Effect of different application methods of *Trichoderma* on malondialdehyde (MDA) content at different time intervals. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

**Assessment of phenol content in plant**

The effect of treatments on PAL and TPC content were estimated over a time spawn of 4 days at 24 hr intervals after of pathogen inoculation.

**4. Phenylalanine ammonia lyase (PAL) activity**

Phenylalanine ammonia lyase (PAL) activity increased consistently and attained its maximum level at 72h followed by 24h after inoculation. The PAL activity was significantly enhanced in all the *Trichoderma* inoculated treatments in comparison to pathogen inoculated and un-inoculated control, while the highest activity was estimated in the seeds primed by high temperature tolerant *T. pseudokoningii* BHUR2 spore suspension. The PAL activity was recorded 1.46 and 1.31 folds increased in T5 and T6 respectively, as compared to pathogen inoculated control (T2) at 72 h (Figure 4.16).

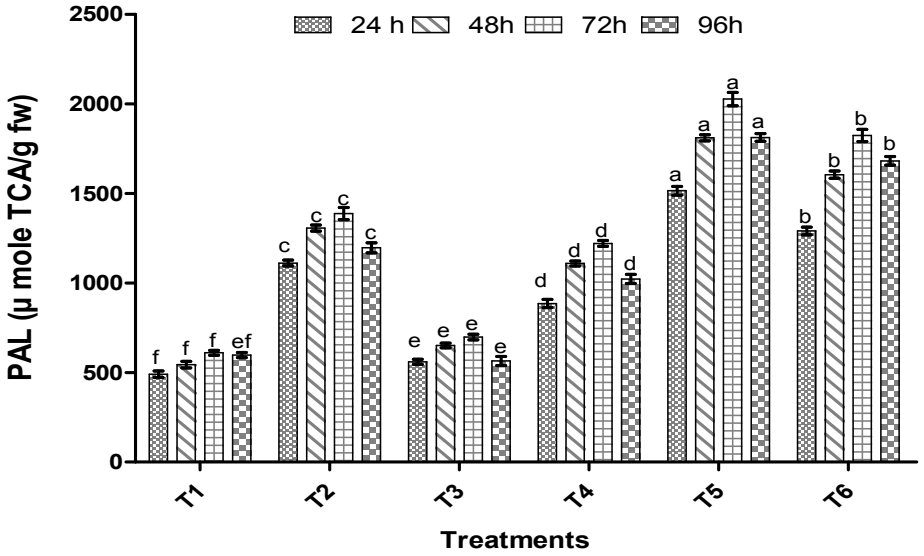



**Figure 4.16** Effect of different application methods of *Trichoderma* on phenylalanine ammonia lyase (PAL) activity at different time intervals. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$. T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged

## 5. Total phenol content (TPC) in plant

Total phenol content (TPC) increased consistently and attained its maximum level at 48h followed by 24h after inoculation. TPC was significantly enhanced in all the *Trichoderma* inoculated treatments in comparison to pathogen inoculated and un-inoculated control, while the highest activity was estimated in the seeds primed by high temperature tolerant *T. pseudokoningii* BHUR2 spore suspension. The TPC was recorded 1.69 and 1.44 folds increased in T5 and T6 respectively, as compared to pathogen inoculated control (T2) at 48 h (Figure 4.17).

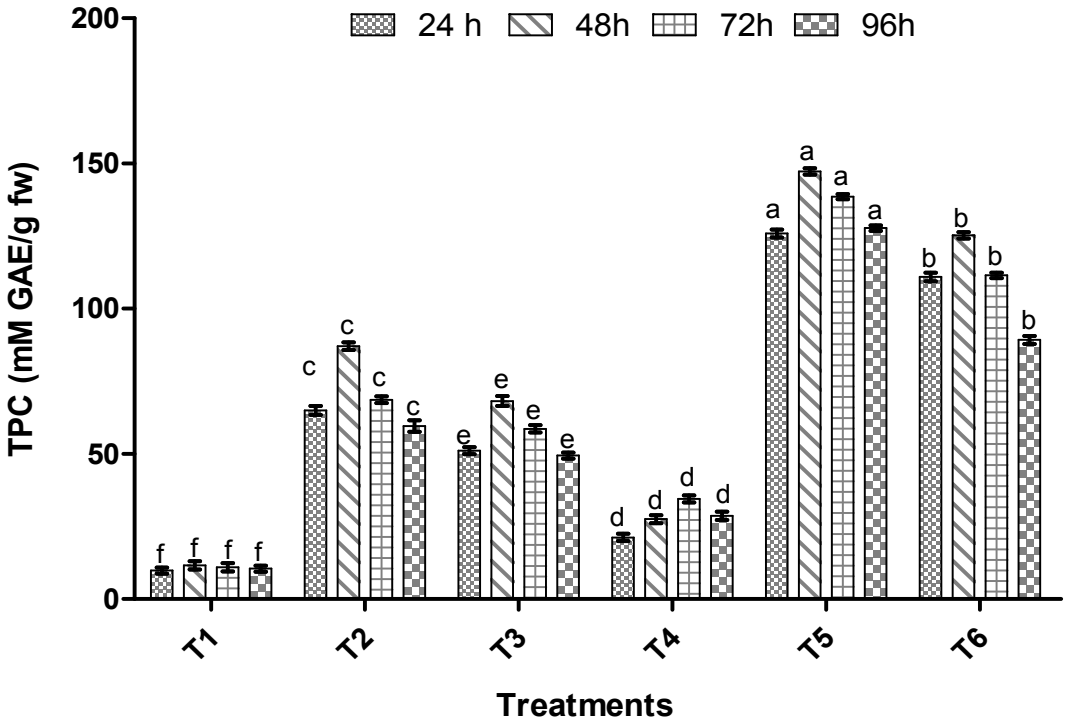



**Figure 4.17** Effect of different application methods of *Trichoderma* on total phenol content (TPC) at different time intervals. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

#### 4.3.1.4 Effect of treatments on nutritional content of tomato fruits

The amount of ascorbic acid, lycopene, total carbohydrate and protein were significantly increased in all *Trichoderma* inoculated treatments as compared to pathogen challenged and non-pathogen challenged control.

- **Ascorbic acid content**

Ascorbic acid content was recorded significantly higher in all the *Trichoderma* inoculated treatments in comparison to pathogen inoculated and un-inoculated control. Ascorbic acid content was recorded significantly increase in treatment T3 (29.11±1.30), followed by T5 (24.27±1.40), T4 (20.26±1.50), T6 (16.50±1.10) in comparison to T1 (12.28±1.50) and T2 (7.73±1.20) [Figure 4.18].

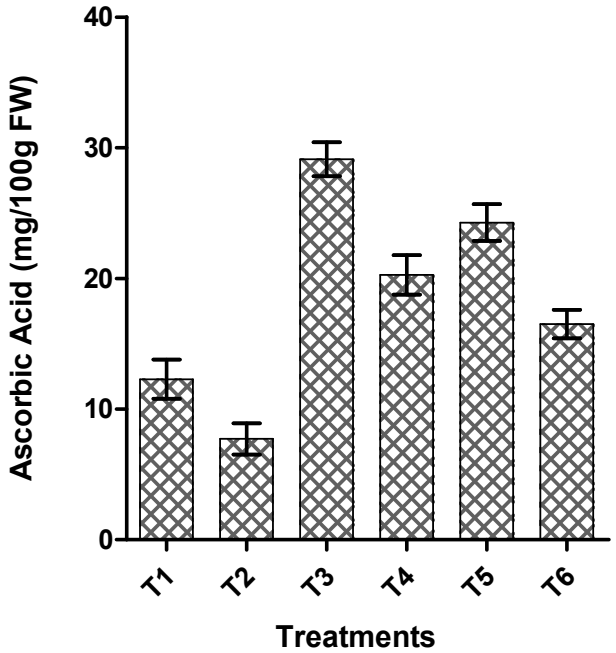



**Figure 4.18** Effect of different application methods of *Trichoderma* on ascorbic acid content of tomato fruit. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

- **Lycopene content**

Lycopene content was significantly enhanced in all the *Trichoderma* inoculated treatments in comparison to pathogen inoculated and un-inoculated control. Lycopene content was significantly increased in treatment T3 (48.11±1.64), followed by T5 (41.48±1.85), T4 (36.05±1.63), T6 (31.85±1.44) in comparison to T1 (17.78±1.27) and T2 (10.59±1.61) [Figure 4.19].

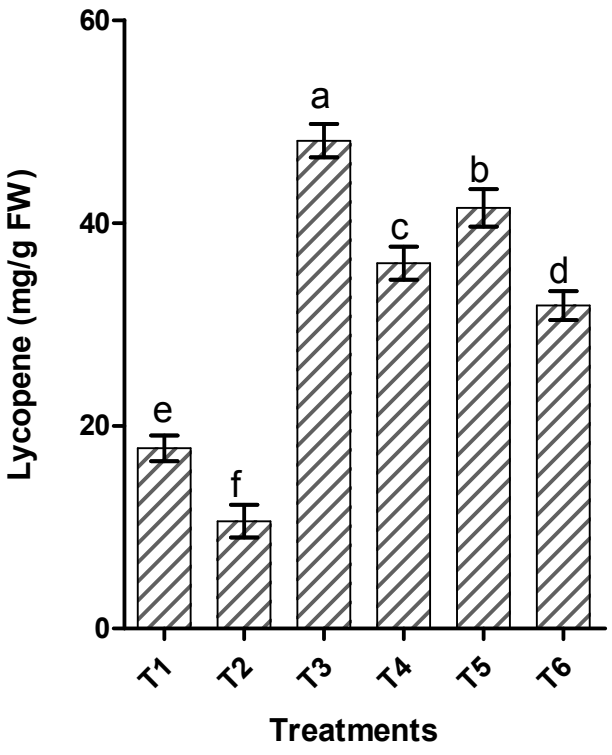



**Figure 4.19** Effect of different application methods of *Trichoderma* on lycopene content of tomato fruit. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

- **Total carbohydrate content**

Total carbohydrate content was recorded significantly increased in all the *Trichoderma* inoculated treatments in comparison to pathogen inoculated and un-inoculated control. Total carbohydrate content was recorded significantly increased in treatment T3 (3.96±0.23), followed by T5 (3.41±0.20), T4 (2.69±0.18), T6 (1.95±0.25) in comparison to T1 (0.70±0.13) and T2 (0.34±0.14) [Figure 4.20].

