Studies on endophytism of *Trichoderma* species in rice against *Rhizoctonia solani*

राइज़ोक्टोनिया सोलेनाई के विरूद्ध धान में *ट्रायकोडर्मा* प्रजातियों की अंतःपादपता का अध्ययन

VERNA COLETTE LEON

वरना कोलेट लेयोन

Division of Plant Pathology
ICAR-Indian Agricultural Research Institute
New Delhi-110012
2015

STUDIES ON ENDOPHYTISM OF *TRICHODERMA* SPECIES IN RICE AGAINST *RHIZOCTONIA SOLANI*

A Thesis

By

VERNA COLETTE LEON

Submitted to the Faculty of the Post-Graduate School,

ICAR- Indian Agricultural Research Institute, New Delhi,

In partial fulfillment of the requirement

For the award of the degree of

MASTER OF SCIENCE

IN

PLANT PATHOLOGY

2015

Approved by:

Chairman: _____
Dr. Pratibha Sharma

Co-Chairman: _____
Dr. Aundy Kumar

Members: _____
Dr. Ashok Kumar Singh

Division of Plant Pathology
ICAR-Indian Agricultural Research Institute
New Delhi –110 012

Dr. Pratibha Sharma
Professor
Psharma032003@yahoo.co.in

CERTIFICATE

This is to certify that the thesis entitled "**Studies on endophytism of *Trichoderma* species in rice against *Rhizoctonia solani***" submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the degree of **Master of Science** in **Plant Pathology** is a record of *bona-fide* research work carried out by **Miss. VERNA COLETTE LEON** under my guidance and supervision. No part of this thesis has been submitted for any other Degree or Diploma.

I further certify that any help or information received during the work on this thesis has been duly acknowledged.

(Pratibha Sharma)
Chairman
Advisory Committee

Place: New Delhi
Date: 05.09.2015

ACKNOWLEDGEMENT

First and foremost i want to thank ***GOD*** *for being with me in this challenging journey to pursue a master's degree in plant pathology. I know that he has been with me all along to give me strength in everything that i had to do .I will be forever grateful to him .*

I wish to express my gratefulness to my country, the ***Republic of Seychelles****, to nurture me into the person i am today. I will be forever a loyal citizen of my country to ensure that we, as a small island state, be a proud and determined nation to move forward to success. I also want to thank my employer,* ***Seychelles Agricultural Agency****, for allowing me educational leave for me to be able to pursue this degree in India. This master's degree will surely help in the capacity building in the agricultural sector for the country in general.*

It has been an honour to work under the competent guidance of my chairman, ***Dr. Pratibha Sharma,*** *Professor & Principal Scientist, Division of Plant Pathology. I am intrigue for her passion she shows for her work and dedication towards this division. Her encouragement during this study and preparation of my thesis has been well appreciated and cherished.*

I am much obliged to my co-chairman ***Dr. Aundy Kumar****, Principal scientist, Division of Plant Pathology, for his guidance and encouragement and most of all his interest in the success of this research work .I am grateful to member of my advisory committee,* ***Dr. Ashok Kumar Singh****, Head & Principal Scientist,Division of Genetics for his kind suggestions and assistance and providing me with inputs to carry out this study .*

I want to express my gratitude and thanks to ***Dr Rashmi Aggarwal*** *Head, National Fellow, and Principal Scientist Division of Plant Pathology for her encouragement and support during my studies.*

My sincere thanks also go to all the principal scientists and scientists who have assisted me in one way or another for this research programme. My sincere thanks go to ***Dr (Mrs.) Bishnu Maya Scientist****,* ***Dr G. Prakash Scientist.***

I will be forever thankful for all the Senior Research Fellow and technical staff of fungal pathology lab No 26, Division of Plant Pathology, for all the help and assistance they gave for this research. My sincere thanks go to ***Ms.Swati Deep (SRF), Mr. Prabhakaran, N. (RA), Mr. Akash Kumar, Mr. Amit Kumar, Mr. Ranjit Kumar, Mr. Mukesh Kumar, Ms. Shaily Javeria and Ms.Jaya Kumain****.*

My deepest gratitude to ***Raja, M.*** *Senior Research Fellow, IARI, New Delhi for his sincere help and devotions in helping me towards this study. He has been the definition of a helpful and good colleague who did not hesitate not only once to be of any assistance from the bottom of my heart I want to thank him and I wish him success in his future endeavors.*

Words are not enough to express my sincere thanks to my senior ***Thava Prakasa Pandian****,* ***R.*** *for his valuable encouragement and support as well as any assistance during my research work and course work.*

My indebted thanks go also to my juniors, ***Veena, Ramya, Sunil, and Rubin*** *for being approachable and available to help in times of difficulty. I have found in them a friend which i will never forget.*

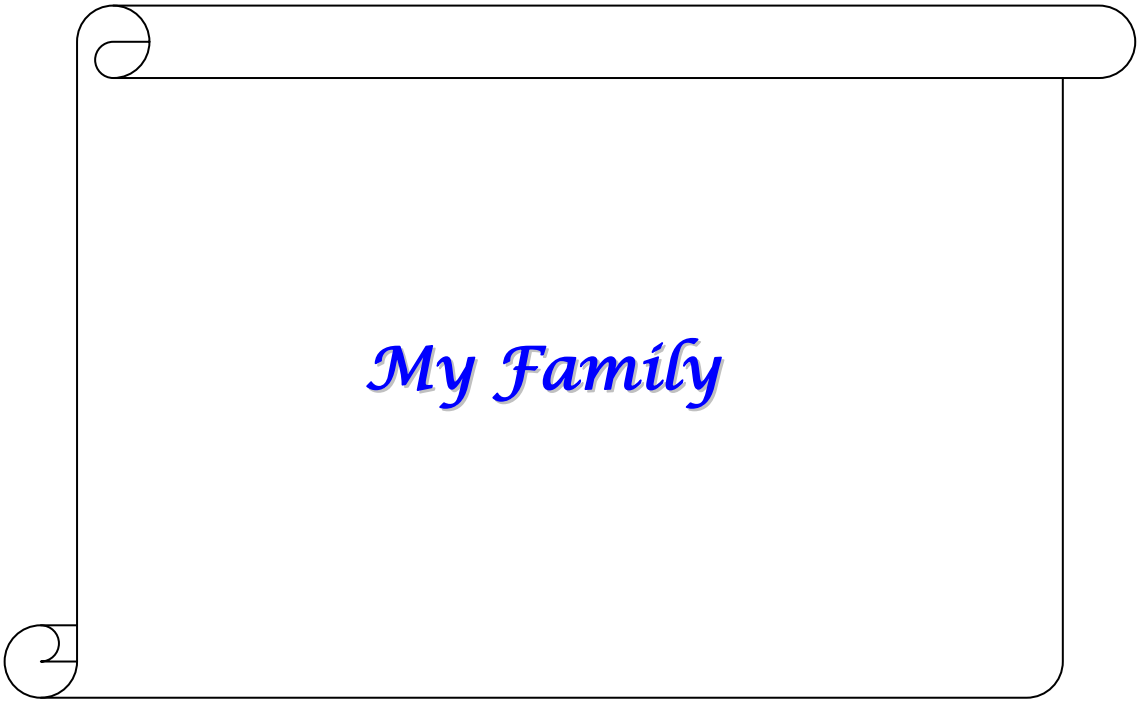
Even though we have been far apart ***my parents*** *have always been in my thoughts and heart .This journey had made me realise how much i love them dearly. It has been equally hard to be without my sister Elsa. It was their encouragement that helped me in times of loneliness to gain courage and persevere.*

Special acknowledgement to ICAR for providing financial assistance during the course of my study. I thank the ***Post-Graduate School, ICAR-IARI*** *for giving an excellent opportunity to learn from this prestigious institute.*

Place: IARI, New Delhi

Date: 05.09.2015 *(Verna Colette Leon)*

Dedicated To



CONTENTS

LIST OF FIGURES

4.8	Phylogenetic relationships of two different *Trichoderma* spp. inferred by analysis of *Tef1* gene sequences. The Maximum Likelihood tree was constructed using two-parameter model implemented in the MEGA 6.06 program.	**25**
4.9	Light microscopy image showing mycelium inside the Pusa Basmasti 1 rice roots soil treated with *Trichoderma* isolates: (A) TaR1 (B) TaR2 (C) TaR3 (D) TaR4 (E) TaR5 at 40X magnification.	**25**
4.10	Light microscopy image showing mycelium inside the Pusa Basmasti 1 rice roots seed treated with *Trichoderma* isolates: (A) TaR1 (B) TaR2 (C) TaR3 (D) TaR4 (E) TaR5 at 40X magnification.	**25**
4.11	(a) Three days old cultures of re-isolated endophytes from the *Trichoderma* treated Pusa Basmati 1 rice roots on PDA plates: (A) TaR1 (B) TaR2 (C) TaR3 (D) TaR4 (E) TaR5; (b) Three days old control plate showing mycelial growth isolated from *Trichoderma* untreated Pusa Basmati 1 rice roots.	**25**
4.12	(a) Seven days old cultures of re-isolated endophytes from the *Trichoderma* treated Pusa Basmati 1 rice roots on PDA plates: (A) TaR1 (B) TaR2 (C) TaR3 (D) TaR4 (E) TaR5; (b) Seven days old control plate showing mycelial growth isolated from *Trichoderma* untreated Pusa Basmati 1 rice roots.	**25**
4.13	SEM images showing conidiophores, phialides and conidial structures of three days old *Trichoderma asperellum* (A) TaR1 (B) TaR2 (C) TaR3 and *Trichoderma asperelloides* isolates (D) TaR4 (E) TaR5 re-isolated from soil treated Pusa Basmati 1 rice roots.	**25**
4.14	SEM images showing conidiophores, phialides and conidial structures of three days old *Trichoderma asperellum* (A) TaR1 (B) TaR2 (C) TaR3 and *Trichoderma asperelloides* isolates (D) TaR4 (E) TaR5 re-isolated from seed treated Pusa Basmati 1 rice roots.	**25**
4.15	ITS region amplification of re-isolated *Trichoderma* isolates from soil treated Pusa Basmati 1 rice roots using primer pair ITS1 and ITS4. M: 1Kb DNA ladder; Lane 1: TaR1; Lane 2: TaR2; Lane 3:	**25**

LIST OF TABLES

CHAPTER 1 INTRODUCTION

History of plant diseases dates back in ancient period. They are the prime most enemies of crop plants which are known to cause severe losses to agriculture and humans. Few diseases like the Irish famine caused by late blight of potato (*Phytophthora infestans*) in Ireland (1845),American chestnut blight (*Cryphonectria parasitica*) in USA (1904), southern corn leaf blight (*Cochliobolus maydis*, anamorph *Bipolaris maydis)* in USA(1969-70),Coffee rust (*Hemileia vastatrix*) in Srilanka (1870) and the great Bengal famine (*Helminthosporium oryzae*) in India (1942) has shattered the world by their severe form of epidemics. In present day agriculture, heavy use of chemicals in plant disease management leads us to face several setbacks; since we are in need of an alternative management strategy. In such scenario, biocontrol agents are the best option available with us. Biological control is the present prospective area in agriculture. It is the use of microorganisms to promote plant growth and to protect the plant hosts from pests and diseases. Biocontrol agents are antagonistic microorganism used against plant pathogens. They employ a series of mode of action *viz.,* competition for nutrients and space, production of cell wall degrading enzymes, production of volatile metabolites and most predominantly mycoparasitism (Singh, 2005; Sharma *et al,* 2014).

Microorganisms that colonize internal tissues of plants are known as endophytes. Endophyte is the new area where scientists are exploring with great interest due to the importance they play in the ecosystem. The major interest with endophytes is that they produce active secondary metabolites which have antimicrobial properties (Stone *et al.,* 2004).Secondary metabolites produced whether in the stems, leaves and roots of plants are mainly used for their survival purpose against abiotic and biotic stress. It helps in water preservation, disease resistance and improved quantity of biomass (Rodriguez *et al* 2009).Endophytes occupy ecological niches in the living internal tissues of their hosts without any adverse effect to the host. Fungal endophytes have been identified in stem, leaves and roots of monocots and dicots (Patel *et al.,* 2013).

Endophytes have now been isolated in various geographic and climatic zones. They have a broader host range. It is thought that endophytes enter the plant tissue through the root zone, flower, leaf and cotyledons. They can be obligate or facultative in

nutrition. Obligate endophytes are defined as being dependent on the host plant for their growth and survival. They undergo vertical transmission to other plants via vectors. Facultative endophytes can be epiphytes at some point of their life cycle (Aishwarya *et al*., 2014).

The fungal genus *Trichoderma* (Teleomorph: *Hypocrea*) is antagonist to various plant pathogens(Akrami,2012; Cotxarrera *et al,*2002; Trillas *et al,*2006; Anees *et al,* 2010). It occupies the rhizosphere in abundance. It can be isolated easily and grows fast on a wide variety of substrate. Also it is known to produce various antibiotic substances *viz.,* Viridins, Trichodermins etc. It is effective in growth promotion and known to have tolerance to abiotic stresses. Strains such as *Trichoderma harzianum* have been seen to efficiently control *Rhizoctonia solani* in rice, the causal agent of sheath blight of rice (Silva *et al*, 2012). Many studies have postulated that *Trichoderma* is believed to occur as endophytes (Chaverri *et al,* 2011; Hanada *et al*, 2008; Bae *et al*, 2009; Mulaw *et al*, 2013; Yuan *et al*, 2008).

Even though few species of *Trichoderma* have been isolated as endophytes, it might be that the other species can be facultative endophytes. *Trichoderma hamatum* for instance are endophytes as well as common inhabitants of soil and rhizosphere. Now, the question arises that whether there are any obligate endophytic *Trichoderma* spp. is available or not. The relationship between endophytic *Trichoderma* spp. and the plant is yet being investigated to understand whether there is some potential fungal prey and plant root-derived nutrients that encourage the internal colonizing of plant roots or whether presence of certain proteases, chitinases and secondary metabolites is playing a role in endophytism (Druzhinina *et al.*, 2011). In depth studies of *Trichoderma* as an endophytes and its importance in antagonistic nature against *R. solani* in rice plants has not been investigated so far.

Rice (*Oryza sativa* L.) in India is the major staple crop similar to other countries over the world. In 2007 the global rice production reached at 645 million tonnes. It is known to be grown in 114 countries with China and India achieving the largest production. Brazil, Thailand, Vietnam are examples of other producing countries (Kumar *et al.,* 2009).

Sheath blight caused by *Rhizoctonia solani* is one of the most important diseases of rice. Sheath blight of rice in India is known to cause losses of about 54.3 per cent. It is not a problem only in India but all over the world (Bhuvaneswari and Ragu, 2012). Sheath blight of rice was first reported from Japan in 1910. It is caused by a fungus called *R. solani*. In India, Tamil Nadu state was the first to report the disease. The disease is seed and soil borne in nature. The fungus *R. solani* once in contact with rice plant will germinate and infect the plant. The fungus penetrates the plant through the cuticle and stomata. Eventually the infection peg and appressoria will be formed and mycelium will grow from the outer surface of the sheath and then through the inner surface. Lesions are initiated during mycelia development on the surface and inside the tissue. Secondary lesions are formed eventually as primary lesions proceeds upwards and laterally (Usharani *et al.,* 2013).

Since biocontrol agents are widely used in disease management programmes, there is a need to decipher its mechanisms employed in biological control. Many workers have worked on different aspects of biological control. The ability to manage the plant diseases once applied through seed or soil treatment makes it more interesting to know about the mechanisms lying inside. First and foremost, we need to understand the endophytic nature of the *Trichoderma* in seed and soil treatment and the role of endophytic *Trichoderma* in controlling sheath blight disease.An outlook on mechanism of colonization in *Trichoderma* can help scientist to better understand and analyse the different management practises in disease management of *R. solani*. Keeping these points in view, the present study has been proposed as "**Studies on endophytism of *Trichoderma* species in Rice against *Rhizoctonia solani*"** with the following objectives

- To investigate endophytism of *Trichoderma* spp. in Rice.
- To understand the role of endophytic *Trichoderma* spp. against sheath blight of rice caused by *R. Solani*

2. 1 Introduction to Endophytes

The term ‘epiphyte’ was introduced by Anton de Bary for fungi that live on the surface of their host whereas endophytes are fungus living inside the tissue of plants.The word endophyte came from two Greek words, "endon" meaning within and "phyton" meaning plant. Endophytes can therefore be defined as plant-associated microorganisms that form association with their host plants, colonizing the internal tissues thus making them valuable for agriculture where crop performance is concerned (Dutta *et al*., 2014).Petrini (1991) defined endophytes as “All organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host”.

Rodriguez*et al*., (2009) pointed out that endophytes differ from mycorrhizae in that they occupy niches entirely within plant tissues and are also present in roots, stems and leaves whereas mycorrhizae are inside colonizers which also occupy the rhizosphere. Endophytes are not only fungi but bacteria as well, such as actinomycetes, which spend the whole or part of their life cycle colonizing inter- or intra- cellularly, inside the healthy living tissues of the host, without any noticeable symptom of disease (Stone *et al*., 2004).

2.1.2 Endophytes as benefial microorganism

Most of the endophytes produce secondary metabolites which are antibiotics having antifungal, antibacterial and insecticidal properties. These act by inhibiting the growth of plant pathogens. Enzymes such as β-1, 3- glucanases and chitinases hydrolyze the plant cell wall and also suppress the plant pathogen activities directly by degrading the cell wall of pathogenic fungi and Oomycetes (Dutta *et al.,* 2014).In a study done by Khan *et al*. (2012), the role of the endophytic fungi were studied in plant growth promoting activity of roots in extreme sand dune environment of coastal regions. The plant height and shoot length of the cultivar *Waito-C* rice was seen to be enhanced by endophytic fungi. This is partly due to the metabolites they produce which are helpful in plant growth and development.Fungal endophytes induces systemic resistance (ISR).It

involves jasmonic acid or ethylene and is not linked with the PR proteins (Dutta *et al.,* 2014).

To have an endophytic niche inside the host plant implies that the microorganism has to compete for space and nutrients, which involve the process of hyperparasites and predation, between the endophyte and plant pathogen. Endophytes usually leave no space for the pathogens to occupy therefore inhibiting the pathogen infection in the plant. Once the endophytes have obtained an ecological niche in the plant they are ensured a reliable source of nutrition from the plant's exudates and leachates and in return the endophytes protect the host against other microorganisms. They are thought to be rapid colonizers and thereby exhausting the limited available substrates for the pathogens to grow. To limit the growth of endophytes when they colonize the plant, lignin is produced along with other cell-wall deposits; this causes endophytes to be virulent. As a result, the cell wall becomes re-reinforced after endophytic colonization, and thus it becomes difficult for pathogens to infest. In hyperparasitism, the pathogen is directly attacked by a specific endophyte that kills it or its propagules. Fungal endophytes parasitize around the hyphae of pathogens by various means such as coiling, twisting, penetrating the hyphae of pathogens and secreting lyase to decompose the cell wall of pathogens. Microbial predation involves the use of enzymes used against the cell walls of fungi to utilize the fragment of pathogens. Thus the major advantages of endophytism to the host plants include the fact that they gain greater access to the nutrients, they are protected from desiccation and from the surface feeding insects and some parasitic fungi (Dutta *et al*., 2014).

Endophytes are known to be anti microbials in that they have the ability to kill or inhibit the growth of microorganisms. Narisawa *et al*. (2000) was able to find that the root endophytic hyphomycete, *Heteroconium chaetospira*, suppressed *Verticillium* sp. in Chinese cabbage in the field. *Verticillium* wilt is one of the most destructive diseasesof aubergine. Endophytic fungal isolates Papochf01 *(Trichocladium* sp.), Papochf02 (*Neonectria* sp.), Papochf03 (Unidentified), Papochf06 (Unidentified) and Papochf08 *(Setophoma* sp.) from the healthy rhizomes of *Paris polyphylla* var. *chinensis* (Trilliaceae), in central China was found to inhibit bacteria such as *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas lachrymans*,

Staphylococcus aureus, *Staphylococcus haemolyticus*, *Salmonella typhimurium*, and *Xanthomonas vesicatoria*(Shan *et al*., 2012).Lehtonen *et al*. (2006), when releasing the viruliferous aphid vectors to endophyte-infected andendophyte-free red clover *Trifolium pretense L* plants in a common garden, found the number of aphids and the percentage of BYDV infections were lower in endophyte-infected plants compared to endophyte-free plants.

2.1.3 Emerging prospective of endophytic Fungi

Endophytic fungi have some important roles in nutrient cycling, biodegradation and bioremediation. Endophytes are involved in nutrient pedaling where dead biomasses gets degraded and is returned back to the environment. Endophytes can also be used to remove contaminants and wastes from the atmosphere. Apart from the clear fact that they help in uptake of essential nutrients necessary for plant growth it is also known that they help in anchorage of plant in soil, absorption of water and ions, nutrient storage. Furthermore endophytes can be anti cancerous.The anticancer agent cajanol is produced by *Hypocrea lixii* from *Cajanus cajan*(Mishra *et al*., 2014). A study done by Kumaresan and Suryanarayanan (2002) showed that a few fungi that existed as endophytes in low frequencies in living leaves appeared in higher frequencies after leaf fall. Such growth activity of these endophytes and their capacity to produce certain enzymes are indicative of their potential role in litter degradation.

2.1.4 Methods of isolating endophytes

Fungal isolation is usually done through surface sterilization of small plant tissues and putting them on PDA plates (Hallmann *et al.,*2006; Zhang *et al.,* 2006; Schulz *et al.,* 1993). It is seen as the most practical method to be used. However the investigator needs to take into consideration the procedures to follow for sampling, the media composition and know about the physiology of the fungus (Stone *et al*., 2004). According to Zhang*et al*. (2006) the isolation of endophytes requires 3–5 mm long segments of plant tissue which are surface sterilized usually with 70 per cent ethanol and /or other disinfectants such as 1 per cent sodium hypochlorite. It is then washed with sterile distilled water before drying and plating them on PDA. The plates are incubated for 2-4 weeks at 28±2^{o}C. Hyphal tips which appear after several days should be re-inoculated on new PDA plates. However, Hyde and Soytong (2008), argued that this technique usually lack

sensitivity and not specific. It takes time and the result is difficult for interpretation. Furthermore it was explained that some endophytes may require more specific and elaborate media for isolation. This will limit the type of endophytes isolated.

2.1.5 Identification of endophytes

When the fungus is grown it can be identified by microscopic visualization for specific colony, branching and mycelium morphology (Zakaria *et al.*,2010; Khan *et al.*,2012).Molecular techniques are used to prevent bias results (Sun and Guo, 2012). DNA sequence analysis is used more frequently to identify fungal endophytes (Mulaw *et al.*, 2013; Macia Vicente *et al*., 2008; Rinu *et al.*, 2013). This is done by the use of polymerase chain reaction (PCR) for internal transcribed spacer (ITS) region amplification. ITS region is more frequently used since it is a highly variable region. It is a region which evolves fast and varies among species within a genus thus it is used to identify closely related species. With the aid of bioinformatics software, the DNA sequences can be read. Basic Local Alignment Search Tool (BLAST) is a bioinformatics tool for finding regions of local similarity between sequences, as well as explores evolutionary relationships between species (Jeewon *et al*., 2013). Further to this, the use of 18S and 28S genes has been used to identify endophytes (Morakotkarn *et al*., 2007). Another important molecular technique is GUS transformation. In a study done by Chatterton *et al.* (2008) the fungal biomass and extent of colonization of the endphytic fungus *Clonostachys rosea f. catenulate* could be investigated by the quantification of GUS activity.

2.1.6 Root endophytes

Root endophytes are nonmycorrhizal microfungi which are found in the roots together with mycorrhizae. Root endophytes have been found more often in forest trees (Stone *et al*.,2004). In a study done by Macia Vicente *et al.* (2008), on fungal endophytes in roots in 24 plant species in the Alicante province, Spain, it was found that the species of *Fusarium* and *Phoma*were most frequently isolated. Other genera include *Aspergillus, Alternaria* and *Acremonium*. The type of fungus isolated depended on soil type. Amin(2013), isolated fungal endophytes from roots of maize plant var. Pulut in Indonesia. The fungal endophytes isolates were: *Trichoderma*sp., *Fusarium* sp., *Acremonium* sp., *Aspergillus* sp., *Penicillium* sp., and *Botryodiplodia* sp. In China

fungal root endophytes in the medicinal plant Panax ginseng were found to be *Nectria, Aspergillus, Fusarium, Verticillium, Engyodontium, Plectosphaerella, Penicillium, Cladosporium,* and Ascomycete (Wu *et al*., 2013).

