“GENOTYPIC FINGERPRINTING OF ANTAGONISTIC *Trichoderma* SPP. AGAINST *Rhizoctonia solani* AND *Sclerotium rolfsii* USING GENE SPECIFIC MOLECULAR MARKER”

M. Sc. (Ag.) THESIS

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RAIPUR, (C.G.)

2011

“GENOTYPIC FINGERPRINTING OF ANTAGONISTIC *Trichoderma* SPP. AGAINST *Rhizoctonia solani* AND

Sclerotium rolfsii **USING GENE SPECIFIC MOLECULAR MARKER”**

Thesis

Submitted to the

Indira Gandhi Krishi Vishwavidyalaya, Raipur

By

RAHATKAR ONKAR VILAS

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Science
In

Agriculture

(Plant Molecular Biology and Biotechnology)

Roll. No. 12507 **ID No. 120109031**

JULY, 2011

CERTIFICATE – I

This is to certify that the thesis entitled **"GENOTYPIC FINGERPRINTING OF ANTAGONISTIC *Trichoderma* SPP. AGAINST *Rhizoctonia solani* AND *Sclerotium rolfsii* USING GENE SPECIFIC MOLECULAR MARKER"**, submitted in partial fulfillment of the requirements for the degree of **"MASTER OF SCIENCE IN AGRICULTURE"** of Indira Gandhi Krishi Vishwavidyalaya, Raipur is a record of the bonafide research work carried out by **Mr. RAHATKAR ONKAR VILAS** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma (Certificate awarded etc.) or has been published/Published part has been fully acknowledged. All the assistance and help received during the course of the investigations have been duly acknowledged by him.

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CERTIFICATE – II

This is to certify that the thesis entitled **"GENOTYPIC FINGERPRINTING OF ANTAGONISTIC *Trichoderma* SPP. AGAINST *Rhizoctonia solani* AND *Sclerotium rolfsii* USING GENE SPECIFIC MOLECULAR MARKER",** submitted by **Mr. RAHATKAR ONKAR VILAS** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur in partial fulfillment of the requirements for the degree of M.Sc. (Ag.) in the Department of Plant Molecular Biology and Biotechnology has been approved by the external examiner and Student's Advisory Committee after oral examination.

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Acknowledgement

I want to dedicate it to my beloved grandfather Late Shri Keshav Rahatkar whose everlasting inspiration propelled throughout my career.

I am fortunate that I got this opportunity to study and conduct my research work under Dr. A.S. Kotasthane major advisor and Professor, Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, Raipur. It is my privilege to acknowledge him for kind guidance, valuable and fruitful suggestions and above all his words of inspiration which imbued me with the unparallel zeal throughout the whole research work.

Most humbly and respectfully I wish to express my profound sense of gratitude to Dr. D. K. Sharma, Professor, Head of Department Plant Molecular Biology and Biotechnology, I.G.K.V., Raipur, for his valuable suggestions and guiding in my research work and for providing necessary facilities.

I express my sincere thanks to respected members of my advisory committee Dr. K.P. Verma and Dr. R. R. Saxena College of Agriculture, Raipur for their useful suggestions, critical comments and kind help rendered as and when needed.

I wish to record my sincere gratitude to Dr. M. P. Pandey, Hon'ble vice Chanceller, Dr. O. P. Kashyap, Dean, Dr. V. K. Mishra, Director of instruction and Dr. S. K. Patil, Director Research Services, I.G.K.V., Raipur for providing me the necessary facilities for research.

I would like to express my sincere thanks to my teacher Dr. G. Chandel sir and also thankful to Dr. (Smt.) Zenu Jha mam.

I am especially thankful to my seniors and other staff members Dr. Toshy madam, Dr. Sweta madam, Renu madam, , Neha madam, Mahima madam, Archana madam, Shiv sir, Rakesh sir, Manju madam, Ravikant bhaiya, Tsukeshi didi and others for their valuable support, advice and genuine guidance, whenever, I sought throughout my course work.

I can't forget the moment shared with my friends Rahul, Sanjeet, Amit, Ravish, Ajit, Ravi, Ganesh, Ashish, Mahendra, Pallavi, Manali, Saroj, and seniors Sada sir, Sandeep sir, Yash sir, Mauli sir also to all my juniors whose support and help always strengthened me.

There aren't enough words to express my gratitude to my beloved parents and family members for their encouragement, sincere prayers, expectations and blessings which have always been the most vital source of inspiration and motivation in my life.

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LIST OF ABBREVIATIONS

%	- per cent
°C	- degree celsius
µl	- microlitre
ADW	- Autoclaved Distilled Water
AFLP	- amplified fragment length polymorphism
Approx.	- approximately
BCA	- Biocontrol agents
bp	- base pair
DNA	- deoxyribonucleicacid
dNTPs	- deoxynucleotide triphosphates
EDTA	- ethylenediamine tetra acetic acid
et al.	- and others
g	- gram
H_2O	- water
HCl	- hydrochloric acid
HCN	- Hydrogen cyanide
i.e.	- that is
IAA	- Indole Acetic Acid
KCl	- potassium chloride
M	- molar
mg	- milligram
$MgCl_2$	- magnesium chloride
ml	- milliliter
mM	- milli molar
NaCl	- sodium chloride
ng	- nanogram
P	- Phosphate
PAGE	- polyacrylamide gel electrophoresis
PCR	- polymerase chain reaction
PDA	- Potato Dextrose Agar
PDB	- Potato Dextrose Broth
RFLP	- restriction fragment length polymorphism
rpm	- revolutions per minute
SDS	- sodium dodecyl sulphate
TAE	- tris acetic acid EDTA buffer
TE	- tris EDTA buffer
TSM	- *Trichoderma* Selective Media
U	- units

CHAPTER I
INTRODUCTION

Trichoderma spp. is well known biocontrol agent against a wide range of soilborne pathogens and some have a plant growth promotion activity (Ozbay *et al*., 2004) and their prevalence as biocontrol agents has stimulated much research into the mechanisms underlying biocontrol, of which mycoparasitism is considered a major component. They have been studied with respect to various characteristics and applications and are known as successful colonizers of their habitats, efficiently fighting their competitors. The complex process of mycoparasitism consists of several events, including recognition of the host, attack and subsequent penetration and killing.

Screening diverse population of biocontrol agents is an important requirement for developing efficient bio control agents. The genus *Trichoderma* has been extensively studied for their abilities to rapidly colonize substrates for the induction acquired systemic resistance in plants (Enkerly *et al*., 1999) , for the growth inhibitory effect of their antibiotics on other pathogens (Keszler *et al*., 2000) and for the production of cell wall degrading enzymes against many plant pathogens. *Trichoderma* strains are able to recognize the host hyphae, to coil around them, develop haustoria and penetrate the cell wall of the host (Abdullah., 2007). Their defense mechanisms comprise both enzymatic and chemical weapons, which make *Trichoderma* spp. efficient mycoparasites, antagonists, and biocontrol agents characteristics that can be exploited by using *Trichoderma* spp. or the metabolites secreted by these fungi as biological fungicides to fight plant diseases caused by pathogenic fungi (Vinale *et al*., 2006). Thereby *Trichoderma* spp. plays an important role in the three-way interaction with the plant and the pathogen (Woo *et al*. 2006).

Interestingly, the efficacy as antifungal agents of the *Trichoderma* enzymes was higher than that described for plant, bacterial or other fungal chitinolytic enzymes assayed under the same conditions (Lorito *et al*., 1996) which supports the hypothesis that these enzymes are specialized for attacking the cell walls of other fungi. Due to their ability to protect plants and contain pathogen populations under different soil conditions, these fungi have been widely studied and commercially marketed as biopesticides, biofertilizers and soil amendments. *Trichoderma* spp. also produces numerous biologically active compounds, including cell wall degrading enzymes, and secondary metabolites (Vinale *et al*., 2008).

Plant diseases play a direct role in the destruction of natural resources in agriculture. In particular, soil-borne pathogens cause important losses, fungi being the most aggressive. The distribution of several phytopathogenic fungi, such as *Phythium, Phytophthora, Botrytis, Rhizoctonia and Fusarium*, has spread during the last few years due to changes introduced in farming, with detrimental effects on crops of economic importance. In addition, not only growing crops but also stored fruits are prey to fungal infections (Chet *et al*., 1997).

Chemical compounds have been used to control plant diseases (chemical control), but abuse in their employment has favored the development of pathogens resistant to fungicides. Unfortunately, the more specific the effect of a chemical on an organism, the greater the probability of decreasing the effect through genetic shifts in the population, whereas fungicides of broad spectrum produce undesirable consequences on non-target organisms (Tjamos *et al*., 1992). By contrast, the use of microorganisms that antagonize plant pathogens (biological control) is risk-free when it results in enhancement of resident antagonists. Moreover, the combination of such biological control agents (BCAs) with reduced levels of fungicide (integrated control)

promotes a degree of disease suppression similar to that achieved with full fungicide treatment (Monte., 2001).

Antagonists of phytopathogenic fungi have been used to control plant diseases, and 90% of such applications have been carried out with different strains of the fungus. Most of these strains are classified as imperfect fungi since they have no known sexual stage (Monte., 2001). However, some *Trichoderma* species are morphologically similar to the anamorph Hypocrea, and their internal transcribed spacer (ITS) sequences have revealed their taxonomic proximity (Monte., 2001; Hermosa *et al*., 2000). The most common BCAs of the *Trichoderma* genus are strains of *T. virens, T. viride* and, above all, *T. harzianum* , which is a species aggregate that includes different strains used as BCAs of phytopathogenic and viral vector fungi (Grondona *et al.,* 1997). Molecular characterization and phylogenetic analysis have allowed strains of *T. harzianum* originally identified as the same species to be assigned to different species clustered into distinct sections and groups. No BCA corresponds to biotypes of *T. harzianum* that is pathogenic to mushrooms (Hermosa *et al*., 2000).

BCAs are living organisms whose activities depend mainly on the different physicochemical environmental conditions to which they are subjected. For this reason, biocontrol exerted by *Trichoderma* strains is sometimes unpredictable. Understanding both the genetic diversity of strains within *Trichoderma* species and their mechanisms of biocontrol will lead to improved application of the different strains as BCAs. These mechanisms are complex, and what has been defined as biocontrol is the final result of different mechanisms acting synergistically to achieve disease control (Kershaw and Talbot, 1998). Biocontrol results either from competition for nutrients and space or as a result of the ability of *Trichoderma* BCAs

to produce and/or resist metabolites that either impede spore germination (fungistasis), kill the cells (antibiosis) or modify the rhizosphere, e.g. by acidifying the soil, so that pathogens cannot grow. Biocontrol may also result from a direct interaction between the pathogen itself and the BCA, as in mycoparasitism, which involves physical contact and synthesis of hydrolytic enzymes, toxic compounds and/or antibiotics that act synergistically with the enzymes. *Trichoderma* BCAs can even exert positive effects on plants with an increase in plant growth (biofertilization) and the stimulation of plant-defense mechanisms.

Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens (Chet *et al*., 1997). For instance, in most filamentous fungi, iron uptake is essential for viability (Eisendle *et al*., 2004), and under iron starvation, most fungi excrete low-molecular-weight ferric-iron-specific chelators, termed siderophores, to mobilize environmental iron (Eisendle *et al*., 2004). Subsequently, iron from the ferrisiderophore complexes is recovered via specific uptake mechanisms. In *Aspergillus fumigatus* and *Aspergillus nidulans*, siderophore biosynthesis is negatively regulated by carbon source (Eisendle *et al*., 2004). In *Ustilago maydis*, gene products related to iron uptake affect the development of plant disease (McIntyre *et al*., 2004). Some *Trichoderma* BCAs produce highly efficient siderophores that chelate iron and stop the growth of other fungi (Chet *et al*., 1994). For this reason, soil composition influences the biocontrol effectiveness of *Pythium* by *Trichoderma* according to iron availability. In addition, *T. harzianum* T35 controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients, with biocontrol becoming more effective as the nutrient concentration decreases (Tjamos *et al*., 1992). Competition has proved to be particularly important for the

biocontrol of phytopathogens such as *Botrytis cinerea*, the main pathogenic agent during the pre- and post-harvest in many countries (Latorre *et al*., 2001). The extraordinary genetic variability of this fungus makes it possible for new strains to become resistant to essentially any novel chemical fungicide it is exposed to (Latorre *et al*., 2001). The advantage of using *Trichoderma* to control *B. cinerea* is the coordination of several mechanisms at the same time, thus making it practically impossible for resistant strains to appear. Among these mechanisms, the most important is nutrient competition, since *B. cinerea* is particularly sensitive to the lack of nutrients.

Trichoderma strains are always associated with plant roots and root ecosystems. Some authors have defined *Trichoderma* strains as plant symbiont opportunistic avirulent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and to produce compounds that stimulate growth and plant defense mechanisms (Haram *et al*., 1996). *Trichoderma harzianum* is an efficient biocontrol agent that is commercially produced to prevent development of several soil pathogenic fungi. Different mechanisms have been suggested as being responsible for their biocontrol activity, which include competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Haram *et al.,* 1996; Zimand *et al.,* 1996). The presence of growth regulators in the soil could come from applications done to the foliar portion of plants or to fruits, where part of applications fall either directly or from plants into the soil. They could also come from treatments of tubers with auxins, which are used to stimulate budding after seeding; or from soil microorganisms. In fact, it has been described that some fungal pathogens are able not only to induce increased levels of

IAA in their respective hosts, but are themselves capable of producing IAA which is directly released into the soil (Agrios, 1997).

Applications of *T. harzianum* in plant production, therefore, can reduce the use of fungicides, growth regulators and labor which eventually will lower the production costs and environmental impact (Phuwiawat and Soytong, 1999). For example, *Trichoderma harzianum* was shown to solubilize phosphate and micronutrients that could be made available to plant (Altomore *et al*., 1999).

Plant growth mechanisms can be grouped as follows: direct like asymbiotic fixation of atmospheric nitrogen, solubilization of minerals such as phosphates and production of plant growth regulators like auxins, gibberellins, cytokinin and ethylene; and indirect like HCN production, antibiotics, siderophores, synthesis of cell wall lysing enzymes and competitions with detrimental microorganisms for sites on plant roots (Ahmad *et al*., 2008) .

Objectives of the current research

The present work, therefore, was aimed at

1) *In vitro* screening of mycoparasitic activity of *Trichoderma* spp. collected from different geographical locations of Chhattisgarh.
2) Evaluation of potential isolates for chitinase production.
3) Morphological and molecular characterization of potential candidate *Trichoderma* isolates.

CHAPTER II

REVIEW OF LITERATURE

The fungus *Trichoderma* was described as early as 1794 by the mycologist Persoon. The potential for using *Trichoderma* as a biocontrol agent was suggested by Weindling (1932), who was the first to demonstrate the parasitic activity the members of this fungus genus to pathogens such as soil borne plant pathogenic fungi e.g., *Rhizoctonia solani*. However, with the increasing interest in biological control, owing to environmental and economic concerns, and with the rapid development of biotechnology, Dennis and Webster (1971) described the antagonistic properties of *Trichoderma* in terms of antibiotic production and hyphal interactions in the control of *Sclerotium rolfsii.* Several *Trichoderma* species were formulated in a commercial production for the protection and growth enhancement of a number of crops in several countries such as the United States (Mcspadden and Fravel, 2002).

The genus *Trichoderma* belongs to the class Deuteromycetes. It was, for the most parts, classified as an imperfect fungus, in that it has no known sexual stage (Gams & Bisset, 1998). Rifai (1969) distinguished nine species differentiated primarily by conidiophore branching patterns and conidium morphology based on microscopic characters; *Trichoderma aureoviride, T. hamatum, T. harzianum*, *T. koningii*, *T. longibrachiatum*, *T. piluliferum, T. polysporum, T. pseudokoningii*, and *T. viride.*

A sectional classification was proposed for *Trichoderma* recognizing the following five sections; section *Trichoderma*, *Longibrachiatum*, *Saturnisporum*, *Pachybasium* and *Hypocreanum* (Bissett, 1991a). Twenty species were assigned to *Trichoderma* section *Pachybasium.* They were described and differentiated on the basis of conidiophore and conidium morphology (Bissett, 1991b). In the section

Trichoderma, Persoon (1794) characterized species by narrow and flexuous conidiophores with branches and phialides uncrowded, frequently paired, and seldom in verticals of more than three. In the section *Longibrachiatum,* Bissett (1984) indicated that conidiophores are sparingly and irregularly branched, with irregularly disposed and not usually in whorls or verticals and species in this section produce distinctive greenish yellow pigments in reverse of cultures. In the section *Saturnisporum*, conidiophores have a branching system with branches and phialides uncrowded and frequently paired and compact conidiogenous pustules as in section *Pachybasium.* However, it was differentiated by the bullate or winglike conidial ornamentation. The section *Pachybasium*, have species with highly ramified, broad conidiophores usually arranged in compact pustules or fascicles, and with branches and phialides broad or inflated, relatively short, and disposed in crowded verticals. Some species are characterized by the production of sterile conidiophores. The section *Hypocreanum*, chractarized by effuse, usually spars conidiation, sparingly branched conidiophores, and cylindrical to subulate phialides frequently borne in Verticillium-like divergent verticals (Bissett, 1991a).

The genus *Trichoderma* is characterized by rapidly growing colonies bearing tufted or postulate, repeatedly branched conidiophore with lageniform phialides and hyaline or green conidia born in slimy heads (Bissett, 1984). The primary branches of conidiophore produce smaller secondary branches that also may produce tertiary branch, and so on. The final branches are very simply constructed, with a majority of singly phialides (Rifai, 1969). Conidiophore may end in sterile appendages with the phialides born on lateral branches in some species. Conidia are hyaline or, more usually, green, smooth – walled or roughened. Hyaline chlamydospores are usually present in the mycelium of older cultures (Domsch *et al*, 1980). Phialides are

ampulliform to lageniform, usually constricted at the base, more or less swollen near the middle, and abruptly near the apex into short subcylindric neck. They are disposed in verticals terminally on branches of the conidiophore, or less frequently singly or in whorls directly beneath septa along the conidiophore and its branches (Bissett, 1991c).

In 1981, Elad, *et al*., found special *Trichoderma* selective media (TSM) that had been recommended for the quantitative isolation of *Trichoderma* from soil. The colony color is white; as the conidia are formed scattered blue-green or yellow-green patches become visible. These patches may sometimes form concentric rings. They are more readily visible on potato dextrose agar compared to other media-Reverse is pale, tan, or yellowish (Sutton *et al.*, 1998; and De Hoog, 2000).

Trichoderma species are ubiquitous in the environment, especially in soils. They have been used or encountered in many human activities, including commercial applications in production of enzymes and biological control of plant disease (Samuels, 1996).

2.1 *Trichoderma* modes of action

No single mode of action for *Trichoderma* species against fungal plant parasites function alone. There are several mechanisms of action suggested for *Trichoderma* spp. mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, induced resistance, solubilization and sequestration of inorganic nutrients, and inactivation of the pathogens enzymes (Samuels, 1996). The first three were the ones by which these fungi have always been considered to function; other mechanisms are suggested but not yet been confirmed (Harman, 2000).

2.2 Mycoparasitism

Mycoparasitism is considered an important mechanism of biological control and probably depends on the production of lytic enzymes including β- 1,3-gluconase, and proteases (Haran *et al*., 1996). Several chitinolytic enzymes have been reported in *T. harzianum* including endochitinases, exochitinases and 1, 4-β-N-acetylglucosaminidases which are induced during growth in liquid medium containing chitin as carbon source (Haran *et al*., 1996). Mycoparasitism is a complex process including several steps. The initial interaction shows that the hypha of the mycoparasites grows directly towards its host (Chet *et al*., 1981). When the mycoparasite reaches the host, its hypha coils it or attaches to it by forming a hook-like structure. Following these interactions hypha sometimes penetrates the host mycelium, apparently, by partially degrading its cell wall (Elad *et al*., 1983). The control of *Rhizoctonia solani* and *Pythium ultimum* and by *Trichoderma* species, including *T. harzianum*, may be affected through direct penetration of host hyphae (Dennis and Webster, 1971; Benhamou and Chet, 1993). They grow toward hyphae of other fungi, coil about them in a lectin mediated reaction, and degrade cell walls of the target fungi by the activity of enzymes, which may be associated with physical penetration of the cell wall (Chet, 1987). Once *Trichoderma* is attached, it coils around the pathogen and forms the appresoria. The following step consists of the production of CWDEs and peptaibols (Howell, 2003), which facilitate both the entry of *Trichoderma* hypha into the lumen of the parasitized fungus and the assimilation of the cell-wall content. The complex process of mycoparasitism consists of several events, including recognition of the host, attack and subsequent penetration and killing. During this process *Trichoderma* secretes CWDEs that hydrolyze the cell wall of the host fungus, subsequently releasing oligomers from the pathogen cell wall

(Howell, 2003; Woo *et al*., 2006). It is believed that *Trichoderma* secretes hydrolytic enzymes at a constitutive level and detects the presence of another fungus by sensing the molecules released from the host by enzymatic degradation (Harman *et al*., 2004; Lorito *et al*., 2006).

2.3 Competition and rhizosphere competence

Competition for space or nutrients has long been considered one of the classical mechanisms of biocontrol by *Trichoderma* spp. (Elad *et al*., 1999). The competition for nutrients, primarily carbon, nitrogen, and iron is one of the methods of the biological control of soilborne plant pathogens (Scher *et al*., 1984). *Trichoderma* species are generally considered to be aggressive competitors and the ability of *Trichoderma* to compete is species dependent (Wardle *et al*., 1993). Competition through rhizosphere competence is a mechanism that has gained adherents in recent years (Howell, 2003). It is an important mechanism because a biocontrol agent cannot compete for space and nutrients if it is unable to grow in the rhizosphere. Lo *et al*., (1996) found that a strain of *T. harzianum* (T-22) was strongly rhizosphere competent and able to control several plant pathogenic fungi including *R. solani* causing brown patch, and it reduced the initial disease severity by as much as 71% on a variety of crops. Some *Trichoderma* strains establish long-lasting colonization of plant roots and penetrate into the epidermis. There, they produce or release compounds that induce localized or systemic plant resistance responses (Harman *et al*., 2004).

2.4 Induced resistance

Induction of resistance in host plant by treatment with the biocontrol agent *Trichoderma* species is another mechanism in biological control (Howell, 2003). Specific strains of fungi in the genus *Trichoderma* colonize and penetrate plant root

tissues and initiate a series of morphological and biochemical changes in the plant, considered to be part of the plant defense response, which finally leads to induced systemic resistance (ISR) in the entire plant (De Meyer *et al*., 1998).