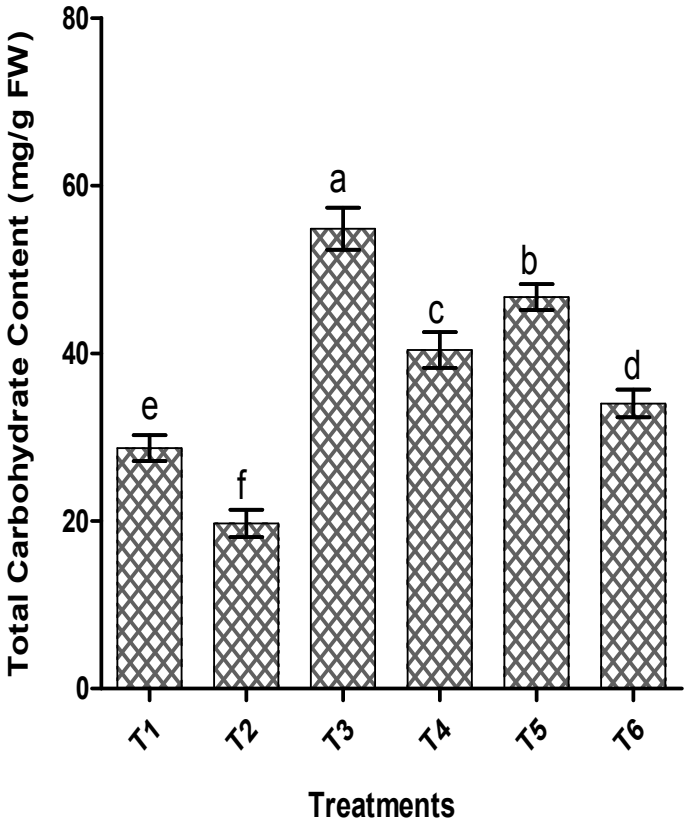



**Figure 4.20** Effect of different application methods of *Trichoderma* on total carbohydrate content of tomato fruit. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

- **Protein content**

Protein content was significantly enhanced in all the *Trichoderma* treated treatments in comparison to pathogen inoculated and un-inoculated control. Protein content was significantly increased in treatment T3 (3.96±0.23), followed by T5 (3.41±0.20), T4 (2.69±0.18), T6 (1.95±0.25) in comparison to T1 (0.70±0.13) and T2 (0.34±0.14) [Figure 4.21].

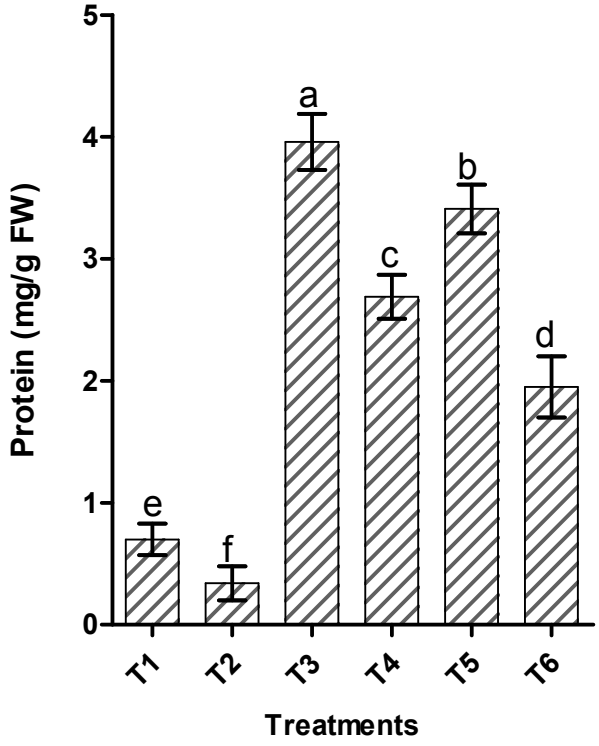



**Figure 4.21** Effect of different application methods of *Trichoderma* on protein content of tomato fruit. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

#### 4.3.1.4 Effect of treatments on mineral content of tomato fruits

A significant increase in minerals content of tomato fruits were noticed in all *Trichoderma* treated treatments as compared to pathogen challenged control. The highest change in nitrogen, phosphorus and potassium content was observed in tomato seeds were primed with high temperature tolerant *Trichoderma* spore suspension as compared to pathogen challenged and non-pathogen challenged control.

- **Nitrogen content**

Nitrogen content was significantly higher in all the *Trichoderma* treatments in comparison to pathogen inoculated and un-inoculated control. Nitrogen content was recorded significantly 2.27, 1.88, 1.66 and 1.45 folds increase in treatment T3, T5, T4 and T6 in comparison to T2 respectively (Figure 4.22).

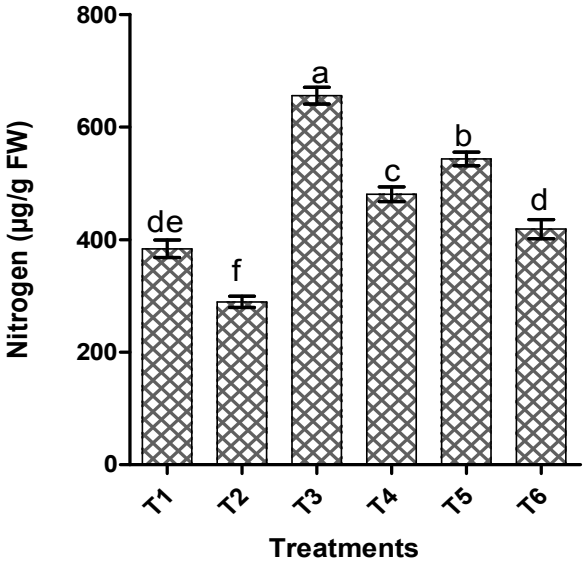



**Figure 4.22** Effect of different application methods of *Trichoderma* on nitrogen content of tomato fruit. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

- **Phosphorus content**

Phosphorus content was significantly enhanced in all the *Trichoderma* treated treatments in comparison to pathogen inoculated and un-inoculated control. Nitrogen content was recorded significantly 2.21, 1.74, 1.93 and 1.40 folds increase in treatment T3, T5, T4 and T6 in comparison to T2 respectively (Figure 4.23).

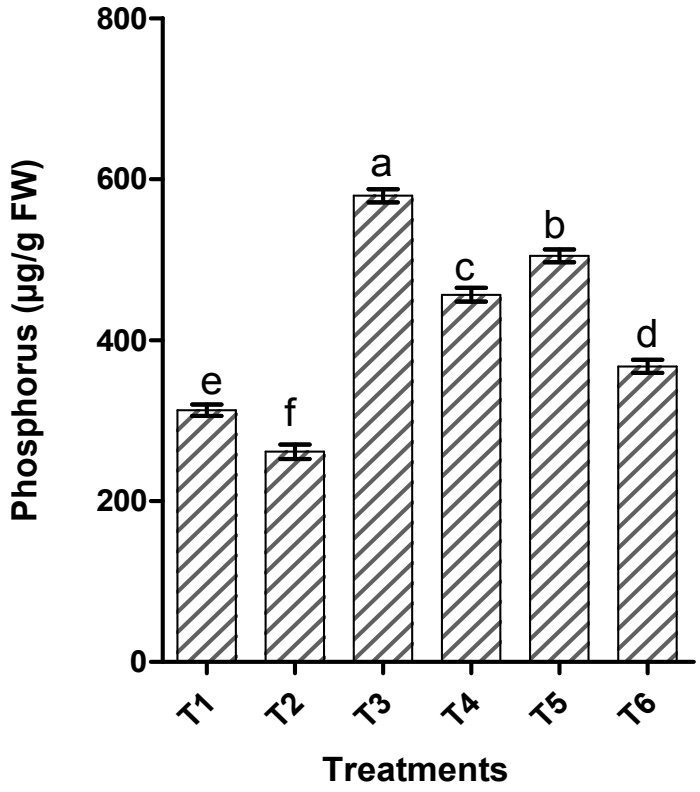



**Figure 4.23** Effect of different application methods of *Trichoderma* on phosphorus content of tomato fruit. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

- **Potassium content**

Potassium content was significantly increased in all the *Trichoderma* treatments in comparison to pathogen inoculated and un-inoculated control. Potassium content was recorded significantly 3.22, 2.80, 2.48 and 1.91 folds increase in treatment T3, T5, T4 and T6 in comparison to T2 respectively (Figure 4.24).

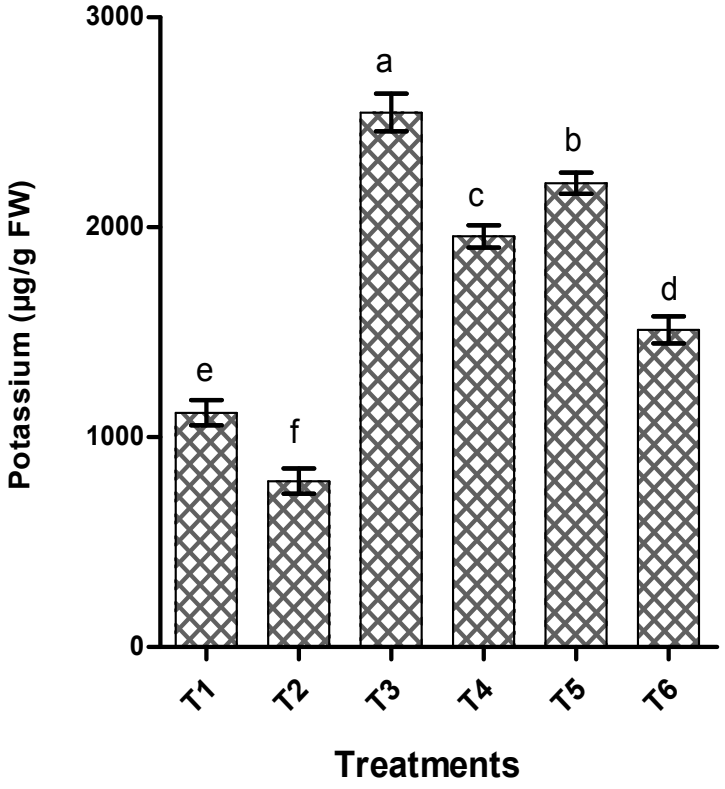



**Figure 4.24** Effect of different application methods of *Trichoderma* on potassium content of tomato fruit. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

- **Calcium content**

Calcium content was significantly enhanced in all the *Trichoderma* treated treatments in comparison to pathogen inoculated and un-inoculated control. Calcium content was recorded significantly 2.84, 2.32, 1.96 and 1.56 folds increase in treatment T3, T5, T4 and T6 in comparison to T2 respectively (Figure 4.25).