2.1.7 Work on Fungal endophytes in India

There is the notion in the present day that endophytic microbes can be of potential importance to humans. This involves the protection of agriculture and plant health and its use in medicine industries. There is a need for antimicrobial compounds in India to deal with alternative means of disease control in farms. Endophytic fungi are potential solutions as antimicrobials compounds. India must embrace this new area of research for its own benefit as a developing country which could at least facilitate some of the existing problems of its huge population (Gond *et al*., 2010). Several papers were published on the biodiversity and distribution patterns of fungal endophytes in various plants in India. Suryanarayanan and Vijaykrishna (2001) from Chennai investigated the diversity of endophytes isolated from leaf tissues and aerial roots of *Ficus benghalensis (Moraceae).* Endophytes were seen from the aerial roots entering the soil. It was concluded that the type of host tissue and the environment play a role in the type of endophyte present throughout the plant. Nalini *et al.* (2014) investigated the quantity of endophytic fungi in seven plant species during winter (November/December) and monsoon (May/June) in Western Ghats. The endophytic fungus was isolated from inflorescence, rhizome, root, and stem. The fungal endophytes included *Fusarium, Acremonium, Colletotrichum, Chaetomium, Myrothecium, Phomopsis,* and *Pestalotiopsis* spp. It was found out that the diversity of fungal endophytes was more in monsoon than in winter. An endophytic fungus was isolated from the lateral roots of lentil (*Lensesculenta* Moench), growing under mountain ecosystem of Indian Himalayan Region (IHR).The fungus identified as *Trichoderma gamsii*, was found to be positive for production of ammonia and salicylic acid, phosphate solubilization, and chitinase activity (Rinu *et al*., 2013). Recently Sandhu *et al*. (2014) reported on the diversity of fungal endophytes in *Calotropis procera* (Linn.). It was an investigationon the colonization frequency of fungal endophytes and also its antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonae, Bacillus subtilis and Staphylococcus epidermidis.* It was found that fungal colonization frequency was equal

in leaves, stems and roots and that *Fusarium solani, Cladosporium herbarum, Curvularia pallescens, Alternaria alternata* and*Drechslera nodulosa* as fungal endophytes, provided broad spectrum antibacterial activity. Raviraja *et al.*(1996) isolated endophytic aquatic hyphomycetes (*Tetracladium furcatum,Triscelophorus acuminatus, T. konajensis* and *T. monosporus*) from coffee plants, rubber plants and ferns near streams in India.

2.2.1*Trichoderma*

Trichoderma (teleomorph: *Hypocrea*, Ascomycota, Dikarya) are the most popular and effective biocontrol control agents in the present day**.** It has been postulated that they have high antagonistic activity against plant pathogens(Mulaw *et al*., 2013). It was Persoon in 1794 that proposed the genus *Trichoderma.* Hewas the first to propose *T. viride, T. nigroscens, T.aureum* and *T. roseum.* In India, it was first time isolated by Thakur and Norris during the year 1928 from Madras (Pandya *et al*., 2011).The potential value of the genus *Trichoderma* as bioagents was first reported by Weindling in 1932.*Trichoderma* Species are diverse. It is found in temperate and tropical soils, as well as in agriculture, forest, prairie, salt marsh and desert soils in all agro-climatic zones. It colonizes decaying wood, decaying bark, roots, litter, and other plant materials at different climatic zones (Pandya *et al*., 2011).*Trichoderma* have high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms. Thus it can be found in any habitat and at high population densities. It usually thrives best in acidic soils (Benitez *et al*., 2004).

Trichoderma uses vary. It manages seed and soil borne pathogens. They can also be used as effluent treatment, degradation of organ chlorine pesticides fabric detergent, animal feed production, fuel production and alternative to conventional bleaching. It suppresses plant parasitic nematodes and there is no issue of resistance by the plant pathogen. It is an eco-friendly control practice which enhances plant growth, induce systemic resistance, solubilization of phosphates and micronutrients and increase soil fertility. Its use can lead to few crop losses, reduces cost of production, increases yield, quality and profit. However it causes damage to mushroom. Because of such limitations

of using only one control measure in isolation, biological based Integrated Disease Management(BIDM) is of great importance today. *Trichoderma* spp. has also the potential to be used in food additives. Enzymes such as β-glucanases from *Trichoderma* are being used in brewing process. Other examples of other enzymes are pectinases, cellulases, Hemicellulases used in fruit juices. Cellulases are used in the production of alcohol and baking. Cell wall-degrading enzymes of *T. harzianum* are being used as food preservatives. Furthermore *T. harzianum* mutanase is being used in toothpaste due to its anti-microbial properties (Schuster and Schmoll, 2010).

2.2.2 Mechanism of action

Trichoderma is foundin root, soil and foliar environments where they control plant diseases**.** *Trichoderma* as biocontrol agent can also be used as biofertilizer because of its ability to establish mycorrhizae-like association with plants. Major mechanisms of *Trichoderma* spp. are competition for space and nutrients, production of diffusible and/or volatile antibiotics and hydrolytic enzymes like chitinase, xylanase and β-1,3-glucanase. Hydrolytic enzymes will degrade the pathogen cell wall and prevent the pathogen's growth. Mycoparasitism is an effective process where *Trichoderma* coils around the pathogen and degrade cell wall of the pathogen. Induce systemic resistance occurs when Specific strains of *Trichoderma* spp. colonize and penetrate plant root tissues and initiate a series of morphological and biochemical changes in the plant. It is considered to be part of the plant defense response. Competition by *Trichoderma* spp. includes exudates from seeds that stimulate the germination of propagules of plant-pathogenic fungi in soil and also compete for nutrients and/or space (Saba *et al.,* 2012). *Trichoderma* spp. has also been identified for the potential to limit yield losses due to Witches Broom caused by phytoplasma (Bailey *et al*., 2009). It is thought that *Trichoderma* spp. can also be opportunistic plant symbionts. Mitogen Activated Protein kinase (MAPK) in the plant as well in *Trichoderma* is required for complete induction of systemic response in plant tissues (Schuster and Schmoll, 2010).

2.2.3 *Trichoderma* as endophytes

Several of *Trichoderma* taxa have been known to be endophytes particularly in the tropical arboreous plants. Chaverri *et al*. (2011) described a new endophytic species of *Trichoderma* named *T. amazonicum*, on sapwood and leaves of *Hevea* sp. It was seen

that it was closely related to the species *T. harzianum*. A new endophyte species *Trichoderma martiale* isolated from sapwood of *Theobroma cacao* (cacao, Malvaceae) in Brazil was found to be a close relative of *T. viride* (Hanada *et al*. 2008). In a study done by Bae *et al.* (2009) the endophytic fungus *Trichoderma hamatum* isolate DIS 219b in cacao was found to delay changes induced by drought and wilt incidence was slower. This fungus was seen to promote growth of roots even in water deficiency. A novel endophytic *Trichoderma* sp. *T. flagellatum* was isolated from coffee plant in Ethiopia. This species was found to successfully antagonized *Fusarium*sp., causing coffee tracheomycosis (Mulaw *et al*., 2013). *Trichoderma chlorosporum* an endophytic fungus was isolatedfrom *Dendrobiumnobile* roots was recorded as a new species in China. This species belonged to the VIII Chlorospora Clade (Yuan *et al*., 2008). According to Samuels (2006) *Trichoderma harzianum* has been seen to be the most common endophytes isolated from stems of *Theobroma* species.

2.2.4 *Trichoderma asperellum* as Biocontrol agents

In a study done by De los Santos-Villalobos *et al*. (2013) the strain T8a of *T. asperellum* was found to control anthracnose through cellulase activity in mango trees. Segarra *et al*.(2010) found that the strain T34 of *T. asperellum* is a useful biological alternative to chemicals for the control of *Phytophtora capsici* in pepper.In spain *Trichoderma asperellum* strain T34 which wasisolated from *Fusarium* suppressive compost was found to suppress Fusarium wilt (*Fusarium oxysporum* f. sp. *dianthi)* on carnations. This strain has been commercialized as T34 Biocontrol® (Sant *et al*.,2010). Furthermore Fusarium wilt (*F. oxysporum* f. sp. *Lycopersici)* in tomato plants was successfully controlled by *Trichoderma asperellum*when applied to the roots (Cotxarrera *etal*., 2002). Trillas *et al*. (2006) demonstrated that damping off caused by *Rhizoctonia solani* in cucumber was controlled by using comosts composed of *T. asperellum* strain T-34. De franca *et al.* (2014) showed that Mixed isolates of *T. asperellum* for the first time were able to reduce disease incidence of sheath blight and increase grain and yield of the rice cultivar BRS tropical.

2.2.5 *Trichoderma asperellum* as endophytes

Recently Rosmana *et al*. (2015) investigated the potential of endophytic *Trichoderma asperellum* isolates as potential control of vascular streak dieback in cacao. The isolates

were successfully isolated from *Theobroma cacoa*. The isolates were re-inoculated in cacao seedlings through the roots and they were recovered in roots and stems after one month. Due to its effectiveness in suppressing Vascular streak dieback it is seen as an alternative to chemical control.

2.2.6 *Trichoderma asperelloides*

Trichoderma asperelloides is a closely related species to *Trichoderma asperellum.* Morphologically it is difficult to distinguish *Trichoderma asperelloides* from *T. asperellum.* Molecular identification such as Internal Transcribed Spacer (ITS 1 and ITS 2) as well as Translation Elongation Factor 1 alpha gene (*TEF*1) has been preferably used to differentiate between the two species (Devi *et al*., 2012).Up to date no teleomorph of the fungus is known.(Samuel *et al*., 2010)Studies have been done to explore the roles of *Trichoderma asperelloides* in plant resistance to abiotic and biotic stresses. Brotman *et al*., (2013) have shown that *T. asperelloides* (T203) assists in salt tolerance in plants. Gupta *et al*. (2014) showed that *T. asperelloides* is able to suppress Nitric Oxide produced by *F. oxysporium* in Arabidopsis roots. In a study done by Doley *et al*. (2014) showed that when *Arbuscular mycorrhizal* was used in combination with *Trichoderma asperelloides* it significantly reduced the activity of *Sclerotium rolfsii* in the roots of Groundnut. Very few studies have been done to explore *Trichoderma asperelloides* as endophytes at present.

2.3.1 Sheath blight disease in rice

Rice (*Oryza sativa* L.) is the most important staple food for hundreds of millions in Africa and Latin America and over two billion people in Asia. Annual rice production must be increased from the present 560 to 750 million tons by 2020 to feed the ever increasing population of the world. The future increase in rice production has to come from the same or even reduced land area and the productivity yield must be greatly enhanced by providing additional nutrient input and through effective control of phytopathogens. Sheath blight disease of rice occurs in most countries in Asia caused by *Rhizoctonia solani*. It was first described from Japan in 1910 (Usharani *et al.,*2013). In India many reports have been made on the occurrence and severity of the disease across different states (Paracer and Chahal, 1963; Kohli 1966; Nair *et al*.,1983; Mathur *et al*.,1999; Chahal,2005).

2.3.2 Causal organism

Rhizoctonia solani Kuhn is the fungus that causes sheath blight in rice. The Perfect stage is *Thanatephorus cucumeris* (Frank) Donk. The hyphae is8-12 µm in diameter and turns from colourless to yellow and brown with maturation. The fungus is characterized by the hyphae branching at right angles. The fungus formscompact mass of hardened fungal mycelium called sclerotia (Palo, 1926).

2.3.3 Symptoms

It was Miyake (1910) that first described the disease symptom of sheath blight of rice. Leaf sheath, leaf blades and panicles of rice are prone to sheath blight disease symptoms. Circular, oblong or ellipsoid, greenish-grey water-soaked spots on leaf sheath are amongst the symptoms seen at the onset of the disease. The spots are greyish white in the centre and the margins are brown (Ou, 1973). Humidity encourages the spread of the disease to the whole length of the leaf sheath. Lesions may coalesce around the culm. Eventually the whole plant rots, dry up and becomes blighted. Sclerotia forms around the lesions (Srinivas *et al*., 2013). Singh *et al*.(1988) has postulated that the plant is most vulnerable at maximum tillering.

2.3.4 Epidemiology

Overwintering of the pathogen is possible through Sclerotial bodies. These sclerotia are sub-globose in shape and change colour from grey white to brown to black and the size is within 0.5-5.0 mm (Palo,1926).As reported by Nandi (1980)the disease is known to be soil borne. Thus it can remain in rice stubbles or attached to alternate hosts in the field. However irrigation water remains the important way of spreading the disease. The optimum temperature and relative humidity is 23-35°C and 96-97 percent respectively. The fungus has good saprophytic survival ability. As shown by their good survival ability on cow dung for several months (Roy, 1979).

2.3.5 Economic importance

In countries such as Sri Lanka, China, Taiwan and Japan, sheath blight disease is a major disease (Chien, 1979). Sheath blight causes considerable damage to rice fields and is prevalent partly because the fungus have a wide hosts range namely green gram, grasses, sugarcane, brinjal water hyacinth beans, tobacco, tomato (Xuebiao *et al.,* 1999; Kohli, 1966; Nayak *et al*., 1979;Kannaiyan and Prasad, 1976; Padwick, 1950).In

Korea and Japan, it was found that the production loss of 0.9 per cent and 0.8 per cent occurred because of sheath blight (IRRI,2001).In India, the disease is prevalent. Chalal *et al*. (2005) reported that grain filling in rice was reduced by 32.3 per cent when diseased top three leaves were 54.3 per cent. Lee and Rush,(1983) reported 20 to 50 percent loss in total infection of sheaths. Hori and Anraku, (1971) reported a 25 per cent yield loss when disease reach to flag leaf. Kozaka(1970) reported 30-40 per cent yield loss of the leaf sheath and leaf blade.

3.1 Experimental Area

The research work was carried out in the net house and growth chamber of Division of Plant Pathology, Indian Council of Agricultural Research-Indian Agricultural Research Institute (ICAR-IARI), New Delhi in the year 2013-2015.

3.2 Equipments

Various lab equipments like inoculation loops, pan, electronic balance, filter papers, centrifuge, blotter papers, forceps, conical flask, test tubes, gel electrophoresis and gel documenetation system, Thermocycler etc. were used.

3.3 Fungal cultures

Trichoderma asperellum isolates (TaR1, TaR2 and TaR3) *and Trichoderma asperelloides* isolates(TaR4 and TaR5)collected from different agro-climatic regions of Rajasthan, India was used in this study. *Rhizoctonia solani* Kuhn. in Pure culture was obtained from Indian Type Culture Collection (ITCC), Division of Plant Pathology, ICAR-IARI, New Delhi. The fungal cultures were maintained at 4°C in a refrigerator on Potato Dextrose Agar (PDA) slants until used.BOD incubator was used to incubate the materials at 28±2° C.

3.4 Source of seeds

Seeds of the Pusa basmati-1 (PB1) were obtained from the Division of Genetics, ICAR-IARI, New Delhi.

3.5 Media

Both liquid and solid media was used. The media ingredients used for culturing *Trichoderma asperellum* isolates*, T. asperelloides* isolates and *Rhizoctonia solani* is provided below. The media constituents for the mass multiplication of *Rhizoctonia solani* have also been provided. All media was sterilized in an autoclave at 15 psi (1.05 kg/cm^2) for 20 minutes before usage.

Potato dextrose agar (PDA) (Riker and Riker, 1936)

Potato	200.0 g
Dextrose	20.0 g
Agar	20.0 g

Distilled water	1000.0 ml
pH	6.0-6.5

Potato dextrose broth (PDB) (Riker and Riker, 1936)

Potato	200.0 g
Dextrose	20.0 g
Distilled water	1000.0 ml
pH	7.0

Typha medium

Peptone	10.0 g
Sucrose	20.0 g
K_2HPO_4	0.1 g
$MgSO_4$	0.1 g
Distilled water	1000.0 ml

***Trichoderma* Selective media**

$MgSO_4$ $7H_2O$	0.2g
K_2HPO_4	0.9 g
KCl	0.150 g
NH_4NO_3	1.0 g
D glucose	3.0 g
Rose Bengal	0.15 g
Distilled water	1000.0 m

3.6 Isolation of endophytic fungi in rice fields

A sum of 15 plants of 28 days old healthy Pusa basmati-1 rice plants were collected from different plots in Division of Genetics, ICAR-IARI, New Delhi. The samples were immediately processed, washed carefully under tap water and leaves, stems and roots were cut into small pieces of 1 cm length. All together 90 segments of plant tissues were used for the isolation of endophytic fungi (30 leaves segments, 30 stem segments and 30 root segments). Surface sterilization was done first in 75 per cent alcohol for 5 minutes followed by 2.5 per cent sodium hypochlorite for 2 minutes.

The plant pieces were washed five times in sterile double distilled water. Sterility checks of the root samples was done by taking 0.1mL from the fifth rinsevand plated out

onto Petri plates of Potato Dextrose Agar and *Trichoderma* specific media for 6 days. The roots were left to dry on sterile blotter paper and plated on potato dextrose agar (PDA). The plates were incubated at 28^{o}C till hyphal tips appear from plant tissues. Pure culture was obtained by sub culturing mycelium emerging from plant tissues. The fungus was identified by their microscopic and macroscopic characters with the help of light microscopy (Zakaria *et al*., 2010;Lakshman *et al*, 2013; Khan *et al.,* 2012). Identification was aided by the practical manual of Mycology by Sharma *et al.* (2014) and the practical manual on Biocontrol of plant diseases by Sharma *et al.* (2013).

3.6.1 Colonization Frequency

Colonization frequency was calculated using the formula below (Petrini *et al*., 1992;Lakshman *et al*, 2013).

$$\text{Colonization Frequency(per cent)} = \frac{\text{Ncol}}{\text{Nt}} \times 100$$

Where,

Ncol = the number of segments colonized by each endophyte

Nt = the total number of segments observed

3.7 Morphological characterization

Morphological identification of *T. asperellum* isolates and *T. asperelloides* isolates was done for the inoculated and re-isolated fungus. The identification was based on the conidia shape, colony appearance, phialides characteristics and branching patterns of conidiophores.

3.8 Preparation of *Trichoderma* inoculum

Treatment with *T. asperellum* isolates and *T. asperelloides* isolates was done by two method *viz* soil inoculation and seed treatment.For soil inoculation the soil was treated with *T. asperellum* and *T. asperelloides* spore suspension with the colony forming unit of 2×10^6 spores/mL each . A disc (5 mm) of *T. asperellum* and *T. asperelloides* was inoculated in 100mL Potato Dextrose Broth (PDB) each one in 250ml flask. The culture flask was kept at 28° C for 5-7 days. The mycelial mat was harvested by using sterile filter cloth. The suspension was used for both soil and seed treatment. Inoculation of *T. asperellum* isolates and *T. asperelloides* isolates in the soil was done five days before

seed sowing. Rice seeds were sown after 5 days of soil treatment of biocontrol agent (Cuevas, 2006).

For seed treatment conidial suspension was used. 3 ml of conidial suspension (cfu of 2×10^6 spores/Ml) was added to 10 grams of seeds in a small plastic container. The container was shaken for uniformity coating (Akrami *et al*., 2012). The seed were kept overnight and the next day it was sown. Seeds for both treatments were sown at the rate of 20 seeds/pots. Control pots were also made.

3.9 Light microscopy

Morphological identification inside the rice roots tissues of *T. asperellum* and *T. asperelloides* was done at 28 days after sowing under light microscope. 28 days old root samples was taken from respective samples and cut into small pieces (0.5-1.0 cm). Root samples were fixed in 50 per cent ethanol for 12 hours. It was further cleared by heating in 5 per cent KOH at 90^{o}C in 5 minutes. Roots samples were washed with tap water and acidified with 0.1N HCl for 5-10 minutes followed by staining with 0.001 per cent acid fuchsin in solution of acid-glycerine-water (87.5 ml lactic acid, 6.3 glycerine and 6.3 ml water) for 1 hour at 55^{o}C. Excess dye amount was removed in 100 per cent glycerine. The root samples were cut in horizontal thin sizes and stained lactophenol cotton blue. The sample were fixed in centre of the slide along with cover slip without any air bubbles and observed under light microscope. *T. asperellum* and *T. asperelloides* were identified on the basis of their morphological conidia, colony texture and shape (Stone *et al*., 2004).

3.10 Isolation of endophytic fungi

Endophytic fungi were isolated from rice roots to investigate endophytism in rice. Roots freshly uprooted were washed under tap water, dried and washed with distilled water. The root was allowed to dry on blotter paper followed by cutting them into approximately 1 cm and then immersed in 70 per cent ethanol (Nalini *et al*., 2014). Surface sterilization process continued by dipping the root pieces in 2.5 per cent sodium hypochlorite for 2 minutes. The roots were rinse five times in sterilized distilled water by picking with sterilized forceps. The plant pieces were washed five times in sterile double distilled water. Sterility checks of the root samples was done by taking 0.1mL

from the fifth rinse and plated out onto Petri plates of Potato Dextrose Agar and *Trichoderma* specific media for 6 days.

Roots were dried on sterilized blotter papers. The roots were crushed in sterile conditions and plated on Potato Dextrose Agar. The petri plates were incubated for 2-3 days at 28 ±2 ^{o}C. After the growth of fungal colony in the sample tissue, colony were transferred to fresh PDA plates for studying morphological characters (Sharma *et al.,* 2013).

3.11 DNA extraction

The *T. asperellum* isolates and *T. asperelloides* isolates were subjected to molecular identification based on DNA sequencing of the ribosomal ITS region (28s rDNA, 18s rDNA, 5.8s rDNA, ITS1 and ITS2*)* and *TEF1* region before inoculating the soil and Pusa Basmati 1 rice seeds through soil and seed treatment. The same procedure was repeated for the re-isolated fungus.DNA was isolated by CTAB method (Selvi and Lakshmi, 2013).

- 0.1g of fungal tissue to be grinded and place in a pestle and mortar was weighed; 4-5 ml of pre-warmed CTAB buffer + 10 µL β-Mercaptoethanol was added and grinded thoroughly.
- It was kept in a water bath at 60^{0}C for 1 hour.
- Following this equal volume of Chloroform was added (Isoamyl alcohol-24:1) to the tube and mixed thoroughly by inverting at least 15 times.
- It has undergone Centrifugation for 20 minutes at 12,000 rpm at 4^{o}C.
- The supernatant was transferred carefully to a new 2.0mL eppendorf tube, and 0.6 volume of 100 per cent Ice cold Isopropanol was added and the tubes were inverted for at least 5 times.
- 0.1 volume of 1 M Sodium acetate, was added (pH 5.2) to the tube and mixed by inverting.
- The tubes were kept in -20^{o}C for 1 hour and centrifuged for 20 minutes at 12,000 rpm at 4^{o}C.
- The supernatant was discarded and 200µL of 70 per cent Ethanol was added to each tube and centrifuged for 5 minutes at 10,000 rpm at 4^{o}C.

- The supernatant was discarded carefully and the pellet was dried at room temperature.
- 50-100µL of TE buffer/MBG water was added to dissolve the pellet.
- The pellet was stored at 4^0C.

3.12 Antagonism test

T. asperellum isolates and *T. asperelloides isolates* were subjected to antagonistic test against *R. solani*. 5 mm diameter mycelial disc from 7 days old culture of *T. asperellum* isolates and *T. asperelloides isolates* as well *R. solani* were placed at equal distance from the periphery on PDA medium in petri plates (60 mm). The petri plates were incubated at 28± 1° C. A control plate of *R. solani* was done simultaneously (Sharma *et al*., 2013).

The inhibition percentage was measured according to Sharma *et al*.(2013),

$$\text{Percentage inhibition (PI)} = \frac{C-T}{C} \times 100$$

Where,

PI = Per cent growth inhibition

C = Colony diameter/radial growth of pathogen in control

T =Colony diameter/radial growth of pathogen in treatment

3.13 Scanning Electron Microscopy (SEM) analysis

The roots were surface sterilized with 75 per cent alcohol for 5 minutes followed by 2.5 per cent sodium hypochlorite for 2 minutes. Sterility checks of the root samples was done by taking 0.1mL from the fifth rinse and plated out onto Petri plates of Potato Dextrose Agar and *Trichoderma* specific media for 6 days. The roots were fixed overnight at room temperature in 4 per cent glutaraldehyde in 0.05 M phosphate buffer (pH7.3). The next day they were washed in phosphate buffer three times for 15 minutes. Samples were dehydrated through 30, 50, 70, 80, 90 and 100 per cent ethanol for 15 minutes. Then three changes were done in 100 per cent ethanol. The samples undergone critical point drying for 5 minutes and they were immediately fixed on the stubs and coated. Samples were immediately observed. Same procedure was followed to observe the morphological characteristics of the re-isolated fungus as well as to investigate

mycoparasitism in dual culture assay of *Trichoderma* spp. and *Rhizoctonia solani* (Garcia *et al*., 2012).

3.14 Multiplication of *R. solani*

Based on the method used by Bhaktavatsalam *et al.* (1978), shoots of water sedge (*Typha angustata*) of 4-5 cm long were washed and soaked in *Typha* medium for 5 minutes. After draining the pieces of typha from excess water, the water sedge were filled in 250 ml conical flask and autoclaved at 1.05 kg/cm^2 for 20 minutes for two consecutive days. A disc (5 mm) *R. solani* was inoculated in the autoclaved typha pieces. The flask was incubated for 15 days at 28±1° C.

3.15 Inoculation Method

Pieces of typha infected with the pathogen were put in-between tillers at the maximum tillering stage just above the water level. The plant was watered everyday to maintain humidity suitable for promote disease development.

3.16 Measurement of disease reaction

Relative lesion height (RLH) was measured after 10 days and 20 days of inoculation. To measure the total length of lesion spread (cm), the distance was measured from base of the plant to the tip of the top most lesions on the stem. RLH was calculated by taking the parameters of the lesion length and plant height by using the following formula:

$$RLH = X \frac{\text{Lesion length (cm)}}{\text{Plant height (cm)}} 100$$

3.17 Measuring Horizontal spread

The infected tillers and the total number of tillers were counted for calculating the horizontal spread.

$$\text{Per cent Horizontal spread} = \frac{\text{No. of infected tillers}}{\text{Total no. of tillers}} \times 100$$

3.18 Disease reaction (Ahn *et al*., 1986)

The RLH was compared with the standard disease scale and the reaction was considered.

