STUDIES ON FUNGAL ENDOPHYTES AGAINST SOIL-BORNE FUNGAL PATHOGENS OF TOMATO

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PALB 7097

DEPARTMENT OF PLANT PATHOLOGY

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BANGALORE **MARCH, 2021**

Affectionately dedicated to backbone of our country

DEPARTMENT OF PLANT PATHOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE

CERTIFICATE

This is to certify that the thesis entitled **"Studies on fungal endophytes against soil-borne fungal pathogens of tomato"** submitted in partial fulfilment of the requirement for the degree of **DOCTOR OF PHILOSOPHY in PLANT PATHOLOGY** to the University of Agricultural Sciences, Bangalore, in a *bona-fide* record of research work done by **Mr. NANDAN, M., ID No. PALB 7097** during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

Bengaluru
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With regardful memories,

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Bengaluru (NANDAN, M.)
March, 2021

STUDIES ON FUNGAL ENDOPHYTES AGAINST SOIL-BORNE FUNGAL PATHOGENS OF TOMATO

NANDAN, M.

ABSTRACT

Studies on fungal endophytes against soil-borne fungal pathogens of tomato was conducted *in-vitro* and *in-vivo*. Thirty-five fungal endophytic OTUs were isolated from leaf, stem and root tissues of *Tridax procumbens*, *Cassia tora* and *Parthenium hysterophorus* and one potential fungal endophyte from previous studies, *i.e.*, *Trichoderma asperellum* isolate 1 (TA1) were evaluated against targeted pathogens, *Sclerotium*, *Fusarium* and *Rhizoctonia* infecting tomato by dual culture technique. OTU's showing more than 50 per cent inhibition against targeted pathogens and three pathogens mentioned above were taken for further characterization through ITS region sequencing and analysis. To decipher the mechanism involved by potential endophyte *Trichoderma asperellum* isolates against selected pathogens, disc diffusion assay and double Petri dish assay was conducted. Further, endophytes showing positive inhibition of pathogen in these assays were processed for LC-ESI-MS/MS and GC-MS analysis and diffusible and volatile compounds involved in interaction were identified, respectively, which showed the presence of antimicrobial diffusible and volatile compounds produced by isolates of *T. asperellum*. To confirm the antimicrobial nature of diffusible compounds *in-silico* docking analysis was performed, which revealed the potentiality of diffusible compounds in binding to β-tubulin and inhibiting the targeted pathogens. Scanning electron microscopic studies showed the parasitizing behaviour of *T. asperellum* against *Rhizoctonia* and *Fusarium* pathogens. Tomato seed primed with endophyte, *T. asperellum* showed delayed in the onset of *Sclerotium* wilt in comparison with control plants. The gene expression studies using qRT-PCR analysis for seven genes involved in resistance pathways showed increase in their expression indicated the role of *T. asperellum* in inducing the plant systemic resistance.

March, 2021 (**C. N. LAKSHMINARAYANA REDDY**)
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ಟೊಮೆಟೊ ಬೆಳೆಯನ್ನು ಬಾಧಿಸುವ ಮಣ್ಣಿನ ಮೂಲದಿಂದ ಬರುವ ಶಿಲೀಂಧ್ರ ರೋಗಕಾರಕಗಳ ವಿರುದ್ಧ ಅಂತರಜೀವಿಗಳ ಅಧ್ಯಯನ

ನಂದನ್, ಎಂ.