The inoculating roots of 7-day-old cucumber seedlings in a hydroponic system with *T. harzianum* (T-203) spores to concentration of 105 per ml initiated plant defense responses in both roots and leaves of treated plants (Yedidia *et al*., 1999). Also they demonstrated that hyphae of the biocontrol fungus penetrated the epidermis and upper cortex of the cucumber root. Cucumber plants were larger in the presence of *Trichoderma*, and the cell wall of roots were strengthened in the area of *Trichoderma* penetration, and both chitinase and peroxidase activities in both root and leaf tissues of treated seedlings were evident (Howell *et al*., 1999).

2.5 Solubilization and sequestration of inorganic plant nutrients

Plant nutrients undergo sometimes transitions in soil from soluble to insoluble forms that influence their accessibility and absorption by roots. These transitions may be influenced by microorganisms (Altomare *et al*., 1999). Iron and manganese have been investigated with regard to both microbial solubilization of oxidized forms of these elements and their influence on plant disease (Graham and Webb, 1991). *In vitro*, strain of *Trichoderma harzianum* produces a large number of chemicals to solubilize rock phosphate, Zn, Mn^{4+}, Fe^{3+}, and Cu^{2+} and increase iron availability and enhance iron uptake (Altomare *et al*., 1999). A direct role for the nutrient solubilization and chelating abilities of *Trichoderma* has not been demonstrated, but circumstantial evidence of its ability to solubilize iron and make it usable to plants is available (Harman, 2000). Rudresh *et al.,* (2005) in their experiments studied nine isolates of *Trichoderma* spp. for their ability to solubilize insoluble phosphate in Pikovskaya's broth and were compared with an efficient phosphate-solubilizing

bacterium *Bacillus megaterium* subsp. *phospaticum* PB that was used as the reference strain in which all nine *Trichoderma* isolates were found to solubilize insoluble tricalcium phosphate to various extents. To understand the role of *Trichoderma* in growth promotion, the ability of *Trichoderma* spp. in solubilizing rock phosphate was tested *in vitro*. Culture filtrates of both species solubilized rock phosphate. *T. harzianum was* more effective than *T. aureoviride* in solubilizing phosphates and producing fungal biomass (Satyavani and Satyaprasad., 2009). Fourteen strains of *Trichoderma* sp. were isolated from the forest tree rhizospheres of pinus, deodar, bamboo, guava and oak on *Trichoderma* selective medium and the isolates were tested for their *in-vitro* P-solubilizing potential using National Botanical Research Institute Phosphate (NBRIP) broth containing tricalcium phosphate (TCP) as the sole P source, and compared with a standard culture of *T. harzianum* in which all the cultures were found to solubilize TCP but with varying potential. (Kapri and Tewari, 2010)

2.6 Inactivation of the pathogen enzymes

Enzymes such as chitinases and glucanases produced by the biocontrol agent are responsible for suppression of the plant pathogen. These enzymes function by breaking down the polysaccharides, chitin, and β- glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity (Howell, 2003). Elad and Kapat (1999) suggested that *T. harzianum* (T39) produce proteases that are capable of degrading the pathogens plant cell wall degrading enzymes, and thereby reducing the ability of the pathogen to infect the plant.

2.7 The fungus *Rhizoctonia Solani*

The fungus *Rhizoctonia solani* had been described on potato by Kühn (1858). It is a very common soil-borne pathogen, with worldwide distribution and with a great

diversity of host plants including bean, alfalfa, peanut, soybean, cucumber, papaya, eggplant, corn and many more (Ogoshi and Ui, 1983). The genus *Rhizoctonia solani* belongs to Form Class Deuteromycetes that does not make vegetative spores and can be present as mycelium, sclerotia or basidiospores. *R. solani* composed of several anastomosis groups distinguished by hyphal anastomosis (Ogoshi, 1972) and also differ morphologically, physiologically, and serologically (Naiki and Ui, 1978).Teleomorphs of *Rhizoctonia* occur naturally in the field on host plant and /or soil surfaces, and appear to sporulate on surface of host and non-host plants alike (Gunnell, 1986). *Rhizoctonia solani* primarily attacks below ground plant parts such as the seeds, hypocotyls, and roots, but is also capable of infecting above ground plant parts (e.g. pods, fruits, leaves and stems) (Ogoshi, 1987). When *R. solani* hyphae come in contact with the plant, they start to grow over the plant surface and their hypha surrounds the host but they are attached to plant surface after 10 to 12 hours (Armentrout and Downer, 1987). The most common symptom of *Rhizoctonia* disease is referred to as "damping-off" characterized by non germination of severely infected seeds whereas infected seedlings can be killed either before or after they emerge from the soil. The fungus can survive as sclerotia in soil and on plant tissue for many years (Sherwood, 1970). Also, it survives as mycelium by colonizing soil organic matter as a saprophyte, particularly as a result of plant pathogenic activity (Ogoshi, 1987).

2.8 The Fungus *Sclerotium Rolfsii*

The fungus had been described by Rolfs (1892), and named *Sclerotium rolfsii* by Saccordo in 1911. It is an economically important pathogen on numerous crops worldwide (Aycock, 1966). It is an omnivorous and destructive parasite of many plants. It has an extensive host range; at least 500 species in 100 families are susceptible, the most common hosts are legumes, crucifers, and cucurbits (Chupp and

Sherf, 1960). *Sclerotium rolfsii* is an imperfect fungus which belongs to Form class Deuteromycetes. It does not produce spores. Growth of *S. rolfsii* on all organic-based and inorganic synthetic media is accompanied by forming of spherical, brown to tan colored sclerotia measuring 0.3 to 3.0 mm in diameter (Edelstein *et al*., 1983). The fungus attacks all plant parts in contact with the soil under favorable environmental conditions including stems, roots, leaves, and fruits (Farr *et al*., 1989).

2.9 Biological control of *R. Solani* and *S. rolfsii* using *Trichoderma*

Biological control of plant diseases, especially soilborne plant pathogens, has been the subject of extensive research in the last two decades. *Trichoderma* spp. is well documented as effective biological control agents of plant diseases caused by soilborne fungi (Sivan *et al*., 1984, Coley-Smith *et al*., 1991). Biological control of soilborne plant pathogens can be achieved by seed treatment with antagonists. Harman *et al*. (1980) reported the biocontrol of *Rhizoctonia solani* and *Pythium* spp. by coating radish and pea seed with *Trichoderma hamatum*. Hadar *et al*., (1979) and Elad *et al*. (1980) found that the application of wheat bran colonized by *Trichoderma harzianum* to soils infested by *Rhizoctonia solani* and *Sclerotium rolfsii* reduced the incidence of disease caused by these pathogens in beans. Control of soil borne plant pathogens including *R. solani* and *S. rolfsii* can be achieved by different fungicides, soil fumigants and bioagents. Because of the concern regarding the toxicity of these compounds, there is a general trend to reduce the amounts applied to soil. Methyl bromide is a typical example of an efficient fumigant about which the public is concerned because its use causes the accumulation of bromide residues, especially in water (Hoffman and Malkomes, 1974). Harman *et al*., (1980) found that *Trichoderma* can survive for long periods of time and propagate in soil when applied with a food base or as a seed coating. Its combination with chemical, cultural, or physical methods

can achieve a long-term controlling effect on soil borne plant pathogenic fungi (Katan *et al*., 1976). Integrating biological and chemical control seems a very promising way of controlling pathogens with minimal interference with biological equilibrium (Baker and Cook, 1982). Hadar *et al*., (1979) applied small non 25 effective doses (1-2 μg/kg) of Pentachloronitrobenzene (PCNB) to soil along with a *Trichoderma* preparation (2g/kg) and the incidence of eggplant disease caused by *R. solani* declined from 40 to 13%, while *T. harzianum* reduced disease incidence to 26%. Combination of heat treatment and *T. harzianum*, both at sublethal doses and under greenhouse conditions, enhanced control of *S. rolfsii* diseases on beans from 90 to 100% (Elad *et al*., 1980). Lewis and Papavizas (1980) found that integrated management by preventing colonization of plant residues, and combining *Trichoderma* with plouing to a depth of 20-25 cm reduced root rot of beans caused by *R. solani.* Transplanting tomato plants treated with *T. harzianum* into soil fumigated with methyl bromide reduced the disease incidence caused by *S. rolfsii* and *R. solani* by 93% and increased yield by 160% (Elad *et al*., 1982). The addition of pregerminated conidia of *Trichoderma lignorum* to soils reduced germination of sclerotial bodies of *R. solani*. In non-rhizosphere and rhizosphere soil of bean seedlings previously enriched with sclerotial bodies of *Rhizoctonia solani* and planted with bean seeds, the germination rate of sclerotial bodies was 21% and 44 %, respectively. However, when *Trichoderma lignorum* was added as seed coating or conidial suspension, the germination rate of *R. solani* was reduced to 30 and 28 %, respectively in rhizosphere soil and to 12 and 8 %, respectively in non-rhizosphere soil (Cook and Baker, 1983).

The addition of bean seed exudates increased the percentage of germinated sclerotial bodies of *R. solani* from 33.7% to 39.0% in absence of *T. lignorum.* Similarly, under greenhouse conditions, the addition of bean exudates to soil infested with *R. solani*

and planted with bean reduced the disease control obtained by *T. lignorum* as either seed coating or conidial suspension. The percentage of damping-off increased from 5.7% to 37.7% in seed coating and from 15.3% to 43.7% in conidial suspension. Addition of germinating bean seed exudate increased the percentage of damping-off from 78.3% to 83.3% (Hussain *et al*., 1990). Aziz *et al*., (1997) found that the application of a wheat bran preparation of *Trichoderma lignorum* conidia (8 × 106 conidia/seed) at a rate of 15 and 20 g/500 g soil decreased greatly the damping-off percentage to 12% and 6%, respectively, as compared to untreated bean seeds. In addition, he found that application of wheat bran preparation of *Trichoderma lignorum* (5 × 106 cfu/g) at a rate of 2.5 g /500 g of soil decreased the damping-off percentage to 45%.

2.10 Cell-wall-degrading enzymes chitinases

The chitinolytic system of *Trichoderma* comprises many enzymes and the list of its components is rapidly being updated as new enzymes and genes are reported. Chitinases are divided into 1,4-β-acetylglucosaminidases (GlcNAcases), endochitinases and exochitinases. Many GlcNAcases and their genes-*exc1* (=*nag1*), *exc2*, *tvnag1*, and *tvnag2* from *T. harzianum* T25-1, *T. atroviride* P1 and *T. virens* Tv29-8 -have been described (Harman *et al*., 2004, Kim *et al*., 2002). The 73-kDa Nag1 represents the main GlcNAcase in *T. atroviride*. Nag1-disruption strain lacks chitinase activity and the endochitinase *chit42* mRNA is absent (Harman *et al*., 2004).This indicates that *nag1* is essential for triggering chitinase gene expression. The pathogen cell wall and chitin induce *nag1*, but it is only triggered when there is contact with the pathogen (Carsolio *et al*., 1999., Mach *et al*., 1999) GlcNAcases CHIT73 and CHIT102 were detected in *T. harzianum* TM and *Trichoderma asperellum* (Haran *et al*., 1996). CHIT102 triggers the expression of other chitinolytic

enzymes (Haran *et al*., 1996). In addition, strain 2413 produces three extracellular endochitinases whose genes, *chit33*, *chit37* and *chit42*, have been cloned from this strain. Other genes coding for Chit42 chitinase-*ech42*, *cht42* and *ThEn4*-have also been cloned from *T. atroviride* IMI206040 (Carsolio *et al*., 1999), Gv2908 (Howell *et al*., 2003) and *T. atroviride* P1 (Howell *et al*., 2003), respectively. Chit37 shows 89% similarity to Chit36 from *T. harzianum* TM at the amino-acid level (Harman *et al*., 2004). Chit36 inhibits *B. cinerea* spore germination and the growth of both *Sclerotium rolfsii* and *Fusarium oxysporum* (Viterbo *et al*., 2001). Other genes homologous to *chit36* have been cloned from *T. harzianum* TM, *T. atroviride* P1 and *T. asperellum* T-203. Endochitinases are regulated by a variety of mechanisms but induction by stress has been reported for *chit33*, *chit36* and *chit42*. However, the induction under mycoparasitic conditions is not clear. *ech42* is induced prior to any physical contact with *R. solani* (Kullnig *et al*., 2000). *chit33* is expressed only during the contact phase and not before overgrowing *R. solani* (Dana *et al*., 2001); and *chit36Y* does not need the direct contact of the pathogen to be expressed. *chit33*, *chit42* and *chit36* have been overexpressed in *Trichoderma* spp. in order to test the role of these chitinases in mycoparasitism, and the 42-kDa chitinase is believed to be a key enzyme (Howell *et al*., 2003). *T. virens* transformants overexpressing Chit42 showed significantly enhanced biocontrol activity compared with the wild-type when assayed against *R. solani* in cotton seedlings experiments (Howell *et al*., 2003). Other *Trichoderma* spp. transformants overexpressing *chit42* resulted in better antagonism than obtained with the wild-type (Carsolio *et al*., 1999; Limon *et al*., 2004). In greenhouse biocontrol tests, however, the activity of chit42 disruptants did not differ from that of the wild-type (Harman *et al*., 2004). *T. harzianum* transformants overexpressing Chit33 chitinase constitutively inhibited the growth of *R. solani* under both repressing and

derepressing conditions; the antagonist tests demonstrated that this chitinase also has an important role in mycoparasitism (Limon *et al*., 1999). *T. harzianum* Rifai TM transformants overexpressing Chit36 chitinase inhibited *F. oxysporum* and *S. rolfsii* more strongly than the wild-type. Moreover, culture filtrates inhibited the germination of *B. cinerea* almost completely (Viterbo *et al*., 2001). The antagonism of Chit33 and Chit42 transformants has been improved by the addition of a cellulose-binding domain to the chitinase genes. As a result, the strains producing the chimeric enzymes increased their specific chitinase activity (Limon *et al*., 2004).

2.11 Cellulase production by *Trichoderma*

Cellulase production is the most important step in the economical production of ethanol, single cell protein and other chemicals from renewable cellulosic materials. To date, the production of cellulase has been widely studied in submerged culture processes, but the relatively high cost of enzyme production has hindered the industrial application of cellulose bioconversion. It has been reported that solid-state fermentation is an attractive process to produce cellulase economically due to its lower capital investment and lower operating expenses. Lignocelluloses degradation represents a considerable challenge to enzymatic hydrolysis on account of its physical structure which resists degradation. Its main component cellulosic composed of long unbranched polymers of glucose packed onto each other to form highly insoluble aggregates. Cellulolytic organisms such as *Trichoderma* produce complex mixtures of enzymes which efficiently solublises this substrate. *Trichoderma reesei* is an impotent industrial fungus known for its ability to efficiently secrete large quantities of protein as well as its wide variety of biomass degrading enzymes. The cellulolytic fungi *Trichoderma viride* and *Trichoderma reesei* has been extensively studied for their cellulase production (Mandels and Weber, 1969; Mandels *et al.,* 1971; Monotenecourt

and Eveleigh, 1979; Gadgil *et al*., 1995; Velkovska *et al* 1997; Domingues *et al.,* 2000). Genome sequencing of the biomass degrading fungus *Trichoderma reesei* has been done, which provides a roadmap for constructing enhanced *T. reesei* strain for industrial application such as biofuel production (Martinez et *al.*, 2008).

2.12 Biofertilization

Root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients (Arora *et al*., 1992). Crop productivity in fields can increase up to 300% after the addition of *Trichoderma hamatum* or *Trichoderma koningii*. In experiments carried out in greenhouses, there was also a considerable yield increase when plant seeds were previously treated with spores from *Trichoderma* (Chet *et al*., 1997). The same increase was observed when seeds were separated from *Trichoderma* by a cellophane membrane, which indicates that *Trichoderma* produces growth factors that increased the rate of seed germination (Benítez *et al*., 1998). However, there are very few reports on strains that produce growth factors which have been detected and identified in the laboratory (auxins, citokinins and ethylene), despite the identification of many filamentous fungi that produce phytohormones, such as indol acetic acid (IAA) and ethylene, whose metabolic pathways have been identified (Arora *et al*., 1992; Osiewacz, 2002). *Trichoderma* strains that produce cytokinin-like molecules, e.g. zeatyn and gibberellin GA3 or GA3 related, have been recently detected.

2.13 Fungal compounds involved in induction of plant responses

Studies revealed many classes of compounds that are released by *Trichoderma* spp. into the zone of interaction and induce resistance in plants. The first class is proteins with enzymatic or other activity. Fungal proteins such as xylanase, cellulase,

and swollenin are secreted by *Trichoderma* species (Anderson *et al.,* 1993, Fuchs *et al.,* 1989, Lotan *et al.,* 1990, Martinez *et al.,* 2001) but seem to induce only localized plant reactions and necrosis (Bailey *et al.,* 1991, Brotman *et al.,* 2008, Martinez *et al.,* 2001). *Trichoderma* endochitinase can also enhance defense, probably through induction of plant defense–related proteins (Harman and Shoresh 2007, Lorito, 1998). Other proteins and peptides that are active in inducing terpenoid phytoalexin biosynthesis and peroxidase activity in cotton, e.g., the small protein, SM1, which has hydrophobin-like properties, were found to be produced by strains of *T. virens* (Djonovic *et al.,* 2006, Djonovic *et al.,* 2007, Hanson and Howell, 2004). Another hydrophobin-like protein produced by T22 that induces both enhanced root development and disease resistance was identified (Ruocco *et al.,* 2007). Another group of proteins that induce defense mechanisms in plants are the products of avirulence-like (Avr) genes (Woo and Lorito, 2007, Woo *et al.,* 2006). These are produced not only by a variety of fungal and bacterial plant pathogens but also by BCF. They usually function as race- or pathovar-specific elicitors of hypersensitive and other defense-related responses in plant species that contain the corresponding resistance (R) gene. At least some of these fungal elicitors of plant defense response could be identified by plants asmicrobe-associated molecular patterns (MAMPs). This recognition plays a key role in innate immunity (Bent and Mackey, 2007). A different group of metabolites that induce plant defense mechanisms against pathogens are peptaibols. Peptaibols are a class of linear short-chain length (≤20 residues) peptides of fungal origin produced by the nonribosomal peptide synthase. The biological role of peptaibols has been demonstrated in few systems, and antimicrobial activity was reported (Chanikul *et al.,* 2008, Chugh and Wallace 2001, Rebuffat *et al.,* 2000, Schirmbock *et al.,* 1994, Szekeres *et al.,* 2005). However, a growing number of

reports indicate that peptaibols can elicit plant defense responses (Chen *et al.,* 2003, Engelberth *et al.,* 2001, Viterbo *et al.,* 2007). Another class of elicitors of plant defense includes oligosaccharides and low-molecular weight compounds. These are released from fungal or plant cell walls by the activity of *Trichoderma* enzymes (Harman *et al.,* 2004, Woo and Lorito, 2007, Woo *et al.,* 2006). Other small secondary metabolites produced by different *Trichoderma* strains were also isolated and shown to induce expression of pathogenesis related (PR) proteins when applied to plants as well as reduce disease symptoms systemically (Vinale *et al.,* 2008).Less-characterized metabolites produced by other BCF induce resistance, induce lignifications at the site of pathogen infection, and elicit generation of reactive oxygen species (ROS) (Koike *et al.,* 2001). Plant responses were also recorded for a cell wall extract from *P. indica*. However, these extracts promote growth but not defense responses (Vadassery *et al.,* 2009). It appears that modulation of Ca2+ signal perception as well as H+- signalling are an early step of plant cells response to the interaction with BCF metabolites (Felle *et al.,* 2009, Navazio *et al.,* 2007, Vadassery *et al.,* 2009).

2.14 Effect on root development and performance

Inoculation of plants roots by *Trichoderma* or *Sebacinales* species results in changes in root development. *Trichoderma*-inoculated roots are deeper and more robust (Harman, 2000). Main and secondary roots of maize increased in size, and the area of the root hair was greater with *Trichoderma*-T22 inoculation (Harman *et al.,* 2004). *P. indica* also induced root developmental changes. Promotion of root growth and increased length of root hairs were detectable even before notable root colonization (Peskan *et al.,* 2004). Root branching can improve soil exploitation and hence result in plant growth promotion. An effector hydophobin-like protein from

T22 has been identified that mimics the effect of the fungus (Ruocco *et al.,* 2007). *Arabidopsis* root colonization by *P. indica* resulted in a stunted but highly branched root system, which is probably mediated by low amounts of auxins produced by *P. indica* (Sirrenberg *et al.,* 2007). Several auxin-like secondary metabolites produced by *Trichoderma* strains were able to induce plant growth and are required for development of lateral roots in *Arabidopsis* (Contreras-Cornejo *et al.,* 2009, Vinale *et al.,* 2008). However, a recent study implicates cytokinins in plant growth promotion (Vadassery *et al.,* 2008). *P. indica* induces relatively high levels of cytokinins, and the cytokinin levels are higher in colonized roots compared with uncolonized controls. Although root colonization was not affected in cytokinin biosynthesis or receptor mutants, no growth response was recorded in mutants possessing decreased levels of *trans*-Zeatin cytokinins. This indicated that cytokinins are required for the plant growth response but not the root interaction with the fungus (Vadassery *et al.,* 2008).

However, many resistance inducing fungi (*Trichoderma* sp.) (Lindsey and Baker, 1967; Chang *et al.,* 1986) and bacteria (plant growth promoting *rhizobacteria*) do increase both shoot and root growth (Kloepper, *et al.*, 1993; Lindsey and Baker, 1967; Pozo *et al*., 2002). *T.harzianum* strain T-22 is widely used for disease control in the greenhouse industry than chemical fungicides, as it is safer to use, its disease control effect last longer, it is less costly and promote root growth that can be as good, or better, than that achieved using pesticide (Harman, 2002). *Trichoderma* treatments therefore have the potential to improve overall crop yields and might be particularly important in sub-potential field condition. Generally the increased yield of plants is more evident under stressful condition; when modern crop plants are grown under near optimal condition there is little opportunity for yield improvement.

IAA is also involved in tomato fruit development, especially during fruit setting and in the final phase of development (Srivastava and Handa, 2005). Indeed, IAA, including microbial, can greatly influence the growth of the root system depending on the amount found in the rhizosphere, through root elongation and the formation of lateral or adventitious roots (Scott, 1972; Patten and Glick, 2002).