Disease grade	Relative lesion height (per cent)	Reaction
0	**0**	Highly resistant (HR)
1	<20	Resistant (R)
3	20-30	Moderately resistant (MR)
5	31-45	Moderately susceptible (MS)
7	46-65	Susceptible (S)
9	>65	Highly susceptible (HS)

3.19 Statistical analysis

OPSTAT (HAU, Haryana) and SPSS software packages were used for statistical analysis of the data obtained. All the experiments were repeated once again to confirm the results. Data deviating from the mean is considered as standard deviation.

4.1 Isolation of endophytic fungi from rice grown under natural conditions

In order to know the number and types of endophytes found in rice under natural conditions, Pusa basmati-1 (PB1) rice samples were collected at the Division of Genetics, ICAR-IARI, New Delhi. In total 10 rice samples were collected in sterile plastic bags and brought to the lab for analysis.

A total of 90 segments of rice tissue were used .30 segments from leaves, 30 segments from stems and 30 segments from roots. Four genera of fungi were isolated: *Penicillium* sp., *Alternaria* sp., *Fusarium* sp and *Aspergillus* sp. (Fig 4.1 and Fig 4.2).

4.1.1 Colonization Frequency

Aspergillus sp. was seen to have the highest colonization frequency in all the plant tissues with a total percentage of 46.66 per cent (Table 4.1),while *Alternaria* sp. had the lowest colonization frequency of 33.32 per cent throughout the plant tissues.No *Trichoderma* species was isolated in this experiment.

4.2 Morphological Characterization of *Trichoderma asperellum* and *Trichoderma asperelloides* isolates

Three *Trichoderma asperellum* isolates (TaR1, TaR2 and TaR3)and two *Trichoderma asperelloides* isolates (TaR4 and TaR5)were used in this study. Light microscopic was done as a preliminary screening to confirm that the fungal isolates were *Trichoderma asperellum and Trichoderma asperelloides.*

Morphological identification of *T. asperellum* and *T. asperelloides* isolates *was* done. The identification was based on the conidia shape, the colony appearance, phialides characters and branching patterns of conidiophores.

4.2.1 Macroscopic Features

Seven days old cultures incubated at 28°C were used to study the colony characters of *T. asperellum* isolates and *T. asperelloides* isolates. At seven days *T. asperellum* isolates and *T. asperelloides* isolates grew colony which was observed to be dark green.

This suggests the fast growth of the isolates. Some colony formed concentric rings and for all the isolates the colour change during the course of the fungal growth went from white to green (Fig 4.3).

4.2.2 Microscopic Features

Light microscope was used to study the microscopic features of *T. asperellum* isolates *and T. asperelloides* isolates. Green colour of sub globose to globose shape conidia was observed for all *T. asperellum* and *T .asperelloides* isolates within 7 days. The conidiophores were seen to be loosely tufted and regularly branched with side branches which were at right angles to the branches. The conidiophores were crowded. The conidiophores terminated with flask shaped phialides which were swollen at the middle. The phialides were either single or was seen to form a whorl of 2-4 divergent phialides (Fig 4.4).

The morphological characters observed through light microscope for both *T. asperellum and T. asperelloides* isolates were more or less similar which made it almost difficult to distinguish the difference between the two species.

4.3 Molecular identification of *T. asperellum* and *T. asperelloides* isolates before root colonization

Morphological studies in isolation cannot be solely used to confirm and identify *T. asperellum* and *T. asperelloides* isolates since there might be limited variation leading to misidentifications between species. Thus molecular technique is more frequently used for species identification because it's more efficient and accurate (Jeewon *et al*., 2013).

4.3.1 DNA Extraction

Genomic DNA of the *T. asperellum* and *T. asperelloides* isolates was extracted by CTAB method with success. DNA quantity and quality was confirmed with the gel and nanodrop reading. In gel a clear distinct band was seen at the top of the gel. In case of the nanodrop, A260/A280 was ranged from 1.94 to 2.01 which show the good quality of the DNA.

4.3.2 PCR Amplification

PCR amplification of all the five isolates were done with the ITS primers and *tef* 1 primers and the results revealed that a band was observed around600bp length and 1000bp length. Amplified fragments were gel purified and sent for sequencing.

4.3.3 Sequencing results

Sequencing of the amplified fragment of PCR was done. They were successfully aligned and Contig was prepared for all the *T. asperellum and T. asperelloides* isolates. To

confirm the isolates as *T. asperellum and T. asperelloides* nBLAST were used. According to the nBLAST results, TaR1, TaR2and TaR3 isolates were matching with *T. asperellum* isolates while the TaR4andTaR5 were matching with *T. asperelloides* for both ITS and *TEF1.*Thisconfirmed the results of the two *Trichoderma* species (Fig 4.5; Fig 4.6; Fig 4.7 and Fig 4.8).

4.4 Light Microscopy

At 28 days after seed and soil treatment with the five isolates the internal colonization of roots was seen through light microscopy for both seed and soil treated. Mycelium was visible but a thorough morphology of the endophytes was not clear to assess as the morphology could have been changed through slide preparation. Conidia were not observed in both instances. The mycelium was seen as straight and sometimes the septation of the hyphae could be visible (Fig 4.9 and Fig 4.10).

4.5 Re-isolation of endophytic fungus

Two replicates were selected at random from each isolates of each treatment. One plant was sample from the control. 10 root segments per isolate were used. Five days after plating the root samples on PDA, individual hyphal tips were re-plated onto new PDA plates to have pure culture. Ninety percent of the 10 samples had endophyte growth of *Trichoderma* species when re isolation was done at 28 days after sowing. At seven days, in all the plates where roots were taken from treated rice plants, dark green colonies were formed while in the control plates where roots were taken from rice plants not treated with *Trichoderma* species *Aspergillus* sp. was observed (Fig 4.11 and Fig 4.12).3 days after replating, morphological characterization was done through SEM analysis to identify fungal endophytes superficially (Fig 4.13 and Fig 4.14). The fungal growth was also re-inoculated on Potato Dextrose Broth for further molecular analysis**.**

4.5.1 Molecular identification of isolated endophytic fungus

ITS region identified *Trichoderma asperellum* isolates (TaR1, TaR2 and TaR3)and two *Trichoderma asperelloides* isolates (TaR4 and TaR5) *as* the re-isolated fungus from soil and seed treatment respectively (Fig 4.15; Fig 4.16 and Fig 4.17).

4.6 Scanning Electron Microscopy

Roots samples were subjected for SEM analysis. *Trichoderma* mycelium was observed in all the root samples for both soil and seed treatment (Fig 4.18 and Fig 4.19).

4.7Antagonism test

Antagonistic effects of *T. asperellum* isolates (TaR1, TaR2 and TaR3) and *Trichoderma asperelloides* isolates (TaR4 and TaR5) were tested against *R. solani* (Fig 4.20) on PDA at 28°C for 7days. In all the dual culture plates, the contact zone was a curve, with concavity oriented towards the pathogenic fungi. In the negative control plates, only *R. solani* species was inoculated (Fig 4.21).

The mean percentage inhibition of the isolate range from 73.49 per cent to 86.84 per cent. All the isolates grew faster than *R. solani.* Significant inhibition of the mycelium pathogen growth was observed by isolate TaR3 with mean percentage inhibition of 86.84 per cent whereas TaR2 showed the least inhibition of 73.49 per cent respectively (Table 4.2,Table 4.3 and Fig 4.22).As the most effective isolate TaR3 was selected for SEM analysis for mycoparasitism assay TaR3was found to parasitize *Rhizoctonia solani* through mycoparasitism (Fig 4.23).

4.8 Disease Measurement of sheath blight disease

To quantify sheath blight disease vertical and horizontal spread through Relative Lesion Height and percent infected tillers were used There were three replication and one control for each isolate for soil and seed treatment of Pusa basmati 1**.**

4.8.1 Measurement of Vertical spread

Relative lesion height (RLH) was recorded after ten days and twenty days of inoculation. At twenty days for soil treatment the highest mean RLH was recorded by the Isolate TaR2 with 51.1per cent. Isolate TaR3 recorded the lowest RLH with 38.9 per cent. For seed treatment isolate TaR4 recorded highest mean RLH with 50.63 per cent. The lowest RLH was seen in isolate TaR3 with 40.2 per cent. According to disease scale given by Ahn *et al*., (1986) the isolates ranged from moderately susceptible to susceptible. The majority of the isolates for the seed and soil treatment were grouped as susceptible.TaR3 was classified as moderately susceptible in both treatments. The controls were seen to be susceptible for both the soil and seed treatment respectively (Table 4.4; Table 4.5; Fig 4.24; and Fig 4.25).

4.8.2 Horizontal spread

Horizontal spread of sheath blight disease in terms of percent infected tillers was also calculated. The mean horizontal spread range from of 72.87 per cent to 92.93 per cent

in the soil treatment and 78.4 per cent to 93.33 per cent in seed treatment. TaR3showed the lowest mean horizontal spread of 72.87 per cent in the soil treatment and 78.4 per cent in the seed treatment. The mean horizontal spread of the controls ranged was 98.58 per cent in the soil treatment and 97.98 per cent in the seed treatment (Table 4.4; Table 4.5; Fig 4.24; and Fig 4.25).

Biological control is one of the most economically and ecologically safest methods for managing plant diseases. *Trichoderma* spp. is the key fungus mostly employed as a biocontrol agent against several plant pathogens. *Trichoderma* has been found to control major diseases of rice (De França*et al*., 2014; Cuevas, 2006; Ali and Nadarajah, 2013;Khalili*et al.*, 2012). Sheath blight of rice as a minor disease has presently become a major disease in all the rice growing regions of the country.

Endophytism is one of the important tools used by biocontrol agents in alleviating the disease effect as well as boost up the plant growth. In India, *Trichoderma harzianum* and *T. viride* has been fully investigated and exploited as potential antagonists. Up to date *T. harzianum* and *T. viride* has been commercialized successfully. *T. asperellum and T. asperelloides* is also an important biocontrol agent which is at its budding stage in exploitation and commercialization. In view of this, a study was conducted to know the effect of *T. asperellum* and *T. asperelloides* as endophytes in rice plants and its effect on the sheath blight disease. This study is amongst other preliminary studies being initiated to shed more light and understanding on the potential use of *T. asperellum* and *T. asperelloides* as a biocontrol agent on a commercial basis. *T. asperellum* has already been commercialized in some countries but not in India (Santos *et al*., 2012; Navaneetha *et al*, 2015).Three isolates of *T. asperellum*(TaR1, TaR2 and TaR3)and two isolates of *T. asperelloides* (TaR4 and TaR5) collected from different agro-climatic zones Rajasthan, India were used.

In this study experiments were designed to investigate endophytism and antagonistic activity of *T. asperellum* and *T. asperelloides* under *in vitro* and *in vivo* conditions. *In vivo* study results revealed that all the *T. asperellum* and *T. asperelloides* isolates showed antagonistic effect against *R. solani* by inhibiting the mycelial growth. This may be attributed to the production of secondary metabolites by the *Trichoderma* isolates (Anees *et al.,* 2010). *Trichoderma* secretes different cell wall degrading enzymes *viz.,* chitinases and glucanases in low level against the plant pathogens before contact with mycelium. Elicitors such as degraded cell wall may induce gene expression of mycoparasiticism as well (Vinale *et al.,*2008). The inhibition percentage of mycelial

growth of plant pathogens is often used as an effective way in the assessment of the antagonistic potential of the beneficial organism (Grondona *et al.,* 1997). Significant inhibition of the mycelium pathogen growth was observed by isolate TaR3 with mean percentage inhibition of 86.84 per cent whereas TaR2 showed the least inhibition of 73.49 per cent respectively. Thus TaR3 (*T. asperellum)* was found to be effective in reducing *Rhizoctonia solani* growth in petri plates.

Under greenhouse conditions, the pot experiments were conducted. Both seed and soil treatment of *T. asperellum* (TaR1, TaR2 and TaR3) and *T. asperelloides* (TaR4, TaR5) were carried out and challenged inoculation with *R. solani* was done. All the isolates shown sheath blight disease reduction when compared to control. De Franca*et al.* (2014) also showed that *T. asperellum* was seen to decrease the disease severity by 19 per cent. Other studies also showed the effective suppression of *R. solani* through the use of different *Trichoderma* spp. (Prasad and Kumar, 2011; Silva *et al*., 2012; Mathivanan *et al*., 2005; Krishnamurthy *et al*., 1999; Ali and Nadarajah, 2013; Khalili *et al.*, 2012;Naeimi*et al*., 2010). Amongst the isolates, *TaR3* was the best isolate in both seed and soil treatment in reducing the disease. Various studies have shown that there may be isolates in a group of isolates under investigation that perform better than the others. El kommy *et al.* (2015) showed that out of 30 isolates of *T. asperellum,* only six were highly antagonistic against *Fusarium* wilt (*Fusarium oxysporum* f. sp. *lycopersici)* in tomato. This was attributed to the ability of the six isolates to produce cell-wall degrading enzymes and showed high antagonistic activity against the pathogen. The Genetic variability and aggressiveness of the six isolate compared to the other isolates was also considered. Same kind of observations has been reported by Anees *et al.* (2010) in which out of sixteen *Trichoderma* isolates they have collected from the sugar beet field affected by *R. solani*, only *Trichoderma gamsii* was found to be the most effective strain against *R. solani* both *in vivo* and *in vitro*.

This was partly attributed to the fact that this strain might have been collected from high disease areas where its antagonistic ability tends to be more pronounced. It may be explained that some isolates might be more efficient in expressing enzymes and genes as compared to other isolates which make them most successful antagonists. It is well known that a biocontrol agent should fulfill some of the important criteria *viz.* quick

in growth, fast in colonization, efficient in expression of cell wall degrading enzymes and genes to weaken the pathogen. In a similar manner, in our study also among the three isolates of *T. asperellum*, TaR3 was efficient with high antagonistic activity in reducing the sheath blight disease. The results of the pot experiments were matching with the earlier reports that inoculation of *Trichoderma* spp. through seed and soil treatment has been found to be effective in seed germination, plant growth in general and most importantly plant disease control (Cuevas, 2006; Jegathambigai *et al*., 2009; Khalili *et al*., 2012; Silva *et al*., 2012; Akrami *et al*., 2012).

The important things in endophytism study is that after treatment (seed or soil) of biocontrol agent it has to be re-isolated again in pure culture from the inoculated plants to prove the endophytic nature. Re-isolation in this study was made possible through surface sterilization, a method which ensures that epiphytic fungi or bacteria are eliminated to prevent biased results. This method have been used in most of the studies on endophytism (Mishra *et al.*, 2014; Mulaw *et al.*, 2013; Wu *et al.*, 2013; Chatterton *et al*., 2008; Hanada *et al.*, 2008; Chaverri *et al*, 2011; Crozier *et al.*, 2006; García *et al.*, 2012). However one important false proof method to prove the endophytic nature of the *Trichoderma* spp. is GUS (β-Gluconiridase)/*gfp* (green fluorescence protein) transformation of the fungus which allows it to colonize the plant tissues so that it will relocate the fungus easily under confocal or fluorescence microscopy. Many studies have successfully used GUS transformation to investigate endophytism in plant tissues (Mulaw *et al*., 2013; Chatterton*et al*., 2008). In our study we have tried to incorporate the *gfp* gene in TaR3 (*T. asperellum*) and colonize the rice plants with the transformed fungus. But we were not able to get successful results in this experiment (Data was not shown).

Morphological characters have been used for the identification of re-isolated fungus (Hallmann *et al.,* 2006). Methods of light microscopy and scanning electron microscopy (SEM) have been used in many studies to investigate fungal endophytes (Chatterton *et al*., 2008; García *et al*, 2012). In this study both the methods were used and hyphae were observed for each isolate in both the treatments. However the best way of confirmation is by molecular methods (PCR confirmation and sequencing) of a known gene. Molecular methods are recommended for its accuracy and specificity

(Jeewon *et al*., 2013). In this study we have amplified and sequenced the ITS region (ITS1 and ITS2) as well as the *TEF1* region of the inoculated isolate and used solely amplification of ITS region for the re-isolated fungus. PCR amplification of ITS1 and ITS2 has also been used in many studies for the identification of endophytic fungi (Chaverri *et al*., 2011; García *et al.,* 2012; Mulaw *et al*., 2013). ITS region is frequently used since it is a variable region. It is a specific locus for species identification (Kullnig-Gradinger *et al*., 2002). It is a region which evolves fast and varies among species within a genus (Jeewon *et al.,* 2013).*TEF1* region on the other hand is used to distinguish closely related *Trichoderma* species. It is more variable than ITS region (Devi *et al*., 2012)

The molecular data confirmed the inoculated and recovered isolates as *T. asperellum* (TaR1, TaR2 and TaR3) and *T. asperelloides* (TaR4 and TaR5).This brought us to the conclusion that all the five isolates are endophytic in nature. This observation is matching with a recent study done by Rosmana *et al*., (2015) where *T. asperellum* isolates were re-inoculated in cacao seedlings through the roots and they were recovered in roots and stems after one month. Up to date there is very few studies done on *T. asperelloides* as endophytes thus this might be a baseline study for more elaborated endophytic study of *T. asperelloides*.

This study has reported that *T. asperellum* and *T. asperelloides*is endophytic in rice upon inoculation through seed and soil treatment. The disease scales of the *in vitro* experiment ranged from moderately susceptible to susceptible in both seed and soil treatment. This indicates the role of *T. asperellum and T. asperelloides* as endophytes against biotic stresses. The isolate TaR3 was proved to be very effective in controlling sheath blight of rice as compared to other isolates. Therefore in near future it is necessary to explore the characteristics of the TaR3 isolate in terms of mycoparasitic genes and some other genes related to the antagonism and plant growth.

CHAPTER 6 SUMMARY AND CONCLUSION

Nowadays pest and disease resurgence poses a problem in agriculture. The use of chemicals is also an issue since in some instances it can be a safety hazard to humans and the environment. There is a need to resort to natural products from microorganisms. Endophytic fungi may play a role in providing ecologically and economically safe biocontrol agents against plant pathogens. Fungi is a vital component in the soil that not only helps in decomposing organic matter which in turn release nutrient for the plant to utilize but also play important roles against plant pathogens and environmental stress in nature (Waller *et al.,* 2005). Rice is an important crop especially in countries around Asia (Kumar *et al*., 2009). Sheath blight of rice is a very important disease of rice which is causing tremendous losses to rice fields (Usharani *et al.,* 2013). Studies on endophytism in rice are a new area of study in order to exploit biocontrol agents in the management of plant diseases.

Trichoderma spp. are effective biocontrol agents for plant diseases (Ali and Nadarajah**,** 2013; Silva *et al*, 2012; Cuevas, 2006; Khalili *et al.*, 2012; Mwangi *et al.*, 2011). The main mechanism involved is antibiosis, mycoparasitism and competition. They also play roles in phosphate solubilization, plant growth promotion, and production of siderophores, drought and salt tolerance, effective soil colonization, plant symbionts in the rhizosphere and play roles in biodegradation (Stone *et al*, 2004). Numerous *Trichoderma* spp. had been reported as endophytes (Bae *et al*., 2009; Samuels, 2006; Hanada *et al.*, 2008; Chaverri *et al*., 2011). Many studies have been done to show the antagonistic effect of *Trichoderma asperellum* against many plant pathogens (De franca *et al*., 2014; Trillas *et al.,* 2006; Sant *et al*., 2010; De los Santos-Villalobos *et al*., 2013).Studies have been done to explore the roles of *Trichoderma asperelloides* in plant resistance to abiotic and biotic stresses (Brotman *et al*., 2013; Gupta *et al*., 2014; Doley *et al*., 2014).

Up to date very few studies have been done on *T. asperellum and T. asperelloides*as an endophyte in India. The scope of this study is part of various projects that is going on in fungal pathology, Biocontrol laboratory, Division of Plant Pathology, ICAR-IARI, New Delhi, on *Trichoderma* as potential biocontrol agents for the farming

community. Through this study we want to elicit further studies of endophytism of *Trichoderma* spp. in India, since there is a lack of studies and information on endophytism of *Trichoderma* spp.

The hypothesis behind this work was that *Trichoderma* spp. is endophytic in nature and they help in antagonistic activity against plant pathogens especially *R. solani* here. This is by the means of secondary metabolites they produce, competition and mycoparatism. This study will serve as a way to incite scientists to do more studies on endophytism of *Trichoderma* spp. in rice as a means of better understanding the biocontrol perspective in management of plant diseases. Summary of the present study is summarized below.

- Isolation of endophytic fungi from Pusa basmati-1 under natural conditions revealed that *Trichoderma* spp. were not present in the rice plant tissues but other fungi were recovered namely *Penicillium* sp., *Alternaria* sp., *Fusarium* sp., *Aspergillus* sp.
- Three *Trichoderma asperellum* isolates (TaR1, TaR2 and TaR3)and two *Trichoderma asperelloides* isolates (TaR4 and TaR5)from different agro-climatic regions in Rajasthan was used for this study.
- Morphological characterization of the isolates was done by observing the conidia, conidiophores and the phialides characteristics to confirm the isolates at species level.
- Molecular characterization of the ITS region (28s rDNA, 18s rDNA, 5.8s rDNA, ITS1 and ITS2) and *TEF1*region revealed thatTaR1, TaR2 and TaR3 were *T. asperellum* isolates and TaR4 and TaR5were*T. asperelloides* isolates.
- At 28 days after sowing of seed and soil treated rice plants, light microscopy was performed and *T. asperellum* and *T. asperelloides* hyphae was seen inside the roots, making indications that the inoculated isolates were growing inside the root zone.
- Further confirmation of the endophytic fungus was carried out by re-isolation of the endophytic fungus from the roots of both treatments. Roots were placed on PDA plates by following the standard protocols for isolation of fungal endophytes. In all the plates fungal growth was observed. Three days after

replating the fungal endophytes was subjected to SEM assay for surface morphology identification. All the fungus showed similar morphological characteristics to the earlier inoculated isolates.

- Molecular identification of re-isolated endophytic fungus through ITS region amplification identified *Trichoderma asperellum* (TaR1, TaR2 and TaR3)and *Trichoderma asperelloides*(TaR4 and TaR5)as the isolated fungus from the root samples of soil and seed treatment.
- The root samples were subjected to SEM assay and *T. asperellum* and *Trichoderma asperelloides* hyphae was seen in all the root samples for both treatments.
- Dual culture test of the five isolates against *Rhizoctonia solani* showed that all the isolates grew faster than *R. solani.* Significant inhibition of the pathogen mycelial growth was observed in isolate TaR3 with mean percentage inhibition of 86.84 per cent, whereas TaR2showed the least inhibition of 73.49 per cent.
- At twenty days for soil treatment the highest mean RLH was recorded by the Isolate *TaR2*with 51.1 per cent. Isolate TaR3 recorded the lowest RLH with 38.9 per cent. For seed treatment isolate TaR4 recorded highest mean RLH with 50.63 per cent. The lowest RLH was seen in isolate TaR3 with 40.2 per cent. According to disease scale given by Ahn *et al*., (1986) the isolates ranged from moderately susceptible to susceptible. The majority of the isolates for the seed and soil treatment were grouped as susceptible.TaR3 was classified as moderately susceptible in both treatments. The controls were seen to be susceptible for both the soil and seed treatment respectively
- Horizontal spread of sheath blight disease in terms of percent infected tillers was also calculated. The mean horizontal spread range from of 72.87 per cent to 92.93 per cent in the soil treatment and 78.4 per cent to 93.33 per cent in seed treatment. TaR3showed the lowest mean horizontal spread of 72.87 per cent in the soil treatment and 78.4 per cent in the seed treatment. The mean horizontal spread of the controls ranged was 98.58 per cent in the soil treatment and 97.98 per cent in the seed treatment.

To conclude, it must be said that. *T. asperellum and T. asperelloides* are endophytic in rice upon inoculation through seed and soil treatment. There is no doubt that TaR3 (*T. asperellum)* is a promising biocontrol agent which can be a potential commercial product available to the farmers in the near future against sheath blight of rice. Since the isolate TaR3 have shown to be effective against *R. solani* both *in vivo* and *in vitro*, it can be further characterized and experiments can be conducted under different climatic conditions where rice is grown. Through all of the related studies mentioned in this thesis, we have seen that *Trichoderma asperellum* is an effective biocontrol agent of sheath blight of rice.

Various experiment referred to in this thesis as well as the results of this study brings us to believe that *Trichoderma* species as an endophytes play multiple roles (easy access to nutrients, production of secondary metabolites, hyperparasitism and predation, inducing plant resistance and occupy ecological niches) in controlling sheath blight (Dutta *et al*., 2014).

More elaborated study should be done to understand interaction between rice, *R. solani* and *Trichoderma* as endophytes. This is essential to ensure that promising biocontrol agents such as *Trichoderma* spp. are well exploited, understood and applied in the correct time, amount and place. In this way biocontrol agents can play an important role in integrated disease management.