ಪ್ರಬಂಧದ ಸಾರಾಂಶ

ಟೊಮೆಟೊ ಬೆಳೆಯಲ್ಲಿ ಮಣ್ಣಿನ ಮೂಲದಿಂದ ಬರುವ ರೋಗಕಾರಕಗಳ ವಿರುದ್ಧ ಅಂತರಜೀವಿಗಳ ಸಾಮರ್ಥ್ಯವನ್ನು ಪರಿಶೀಲಿಸಲು ಇನ್-ವಿಟ್ರೋ ಮತ್ತು ಇನ್-ವಿವೋ ಅಧ್ಯಯನವನ್ನು ನಡೆಯಿಸಲಾಯಿತು. *ಟ್ರೈಡ್ಯಾಕ್ಸ್ ಪ್ರೊಕ್ಯೂಮ್ಬೆನ್ಸ್*, *ಕ್ಯಾಷಿಯ ತೋರ*ಮತ್ತು *ಪಾರ್ಥೇನಿಯಂ ಹೈಸ್ಟೆರೊಫೋರೋಸ್*ಸಸ್ಯಗಳ ಎಲೆ, ಕಾಂಡ ಮತ್ತು ಬೇರನ್ನು ಸಂಸ್ಕರಿಸಿ, ೩೫ ಶಿಲೀಂಧ್ರ ಅಂತರಜೀವಿಗಳನ್ನು ಪ್ರತ್ಯೇಕಿಸಿ ಹಾಗು ಒಂದು ಹಿಂದಿನ ಅಧ್ಯಯನದ ಸಾಮರ್ಥ್ಯವುಳ್ಳ *ಟ್ರೈಕೋಡರ್ಮ ಅಸ್ಪೆರೆಲ್ಲಮ್* ಐಸೋಲೇಟ್-೧, ಒಟ್ಟು ೩೬ ಶಿಲೀಂಧ್ರ ಅಂತರಜೀವಿಗಳ ಓಟಿಯು ಗಳನ್ನು *ಸ್ಕ್ಲೆರೋಟಿಯಂ*, *ಫ್ಯುಸೆರಿಯಂ* ಮತ್ತು *ರೈಥೋಕ್ಟೋನಿಯಾ* ರೋಗಕಾರಕಗಳ ವಿರುದ್ಧ ಉಭಯ ಸಂಸ್ಕೃತಿ ಪದ್ಧತಿಯಿಂದ ಮೌಲ್ಯಮಾಪನ ಮಾಡಲಾಯಿತು. ಮೂವತ್ತಾರು ಓಟಿಯು ಗಳಲ್ಲಿ, ೫೦% ಗಿಂತ ಹೆಚ್ಚು ಪ್ರತಿರೋಧ ತೋರಿಸಿದ ಓಟಿಯು ಗಳು ಮತ್ತು ಮೂರು ರೋಗಕಾರಕಗಳನ್ನು ಆಣ್ವಿಕ ಗುಣಲಕ್ಷಣವನ್ನು ಐಟಿಎಸ್ಸ ಪ್ರೈಮರ್ ಗಳನ್ನು ಬಳಸಿ ಧೃಡಿಪಡಿಸಲಾಯಿತು. ರೋಗಕಾರಕಗಳನ್ನು ಪ್ರತಿರೋಧಿಸುವ ಸಾಮರ್ಥ್ಯವುಳ್ಳ *ಟ್ರೈಕೋಡರ್ಮ ಅಸ್ಪೆರೆಲ್ಲಮ್* ಐಸೋಲೇಟ್ಸ್ನ ಕಾರ್ಯವಿಧಾನ ಅರಿಯಲು ಡಿಸ್ಕ್ ಡಿಫ್ಯೂಸನ್ ಮತ್ತು ಡಬಲ್ ಪೆಟ್ರಿಡಿಶ್ ಅಸ್ಸೆಯ ಮೂಲಕ ಮೌಲ್ಪಿಕರಿಸಲಾಯಿತು. ಈ ಮೌಲ್ಯಮಾಪನದಲ್ಲಿ ಸಕಾರಾತ್ಮಕತೆಯನ್ನು ತೋರಿಸಿದ ಅಂತರಜೀವಿಗಳನ್ನು ಎಲ್ಸಿಎಂಎಸ್ ಮತ್ತು ಜಿಸಿಎಂಎಸ್ ಮೂಲಕ ವಿಶ್ಲೇಷಿಸಿ ಆಂಟಿಮೈಕ್ರೋಬಿಯಲ್ ರಾಸಾಯನಿಕಗಳನ್ನು ಖಚಿತ ಪಡಿಸಿಕೊಳ್ಳಲಾಯಿತು. ಇದಕ್ಕೆ ಅನುಗುಣವಾಗಿ, ಇನ್ ಸಿಲಿಕೋ ಡಾಕಿಂಗ್ ವಿಶ್ಲೇಷಣೆಯ ಮೂಲಕ ಬಿ-ಟ್ಯುಬ್ಯುಲಿನ್ ಅನ್ನು ಬಂಧಿಸುವ ರಾಸಾಯನಿಕಗಳ ಸಾಮರ್ಥ್ಯವನ್ನು ಖಚಿತಪಡಿಸಲಾಯಿತು. ಸ್ಕ್ಯಾನಿಂಗ್ ಎಲೆಕ್ಟ್ರಾನ್ ಮೈಕ್ರೋಸ್ಕೋಪಿ ಅಧ್ಯಯನದ ಮೂಲಕ *ಟ್ರೈಕೋಡರ್ಮ ಅಸ್ಪೆರೆಲ್ಲಮ್* ಐಸೋಲೇಟ್-೨ (ಟಿಎ2) ನಾ ಪರಾವಲಂಬಿ ವರ್ತನೆಯನ್ನು *ಫ್ಯುಸೆರಿಯಂ* ಮತ್ತು *ರೈಥೋಕ್ಟೋನಿಯಾ* ವಿರುದ್ಧ ಧೃಡಿಪಡಿಸಲಾಯಿತು. *ಟ್ರೈಕೋಡರ್ಮ ಅಸ್ಪೆರೆಲ್ಲಮ್* ಐಸೋಲೇಟ್-೨ ನಾ ವಸಾಹತುಶಾಹಿ ಅಧ್ಯಯನದಲ್ಲಿ ಟಿಎ೨ ಯಶಸ್ವಿಯಾಗಿ ಟೊಮೆಟೊ ಕಾಂಡ ಮತ್ತು ಬೇರುಗಳಲ್ಲಿ ವಸಾಹತುಗೊಂಡು *ಸ್ಕ್ಲೆರೋಟಿಯಂ* ಆಕ್ರಮಣವನ್ನು ವಿಳಂಬಗೊಳಿಸಿದ್ದು, ಮತ್ತು ಟಿಎ೨ ವಸಾಹತುಗೊಂಡಿರುವ ಟೊಮೆಟೊ ಸಸ್ಯದಲ್ಲಿ ರಕ್ಷಣಾತ್ಮಕ ಕಾರ್ಯ ಹೆಚ್ಚಾಗಿರುವುದು ಆರ್ಟ್-ಪಿಸಿಆರ್ ಜೀನ್ ಅಭಿವ್ಯಕ್ತದ ಅಧ್ಯಯನದಲ್ಲಿ ಖಚಿತವಾಗಿರುತ್ತದೆ.