Several auxin-like secondary metabolites produced by *Trichoderma* strains were able to induce plant growth and are required for development of lateral roots in *Arabidopsis* (Contreras *et al*., 2009, Vinale *et al*., 2008). The observed effect of *Trichoderma* in promoting lateral root development is similar to that described for auxins in plants (Casimiro *et al*., 2001) IAA is a molecule that is synthesized by plants and a few microbes (Woodward and Bartel, 2005). In plants IAA plays a key role in root and shoot development. The hormone moves from one part of the plant to another by specific transporter systems that involve auxin importer (AUX1) and efflux (PIN1-7) proteins. IAA is a key regulator of lateral root development and root hair development (Casimiro *et al*., 2001). Expression studies of the auxin-inducible marker DR5: uidA suggested that *T. virens* inoculation increases the auxin response in Arabidopsis seedlings.

Another component of growth induction could be due to increase in nutrient uptake. *Trichoderma* spp. have significant abilities to solubilize a range of plant nutrients, such as phosphorus and micronutrients including iron, copper, zinc, and manganese, thus rendering them available for plants (Altomare *et al*., 1999). Thus, BCF can solubilize plant nutrients (indirect effect) and also induce plants to uptake more nutrients (direct effect). In soil, plant roots normally coexist with bacteria and fungi which may produce siderophores capable of sequestering the available soluble iron and hence interfere with plant growth and function. Siderophores are produced

during extreme iron-depleted conditions for the solubilization of extracellular ferric iron by most bacteria and fungi. The acquisition of iron by microorganisms in organic environments (Lindsay and Schwab, 1982) presents a difficult problem since the solubility products constant for ferric hydroxide is about 10^{-38}. Thus at pH 7, the freely available iron is at a concentration of no more than 10^{-17} M, which is far below that required for microbial and plant growth. Iron in an aerated environment exists in the ferric form and hence is highly insoluble in neutral or alkaline soil (Shenker *et al*., 1995). The synthesis and secretion of a low molecular weight ferric-specific chelation agent to solubilize iron is termed as Siderophore (Neilands, 1981).

2.15 Molecular markers in *Trichoderma* spp.

The anamorphic fungal genus *Trichoderma* (Hypocreales, Ascomycota) contains cosmopolitan soil born fungi frequently also found on decaying wood (Samuels, 1996; Klein and Eveleigh, 1998), of which some are economically important producers of industrial enzymes (*Trichoderma reesei = Hypocrea jecorina*) (Kubicek and Penttila,1998) and antibiotics (Sivasithamparam and Ghisalberti, 1998), or have application as biocontrol agent against plant pathogens (i.e. *T. harizanum =H. lixii; T. atroviride =H. atrovirides; T. asperellum*) (Hjeljord and Tronsmo,1998). *Trichoderma* has been recognized to comprise a significant amount of fungal biomass in soil (Widen and Abitbol, 1980) and is frequently present as an indoor contamination (Thrane *et al*., 2001). These diverse implications of *Trichoderma*/Hypocrea with human society render an accurate species and strain identification to be an important issue. However, classical approaches based on the use of morphological criteria are, as in several other fungi, difficult to apply to *Trichoderma,* due to the plasticity of characters.

Species in the fungal genus *Trichoderma* (Ascomycetes, Hypocreales) are of great economic importance as sources of enzymes, antibiotics, as plant growth promoters, xenobiotic degraders, and most importantly, as commercial biofungicides (Mukherjee, 1999). Until recently, *Trichoderma* spp. were being identified based on morphological data like cultural characteristics, structure of conidiophores/conidia, etc. (Rifai, 1969; Bissett, 1984). However, subsequent molecular analysis of several strains, including some ex-type strains revealed that classification based on morphological data has been, to a great extent, erroneous resulting in re-classification of several isolates and species (Castle *et al.,* 1998; Kindermann, *et al*., 1998; Lieckfeldt *et al*., 1999). For example, three Indian isolates of *Trichoderma* that are deposited at Microbial Type culture collection, Chandigarh as *T. harzianum*, were examined by (Hermosa *et al*., 2000). Two of them were found to be *in T. hamatum*, and one was classified as *T. longibrachiatum*, using molecular tools. The exact characterization and identification of *Trichoderma* strain at the species level is an important first step in systematically utilization the full potential of fungi in specific application (Lieckfeldt *et al.,* 2001). Species designation based on the evolution of 40 or more morphological traits (Hermosa *et al.,* 2001) have been shown to provide a reliable diagnostic method in expert hands. However, this presents a major time and cost bottleneck for raised large-scale screening to identify agriculturally useful isolates (Kubicek *et al*., 2002). DNA data, and to a limited extent also protein data, reflect the genotype of the organism and may give a clearer picture of relationships than do morphological characters. Molecular methods based on the characterization of proteins and/or nucleic acid and their polymorphisms provide an almost unlimited number of potential markers for taxonomic studies. A frequently used method for estimating genetic variation is isozyme electrophoresis. The first characterization of

Trichoderma species by isozyme patterns was done by Zamir and Chet (1985). Twenty tree geographically diver's isolates of *Trichoderma harzianum* were grouped into five types according to the isozyme profiles. Stasz *et al.,* (1989) used 16 enzyme loci that resolved 109 alleles in a study of 71 strain that be are distributed between five morphological species (Rifai, 1969), namely *Trichoderma harzianum, Trichoderma hamatum, Trichoderma kiningii, Trichoderma pseudokoningii* and *Trichoderma viride*. The close morphological resemblance that exists between the species of *Trichoderma harzianum* and *Trichoderma inhamatum; Trichoderma viride* and *Trichoderma asperllum*; and *Trichoderma Koningii* and *Trichoderma Konilangbr*a has been resolved clearly without any controversy using molecular and biochemical analysis (Samuels,1996; Latha *et al.,* 2002). Polymorphism at the DNA level can be studied by several means, the most common of which is the analysis of restriction fragment length polymorphisms. Meyer (1991) used mitochondrial DNAs and plasmids as taxonomic characteristics for *Trichoderma viride*. He investigated 12 strains of the *Trichoderma viride* aggregated species and result of the molecular analysis was discussed in comparison to conidial ornamentation (Meyer and Plaskowiz, 1989). Morawetz *et al.,* (1992) and Kubicek *et al.,* (1996) produced RFLP patterns of a DNA fragment representing the cellobiohydrolase I-encoding gene *cbh*1 from different *Trichoderma* species that were species-specific. Internal transcribed sequences (ITS) of the ribosomal DNA (rDNA) analysis (rDNA-ITS1) and universally primed polymerase chain reaction (PPCR) have used to characterize isolates of *Trichoderma* (Cumagun *et al*., 1999). The polymerase chain reaction (PCR) technique has created new ways of revealing DNA polymorphisms among closely related genotypes with high sensitivity via a fast and easy to perform protocol. PCR-based fingerprinting involves the amplification of unknown DNA fragment

using arbitary CG-rich primers known as RAPD analysis (Williams *et al.,* 1990) or microsatelite- complementary oliginucleotides: (GACA).4, (GTG) 5 and 13 core sequence (Lieckfeldt, *et al*., 1993; Meyer *et al.,* 1992). PCR-based fingerprinting is widely applied for the characterization of *Trichoderma* strain and species for various purposes. Schlock *et al.,* (1994) Khuls *et al*., (1995) used PCR fingerprinting for *Trichoderma* strain identification and detection of culture impurities. The RAPD technique was used by Muthumeenakshi *et al*., (1994) to investigate *Trichoderma harzianum* strain that are antagonistic to the commercial production of mushrooms. Kuhls *et al.,* (1996) combined PCR fingerprinting with rDNA sequence studies to prove the relation between a strictly asexual *Trichoderma* species and the ascomycete *Hypocrea jecorina*. The RAPD technique in an extensive study of molecular taxonomy of *Trichoderma* that included 145 strain of *Trichoderma* spp. (Turner *et al*., 1996). Random markers as products of the PCR-based randomly amplified polymorphic DNA (RAPD) technique (Williams*, et al*., 1990) haven developed to differentiate numerous fungi, including *Trichoderma* species (Arisan, *et al*., 1995; Elad, *et al.,* 1981). Since the genome of *Trichoderma hamatum* 382 is poorly understood, RAPD analysis may prove to be an ideal method for DNA fingerprinting. Recently, sequence characterized amplified region (SCAR) marker (Ohmori, *et al*., 1996; Paran, *et al*., 1993) or sequence-tagged site markers (Brisban *et al*., 1995; Kapalan *et al.,* 1996) have been derived from RAPD markers. SCAR and sequence-tagged site markers offer advantages over, and are distinct from, RAPD markers because they are PCR amplified with specific primers and may represent a single locus in the genome. Initial classification of 48 *Trichoderma* isolates, derived from four different groundnut cultivation sites in India was based on alignment of 28S rDNA sequences to Gene Bank sequences of ex-type strains. This was found to be

substantially more reliable than the routine morphological characterization, but did not provide a comprehensive diagnostic solution as unique single nucleotide polymorphism (SNP) analysis, based on six primer pair combination, which generated 234 polymorphic bands. Genetic classification using internal transcribed spacer 1 and 2 (ITS1 and ITS2) sequences of the rDNA gene cluster allowed the separation of the former *Trichoderma harzianum* Rifai aggregate into *Trichoderma asperellum* (Samuels *et al*., 1999), *Trichoderma atroviride*, *T. harzianum* sensu stricto, and *Trichoderma longibrachiatum* (Hermosa *et al*., 2000).

However, ITS1/ITS2 sequence differences were unable to consistently distinguish between very close species, and thus multigene approaches, including analysis of different fragments of the translation elongation factor EF- 1a (*tef1* gene) (Druzhinina *et al*., 2005), were carried out to separate and place new isolates in appropriate species (Druzhinina *et al*., 2006; Hermosa *et al*., 2004), study the frequency of biocontrol agents in the genus, (Hermosa *et al*., 2004) identify new species (i.e.,*Trichoderma gamsii*) (Jaklitsch *et al*., 2006), or establish teleomorph/anamorph associations such as *Hypocrea virens/T. virens* (Chaverri *et al*., 2001, *Hypocrea atroviridis/T. atroviride* (Dodd *et al*., 2003). Among 11 gene loci or fragments tested in *Hypocrea/Trichoderma*, the most promising ones appear to be the 4th and 5th introns of translation elongation factor 1-alpha (*tef1*, ~EF-1α,), and the coding portions of endochitinase 42 (*ech42*) (Druzhinina *et al*., 2006). Resolution of some clades can be obtained by the use of *rpb2* and the ITS1 and ITS2 diagnostic regions. Integrated physiological and molecular investigations served to separate *T. harzianum* from the mushroom pathogens (Grondona *et al*., 1997, later described as *Trichoderma aggressivum* (Samuels *et al*., 2002) or *Trichoderma pleurotum* and *Trichoderma pleuroticola* (Komon *et al*., 2007).

The International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH) has developed methods for quick molecular identification of *Hypocrea* and *Trichoderma* species, available at **http://www.isth.info**, that are based on DNA oligonucleotide sequence hallmarks of the genera and species (Druzhinina *et al*., 2005). The BarCode identification platforms use ITS1 and ITS2, *tef1* (fourth intron, fifth intron, sixth exon) and/or an RNA polymerase gene (*rpb2* exon) for the analysis in TrichOKEY, TrichoBLAST, and TrichoCHIT, (Kopchinskiy *et al*., 2005; Nagy *et al*., 2007). The majority of *Trichoderma* isolates are easily identified by TrichOKEY and TrichoBLAST, but the existence of new species is still indicated by the occasional lack of sequence match. Regardless, the number of species now recognized is more than 100 (Druzhinina *et al*., 2006). In addition, molecular characterization has become necessary to monitor the activity and register agents for biocontrol and other commercial applications. Reporter genes have been used to study *Trichoderma* plant-pathogen interactions in vivo, also with commercial strains (Lo *et al*., 1998; Lu *et al*., 2004). Identification of specific biocontrol strains *in situ* was achieved by using random amplified polymorphic DNA (RAPD) analysis, sequence-characterized amplified region (SCAR) markers, and realtime polymerase chain reaction (PCR) (Abbasi *et al*., 1999; Hermosa *et al*., 2001; Savazzini *et al*., 2008). The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett., 1992). They also occur in multiple copies with up to 200 copies per haploid genome (Bruns *et al*.,1991; Yao *et al*.,1992) arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan *et al*., 1995).

CHAPTER III

MATERIALS AND METHODS

The present investigation entitled **"Genotypic fingerprinting of antagonistic *Trichoderma* spp. against *Rhizoctonia solani* and *Sclerotium rolfsii* using gene specific molecular marker"** was conducted at the Molecular Plant Pathology laboratory of Department of Plant Molecular Biology and Biotechnology, College of Agriculture, IGKV, Raipur (Chhattisgarh). During the course of study, potato dextrose agar was used for maintaining the culture of different species of *Trichoderma*, *R.solani, S.rolfsii* unless and until stated otherwise. Prior to the use glassware's were cleaned with labolin, and rinsed with tap water and / or distilled water. Dried glassware's were sterilized in hot air oven at 80˚C for two hours, and forceps, inoculation needle and other metallic instruments were sterilized by dipping them in alcohol followed by heating over the flame. Until and otherwise stated media was sterilized by autoclaving at 121˚c at 15psi for 30 min.

3.1. Materials

During the course of investigation twenty genetically purified isolates of *Trichoderma* spp. (Table 3.1) kindly provided by Dr. A.S.Kotasthane, Professor Department of Plant Molecular Biology and Biotechnology, College of Agriculture, IGKV, Raipur were used. Isolates of *Rhizoctonia solani* and *Sclerotium rolfsii* were isolated from infected rice (*Oryza sativa*) plants (Table 3.1).

Table 3.1 Isolates of *Trichoderma* spp. obtained from soil samples collected from different geographical location of Chhattisgarh.

S.No.	Iso No.	Location
1	N	Raipur
2	T1	Guava,IGKV,Horticulture
3	T7	Rice Field, Raipur
4	T14	Raipur field
5	T15	Patan,Mango
6	T16	Raipur,Rice field
7	T17	Raipur,Rice field
8	T27	Telidabra,Amarkantak
9	T29	Abhanpur
10	T31	Abhanpur
11	T66	Pareshgaon(Jagdalpur)
12	T73	Dhamtari road, Raipur
13	T93	Jaisekara
14	T101b	Kanker,Forest
15	T110	Kirda
16	T114	Kodebor
17	T120	Kurdha
18	T132	Purur
19	T158a	Jagdalpur city
20	T174	VIP road, Raipur
Isolates of *Rhizoctonia solani and Sclerotium rolfsii* derived from infected plants of rice		
1	*R. solani*	Rice (*Oryza sativa)*
2	*S. rolfsii*	Rice(*Oryza sativa)*

3.2. Methods

3.2.1 Morphological characterization of the isolates of *Trichoderma* spp.

Cultural characters of all the single spore isolates under investigation were studied on 20 ml sterilized PDA in petri plates. Mycelial disc of approximately 5 mm diameter was transferred aseptically from culture slants at the center of each petri dish and incubated at 282 ^{o} C for 4 days. The petri dishes with the culture were observed for growth pattern (presence or absence of aerial mycelium or subdued growth etc.), pigmentation of varying shades of green of the vegetative growth and pigmentation of the secreated metabolite. in the substrate medium against white background under sunlight. The cultural characteristics were photographed using digital camera.

3.2.2 Microscopic characterization of the isolates of *Trichoderma* spp.

Conidiophores and conidial morphology, branching pattern, critical for identification to the species level are best observed before the conidia are completely mature. Mounts from the actively growing (fungal growth from the growing colony margin) isolates of *Trichoderma* spp.were prepared in lactophenol cotton blue on glass microscopic slide (preferably young tufts where the conidia were just beginning to develop pigment in actively growing cultures). Mounts were prepared using a cello tape. A strip of cellotape was held in thumb and forefinger and the gum coated surface was impressed against the sporulating growth of the isolate. The cellotape thus lifted the intact sporulating growth (including conidiophores, philides, sporulation) without forming any clump of mycelium and injuring the substrate. Microscopic morphological features of all the selected isolates were observed using a Leica binocular microscope and were micro photographed digitally.

3.3 Screening of isolates of *Trichoderma* spp. for *In vitro* antagonism

3.3.1 Dual culture interaction

Mycoparasitic activity of twenty *Trichoderma* isolates was studied *in vitro* following dual culture method (Bell *et al*., 1982) against the two rice pathogens *R.solani* and S. *rolfsii* (Table 3.2). For dual culture 3-mm diameter mycelial block (derived from 7-days-old culture of each *Trichoderma* isolates and of two rice pathogen(s)) were placed 3 cm apart on the pre sterilized and cooled PDA surface under aseptic conditions and care was taken to avoid contamination. Petri dishes containing dual cultures were incubated at 28±2°C under continuous light and were observed at regular intervals until mycelia growth of the oppositely paired cultures merged. Mycoparasitic activity of the respective *Trichoderma* isolate was assessed as follows:

1= *Trichoderma* completely overgrew the pathogen and covered the entire medium surface

2= *Trichoderma* overgrew at least 2/3 of the medium surface

3= *Trichoderma* and pathogen each colonized approximately ½ of the medium surface (more than 1/3 and less than 2/3), and neither organism appeared to dominate the other

4= The pathogen colonized at least 2/3 of the medium surface and appeared to withstand encroachment by Trichoderma

5= The pathogen completely overgrew the *Trichoderma* and covered the entire medium surface

Table 3.2 Possible combination(s) for dual culture to evaluate the mycoparasitic activity of *Trichoderma* spp. against rice pathogen(s) *R. solani* and *S. rolfsii.*

Isolates of *Trichoderma* spp.	Rice derived pathogens	
	R. solani	*S. rolfsii*
T1	*	*
T7	*	*
T14	*	*
T15	*	*
T16	*	*
T17	*	*
T27	*	*
T29	*	*
T31	*	*
T66	*	*
T73	*	*
T93	*	*
T110	*	*
T114	*	*
T120	*	*
T132	*	*
T174	*	*
T101b	*	*
T158a	*	*
N	*	*

3.3.2 Microscopic examination of Hyphal Interaction on thin films of agar

Hyphal interaction was made on sterile glass cover slips coated with 2% water agar (Laing and Deacon, 1991). Each glass slide was sterilized by autoclaving which was then poured with a thin layer of presterilized melted 2% water agar. To evaluate the mycoparasitic activity of *Trichoderma* spp. against rice pathogen(s) *R. solani* and *S. rolfsii* following microscopic examinations isolates of *Trichoderma* spp. were paired (as described earlier in section 3.3.1 Dual Culture Interaction) against rice pathogen(s) *R. solani* and *S. rolfsii* on the thin layer of solidified 2% water Agar. Petri dishes containing dual cultures were incubated at 28±2°C under continuous light and were observed at regular intervals until mycelia growth of the oppositely paired cultures merged. Microscopic observations were performed using Leica binocular microscope and the selected specimen were micro photographed digitally.

- **Screening isolates of *Trichoderma* spp. for chitinase activity**

3.4.1. Preparation of colloidal chitin:

Colloidal chitin was prepared from commercial chitin (Hi Media) and was used for chitinase detection test.

- **Acid hydrolysis of chitin:-**

To prepare colloidal chitin , 5.0 g of chitin was suspended in 60 ml conc. HCl and was acid hydrolyzed by constant stirring using a magnetic stirrer at 4ºC (refrigerator) overnight.

- **Extraction of colloidal chitin by ethanol neutralization:-**

To the resulting slurry 2000 ml ice cold 95% ethanol was added and was kept at 28±2°C for overnight to neutralize the acid hydrolyzed chitin, which was then centrifuge at 3000 rpm for 20 min at 4ºC. Supernatant was discarded and the pellet

was washed with sterile distilled water by centrifugation at 3000 rpm for 5 min at 4ºC. The washing of pellets was repeated till the smell of alcohol vanished. Colloidal chitin thus obtained was stored at 4ºC until further use.

3.4.2. Screening of isolates of *Trichoderma* spp. for chitinase activity:

For estimation of chitinase activity a simple technique developed by Agrawal and Kotasthane (2008) was followed. Colloidal chitin and a pH indicator was supplemented to the minimal media, 20 ml of the molten chitin supplemented media was poured in pre sterilized petri dishes after solidification, the plates were inoculated with culture plugs containing young actively growing mycelium of *Trichoderma* isolates (Table 3.3). The plates were then incubated at 28±2°C. Chitinase activity was identified due to the formation of purple coloured zone. Observations were recorded for the colour intensity and diameter of the purple coloured zone until 3rd day after inoculation.

3.5 Screening isolates of *Trichoderma* spp. for cellulose hydrolysis

Cellulose hydrolysis by isolates of *Trichoderma* spp. was studied by using phosphoric acid swollen cellulose (PASC) as a substrate (Tansey,1971). PASC was prepared by following the method of Wood (1971) with slight modifications. To prepare PASC 5.0 g of cellulose powder (Hi media) was suspended in 200 ml Ortho Phosphoric Acid on a magnetic stirrer by slowly adding small amounts of powder taking care that no clump was formed(Table 3.3). The cellulose was acid swollen by constant stirring using magnetic stirrer at 4ºC (refrigerator) overnight. To the resulting slurry 800 ml pre-chilled sterile distilled water was added and centrifuged at 3000 rpm for 20 min at 4ºC. Supernatant was discarded and the pellet was washed with sterile distilled water by centrifugation at 3000 rpm for 10 min at 4ºC. The washing of

the pellets was then repeated using 50 mM NaH_2PO_4 solution (pH 7.5) several times till the pH of the supernatant become 7.5. PASC then obtained was stored at 4ºC until further use . Cellulose detectipion was performed in the Mandel and Weber's medium (Mandel's *et al*., 1974) supplemented with PASC and congo red. The Plates were inoculated with young actively growing cultures and incubated at 28±2°C for 3 days followed by 18 hours incubation at 50ºC. Hypercellulolytic isolates were selected on the basis of diameter of the colonies.