Studies on endophytism of *Trichoderma* species in Rice against *Rhizoctonia solani*

ABSTRACT

Microbes that colonize internal tissues of plants are known as endophytes. The major interest with endophytes is that they play functional roles against abiotic, biotic stress and are involved in plant growth promotion. The fungus *Trichoderma* spp. (Teleomorph: *Hypocrea*) are well known antagonists as well as endophytes. Sheath blight caused by *Rhizoctonia solani* is one of the most important diseases of rice. *Trichoderma asperellum* isolates (TaR1, TaR2 and TaR3) *and Trichoderma asperelloides* isolates (TaR4 and TaR5) collected from different agro-climatic zones in Rajasthan was used for this study. Pusa basmati 1 was subjected to soil and seed treatment with the five isolates. After 28 days, the microscopic analysis (light and SEM) and isolation of endophytic fungi revealed that the isolated endophytic fungus were identical to the inoculated ones. These results were further confirmed by PCR amplification of the rDNA region (18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA) with the newly isolated *Trichoderma asperellum* and *T. asperelloides.* Consequently, the *in vivo* (Dual culture assay) and *in vitro* (Plant infection) studies of the antagonistic activity of the five isolates against *R. solani* showed that all the isolates significantly inhibited the growth of the pathogen. In both treatments, TaR3 proved to be the best isolate. In dual culture testTaR3 mean percentage inhibition was 86.84 per cent while its relative lesion height at twenty days in soil and seed treatment was the lowest at 38.9 per cent and 40.2 per cent respectively. The mean horizontal spread range from of 72.87 per cent to 92.93 per cent in the soil treatment and 78.4 per cent to 93.33 per cent in seed treatment. *TaR3* showed the lowest mean horizontal spread of 72.87 per cent in the soil treatment and 78.4 per cent in the seed treatment. The mean horizontal spread of the controls range was 98.58 per cent in the soil treatment and 97.98 per cent in the seed treatment. In this study, it has been reported that *T. asperellum and T. asperelloides* is endophytic in rice upon inoculation through seed and soil treatment.TaR3 was proved to be very effective in controlling sheath blight as compared to other isolates. This may indicate the role of *T. asperellum and T. asperelloides* as endophytes against biotic stress.

राइज़ोक्टोनिया सोलेनाई के विरूद्ध धान में *ट्रायकोडर्मा* प्रजातियों की अंतःपादपता का अध्ययन

सार

पौधों के आंतरिक ऊतकों में वास करने वाले सूक्ष्ममजीव अंतःपादप जीव कहलाते हैं। इन अंतःपादपों रूचि होने का प्रमुख कारण यही है कि वे अजैव एवं जैव प्रतिबलों के विरूद्ध कार्यात्मक भूमिका निभाते है और इनकी पादप वृद्धि के प्रोत्साहन में सहभागिता होती है। *ट्रायकोडर्मा* प्रजातियाँ (टीलोमार्फ : हायपोक्रेया) उत्तम जीवविरोधी होने के साथ–साथ अंतःपादप भी हैं। *राइज़ोक्टोनिया सोलेनाई* द्वारा उत्पन्न पर्णाच्छद झुलसा, धान के सर्वाधिक महत्वपूर्ण रोगों में से एक है। राजस्थान के विभिन्न कृषि–जलवायु संबंधी क्षेत्रों से एकत्रित *ट्रायकोडर्मा एस्परेलम* विलगों (TaR1, TaR2, TaR3) एवं *ट्रायकोडर्मा एस्परेलॉयड़्स* विलगों (TaR4, TaR5) का इस अध्ययन में उपयोग किया गया। धान की पूसा बासमती 1 क़िस्म का पाँच विलगों के साथ बीजोपचार एवं मृदा–उपचार किया गया। बुआई के 28 दिन पश्चात सूक्ष्मदर्शी द्वारा विश्लेषण (प्रकाश सूक्ष्मदर्शी एवं क्रमवीक्षण इलेक्ट्रॉन सूक्ष्मदर्शी) तथा अंतःपादप कवकों के विलगन ने दर्शाया कि ये विलगित कवक एवं निवेशित कवक एक समान थे। नवीन विलगित *ट्रायकोडर्मा एस्परेलम* एवं *ट्रा. एस्परेलॉयड़्स* के साथ आर डी एन ए क्षेत्र (18S rRNA, ITS1, 5.8S rRNA, ITS2 एवं 28S rRNA) के पी सी आर प्रर्वधन द्वारा भी इन परिणामों की पुष्टि की गई। तत्पश्चात *राइज़ोक्टोनिया सोलेनाई* के विरूद्ध पाँच विलगों की जीवविरोधी सक्रियता के सजीव कोशिकाओं के भीतर (दोहरा संवर्ध आमापन) सभी विलगों ने रोगजनक की वृद्धि का महत्वपूर्ण रूप से संदमन किया। इन दोनों उपचारों में, TaR3 बेहतर विलग पाया गया। दोहरे संवर्ध परीक्षण में TaR3 माध्य प्रतिशत संदमन 86.84 प्रतिशत था जबकि मृदा एवं बीजोपचार में, बीस दिन पर आपेक्षित क्षत ऊँचाई क्रमशः 38.9 प्रतिशत 40.2 प्रतिशत पर न्यूनतम थी। मृदा उपचार में माध्य/क्षैतिज प्रसार 72.87 प्रतिशत से 93.33 प्रतिशत की सीमा में था तथा बीजोपचार में 78.4 प्रतिशत से 93.33 प्रतिशत पायी गई। TaR3 ने मृदा–उपचार में 72.87 प्रतिशत का तथा बीजोपचार में 78.4 प्रतिशत का न्यूनतम क्षैतिज प्रसार दर्शाया। कंट्रोल के मृदा–उपचार में माध्य क्षैतिज प्रसार 98.58 प्रतिशत एवं बीजोपचार में माध्यम से निवेशित करने पर धान में *ट्रायकोडर्मा एस्परेलम* एवं *ट्रायकोडर्मा एस्परेलॉयड़्स* अंतःपादप के रूप मे होते हैं। अन्य विलगों की तुलना में, धान के आच्छद झुलसा रोग को नियंत्रित करने में TaR3 अत्यधिक प्रभावी सिद्ध हुआ। इससे जैवप्रतिबल के विरूद्ध अंतःपादप के रूप में *ट्रायकोडर्मा एस्परेलम* एवं *ट्रायकोडर्मा एस्परेलॉयड़्स* की भूमिका ज्ञात होती है।

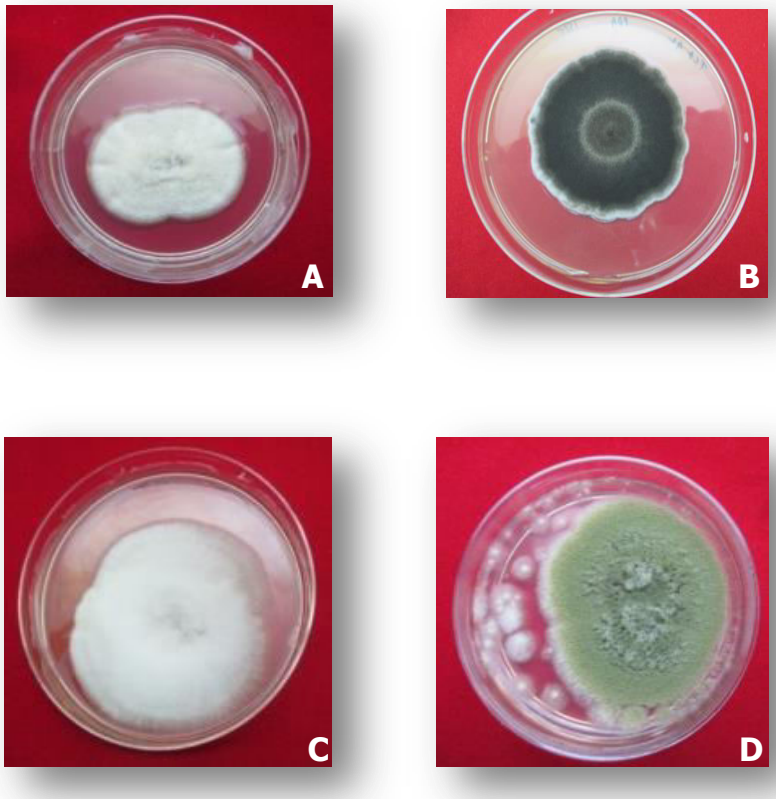


Fig. 4.1: Seven days old cultures of endophytic fungi on Potato Dextrose Agar isolated from Pusa Basmati 1 rice plants grown under natural condition (A) *Penicillium* sp. (B)*Alternaria* sp. (C) *Fusarium* sp. (D) *Aspergillus* sp.

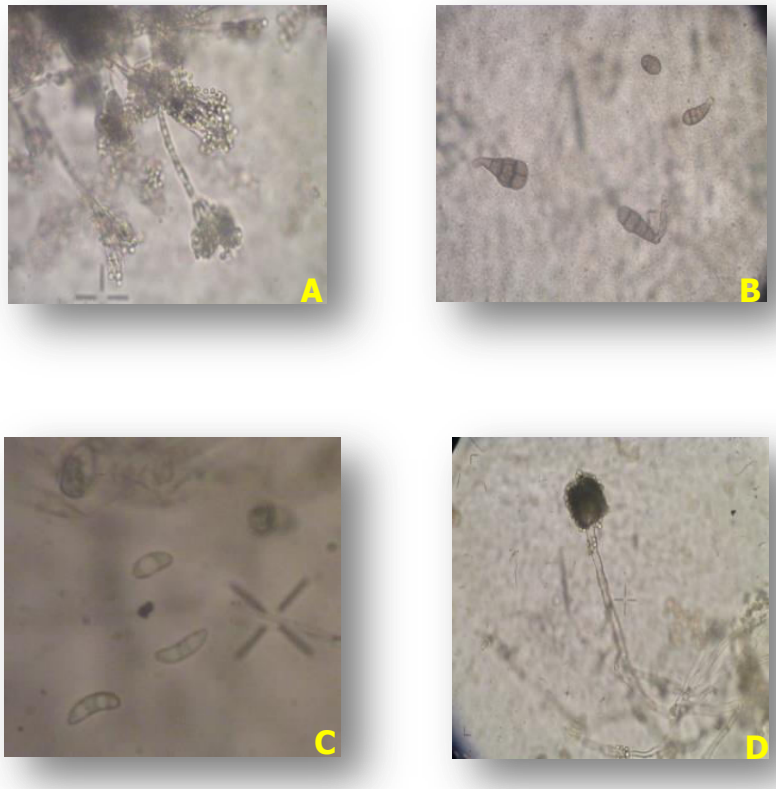


Fig. 4.2: Light microscopy image of endophytic fungi isolated from Pusa Basmati 1 rice plants grown under natural condition (Magnification at 40X) (A) *Penicillium* sp. (B) *Alternaria* sp (C) *Fusarium* sp. (D) *Aspergillus* sp.

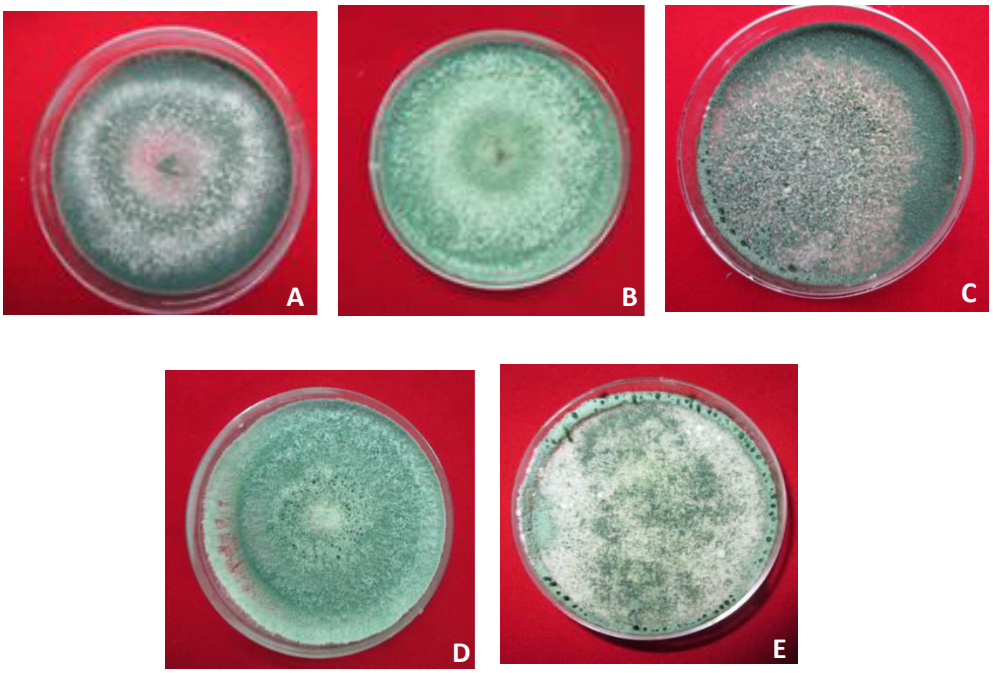


Fig. 4.3: Seven days old cultures of *Trichoderma asperellum*: (A) TaR1 (B) TaR2 (C) TaR3 and *Trichoderma asperelloides*: (D) TaR4 (E) TaR5 grown on PDA plates collected from different agro-climatic zone in Rajasthan, India.

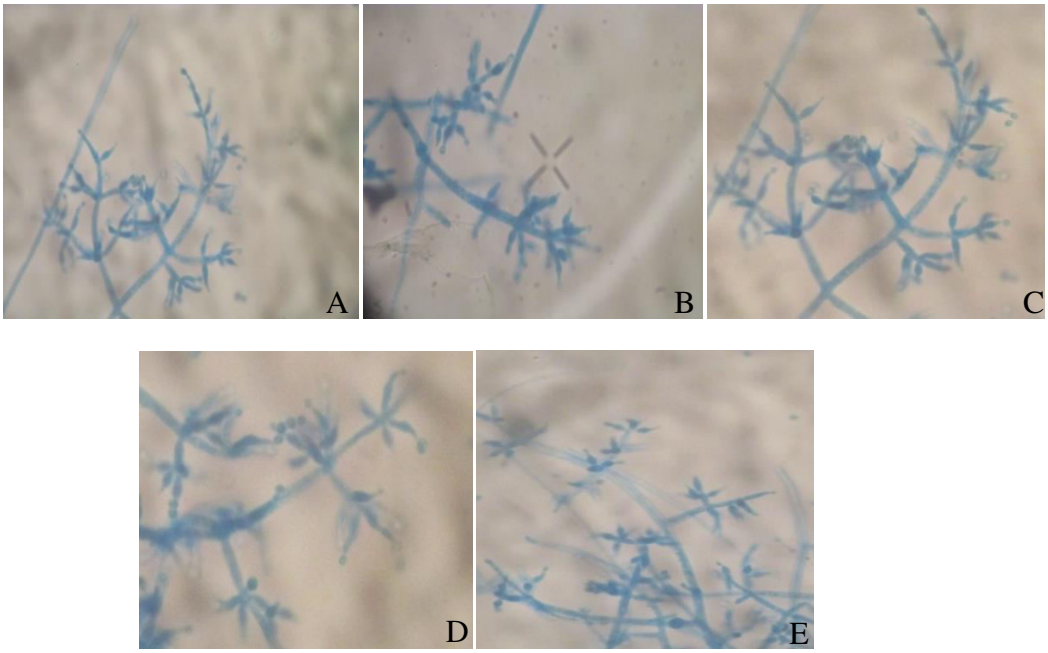


Fig. 4.4: Light microscope images of mycelial and conidiophores characteristics of *Trichoderma asperellum* isolate: (A) TaR1 (B) TaR2 (C) TaR3 and *Trichoderma asperelloides* isolate: (D) TaR4 (E) TaR5 at 40X magnification.

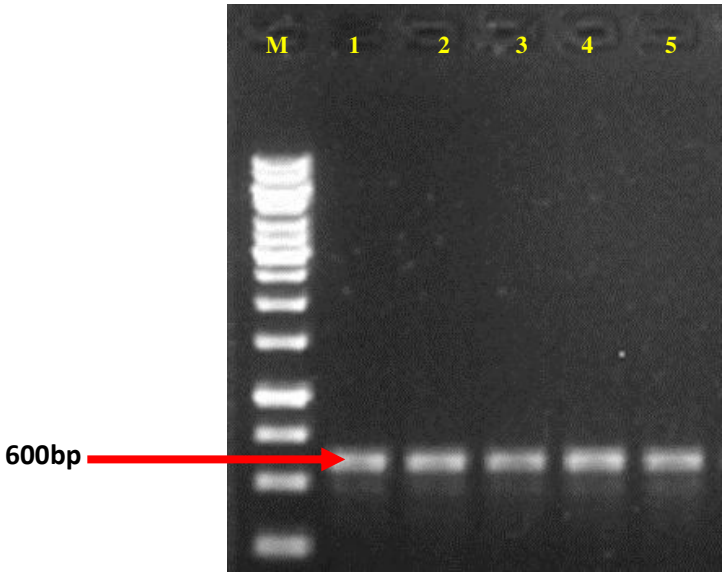


Fig. 4.5: ITS region amplification of *Trichoderma* isolates using primer pair ITS1 and ITS4. M: 1Kb DNA ladder; Lane 1: TaR1; Lane 2: TaR2; Lane 3: TaR3; Lane 4: TaR4 and Lane 5: TaR5.

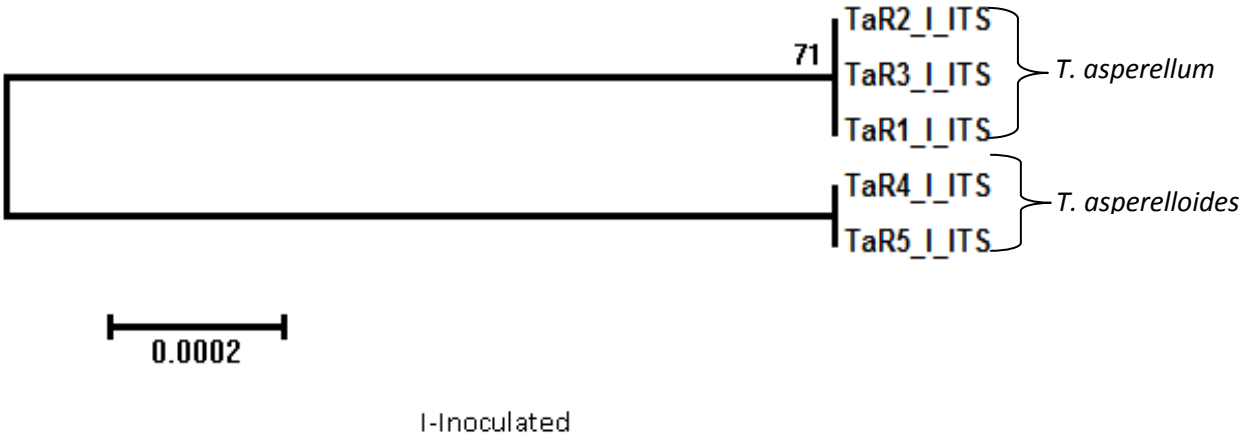


Fig. 4.6: Phylogenetic relationships of two different *Trichoderma* spp. inferred by analysis of ITS gene sequences. The Maximum Likelihood tree was constructed using two-parameter model implemented in the MEGA 6.06 program.

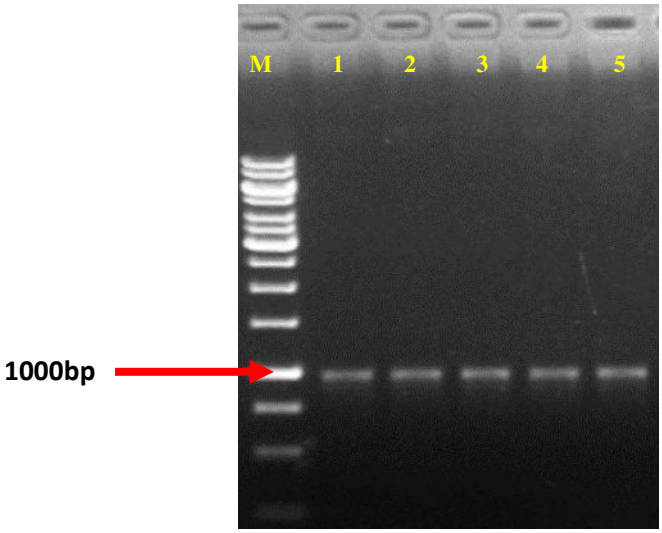


Fig. 4.7: Tef1 region amplification of *Trichoderma* isolates using primer pair EF1F and Tef1R. M: 1Kb DNA ladder; Lane 1: TaR1; Lane 2: TaR2; Lane 3: TaR3; Lane 4: TaR4 and Lane 5: TaR5.

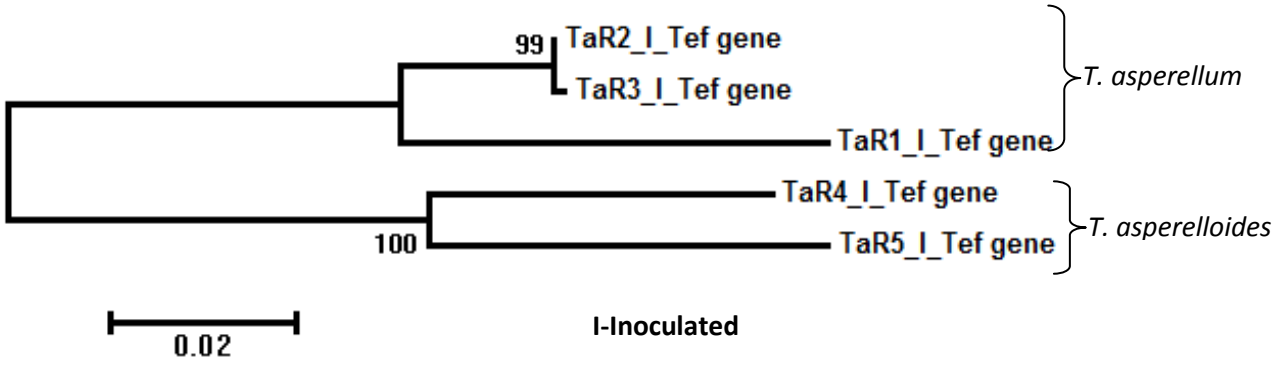


Fig. 4.8: Phylogenetic relationships of two different *Trichoderma* spp. inferred by analysis of *Tef1* gene sequences. The Maximum Likelihood tree was constructed using two-parameter model implemented in the MEGA 6.06 program.

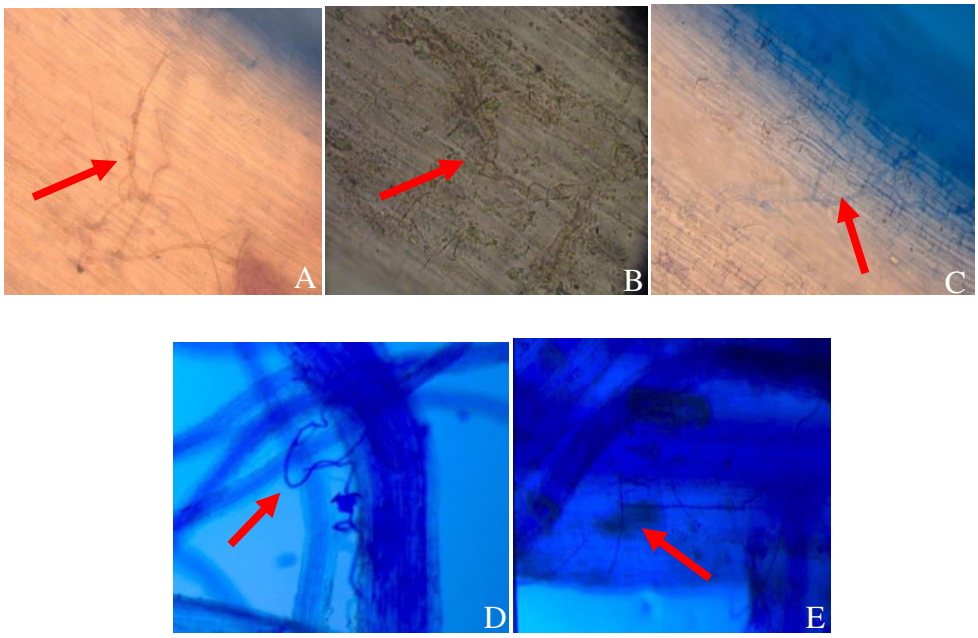


Fig. 4.9: Light microscopy image showing mycelium inside the Pusa Basmasti 1 rice roots soil treated with *Trichoderma* isolates: (A) TaR1 (B) TaR2 (C) TaR3 (D) TaR4 (E) TaR5 at 40X magnification.