ಮಾರ್ಚ್ ೨೦೨೧

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ಪ್ರಮುಖ ಸಲಹೆಗಾರ

ಸಸ್ಯ ರೋಗ ಶಾಸ್ತ್ರ ವಿಭಾಗ
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I. INTRODUCTION

The cultivated tomato (*Solanum lycopersicum* L.) is the world's highly consumed vegetable due to its status as a basic ingredient in a wide range of fresh, cooked, and/or processed foods. It belongs to the family Solanaceae, which also includes several other commercially important species. Cultivated tomato was originated from Peru, Ecuador, and other parts of South America including the Galapagos Islands. The centre of its domestication and diversification is Mexico (Rick, 1978; Jenkins, 1948; Peralta *et al.*, 2008). It is widely cultivated in almost 140 countries with diverse climatic conditions.

Tomato is a herbaceous perennial plant, however biennial and perennial forms exist and mostly grown as an annual crop. Tomato is cultivated in tropical and temperate climates in the open field or under greenhouse. It is easily and widely cultivated, not limited by day length or any other special condition for its growth and reproduction (Leonardi *et al*., 1999).

In world, during 2018-19 tomato is cultivated in 4.76 m ha area with 182.25 mt of production. India has 0.781 m ha area of tomato cultivation with 19.007 mt of production and ranks second in production among the vegetables. In Karnataka during 2018-19 tomato is cultivated in 0.06425 m ha with 2.0815 mt of production (Anon, 2019).