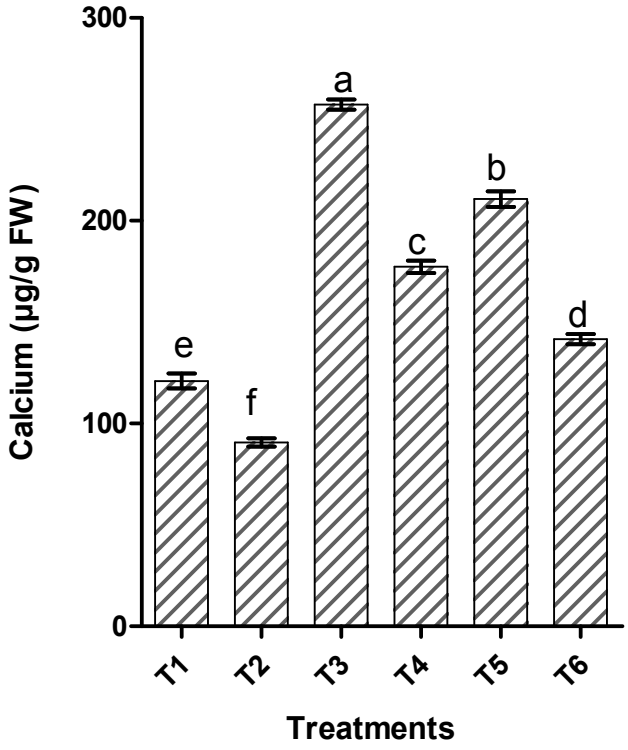



**Figure 4.25** Effect of different application methods of *Trichoderma* on calcium content of tomato fruit. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 = Pathogen challenged only; T3 = Biopriming only; T4 = Root dipping only; T5 = Biopriming + Pathogen challenged; T6 = Root dipping + Pathogen challenged.

### 3.9.2. Effect of encapsulated bioprimed seeds on nutrient use efficiency and plant growth promotion

Effect of encapsulated bioprimed seeds on plant growth promoting traits under different doses of fertilizer were estimated after 30 days of seed germination. Results showed increase in plant biomass, shoot length, root length and lateral root numbers in treatment T2 in comparison to control T1 treatment (Plate 6).

**Plant growth promoting traits**

- **Shoot length (cm)**

  Shoot length was significantly higher in treatment T2 (22.65±1.53), followed by T4 (19.38±1.69), T3 (15.43±1.44) in comparison to T1 (10.93±1.73) respectively (Figure 4.26).

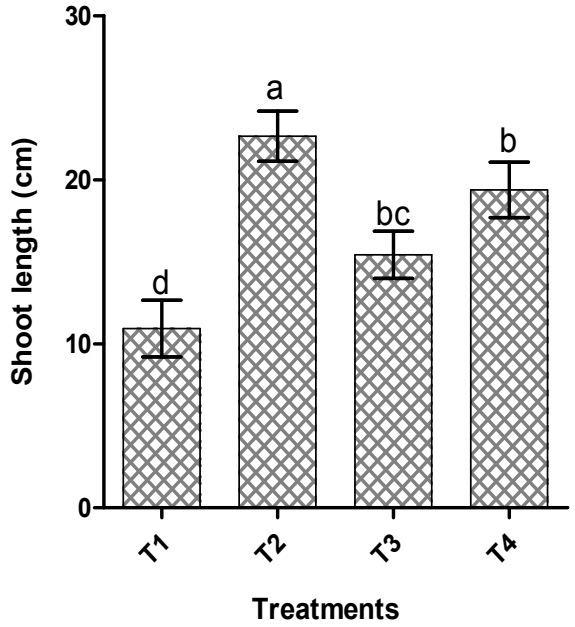



**Figure 4.26** Effect of encapsulated bioprimed tomato seeds under different fertilizer dose on shoot length. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 =75% RDF+EBS; T3 = RDF + Unprimed seed; T4 = Only EBS. RDF (Recommended dose of fertilizer); EBS (Encapsulated bioprimed seed)

- **Root length (cm)**

  Root length was significantly enhanced in treatment T2 (18.86±1.19), followed by T4 (15.21±1.14), T3 (12.39±1.21) ,T1 (9.60±1.15) [Figure 4.27].

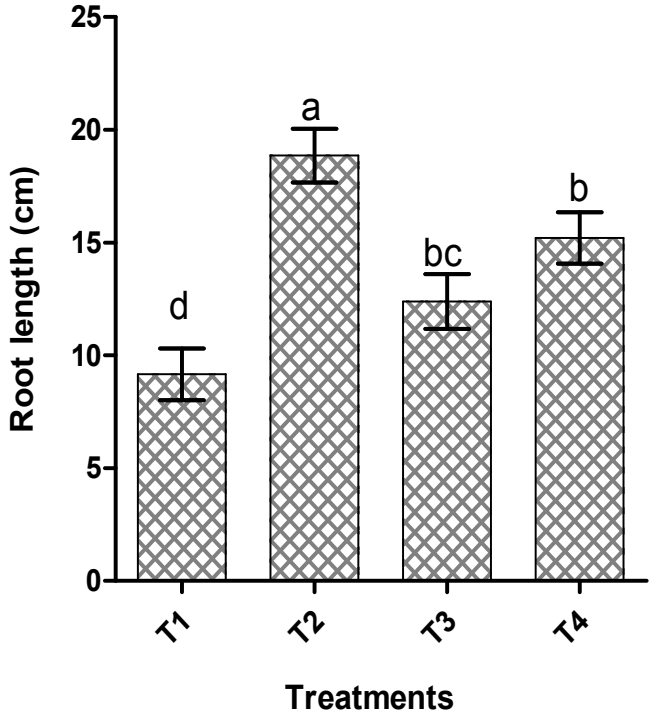



**Figure 4.27** Effect of encapsulated bioprimed tomato seed under different fertilizer dose on root length. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 =75% RDF+EBS; T3 = RDF + Unprimed seed; T4 = Only EBS. RDF (Recommended dose of fertilizer); EBS (Encapsulated bioprimed seed)

- **Lateral root numbers**

Lateral root numbers was significantly increased in treatment T2 (21.66±1.18), followed by T4 (18.92±1.14), T3 (14.24±1.16) in comparison to T1 (10.46±1.12) [Figure 4.28].

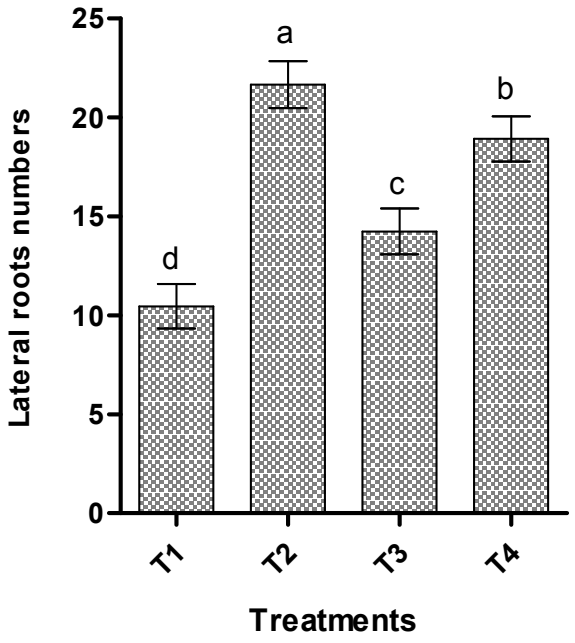



**Figure 4.28** Effect of encapsulated bioprimed tomato seed under different fertilizer dose on lateral root numbers. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 =75% RDF+EBS; T3 = RDF + Unprimed seed; T4 = Only EBS. RDF (Recommended dose of fertilizer); EBS (Encapsulated bioprimed seed)

- **Determinations of fresh and dry weight (g) of shoot**

  Shoot fresh weight was significantly enhanced in T2 (2.48±0.14), followed by T4 (1.97±0.13), T3 (1.62±0.12) in comparison to T1 (1.28±0.10) respectively [Figure 4.29 (a)]. Similarly, shoot dry weight was significantly increased in treatment T2 (0.193±0.007), followed by T4 (0.142±0.004), T3 (0.116±0.005), T1 (0.084±0.006) [Figure 4.29 (b)].