3.6 Quantitative spectrophotometric assay for siderophore production (liquid assay)

The chrome azurol sulfonate (CAS) assay – Payne, (1994) was used to evaluate the siderophore production. For siderophore quantification, actively growing cultures of *Trichoderma* sp. with four discs (3 mm diameter) was inoculated to 20 ml PDB (Hi Media) media in tarson tubes and incubated for 3 days at 28±2°C. The mycelium growth was removed by centrifugation at 3000 rpm for 5 min. 0.5 ml of the culture supernatant was then mixed with 0.5 ml CAS solution and 10µl shuttling solution (sulfosalicyclic acid). The colour obtained was determined using the spectrophotometer at 630 nm after 20 mins of incubation. Blank as PDB and reference solution as PDB, CAS dye and shuttle solution were mixed and absorbance was recorded. Values of siderophore released in PDB was expressed in per cent siderophore units and calculated using the formula

Where,

Ar = OD of reference solution,

As = OD of samples

3.7 Screening isolates of *Trichoderma* spp. for phosphate solubilisation ability

Three different qualitative procedures as described by Nautiyal, 1999 were used to screen phosphate solubilising efficacy of *Trichoderma* spp. with slight modification. Isolates of *Trichoderma* spp. were inoculated on three different media (Table 3.3) to evaluate its ability to solubilize supplemented phosphate *in vitro.* The first substrate medium used yeast extract in Pikovskaya agar medium (Pikovskaya, 1948) while other two procedures makes use of NBRIP agar medium (National Botanical Research Institutes Phosphate growth medium) and NBRIY agar medium (NBRI'S Phosphate growth medium devoid of yeast extract) without yeast extract. All the three substrates had tri-calcium phosphate as one of the ingredient as phosphate source. Presterilized molten media were also supplemented aseptically with BCP (bromocresol purple) (0.1 gL^{-1}) as pH indicator (Vasquez et al., 2000) and poured in petri dishe(s). Plates were inoculated with young actively growing cultures of *Trichoderma* spp. and incubated at 28±2°C for 3 days. After 3 days of incubation at 28±2°C, phosphate solubilising positive isolates turned the media from purple to yellow in the zone of acidification which indicated positive response for phosphate solubilising efficacy of *Trichoderma* spp.

3.8 Quantification of indole acetic acid production by *Trichoderma* spp.

For the production of indolic compounds, an active cultures of *Trichoderma* spp. with four discs (3 mm diameter) was inoculated to 20 ml DF salts minimal media (Dworkin and Foster, 1958) in 100ml conical flasks and incubated for 3 days at 28±2°C. The medium was supplemented with L-tryptophan at a concentration of 1.02 g/L from a 5mM filter sterilized stock prepared in warm distilled water. After incubation for 72 hours, the mycelium were removed from the culture medium by centrifugation at 5,000 rpm for 5 min and then 1 ml of aliquot of the supernatant was

mixed vigorously with 4 ml of Salkowski's reagent (Gordon and Webber, 1951) with blank as DF salts minimal media and allowed to stand at RT for 20 min. before the absorbance at 535 nm was measured in spectrophotometer. The standard curve was prepared by adding the 2 mg/ml stock IAA in the increasing concentration : 0.0, 0.5, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50µl in DF salts minimal media with decreasing concentration : 1ml, 995µl, 990µl, 985µl, 980µl, 975µl, 970µl, 965µl, 960µl, 955µl, 950µl, respectively. Then 4 ml of Salkowski's reagent was mixed vigorously in each tube followed by incubation at RT for 20 min after which absorbance at 535 nm was measured in colorimeter. The concentration of IAA in each culture was determined by comparison with standard curve.

3.9 Estimation of hydrogen cyanide (volatile inhibitory compound)

The production of HCN compound was estimated by method of Wei. *et al* (1991). The cultures were grown on PDA plates supplemented with 4.4 g/L glycine as a precursor molecule for hydrogen cyanide production and the filter paper strips soaked in saturated picric acid solution were exposed to the growing *Trichoderma* isolates. The plates were incubated for 7 days at 28±2°C and observations were recorded as colour of filter paper turning to brown recorded as positive for HCN production.

3.10 Screening of selected species of *Trichoderma* isolates for plant growth promoting activity on bottle gourd, cucumber and bitter gourd

3.10.1 Preparation of *Trichoderma* isolates for seed treatment

For seed treatment spores were harvested from sporulating plates of different isolates of *Trichoderma* spp. in 10 ml autoclaved distilled water +10 ml (1%) CMC. Harvasted spores from each isolate were transferred to specified 50 ml tarson tubes containing seeds of bottle gourd (F1 hybrid VNR seeds), cucumber (Local variety)

and bitter gourd (F1 hybrid VNR seeds). The tubes were then fixed on rotary shaker set at 50 rpm for one hour. In the present investigation 12 isolates of *Trichoderma* spp. were assayed to evaluate the plant growth promotion activity. Treated seeds were sown in autoclaved potting mixture filled in pots. From different treatment combinations seedlings were harvested one month after sowing and observations were recorded of root length and shoot length.

3.11 Estimation of chlorophyll content

Chlorophyll (Total chlorophyll, chl a and chl b) contents was determined following Arnon (1949) on freshly harvested leaves derived from 30 day old plant. Fresh fully opened leaves harvested from apical portion of the plant were cut into small pieces of which 100 mg from each sample wer homogenized in 80 per cent acetone and volume make up to 10 ml. Extract was centrifuged at 5000 rpm for 5 min and supernantant was transferred to another tube and final volume of the extract was made up to 20 ml. Absorbance of the extract was recorded at 645, 652 and 663 nm in colorimeter with 80 per cent acetone used as blank.

Chlorophyll (Chl a, Chl b and Total Chl) content was then estimated using the formula:

$$\text{Chlorophyll a} = 12.7\,(A663) - 2.69\,(A645) \times \frac{V}{100 \times w \times a}\ (\text{mg/g r. wt.})$$

$$\text{Chlorophyll b} = 22.9\,(A645) - 4.68\,(A663) \times \frac{V}{100 \times w \times a}\ (\text{mg/g r. wt.})$$

Total chlorophyll was rechecked using the formula.

$$\text{Total Chlorophyll} = 27.8\,(A652) \times \frac{V}{100 \times w \times a}\ (\text{mg/g r. wt.})$$

Where,

A = Absorbance at different wavelength

V = Final volume (20 ml)

w = Fresh weight of the sample (0.1g)

a = path length (1 cm)

Table 3.3 Composition of different media used in present investigation

A	**Potato dextrose Agar (PDA) Medium.**	
1	Potato	250g
2	Dextrose	18g
3	Agar agar	15g
4	Distilled water	1000ml
5	Added oxytetracycline before pouring the media in plates	
B	**Chitin amended medium(pH4.7)**	
1	Colloidal chitin	4.5 g
2	$MgSO_4.7H_2O$	0.3 g
3	$(NH_4)_2SO_4$	3 g
4	KH_2PO_4	2 g
5	Distilled water	1000ml
6	Agar	15 g
7	Citric acid monohydrate	1 g
8	Tween 80	200µl
9	Bromo cresol purple	0.15 g
10	Agar	15 g
11	Distilled water	1000ml
C	**Cellulase detection medium**	
1	PASC	7 g
2	$MgSO_4.7H_2O$	0.3 g
3	$(NH_4)_2SO_4$	2.1 g

4	KH_2PO_4	2 g
5	$CaCl_2$(fused)	1 g
6	Yeast extract	0.5 g
7	$FeSO_4.7H_20$	5 mg
8	$MnSO_4.H_2O$	156 mg
9	$ZnSO_4.7H_2O$	1.40 mg
10	$CoCl_2.6H_2O$	2.66mg
11	Triton X-100	0.1%(v/v)
12	Distilled water	1000ml
13	Agar	15 g
14	Congo red	0.15g
15	Distilled water	1000ml
Composition of medium for Phosphate solubilization		
D	**Pikovskaya agar (P1) (Pikovskaya, 1948)**	
1	Glucose	10g
2	$Ca_3(PO_4)_2$	5g
3	$(NH_4)_2SO_4$	0.5g
4	NaCl	0.2g
5	$MgSO_4.7H_2O$	0.1g
6	KCl	0.2g
7	Yeast extract	0.5g
8	$MnSO_4$. $1H_2O$	0.002g
9	$FeSO_4.7H_2O$	0.002g
10	Agar	15 g
11	Distilled water	1000ml
E	**NBRIP agar (P2) (Nautiyal., 1999)**	
1	Glucose	10g
2	$Ca_3(PO_4)_2$	5g
3	$(NH_4)_2SO_4$	0.1g
4	$MgSO_4.7H_2O$	0.25g
5	$MgCl_2.6H_2O$	5g

6	KCl	0.2g
7	Agar	15 g
8	Distilled water	1000ml
F	**NBRIY agar (P3) (Nautiyal, 1999)**	
1	Glucose	10g
2	$Ca_3(PO_4)_2$	5g
3	$(NH_4)_2SO_4$	0.5g
4	$MgSO_4.7H_2O$	0.1g
5	$MnSO_4.1H_2O$	0.002g
6	$FeSO_4.7H_2O$	0.002g
7	KCl	0.2g
8	NaCl	0.2g
9	Agar	15 g
10	Distilled water	1000ml
G	**Minimal Medium, DF salts (Dworkin and Foster, 1958)**	
1	KH_2PO_4	4g
2	Na_2HPO_4	6g
3	$MgSO_4.7H_2O$	0.2g
4	Glucose	2.0g
5	Gluconic acid	2.0g
6	Citric acid	2.0g
7	$(NH_4)_2SO_4$	2.0g
8	$FeSO_4.7H_2O$	0.001g
9	H_3BO_3	1mg
10	$MoSO_4.H_2O$	11.19µg
11	$ZnSO_4.7H_2O$	124.6µg
12	$CuSO_4.5H_2O$	78.22µg
13	MoO_3	10µg

3.12 Molecular characterization of the isolates of *Trichoderma* spp.

3.12.1 Fungal growth conditions and DNA extraction

- To obtain the vegetative growth of all the isolates of *Trichoderma* spp., 3 to 4 mycelial blocks from one week old culture (approx. 3mm × 3mm) were transferred separately in 50 ml potato dextrose broth (HiMedia) medium contained in 100 ml flasks. The flasks were incubated at 28±2°C for 7 days.
- Vegetative growth (mycelia) was harvested from the broth by suction filtration through Whatman no. 1 filter paper using a Buchner filtration apparatus connected to a vacuum pump. The mycelia mat was removed from the filter paper, blot dried and was then used for DNA extraction.
- Mycelia mat was grind to uniform consistency in 2000 µl resuspension buffer using a scintered glass tissue homogenizer.
- The grinded sample (500 µl) were collected in an 1.5 ml eppendorf tube to which 150µl 5M potassium acetate buffer was added, kept in water bath at 75°C for 10 min then vortexed briefly and then centrifuged at 13,000 rpm for 10 min.
- To the supernatant added 600µl of chloroform and inverted the tubes for 10min. and centrifuged at the 12,000rpm for 10min. Supernatant was taken carefully and transfered to another new eppendorf tubes.
- Added equal volume of pre-chilled isopropanol and inverted tubes gently, kept the tubes in deep freezer for half hour and then centrifuged again for 12,000 rpm for 10min. and supernatant was taken carefully.
- The pellets was washed with 300µl of 70% ethanol, air dried and resuspended in 50µl of TE buffer and left overnight.

- 5 µl RNase (10 mg/ml) was added to the samples and incubated at 37°C for 1 hour. The samples were precipitated with 2X volume of pre chilled absolute alcohol and incubated at -20°C deep freezer for 30 min. followed by spinning at 13000 rpm for 10 minutes.
- Supernatant was discarded and the pellet was washed with 70% ethanol and then air dried. The DNA pellet was dissolved in 100 µl of TE buffer and stored at -20°C till further use.

3.12.2 Quantification of genomic DNA

The DNA samples were quantified using Nanodrop Spectrophotometer (ND 100). One microliter of isolate DNA was placed over lower pedestal of nanodrop to record absorbance at 260nm and concentration was estimated using TE buffer as blank reference. After quantification, the DNA was diluted with sterile water to get a final concentration 40µg DNA/l. The absorbance ratio (A260/A280) was recorded for each sample to find out the purity of DNA. The acceptable absorbance ratio (A260/A280) for pure DNA is 1.8.

3.12.3 Polymerase Chain Reaction (PCR) analysis of 20 Isolates of *Trichoderma* spp.

A set of 9 primers (Table 3.4) were used for PCR based DNA fingerprinting analysis of 20 isolates of *Trichoderma* spp. The reaction mixture and the temperature profiles used were summarized in Table 3.5 to Table 3.13 respectively.(Corbett Life Sciences, Sydney, Australia DNA) 6% denaturing PAGE was performed to separate the PCR amplified products.

Table 3.4 DNA primer and their sequences which were used in present investigation.

Sr.No.	Primers	Tm	Bases	Sequence (5'-3')
1.	TR1	49.2ºC	16	(5'-GACAGACAGACAGACA-3')
2.	B21(F)	58.2º	18	(5'-TGAAGAGCGCCTCGACGA-3')
	BR2(R)	56ºC	18	(5'GGGTGATGATTTGCTGGC-3')
3.	Tef 71F	61ºC	24	(5'-CAAAATGGGTAAGGAGGACAAGAC-3')
	Tef 997R	62.1ºC	22	(5'-CAGTACCGGCAGCGATAATCAG-3')
4.	Tef 85F	57.9ºC	21	(5'-AGGACAAGACTCACATCAACG-3)
	Tef 954R	56.5ºC	22	(5'AGTACCAGTGATCATGTTCTTG-3')
5.	fRPB2-5F1	51.2ºC	20	(5'-GATGATAGAGATCATTTTGG-3')
	fRPB2-7cR1	55.3ºC	20	(5'-CCCATAGCTTGTTTACCCAT-3')
6.	fRPB2-5F2	61.4ºC	20	(5'-GACGACCGTGATCACTTCGG-3')
	fRPB2-7cR2	61.4ºC	20	(5'CCCATGGCTTGCTTGCCCAT-3')
7.	ITS 1n	61ºC	19	(5'-TCCGTAGGTGAACCTGCGG-3')
8	ITS 5	56.5ºC	22	(5'-GGAAGTAAAAGTCGTAACAAGG-3')
9.	ITS 4	58.35ºC	20	(5'TCCTCCGCTTATTGATATGC-3')
10.	ITS 1	62.32ºC	19	(5'-TCTGTAGGTGAACCTGCGG-3')
11.	ALRO	60.61ºC	21	(5'-CATATGCTTAAGTTCAGCGGG-3')

Following are the primer pairs used for fingerprinting

- TR1
- BR1+BR2
- Tef 71F+Tef 997R
- Tef 85F+Tef 954R
- fRPB2-5F1+fRPB2-7cR1
- fRPB2-5F2+fRPB2-7cR2
- ITS 5+ITS 4
- ITS 1n+ITS 4
- ITS 1+ALRO

Table 3.5 Reaction mixture used for polymerase chain reaction (PCR) for TR1 primers

Sr.No.	Component	Stock concentration	Volume/reaction
1	DNA	40µg/ml	2µl
2	ADW		12.5 µl
3	10X Buffer	10X	2 µl
4	dNTP	1mM	2 µl
5	Primer TR1	10µM	1 µl
6	*Taq* polymerase	1 U/µl	0.5 µl

Stored the samples at- 20ºC after PCR.

Table 3.6 Thermal profile for amplification of primer TR1

Steps	Activity	Temperature(˚C)	Time (min)	Repeats
1	Initial denaturation	93	3	1
2	Denaturation	93	1	30
3	Annealing	50	1	
4	Extension	72	1	
5	Final extension	72	10	1
6	Storage	4	-	-

Stored the samples at- 20ºC after PCR.

Table 3.7 Reaction mixture used for Polymerase chain reaction with BR1-BR2, Tef 71F+Tef 997R and Tef 85F+Tef 954R, fRPB2-5F1+fRPB2-7cR1 and fRPB2-5F2+fRPB2-7cR2, ITS5+ITS4, ITS1+ALRO, ITSIn+ITS4 pair of primers

Sr.No.	Component	Stock concentration	Volume/reaction
1	DNA	40µg/ml	2.5µl
2	ADW		11 µl
3	10X Buffer	10X	2 µl
4	dNTP	1mM	2 µl
5	Forward Primer	10µM	1 µl
6	Reverse primer	10µM	1 µl
7	*Taq* polymerase	1 U/µl	0.5 µl

Table 3.8 Thermal profile for amplification of primers BR1-BR2

Steps	Activity	Temperature(˚C)	Time (min)	Repeats
1	Initial denaturation	94	5	1
2	Denaturation	94	1	30
3	Annealing	55	1.5	
4	Extension	72	2	
5	Final extension	72	7	1
6	Storage	4	-	-

Stored the samples at- 20ºC after PCR.

Table 3.9 Thermal profile (touch down profile) for amplification of primers Tef 71F+Tef 997R and Tef 85F+Tef 954R

Steps	Activity	Temperature(˚C)	Time (min)	Repeats
1	Initial denaturation	94	4	1
2	Denaturation	94	1	4
3	Annealing	70	1.5	
4	Extension	72	1.5	
5	Denaturation	94	1	26
6	Annealing	68(lower by 0.5ºC per cycle)	1.5	
7	Extension	72	1.5	
8	Denaturation	94	1	12
9	Annealing	55	1.5	
10	Extension	72	1.5	
11	Final Extension	72	7	1
12	Storage	4		

Stored the samples at- 20ºC after PCR.

Table 3.10 Thermal profile (touch down profile) for amplification of primers fRPB2-5F1+fRPB2-7cR1and fRPB2-5F2+fRPB2-7cR2:

Steps	Activity	Temperature(ºC)	Time (min)	Repeats
1	Initial denaturation	94	3	1
2	Denaturation	94	45s	5
3	Annealing	60	45s	
4	Extension	72	2	
	Denaturation	94	45s	5
5	Annealing	58(lower by 1ºC per cycle)	45s	
6	Extension	72	2	
7	Denaturation	94	45s	30
8	Annealing	54	45s	
9	Extension	72	2	
10	Final Extension	72	10	
10	Storage	4		

Stored the samples at- 20ºC after PCR.

Table- 3.11 Thermal profile for amplification of primers ITS5+ITS4, ITSIn+ITS4

Steps	Activity	Temperature(˚C)	Time (min)	Repeats
1	Initial denaturation	94	3	1
2	Denaturation	94	1	35
3	Annealing	46	1	
4	Extension	72	1	
5	Final extension	72	10	1
6	Storage	4	-	-

Stored the samples at- 20ºC after PCR.

Table- 3.12 Thermal profile for amplification of primers ITS1+ALRO

Steps	Activity	Temperature(˚C)	Time (min)	Repeats
1	Initial denaturation	94	15s	1
2	Denaturation	94	10	40
3	Annealing	56	15s	
4	Extension	72	45s	
5	Final extension	72	2	1
6	Storage	4	-	-

Stored the samples at- 20ºC after PCR.

Table- 3.13 Reaction mixture used for performing restriction enzyme digestion of PCR product

Sr.No.	Component	Stock concentration	Working concentration
1	PCR product	**-**	7-10µl
2	Autoclaved nano water (ANW)	-	7.9µl
3	OPA	10X	1µl
4	BSA	100X	0.1µl
5	*Mse* I	10U/µl	0.5µl
6	*Eco* RI	20U/µl	0.5µl

Kept at 37°C for 3 hours

3.12.4 Visualizing PCR products on denaturing polyacrylamide gel electrophoresis (PAGE)

- The Bangalore genei Sequencing gel apparatus was used for PAGE of PCR products. The plates and spacers were cleaned thoroughly with three washes of distilled water, followed by two washes with 75% alcohol and then with 100% alcohol respectively.
- One ml bind silane solution was applied on unnotched plated, uniformly spread by wiping with a tissue paper, and kept for drying for 30 min. After drying, the plate was wiped with 100% alcohol.
- One ml of sigma cote (sigma. cat. # SL-2) was applied to the notched plate, uniformly spread with a tissue paper and kept for drying for 30 min.
- Post-drying the gel plates were assembled as per manufacturer's instructions so that the processed surfaces of both plates face each other.
- To 50 ml of the 6% urea-acrylamide gel solution, 35µl of freshly prepared 10% ammonium persuiphate (APS) solution and 30 µl TEMED were added and swirled gently. The solution was gently poured in the gap between the two glass plates by taking care to prevent the formation of air bubbles. The comb was inserted immediately and the gel was allowed to polymerize for at least an hour.
- After one hour, the comb was removed gently without disrupting the gel line and the well was washed with distilled water by using a syringe.
- The gel plates were fixed to the electrophoretic mold/gel tank and 1X TBE (running buffer) was added to the buffer chamber of the mold.
- The gels were put for pre run at 1200 V for 1 hour, until the temperature of the plates reached 50°C and the gel line was washed with lX TBE buffer. The comb was inserted again to form the wells by ensuring that the teeth of comb just touched the gel line uniformly.
- The PCR reaction mixture from PCR were mixed with 3 µl of formamide dye and denatured at 95˚C in Applied Biosystems 9700 PCR thermal cycler, and immediately kept in ice. 3µl of each denatured sample was loaded and the

samples were electrophoresed at a constant 1200 V for 3.5 h. After completion of the run, the plates were allowed to cool to room temperature and then the bands were visualized by silver staining.

Silver Staining

- After electrophoresis, the power supply was disconnected, the comb was removed and the notched plate was separated from the un notched plate. The gel adheres to the un notched plate coated with bind silane.
- The plate transferred to a tray containing 1 liter fix/stop solution and kept for 10 min at room temperature in the solution with shaking at 90 rpm. The gel was then given a quick wash with distilled water for 30 s.
- Then the gel plate was transferred to a tray containing 0.2% silver nitrate solution, and kept for 10 min in dark with slow shaking, followed by a quick wash with distilled water for 30 s.
- The gel plate was then placed in the tray containing one liter developer solution and kept for 5 to 7 min until bands began to appear. After bands with detectable intensity appeared, the gel was transferred to a tray containing distilled water and rinsed for few minutes.
- The gel was kept for air-drying and subsequently the bands were captured by scanning and/or photography.

3.13 Statistical analysis of primer data

3.13.1 Data scoring

The band appeared in Gene specific primers were scored in the form a matrix with '1' and '0', which indicate the presence and absence of bands in each isolate respectively.

3.13.2 Cluster analysis

The binary data scored was used for computing similarity matrix similarity indices. Similarity matrix generated using the SIMQUAL programme of NTSYS-pc software, version 2.02 (Rohlf, 1998). The similarity coefficients were used for cluster analysis and dendrograms was constructed by the Unweighted Pair-Group method (UPGMA) (Sneath and Sokal, 1973).

BUFFER, SOLUTION AND REAGENTS

A. Solutions used for preparation of PASC

- 1M NaH_2PO_4(pH) sock solution:

  Dissolved 13.799 g of NaH2PO4 inn 60 ml autoclaved distilled water. Adjusted the pH to 7.5 with 10 mM NaOH. Made up the final volume to 100 ml with autoclaved distilled water.

- 50mM NaH_2PO_4 (pH) working solution:

  Diluted 50 ml of 1M NaH_2PO_4 to 1000 ml with autoclaved distilled water.