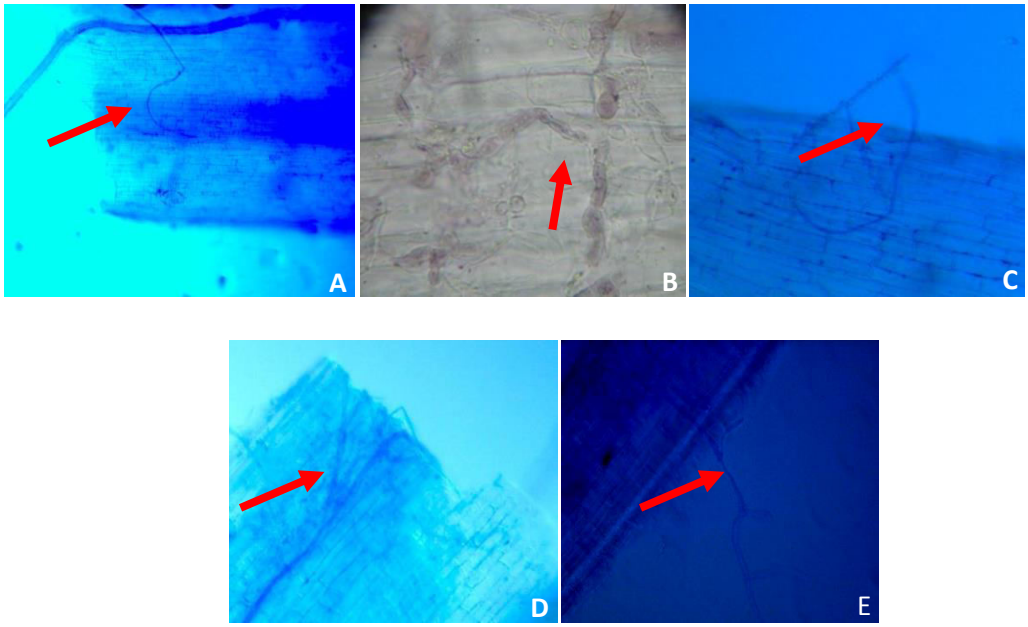


Fig. 4.10: Light microscopy image showing mycelium inside the Pusa Basmasti 1 rice roots seed treated with *Trichoderma* isolates: (A) TaR1 (B) TaR2 (C) TaR3 (D) TaR4 (E) TaR5 at 40X magnification

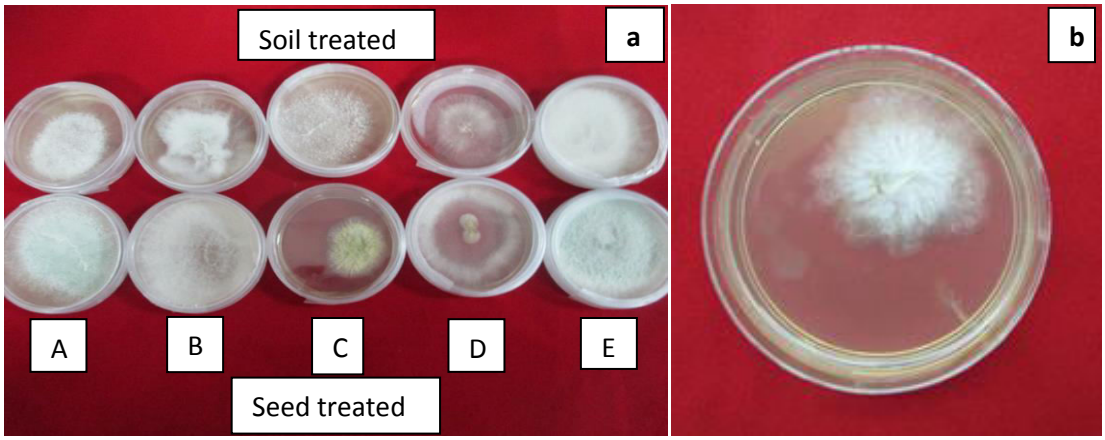


Fig. 4.11: (a) Three days old cultures of re-isolated endophytes from the *Trichoderma* treated Pusa Basmati 1 rice roots on PDA plates: (A) TaR1 (B) TaR2 (C) TaR3 (D) TaR4 (E) TaR5; (b) Three days old control plate showing mycelial growth isolated from *Trichoderma* untreated Pusa Basmati 1 rice roots.

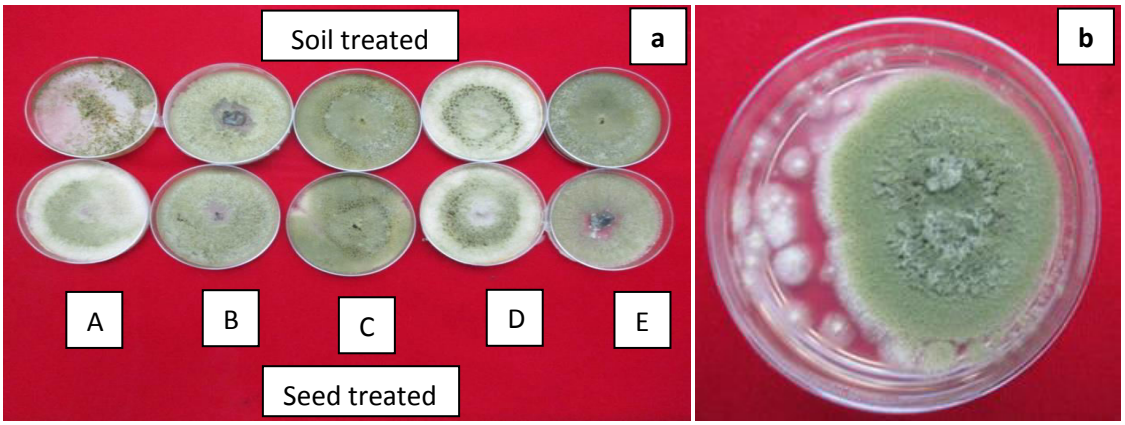


Fig. 4.12: (a) Seven days old cultures of re-isolated endophytes from the *Trichoderma* treated Pusa Basmati 1 rice roots on PDA plates: (A) TaR1 (B) TaR2 (C) TaR3 (D) TaR4 (E) TaR5; (b) Seven days old control plate showing mycelial growth isolated from *Trichoderma* untreated Pusa Basmati 1 rice roots.

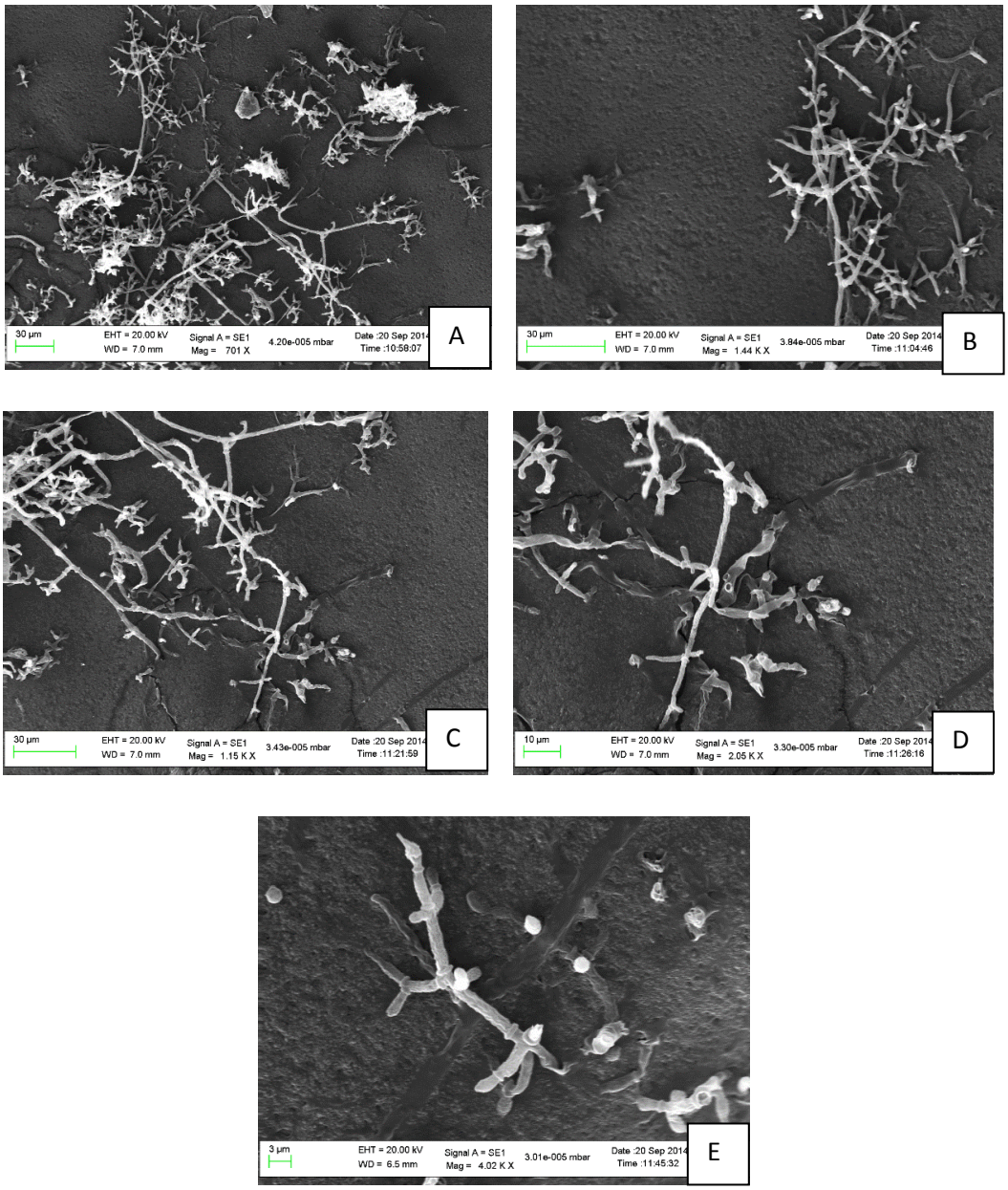


Fig. 4.13: SEM images showing conidiophores, phialides and conidial structures of three days old *Trichoderma asperellum* (A) TaR1 (B) TaR2 (C) TaR3 and *Trichoderma asperelloides* isolates (D) TaR4 (E) TaR5 re-isolated from soil treated Pusa Basmati 1 rice roots.

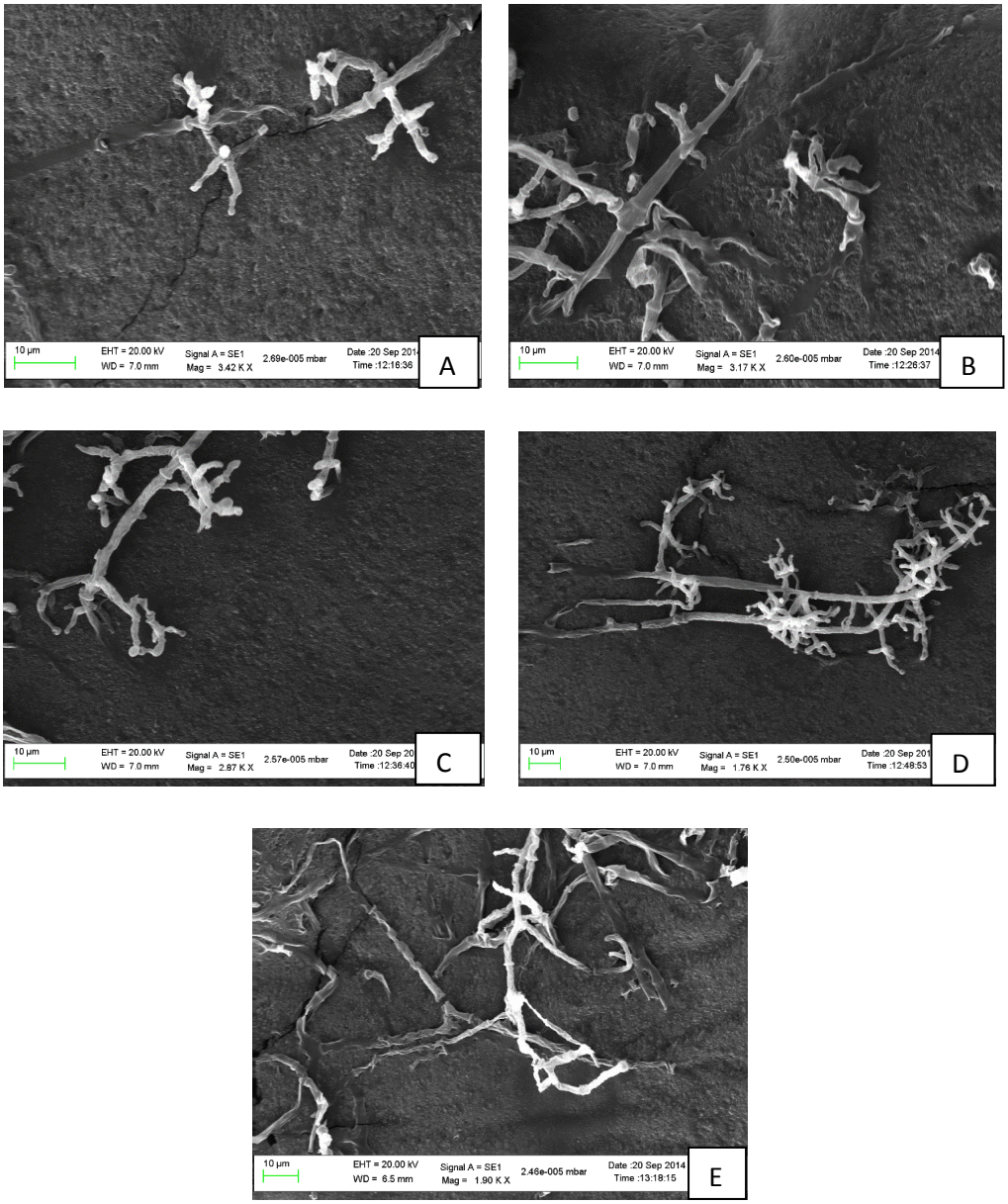


Fig. 4.14: SEM images showing conidiophores, phialides and conidial structures of three days old *Trichoderma asperellum* (A) TaR1 (B) TaR2 (C) TaR3 and *Trichoderma asperelloides* isolates (D) TaR4 (E) TaR5 re-isolated from seed treated Pusa Basmati 1 rice roots.

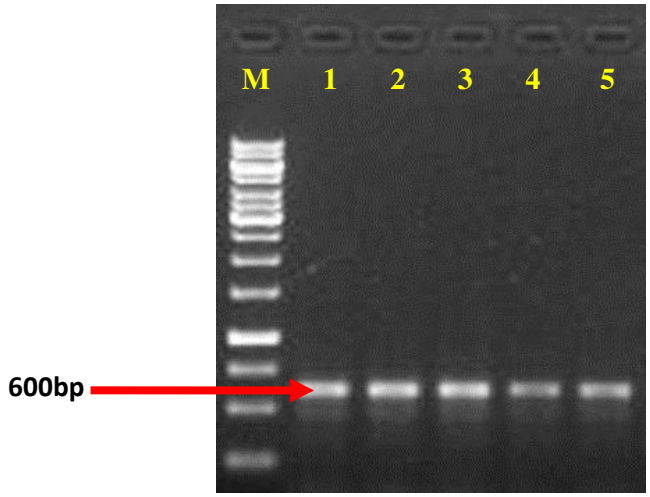


Fig. 4.15: ITS region amplification of re-isolated *Trichoderma* isolates from soil treated Pusa Basmati 1 rice roots using primer pair ITS1 and ITS4. M: 1Kb DNA ladder; Lane 1: TaR1; Lane 2: TaR2; Lane 3: TaR3; Lane 4: TaR4 and Lane 5: TaR5.

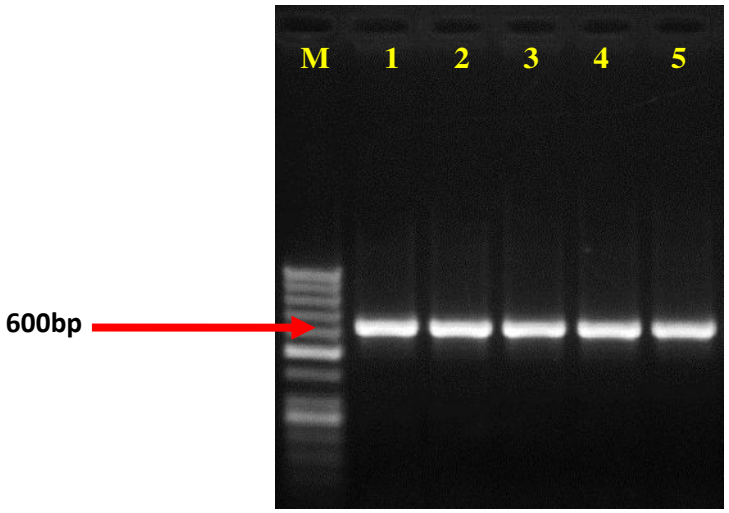


Fig. 4.16: ITS region amplification of re-isolated *Trichoderma* isolates from soil treated Pusa Basmati 1 rice roots using primer pair ITS1 and ITS4. M: 1Kb DNA ladder; Lane 1: TaR1; Lane 2: TaR2; Lane 3: TaR3; Lane 4: TaR4 and Lane 5: TaR5.

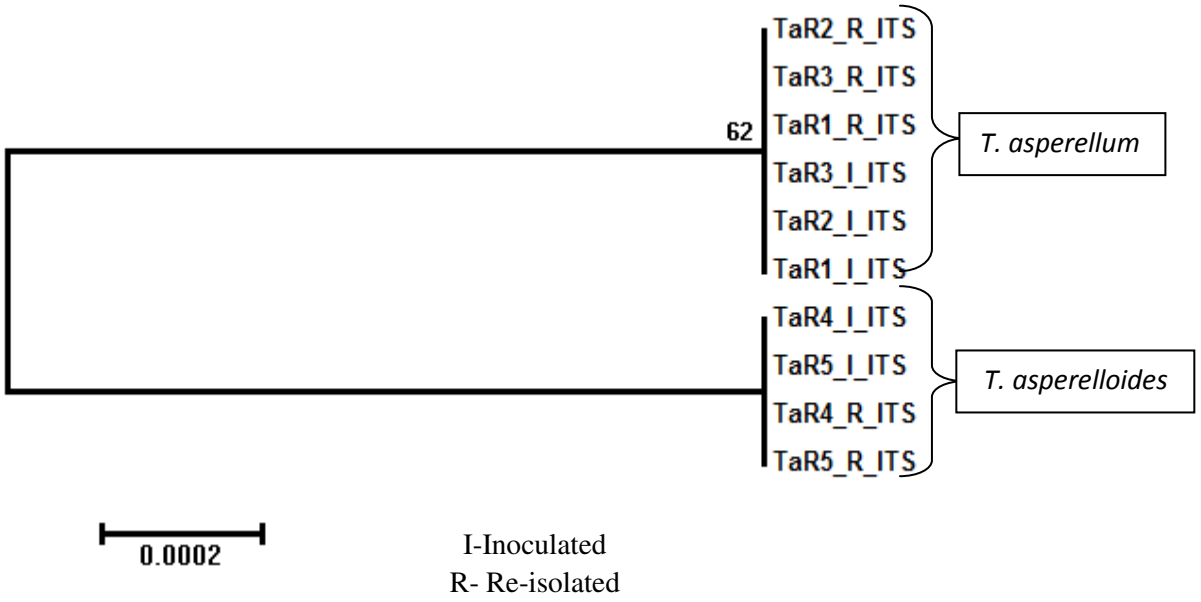


Figure 4.17: Phylogenetic relationships of two different *Trichoderma* spp. inferred by analysis of ITS sequences re-isolated from Pusa Basmati 1 rice roots from both soil and seed treatment. The Maximum Likelihood tree was constructed using two-parameter model implemented in the MEGA 6.06 program.

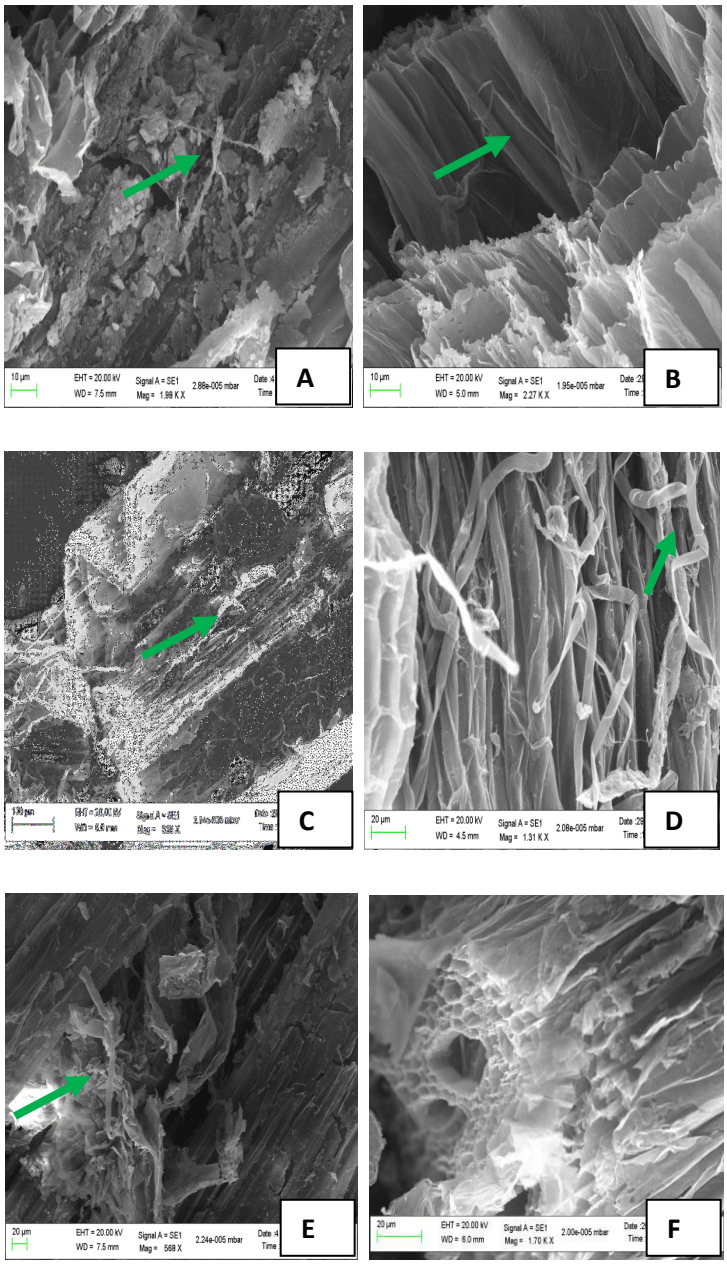


Fig 4.18: SEM photos showing internal colonization of Pusa Basmati 1 soil treated rice roots with re-isolated *Trichoderma asperellum:* (A) TaR1, (B) TaR2 (C) TaR3; *Trichoderma asperelloides* isolates: (D) TaR4 (E) TaR5 and (F) Control image showing no mycelial growth isolated from *Trichoderma* untreated Pusa Basmati 1 rice roots.

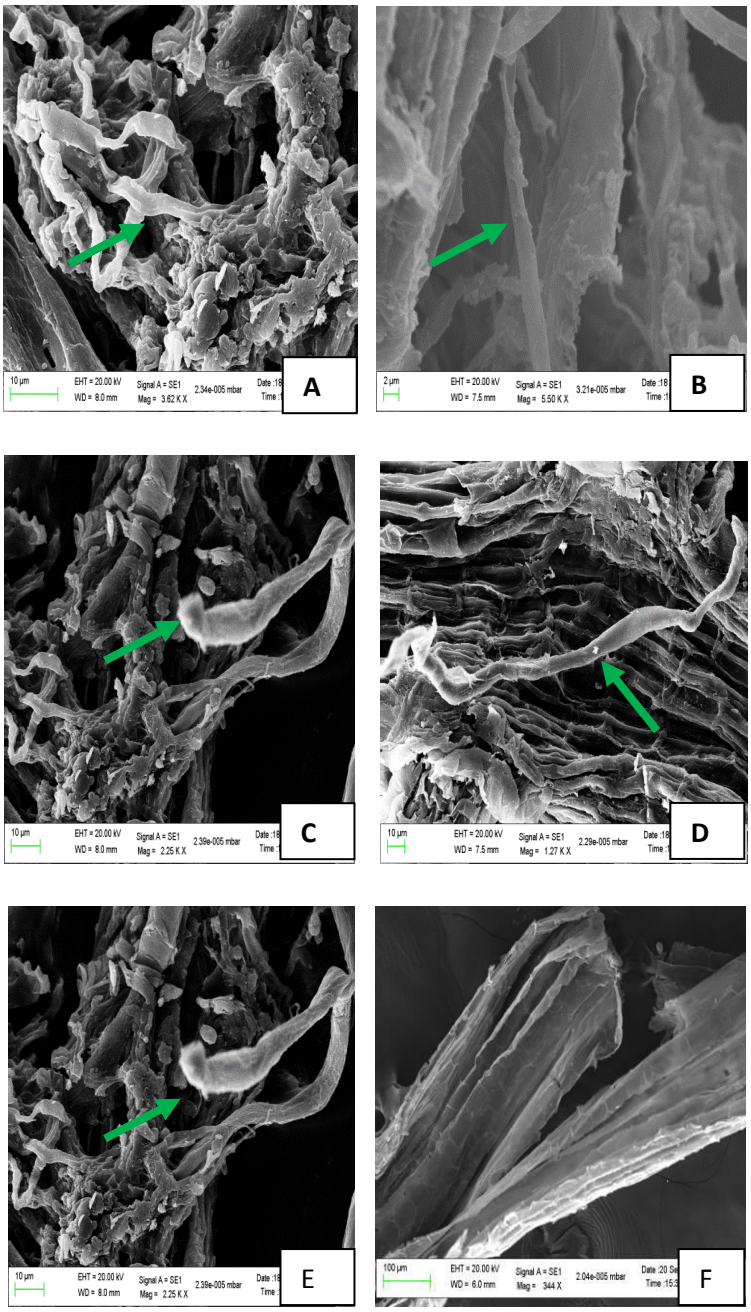


Fig 4.19: SEM photos showing internal colonization of Pusa Basmati 1 seed treated rice roots with re-isolated *Trichoderma asperellum:* (A) TaR1, (B) TaR2 (C) TaR3; *Trichoderma asperelloides* isolates: (D) TaR4 (E) TaR5 and (F) Control image showing no mycelial growth isolated from *Trichoderma* untreated Pusa Basmati 1 rice roots.