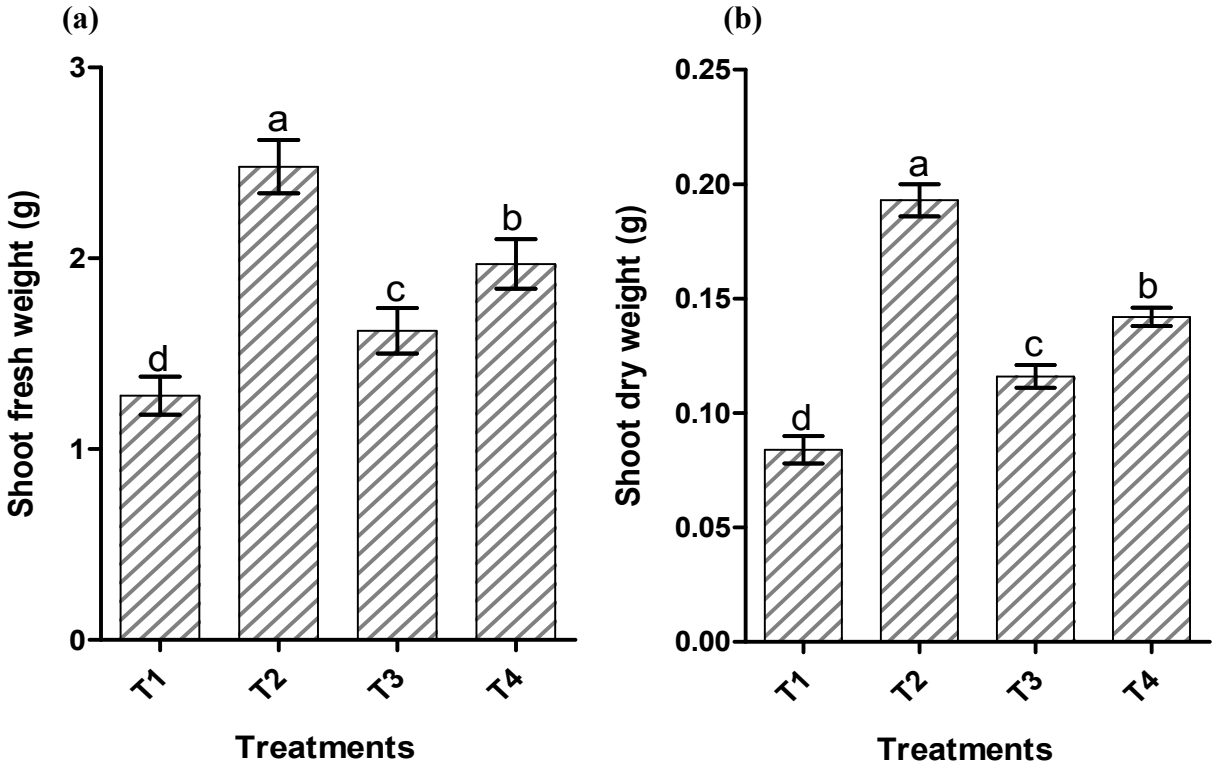



**Figure 4.29** Effect of encapsulated bioprimed tomato seed under different fertilizer dose on shoot fresh (a) and dry (b) weight. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 =75% RDF+EBS; T3 = RDF + Unprimed seed; T4 = Only EBS. RDF (Recommended dose of fertilizer); EBS (Encapsulated bioprimed seed)

- **Determinations of fresh and dry weight (g) of root**

  Root fresh weight was significantly increased in T2 (2.14±0.14), followed by T4 (1.79±0.12), T3 (1.54±0.12) in comparison to T1 (1.18±0.10) respectively [Figure 4.30 (a)]. Similarly, root dry weight was significantly increased in treatment T2 (0.051±0.015), followed by T4 (0.124±0.013), T3 (0.098±0.012), T1 (0.051±0.015) [Figure 4.30 (b)].

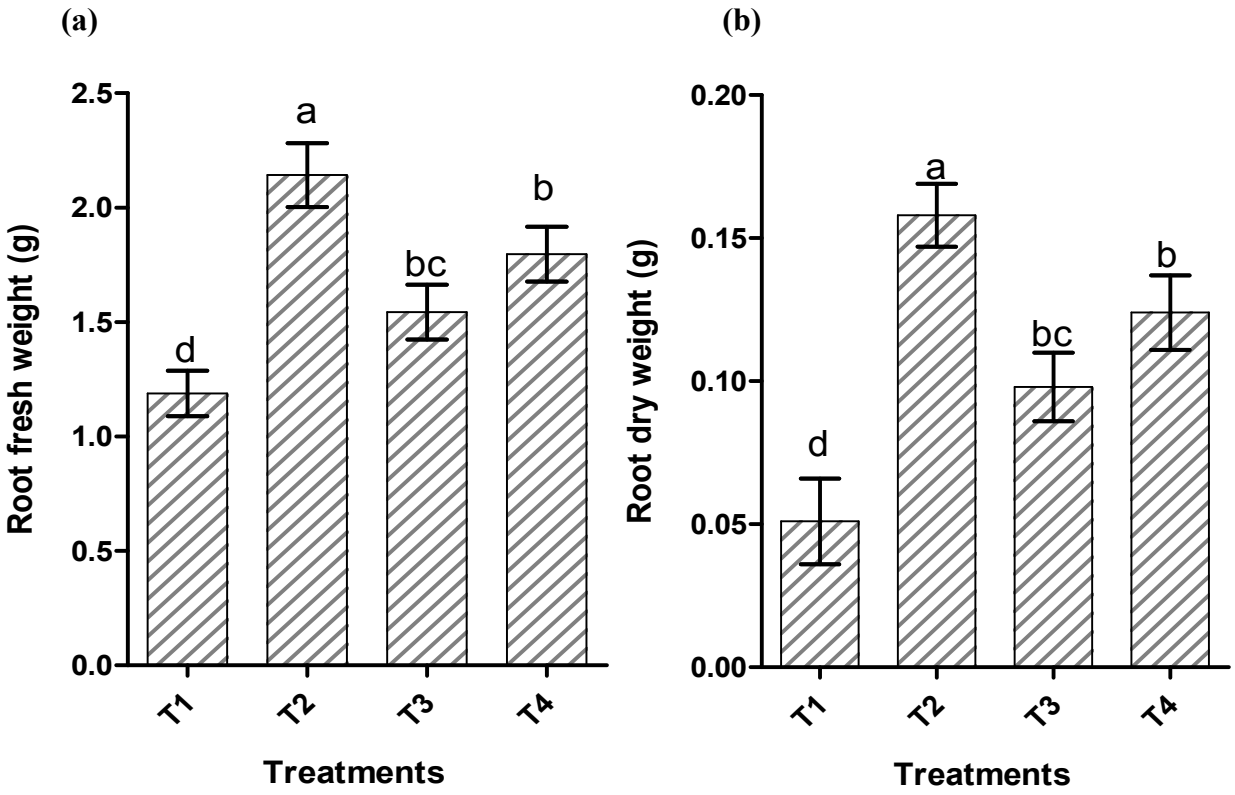



**Figure 4.30** Effect of encapsulated bioprimed tomato seed under different fertilizer dose on root fresh (a) and dry (b) weight. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Different letters indicates significant differences between treatments taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$; T1 =Control; T2 =75% RDF+EBS; T3 = RDF + Unprimed seed; T4 = Only EBS. RDF (Recommended dose of fertilizer); EBS (Encapsulated bioprimed seed)

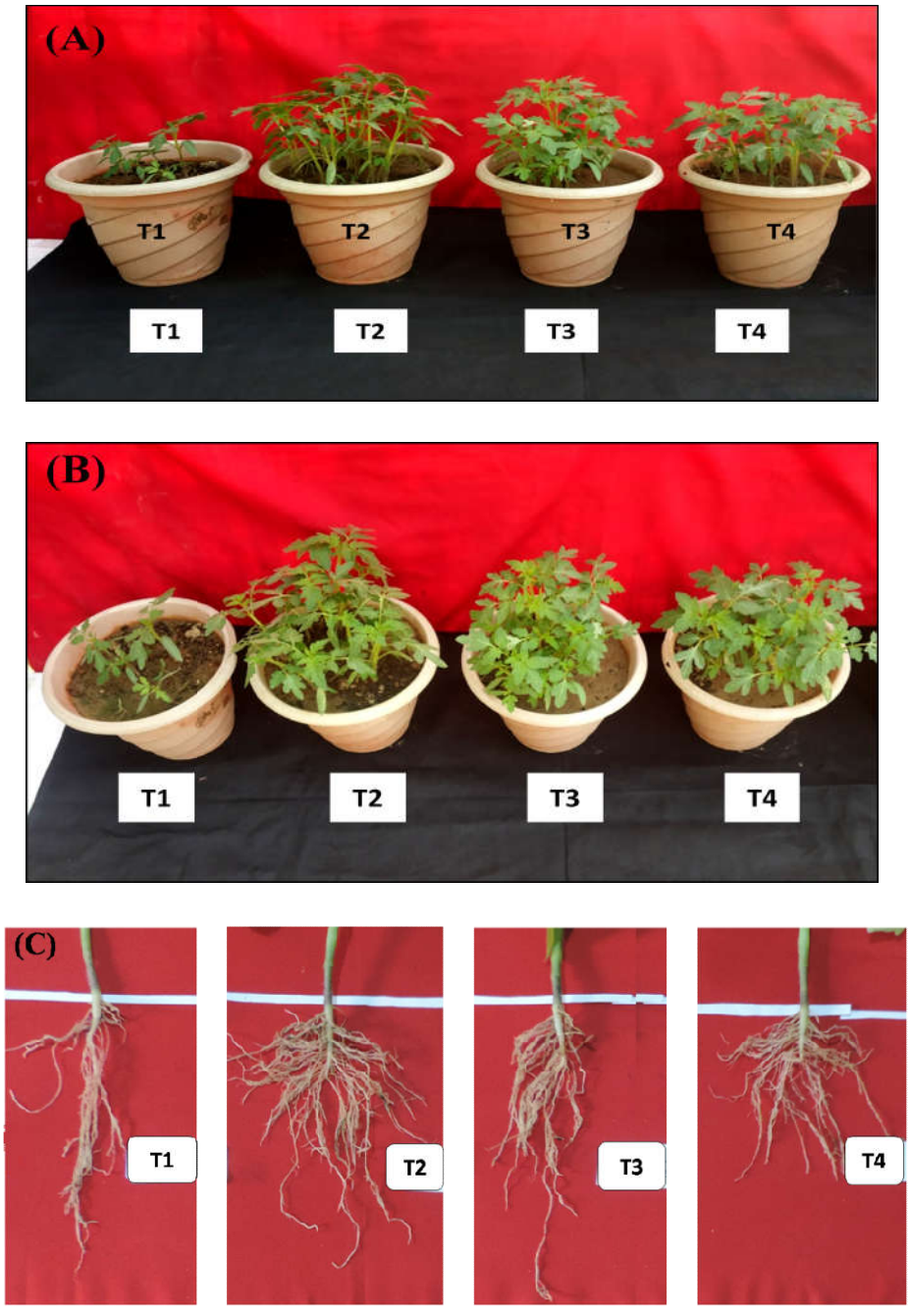



**Plate 6** Effect of encapsulated bioprimed tomato seeds on plant growth (A) front view of pots (B) upper view of pots and (C) root growth. T1 =Control; T2 =75% RDF+EBS; T3 = RDF + Unprimed seed; T4 = Only EBS. RDF (Recommended dose of fertilizer); EBS (Encapsulated bioprimed seed)