B. Chrome Azurol assay (CAS) solution

- 1M $FeCl_3.6H_2O$(stock solution)

Dissolve 2.703g of $FeCl_3.6H_2O$ in 10ml of distilled water.

- 1M HCl(stock)

Diluted 0.86ml of conc. HCl to 10ml with distilled water

- Iron (Ш) solution

Chemicals	Stock Concentration	Working concentration	Volume required per 1000ml
$FeCl_3.6H_2O$	1M	1mM	10ul
HCl	1M	1mM	100µl

Mix both the components and make up the volume to 10ml

- CAS solution

  In a beaker 60.5mg of CAS was dissolved in 50ml autoclave distilled water and mixed with 10ml Iron (Ш) solution (1mM $FeCl_3.6H_2O$+10mM HCl) stirred and to it HDTMA solution (72.9mg dissolved in 40ml of autoclave distilled water) was added slowly. This resulted in a formation of a dark blue liquid.

C. Salkowski's reagent (Gordon and Weber, 1951)

S. No.	Component	Amounts (g/L)

1	Conc. H_2SO_4	150 ml
2	Double distilled H_2O	250 ml
3	$FeCl_3 \cdot 6H_2O$	7.5 ml of 0.5 M

pH was set at 7.5.

D. Genomic DNA extraction buffer:

1. 1M Tris-HCl solution (pH 8)

   121.1gm Tris-HCL (pH 0.9)

   800 ml distill water

   Adjust the pH by adding conc. Made up the final volume to 1000 ml with distilled water. Sterilized by autoclaving.

2. 0.5M EDTA solution (pH 7.5)

   186.1 gm EDTA (pH 7.5) in 800ml water

   Stire vigorously on a magnetic stirrior adjust the pH to7.5 respectively with NaOH pellets itself. Made up the final volume to 1000 ml with distilled water. Sterilized by autoclaving.

3. Chloroform

4. Ethanol (100%&70%)

5. 5M Potassium acetate-Acetic acid buffer (pH-4.8)

Sr No.	Chemicals	Volume required per 100ml
1	5M Potassium acetate	60ml
2	Glacial acetic acid	11.5ml

Mixed both the components and adjusted the final volume to 100ml with autoclaved distilled water.

6. 5M potassium acetate was prepared by dissolving 49.07 g of potassium acetate in 100ml autoclaved distilled water.

7. DNA resuspension buffer

Chemicals	Stock Concentration	Working concentration	Volume required per 1000ml
Tris-HCl(pH 7.5)	1M	50mM	50ml
EDTA(pH 7.5)	0.5M	10mM	20ml
SDS	10%	1%	100ml

7. T.E buffer (1 litre) (pH-8.0)

10 ml 1M Tris HCl

2 ml 0.5 M EDTA

Make up final volume to 1000ml with autoclaved distilled water.

E. Stocks and solutions for PAGE and silver staining

A 6% Urea –Acrylamide gel solution (1000 ml)

S.NO.	Stock	For 1000ml
1	Urea	420g
2	Acrylamide	57g
3	Bis Acrylamide	3g
4	10X TBE	100ml

Autoclaved nanopure water (milliQ) water (to make up volume to 1000 ml)

Urea was first dissolved in 400 ml warm distilled water. Acrylamide bisacrylamide were weighed in fumehood and dissolved in 100 ml distilled separately and then added to the beaker containing urea solution. One hundred ml of 10X TBE was added to the solution and the volume was made upto 1000 ml by adding autoclaved milliQ water. The solution was sterilized by passing through 0.22-micron and stored in amber colour bottle at 4°C.

B Fix/Stop solution was prepared by mixing following components

Sr.No.	Stock	For 1000ml
1	100% ethanol	100ml
2	Glacial acetic acid	5ml
3	Nanopure water	895ml

C Staining Solution was prepared by mixing following components

Sr.No.	Stock	Final concentration	For 1000ml
1	Silver nitrate	0.2%	2g
2	Nanopure water		1000ml

D Developer was prepared by mixing following components and final volume made upto 1000 ml with water

Sr.No.	Stock	Final concentration	For 1000ml
1	Sodium hydroxide	3%	30g
2	Formaldehyde	0.5%	5ml

E Ammonium persuiphate (APS) – l0% solution was prepared by mixing following components

Sr.No.	Component	Final concentration
1	Ammonium persuiphate	1.0g
2	Ditilled water	10ml

F Bind silane solution

Bind Silane: (3- (Trimethoxysilyl) propyl methacrylate methacrylsaure-3 trimethoxysilyl propylester) was prepared by mixing following components.

S.NO.	Stock	For 1 ml
1	100% Ethanol	945µl
2	Bind saline	5µl
3	Glacial acetic acid	5µl
4	Ditilled water	45µl

G. Formamide dye was prepared by mixing following components

S.NO.	Stock	Final concentration	For 10ml
1	Formamide	98%	9.8ml
2	0.5 M EDTA	10mM	200µl
3	Xylene cyanol	0.1%	0.01g
4	Bromophenol blue	0.1%	0.01g

CHAPTER IV

RESULT AND DISCUSSION

Trichoderma pres. Ex., Fr., a genus under Deuteromycotina, Hyphomycetes, Philasporace, Hyphales, Dematiaceae, characterized as "rapidly growing colonies bearing tufted or postulate, repeatedly branched conidiophores with lageniform phialides and hyaline or green conidia born in slimy heads". (Bisset,*et al*., 1984).*Trichoderma* spp. are cosmopolitan soil fungi, remarkable for their rapid growth, capability of utilizing diverse substrates, and resistant to noxious chemicals. They are often predominant components of the mycoflora in various soils, such as agriculture, prairie, forest salt marsh, and desert soil in all climatic zones (Danielson, 1973; Klein and Eveleigh, 1998; Roiger, *et al*., 1991; Wardle, *et al*., 1993), where they are significant decomposers of woody and herbaceous material, and are also necrotrophic against the primary wood decomposers (Rossman, 1996). Some of the about 35 established species of *Trichoderma* (Gams and Bissett, 1998) are also of economic importance because of their production of enzymes and antibiotics, or use as biocontrol agents (Hjeljord and Tronsmo,1998; Kubicek and Penttilä, 1998; Sivasithamparam and Ghisalberti, 1998).

Trichoderma spp. are the most common biocontrol agent of plant pathogenic fungi that caused soil borne, air borne and post harvest disease in several crops. Generally, the beneficial effects obtained with *Trichoderma* to increase plant response have important economic implication, such as shortening the plant growth period and time in the nursery there by increasing production capacity. *Trichoderma* spp. is highly diverse and ecologically successful fungi. Members of the genera have

long been known to act as biocontrol agents of plant pathogens. Recently, these fungi have been used in quite significant amounts in commercial agriculture. Until recently, *Trichoderma* spp. was believed to achieve biocontrol by direct effects on fungal pathogens, particularly via mycoparasitism, antibiosis and competition. While these mechanisms are important, direct effects on plants are no doubt equally important. Some strain of *Trichoderma* effectively colonizes the roots of most crops and in most soils, increases the density and depth of rooting of many plants, enhances nutrient uptake and solubilizes some mineral plant nutrients. Some *Trichoderma* strains have been reported to induce systemic resistance in plants. For many years the ability of these fungi to increase the plant growth and development including especially their ability to cause production of more robust roots has been known. *Trichoderma* species is isolated from areas of plant and soil where it is expected to function in disease control and where it is growing under condition of temperature, moisture and nutrient availability that approximate those found in nature. *Trichoderma* species exhibit other characteristics during interaction with host plants that may contribute to disease resistance or tolerance. These characteristics manifest themselves by increase in plant root and shoot growth, changes in plant root and shoot growth, resistance to biotic and abiotic stress, and changes in the nutritional status of the plant with the present investigation were identified potential candidate isolates of *Trichoderma* spp. They are able to evaluate the plant growth promoting activity on different vegetable crops.

4.1 Cultural characterization of the isolates of *Trichoderma* spp.

In recent year a growing number of telomorphs in Hypocrea have been linked to commonly occurring *Trichoderma* anamorphs through macromolecular investigations. However in spite of these significant advances in our knowledge of the genus, the taxonomy of *Trichoderma* and the distinction of species in the genus *Trichoderma* are still rather incomplete. A refined concept may eventually by correlate unequivocally with telomorphs species. Samuels (1996) also provided detailed observation and components on the utility of morphological characters to define species in *Trichoderma*. The patterns of conidiophore branching and aggregation of conidiophores into fascicles and pustules are useful for identification of strain *Trichoderma* to section and species aggregates (Kubicek and Harman, 1998). Phialides shape is character of the section; short and plums in section *Pachybasium*, where as in section: *Longibrachiatum* they are elongate and lageniform to nearly cylindrical (Kubicek and Harman, 1998). Conidia shape varies from globose, to ellipsoidal, obovoidal, or short cylindrical with the best end more or less tapering and truncate. *Trichoderma* sect. *Longibrachiatum* is distinguished by having aggregated conidiophores forming weakly developed pustules. The conidiophores consist of long primary branches and short, unbranched secondary branches. The phialides are solitary, rarely in verticils, and they produce ellipsoidal to oblong, green conidia. The conidial surface appears smooth in most species in light microscope observation, although many species with apparently smooth conidia are delicately ornamented when examined by SEM (Kubicek and Harman, 1998).

The dominant components of the soil micro flora, the species of *Trichoderma* are observed in widely varying habitats owing to the diverse metabolic capabilities and their aggressively competitive nature. Identifications based on morphological

characteristics remain the primary method for identification and verification of species in *Trichoderma*. Colony characteristics can be distinctive and characteristic of a species. However, colony appearance is difficult to describe with sufficient precision for it to be very useful for identification. The production of conidia from effused conidiophores, or from conidiophores aggregate into fascicles or pustules is usually characteristic of a species. Diffusible pigments and aromatic odor can also be characteristic feature for a species in *Trichoderma*. In the present investigation 20 isolates of *Trichoderma* collected from different geographical locations of Chhattisgarh were characterized primarily on the basis of morphological characteristics. (Table 4.1, Table 4.2, Plate 4.1)

Comment [AK1]: Write the cultural characteristics of the 20 *Trichoderma* isolates.In tabular Form

4.2 Morphological characterization of the isolates of *Trichoderma* spp. by light microscopy

Through light microscopy we observed the typical characteristics features of the phialides and conidia. This helped us to classify / allocate the *Trichoderma* isolates collected from different geographical locations to different sections as per the characteristics key feature of the sections.

4.2.1 Keys to the sections of *Trichoderma* (Bisset, 1991a; Samuels *et al.*, 1998)

Conidiophores and branches narrow and flexous (wavy) (main axes to 6um wide); phialides mostly in verticils (Whorls) of 2 or 3(-5), lageniform (flask shaped) to subulate; conidia always green.

Section *Trichoderma*: Based on the characteristic features of the section the isolates categorized within were further observed for the microscopic details and as

per the typical characteristic of the conidiophores, phialides and conidia the isolates designated the species identity.

- Conidiation forming compact tufts or more effuse glaucous to dark bluish green. Reverse typically uncolored, less often pale yellowish. Odor ususlly distinctly aromatic as of coconut. Phialides cylindrical, often sinuous or hooked. Fully mature conidia more or less roughened or warted, globose to subglobose. ***T. viride*** aggregate. (Plate 4.2)
- Conidiation entirely effuse, or conidiophores lacking sterile apical elongations; Reverse colourless, or slowly developing dull yellowish to amber shades. Conidiophores in areas of effuse conidiation arising as lateral branches ; phialides convergent in penicillate manner. ***T. virens*** aggregate. (Plate 4.2)
- Conidiation predominantly effuse, appearing granular or powdery due to dense conidiation; rapidly turning yellowish green to dark green or producing tuft or pustules fringed by sterile white mycelium. Reverse colourless to dull yellowish, buff or drab. Coloured indistinctly or faintly earthly. Fully mature conidia sub globose (not quite round or spherical) to obovoid, pale green. ***T. harzianum*** aggregate.(Plate 4.2)
- Conidiation forming compact dull green tufts or pustules. Colony reverse conspicuously discolored brownish yellow, in part due to the production of yellow crystals. Phialides in false verticals (Having branches arranged in verticils or whorls.), phialospores obovoid with truncate (Cut off sharply) base. ***T. aureoviride*** aggregate. (Plate 4.3)

4.2.2 Key to the species in *Trichoderma* section *Pachybasium*

Conidiophores and branches relatively broad (main axes to 10µm wide); phialides in verticils of 2-7, ampulliform to ageniform; conidia green, brownish or hyaline. **Section *pachybasium.*** Conidiation entirely effuse, or conidiophores arranged in loosely organized flat pustules or small irregular fascicles; conidiophores sparingly branched with principal brenches most often arising singly or paired. (Plate 4.4)

- Conidiophores organized in compact, hemispherical to cushion-shaped pustules; conidiophores usually highly branched with branches 2-4 verticillate.
- Conidiophores with sterile elongations; phalides divergent; conidia ellipsoidal. ***T. polysporum***
- Conidiophoreslacking sterile elongations phialides more or less convergent; conidia suglobose. ***T. piluliferum***

Conidiogenous pustules bluish green appearing velvety due to presence of strongly undulate or hamate, conidiophores apices, ***T. hamatum.***

4.2.3 Key to the species in *Trichoderma* section *Longibrachiatum.*

Conidiophores main axes long with short secondary branches, not extensively rebranching; branches and phialides frequently arising singly, particularly the terminal ones; conidia smooth but sometimes with conspicuous, sinuate, wing-like or bullate ornamentation: **section *Longibrachiatum.*** (Plate 4.5)

Colonies with conidial areas widely effused and not forming pustule; conidiation mostly in bluish green shades and not darkening appreciably in age; conidia ellipsoid to atly cylindrical.***T pseudokoningii***

- Conidia ellipsoidal and consistenty smooth-walled. ***T. parceramosum***

- Conidia obovoid to ellipsoid; colony exuding yellow pigment into the agar. ***T. reesei***
- Conidia short-cylindrical to obovoid, with a conically tapering base; reverse uncolored. ***T. longibrachiatum***.

All the isolates were characterized for cultural morphology. However, colony appearance was observed to be difficult to describe with sufficient precision which is in support of the observations of Rifai (1969) and Bissett (1991a) who have discussed the morphological characters that they used to characterize and differentiate species of *Trichoderma*. Both authors emphasized the difficulties inherent in defining morphological species *Trichoderma*. Samuels (1996) also provided detailed observations and comments on the utility of morphological characters to define species in *Trichoderma.* In the present investigation 20 isolates of *Trichoderma* were characterized primarily on the basis of morphological characters. All the isolates were grown on PDA medium and were observed for diffusible pigments. One of the convincing evidence for the identification of *Trichoderma* spp. belonging to different sections and species was the diffuseble pigmentations released by the isolates in the substrate. Characteristic crystals are produced commonly by strains of *Trichoderma aureoviride*. Strains referable to section *Longibrachiatum* typically have conspicuous bright greenish-yellow pigments, at least when first isolated. Kubicek and Harman, 1998 have also reported similar observation for the identification of the strains referable to the section *longibrachiatum*. Similarly characteristic crystals in the media and has only been reported for *Trichoderma aureoviride*. Light microscopy was performed to observe the microscopic details of the 20 isolates under investigation. It was observed that conidiophores differed in branching patterns. The

key characteristics features of the isolates for conidiophore branching and the phialides corroborates with the keys to sections of *Trichoderma* as reported by Bisset, 1991a and Samuels *et al*., 1996, which justifies to group the isolates with similar features of conidiophore and phialides to be grouped under different sections as *Trichoderma, Longibrachiatum* and *Pachybasium.*

Table 4.1 Grouping of isolates of *Trichoderma* spp. based on cultural and microscopic characteristics

Sr. #	Species of *Trichoderma* isolate(s)	Isolate number / Total
1	*Trichoderma viride*	T14,T17 = (2)
2	*Trichoderma aureoviride*	T27,T29,T114,T120 = (4)
3	*Trichoderma virens*	N,T101b = (2)
4	*Trichoderma harzianum*	T15,T16 = (2)
Isolates belonging to section		
1	*Pachybasium*	T66, T93, T110, T73, T174, T158a, T132 = (7)
2	*Longibrachiatum*	T1, T7,T31 = (3)
	Total	20

Table 4.2 Morphological characteristics of isolates based on key characters described for the identification of *Trichoderma* species

Iso#	Colony colour	Growth pattern	Appearance	Pustule	Different pigments	Odour
T14	Dark green	Aereial and subdued	Uniform velvety	Absent	Absent	Coconut
T17						
T15	Green	Subdued	Slightly velvety and ringed	Absent	Absent	Earthy smell
T16						
T27	Light green	Subdued	Velvety	Minute centered	Yellow crystal	Absent
T29	Dark green	Subdued	Uniform ringed	Absent		

			velvety			
T110	Dark green	Subdued	Uniform and smooth	Absent	Yellow crystal	Absent
T114	Dark green	Subdued		Absent		
N	Blackish green	Effuse	Slightly cottony	Absent	Absent	
T101b		Subdued	smooth and sectered		Absent	
T1	Light green	Subdued	Rough	Minute marginal	Yellow pigmentation	Absent
T7	Blueish green	Subdued		Compact		
T31	Dark green	Subdued		Scattered		
T66	Green	Effuse and subdued	cottony	Irregularly disposed	Absent	Absent
T93		Subdued	Slightly cottony uniform	absent		
T110		Subdued		absent		
T132	Yellowish green	Aerial and subdued	cottony	Loosely organised		
T158a		Slightly aerial	velvety	Compact		
T73	Green	Subdued	Rough and Scattered	Irregularly disposed	Media coloured to dull brown	
T174						

4.3 *In vitro* antagonistic reactions of *Trichoderma* spp*.*

In vitro antagonistic potential of different isolates of *Trichoderma* spp. was studied against fungal plant pathogens *Rhizoctonia solani* and *Sclerotinium rolfsii* of rice following dual culture method and was assessed after 7 days of growth using a 1-5 scale of reaction type. **1**: *Trichoderma* completely overgrew the pathogen and covered the entire medium surface; **2**: *Trichoderma* overgrew at least two thirds of the medium surface; **3**: *Trichoderma* and pathogen each colonized approximately one half of the medium surface (more than one third and less than two thirds) and neither organism dominate to each other; **4**: the pathogen colonized at least two thirds of the medium surface and appeared to withstand encroachment by *Trichoderma*; **5**: the pathogen completely overgrew the *Trichoderma* and occupied the entire medium surface.

Screening of *Trichoderma* isolates for antagonism showed diversify results (Table 4.3) from scale 1-5 against the two rice phytopathogens *Rhizoctonia solani* and *Sclerotinia rolfsii.* An isolate of *T.virens* # N was identified as strong antagonist with reaction type 1 as it completely overgrew both *R.solani* and *S.rolfsii* where as # T 66 belonging a *Trichoderma* isolate to section *Pachybasium* was weak in antagonistic potential. Isolates showing reaction type 1 against the *R.solani* were #T101b (*T.virens*), #T17 (*T.viride*), #T15 and #T16 (*T.harzianum*), #T27, #T29, #T114, #T120 (*T. aureoviride*) and #T93, #T132 (section Pachybasium). (Plate 4.6, Plate 4.7, Plate 4.8)

Isolates no.T16, T27, T120, T93 and T132 *Trichoderma* spp. completely overgrew *R. solani* and showed reaction type 1 results antagonism was not apparent against S*. rolfsii* and 60% of the *Trichoderma* isolates were observed to

show the reaction type 4 / 5. It was realized that *in vitro* screening with the arbitrary rating system for biological antagonist effective against soilborne plant pathogens is a simplistic approach to understand a small sector of biological system in disease control. However, controlling a large sector of the environment, excluding other soil microflora and supplying a uniform food base, temperature, moisture, and light should yield useful information on the degree of antagonistic variability within *Trichoderma* and the diversity of ability among soilborne pathogens to resist antagonism. Kapil and Kapoor (2005) reported that the culture filtrate of *T. viride* inhibited the mycelial growth of *Sclerotinia sclerotiorum* due to production of antibiotic like substance. Lee and Wu (1984) observed that *T. viride* produced metabolites that inhibited the mycelial growth of *Sclerotinia sclerotiorum*. Elad *et al.* (1982) reported that the isolates of *T. harzianum*, which were found to differ in their ability to attack *Sclerotium rolfsii*, *Rhizoctonia solani* and *P.aphanidermatum,* also differed in the levels of mycolytic enzymes produced by them. The antagonistic fungus *Trichoderma* sp. led to break the outer shell of sclerotia causing its destruction along with several histological changes such as degeneration and decay of cytoplasmic content, deformation and lysis of cell wall of hypha (Rawat and Tewari, 2011). Seventy *Trichoderma* isolates collected from different regions of Morocco were tested for their capacity to inhibit *in vitro* mycelia growth of *Sclerotium rolfsii* (Khattabi *et al*, 2004). Four of these isolates (Nz, Kb2, Kb3 and Kf1) showed good antagonistic activity against *S. rolfsii* and were also highly competitive in natural soil. These isolates would therefore be candidates for development in biological control. However, Shalini and Kotasthane (2007) screened seventeen *Trichoderma* strains against *Rhizoctonia solani in vitro*. All strains including

T.harzianum, T.viride and *T. aureoviride* were more or less inhibited the growth of *R. solani*.

There were significant differences in responses to antagonism by 20 isolates of *Trichodema* against the two soilborne pathogen. However, within the limited parameter studied, the high level of significant interactions observed indicate that several genes of both the antagonist and the pathogen must be involved in regulating the different levels of antagonism observed in the study. If these and other genetic factors interact with the environment, the likelihood of finding a specific biological antagonist that has wide adaptability is remote. Therefore, it may be more prudent to search for biological antagonists against specific diseases and evaluate blends of antagonists for wider application.