Tomato is having outstanding nutritional profile owing largely to vitamins (A, B1, B2, B6, folic acid, biotin, pantothenic acid, nicotinic acid, C, and E), antioxidants, such as carotenoids, lycopene, polyphenolic compounds, and carbohydrates. Because of its nutritive value, tomato is regarded as the world's major vegetable crop. High nutritional value and overall ease of growing have made tomato the world's second most consumed crop (Beecher, 1998). In combination with a variety of vegetables, fresh ripe tomatoes are usually eaten raw in salad and in addition to its usage curries. A wide variety of value-added items such as soups, juices, pastes, sauces, and ketchup can also be processed and canned using tomatoes (Elbadrawy and Sello, 2016).

Tomato crop suffers from various biotic and abiotic stresses, which are becoming limiting factors for its production. Among the biotic stresses, diseases such as early blight, late blight, powdery mildew, fusarium wilt, sclerotium wilt, rhizoctonia root rot,

leaf curl, tospow virus, etc., are known to have a devastating effect on production of tomato in both quality and quantity (Pico *et al*., 1996 and Picanco *et al*., 2007). The soil-borne diseases are behaving like hidden enemies, because, unless some symptom expresses on the foliar parts of the plants it is difficult to make out what happening to below-ground part of plant tissues. Soil-borne diseases like sclerotium wilt, rhizoctonia root rot, and fusarium wilt are known to cause huge yield losses in tomato cultivation systems (Mao *et al*., 1998).

Sclerotium rolfsii Sacc. is a devastating soil-borne plant pathogenic fungus having a wide host range, belongs to the family *Atheliaceae* and division *Basidiomycota* with the telomorphic stage as *Athelia rolfsii*. Upon infection, it causes drooping of leaves with loss of rigidity in stem and foliar parts followed by complete wilting of tomato plants (Ramakrishnan, 1930).

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* belongs to the family *Nectriaceae* and the division *Ascomycota*, is also a serious threat for tomato production. The disease is characterized by symptoms, like yellowing of leaves followed by drooping appearance and finally wilting of entire plants. The most prominent symptom, brownish discoloration of vascular tissues can be observed upon the longitudinal splitting of infected plant stem region (Jones *et al*., 1997).

Similarly, another soil-borne pathogen, *Rhizoctonia solani* Khun. belongs to the family *Ceratobasidiaceae* and the division *Basidiomycota* was reported to cause root rot disease in tomato crop. The typical symptoms induced by this pathogen were initial yellowing of older leaves, necrotised sunken area at the collar region and also on the root tissues and finally the whole plant dries leading to the death of the plant (Wokoma, 2008).

These three soil-borne pathogens were reported to survive in the soil for several years. *Sclerotium* and *Rhizoctonia* were known to survive in the form of dormant mycelia and sclerotia. However, in case of *Fusarium*, chlamydospores and dormant mycelium on the infected tissues survive in the soil for many years (Young and Ashford, 1995; Ford *et al*., 1970).

Different management approaches were followed in the farmer's field to combat these diseases such as summer ploughing, clean cultivation, use of resistant varieties, application of biocontrol agent's and drenching with the fungicide molecules (Madhavi and Bhattiprolu, 2011). Every management approach has both pros and cons. Even though the use of pesticides is giving effective control, they are resulting in soil and environment pollution, destroying the beneficial flora and fauna in the soil, resistance development of pathogens to chemicals. Due to this biological control of plant disease management is gaining great impetus because of its in eco-friendly nature, apart from reducing cost of cultivation (Deacon and Berry, 1993). Among the biocontrol approaches for plant disease management, utilization of endophytes for plant disease management is emerging as most promising alternative to manage the soil-borne diseases in the recent days (Pablo *et al*., 2015).