# CHAPTER V

# DISCUSSION

*Chapter-V*

# DISCUSSION

Agricultural sector has an important role in economic growth and development by contributing significantly to food security, national income and employment. However, despite this importance, agriculture sector continuously faces multiple challenges especially the climate change, affecting negatively both crop and livestock production system with increases in temperatures, rainfall variation and the frequency and the intensity of extreme weather events (Fellmann *et al*., 2018). Changes in environment significantly affect plant growth, pathogen resurgence and their antagonist's activities. It also regulates the level of loss by a given disease and/or may facilitate disease spread into new areas (Bandyopadhyay and Frederiksen, 1999; Anderson *et al*., 2004; Fisher *et al*., 2012; Kamoun *et al*., 2015; Sicard *et al*., 2018). Continuously incensement is seen in new, emerging, re-emerging and threatening plant diseases which have potential to cause significantly yield loss. Among this re-emerging plant diseases, collar rot caused by *Sclerotium rolfsii* is a major threat to crop production and has been a challenge to both farmers and scientists. *S. rolfsii* is a facultative soilborne parasite having an extensive host range over 500 plant species (Punja, 1985; Banyal *et al*., 2008) and forms sclerotia which aids in maintain continuity of its generation under adverse situation (Ahmed, 1980; Siddique *et al*., 2018). It attacks plant at any growth stage and forms characteristic dark brown lesions which appear on basal part of stem followed by wilting of leaves and gradually whole plant dies. Several researchers and scientists have attempted to manage the collar rot pathogen. The indiscriminate use of chemical fungicides for disease

management leads to pollute soil and water and also causing deleterious effects on human health. Apart from that, these chemical also break the natural ecological balance by killing beneficial and/or antagonists microorganisms and also leads to development of fungicide resistance amog the pathogen isolates. The hazardous effect of these fungicides on our agro-ecosystem generates concern for use of a safer alternative method to protect plants. Thus, it paved the path for utilization of different biological control agents (BCAs) for developing efficient disease management strategies (Singh *et al.*, 2013; Abhilash *et al.*, 2016; Singh, 2016). In addition to biotic stresses, biocontrol agents also help plant system to cope up with various abiotic stresses i.e. heat, drought, salinity etc. and improve their growth promoting attributes as well as nutritional status (Singh *et al*., 2016; Chanu *et al*., 2018; Hirpara *et al*., 2019).

**5.1 Collection, identification and purification of the pathogen causing collar rot of tomato from different districts of Eastern Uttar Pradesh.**

A roving survey was conducted during March to April, 2016 in 5 districts of Eastern Uttar Pradesh to analyze the status of collar rot incidence and to collect diseased samples infected by *S. rolfsii* under field condition. The areas having maximum collar rot incidence were selected and infected samples were collected from those areas on the basis of disease severity rating scale of 0-5. The most characteristic symptoms are basal part of stem turn pale brown and soft, wilting of leaves, bronzing of the leaves on one or more of the lower branches, leaves became yellow to brown in plants showing advanced disease symptoms. At progression of disease, whole plant collapses or die and hard resting structures (sclerotia) are formed on plant surface. Kwon and Park (2002) reported similar symptoms on tomato plant during *S. rolfsii* infection i.e. white mycelium gets

spread over stems and forms abundant sclerotia on the old lesions nearby soil surface. The diseases caused by *S. rolfsii* primarily occur in warm climates, especially under a high temperature (30°±4°C) with humid condition (Kwon *et al*., 2008). The existing temperature range and high humidity match with the optimum temperature (30°C) and moisture requirements for the growth of *S. rolfsii* (Zape *et al.*, 2013) and may be the possible reasons for large scale presence of the disease.

Twenty different isolates of *S. rolfsii* were isolated from different areas which vary considerably in their cultural and morphological patterns like colony diameter, colony colour, colony texture and sclerotial development. Results of the pathogenicity test revealed that all of twenty isolates were found able to produce collar rot symptom in tomato cultivar "Kashi Amrit". Similar studies on cultural and morphological characters of *S. rolfsii* were conducted by Sharma *et al.* (2012) and Gupta *et al.* (2015).

Isolates showed different time for initiation of sclerotium on growth media. Only two i.e. Sr 9 and Sr 17 showed early sclerotial development on 6$^{th}$ day in PDA plates while majority of the isolates showed delayed sclerotial development. Kokub (2007) also noticed that the difference in formation of sclerotia and initiated after 72 hrs of incubation which continued till 168 hrs for *S. rolfsii* isolates.

Agakhani and Dubey (2009) isolated Twenty-three isolates of *S. rolfsii* collected from ten different major chickpea growing states of India having high variability in their morphological and cultural characters along with pathogenicity/ virulence.

Naresh *et al*. (2017) reported variability among ten isolates of *S. rofsii* collected from different locations of chilli host is reported during 2009-10 and 2010-11 and these isolates varied in different cultural characters like colony diameter, no. of sclerotia,

colony character i.e. colony appearance and colony colour. In cultural variability, out of ten isolates of *S. rofsii* have revealed that maximum mycelial growth (88.0 mm) was recorded in SR1 (Sarsaul) isolate followed by SR9 (Maudaha) isolate while the lowest mycelial growth (79.00 mm) was recorded in SR6 (Kucharia) isolate. It was also found that most of the isolates produced sclerotia on large scale, while some isolates were produced sclerotia. The colony appearance from fluffy (SR1, SR2, SR3, SR4, SR8, SR9 and SR10) to compact (SR5, SR6 and SR7) while colony colours were dark brown, the sclerotial shapes were round while colours of sclerotia were light brown (SR1, SR2, SR3 and SR4) to dark brown (SR5, SR6 and SR7) and brown (SR8, SR9 and SR10). Sclerotial patterns were scarred to and groups. In pathogenic variability, among the ten isolates were also exist among the isolates representing the incidence of stem rot was recorded after one month of seedling 33.33 to 57.57% seedling mortality. The highest mortality (57.57%) was recorded in the isolates of (SR1) Sarsaul, Kanpur location and minimum 33.33% in the isolates of (SR5) Mallavan district, Hardoi.

Kumari and Ghatak (2018) identified the cultural and pathogenic variability of the two chickpea rot-causing necrotrophic soil-borne pathogens i.e. *S. rolfsii* and *M. phaseolina* which causes significant damage to chickpea production. Among twelve isolated *S. rolfsii* isolates, BAUSr4 and Ag2 produced the highest infection on genotype L550. They reported that there was No relationship was found among the cultural characters and pathogenicity of the isolates. All isolates share differed in aggressiveness across different locations and host crops. Manu *et al*. (2018) reported the variation among 12 isolates of *S. rolfsii* collected from different location and concluded that the fluffy colony characteristic was more predominant in all isolates and variations in all isolates

depends on the host plant, geographical location of the isolate and the media composition used for experimental studies.

Kwon *et al*. (2018) studied on purple wood sorrel (*Oxalis purpurea*) over a 3-year period (2015–2017) for sclerotium rot infection in Jinju, South Korea. The *Sclerotium rolfsii* infected plants showed rot and blight symptoms and white mycelial mats spread over lesions. Numerous sclerotia formed on the adjoining region near the soil line which white to brown in colour with globoid shape and 1-3 mm in size. The optimum temperature for mycelial growth and sclerotium formation on potato dextrose agar (PDA) was 30 °C and the hyphal width was 4–8 µm. Typical clamp connections were observed in the hyphae of fungi grown on PDA.

Ünal *et al.* (2019) surveyed nine provinces of Turkey for *S. rolfsii* infection in turfgrass growing areas and 32 *Sclerotium rolfsii* isolates were obtained from these areas. The Disease severity in pathogenicity tests carried out in the greenhouse experiment ranged from 83.74 to 92.87%.

**5.2 Sample collection, isolation, characterization and identification of abiotic stress tolerant *Trichoderma* species from various agro ecosystems.**

A roving survey was conducted during April to June, 2016 in 10 districts of different states i.e. Uttar Pradesh, Madhya Pradesh, Chhattisgarh and Rajasthan for collection of soil and composting samples for isolation of thermotolerant (high temperature) *Trichoderma.* The problem of increasing temperature as a result of global warming can be considered as one of the prominent hindrance in crop production as an increase in temperature beyond the optimum level hampers growth, development and economic yield of the crop. The prime objective of this study was to isolate

thermotolerant *Trichoderma* spp. which helps to protect plant from biotic as well abiotic stress. Total eighty samples were collected from the different locations of agriculture fields and composting sites of Allahabad, Varanasi and Mirzapur from Uttar Pradesh; Damoh and Hoshangabad from Madhya Pradesh; Raipur and Bilaspur from Chhattisgarh; Jodhpur and Shri Ganganagar from Rajasthan. A total of one hundred fifty two isolates were recovered from TSM agar plates after subjecting samples to serial dilution. The fungal colonies were differentiated on the basis of their colony colour, appearance and morphology which were further characterized as *Trichoderma* according to the historic species concept established by Rifai (1969). These isolates were again transferred aseptically to Potato dextrose agar (PDA) plates and pure isolates were also maintained on PDA slants at 4°C for further studies.