Table 4.3 *In vitro* mycoparasitic activity of different *Trichoderma* spp. against rice pathogen(s) *R. solani* and *S. rolfsii* following dual culture

Species of *Trichoderma* isolate(s)	Reaction type		Isolate # / section	Reaction type	
	S. rolfsii	*R. solani*		*S. rolfsii*	*R. solani*
Trichoderma virens			Longibrachiatum		
N	**1**	**1**	T1	5	2
T101b	2	1	T7	4	2
Trichoderma viride			T31	4	2
T14	3	4	Pachybasium		
T17	3	1	**T66**	**5**	**5**
Trichoderma harzianum			T73	4	2
T15	2	1	T93	4	1
T16	4	1	T110	2	4
Trichoderma aureoviride			T132	4	1
T27	4	1	T158a	4	2
T29	2	1	T174	5	3
T114	2	1			
T120	4	1			

1=*Trichoderma* completely overgrew the pathogen and covered the entire medium surface;2=*Trichoderma* overgrew at least 2/3 of the medium surface; 3=*Trichoderma*

and pathogen each colonized approximately ½ of the medium surface (more than 1/3 and less than 2/3), and neither organism appeared to dominate the other; 4=The pathogen colonized at least 2/3 of the medium surface and appeared to withstand encroachment by *Trichoderma*; 5=The pathogen completely overgrew the *Trichoderma* and covered the entire medium surface

4.4 Screening of Isolates of *Trichoderma* spp. for chitinase activity

Twenty isolates of Trichoderma spp. were screened for their ability to hydrolyse chitin and were then grouped according to the diameter (mm) of the purple coloured zone observed as: 1- low chitinase activity (7-25mm); 2- medium chitinase activity (26-41mm) and 2- high chitinase activity (42-85mm).

Isolates of *Trichoderma* spp. used in the present investigation (*Trichoderma aureoviride, Trichoderma harzianum, Trichoderma virens, Trichoderma viride,* section *Longibrachiatum* and section *Pachybasium* showed variable responses for chitinase expression (Table 4.4,Table 4.5) for utilization of colloidal chitin supplemented as substrate in the agar medium. Isolates with high chitinase activity are the isolates of choice for their application as biological control agent and were also identified after screening on colloidal chitin supplemented as substrate in the agar medium: 1) *T.virens* isolates N and T101b, *T.viride* isolates T14 and T17, *T.harzianum* isolate T16, *T. aureoviride* isolate T27, isolates belonging to section Pachybasium isolate # T66, T73, T93, T110, T132, T158a and T174 and section Longibrachiatum isolate # T1, T7 and T31. Medium chitinase activity was observed for *T.harzianum* isolate T15 and *T. aureoviride* isolates T29, T114 and T120. No isolate was observed with low chitinase activity. *Trichoderma virens* # N measured (88.9 mm radial diameter) and was assessed (following dual culture) effective (reaction type 1) against two rice phytopathogens *Rhizoctonia solani* and *Sclerotium rolfsii*. Fungal Strains assigned to the genera *Trichoderma* are well known producers of chitinolytic enzymes and are used commercially as sources of these proteins. Additional interest in these enzymes is stimulated by the fact that chitinolytic strains of *Trichoderma* are among the most effective agents for biological control of plant diseases and can be

serious pathogens. (Chet, 1987; Harman, 1990; Harman *et al*., 1993; Komatsu, 1976; Muthumeenakshi *et al*., 1994; Samuels, 1996). Research on chitinolytic enzymes from *Trichoderma* has flourished in recent years (Carsolio *et al*., 1994; Chet *et al*., 1993; Draborg *et al*., 1996; Fekete *et al*., 1996). To date, several laboratories around the world are applying these genes to a variety of biocontrol strategies and studying the mechanism of fungal antagonism and mycoparasitism. There are several mechanisms involved in *Trichoderma* antagonism namely antibiosis whereby the antagonist fungus shows production of antibiotics; competition for nutrients; and mycoparasitism whereby *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, ß-1,3 glucanases and proteases (Haran *et al*., 1996). Because the skeleton of filamentous fungi cell walls contains chitin, glucan and proteins, enzymes that hydrolyze these components have to be present in a successful antagonist in order to play a significant role in cell wall lysis of the pathogen (Lorito *et al*. 1994; Carsolio *et al*. 1999). Several distinct chitinolytic enzymes have been reported in *T. harzianum* (De la Cruz *et al*., 1992; Haran *et al*. 1996). These include endochitinases, exochitinases and 1,4-ß-N-acetylglucosaminidases, which are induced during growth of *T. harzianum* in liquid medium containing chitin as carbon source.

4.5 Screening of isolates of *Trichoderma* spp. for cellulose hydrolysis

Twenty isolates of *Trichoderma* spp. were screened for their ability to hydrolyse phosphoric acid swollen cellulose. Isolates were grouped as low cellulase activity (7-25mm); medium cellulase activity (26-41mm) and high cellulose activity (42-85mm) (Table 4.4). Out of twenty isolates screened only five isolates of *Trichoderma* spp. were observed to have high cellulase activity (*T.viride* #T17,

T.harzianum #T16, *T.aureoviride* #T27, *Trichoderma* belonging to section Pachybasium #T158a and section Longibrachiatum #T1) and three with medium cellulase activity (*T.virens* #T101b, *Trichoderma* belonging to Section Pachybasium #T73 # T174) while low cellulase activity was observed in rest of the twelve isolates belonging to different species of *Trichoderma* (*Trichoderma virens* isolate N, *Trichoderma viride* isolate T14, *Trichoderma harzianum* isolate T15, *Trichoderma aureoviride* isolates T29, T114 and T120, Section Pachybasium belonging isolates T66, T93, T110, T132 and Section Longibrachiatum belonging isolates T7 and T31).

Trichoderma viride #T17, *Trichoderma harzianum* #T16, *Trichoderma aureoviride* #T27 section Longibrachiatum belonging #T1 and isolate belonging to section Pachybasium # T158a expressed high chitinase and cellulase activity (Table 4.5) and are therefore candidate isolates will contribute towards the development of a second line of bio-inoculants with enzymatic system that is involved in is β-glucan degrading enzymes as well as for biocontrol activity.

Several lines of evidence indicate that members of the fungal genus *Trichoderma* have been extensively studied, for their ability to secrete cellulose degrading enzymes or to act as biocontrol agents (Mandels and Andreotti, 1978; Nevalainen *et al*., 1980; Szengyel *et al*., 2000). Identification of isolates of *Trichoderma* spp. with high and medium cellulose activity will contribute towards the development of a second line of bio-inoculants with enzymatic system that is involved in is β-glucan degrading enzymes. Studies of the cellulolytic enzymes systems of *Trichoderma* species have a long history (Beguin and Aubert, 1994; Teeri, 1997; Wood and Garcia campayo, 1979). Several reports indicate cellulolytic fungi as *Trichoderma viride* and *Trichoderma reesei* (Mandels and Weber, 1969; Mandels *et*

al., 1971; Montenecourt and Eveleigh, 1979; Gadgil *et al*., 1995; Velkovska *et al*., 1997; Domingues *et al*., 2000). Genome sequencing of the biomass degrading fungus *Trichoderma reesei* has been done, which provides a roadmap for constructing enhanced *T.reesei* strains for industrial applications such as biofuel production (Martinez *et al.,* 2008). To enhance the cellulase titer, various mutants of *Trichoderma* have been developed, among which *T. reesei* RUT C30 is of industrial interest because of its high cellulase production level (Montenecourt and Eveleigh, 1979) as well as its ability to grow on waste cellulosic material (Reczey *et al*., 1996; Ju and Afolabi,1999; Domingues *et al*., 2000). Although various *Trichoderma reesei* strains have been shown to be among the most cellulolytic strains currently available (Mandels, 1982; Montenecourt and Eveleigh, 1977) most applications involving hydrolysis of lignocellulosic substrates have required supplementing the culture filtrates with a separate fl-glucosidase source (Sternberg *et al*., 1977).Other workers (Gracheck *et al*., 1980) have tried to circumvent this deficiency by using whole cultures as most of the fl-glucosidase activity was found tobe associated with the mycelia.Cellulolytic enzymes were produced on four carbon sources by *T. reesei* RUT C30, characterized by various enzyme activity measurements and compared to commercial cellulases (Juhasz *et al*., 2005).

Table 4.4 Screening of isolates of *Trichoderma* spp. for chitinase and cellulase producing ability

Species of *Trichoderma* isolate(s)	Enzymatic activity		Isolate # / section	Enzymatic activity	
	Chitinase	Cellulase		Chitinase	Cellulase
Trichoderma virens			Longibrachiatum		
N	88.9 (H)	22(L)	T1	81.3(H)	71(H)
T101b	71.1(H)	28(M)	T7	55.9(H)	21(L)
Trichoderma viride			T31	43.2(H)	25(L)

T14	53.4(H)	18(L)	Pachybasium		
T17	78.8(H)	43(H)	T66	50.8(H)	22(L)
Trichoderma harzianum			T73	71.1(H)	29(M)
T15	40.7(M)	23(L)	T93	63.5(H)	21(L)
T16	76.2(H)	56(H)	T110	68.6(H)	18(L)
Trichoderma aureoviride			T132	76.2(H)	23(L)
T27	89(H)	43(H)	T158a	68.6(H)	51(H)
T29	35.6(M)	25(L)	T174	60.1(H)	27(M)
T114	28(M)	23(L)			
T120	38.1(M)	24(L)			

DAI=Days after inoculation

Table 4.5 Isolates of *Trichoderma* spp. with high chitinase and cellulase producing ability

Species of *Trichoderma* isolate(s)	Enzymatic activity	
	Chitinase	Cellulase
Trichoderma viride		
T17	78.8(H)	43(H)
Trichoderma harzianum		
T16	76.2(H)	56(H)
Trichoderma aureoviride		
T27	89(H)	43(H)
Longibrachiatum (Section)		
T1	81.3(H)	71(H)
Pachybasium (Section)		
T158a	68.6(H)	51(H)

DAI=Days after inoculation

4.6 Quantitative spectrophotometric assay for siderophore production (liquid assay)

In soil, plant roots normally coexist with bacteria and fungi which may produce siderophores capable of sequestering the available soluble iron and hence interfere with plant growth and function. Siderophores are produced during extreme iron-depleted conditions for the solubilization of extracellular ferric iron by most bacteria and fungi. In the present investigation 20 isolates were screened for the siderophore production following the method by Payne 1994. The change in color of the blue dye chrome azurol sulphonate assay solution to purple-orange indicates the presence of siderophore.

Siderophore production ranged from 10.18% to 94.61% Isolates #T110 belonging to section Pachybasium and *T. harzianum* isolate #T15 showed 94.61% siderophore units and were highest siderophore producing isolates, while isolate #

T73 belonging to section Pachybasium showed the least 10.18% siderophore units (Table 4.6, Figure 4.1). Dutta *et al.,* (2006) studied production of siderophores by 9 different soil fungi and wood-decay fungi following CAS-assay and CAS-agar plate assay. Siderophores can promote rhizosphere colonization and plant growth in synergy with other substances, a mechanism also suggested in plant growth enhancement by pseudomonads (Anke *et al*., 1991; Lugtenberg *et al*., 2001; Sharma *et al*., 2003). Altomare *et al*. (1999) found that *T. harzianum* strain T22 could produce soluble forms of manganese, metallic zinc and calcium phosphate *in vitro*, and also that the fungus produced metabolites that reduced ferric iron (III) to the ferrous form readily assimilated by plants. Studies by Jalal *et al*. (1986, 1987) showed that *T. virens* produces the siderophores mono- and di-hydroxamates as mechanisms of Fe III chelation. According to these investigators, the conversion of metal oxides to soluble forms by *Trichoderma* spp. involved chelation and reduction, both mechanisms also implicated in the control of plant pathogens and components of growth stimulation and biocontrol (Anusuya and Jayarajan, 1998; Harman, 2006; Woo *et al*., 2006).

4.7 Screening isolates of *Trichoderma* spp. for *in vitro* phosphate solubilization ability

Phosphate solubilization efficacy of isolates of *Trichoderma* spp. was performed on three different media 1) Pikovaskaya agar (P1) (Pikovaskya's, 1948), NBRIP agar (National Botanical Research Institutes Phosphate growth medium) (P2) (Nautiyal, 1999) and NBRIY agar (NBRI'S Phosphate growth medium devoid of yeast extract) (P3) (Nautiyal, 1999). Inoculated media plates after 3 days of incubation at

28±2ºC, indicated phosphate solubilising positive isolates which turned the media from purple to yellow in zone of acidification. Diameter of the zone determined the phosphate solubilization ability. Phosphate solubilization ability was determined by measuring the radial diameter on colour change in zone of acidification. The phosphate solubilizing ability of the isolates tested on P1, P2 and P3 ranged from 29 to 80 mm diameter, 15 to 43 mm diameter and 22 to 55 mm diameter respectively (Table 4.7) (Plate 4.9). Screening of isolates showed variation in their ability to utilized tri calcium phosphate supplemented in three different media P1, P2 and P3 containing different nutrient constituents. Higher efficacy to utilize tri calcium phosphate varied and are enumerated as follows:- **P1 (Pikovaskya agar) =** *Trichoderma harzianum*(# T15), *Trichoderma aureoviride*(# T29, # T27, # T114), Pachybasium(# T132), Longibrachiatum(# T1); **P2 (NBRIP agar) =**Pachybasium(# T110), Longibrachiatum(# T1), *Trichoderma viride*(# T17), *Trichoderma aureoviride*(# T27, #T114); **P3 (NBRIY agar) =** Pachybasium(# T73, #T174), *Trichoderma viride*(# T17), *Trichoderma aureoviride*(# T114, # T120, # T27), *Trichoderma virens*(# N, # T101b), *Trichoderma viride*(#T17). *Trichoderma aureoviride*(#T114) exhibited higher phosphate solubilising ability in all the three media used for screening and therefore can be considered as promising inducer of phosphate mobilization. Present investigation are in support of the earlier reports that the isolates belonging to sections Pachybasium and Longibrachiatum and isolates of *T. viride, T. aureoviride, T. virens and T. harzianum* as promising inducer of phosphate mobilization. Experiments carried out by Dunaitsev *et al* (2008), showed the strains no. 13 *T.*

viride, no. 20 *T. asperellum*, no. 29 *T. longibrahiatum* and for the strain *Bacillus subtilis* IPM 215 also, the ability to release phosphorus from mineral raw materials was revealed which can be considered as promising producers of a biological preparation of combined operating-biofungicide and inducer of phosphate mobilization. Rudresh *et al*, (2005) showed in his investigation that *Trichoderma viride* (TV 97) (9.03 µg mL–1), *Trichoderma virens* (PDBCTVs 12) (9.0 µg·mL–1), and *Trichoderma virens* (PDBCTVs 13) (8.83 µg·mL–1) solubilized 70% of that solubilized by the reference strain *Bacillus megaterium* (12.43 µg·mL–1). Satyavani and Satyaprasad, (2009) demonstrated that two species of *Trichoderma viz*., *T. harzianum* and *T. aureoviride* isolated from rhizosphere of pigeon pea promoted plant growth so to understand the role of *Trichoderma* in growth promotion, the ability of *Trichoderma* spp. in solubilizing rock phosphate was tested *in vitro*. Culture filtrates of both species solubilized rock phosphate in which *T.harzianum* was more effective than *T.aureoviride* in solubilizing phosphates and producing fungal biomass. For example, *Trichoderma harzianum* was shown to solubilize phosphate and micronutrients that could be made available to plant (Altomore *et al*., 1999).

Table 4.6 Efficacy of different *Trichoderma* spp. grown at 37°C for 3 days to produce siderophore

Species of *Trichoderma* isolate(s)	OD (635nm)	% siderophore units	Isolate # / section	OD (635nm)	% siderophore units
T.virens			Longibrachiatum		
N	0.0075	86.53%	T1	0.013	76.65%
T101b	0.008	85.63%	T7	0.015	73.05%
T. viride			T31	0.00733	86.83%
T14	0.0055	90.12%	Pachybasium		
T17	0.00466	91.63%	T66	0.0105	81.14%
T.harzianum			T73	0.05	10.18%
T15	0.003	94.61%	T93	0.0045	91.92%
T16	0.0215	61.38%	T110	0.003	94.61%
T.aureoviride			T132	0.0075	86.53%
T27	0.0065	88.32%	T158a	0.0065	88.32%
T29	0.005	91.02%	T174	0.00733	86.83%
T114	0.034	38.92%			
T120	0.0095	82.93%			

Table 4.7 Screening isolates of *Trichoderma* spp. *in vitro* for phosphate solubilizing ability

Species of *Trichoderma* isolate(s)	Phosphate solubilisation (mm)			Isolate # / section	Phosphate solubilisation (mm)		
	P1	P2	P3		P1	P2	P3
Trichoderma virens				Longibrachiatum			
N	47	24	49	T1	80	31	35
T101b	29	25	52	T7	41	16	25
Trichoderma viride				T31	48	18	39
T14	40	23	34	Pachybasium			
T17	54	33	47	T66	44	25	22
Trichoderma harzianum				T73	52	20	45
T15	62	15	40	T93	56	29	37
T16	50	20	39	T110	42	31	36
Trichoderma aureoviride				T132	70	22	35
T27	62	33	42	T158a	50	20	35
T29	62	23	34	T174	40	20	55
T114	65	43	47				
T120	51	27	50				

P1= Pikovaskaya agar (Pikovaskya's, 1948), **P2**= NBRIP agar (National Botanical Research Institutes Phosphate growth medium) and **P3** = NBRIY agar (NBRI'S Phosphate growth medium devoid of yeast extract) (Nautiyal, 1999).

4.8 Production of IAA and IAA-related compounds by *Trichoderma* spp.

Production of IAA and IAA-related compounds was evaluated for 20 isolates of *Trichoderma* spp. in culture medium amended with 1.02 g/L from 5mM stock of L-tryptophan as precursor molecule and without any IAA precursor as control. The mixture of culture supernatant and salkowski's reagent was incubated at room temperature for 20 min and the absorbance was measured at 535 nm. The concentration of IAA and IAA related compounds was evaluated by comparison with a standard curve prepared using serial dilutions (0-50 µg/ml) prepared from commercially available IAA. Interpolation of the colorimeter readings with standard curve were used to quantify the amount of IAA produced by different isolates of *Trichoderma* in the media which ranged from 0.793 to 3.560 µg/ml (Table 4.8, Figure 4.2). The highest IAA was produced by *Trichoderma viride* isolate # T14 (3.560 µg/ml) where as *T. aureoviride* isolate # T27 (0.793 µg/ml) was the lowest producer. *Trichoderma viride isolate* # T14 also induced highest plant growth promoting activity , chlorophyll a, b and total content when applied as seed treatment to cucumber and bottle gourd. Isolate # T15 and T16 (*T.harzianum*) were also identified as promising producers of Indole acetic acid 3.422µg/ml and 3.0 µg/ml respectively. *T.harzianum* isolate# T16 treated plants expressed highest chlorophyll a and total chlorophyll content in bitter gourd. Isolate # T15 high siderophore producer with plant growth promotion activity. Efficacy of different isolates of *Trichoderma* spp. used in the present investigation to produce IAA from L-tryptophan as a precursors varied, the *Trichoderma viride* isolate # T14 and *T.harzianum* isolate # T15 and T16 were identified as the highest producer of IAA thus indicating their ability to synthesize IAA from L-tryptophan as a precursors *in vitro.* This further supports the

theory that microbial IAA could be involved in the growth stimulation observed in our greenhouse assay. Production of plant growth regulators by the microorganisms is another important mechanism often associated with growth stimulation (Vessey, 2003). The balance between vegetative and reproductive growth is controlled by hormone signaling within the plant and can therefore be highly influenced by it (Taiz and Zeiger, 1991). At relatively high concentrations, natural auxins, such as IAA, stimulate shoot elongation and root induction while reducing root elongation (Tanimoto, 2005). *Trichoderma* spp. have been also shown to exhibit plant growth-promoting activity on numerous cultivated plants (Kleifeld and Chet, 1992; Ousley *et al*.,1994; Altomare *et al*., 1999; Harman, 2000; Yedidia *et al*.,2001). Gravel *et al* (2007) in his results, showed that *P. putida* subgroup B strain 1 and *T. atroviride* are able to synthesize IAA from different precursors *in vitro*, which supports the theory that microbial IAA could be involved in the growth stimulation observed in our greenhouse assay.

Table 4.8 Efficacy of different *Trichoderma* spp. for IAA production

Species of *Trichoderma* isolate(s)	OD (Y)	µg/ml	Isolate # / section	OD (Y)	µg/ml
Trichoderma virens			Longibrachiatum		
N	0.114	0.982759	T1	0.106	0.913793
T101b	0.148	1.275862	T7	0.199	1.715517
Trichoderma viride			T31	0.154	1.327586
T14	0.413	3.560345	Pachybasium		
T17	0.279	2.405172	T66	0.123	1.060345
Trichoderma harzianum			T73	0.133	1.146552
T15	0.397	3.422414	T93	0.097	0.836207
T16	0.348	3	T110	0.109	0.939655
Trichoderma aureoviride			T132	0.109	0.939655
T27	0.092	0.793103	T158a	0.138	1.189655

T29	0.158	1.362069	T174	0.116	1
T114	0.131	1.12931			
T120	0.106	0.913793			

4.9 Screening of different isolates of *Trichoderma* spp. for hydrogen cyanide production

In the present investigation twenty isolates of *Trichoderma* spp. were screened for its ability to produce hydrogen cyanide. The production of hydrogen cyanide by *Trichoderma* spp. was screened using glycine as its precursor molecule. The plates were incubated for 7 days at 28±2°C and observations were measured as colour of filter paper turning to brown measured as positive for HCN production. *Trichoderma harzianum* isolate # T15 and *Trichoderma viride* isolate # T17 were observed as positive for its ability to produce HCN (Plate 4.10, Table 4.9). Microbial cyanogenesis has been demonstrated in many species of fungi (Hutchinson *et al.*, 1973), but only in a few species of bacteria in the genera Chromobacterium and *Pseudomonas* (Michaels and Corpe, 1965; Patty, 1921)**.** Glycine has usually been the indicated precursor of cyanide in fungi and bacteria (Brysk *et al.*, 1969; Ward *et al.*, 1971; Wissing *et al.*, 1974)**.**

Table 4.9 Screening isolates of *Trichoderma* spp. for its ability to produce HCN

Species of *Trichoderma* isolate(s)	Observations	Isolate # / section	Observations
T.virens		Longibrachiatum	
N	(–)	T1	(–)
T101b	(–)	T7	(–)
T. viride		T31	
T14	(–)	Pachybasium	
T17	**(+)**	T66	(–)
T.harzianum		T73	(–)
T15	**(+)**	T93	(–)

T16	(–)	T110	(–)
T.aureoviride		T132	(–)
T27	(–)	T158a	(–)
T29	(–)	T174	(–)
T114	(–)		
T120	(–)		

(+) = Isolates Producing HCN; (–) = Isolates not Producing HCN.

4.10 Efficacy of *Trichoderma* spp. for plant growth promoting activity:

In the present investigation twelve isolates were screened for the plant growth promotion on three different vegetable crops Cucumber (*Cucumis sativus* L.), Bottle gourd (*Lagenaria vulgaris*) and Bitter gourd (*Momordica charantia*). Root length and shoot length were assessed to evaluate the efficacy of different *Trichoderma* spp. to promote plant growth. Observations were recorded 30 days after sowing.