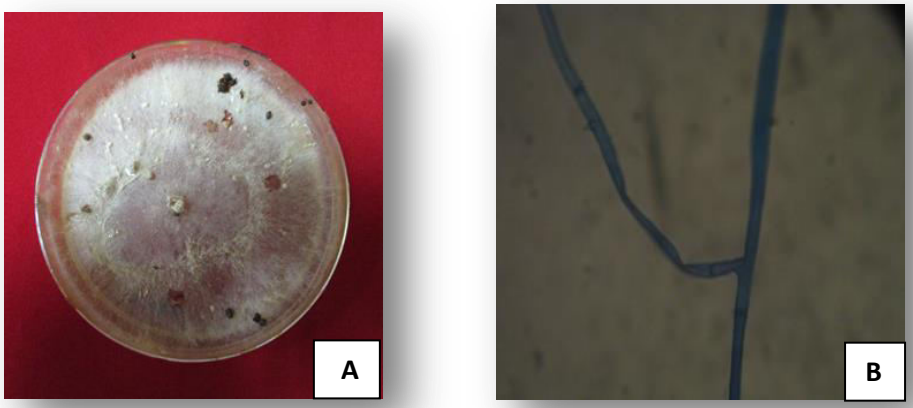


Fig. 4.20: (A) PDA plates showing *Rhizoctonia solani* in pure culture (B) Light microscopy image of *Rhizoctonia solani* mycelium at 40X magnification

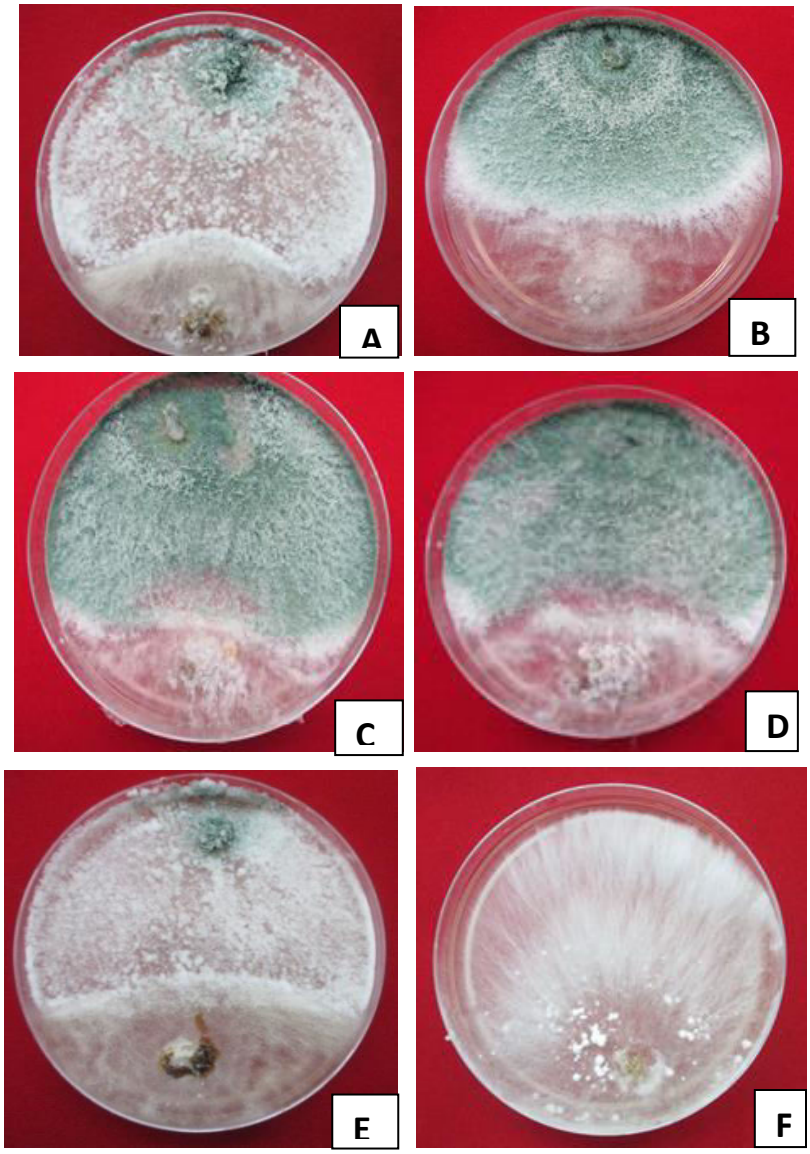


Fig. 4.21: PDA plates showing the dual culture assay between *Trichoderma asperellum*: (A) TaR1 (B) TaR2 (C) TaR3 and *Trichoderma asperelloides* isolates: (D) TaR4 (E) TaR5 against *Rhizoctonia solani* F) Control plate having only *Rhizoctonia solani* culture.

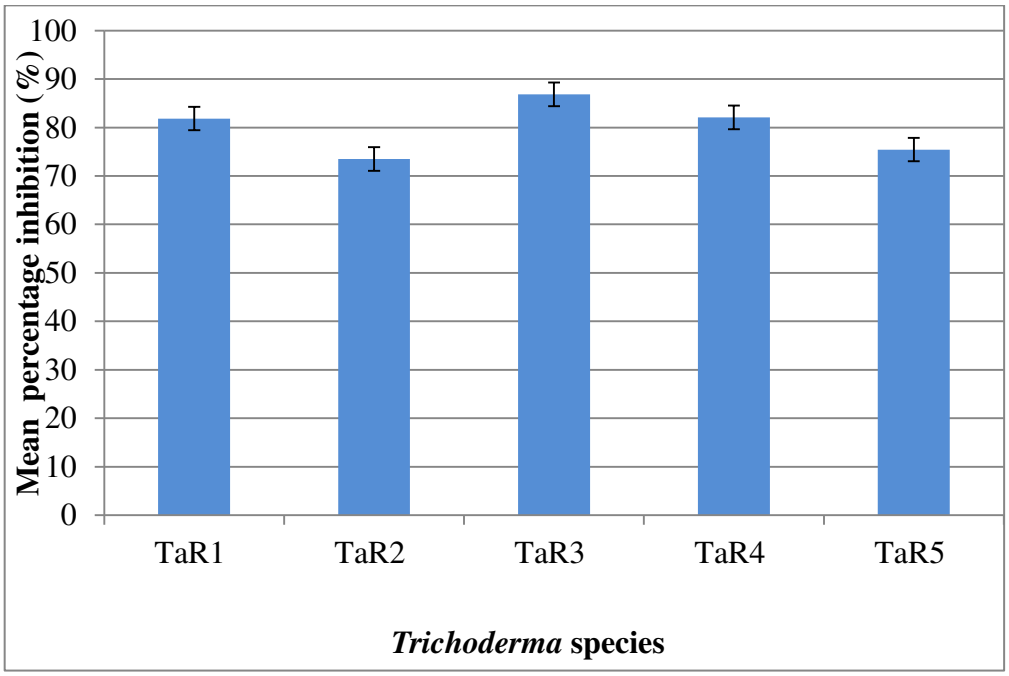


Fig. 4.22: Graphical representation of mean percentage inhibition of *Rhizoctonia solani* by five different *Trichoderma* isolates.

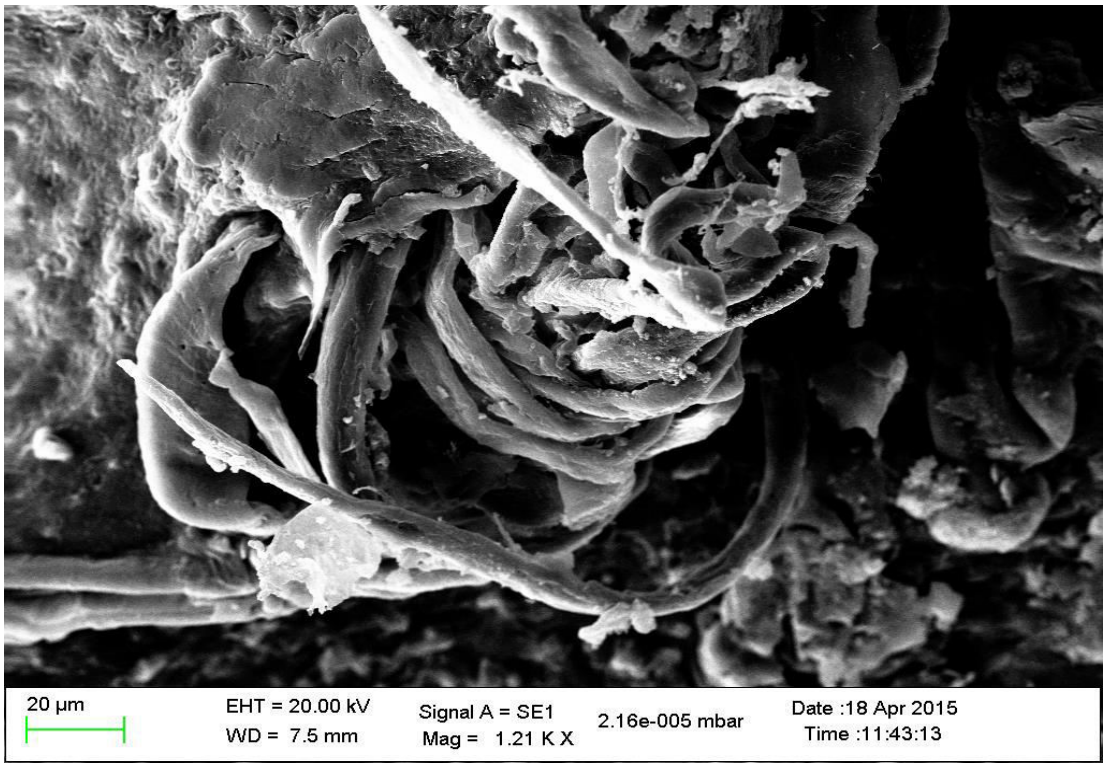


Fig. 4.23: SEM image showing the mycoparasitism of *Rhizoctonia solani* by *Trichoderma asperellum* isolate (TaR3)

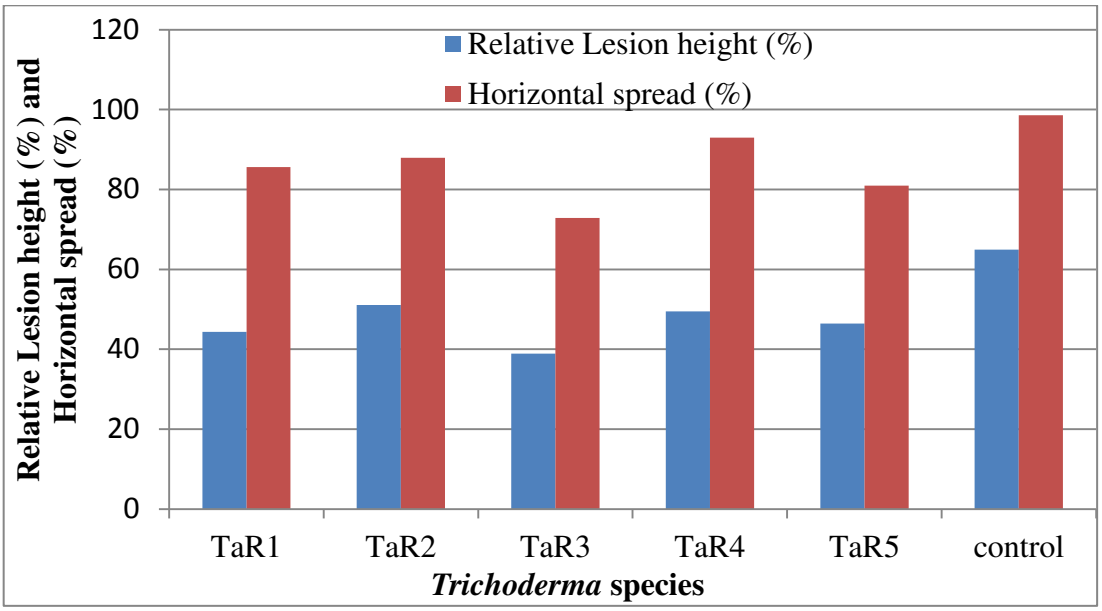


Fig. 4.24: Graphical representation of the effect of five different *Trichoderma* isolates on Relative Lesion Height (%) and horizontal spread (%) of *Rhizoctonia solani* in soil treated Pusa Basmati 1 rice plants.

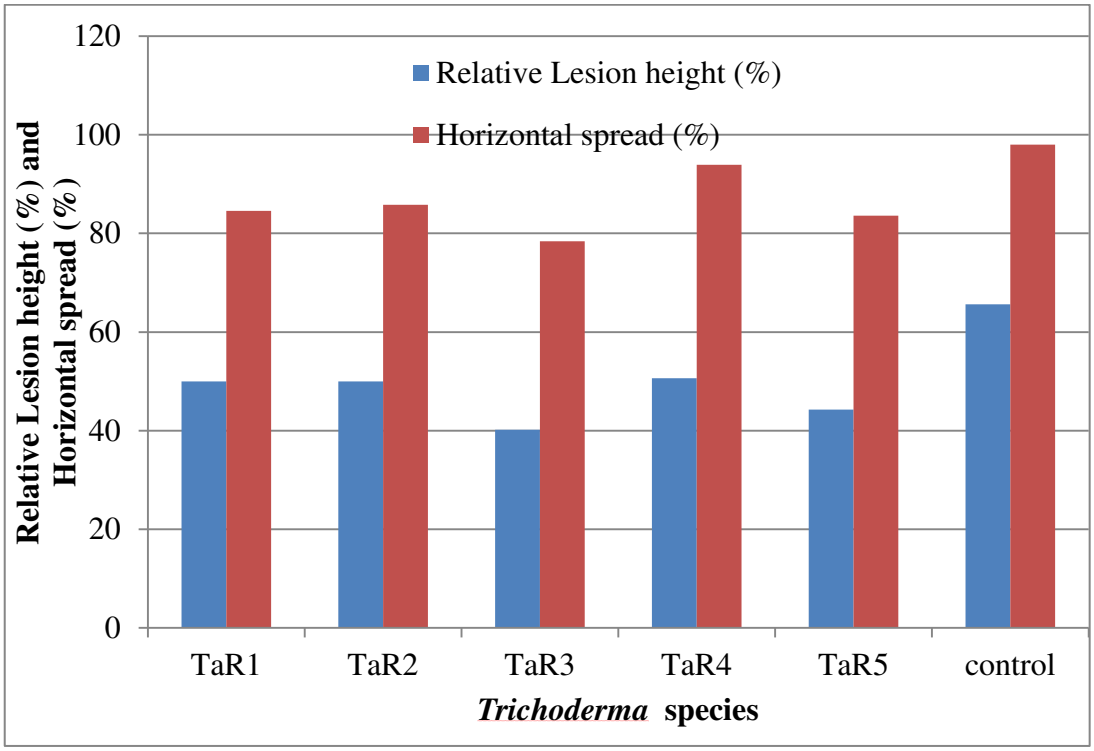


Fig. 4.25: Graphical representation of the effect of five different *Trichoderma* isolates on Relative Lesion Height (%) and horizontal spread (%) of *Rhizoctonia solani* in seed treated Pusa Basmati 1 rice plants.

Table 4.1: Colonization frequency of endophytes isolated from different plant tissues of Pusa basmati-1 (PB1)

Endophytic fungi	**Plant tissues**						**Total isolates**
	Leaves	**CF (%)**	**Stems**	**CF (%)**	**Roots**	**CF (%)**	
Fusarium	5	**16.66**	3	**10**	2	**6.66**	32.32
Aspergillus	5	**13.33**	5	**13.33**	6	**20**	46.66
Alternaria	4	**13.33**	5	**16.66**	1	**3.33**	33.32
Penicillium	6	**20**	1	**3.33**	3	**10**	33.33

Table 4.2 Measurement of mean inhibition of *Rhizoctonia solani* against *Trichoderma* isolates in dual culture

***Trichoderma* spp.**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**Mean inhibition (mm)**
TaR1	10.7	11.3	11	10.9	11.5	10.5	10.983
TaR2	15.9	16.2	16	15.8	16.1	16.3	16.05
TaR3	7.9	8.2	8.1	8	7.5	8.1	7.967
TaR4	10.5	10	9	12.5	12	11	10.833
TaR5	15	15.3	15.7	14.5	14.7	14	14.867
Control	60.5	60.3	61.2	60.5	60	60.7	60.533
CD (0.05%)							0.754
SE (d)							0.367

Table 4.3 Measurement of percentage inhibition (%) in dual culture

Sl. No.	*Trichoderma* isolates	Radial growth in control in mm (C)	Average radial growth in test petri dish plate in mm (T)	C-T	Mean Percentage inhibition (C-T/C*100) in %
1	TaR1	60.533	10.983	49.55	81.86
2	TaR2	60.533	16.05	44.483	73.49
3	TaR3	60.533	7.967	52.566	86.84
4	TaR4	60.533	10.833	49.7	82.1
5	TaR5	60.533	14.867	45.666	75.44

Table 4.4: *Rhizoctonia solani* disease measurement in terms of RLH (%) and horizontal spread (%) for the soil treated Pusa Basmati-1 (PB1) plants

Trichoderma Isolates	Mean RLH (%) at 10 Days after inoculation (DAI)	Mean RLH (%) at 20 Days after inoculation (DAI)	Mean Horizontal spread (%) at 20 days after inoculation
TaR1	8.067	38.9	92.933
TaR2	8.933	49.5	80.967
TaR3	8.667	46.4	85.567
TaR4	7.9	44.367	87.933
TaR5	9.467	51.1	72.867
Control	11.867	64.967	99.2
CD (0.005%)	1.379	6.732	4.469
SE (d)	0.626	3.056	2.029

Table 4.5: *Rhizoctonia solani* disease measurement in terms of RLH (%) and horizontal spread (%) for the seed treated Pusa Basmati-1 (PB1) plants

***Trichoderma* Isolates**	**Mean RLH (%) at 10 Days after inoculation (DAI)**	**Mean RLH (%) at 20 Days after inoculation (DAI)**	**Mean Horizontal spread (%) at 20 days after inoculation**
TaR1	8.6	50	93.933
TaR2	8.77	47.6	83.633
TaR3	8.47	40.2	84.567
TaR4	9.6	50.63	85.8
TaR5	7.17	44.3	78.4
Control	12.6	65.63	98.033
CD (0.005%)	1.747	6.557	3.916
SE (d)	0.793	2.977	1.777

Ahn, S. W., DenaDela, R. C., Candole, B. L. and Mew, T. W. (1986). A new scale for rice sheath blight disease assessment. *International Rice Research Newsletter*. **11**: 17.

Aishwarya, S., Venkateswarlu, N., Chandramouli, K. and Vijaya, T. (2014). Role of endophytic fungi in restoration of heavy metal contaminated soils. *Indo American Journal of Pharmaceutical Research*. **4**(11): 5427-5436.

Akrami, M., Sabzi, M., Mehmandar, F. B. and Khodadadi, E. (2012). Effect of seed Treatment with *Trichoderma harzianum* and *Trichoderma asperellum* species for controlling *Fusarium* rot of common bean. *Annals of Biological Research*. **3**(5): 2187-2189

Ali, H. Z. and Nadarajah, K. (2013). Evaluating the efficacy of *Trichoderma* isolates and *Bacillus subtilis* as biological control agents against *Rhizoctonia solani*. ***Research Journal of applied Sciences.*** **8**(1): 72-81.

Amin, N. (2013). Diversity of endophytic fungi from root of Maize var. Pulut (waxy corn local variety of South Sulawesi, Indonesia) .*International Journal of Current Microbiology and Applied Sciences.* **2**(8): 148-154.

Anees, M., Tronsmo, A., Edel-Hermann, V., Hjeljord, L. G., Héraud, C. and Steinberg, C. (2010). Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. *Fungal Biology.* **114**(9): 691-701.

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

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

Bailey, B. A., Strem, M. D. and Wood, D. (2009).*Trichoderma* species form endophytic associations within *Theobroma cacao* trichomes. *Mycological Research.* **113**: 1365-1376

Benítez, T., Rincón, A. M., Limón, M. C. and Codón, A. C. (2004). Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology*. **7**: 249-260

Bhaktavatsalam, G., Satyanarayana, K., Reddy, A. P. K. and John, V. T. (1978). Evaluation of sheath blight resistance in rice. International Rice Research Newsletter. 3: 9-10.

Bhuvaneswari, V. and Raju, S. K. (2012). Efficacy of New Combination Fungicide against Rice sheath blight caused by *Rhizoctonia solani* (Kuhn). *Journal of Rice Research.* **5**: 1-2.

Brotman, Y., Landau, U., Cuadros-Inostroza, A., Takayuki, T., Fernie, A. R., Chet, I., Viterbo, A., and Willmitzer, L. 2013. Trichoderma-plant root colonization: escaping early plant defense responses and activation of the antioxidant machinery for saline stress tolerance. PLoS Pathog.9:e1003221. Published online.

Butler, E. J. (1918). Fungi and Diseases in Plants. Thacker Spink and Co., Calcutta. 410p.

Chahal, S. S. (2005). Disease scenario in rice during post revolution era in Punjab. *Plant Disease Research.* **20**(1): 1-11.pp 491–509.

Chatterton, S., Jayaraman, J. and Punja, Z. K. (2008).Colonization of cucumber plants by the biocontrol fungus *Clonostachysrosea* f. *catenulate*. Biological Control. 46 : 267–278.

Chaverri, P., Gazis, R. O. and Samuels, G. J. (2011). *Trichoderma amazonicum*, a new endophytic species on *Hevea brasiliensis* and *H. guianensis* from the Amazon basin. *Mycologia*. **103**(1): 139–151.

Chien, C. C. (1979). Occurrence and influence of rice diseases on yield in the first and second crops in Taiwan. In the causes of low yield of second rice crops in Taiwan and measurement for crop improvement. Tapei, Taiwan, National Science Council. 177-189.

Cotxarrera, L., Trillas-Gay, M. I., Steinberg, C. and Alabouvette, C. (2002). Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress Fusarium wilt of tomato. *Soil Biology and Biochemistry.* **34**: 467–476

Crozier, J., Thomas, S. E., Aime, M. C., Evans, H. C. and Holmes, K. A. (2006). Molecular characterization of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao. Plant Pathology.* **55**: 783–791

Cuevas, V. C. (2006). Soil Inoculation with *Trichoderma pseudokoningii* Rifai Enhances Yield of Rice. *Philippine Journal of Science.* **135**(1): 31-37

De França, S. K. S., Cardoso, A. F., Lustosa, D. C., Ramos, E. M. L. S., De Filippi, M. C. C. and Da Silva, G. B. (2014). Biocontrol of sheath blight by *Trichoderma asperellum* in tropical lowland rice. *Agronomy for Sustainable Development.* **35**: 317-324

De los Santos-Villalobos, S., Guzmán-Ortiz, D. A., Gómez-Lim, M. A., Délano-Frier, J. P., De-Folter, S., García, P. S. and Peña-Cabriales, J. J. (2013). Potential use of *Trichoderma asperellum* (Samuels, Liechfeldt et Nirenberg) T8a as a biological control agent against anthracnose in mango (*Mangifera indica* L.). *Biological Control.* **64**: 37–44

Devi, P., Prabhakaran, N., Kamil, D., Pandey, P. and Borah, J. L. (2012). Characterization of Indian native isolates of *Trichoderma* spp. and assessment of their bio-control efficiency against plant pathogens. *African Journal of Biotechnology.* **11**(85):15150-15160.

Doley,K.,Dudhane,M.,Borde,M.,Jite,P.K.(2014).Effects of *glomus fasciculatum* and *Trichoderma asperelloides* in roots of groundnut (cv. western-51) against pathogen *Sclerotium rolfsii*.*Int. J. Phytopathol*.**3**(2): 89-100

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., Mukherjee, P. K., Zeilinger, S., Grigoriev, I. V. and Kubicek, C. P. (2011). Trichoderma: the genomics of opportunistic success. *Natural Review of Microbiology.* **9**: 749–759.

Dutta, D., Puzari , K. C., Gogoi, R. and Dutta, P.(2014). Endophytes: exploitation as a tool in plant protection. *Brazilian Archives of Biology and Technology*. **57**(5): 621-629.