Elad *et al*. (1982) developed a *Trichoderma*-selective agar medium (TSM) was developed for quantitative isolation of *Trichoderma* spp. from natural soil. Selectivity was obtained by using chloramphenicol (bacterial inhibitor) and rose-bengal (selective fungal inhibitors). TSM also contained low concentration of glucose which allows relatively rapid growth and sporulation of *Trichoderma* and enabling convenient rapid identification of *Trichoderma* colonies.

Mukherjee *et al.* (1993) reported a baiting technique for selective isolation of *Gliocladium virens* from natural soil by mixing *Sclerotium rolfsii* colonized sorghum grains with moist natural soil which incubated at 30 ± 5°C for 6–10 days. *G. virens* developed large, distinct colonies on the soil surface by colonizing *S. rolfsii* and easily isolated for use managing of phytopathogen.

Similarly Mukherjee *et al.* (2014) isolated three novel strains of *Trichoderma* (two *T. harzianum* and one *T. atroviride*) from wild mushroom and tree bark, and evaluated their biocontrol potential against *Sclerotium delphinii* which destroy cotton seedlings.

Assessment of thermotolerance ability of isolated *Trichoderma* spp. was done by subjecting them at high temperature stress (47°C) for continuous 5 hr and their spore survivability was checked regularly at 1 hr interval (Mukherjee and Raghu, 1997; Poosapati *et al*., 2014; Keswani, 2015). All the *Trichoderma* isolates showed variations in spore viability on thermal stress at 47°C. Most of the spores of *Trichoderma* isolates lost their survivability which declined to zero or near zero within a period from 60 min, while some isolates retained their viability up to 300 min. Sixteen *Trichoderma* isolates were selected which retained the spore viability for more than 90 min of heat stress and showed high CFU per ml. The selected *Trichoderma* isolates showed distinct variations in colony morphology and sporulation pattern at the different temperatures (20, 25, 30, 35, 40 and 45°C). The growth pattern of mycelia was changed with temperature. The isolate BHUR2 showed the maximum temperature tolerance with mycelial growth up to 45°C. Among them, BHUR2 isolate showed maximum temperature tolerance with mycelial growth up to 45°C. Thereafter, five *Trichoderma* isolates namely BHURP3, BHUP4, BHUR2, BHUR5, BHUV3 were subjected to antagonism test against *S. rolfsii* through dual culture assay. Highest reduction in mycelium growth of *S. rolfsii* on dual culture plates was showed by BHUR2. The microscopic observations of mycelium interaction between *Trichoderma* BHUR2 and *S. rolfsii* showed mycoparasitic activity as mode of action against the pathogen

Chet *et al*. (1981) reported hyphal interaction and parasitism between *Trichoderma hamatum* and plant pathogenic fungi *Pythium* spp. and *Rhizoctonia solani* as mode of action. Similarly Upadhyay and Mukhopadhyay, (1983) reported that an isolate of *Trichoderma harzianum* (IMI no. 238493) lysed the mycelium and sclerotia of *Sclerotium rolfsii* when the two fungi were grown in dual culture and type of interactions between the antagonist and pathogen were seen as hyphal coiling.

Singh *et al.* (2014) also evaluated that the antagonistic effect of *T. harzianum* against *S. rolfsii. T. harzianum* inhibited the growth of *S. rolfsii* by 68.7%. Therefore, in the present case, the antagonistic interaction between the biocontrol agents and pathogen may be termed as effective and can be considered viable and alternative to chemical management.

The potential antagonist and high temperature tolerant *Trichoderma* isolates BHUR2, BHUP4, BHUR5 and BHUV3 was further selected to analyze different plant growth promoting (PGP) traits and extra- cellular enzyme production activity under 40˚C, 45˚C and control (30±2˚C) conditions. BHUR2 strain showed higher activity of IAA production and phosphate solubilization in comparison with other strains (Jain *et al*., 2013; Singh *et al*., 2013; Mishra *et al*., 2015). Similarly, siderophore, amylolytic, proteolytic and chitinase activity were found higher in BHUR2 strain compared to other strains. Production of hydrolytic enzymes by *Trichoderma* spp. has been reported previously by various researchers (Elad *et al*., 1982; Howell, 2003; Harman *et al*., 2004; Harman *et al*., 2007; Mukherjee *et al*., 2012). Molecular identification was done by using ITS sequencing and BHUR2 strain showed closest relationship with *Trichoderma pseudokoningii* with 99.67 % homology. The accession number MH729058 was

generated for *Trichoderma* strain BHUR2 after sequence submission to NCBI GenBank database. Results revealed that BHUR2 possess both PGP and enzyme activity up to 45˚C, while other isolates showed less activity up to same temperature. All these characteristics clearly indicate that the BHUR2 isolate can tolerate high temperature and can be used as bioinoculant in field after greenhouse trials. Use of thermotolerant *Trichoderma* spp. for development of hard gelatin encapsulated formulation is a novel work and has not been reported by any researchers.

**5.2 Development of commercially viable, cost effective formulation using potential strain of *Trichoderma* and evaluate the shelf life of formulation**

Two different types of hard gelatin encapsulated formulations Tricho Capsule and encapsulated bioprimed seed (EBS) were developed by using thermotolerant *T. pseudokoningii* BHUR2. For development of encapsulated formulation three different ratio of carrier materials (rice bran, coal powder, rutin, okra gum powder); Mix A, Mix B and Mix C with *Trichoderma pseudokoningii* BHUR2 spores was filled in hard gelatin capsules separately. Capsules (Tricho Capsule & EBS) were stored in room temperature and the shelf life of formulations was assessed through CFU count method at 1 month interval for 6 months. At initial CFU count was recorded similar in all the capsule formulations. After 30 days of storage, CFU count was found significantly higher in capsule formulations in comparison to control. The shelf life of Mix C containing Tricho Capsule formulation was found significantly higher in comparison of control after 180 days of storage in room temperature. Previously researchers used various carrier materials for improve the shelf life *Trichoderma* formulation (John *et al*., 2011; Jin and Custis, 2011; Yang *et al*., 2011; Woo *et al*., 2014; Keswani *et al*., 2016;) and found

effective but hard gelatin encapsulation was never done with use of plant based anti-oxidative compound like rutin and okra gum powder which act as adhesive agent. It provides protection from adverse condition of storage to *Trichoderma* spores for better survivability and also provides an easy storage, application and transport option from existing formulations.

**5.3 Evaluate the impact of encapsulated formulations on plant growth promotion and biological control potential against *Sclerotium rolfsii* in tomato.**

**5.3.1. Effect of Tricho-capsule treatments on plant growth promotion and biological control potential**

The experiments on plant growth promotion and disease management on tomato plants were conducted in greenhouse and a significant increase in the morpho-physiological parameters of the crop was observed. Also the isolated *Trichoderma* strain BHU R2 showed its potential for imparting resistance to tomato against collar rot. There was a significant increase observed in all the growth parameters in the BHU R2 treated plants compared to the pathogen challenged plants. Results also revealed that treatment where pathogen stress were applied (T2), showed the least value of all the studied traits. The highest growth promotion was seen in T3 where tomato seeds were primed with BHUR2 spore suspension. The results of plant growth promotion and protection from disease in plants by PGPM (plant growth promoting microbuses) have been found to support the results of numerous researchers (Callan *et al*., 1990; Sarma *et al*., 2002; Reddy *et al*., 2013; Yadav *et al*., 2013; Saxena *et al*., 2015; Singh *et al*., 2016). The increase in yield attributing characters like number fruits per plants was recorded high in

*Trichoderma* spp. treatment in comparison to control in tomato, pepper and lettuce crop under greenhouses conditions (Vinale *et al*., 2008).

**5.3.2. Effect of Tricho-capsule treatments on anti-oxidative enzyme activities and phenol content in plant**

The resistance response was evaluated by studying the various biochemical parameters of plant pathogen interaction. All the major enzymes which contribute inducing systemic resistance were studied and their synthesis amount was checked. Application of *T. pseudokoiningii* BHUR2 induced accumulation of high level of phenol, activities of SOD, POx and PAL in tomato plants when compared with pathogen inoculated control. Induction of systemic resistance has been established as a new mechanism by which plants defend themselves against pathogen attack (Van Loon *et al*., 1998). Various reports confirm the induction of systemic resistance by biocontrol agents against fungal pathogens (Yedidia *et al*., 1999: Meena *et al.,* 2000). Superoxide dismutase (SOD) catalyzes dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. In higher plants, SOD act as antioxidants and protect cellular components from being oxidized by ROS. At 72 h, SOD activity in the T7 treatment was found to be highest than those in the other challenged control and unchallenged healthy control. The enzymes, SOD and PO work together with other enzymes of the ascorbate–glutathione cycle to promote scavenging of free radicals (Hernandez *et al.,* 2001). Analysis of plants post infection indicated that pea seeds treated with the three-microbe consortium followed by two-microbe consortium of *B. subtilis* and *P. aeruginosa* exhibited maximum activities of PO and SOD compared with the plants treated with single BCAs

and untreated control (Jain *et al.,* 2011; 2014; Mastouri *et al*., 2017). Similar results were also obtained in a previous study, where *Trichoderma* inoculation was found to induce maximum SOD and PO activity compared with untreated pathogen inoculated control (Jetiyanon, 2007; Van der Ent *et al*., 2009; Zehra *et al*., 2017). Several individual PGPR strains have been demonstrated to induce generation of antioxidants including SOD and PO in the host plants upon pathogen attack as an ISR response that provides protection against diverse pathogens (Silva *et al.,* 2004; Singhai *et al.,* 2011). Some amount of natural SOD activity in unchallenged healthy control was consistently present throughout the assay. Increase in SOD and POx activity elicited by the treatments could explain the observed disease suppression in all the tested pathosystems. Phenylalanine ammonia-lyase (PAL) plays an important role in the biosynthesis of various defense chemicals in phenylpropanoid metabolism. Activity of PAL could be induced in plant-pathogen interactions and fungal elicitor treatment (Segarra *et al*., 2007; Tucci *et al*., 2011). Malolepsza *et al.* (2017) reported that rhizosphere colonization by *Trichoderma* TRS106 stimulates systemic defense response in tomato plant by activating defense enzyme production including Guaiacol peroxidase (GPx) and PAL and also enhances phenolic content in plant. In the present study, activity of PAL was increased up to 72 h and then declined thereafter. The maximum level of PAL was recorded in treatment T5 which indicate that association with the pathogen resulted in more PAL production in order to extent the defense mechanism to its zenith. The time required to activate the defense mechanisms is important for suppression of the invading pathogen. Similarly, TPC content may contribute their role by enhancing the mechanical strength of the host cell wall and due to its fungitoxic nature may also suppress the fungal growth. The induction

of phenolic compound development might resulted due to the activation of the shikimic acid pathway, through which the other aromatic amino acids, tyrosine and phenylalanine are formed and channeled for the synthesis of different type of phenolics. In the present study maximum accumulation of total phenol content was observed in tomato seeds primed with *T. pseudokoningii* BHUR2. Singh *et al.* (2013) also reported higher accumulation of phenols in tomato at 48 h, with maximum accumulation in the consortium of *Trichoderma* and *Pseudomonas* against *S. rolfsii*. Similar results were reported for cucumber plant against *Rhizoctonia solani* (Nawrocka *et al.*, 2000). Salas-Marina *et al*. (2015) reported that roots colonization of *Arabidopsis* plant through *T. atroviride* promotes defense and enhances systemic disease resistance by jasmonic acid/ ethylene and salicylic acid pathways.