Trichoderma treated seed derived 30 day old cucumber plants were evaluated for root and shoot length (Table 4.10). Efficacy of different species of *Trichoderma* isolates varied to induce root and shoot length which ranged 20.7 cm to 43.55 cm and 6.33 cm to 22.85 cm respectively. No significant differences were observed to induce increase root length by different isolates of *Trichoderma* on cucumber, bottle gourd and bitter gourd plants as compared to control. Significant differences were observed for increase in shoot length as compared to control by the *T. viride isolate* # T 14 on cucumber and on bitter gourd with different species of *Trichoderma* isolates # T27, T120, T114 (*T.aureoviride*) and # T14 , T 17 (*T. viride)*(Table 4.11)(Plate 4.11, Plate 4.12).

Biocontrol fungi (BCF) are beneficial organisms that reduce the negative effects of plant pathogens and promote positive responses in the plant. Recent data indicate that their abilities to control plant diseases are only a subset of their capabilities. They do control diseases and in addition have other benefits, including

amelioration of intrinsic physiological stresses in seeds and alleviation of abiotic stresses. They can also improve photosynthetic efficiency, especially in plants subjected to various stresses. Finally, several fungi also increase nitrogen use efficiency in plants. As a consequence, plants treated with beneficial fungi may be larger and healthier and have greater yields than plants without them. Mechanisms by which these changes occur are becoming known. The PGPR are known to participate in many important ecosystem processes, such as the biological control of plant pathogens, nutrient cycling, and/or seedling growth (Persello-Cartieaux *et al*., 2003; Barea *et al*., 2004; Zahir *et al*., 2004). It has long been known that BCF can enhance plant growth. *Trichoderma* and *Sebacinales* species inoculation induces root and shoot growth (Barazani *et al.,* 2005, Harman, 2000, Harman *et al.,* 2008, Harman *et al.,* 2004a, Harman *et al.,* 2004a, Peskan-Berghoefer *et al.,* 2004, Rai *et al.,* 2001a). *Trichoderma* even promoted growth of trees (Adams and De-Lij 2007, Bae *et al.,* 2009). BCF also increase percentages of germination and rates of germination of seeds (Barazani *et al.,* 2005, Bjorkman *et al.,* 1998, Chang *et al.,* 1986; Mastouri, Bj¨ orkman, G Harman, unpublished data). More importantly, the effect of BCF on plant growth has a long duration and even lasts for the entire life of annual plants (Barazani *et al.,* 2005, Harman, 2000, Harman *et al.,* 2004, Waller *et al.,* 2005). However, *Trichoderma* and *Sebacinales* species were shown to induce growth under sterilized and nonsterilized conditions (Harman *et al.,* 2004, Lindsey and Baker 1967, Peskan-Berghoefer *et al.,* 2004, Rai *et al.,* 2001a, Singh *et al.,* 2000, Yedidia *et al.,* 2001), suggesting a direct mechanism through plant response. Harman, 2002 demonstrated that the presence of root-colonizing T-22 induced about twice as many deep-root intercepts 25-75 cm below the soil surface which resulted in increased drought tolerance, and probably resistance to compact soil. Seeds exposed

to abiotic stresses, including osmotic, salt, heat, and cold stresses, in the presence of T22 have much higher percentages of germination and improved seedling vigor (F Mastouri, T Bj ¨ orkman, G Harman,unpublished data). Tomato seed lots with reduced vigor caused by various aging regimes exhibit higher percentages of germination and improved seedling vigor compared with nontreated seeds (F Mastouri, T Bj ¨ orkman, G Harman, unpublished data). *Trichoderma* treated plants were shown to have enhanced nutrient uptake, increased root and shoot growth, and improved plant vigor (Inbar *et al.*, 1994; Yedidia *et al.*, 2001; Harman *et al.*, 2004).

Table 4.10 Efficacy of different *Trichoderma* spp. to induce shoot and root growth in Cucumber, Bottle gourd and Bitter gourd.

**Treatment	Cucumber	Bottle gourd	Bitter gourd
Root Length			
Control	42.23±*9.43	37.68±4.38	23.44±1.15
T14	43.55±6.45	32.46±1.36	16.2±0.58
T15	38.57±4.19	24.26±3.03	24.5±1.98
T16	32.8±0.7	25.96±2.47	23.28±1.60
T17	30.52±2.96	22.94±1.88	24.75±2.75
T27	30	32.9±7.23	22.1±1.09
T29	No germination	36.85±4.48	25.82±1.13
T93	37.65±9.12	29.98±3.15	26.96±2.18
T101b	20.7±4.99	22.13±6.00	28.08±0.45
T110	42.5±7	25.65±5.66	23.62±3.62
T114	26.95±3.55	30.78±2.18	26.08±2.55
T120	41.4±7.86	25.53±1.07	27.38±2.48
N	43.5	27.55±5.23	24.13±1.30
Shoot Length			
Control	11.3±1.62	9.37±1.13	12.8±0.64
T14	22.85±8.75	14.78±0.59	10.94±1.00
T15	7.83±1.00	9.3±0.52	16.3±1.60
T16	10±0.2	9.2±0.63	10.96±0.68
T17	9.7±0.98	9.38±0.45	12.65±1.85
T27	6.4	10.46±0.29	19.72±1.55

T29	No germination	8.53±0.81	14.84±1.95
T93	9.05±1.08	10.18±0.89	13.66±1.05
T101b	6.33±0.94	7.7±2	12.7±0.43
T110	10.8±1.3	10.2±1.48	10.92±2.02
T114	9.7±0.6	9±0.54	11.12±0.73
T120	11.24±1.17	11.35±1.19	16.3±2.14
N	10.6	9.45±0.03	14.19±0.72

*values after ± represents standard error of mean;
**Treatment = Seed treatment with different isolates of *Trichoderma* spp.

Table 4.11 Species of *Trichoderma* isolates which induced highest root and shoot length on different crops following seed treatment

Sr. No.	Crops	Root length	Shoot length
1	Cucumber	T14	T14
2	Bottle gourd	control	T14
3	Bitter gourd	T101b	T27

4.11 Estimation of chlorophyll content

Chlorophyll (Total chlorophyll, chl a and chl b) contents was determined following Arnon (1949) on freshly harvested leaves derived from 30 day old plant which were grown following seed treatment with 12 different isolates of *Trichoderma* spp. (Table 4.12).

Total chlorophyll content in Cucumber ranged from 5.81 mg/g to 29.33 mg/g and chlorophyll a and b ranged from 3.65 mg/g to 12.71 mg/g and 2.20 mg/g to 11.86 mg/g, respectively. Chlorophyll a, b and total chlorophyll content for control plants was 5.96 mg/g, 3.09 mg/g and 7.65 mg/g, respectively. *T. viride* (# T14) treated plants measured highest chlorophyll a (12.71 mg/g), b (11.86 mg/g) and total chlorophyll (29.33 mg/g) content as, compared control. Total chlorophyll content in Bottle gourd ranged from 10.81 mg/g to 22.32 mg/g. Chlorophyll a and b ranged from 7.31 mg/g to 14.74 mg/g and 3.57 mg/g to 7.25 mg/g, respectively. The chlorophyll a, b and total chlorophyll content for control plants was 8.29 mg/g, 3.91 mg/g and 12.26 mg/g, respectively. *T.viride* (# T14) treated plants measured highest

chlorophyll a, b and total content as 14.74 mg/g, 7.25 mg/g and 22.32 mg/g, respectively as compared to chlorophyll content of control. Total chlorophyll content in Bitter gourd ranged from 9.15 mg/g to 18.35 mg/g. Chlorophyll a and b ranged from 5.51 mg/g to 12.88 mg/g and 3.28 mg/g to 5.66mg/g, respectively. Chlorophyll a, b and total chlorophyll content for control plants was 5.51 mg/g, 4.78 mg/g and 13.65 mg/g, respectively. *T.harzianum* (iso # T16) treated plants measured highest chlorophyll a and total content as 12.88 mg/g and 18.35 mg/g, as compared to control. *T. aureoviride* (iso. # T120) treated plants measured highest chlorophyll b content as 5.66 as compared to control.

Seed treatment with *T.viride* iso#T14 induced comparatively larger effects similar to hormonal application for plant growth promotion as compared to other isolates (Table 4.12) used in the present investigation which was measurable in terms of high chlorophyll a, b and total chlorophyll content , indole acetic acid production (3.560345 µg) and siderophore production. (90.12%). Similarly *T.harzianum* (iso # T16) and *T. aureoviride* (iso. # T120) treated bitter gourd plants expressed higher chlorophyll content. Several lines of evidence indicate that filamentous fungi induce phytohormones like effect in the treated plants. *Trichoderma* strains that produce cytokinin-like molecules, e.g. zeatyn and gibberellin GA3 or GA3 related, have been recently detected. IAA is also involved in tomato fruit development, especially during fruit setting and in the final phase of development (Srivastava and Handa, 2005). Indeed, IAA, including microbial, can greatly influence the growth of the root system depending on the amount found in the rhizosphere, through root elongation and the formation of lateral or adventitious roots (Scott, 1972; Patten and Glick, 2002). Several auxin-like secondary metabolites produced by *Trichoderma* strains were able to induce plant growth and are required

for development of lateral roots in *Arabidopsis* (Contreras *et al*., 2009, Vinale *et al*., 2008). The observed effect of *Trichoderma* in promoting lateral root development is similar to that described for auxins in plants (Casimiro *et al*., 2001) IAA is a molecule that is synthesized by plants and a few microbes (Woodward and Bartel, 2005). In plants IAA plays a key role in root and shoot development. The hormone moves from one part of the plant to another by specific transporter systems that involve auxin importer (AUX1) and efflux (PIN1-7) proteins. IAA is a key regulator of lateral root development and root hair development (Casimiro *et al*., 2001). Expression studies of the auxin-inducible marker DR5: uidA suggested that *T. virens* inoculation increases the auxin response in Arabidopsis seedlings.

Root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients (Arora *et al*., 1992). Crop productivity in fields can increase up to 300% after the addition of *Trichoderma hamatum* or *Trichoderma koningii*. In experiments carried out in greenhouses, there was also a considerable yield increase when plant seeds were previously treated with spores from *Trichoderma* (Chet *et al*., 1997). The same increase was observed when seeds were separated from *Trichoderma* by a cellophane membrane, which indicates that *Trichoderma* produces growth factors that increased the rate of seed germination (Benítez *et al*., 1998). However, there are very few reports on strains that produce growth factors which have been detected and identified in the laboratory (auxins, citokinins and ethylene), despite the identification of many filamentous fungi that produce phytohormones, such as indol acetic acid (IAA) and ethylene, whose metabolic pathways have been identified (Arora *et al*., 1992; Osiewacz , 2002).

Table 4.12 Estimation of chlorophyll (Chl. A, b and Total) content in Cucumber,Bottle gourd and Bitter gourd plants derived following seed treatment with different species of *Trichoderma*

Species of *Trichoderma* isolate(s)	Chlorophyll content (mg/g)								
	a	b	Total	a	b	Total	a	b	Total
	Cucumber			Bottle gourd			Bitter gourd		
Trichoderma virens									
N	3.65	2.20	5.81	8.81	4.16	12.95	9.46	4.29	13.84
T101b	7.94	3.87	11.84	10.01	4.64	14.71	7.94	3.87	11.56
Trichoderma viride									
T17	7.21	4.59	11.56	7.02	3.57	10.59	8.10	3.90	11.98
T14	12.71	11.86	29.33	14.74	7.25	22.32	10.34	4.82	15.35
Trichoderma harzianum									
T15	8.09	4.05	12.29	10.37	4.79	15.40	9.19	4.39	13.65
T16	6.43	3.46	9.67	10.91	4.83	15.85	12.88	5.44	18.35
Trichoderma aureoviride									
T27	8.99	4.25	13.68	8.15	3.94	12.07	9.00	4.15	13.23
T29	No germination			9.34	4.33	13.79	9.61	4.52	14.07
T114	5.68	2.92	8.62	8.35	4.11	12.54	7.42	3.66	11.04
T120	4.28	2.59	6.76	11.13	3.17	13.98	12.10	5.66	17.82
Section Pachybasium									
T93	5.60	2.97	8.53	7.60	3.88	11.51	11.39	5.36	16.79
T110	5.36	2.84	8.15	8.23	4.24	12.40	5.97	3.28	9.15
CONTROL	5.96	3.09	7.65	8.29	3.91	12.26	5.51	4.78	13.65
Minimum	**3.65**	**2.20**	**5.81**	**7.02**	**3.17**	**10.59**	**5.51**	**3.28**	**9.15**
Maximum	**12.71**	**11.86**	**29.33**	**14.74**	**7.25**	**22.32**	**12.88**	**5.66**	**18.35**
Average	**6.30**	**3.75**	**10.30**	**9.46**	**4.37**	**13.87**	**9.15**	**4.47**	**13.88**

4.12 DNA fingerprinting analysis of population and sub population of the isolates representing *Trichoderma* spp. following cluster analysis

DNA fingerprinting involves the display of a set of DNA fragments from a specific DNA sample. Total genomic DNA was extracted from 20 selected isolates of *Trichoderma* spp. and was subjected to PCR amplification using gene specific markers and rDNA markers. A total of 9 primer pairs were screened on twenty *Trichoderma* isolates to identify the primer pairs that produced the most polymorphic fragments. For each primer pair as well as specific digestion products

the presence or absence of bands in each isolate was visually scored and set in a binary matrix. All polymorphic bands were scored as presence (1) or absence (0) and used for statistical analysis to calculate the similarity coefficient and generate dendograms using unweighted pair-group method of arithmetic average (UPGMA) with a SAHN module of NTSYSpc (Numerical Taxonomy System Biostastiscs) software.

In the present work, we report on the application of combined molecular markers to study genetic variability among 20 isolates of *Trichoderma* spp. The number of bands amplified by every primer combinations varied among the isolates. Number of amplified bands in primers were: TR1 (1 to 10); BR1-BR2 (2 to 17); (1to 9); (1 to 6) (Plate 4.13); fRPB2-5F1-fRPB2-7CR1 (1 to 11); fRPB2-5F2-fRPB2-7CR2 (2 to 6). Primers TR1 and BR1-BR2 gave distinct amplification patterns for all the three isolates (T1, T7 and T31) belonging to section Longibrachitum resulting in distinguishable clustering of the section from all other isolates of *Trichoderma* spp. These primers were previously found to be useful in analysis of fungi at the species level and even for strain identification (Kuhls *et al* 1996).

Major bands generated by primer pairs Tef71F-Tef997R and Tef85F-Tef954R were in between 800 to 1000 bp. As reported by International Subcommission on *Trichoderma* and Hypocrea (ISTH) these primers amplify a 0.9-kb fragment of the 5' end of the translation elongation factor-1α (*tef*) gene (eEF1a1) containing three major introns which is confirmed in the present work. Similarly primer pair fRPB2-5F2 + fRPB2-7cR2 generated major band above 1000 bp confirming the amplification of a 1.2-kb fragment of subunit 2 of the RNA polymerase B gene (RPB2) containing an ITS-like region. However these bands should be further sequenced to work out

taxonomic identification of the *Trichoderma* isolates (this part was not carried out in the present work due to lack of resources).

PCR amplification of ITS region of 5.8s rRNA gene with three primers (ITS5 + ITS4, ITS1n + ITS4 and ITS1 + ITS ALRO) yielded ITS fragment 600-500 bp length in the most of the isolates of *Trichoderma*. No distinct inter or intra specific ITS length diversity was detected. This is due to the fact that 5.8s rRNA gene is known to be highly conserved at genus level and this only confirmed that all the isolates belonged to single genus (Singh *et al*., 2006). However, double digestion of its amplicons with restriction endonuclease *Mse* I and *Eco* RI revealed polymorphism in ITS1-5.8S-ITS2 region (Plate 4.14). The resulting dendograms clustered some isolates were specifically into its respective species whereas overall homogeneity was observed between different sections of the genus *Trichoderma*. Similar results were observed by Mukherjee *et al* (2002).

When the analytical approach using pooled data of all the nine primer pairs was repeated using our morphological based species designations, that data didn't support the resultant hierarchal population structure. However, sub populations were able to provide species or species complex designations analysis. Pair wise genetic similarities based on Jaccard's coefficient of selected sub-populations suggest distinct subgroups within the isolates. There was presence of distinct subgroups in the following combinations of subpopulations: (1) Isolates belonging to section Longibrachiatum Vs Pachybasium (2) Isolates belonging to section Longibrachiatum Vs *T. aureoviride* Vs *T. virens* (3) Isolates belonging to section Longibrachiatum Vs *T. aureoviride*.

4.12.1 Cluster analysis of all 20 isolates of *Trichoderma* spp. using pooled binary matrix generated from all the nine primer pairs as well as double digested amplicons of three ITS primers

Assesment of genetic relationships by UPGMA analysis of *Trichoderma* population illustrated genetic variation among the isolates with the similarity coefficient ranging from 0.71 to 0.92 (Plate 4.15). There were two clusters, major cluster A with 17 isolates and minor cluster B with two isolates. All the three isolates (# T73, T93 and T110) of minor cluster B represented section Pachybasium. However the major cluster A was subdivided into four subclusters: subcluster 1 represented all the three isolates (# T1,T31,T7) belonging to section Longibrachiatum; subcluster 2 represented four isolates (#T66 of section Pachybasium, *T.harzianum* # T16, and two *T.virens* isolates #T101b,N) out of which the two *T.virens* isolates #T101b,N showed maximum similarity of 92%; subcluster 3 represented four isolates (# T158a,T174 of section Pachybasium; and two *T.aureoviride* isolates T27,T29); subcluster 4 consisted of two isolates (one each *T.harzianum* # T15 and *T.viride* # T17; representing overlapping silmilarity among them). The remaining isolates *T.viride* # T14, *T.aureoviride* # T114 and # T120; Iso # T132 of section Pachybasium did not belonged to any subcluster and represented distinct molecular identity.

Cluster analysis of subpopulations of *Trichoderma* spp.

4.12.2 Cluster analysis of 10 isolates of *Trichoderma* spp. representing subpopulations of Section Pachybasium and Section Longibrachiatum using pooled binary matrix generated from all the nine primer pairs as well as double digested amplicons of three ITS primers

Subpopulations of Section Pachybasium Vs Section Longibrachiatum appear to form subclusters between similarity coefficient range 0.70 to 0.88 (Plate 4.16 A). Distinct subcluster of all the three isolates of section Longibrachiatum (# T1, T31, T7) was formed at 84% similarity level with isolates T1 and T31 showing maximum similarity (88%) to each other. Seven isolates of section Pachybasium were subclustered into two subclusters one of Iso# T132,T158a,T174 and the other of Iso# T73,T93,T110. However the isolate T66 was out of cluster due to its molecular distinctness.

4.12.3 Cluster analysis of 9 isolates of *Trichoderma* spp representing subpopulations of Section Longibrachiatum Vs. *T. aureoviride* Vs *T. virens* using pooled binary matrix generated from all the nine primer pairs as well as double digested amplicons of three ITS primers

Subpopulations of Section Longibrachiatum Vs. *T. aureoviride* Vs *T. virens* appear to form subclusters between similarity coefficient range 0.73 to 0.92 (Plate 4.16 B). Distinct subcluster 1 of all the three isolates of section Longibrachiatum (# T1, T31, T7) was formed at 87% similarity level with isolates T1 and T31 showing maximum similarity (88%) to each other. Cluster II represented two *T.aureoviride* (Iso# T27, T29) and Cluster III *T.virens* (Iso#N, T101b). *T.virens* isolates T101b and N

(92%) similarity. The graphic dendogram distinctly placed *T.aureoviride* isolates # T114 and #T120 from rest of the isolates.

4.12.4 Cluster analysis of 9 isolates of *Trichoderma* spp representing subpopulations of Section Pachybasium Vs. *T. virens* using pooled binary matrix generated from all the nine primer pairs as well as double digested amplicons of three ITS primers

Assesment of genetic relationships by UPGMA analysis of subpopulation (section Pachybasium and *T.virens*) illustrated genetic variation among the isolates, as they were mainly divided into two major clusters representing two different subpopulations of *Trichoderma* spp. (Plate 16 C) Cluster **I** represented section Pachybasium (Iso. # T158a,T132,T174,T66,N,T101b) and cluster II represented section Pachybasium (Iso# T73,T93,T110). Cluster I showed grouping of six isolates with 76% similarity and further subcluster as sub group cluster I(a) consisting isolates T158a,T174,T66 belonging to section Pachybasium (79% similarity) and cluster I(b) consisting isolate Iso.#T132 belonging to section Pachybasium. In cluster I(a) *T.virens* isolates showed 100% similarity. Cluster II showed grouping of three isolates with 76.5% similarity. Sub group cluster II (a) consisting isolate T73 and T93 belonging to section Pachybasium (81.5% similarity) and cluster II (b) consisting isolates T110 belonging to section Pachybasium. The isolates were clearly grouped according to the section as Pachybasium and *T.virens* into two respective groups. Grouping the isolates based on cluster analysis of their DNA profiles matched that based on their morphological taxonomy except for isolate # T66 showed diversity and grouped separate sub cluster with section *T.virens* in cluster I.

4.12.5 Cluster analysis of 7 isolates of *Trichoderma* spp representing subpopulations of Section Longibrachiatum Vs. *T. aureoviride* using pooled binary matrix generated from all the nine primer pairs as well as double digested amplicons of three ITS primers

Similarity coefficient ranged from 0.71 to 0.89 among subpopulation of Section Longibrachiatum and *T. aureoviride* (Plate 4.16 D). Cluster **I** represented section Longibrachiatum (Iso. # T1, T31, T7) with 85% similarity. Cluster II showed grouping of three *T.aureoviride* isolates (Iso. # T27, T29 and T114) with 78.4% similarity among which T27 and T29 represented maximum similarity of 89%. The isolates were clearly grouped according to the section as one group of Longibrachiatum and *T.aureoviride* into respective groups. Grouping the isolates based on cluster analysis of their DNA profiles matched that based on their morphological taxonomy except for isolate # T120 showed diversity and formed separate cluster with section *T.aureoviride* in cluster III.