ElKomy, M. H., Saleh, A. A., Eranthodi, A. and Molan, Y. Y. (2015). Characterization of Novel *Trichoderma asperellum* isolates to select effective biocontrol agents against tomato *Fusarium* Wilt. *Plant Pathol*ogy. **31**(1): 50-60.

Fisher, P. J. and Petrini, O. (1992). Fungal saprobes and pathogens as endophytes of rice *(Oryza sativa* L.) *New Phytologist.* **120**: 137-143.

García, A., Rhoden, S. A., Filho, C. J. R., Nakamura, C. V. and Pamphile, J. A. (2012). Diversity of foliar endophytic fungi from the medicinal plant *Sapindus saponaria* L . and their localization by scanning electron microscopy. *Biological Research.* **45**: 139-148

Gazis, R. and Chaverri, P. (2010). Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. *Fungal Ecology*. **3**: 240-254.

Gond, S. K., Verma, V. C., Mishra, A., Kumar, A. and Kharwar, R. N. (2010). Management of Fungal Plant Pathogens, Chapter: Role of fungal endophytes in plant protection, Publisher: CAB International, UK, pp 183-200.

Grondona, I., Hermosa, R., Tejada, M., Gomis, M. D., Mateos, P. F., Bridge, P. D., Monte, E. and Garcia-acha, I. (1997). Physiological and biochemical characterization of *Trichoderma harzianum*, a biological control agent against soilborne fungal pathogens. *Applied and Environmental Microbiology.* **63**(8): 3189-3198.

Gupta,K.J., Mur,L.A.J., Brotman,Y.(2014).*Trichoderma asperelloides* Suppresses Nitric Oxide Generation Elicitedby *Fusarium oxysporum* in *Arabidopsis* Roots.*MPMI* **27**(4): 307–314

Hallmann, J., Berg, G. and Schulz, B. (2006). Isolation Procedures for Endophytic Microorganisms. Microbial Root Endophytes (Eds.). *In Soil Biology*. **9**: 299-319.

Hanada, R. E., Jorge souza, T., Pomella, A. W. V., Hebbar, K. P., Pereira, J. O., Ismaiel, A. and Samuels, G. J. (2008). *Trichoderma martiale* sp. nov., a new endophyte from sapwood of *Theobroma cacao* with a potential for biological control. *Mycological research*. **112**: 1335-1343.

Hori, M. and Anraku, M. (1971). Studies on the forecasting techniques of sheath blight of rice plant. Special Bulletin. Yamaguchi Agricultural Experiment Station No. 24, 138 p. House, Farnhain Royal, Slough.

Hyde, K. D. and Soytong, K. (2008).The fungal endophyte dilemma. *Fungal Diversity.* **33**: 163-173.

IRRI, (2001). Annual Reports for (2000). International Rice Research Institute, P.O. Box 933. Manila, Philippines. p55.

Jeewon, R., Ittoo, J., Mahadeb, D., Jaufeerally-Fakim, Y., Wang, H. and Liu, A. (2013). DNA Based Identification and Phylogenetic Characterisation of Endophytic and Saprobic Fungi from *Antidesma madagascariense*, a Medicinal Plant in Mauritius. *Journal of Mycology.* **2013**: 1-10. http://dx.doi.org/10.1155/2013/781914.

Jegathambigai, V., Wilson, W. R. S. and Wijesundera, R. L. C. (2009). *Trichoderma* as a Seed Treatment to Control *Helminthosporium* Leaf Spot Disease of *Chrysalidocarpus lutescens. World Journal of Agricultural Sciences*. **5**(6): 720-728.

Kannaiyan, S. and Prasad, N. N. (1976). Sheath blight disease of rice in Tamil Nadu. *Kisan World***. 3**: 17.

Khalili, E., Sadravi, M., Naeimi, S. and Khosravi, V. (2012). Biological control of rice brown spot with native isolates of three *Trichoderma* species. *Brazilian Journal of Microbiology.* **43**(1): 297–305.

Khan, S. A., Hamayun, M., Khan, A. L., Lee, I., Shinwari, Z. K. and Kim, J. (2012). Isolation of plant growth promoting endophytic fungi from dicots inhabiting coastal sand dunes of Korea. *Pakistan Journal of Botany*. **44**(4): 1453-1460

Kohli, C. K. (1966). Pathogenicity and host range studies on the paddy sheath blight pathogen (*Rhizoctonia solani* Kuhn.). *Journal of Research.* **3**: 37-40.

Kozaka, T. (1970). *Pelliculuria* sheath blight of rice plants. *Japan Agricultural Research Quarterly*. 5: 12-16.

Krishnamurthy, J., Samiyappan, R., Vidhyasekaran, P., Nakkeeran, S., Rajeswari, E., Raja, J. A. J. and Balasubramanian, P. (1999). Efficacy of *Trichoderma* chitinases against *Rhizoctonia solani* the rice sheath blight pathogen. *Journal of Biosciences.* **24**: 207–213.

Kullnig-Gradinger, C. M., Szakacs, G. and Kubicek, C. P. (2002). Phylogeny and evolution of the genus *Trichoderma*: a multigene approach. *Mycological Research.* **106**: 757-767.

Kumar, K. V. K., Reddy, M. S., Kloepper, J. W., Lawrence, K. S., Groth, D. E. and Miller, M. E. (2009).Sheath blight disease of rice (*Oryza sativa* L.) An overview. *Biosciences, Biotechnology Research Asia.* **6**(2): 465-480

Kumaresan, V. and Suryanarayanan, T. S. (2002). Endophyte assemblages in young, mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. *Fungal Diversity.* **9**: 81-91

Lakshman, H. C. and. Kurandawad, J. M. (2013). Diversity of the endophytic fungi isolated from spilanthesacmellalinn. - a promising medicinal plant. *International Journal of Pharma and Bio Sciences.* **4**(2): 1259-1266.

Lee, F. N. and Rush, M. C. (1983). Rice sheath blight: a major rice disease. *Plant Disease.* **67**: 829–832.

Lehtonen, P. T., Helander, M., Siddiqui, S. A., Lehto, K. and Saikkonen, K. (2006). Endophytic fungus decreases plant virus infections in meadow ryegrass (*Lolium pratense*). *Biology Letters.* **2**: 620–623.

Macia -Vicente, J. G., Jansson, H., Abdullah, S. K., Descals, E., Salinas, J. and Lopez-Llorca, L. V. (2008). Fungal root endophytes from natural vegetation in Mediterranean environments with special reference to *Fusarium* spp. *FEMS Microbiology Ecology.* **64:** 90–105.

Mathivanan, N., Prabavathy, V. R. and Vijayanandraj, V. R. (2005). Application of talc formulations of *Pseudomonas fluorescens* Migula and *Trichoderma viride* Pers. Ex S.F. gray decrease the sheath blight disease and enhance the plant growth and yield in rice. *Journal of Phytopathology*. **153**: 697–701.

Mathur, K. C., Reddy, P. R., Rajamani, S. and Moorthy, B. T. S. (1999). Integrated pest management in rice to improve productivity and sustainability. *Oryza*. **36**(3): 195-207.

Mishra, Y., Singh, A., Batra, A. and Sharma, M. M. (2014). Understanding the Biodiversity and Biological Applications of Endophytic Fungi: A Review. *Journal of Microbial and Biochemical Technology.* **S8**: 004.

Miyake, I. (1910). Studiezn uber die Pilze dor Reisflanze in Japan. *Journal of the College of Agriculture,* Tokyo. **2**: 237-276.

Morakotkarn, D., Kawasak, H. and Seki, T. (2007). Molecular diversity of bamboo-associated fungi isolated from Japan. *FEMS Microbiology Letters*. **266**: 10–19.

Mulaw, T. B., Druzhinina, I. S., Kubicek, C. P. and Atanasova, L. (2013). Novel Endophytic *Trichoderma* spp. isolated from Healthy *Coffea arabica* Roots are Capable of Controlling Coffee Tracheomycosis. *Diversity*. **5**: 750-766.

Mwangi, M. W., Monda, E. O., Okoth, S. A. and Jefwa, J. M. (2011). Inoculation of tomato seedlings with *Trichoderma harzianum* and Arbuscular Mycorrhizal Fungi and their effect on growth and control of wilt in tomato seedlings. *Brazilian Journal of Microbiology.* **42**(2): 508-513.

Naeimi, S., Okhovvat, S. M., Javan-Nikkhah, M., Vágvölgyi ,C., Khosravi ,V. and Kredics, L. (2010). Biological control of *Rhizoctonia solani* AG1-1A, the causal agent of rice sheath blight with *Trichoderma* strains. *Phytopathologia Mediterranea.* **49**: 287–300

Nair, M. C., Balakrishana, B., Pillai, M. V. R., Gokulpalan, C. and Aiyar, R. S. (1983). New strategies for control of sheath blight of rice. *Indian Phytopathology*. **36**: 187.

Nandi, S. (1980). Studies on sheath blight disease of rice Ph.D. Thesis, Utkal University, Bhubaneswar, Orissa.

Nalini, M. S., Sunayana, N. and Prakash, H. S. (2014). Endophytic Fungal Diversity in Medicinal Plants of Western Ghats, India. *International Journal of Biodiversity*.

Narisawa, K., Ohki, K. T. and Hashiba, T. (2000). Suppression of clubroot and *Verticillium* yellows in Chinese cabbage in the field by the root endophytic fungus, *Heteroconium chaetospira. Plant Pathology.* **49**(1): 141–146.

Navaneetha, T., Prasad, R. D. and Venkateswara, R. L. (2015). Liquid Formulation of *Trichoderma* Species for Management of Gray mold in Castor (*Ricinus communis* L.) and Alternariaster Leaf Blight in Sunflower (*Helianthus annuus* L.). *Journal of Biofertilizers and Biopesticides.* **6**: 149.

Nayak, P., Kameswara Row, K. V. S. R. and Sridhar, R. (1979). Host range of *Rhizoctonia solani,* the causal organism of sheath blight of rice. *Indian Phytopathology.* **32**: 604-605.

Ommati, F. and Zaker, M. (2012). Evaluation of some *Trichoderma* isolates for biological control of potato wilt disease (*Fusarium oxysporum*) under lab and green house conditions. *Journal of Crop Protection.* **1**(4): 279-286.

Ou, S. H. (1973). A handbook of rice disease in the tropics. International Rice Research Institute, Los Banos, Philippines, p58.

Padwick, G. W. (1950). *Manual of rice diseases*. CMI, New Surrey England. 198 p.

Palo, M. A. (1926). *Rhizoctonia* diseases of rice. I. A study of the disease and of the influence of certain conditions upon the viability of the sclerotial bodies of causal fungus. *Philippine Agriculturist.* **15**: 361-375.

Pandya, J. R., Sabalpara, A. N. and Chawda, S. K. (2011). *Trichoderma*: a particular weapon for biological control of phytopathogens. *Journal of Agricultural Technology.* **7**(5): 1187-1191.

Paracer, C. S. and Chahal, D. S. (1963). Sheath blight of rice caused by *R. solani*- A new record in India. *Current science*. **32**: 328-329.

Park, M. and Bertus, L. S. (1932). Sclerotial diseases of rice in Ceylon I. *Rhizoctonia solani* Kuhn. *Ceylon Journal of Science*. **11**: 319-331.

Patel, C., Yadav, S., Rahi, S. and Dave A. (2013).Studies on Biodiversity of fungal endophyes of indigenous Monotaceous and Dicotaceous plants and evaluation of their enzymatic Potentialities. *International Journal of Scientific and Research Publications.* **3**:1-5.

Petrini, O. (1991). Fungal endophytes of tree leaves. In: *Microbial ecology of the leaves* (eds. N.J. Fokkema and 1. van den Heuvel). Cambridge University Press, Cambridge: 185187.

Petrini, O., Fisher, P. J. and Petrini, L. E. (1992). Fungal endophytes of bracken (*Pteridium aquilinum*), with some reflections on their use in biological control. *Sydowia.* **44**: 282-293.

Prasad, B. N. and Kumar, M. R. (2011). Comparative Efficacy of Different Isolates of *Trichoderma* Spp. against *Rhizoctonia Solani*, incitant of Sheath Blight of Rice. *Indian Journal of Fundamental and Applied Life Sciences*. **1**(3): 107-111.

Prisa, D., Sarrocco, S., Forti, M., Burchi, G. and Vannacci, G. (2013). Endophytic ability of *Trichoderma* spp. as inoculants for ornamental plants innovative substrates. *Biocontrol of plant pathogens in sustainable agriculture Bulletin.* **86**: 169-174.

Rangaswamy, S. and Mahadevan, S. (1998). The disease of crop plants in India. Cheran Publisher, Coimbatore, India. 273-278 p.

Raviraja, N. S., Sridhar, K. R. and Bärlocher, F. (1996). Endophytic aquatic hyphomycetes of roots of plantation crops and ferns from India. *Sydowia*. **48**(1): 152-160.

Riker, A. J. and Riker, R. S. (1936). Introduction to research on plant disease. John S. Swift co. Inc., New York, p117.

Rinu, K., Sati, P. and Pandey, A. (2013). *Trichoderma gamsii* (NFCCI 2177): A newly isolated endophytic, psychrotolerant, plant growth promoting, and antagonistic fungal strain. *Journal of Basic Microbiology*. **00**: 1–10

Rodriguez, R. J., White, Jr. J. F., Arnold, A. E. and Redman, R. S. (2009). Fungal endophytes: diversity and functional roles. *New Phytologist*. **182**(2): 314-330.

Rosmana, A., Samuels, G. J., Ismaiel, A., Ibrahim, E. S., Chaverri, P., Herawati, Y. and Asman, A. (2015). *Trichoderma asperellum*: A Dominant Endophyte Species in Cacao Grown in Sulawesi with Potential for Controlling Vascular Streak Dieback Disease.*Tropical Plant Pathology.* **40**: 19-25.

Roy, A. K. (1979). Sheath blight of rice in India. *Indian Phytopathology*. **46**(3): 197-205.

Roy, A. K. (1979). Susceptibility of rice plants to sheath blight at different stages of growth. *Kavaka.* **7**: 25-26.

Saba, H., Vibhash, D., Manisha, M., Prashant, K. S., Farhan, H. and Tauseef, A. (2012). *Trichoderma* a promising plant growth stimulator and biocontrol agent. *Mycosphere*. **3**(4): 524–531.

Samuels, G. J. (2006). *Trichoderma*: Systematics, the sexual state, and ecology. *Phytopathology.* **96**: 195-206.

Samuels, G. J., Ismaiel, A., Bon, M-C., Respinis, S. D., Petrini, O. (2010). *Trichoderma asperellum* sensu lato consists of two cryptic species. *Mycologia.* **102**: 944-66.

Sandhu, S. S., Kumar, S. and Aharwal, R. P. (2014). Isolation and antibacterial property of endophytic fungi isolated from Indian medicinal plant *Alotropis procera* (linn.) r.br. *world journal of pharmacy and pharmaceutical sciences.* **3**(5): 678-691.

Sant, D., Casanova, E., Segarra, G., Aviles, M., Reis, M. and Trillas, M. (2010). Effect of *Trichoderma asperellum* strain T34 on *Fusarium* wilt and water usage in carnation grown on compost-based growth medium. *Biological Control.* **53**: 291–296.

Santos, A ., García, M., Cotes, A. M. And Villamizar, L. (2012). The effect of the formulation on the shelf-life of biopesticides based on two Colombian isolates of *Trichoderma koningiopsis* Th003 and Trichoderma asperellum Th034. *Revista Iberoamericana de Micología.* **29**(3): 150-6.

Satya, A. K., Babu, P. J., Sowjanya, K. M., Narendra, K., Swathi, J. and Padmaja, M. (2013). T*richoderma spp.* as a microbial antagonist against *Rhizoctonia solani. International Journal of Pharmacy and Pharmaceutical Sciences.* 5(4): 322-325.

Sawada, K. (1912). On the Shirakinibuy of Camphor. Special Bulletin of the Formosan Agricultural experiments Station, No. 4: 805246.

Scherm, B., Schmoll, M., Balmas, V., Kubicek, C. P. and Migheli, Q. (2009). Identification of potential marker genes for *Trichoderma harzianum* strains with high antagonistic potential against *Rhizoctonia solani* by a rapid subtraction hybridization approach. *Current Genetics.* **55**(1): 81-91.

Schulz, B., Wanke, U., Draeger, S. and Aust, H. J. (1993). Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycological Research*. **97**(12): 1447-1450.

Schuster, A. and Schmoll, M. (2010). Biology and biotechnology of *Trichoderma. Applied Microbiology Biotechnology*. **87**: 787–799.

Segarra, G., Casanova, E., Avilés, M. and Trillas, I. (2010). *Trichoderma asperellum* strain T34 controls Fusarium wilt disease in tomato plant in soilless culture through competition for iron. *Microbial Ecology.* **59**(1): 141-149.

Selvi, K. V. and Lakshmi, P. J. (2013). Anticancer potentials of secondary metabolites from endophytes of *Barringtonia acutangula* and its molecular characterization. *International Journal of Current Microbiology and Applied sciences*. **2**(2):44-45.

Shan, T., Lou, J., Gao, S., Zhou, Y., Weibo Sun, W., Chao Luo, C. and Ligang Zhou, L. (2012). Antibacterial activity of the endophytic fungi from a traditional Chinese herb *Paris polyphylla* var. *chinensis.* *African Journal of Microbiology Research.* **6**(14): 3440-3446.

Sharma, N. R., Teng, P. S. and Olivares, P. M. (1990a). Comparison of assessment methods for rice sheath blight disease. *Philippines Phytopathology*. **26**: 20-24.

Sharma, P., Singh, D. and Aggarwal, R. (2013). A practical manual on Biocontrol of plant diseases. Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India.

Sharma, R. K., Kamil, D., Devi, T. P. and Sharma, P. (2014). Practical Manual of Mycology, Indian Agricultural Research Institute, New Delhi, India.

Silva, J. C., Torres, D. B., Lustosa, D. C., Filippi, M. C. C., Silva, G. B. (2012).Rice sheath blight biocontrol and growth promotion by *Trichoderma* isolates from the Amazon. *Amazonian Journal of Agricultural and Environmental Sciences.* **55** (4): 243-250.

Singh, H. K. and Singh, U. D. (2012) Evaluation of vertical and horizontal spread of sheath blight in rice varieties for resistance against *Rhizoctonia solani. International Journal of Agriculture, Environment and Biotechnology.* **5**(4): 367-372.

Singh, N. I., Devi, R. K. and Singh, K. U. (1988). Occurrence of rice sheath blight (ShB) *Rhizoctonia solani* Kuhn. on rice panicles in India. *International Rice Research Newsletter.* **13**: 29.

Singh, R. A. and Pavgi, M. S. (1969). Oriental sheath and leaf spot of rice. *Plant Disease*. **53**: 444-445.

Singh, R. P. (2005). Plant Pathology, Kalyani Publishers, Ludhiana, Kolkata, New Dehli, Chennai, Noida, Hyderabad.

Srinivas, P., Ratan, V., Prakash, A., Patel, A. P. and Madhavi, G. B. (2013). Review on banded leaf and sheath blight of rice caused by *Rhizoctonia solani* KÜHN. *International Journal of Applied Biology and Pharmaceutical Technology.* **4**(4): 178-185.

Stone, J. K., White, J. F. and Polishook, J. D. (2004). Endophytic fungi. In: Measuring and Monitoring Biodiversity of Fungi. Inventory and monitoring methods. G. Mueller, G. Bills, M. Foster (Eds.). Elsevier Academic Press, Boston, MA. pp. 241-270.

Sun,X. and Guo, L. (2012). Endophytic fungal diversity: review of traditional and molecular techniques. *Mycology: An International Journal on Fungal Biology.* **3**(1): 65-76.

Suryanarayanan, T. S. and Vijaykrishna, D. (2001). Fungal endophytes of aerial roots of *Ficus.*

Trillas, M. I., Casanova, E., Cotxarrera, L., Ordovas, J., Borrero, C. and Aviles, M. (2006). Composts from agricultural waste and the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. *Biological Control.* **39**: 32–38.

Usharani, G., Kanchana, D., Jayanthi, M., Saranraj, P. and Sujitha, D.(2013).Evaluation of Certain Resistance Inducing Chemicals against Sheath Blight Incidence in Paddy (Oryza sativa L.). *International Journal Microbiology Research.* **4**(3): 333-335.

Vinale, F., Sivasithamparamb, K., Ghisalbertic, E. L., Marraa, R., Wooa, S. L. and Loritoa, M. (2008). *Trichoderma* plant-pathogen interactions. *Soil Biology and Biochemistry.* **40**: 1-10.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Huckelhoven, R., Neumann, C., Wettstein, D. V., Franken, P. and Kogel, K.H. (2005). The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proceedings of the National Academy of Sciences*. **102**(38): 13386–13391.

Wu, H., Yang, H., You, X. and Li, Y. (2013). Diversity of endophytic fungi from roots of Panax ginseng and their sapon in yield capacities. *SpringerPlus*. **2**: 107.

www.isth.info.in

Xuebiao, P. A. N., Junhuang, Z., Ongxiang, C., Jufeil, L., Hengxiul, L., Haitaol, L., Zibinl, W., Xingyuanl, P. A. N., Rush, M. C. and Lihuang, Z. (1999). Tagging

major quantitative trait loci for sheath blight resistance in a rice variety, Jasmine 85. *Chinese Science Bulletin*. **44**: 19.

Yuan, Z., Chen, Y., Zhang, C., Lin, F. and Chen, L. (2008). *Trichoderma chlorosporum*, a new record of endophytic fungi from *Dendrobium nobile* in China. *Mycosystema.* **27**(4): 608-610.

Zakaria, L., Yaakop, A. S., Salleh, B. and Zakaria, M. (2010). Endophytic Fungi from Paddy. *Tropical Life Sciences Research.* **21**(1): 101–107.

Zhang, H. W., Song, Y. C. and Tan, R. X. (2006). Biology and chemistry of endophytes. *Natural Product Reports.* 23: 753-771.