**5.3.3. Effect of Tricho-capsule treatments on Mineral content of tomato fruits**

Tomato fruits are rich in macronutrients contents, especially potassium (K), phosphorus (P), magnesium (Mg) and calcium (Ca) (Paiva *et al*., 1998; Wilcox *et al*., 2003; Súarez *et al*., 2008; Odriozola-Serrano *et al*., 2009;) and also contains high amounts of trace elements such as iron(Fe), Manganese (Mn), Zinc (Zn), and copper (Cu) (Ahmed *et al*., 2011). Tomato fruit play an important role in human health (Rao and Agarwal, 2002; Chapagain and Wiesman, 2004). Various abiotic and biotic stresses hampers the fruit production and quality of tomato crop and there is need of effective strategies for increasing fruit production and quality which will be helpful for growers (Gruda, 2005, Dorais *et al*., 2008). There are various studies which suggest that mineral nutrients can affect the antioxidant content of tomato fruit and overall tomato fruit quality. Ca is an essential element for fruit firmness and extended shelf life (Cooper and

Bangerth, 1976). Paiva *et al*. (1998) reported that increased Ca levels in soil solution enhance the Ca content in tomato fruit, but reduces carotene content and lycopene levels. Increasing K also increases carotenoid concentration and more particularly the lycopene content (Ulrichs *et al*., 2008). Lester (2006) reported that ascorbic acid increases with increasing levels of Mn, K, B, Cu and Zn mineral content. Phosphorus may also increase the concentration of phytochemicals such as ascorbic acid, flavonoids and lycopene in fruits (Dorais *et al*., 2008). The need for production of high quality food, while mitigating deleterious environmental impact of excessive use of chemical fertilizers for enhanced crop production (Mader *et al*., 2002) generates the need for use of plant beneficial microbial inoculants. In the present study, enhanced mineral contents (N, P, K and Ca) were recorded in treatment of seed bioprimed with *T. pseudokoningii* spore suspension in comparison to pathogen inoculated and uninoculated control. Various scientific reports showed that microbial inoculant can alter the nutrient quality of crop products (Worthington, 2001; Meena *et al*., 2004; Mishra and Nautiyal, 2018; Singh *et al*., 2018). Kaya *et al*. (2009) found that *T. harzianum* can improve the solubility of soil micronutrients, such as Cu, Fe, Zn and ultimately enhance their uptake in plant. Marra *et al*. (2019) reported that combine application of *Trichoderma* spp. with secondary metabolites found effective in increase of yield as well as enhance the nutrient quality in soybean crop.

**5.3.4. Effect of Tricho-capsule treatments on nutritional content of tomato fruits**

The amount of ascorbic acid, lycopene, total carbohydrate and protein were significantly increased in all *Trichoderma* inoculated treatments as compared to pathogen challenged and non-pathogen challenged control. Tomato fruits are an excellent source of

health benefiting compounds due to they have a balanced mixture of antioxidants including ascorbic acid and tocopherol, beta-carotene, lycopene, lutein and flavonoids (c *et al*., 2008), amino acids, proteins, carbohydrates and fatty acids (Heeb, 2005; Javanmardi, J., Kubota, 2006). Various nutritional studies have suggested that regular consumption of vegetables and fruits, including tomatoes, can play an important role in preventing cardiovascular diseases in humans (Rao and Agarwal, 2000, Toor and Savage, 2005; Toor *et al*., 2006). There are several studies which showed the intervention of plant growth promoting microbes with plant enhances the nutrient quality of food products. In present study, the highest content of ascorbic acid, lycopene, protein and total carbohydrate were observed in treatment T3 (seed bioprimed) in comparison to T1 (without pathogen challenged control). Similar studies was reported by Gravel *et al*., (2007); Haque *et al*. (2012) ; Nzanza *et al*. (2012) in tomato; Haque *et al*. (2012) in mustard; Jain *et al*. (2014) in pea; Meena *et al*. (2016) in wheat.

### 5.3.5 Effect of encapsulated bioprimed seeds on nutrient use efficiency and plant growth promotion

Seed biopriming is novel concept of seed treatment which protect plants from various biotic and abiotic in a better way. Application of PGPMs (plant growth promoting microbes) for seed biopriming to manage soilborne and seedborne phytopathogens is a model delivery system as it brings the microbial inoculum directly into the rhizosphere. Wide ranges of fungal and bacterial antagonists have been commercially exploited for this purpose (Nelson 2004; Berg 2009, Bajpai *et al*., 2018) but their applications as seed biopriming is very limited. The encapsulation of bioprimed seed provide a ready to use method for sowing of primed seed in to field or greenhouse

conditions that helps growers to protect their crops in a sustainable and ecofriendly manner. Previously several studies and experiments have been done on seed biopriming with PGPMs in various crops including sweet corn (Callan et al. 1991), carrot (Murunde and Wainwright, 2018) and tomato (Harman and Taylor 1988). Seed biopriming has also been reported to facilitate the survival of microbial agents in/on seed surface thus provide better plant growth and yield (Bhatt *et al*., 2015; Shukla *et al*., 2015; Singh *et al*., 2016; Singh 2016; Hernández-Herrera *et al*., 2019). Molla *et al*. (2012) reported that use of *Trichoderma* improves both yield and quality of tomato crop. The combine application of *Trichoderma* enriched biofertilizer with chemical fertilizer at 50:50 ratios increased the plant growth, yield and nutritional quality in tomato plant due to slow and continuous release of nutrients to plants rather than quick release of nutrients as during application of chemical fertilizers. This strategy has also been helpful in reducing the cost of cultivation and minimizes pollution by cutting down the amount of excessive nitrogen fertilizers.

In present study, it has been found that encapsulated bioprimed seed (EBS) with 75% of RDF (T2) showed better performance under greenhouse condition in comparison with control (without fertilizer application) unencapsulated seed (T1). Results revealed that increase in plant biomass, shoot length, root length and lateral root numbers were observed in treatment T2 in comparison to control T1 treatment. Biopriming has several mechanisms to stimulate plant growth and plant immunity, *viz*., induced expression of plant growth-promoting genes, production of phytohormones, increased nutrient status into the plant, antibiosis, mycoparasitism, activation of antioxidant production, induced phenolic production and systemic defense activation (Chatterjee *et al*., 2018; Pehlivan *et al*., 2018; Singh, *et al*., 2018;Vinci *et al*., 2018; Rakshit, 2019).

# CHAPTER VI

# SUMMARY AND CONCLUSION

*Chapter-VI*

# SUMMARY AND CONCLUSION

The present investigation was undertaken to meet the following objectives:

1. Isolation, characterization and identification of abiotic stress tolerant *Trichoderma* species from various agro ecosystems
2. Development of commercially viable and cost effective formulation using potential strains of *Trichoderma* species
3. To study the shelf life of *Trichoderma* formulation at room temperature.
4. To study the impact of *Trichoderma* based formulation on plant growth promotion and biological control potential (*viz*. SOD, POx, MDA, PAL, TPC) against *Sclerotium rolfsii* in tomato under green house conditions

The *in vitro* experiment was conducted in the Plant Health Clinic and Biocontrol Laboratory of Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India. The *in vivo* experiment was carried out in the greenhouse and field of the same department.

**1. Collection, identification and purification of the pathogen causing dry root rot of chickpea from different districts of Eastern Uttar Pradesh.**

A roving survey was conducted during March to April, 2016 in 5 districts of Eastern Uttar Pradesh to analyze the status of collar rot incidence and to collect diseased samples

infected by *S. rolfsii* under field condition. The areas having maximum collar rot disease incidence were selected and infected samples were collected from those areas on the basis of disease severity rating scale of 0-5. 20 different isolates of *S. rolfsii* were isolated and were subjected to pathogenicity test. All of them were found to prove pathogenicity on tomato cultivar "Kashi Amrit". The percent disease severity (% DS) incidence of all isolates were checked and it was found that at 60 DAI, *S. roflasii* isolate Sr 17 showed maximum % DS of 89.63% amongst all isolate and thus was selected for further experiments.