The molecular studies essentially support the morphologically based taxonomy proposed by Bissett (1984; 1991 a,b,c; 1992), although the arrangement of species in morphologically defined sections is not supported and individual species may be too broadly conceived. Two of the sections that they proposed, sects. *Longibrachiatum* and *Saturnisporum*, are phylogenetically and morphologically homogeneous. The phylogenetic tree for *Trichoderma* is growing. Many *Trichoderma* species are known under two names, viz. the name of the *Trichoderma* anamorph and that of the *Hypocrea* teleomorph. *Trichoderma* sect. *Pachybasium*, in its morphological sense, so far contains the anamorphs of the majority of the described

Hypocrea/Trichoderma species. *Pachybasium* has since been shown to be polyphyletic (Kindermann *et al* 1998,

Lieckfeldt and Seifert 2000, Kullnig-Gradinger *et al.,* 2002). A serious drawback of the use of ITS1 and ITS2 is that it provides only poor phylogenetic resolution in some clades, particularly *Pachybasium* B (Kullnig-Gradinger *et al* 2002; Chaverri *et al.,* 2003). ITS1 and ITS2 sequence differences were also unable to consistently distinguish between three taxa from the "*H. rufa* species complex" namely *T. viride*, *T. koningii* and *T. atroviride* (Kubicek and Druzhinina, 2005). DNA sequence analyses indicates that *T. virens* (= *Gliocladium virens*) is a species of *Trichoderma*; it is the anamorph of *H. virens* (Chaverri *et al.,* 2001) and is closely related to *T. harzianum* (Chaverri *et al.,* 2003). Currently, there is no acceptable formal subdivision of *Trichoderma* or *Hypocrea*, and as new species are added, the genus appears to become more complex. Today, only sect *Longibrachiatum* (including sect. *Saturnisporum*) is monophyletic. In this study, isolates in the sub-population were analyzed in combination to discriminate them to provide species or species complex designations. Association of more molecular markers will have to be analyzed to clearly resolve and provide the species or species complex designations. Association of molecular markers with morphological based diagnostics indicated that it was able to differentiate the inter specific sub-population to some extent.

CHAPTER V

SUMMARY, CONCLUSION AND SUGGESTION FOR FUTURE RESEARCH WORK

The genus *Trichoderma* comprises a great number of fungal strains that act as biological control agents, the antagonistic properties of which are based on the activation of multiple mechanisms. *Trichoderma* strains exert biocontrol against fungal phytopathogens either indirectly, by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis, or directly, by mechanisms such as mycoparasitism. These indirect and direct mechanisms may act co-ordinately and their importance in the biocontrol process depends on the *Trichoderma* strain, the antagonized fungus, the crop plant, and the environmental conditions, including nutrient availability, pH, temperature, and iron concentration. Activation of each mechanism implies the production of specific compounds and metabolites, such as plant growth factors, hydrolytic enzymes, siderophores, antibiotics, and carbon and nitrogen permeases. During the course of present investigation, based on our observation, we summerize the results as below.

5.1 Summary

5.1.1 Screening isolates of *Trichoderma* spp. for its antagonistic ability

- In the present study, twenty isolates of *Trichoderma* spp. were screened *in vitro* by dual culture plate method for its ability to show antagonism against two phytopathogens *R.solani* and *S.rolfsii.* Iso# N (*T.virens*) was strong antagonistic as it completely overgrew both the pathogens while iso# T66 (section Pachybasium) was weak antagonistic as pathogen overgrew.

- Microscopic examination revealed differences in mechanism of mycoparasitism by species of *Trichoderma* against *R. solani* and *S. rolfsii*.

5.1.2 Screening isolates of *Trichoderma* spp. for enzymatic activity of chitinase and cellulase

- Screening of twenty isolates of *Trichoderma* spp. were found to hydrolyse chitin and cellulose with varying potential.

- Out of twenty isolates, five isolates iso# T17 (*T. viride*), T16 (*T. harzianum*), T27 (*T.aureoviride*), T1 (section Longibrachiatum) and T158a (section Pachybasium) showed high chitinase and cellulase enzymatic activity.

5.1.3 Screening isolates of *Trichoderma* spp. for siderophore production and Phosphate solubilisation

- *Trichoderma* spp. has been known for biocontrol agent and plant growth promoter as it secrets and solubilizes nutrients to enhance the root and shoot growth, seed germination which helps to increase the crop productivity.

- *Trichoderma* spp. producing siderophore molecules involved in iron solubilisation were screened. Iso# T15 (*T. harzianum*) and Iso# T110 (Section Pachybasium) were identified as high producers of siderophore (94.61% siderophore units) while T73 (Section Pachybasium) showed 10.18% as the least.

- Isolates of *Trichoderma* spp. were tested for their in vitro phosphate solubilising potential in three types of media containing tri calcium phosphate as the sole phosphorus source. All the isolates were found to solubilise

phosphate but with varying potential. Iso# T114 (*T. aureoviride*) showed highest for phosphate solubilisation in all three types of media.

5.1.4 Screening isolates of *Trichoderma* spp. for indole acetic acid (IAA) and hydrogen cyanide (HCN) production

- Plant growth regulator IAA synthesis by *Trichoderma* spp. was determined by L-Tryptophan supplemented as precursor under *in vitro* conditions. Isolate of *T. viride* iso# T14 produced highest (3.56µg/ml) while *T. aureoviride* iso# T27 was lowest in IAA production.

- Hydrogen cyanide as avolatile inhibitory compound produced by *Trichoderma* spp. was screened which indicated positive results for HCN production by iso# T17 (*T. viride*) and iso# T15 (*T. harzianum*).

5.1.5 *Trichoderma* spp. for plant growth promoting activity

- In this study, efficacy of twelve isolates of *Trichoderma* spp. were screened for plant growth promoting activity. Iso# T14 (*T. viride*) induced and increase in shoot length in cucumber and bottle gourd as compared to control while iso# T27 increased the shoot length of bottle gourd.

- Cholorophyll content estimated was high in T14 and T16 treated seeds in cucumber, bottle gourd and bitter gourd as compared to control.

- **Molecular characterization of twenty isolates of *Trichoderma* spp.**

- The genetic relatedness among twenty isolates of *Trichoderma* spp. were analyzed derived by using nine gene specific markers. The number of

amplified bands showed varied for each different primer pair as TR1(1-10 amplified bands), BR1-BR2 (2-17 amplified bands). Isolates belonging to section Longibrachiatum (T1, T7, T31) gave distinct amplification pattern by markers that resulted different cluster formation.

- ITS markers showed nodistinct amplification pattern for different species but showed polymorphic bands when digested with restriction endonuclease *Mse* I and *Eco* RI.

5.2 Conclusion

- The antagonistic ability varied with different *Trichoderma* spp. against *R.solani* and *S.rolfsii*.

- The experimental results showed that seed treatment if iso# T14 (*T.viride*) and Iso# T16 (*T. harzianum*) significantly produced IAA and were therefore effective as plant growth promoting agents an vegetable crops.

- DNA fingerprinting resolved genetic relatedness of *Trichoderma* spp. derived by gene specific markers in sub population.

- Some gene specific molecular markers were able to discriminate sub population of *Trichoderma* sp. and therefore can be the useful marker from the taxonomic point of view.

5.3 Suggestion for future research work

- Those isolates which possessed high phosphate solubilising ability, IAA production and strong antagonism will be further exploited for their above mentioned characteristics.

- Studying the relationship between plant growth regulators produced by *Trichoderma* spp. for increased growth response of plant.

- Gene specific markers are required to characterize the species of *Trichoderma* as well as to resolve new species and to generate a molecular marker of taxonomic importance.

"Genotypic fingerprinting of antagonistic *Trichoderma* spp. against *Rhizoctonia solani* and *Sclerotium rolfsii* using gene specific molecular marker"

BY

RAHATKAR ONKAR VILAS

ABSTRACT

Trichoderma sp. is well known as biofungicide as well as plant growth promoter which has been engaged in producing various extracellular antifungal enzymes as chitinases, cellulases and recognized to promote the plant growth by solubilisation of various macro and micro nutrients, its uptake and production of growth regulators such as indole acetic acid (IAA), gibberelic acid (GA_3). In the present study, antagonistic potential of the twenty isolates against the soilborne rice pathogen *Rhizoctonia solani* and *Sclerotium rolfsii* was investigated in dual culture and microscopic study which revealed mycoparasitic activity of *Trichoderma* spp. In dual culture method, isolate# N (*T. virens*) was found to be an eminent antagonistic isolate which completely overgrew both the pathogens. Most of the isolates showed high chitinse enzymatic activity as compared to enzymatic activity of cellulase was low. The siderophore producing ability of iso# T15 (*T. harzianum*) and iso# T110 (Section Pachybasium) was found to be highest producing 94.61% siderophore units. Iso# T114 (*T.aureoviride*) showed high level of phosphate solubilisation in three different kinds supplemented with tricalcium phosphate whereas others showed variable phosphate solubilising ability. Cyanogenesis activity of *Trichoderma* spp. produces hydrogen cyanide (HCN) using glycine as precursor molecule as a volatile inhibitory compound. In the present investigation, HCN production was demonstrated with brown colour formation on the filter paper dipped in picric acid. Iso# T14 (*T. viride*) and Iso# T17 (*T. harzianum*) indicated positive results to produce HCN. The isolates of *Trichoderma* spp. produced growth regulators such as indole acetic acid (IAA) to induce the plant growth for enhanced root development which ultimately increases the nutrient uptake and crop production. In *in vitro* studies iso# T14 (*T. viride*) produced highest IAA followed by iso# T15, T16 (*T. harzianum*). The responsiveness of *Trichoderma* isolates for plant growth promoting activity was conducted against the vegetable crops. Molecular characterization of isolates of *Trichoderma* spp. revealed diversity in isolates of *Trichoderma* spp.

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Table 4.1 Grouping of isolates of *Trichoderma* spp. based on cultural and microscopic characteristics

Sr. #	Species of *Trichoderma* isolate(s)	Isolate number / Total
1	*Trichoderma viride*	T14,T17 = (2)
2	*Trichoderma aureoviride*	T27,T29,T114,T120 = (4)
3	*Trichoderma virens*	N,T101b = (2)
4	*Trichoderma harzianum*	T15,T16 = (2)
Isolates belonging to section		
1	*Pachybasium*	T66, T93, T110, T73, T174, T158a, T132 = (7)
2	*Longibrachiatum*	T1, T7,T31 = (3)
	Total	20

Table 4.2 Morphological characteristics of isolates based on key characters described for the identification of *Trichoderma* species

Iso#	Colony colour	Growth pattern	Appearance	Pustule	Different pigments	Odour
T14	Dark green	Aereial and subdued	Uniform velvety	Absent	Absent	Coconut
T17						
T15	Green	Subdued	Slightly velvety and ringed	Absent	Absent	Earthy smell
T16						
T27	Light green	Subdued	Velvety	Minute centered	Yellow crystal	Absent
T29	Dark green	Subdued	Uniform ringed velvety	Absent		
T110	Dark green	Subdued	Uniform and smooth	Absent	Yellow crystal	Absent
T114	Dark green	Subdued		Absent		
N	Blackish green	Effuse	Slightly cottony	Absent	Absent	
T101b		Subdued	smooth and sectered		Absent	
T1	Light green	Subdued	Rough	Minute marginal	Yellow pigmentatio n	Absent
T7	Blueish green	Subdued		Compact		
T31	Dark green	Subdued		Scattered		
T66	Green	Effuse and subdued	cottony	Irregularly disposed	Absent	Absent
T93		Subdued	Slightly cottony uniform	absent		
T110		Subdued		absent		
T132	Yellowish green	Aerial and subdued	cottony	Loosely organised		
T158a		Slightly aerial	velvety	Compact		
T73	Green	Subdued	Rough and Scattered	Irregularly disposed	Media coloured to dull brown	
T174						

Table 4.3 *In vitro* mycoparasitic activity of different *Trichoderma* spp. against rice pathogen(s) *R. solani* and *S. rolfsii* following dual culture

Species of	Reaction type	Isolate # /	Reaction type

Trichoderma isolate(s)	*S. rolfsii*	*R. solani*	section	*S. rolfsii*	*R. solani*
Trichoderma virens			Longibrachiatum		
N	1	1	T1	5	2
T101b	2	1	T7	4	2
Trichoderma viride			T31	4	2
T14	3	4	Pachybasium		
T17	3	1	T66	5	5
Trichoderma harzianum			T73	4	2
T15	2	1	T93	4	1
T16	4	1	T110	2	4
Trichoderma aureoviride			T132	4	1
T27	4	1	T158a	4	2
T29	2	1	T174	5	3
T114	2	1			
T120	4	1			

1=*Trichoderma* completely overgrew the pathogen and covered the entire medium surface;2=*Trichoderma* overgrew at least 2/3 of the medium surface; 3=*Trichoderma* and pathogen each colonized approximately ½ of the medium surface (more than 1/3 and less than 2/3), and neither organism appeared to dominate the other; 4=The pathogen colonized at least 2/3 of the medium surface and appeared to withstand encroachment by *Trichoderma*; 5=The pathogen completely overgrew the *Trichoderma* and covered the entire medium surface

Table 4.4 Screening of isolates of *Trichoderma* spp. for chitinase and cellulase producing ability

Species of *Trichoderma* isolate(s)	Enzymatic activity		Isolate # / section	Enzymatic activity	
	Chitinase	Cellulase		Chitinase	Cellulase
Trichoderma virens			Longibrachiatum		
N	88.9 (H)	22(L)	T1	81.3(H)	71(H)
T101b	71.1(H)	28(M)	T7	55.9(H)	21(L)
Trichoderma viride			T31	43.2(H)	25(L)
T14	53.4(H)	18(L)	Pachybasium		
T17	78.8(H)	43(H)	T66	50.8(H)	22(L)
Trichoderma harzianum			T73	71.1(H)	29(M)
T15	40.7(M)	23(L)	T93	63.5(H)	21(L)
T16	76.2(H)	56(H)	T110	68.6(H)	18(L)
Trichoderma aureoviride			T132	76.2(H)	23(L)
T27	89(H)	43(H)	T158a	68.6(H)	51(H)
T29	35.6(M)	25(L)	T174	60.1(H)	27(M)
T114	28(M)	23(L)			
T120	38.1(M)	24(L)			

DAI=Days after inoculation

Table 4.5 Isolates of *Trichoderma* spp. with high chitinase and cellulase producing ability

Species of *Trichoderma* isolate(s)	Enzymatic activity	
	Chitinase	Cellulase
Trichoderma viride		
T17	78.8(H)	43(H)
Trichoderma harzianum		
T16	76.2(H)	56(H)
Trichoderma aureoviride		
T27	89(H)	43(H)
Longibrachiatum (Section)		
T1	81.3(H)	71(H)
Pachybasium (Section)		
T158a	68.6(H)	51(H)

DAI=Days after inoculation

Table 4.6 Efficacy of different *Trichoderma* spp. grown at 37°C for 3 days to produce siderophore

Species of *Trichoderma* isolate(s)	OD (635nm)	% siderophore units	Isolate # / section	OD (635nm)	% siderophore units
T.virens			Longibrachiatum		
N	0.0075	86.53%	T1	0.013	76.65%
T101b	0.008	85.63%	T7	0.015	73.05%
T. viride			T31	0.00733	86.83%
T14	0.0055	90.12%	Pachybasium		
T17	0.00466	91.63%	T66	0.0105	81.14%
T.harzianum			T73	0.05	10.18%
T15	0.003	94.61%	T93	0.0045	91.92%
T16	0.0215	61.38%	T110	0.003	94.61%
T.aureoviride			T132	0.0075	86.53%
T27	0.0065	88.32%	T158a	0.0065	88.32%
T29	0.005	91.02%	T174	0.00733	86.83%
T114	0.034	38.92%			
T120	0.0095	82.93%			

Table 4.7 Screening isolates of *Trichoderma* spp. *in vitro* for phosphate solubilizing ability

Species of *Trichoderma* isolate(s)	Phosphate solubilisation (mm)			Isolate # / section	Phosphate solubilisation (mm)		
	P1	P2	P3		P1	P2	P3
Trichoderma virens				Longibrachiatum			
N	47	24	49	T1	80	31	35
T101b	29	25	52	T7	41	16	25
Trichoderma viride				T31	48	18	39
T14	40	23	34	Pachybasium			
T17	54	33	47	T66	44	25	22
Trichoderma harzianum				T73	52	20	45
T15	62	15	40	T93	56	29	37
T16	50	20	39	T110	42	31	36
Trichoderma aureoviride				T132	70	22	35
T27	62	33	42	T158a	50	20	35
T29	62	23	34	T174	40	20	55
T114	65	43	47				
T120	51	27	50				

P1= Pikovaskaya agar (Pikovaskya's, 1948), **P2**= NBRIP agar (National Botanical Research Institutes Phosphate growth medium) and **P3** = NBRIY agar (NBRI'S Phosphate growth medium devoid of yeast extract) (Nautiyal, 1999).

Table 4.8 Efficacy of different *Trichoderma* spp. for IAA production

Species of *Trichoderma* isolate(s)	OD (Y)	µg/ml	Isolate # / section	OD (Y)	µg/ml
Trichoderma virens			Longibrachiatum		
N	0.114	0.982759	T1	0.106	0.913793
T101b	0.148	1.275862	T7	0.199	1.715517
Trichoderma viride			T31	0.154	1.327586
T14	0.413	3.560345	Pachybasium		
T17	0.279	2.405172	T66	0.123	1.060345
Trichoderma harzianum			T73	0.133	1.146552
T15	0.397	3.422414	T93	0.097	0.836207
T16	0.348	3	T110	0.109	0.939655
Trichoderma aureoviride			T132	0.109	0.939655
T27	0.092	0.793103	T158a	0.138	1.189655
T29	0.158	1.362069	T174	0.116	1
T114	0.131	1.12931			
T120	0.106	0.913793			

Table 4.9 Screening isolates of *Trichoderma* spp. for its ability to produce HCN

Species of *Trichoderma* isolate(s)	Observations	Isolate # / section	Observations
T.virens		Longibrachiatum	
N	(--)	T1	(--)
T101b	(--)	T7	(--)
T. viride		T31	
T14	(--)	Pachybasium	
T17	**(+)**	T66	(--)
T.harzianum		T73	(--)
T15	**(+)**	T93	(--)
T16	(--)	T110	(--)
T.aureoviride		T132	(--)
T27	(--)	T158a	(--)
T29	(--)	T174	(--)
T114	(--)		
T120	(--)		

(+) = Isolates Producing HCN; (--) = Isolates not Producing HCN.

Table 4.10 Efficacy of different *Trichoderma* spp. to induce shoot and root growth in Cucumber, Bottle gourd and Bitter gourd.

**Treatment	Cucumber	Bottle gourd	Bitter gourd
Root Length			
Control	42.23±*9.43	37.68±4.38	23.44±1.15
T14	43.55±6.45	32.46±1.36	16.2±0.58
T15	38.57±4.19	24.26±3.03	24.5±1.98
T16	32.8±0.7	25.96±2.47	23.28±1.60
T17	30.52±2.96	22.94±1.88	24.75±2.75
T27	30	32.9±7.23	22.1±1.09
T29	No germination	36.85±4.48	25.82±1.13
T93	37.65±9.12	29.98±3.15	26.96±2.18
T101b	20.7±4.99	22.13±6.00	28.08±0.45
T110	42.5±7	25.65±5.66	23.62±3.62
T114	26.95±3.55	30.78±2.18	26.08±2.55
T120	41.4±7.86	25.53±1.07	27.38±2.48
N	43.5	27.55±5.23	24.13±1.30
Shoot Length			
Control	11.3±1.62	9.37±1.13	12.8±0.64
T14	22.85±8.75	14.78±0.59	10.94±1.00
T15	7.83±1.00	9.3±0.52	16.3±1.60
T16	10±0.2	9.2±0.63	10.96±0.68
T17	9.7±0.98	9.38±0.45	12.65±1.85
T27	6.4	10.46±0.29	19.72±1.55
T29	No germination	8.53±0.81	14.84±1.95
T93	9.05±1.08	10.18±0.89	13.66±1.05
T101b	6.33±0.94	7.7±2	12.7±0.43
T110	10.8±1.3	10.2±1.48	10.92±2.02
T114	9.7±0.6	9±0.54	11.12±0.73
T120	11.24±1.17	11.35±1.19	16.3±2.14
N	10.6	9.45±0.03	14.19±0.72

*values after ± represents standard error of mean;

**Treatment = Seed treatment with different isolates of *Trichoderma* spp.

Table 4.11 Species of *Trichoderma* isolates which induced highest root and shoot length on different crops following seed treatment

Sr. No.	Crops	Root length	Shoot length
1	Cucumber	T14	T14
2	Bottle gourd	control	T14
3	Bitter gourd	T101b	T27

Table 4.12 Estimation of chlorophyll (Chl. A, b and Total) content in Cucumber,Bottle gourd and Bitter gourd plants derived following seed treatment with different species of *Trichoderma*

Species of *Trichoderma* isolate(s)	Chlorophyll content (mg/g)								
	a	b	Total	a	b	Total	a	b	Total
	Cucumber			Bottle gourd			Bitter gourd		
Trichoderma virens									
N	3.65	2.20	5.81	8.81	4.16	12.95	9.46	4.29	13.84
T101b	7.94	3.87	11.84	10.01	4.64	14.71	7.94	3.87	11.56
Trichoderma viride									
T17	7.21	4.59	11.56	7.02	3.57	10.59	8.10	3.90	11.98
T14	12.71	11.86	29.33	14.74	7.25	22.32	10.34	4.82	15.35
Trichoderma harzianum									
T15	8.09	4.05	12.29	10.37	4.79	15.40	9.19	4.39	13.65
T16	6.43	3.46	9.67	10.91	4.83	15.85	12.88	5.44	18.35
Trichoderma aureoviride									
T27	8.99	4.25	13.68	8.15	3.94	12.07	9.00	4.15	13.23
T29	No germination			9.34	4.33	13.79	9.61	4.52	14.07
T114	5.68	2.92	8.62	8.35	4.11	12.54	7.42	3.66	11.04
T120	4.28	2.59	6.76	11.13	3.17	13.98	12.10	5.66	17.82
Section Pachybasium									
T93	5.60	2.97	8.53	7.60	3.88	11.51	11.39	5.36	16.79
T110	5.36	2.84	8.15	8.23	4.24	12.40	5.97	3.28	9.15
CONTROL	5.96	3.09	7.65	8.29	3.91	12.26	5.51	4.78	13.65
Minimum	**3.65**	**2.20**	**5.81**	**7.02**	**3.17**	**10.59**	**5.51**	**3.28**	**9.15**
Maximum	**12.71**	**11.86**	**29.33**	**14.74**	**7.25**	**22.32**	**12.88**	**5.66**	**18.35**
Average	**6.30**	**3.75**	**10.30**	**9.46**	**4.37**	**13.87**	**9.15**	**4.47**	**13.88**