Appendix A: Sequence for ITS region Inoculated and Re-isolated fungus (Soil treatment)

>Inoculated *Trichoderma asperellumTaR1* ITS gene (GenBank No.-KT001076)

CGGCATTCCTACATGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGACGTGG
ACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAG
ACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGAT
CCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACT
GGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTA
CTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA
GTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGACT
ACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACC
CGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTTG
TAAACTCGGTAATGATCCCTCCGAGCCCCCCCCCAAGAAAAAAT

>Re-isolated*Trichoderma asperellum TaR1* ITS gene

CGGCATTCCTACATGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGACGTGG
ACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAG
ACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGAT
CCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACT
GGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTA
CTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA
GTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGACT
ACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACC
CGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTTG
TAAACTCGGTAATGATCCCTCCGAGCCCCCCCCCAAGAAAAAAT

>Inoculated*Trichoderma asperellumTaR2* ITS gene (GenBank No.-KT001077)

CGGGCATTCCTACCTGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTTACGGACGT
GGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCG
AGACCGCCACTGTATTTAGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCG
ATCCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATA
CTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAT
TACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGA
AAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAAGAAATACGTCCGCGAGGGG
ACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGC
ACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAG
TTGTAAACTCGGTAATGATCCCTCCGACCCCCCCCCCACACGAAAAAA

>Re-isolated*Trichoderma asperellumTaR2* ITS gene

CGGGCATTCCTACCTGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTTACGGACGT
GGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCG
AGACCGCCACTGTATTTAGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCG
ATCCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATA
CTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAT
TACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGA
AAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAAGAAATACGTCCGCGAGGGG
ACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGC
ACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAG
TTGTAAACTCGGTAATGATCCCTCCGACCCCCCCCCCACACGAAAAAA

>Inoculated *Trichoderma asperellumTaR3* ITS gene (GenBank No.-KT001078)

CTTGGGGCATTCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGAC
GTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGG
CGAGACCGCCACTGTATTTAGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGC
CGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAA
TACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCAC
ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT
GAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAAGAAATACGTCCGCGAGG
GGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGAC
GCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGG
AGTTGTAAACTCGGTAATGATCCCTCCGCATCCCCCCCCAAAGGAGAGA

>Re-isolated *Trichoderma asperellumTaR3* ITS gene

CTTGGGGCATTCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGAC
GTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGG
CGAGACCGCCACTGTATTTAGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGC
CGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAA
TACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCAC
ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT
GAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAAGAAATACGTCCGCGAGG
GGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGAC
GCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGG
AGTTGTAAACTCGGTAATGATCCCTCCGCATCCCCCCCCAAAGGAGAGA

>Inoculated *Trichoderma asperelloidesTaR4* ITS gene (GenBank No.-KT001079)

CTTGGGCATTCCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGAC
GTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGG
CGAGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGC
CGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAA
TACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCAC
ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT
GAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGG
GACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACG
CACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGA
GTTGTAAACTCGGTAATGATCCCTCCGACCACCCCTGGACGAAAAT

>Re-isolated*Trichoderma asperelloidesTaR4* ITS gene

CTTGGGCATTCCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGAC
GTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGG
CGAGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGC
CGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAA
TACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCAC
ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT
GAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGG
GACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACG
CACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGA
GTTGTAAACTCGGTAATGATCCCTCCGACCACCCCTGGACGAAAAT

>Inoculated *Trichoderma asperelloides TaR5* ITS gene (GenBank No.-KT001080)

TGGCTGGGCATTCCTACCTGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGA
CGTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCG
GCGAGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACG
CCGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGA
ATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCA
CATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGT
TGAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGG
GGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGAC
GCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGG
AGTTGTAAACTCGGTAATGATCCCTCCGCAGGTACCCCTTAACGGGGA

>Re-isolated*Trichoderma asperelloidesTaR5* ITS gene

TGGCTGGGCATTCCTACCTGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGA
CGTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCG
GCGAGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACG
CCGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGA
ATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCA
CATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGT
TGAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGG
GGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGAC
GCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGG
AGTTGTAAACTCGGTAATGATCCCTCCGCAGGTACCCCTTAACGGGGA

Appendix B:Sequence for ITS region Inoculated and Re-isolated fungus (Seed treatment)

>Inoculated *Trichoderma asperellumTaR1* ITS gene (GenBank No.-KT001076)

GCATTCCTACATGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGACGTGGAC
GCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGAC
CGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGATCC
CCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACTGG
CGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACT
TATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGT
TTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGACTAC
AGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACCCG
GGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTTGTA
AACTCGGTAATGATCCCTCCGAGCCCCCCCCCAAGAAAAAAT

>Re isolated *Trichoderma asperellum TaR1* ITS gene

TTCCTACATGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGACGTGGACGCG
CCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGC
CACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGATCCCCC
GGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACTGGCGG
GCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTAT
CGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTT
GATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGACTACAGA
AAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACCCGGGG
CGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTTGTAAAC
TCGGTAATGATCCCTCCGAGCCCCCCCCCAAGAA

>Inoculated *Trichoderma asperellum TaR2* ITS gene (GenBank No.-KT001077)

GCATTCCTACCTGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTTACGGACGTGGA
CGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGA
CCGCCACTGTATTTAGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGATC
CCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACTG
GCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTAC
TTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG
TTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAAGAAATACGTCCGCGAGGGGACT
ACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACC
CGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTTG
TAAACTCGGTAATGATCCCTCCGACCCCCCCCCCACACGAAAAAA

>Re-isolated *Trichoderma asperellum TaR2* ITS gene

CGGGCATTCCTACCTGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTTACGGACGT
GGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCG
AGACCGCCACTGTATTTAGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCG
ATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATA
CTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAT
TACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGA
AAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAAGAAATACGTCCGCGAGGGG
ACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGC
ACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAG
TTGTAAACTCGGTAATGATCCCTCCGACCCCCCCCCCACACGAAAAAA

>Inoculated*Trichoderma asperellum TaR3* ITS gene (GenBank No.-KT001078)

CTTGGGGCATTCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGAC
GTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGG
CGAGACCGCCACTGTATTTAGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGC
CGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAA
TACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCAC
ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT
GAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAAGAAATACGTCCGCGAGG
GGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGAC
GCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGG
AGTTGTAAACTCGGTAATGATCCCTCCGCATCCCCCCCCCAAAGGAGAGA

>Re-isolated *Trichoderma asperellum TaR3* ITS gene

GGGGCATTCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGACGTG
GACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGA
GACCGCCACTGTATTTAGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGA
TCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATAC
TGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT
ACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAA
AGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAAGAAATACGTCCGCGAGGGGA
CTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCA
CCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGT
TGTAAACTCGGTAATGATCCCTCCGCATCCCCCCCCCAAAGGAGAGA

>Inoculated*Trichoderma asperelloidesTaR4* ITS gene (GenBank No.-KT001079)

GGGCATTCCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGACGTG
GACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGA
GACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGA
TCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATAC
TGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT
ACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAA
AGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGAC
TACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCAC
CCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTT
GTAAACTCGGTAATGATCCCTCCGACCACCCCTGGACGAAAAT

>Re-isolated *Trichoderma asperelloides TaR4* ITS gene

CTTGGGCATTCCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGAC
GTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGG
CGAGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGC
CGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAA
TACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCAC
ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT
GAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGG
GACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACG
CACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGA
GTTGTAAACTCGGTAATGATCCCTCCGACCACCCCTGGACGAAAAT

>Inoculated*Trichoderma asperelloides TaR5* ITS gene (GenBank No.-KT001080)

TGGCTGGGCATTCCTACCTGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGA
CGTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCG
GCGAGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACG
CCGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGA
ATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCA
CATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGT
TGAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGG
GGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGAC
GCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGG
AGTTGTAAACTCGGTAATGATCCCTCCGCAGGTACCCCTTAACGGGGA

>Re-isolated *Trichoderma asperelloides TaR5* ITS gene

CTGGGCATTCCTACCTGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGACGT
GGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCG
AGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCG
ATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATA
CTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAT
TACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGA
AAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGA
CTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCA
CCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGT
TGTAAACTCGGTAATGATCCCTCCGCAGGTACCCCTTAACGGGGA

Appendix C: Sequence for *Tef1*region for inoculated isolates (Soil treatment)

>*TaR1*_Inoculated_*Tef1* gene

ACCGCTCCGGGCGCGGTCATTTTGGCTAGCGCAGTCAGCCTGGGAGGTACCAGTGATCA
TGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGTGGGAAGAGCATGTTAGAATGGC
GGACTTGGAATGTCGATAGCTATAGAGAAGAGTCCAAAACATACCAATGACGGTGACAT
AGTACTTGGGAGTCTCGAACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACGC
TCGGCCTTGAGCTTGCCAAGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGGC
GGCTTCCTATTGATTGAAGCATGATCAGCATAATGAACAAAAAAAGACGACGCGATTGA
GCAAATAGCATGTGGTGGGTGATGGAAGAGGTGCAGTGACGATAGCGGGGTTGCTGCCA
AAAAATTTGACACCCCACTAAAAGCCAAACAAGGCAGCCAGAAAATTTTTGCTGGGCCC
CTCGAGGCGGTGGAAAGACTGGCAAAGCGGGGTGACGCTGGATAAATATAGACAAAAAG
TGAGAGCAGAATTGTCGGGCATATGATTGTGCCAAAAATTGATGGGAAAAGCAGTGCAA
ATGAGCTTACCTTCTCGAACTTCTCGATGGTACGCTTTGTCAATACCACCGCACTGGTA
GATCAAGTGACCAGTCTATTGTGAAAAACGTTACCTGCATGTTGCTACAATCAAGTTGG
CCCCGCCCCTGCCCGACAGAGTCGATAGCAGAGGGTGCTCGAGGTACTTCTATATTACT
CACGGTGGTAGACTTGCTCGGAGTCGACGTGTCTGTTGTATCAAGTCAGTATTCGAGTC
GATCGAAAGCAGATTGCCGGACGATATTACGACGCCCTATAGACGATGATGGTCCAGCA
CAATGTCATTTCGCATGACAACGACAGATCAGTAGTGCTCGCTCGCTGGTAGAATGGGC
ATTACAAGCTACGATCGACGACGACGTTGATGTAGTCATGTCGTCTCCTACCCTAA

>*TaR2*_Inoculated_*Tef1* gene

CATACCCGTCCGGGGGCGGTATCAGGATAGCGCAGTCAGCCTGGGAGGTACCAGTGATC
ATGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGTGGGAAGAGCATGTTAGAATGG
CGGACTTGGAATGTCGATAGCTAGAGAGAAGAGTCCAAAACATACCAATGACGGTGACA
TAGTACTTGGGAGTCTCGAACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACG
CTCGGCCTTGAGCTTGTCAAGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGG
CGGCTTCCTATTGATTGAAGCATGATCAGCATAATGAACAAAAAAAGACGACGCGATTG
AGCAAATAGCATGTGGTGGGTGATGGAAGAGGTGCAGTGGCGATAGCGGGGTTGCTGCC
AAAAAATTTGACACCCCACTAAAAGCCAAACAAGGCAGCCAGAAAATTTTTGCTGTGCC
AAAGGAGGGGTAGGAAGACTGGCAAAGCGGGGTGACGCTGGAAAAAAAAAGACAAAAAC
TGAGAGCAGAATTGTCGGGCATATGATTGTGCCAAAAATTGATGGGAAAAGCAGTGAAA
TGAGCTTACCTTCTCGAACTTCTCGATGGTACGCTTGTCAATACCACCGCACTGGTAGA
TCAAGTGACCAGTCTAATTGGCAAAAACGTTACCTGGATGTTGCTACAATCAAGTTGCC
CCGCGCTGCCCGACAGAGTCGATAGCAGAGGGTGCTCGAGGAATTGGCTATTACTCACG
GGTGTTAGACTTGCCGTAGTCGACGTGTCTGTTGTAGCAAGACAGTATTAGAGTCGATC
GAAAGCAGATTGCCGGAGAAATACGAACCCCCTAGAGACGATGATGGTCAGCAACAATG
TCATTTGCAAGACAACGCACAGAGACGAATGTCCGCTCGTACGGCAGTAAAGCGCATTA
CTAGCTTACGACTGACGACGACCGTTGATGTGAGTCTGGTCGTCCTTACCATATGA

>*TaR3*_Inoculated_*Tef1* gene

GTCCGGGGGCGGTATCAGGATAGCGCAGTCAGCCTGGGAGGTACCAGTGATCATGTTCT
TGATGAAATCACGGTGACCGGGAGCGTCTGTGGGAAGAGCATGTTAGAATGGCGGACTT
GGAATGTCGATAGCTAGAGAGAAGAGTCCAAAACATACCAATGACGGTGACATAGTACT
TGGGAGTCTCGAACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACGCTCGGCC
TTGAGCTTGTCAAGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGGCGGCTTC
CTATTGATTGAAGCATGATCAGCATAATGAACAAAAAAAGACGACGCGATTGAGCAAAT
AGCATGTGGTGGGTGATGGAAGAGGTGCAGTGGCGATAGCGGGGTTGCTGCCAAAAAAT
TTGACACCCCACTAAAAGCCAAACAAGGCAGCCAGAAAATTTTTGCTGTGCCAAAGGAG

GGGTAGGAAGACTGGCAAAGCGGGGTGACGCTGGAAAAAAAAAGACAAAAACTGAGAGC
AGAATTGTCGGGCATATGATTGTGCCAAAAATTGATGGGAAAAGCAGTGAAATGAGCTT
ACCTTCTCGAACTTCTCGATGGTACGCTTGTCAATACCACCGCACTGGTAGATCAAGTG
ACCAGTCTAATTGGCAAAAACGTTACCTGGATGTTGCTACAATCAAGTTGCCCCGCGCT
GCCCGACAGAGTCGATAGCAGAGGGTGCTCGAGGAATTGGCTATTACTCACGGGTGTTA
GACTTGCCGTAGTCGACGTGTCTGTTGTAGCAAGACAGTATTAGAGTCGATCGAAAGCA
GATTGCCGGAGAAATACGAACCCCCTAGAGACGATGATGGTCAGCAACAATGTCATTTG
CAAGACAACGCACAGAGACGAATGTCCGCTCGTACGGCAGTAAGCGCATTACTAGCTTA
CGACTGACGACGACCGTTGATGTGAGTCTGGTCGTCCTT

>*TaR4*_Inoculated_*Tef1* gene

CCCCCCGTCCGGCAGCGATAATCAGGATAGCGCAGTCAGCCTGGGAGGTACCAGTGATC
ATGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGTGGGGAGAGTATGTTAGAATGA
CGATCTTGCAATGTCGACTGAAGTGTCCAAAACATACCAATGACGGTGACATAGTACTT
GGGAGTCTCGAACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACGCTCGGCCT
TGAGCTTGTCAAGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGGCGGCTTCC
TATTGATTGAAGCATGATCAGCATAATGAACAAGAGATATTGAAAATAGACGATGCGAT
TGAACAAAAGCATGTGGTGGATGATGGAAAGAGGTACAGTGGCGATAGTGGGGTTGCCG
TCAAACAAAATTGACACCCCACTAAAAGCCAAACAAGGCAGCGAGAAAATTTTTTGCTG
TGCCAAAGGAGGGGTAGGTAGGCTGGCAAAGCGGGGTGATGCTGAAAAAAGAAAGACAA
AACTGAGAACAGAATTGTCGGGCATATAATTGTGCCAAAAATTGATGGGAAAAGCAGTG
AAATTAGCTTACCTTCTCGAACTTCTCGATGTTACGCTCGTCTGTACCACAGCACTGGT
AGATCAAGTGACCAGTCTATTCGGAAAAACGTTAGCTGGATGCTTCCATATTCAAGTTG
CCCCCGCGACACCCGACAGAGTCGAGAGCAGACGGTGCTTCGCGGAATTGTGTATCACT
CGCGGTGTTAGACTTGCCGGAGTCGACGTGGCCTGTTGTAGCAAGTCAGTATTGGAGTC
GGTCGATAGTCAGTCTGATGGACGAAATGACGCATCCTCCACACACGATGAAGGTCAGC
ACAACTGTCGTTTCACACTTCAACGGTCAAAGAAAAGATCGCTGATGGTAACAATGGGG
AATTGCTGCTTACGACTGACGACGACGGTGCATGTCGTCGTGTGCTACCTTCGCCG

>*TaR5*_Inoculated_*Tef1* gene

TGCTATGATTCAGGATAGCGCAGTCAGCCTGTGGAGGTACCAGTGATCATGTTCTTGAT
GAAATCACGGTGACCGGGAGCGTCTGTGGGGAGAGTATGTTAGAATGACGATCTTGCAA
TGTCGACTGAAGTGTCCAAAACATACCAATGACGGTGACATAGTACTTGGGAGTCTCGA
ACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGTCA
AGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGGCGGCTTCCTATTGATTGAA
GCATGATCAGCATAATGAACAAGAGATATTGAAAATAGACGATGCGATTGAACAAAAGC
ATGTGGTGGATGATGGAAAGAGGTACAGTGGCGATAGTGGGGTTGCCGTCAAACAAAAT
TGACACCCCACTATAAGCCAAACAAGGCAGCGAGAAAATTTTTTGCTGTGCCCAAGGAG
GGGTACGTAGGCTGGCAAAGCGGGGTGATGCTGAAAAAAGAAAGACAAAACTGAGAACA
GAATTGTCGGGCATATAATTGTGCCAAAAATTGATGGGAAAAGCAGTGAAATGAGCTTG
CCCTTCTCGAACTTCTCGATGGTACGCTTGCCAGTACCACCGCACTGGTAGATCAACGT
GACCAGTCTATTAGGACAAAACGCTACCTGGATGCTTCCATAATCAAGTTGCCCCGCGC
CACCCGACAGAGTCGAGAGCAGACGGGGATTGGGGAATTGGGTATCACTCACGGTGTGT
AGACACTGCCGGGAGTCGATGTGGCTGTTGTAGCTAGTGAGTATTGAAGTTCGGTCGAT
ATTAGTCCTGATTCGTACGAATCTACGAACTCCCTACAGACGATGCAGGGCCAGGCACC
AATGTCGGATTCACAGTCAACGGCCAGAGCAATAGTTCCGCCGATGGTTGACAATGCGC
ATCGCAGACTTACGACTGACGGACGTACGTCGATAGTGACGTCGTTGTGCTACCTC

Appendix D: Sequence for *Tef1*region for inoculated isolates (Seed treatment)

>*TaR1*_Inoculated_*Tef1* gene

ACCGCTCCGGGCGCGGTCATTTTGGCTAGCGCAGTCAGCCTGGGAGGTACCAGTGATCA
TGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGTGGGAAGAGCATGTTAGAATGGC
GGACTTGGAATGTCGATAGCTATAGAGAAGAGTCCAAAACATACCAATGACGGTGACAT
AGTACTTGGGAGTCTCGAACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACGC
TCGGCCTTGAGCTTGCCAAGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGGC
GGCTTCCTATTGATTGAAGCATGATCAGCATAATGAACAAAAAAAGACGACGCGATTGA
GCAAATAGCATGTGGTGGGTGATGGAAGAGGTGCAGTGACGATAGCGGGGTTGCTGCCA
AAAAATTTGACACCCCACTAAAAGCCAAACAAGGCAGCCAGAAAATTTTTGCTGGGCCC
CTCGAGGCGGTGGAAAGACTGGCAAAGCGGGGTGACGCTGGATAAATATAGACAAAAAG
TGAGAGCAGAATTGTCGGGCATATGATTGTGCCAAAAATTGATGGGAAAAGCAGTGCAA
ATGAGCTTACCTTCTCGAACTTCTCGATGGTACGCTTTGTCAATACCACCGCACTGGTA
GATCAAGTGACCAGTCTATTGTGAAAAACGTTACCTGCATGTTGCTACAATCAAGTTGG
CCCCGCCCCTGCCCGACAGAGTCGATAGCAGAGGGTGCTCGAGGTACTTCTATATTACT
CACGGTGGTAGACTTGCTCGGAGTCGACGTGTCTGTTGTATCAAGTCAGTATTCGAGTC
GATCGAAAGCAGATTGCCGGACGATATTACGACGCCCTATAGACGATGATGGTCCAGCA
CAATGTCATTTCGCATGACAACGACAGATCAGTAGTGCTCGCTCGCTGGTAGAATGGGC
ATTACAAGCTACGATCGACGACGACGTTGATGTAGTCATGTCGTCTCCTACCCTAA

>*TaR2*_Inoculated_*Tef1* gene

CATACCCGTCCGGGGGCGGTATCAGGATAGCGCAGTCAGCCTGGGAGGTACCAGTGATC
ATGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGTGGGAAGAGCATGTTAGAATGG
CGGACTTGGAATGTCGATAGCTAGAGAGAAGAGTCCAAAACATACCAATGACGGTGACA
TAGTACTTGGGAGTCTCGAACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACG
CTCGGCCTTGAGCTTGTCAAGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGG
CGGCTTCCTATTGATTGAAGCATGATCAGCATAATGAACAAAAAAAGACGACGCGATTG
AGCAAATAGCATGTGGTGGGTGATGGAAGAGGTGCAGTGGCGATAGCGGGGTTGCTGCC
AAAAAATTTGACACCCCACTAAAAGCCAAACAAGGCAGCCAGAAAATTTTTGCTGTGCC
AAAGGAGGGGTAGGAAGACTGGCAAAGCGGGGTGACGCTGGAAAAAAAAAGACAAAAAC
TGAGAGCAGAATTGTCGGGCATATGATTGTGCCAAAAATTGATGGGAAAAGCAGTGAAA
TGAGCTTACCTTCTCGAACTTCTCGATGGTACGCTTGTCAATACCACCGCACTGGTAGA
TCAAGTGACCAGTCTAATTGGCAAAAACGTTACCTGGATGTTGCTACAATCAAGTTGCC
CCGCGCTGCCCGACAGAGTCGATAGCAGAGGGTGCTCGAGGAATTGGCTATTACTCACG
GGTGTTAGACTTGCCGTAGTCGACGTGTCTGTTGTAGCAAGACAGTATTAGAGTCGATC
GAAAGCAGATTGCCGGAGAAATACGAACCCCCTAGAGACGATGATGGTCAGCAACAATG
TCATTTGCAAGACAACGCACAGAGACGAATGTCCGCTCGTACGGCAGTAAAGCGCATTA
CTAGCTTACGACTGACGACGACCGTTGATGTGAGTCTGGTCGTCCTTACCATATGA

>*TaR3*_Inoculated_*Tef1* gene

GTCCGGGGGCGGTATCAGGATAGCGCAGTCAGCCTGGGAGGTACCAGTGATCATGTTCT
TGATGAAATCACGGTGACCGGGAGCGTCTGTGGGAAGAGCATGTTAGAATGGCGGACTT
GGAATGTCGATAGCTAGAGAGAAGAGTCCAAAACATACCAATGACGGTGACATAGTACT
TGGGAGTCTCGAACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACGCTCGGCC
TTGAGCTTGTCAAGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGGCGGCTTC
CTATTGATTGAAGCATGATCAGCATAATGAACAAAAAAAGACGACGCGATTGAGCAAAT
AGCATGTGGTGGGTGATGGAAGAGGTGCAGTGGCGATAGCGGGGTTGCTGCCAAAAAAT

TTGACACCCCACTAAAAGCCAAACAAGGCAGCCAGAAAATTTTTGCTGTGCCAAAGGAG
GGGTAGGAAGACTGGCAAAGCGGGGTGACGCTGGAAAAAAAAAGACAAAAACTGAGAGC
AGAATTGTCGGGCATATGATTGTGCCAAAAATTGATGGGAAAAGCAGTGAAATGAGCTT
ACCTTCTCGAACTTCTCGATGGTACGCTTGTCAATACCACCGCACTGGTAGATCAAGTG
ACCAGTCTAATTGGCAAAAACGTTACCTGGATGTTGCTACAATCAAGTTGCCCCGCGCT
GCCCGACAGAGTCGATAGCAGAGGGTGCTCGAGGAATTGGCTATTACTCACGGGTGTTA
GACTTGCCGTAGTCGACGTGTCTGTTGTAGCAAGACAGTATTAGAGTCGATCGAAAGCA
GATTGCCGGAGAAATACGAACCCCCTAGAGACGATGATGGTCAGCAACAATGTCATTTG
CAAGACAACGCACAGAGACGAATGTCCGCTCGTACGGCAGTAAGCGCATTACTAGCTTA
CGACTGACGACGACCGTTGATGTGAGTCTGGTCGTCCTT

>*TaR4*_Inoculated_*Tef1* gene

CCCCCCGTCCGGCAGCGATAATCAGGATAGCGCAGTCAGCCTGGGAGGTACCAGTGATC
ATGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGTGGGGAGAGTATGTTAGAATGA
CGATCTTGCAATGTCGACTGAAGTGTCCAAAACATACCAATGACGGTGACATAGTACTT
GGGAGTCTCGAACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACGCTCGGCCT
TGAGCTTGTCAAGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGGCGGCTTCC
TATTGATTGAAGCATGATCAGCATAATGAACAAGAGATATTGAAAATAGACGATGCGAT
TGAACAAAAGCATGTGGTGGATGATGGAAAGAGGTACAGTGGCGATAGTGGGGTTGCCG
TCAAACAAAATTGACACCCCACTAAAAGCCAAACAAGGCAGCGAGAAAATTTTTTGCTG
TGCCAAAGGAGGGGTAGGTAGGCTGGCAAAGCGGGGTGATGCTGAAAAAAGAAAGACAA
AACTGAGAACAGAATTGTCGGGCATATAATTGTGCCAAAAATTGATGGGAAAAGCAGTG
AAATTAGCTTACCTTCTCGAACTTCTCGATGTTACGCTCGTCTGTACCACAGCACTGGT
AGATCAAGTGACCAGTCTATTCGGAAAAACGTTAGCTGGATGCTTCCATATTCAAGTTG
CCCCCGCGACACCCGACAGAGTCGAGAGCAGACGGTGCTTCGCGGAATTGTGTATCACT
CGCGGTGTTAGACTTGCCGGAGTCGACGTGGCCTGTTGTAGCAAGTCAGTATTGGAGTC
GGTCGATAGTCAGTCTGATGGACGAAATGACGCATCCTCCACACACGATGAAGGTCAGC
ACAACTGTCGTTTCACACTTCAACGGTCAAAGAAAAGATCGCTGATGGTAACAATGGGG
AATTGCTGCTTACGACTGACGACGACGGTGCATGTCGTCGTGTGCTACCTTCGCCG

>*TaR5*_Inoculated_*Tef1* gene

TGCTATGATTCAGGATAGCGCAGTCAGCCTGTGGAGGTACCAGTGATCATGTTCTTGAT
GAAATCACGGTGACCGGGAGCGTCTGTGGGGAGAGTATGTTAGAATGACGATCTTGCAA
TGTCGACTGAAGTGTCCAAAACATACCAATGACGGTGACATAGTACTTGGGAGTCTCGA
ACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGTCA
AGAACCCACGCATACTTGAAGGAACCCTTGCCGAGTTCGGCGGCTTCCTATTGATTGAA
GCATGATCAGCATAATGAACAAGAGATATTGAAAATAGACGATGCGATTGAACAAAAGC
ATGTGGTGGATGATGGAAAGAGGTACAGTGGCGATAGTGGGGTTGCCGTCAAACAAAAT
TGACACCCCACTATAAGCCAAACAAGGCAGCGAGAAAATTTTTTGCTGTGCCCAAGGAG
GGGTACGTAGGCTGGCAAAGCGGGGTGATGCTGAAAAAAGAAAGACAAAACTGAGAACA
GAATTGTCGGGCATATAATTGTGCCAAAAATTGATGGGAAAAGCAGTGAAATGAGCTTG
CCCTTCTCGAACTTCTCGATGGTACGCTTGCCAGTACCACCGCACTGGTAGATCAACGT
GACCAGTCTATTAGGACAAAACGCTACCTGGATGCTTCCATAATCAAGTTGCCCCGCGC
CACCCGACAGAGTCGAGAGCAGACGGGGATTGGGGAATTGGGTATCACTCACGGTGTGT
AGACACTGCCGGGAGTCGATGTGGCTGTTGTAGCTAGTGAGTATTGAAGTTCGGTCGAT
ATTAGTCCTGATTCGTACGAATCTACGAACTCCCTACAGACGATGCAGGGCCAGGCACC
AATGTCGGATTCACAGTCAACGGCCAGAGCAATAGTTCCGCCGATGGTTGACAATGCGC
ATCGCAGACTTACGACTGACGGACGTACGTCGATAGTGACGTCGTTGTGCTACCTC