**2. Sample collection, isolation, characterization and identification of abiotic stress tolerant *Trichoderma* species from various agro ecosystems.**

A roving survey was conducted during April to June, 2016 in 10 districts of different states i.e. Uttar Pradesh, Madhya Pradesh, Chhattisgarh and Rajasthan for collection of soil and composting samples for isolation of thermotolerant (high temperature) *Trichoderma.* A total of one hundred fifty two isolates were recovered from TSM agar plates after subjecting samples to serial dilution. The fungal colony were differentiated on the basis of their colony colour, appearance and morphology which were further characterized as *Trichoderma* according to the historic species concept established by Rifai (1969). These isolates were again transferred aseptically to Potato dextrose agar (PDA) plates and pure isolates were also maintained on PDA slants at 4˚C for further studies. Sixteen *Trichoderma* isolates were selected which retained the spore viability for more than 90 min of heat stress and showed high CFU per ml. The selected *Trichoderma* isolates showed distinct variations in colony morphology and sporulation pattern at the different temperatures (20, 25, 30, 35, 40 and 45˚C). Among them, BHUR2

isolate showed maximum temperature tolerance with mycelial growth up to 45˚C. Thereafter, five *Trichoderma* isolates namely BHURP3, BHUP4, BHUR2, BHUR5, BHUV3 were subjected to antagonism test against *S. rolfsii* through dual culture assay. Highest reduction in mycelium growth of *S. rolfsii* on dual culture plates was showed by BHUR2. The plant growth promoting (PGP) traits and extra- cellular enzyme production activity checked under 40°C, 45°C and control (30±2°C) conditions. BHUR2 strain showed higher activity of IAA production, phosphate solubilization, siderophore production, amylolytic, proteolytic and chitinase activity in comparison with other strains. Molecular identification was done by using ITS sequencing and phylogenetic tree was conducted. BLAST results revealed that BHUR2 showed 99.67 % homology *with T. pseudokoningii*. The accession number MH729058 was generated after sequence submittion to NCBI GenBank database.

**2. Development of commercially viable, cost effective formulation using potential strain of *Trichoderma* and evaluate the shelf life of formulation**

Two different types of hard gelatin encapsulated formulations Tricho Capsule and encapsulated bioprimed seed (EBS) were developed by using of high temperature tolerant *T. pseudokoningii* BHUR2. For development of encapsulated formulation, three different ratio of carrier materials (rice bran, coal powder, rutin, okra gum powder); Mix A, Mix B and Mix C with BHUR2 spores was used and filled in hard gelatin capsules separately. Capsules (Tricho Capsule & EBS) were stored in room temperature and the shelf life of formulation was assessed through CFU count method at 1 month interval for 6 months. The shelf life of Mix C containing Tricho Capsule formulation was found significantly higher in comparison of control after 180 days of storage in room temperature. Similarly,

EBS showed high CFU count in comparison of control at 180 days of storage in room temperature. Hard gelatin encapsulation provides protection to *Trichoderma* spores from adverse condition of storage for better survivability and also provides an easy storage, application and transport option from existing formulations.

**3. Evaluate the impact of encapsulated formulations on plant growth promotion and biological control potential against *Sclerotium rolfsii* in tomato.**

**3.1. Effect of Tricho-capsule treatments on plant growth promotion and biological control potential**

Experimental findings showed that Tricho-capsule treatment enhances plant growth promotion and disease management on tomato plants. *Trichoderma* strain BHUR2 showed the potential for imparting resistance to tomato against collar rot through seed priming. A significant increase was observed in T3 where tomato seeds were primed with BHUR2 spore suspension; compared to the pathogen challenged (T2) plants.

**3.2. Effect of Tricho-capsule treatments on anti-oxidative enzyme activities and phenol content in plant**

Application of BHUR2 induced accumulation of high level of phenol, activities of SOD, POx and PAL in tomato plants when compared with pathogen inoculated control. The treatment T5 showed highest value of all these enzymes at different hours after pathogen challenge varying from 48-72 hr and then declined. Increased levels of plant defense enzymes upon pathogen attack suggest that they may lead to initiate induced systemic response (ISR) and systemic acquired resistance (SAR) mechanisms of the plants and thus will trigger a defense response in the host. The antioxidant enzymes i.e. SOD and POx activity quench the toxic ROS and protect plants from oxidative damage.

PAL activity precedes rapid recognition of a microbial invader which potentiates the accumulation of disease resistance factors including phenolics, phytoalexins and lignin. An increase in lignification is often observed in response to attack by pathogen and is believed to represent one of the chief mechanisms adopted to block pathogen invasion due to its highly non-degradable and antimicrobial nature. Similarly, total phenolic content aid in enhancing the mechanical strength of the host cell wall and suppress fungal growth owing to its fungitoxic nature. The induction of phenolic compound might occur due to activation of shikimic acid pathway, through which the other aromatic amino acids *viz*., tyrosine and phenylalanine are formed and channeled for the synthesis of different type of phenolics. In the present study maximum accumulation of TPC was observed in tomato seeds primed with *T. pseudokoningii* BHUR2. The MDA is a byproduct of lipid peroxidation reaction and also used as an indicator of oxidative damage to membranes. In the present study, T5 treated plants exhibited less MDA content as compared to other treatments indicating that vermiwash and *T. pseudokoningii* BHUR2 synergistically reduced pathogen-induced oxidative damage effect.

**3.3. Effect of Tricho-capsule treatments on mineral content of tomato fruits**

In present study, the enhanced mineral contents (N, P, K and Ca) were recorded in treatment of seeds bioprimed with *T. pseudokoningii* spore suspension (T5) in comparison to pathogen inoculated (T1) and uninoculated (T2) control. The increased mineral content especially Ca, increases the shelf life of tomato fruits which ultimately improve their market utility.

**3.4. Effect of Tricho-capsule treatments on nutritional content of tomato fruits**

Tomato fruits are an excellent source of health benefiting compounds as they possess a mixture of antioxidants including ascorbic acid and tocopherol, beta-carotene, lycopene and flavonoids. Abiotic and biotic stresses lead to detrimental effect on nutritional content and quality of fruits. PGPMs application on/in plants can help to improve the nutritional status in crop produces. In present study, highest content of ascorbic acid, lycopene, protein and total carbohydrate were observed in treatment T3 (seed bioprimed) in comparison to T1 (without pathogen challenged control) and T2 (pathogen challenged control).

**3.5 Effect of encapsulated bioprimed seeds on nutrient use efficiency and plant growth promotion**

A lot of research has been carried out which validates the beneficial effect of seed bioprimg in various crops, but still lack of utilization of this technique in field level have been a major issue. Currently, no literature is available citing encapsulation of bioprimed seed and their effect on plant growth promotion as well as the survivability of bioinoculant spore in/on primed seed. The present findings revealed that encapsulation of bioprimed seed aids in bioinoculant's spore survivability and provide an easy method to use bioprimed seeds for sowing purpose. The encapsulated bioprimed seed (EBS) with 75% of RDF (T2) showed better performance under greenhouse condition in comparison with control (without fertilizer application) unencapsulated seed (T1). Results demonstrated that increase in plant biomass, shoot length, root length and lateral root numbers were observed in treatment T2 in comparison to control T1 treatment.

**Conclusions**:

Following conclusions have been drawn from the present investigation

- Novel *Trichoderma pseudokoningii* strain BHUR2 has been reported to be thermotolerant and possess greater biocontrol potential which can be further utilized for managing plant disease at tropical and subtropical regions.

1. The hard gelatin encapsulation of *T. pseudokoningii* spores with Mix C [Rice bran (39.95 g) + Coal powder (39.95 g) + Okra gum powder (10 g) + Rutin (0.1 g)] shown enhanced shelf life in the formulation and provides protection to spores from harsh environmental conditions at storage. It also reduced the amount of biopesticide needed for treatment of seeds / planting during conventional biological treatment process.

- Encapsulation of bioprimed seeds has emerged as a novel method as the seeds can be applied directly in to field. It is an easy and ready to use method to protect plant from various biotic and abiotic stresses during germination. In addition, it also showed a significant effect on different physiological and morphological characters of tomato plants
- Tomato plants treated with *T. pseudokoningii* BHUR2 strain showed significant augmentation in levels of antioxidant enzymes and phenylpropanoid pathway. It positively modulated the oxidative and defense network in the host plant.
- The nutrient and mineral contents of tomato fruits has been enhanced in *T. pseudokoningii* BHUR2 treated plants challenged with *S. rolfsii*.

- Based on above observations, it may be concluded that *T. pseudokoningii* BHUR2 has proved to be a promising biocontrol agent for the management of collar rot disease of tomato under glass and field conditions.

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# LIST OF PUBLICATIONS

**Research Papers**

- Rajput, R. S., Singh, P., Singh, J., Vaishnav, A., Ray, S. and Singh, H. B. (2019) *Trichoderma* mediated seed biopriming augments antioxidant and phenylpropanoid activities in tomato plant against *Sclerotium rolfsii*. *Journal of Pharmacognosy and Phytochemistry*. 8(3): 2641-2647.
- Rajput, R. S., Singh, J., Singh, P., Vaishnav, A. and Singh, H. B. (2019) Synergistic effect of *Trichoderma pseudokoningii* BHUR2 and vermiwash improve defense system and fruit quality of tomato plants challenged by *Sclerotium rolfsii* under heat stress. *Biological Control*. (Communicated July, 2019)

**Patent filed**

- Singh, H.B., **Rajput, R.S.**, Vaishnav, A., Singh, A and Sarma, B.K. Method for development of encapsulated bioprimed seeds comprising microbial consortium and uses thereof. Indian Patent (Filing no. 201811043111; CBR No. 30536)

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**Academic Credentials**

| S. No | Degree | Year of passing | Name of University/Board | Marks (%) | Division |
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| 1 | Ph. D. | 2019 (Submitted on 10/08/2019) | B.H.U, Varanasi | 82.5% | First |
| 2 | M.Sc. (Ag) Plant Pathology | 2014 | I.G.K.V, Raipur | 82.3% | First |
| 3 | B.Sc. (Agriculture) | 2012 | I.G.K.V, Raipur | 77.9% | First |
| 4 | Intermediate ($12^{th}$) | 2007 | C.G.B.S.E, Raipur | 69.60% | First |
| 5 | High School ($10^{th}$ ) | 2005 | C.G.B.S.E, Raipur | 70% | First |
| 6 | ICAR- NET | 2017 | A.S.R.B., New Delhi | 63.78 | Qualified |

## DECLARATION

I hereby affirm that the information provided in this document is accurate and true to the best of my knowledge.

Date:
Place: B.H.U., Varanasi **(Rahul Singh Rajput)**