Endophytes are the living organisms may be fungi or bacteria or actinomycetes, which lives inside the plant tissues without causing any apparent diseases and with more of beneficial effects to its host plant (Pablo *et al*., 2015). Among them, fungal endophytes have gained huge importance due to their wide range of functional activities in living host system *i.e.*, production of secondary metabolites and safeguarding host plant against many biotic and abiotic stresses, which has made eyesight towards these organisms for exploiting them as a promising source of new natural sources that could hold the key to mitigate the plant diseases (Souvik *et al*., 2012). Many fungal endophytes were reported to inhibit the plant pathogens in both *in-vitro* and *in-vivo* condition by employing antibiosis through diffusible and volatile compounds, competition for space and nutrients, hyper parasitism as direct mechanisms and inducing systemic resistance and attributing towards the growth promotion in host plants as indirect mechanisms (Gao *et al*., 2010).

Considering the status of tomato soil-borne diseases, management approaches and the potentiality of fungal endophytes in mitigating the plant diseases, the current study was undertaken to study the effect of fungal endophytes as potential biocontrol agents against *Sclerotium*, *Fusarium*, and *Rhizoctonia* pathogens infecting tomato crop with the following objectives,

Objectives:

1. To isolate and evaluate the fungal endophytes against soil-borne fungal pathogens of tomato

2. To decipher the mechanisms of action of fungal endophytes against soil-borne fungal pathogens of tomato

3. To study the ability of endophytes to serve as biocontrol agents in tomato crop system

II. REVIEW OF LITERATURE

Tomato crop affected by various soil-borne fungal plant pathogens *viz.*, *Fusarium*, *Sclerotium* and *Rhizoctonia*, which are major constraints in tomato production (Mao *et al*., 1998). Fungal endophytes have gained great importance in mitigating biotic and abiotic stresses in the recent decade (Chaudhari *et al.*, 2014). Further, exploitation of fungal endophytes for management of soil-borne fungal pathogens in various crops has made a new promising way in biological management approach (Abro *et al*., 2019 and Gonzalez *et al*., 2020).

Endophytes are living organisms that may be fungi or bacteria which live inside the plant tissues for at least a part of its life cycle without causing any apparent symptoms and with more beneficial effects to its host plant (Pablo *et al*., 2015). Among those, fungal endophytes have gained huge importance with multifunctional activities like imparting stress tolerance against many biotic and abiotic stresses. Further, exploitation of beneficial endophytic organisms for plant disease management will have a greater impact on eco-friendly farming with reduced risk of resistance development in pathogens, which is gaining momentum worldwide (Luis *et al*., 2008). The various proved multifunctional activities of fungal endophytes that confer tolerance against invading plant pathogens are reviewed in this chapter.

2.1 Soil-borne plant pathogens

Sclerotium rolfsii Sacc. is the most destructive soil-borne fungus, first time reported by Rolfs (1892) causing tomato blight in Florida. Later, Saccardo (1911) named the fungus *S. rolfsii*. In India, Shaw and Ajrekar (1915) isolated the fungus from rotten potatoes and identified it as *Rhizoctonia destruens* Tassi. However, later studies showed that the fungus involved was *S. rolfsii* (Ramakrishnan, 1930). In tomato, it was reported to cause southern blight and also wilt disease, which was responsible for reduction in yield. *S. rolfsii* is generally distributed in tropical and subtropical countries where high temperature prevails during the rainy season (Weber, 1931). The fungus can survive in the soil for years by producing sclerotial bodies causing-foot rot, stem rot, collar rot, and leaf blight in many

economically important host crops belonging to diverse taxonomic families (Young and Ashford, 1995).

Fusarium wilt disease in tomato is caused by *Fusarium oxysporum* f. sp. *lycopersici* (Fol). Fusarium wilt of tomato was first described by G.E. Massee in England in 1895. Many reports also claim that *F. solani* was known to cause wilt in tomato plants (Karima and Nadia, 2012). The hyphae of these vascular fungi can enter the root by penetrating directly through the cortical root cells or via any mechanical damages. After the invasion, the fungal mycelium colonizes intercellularly and intracellularly through the root cortex until it reaches the xylem vessels. Finally, browning of the vascular system occurs which is characteristic of the disease and generally can be used for the identification (Jones *et al*., 1997).

Rhizoctonia solani Khun is most devastating and destructive pathogen that causes root rot, and became the major constraint for the production of tomato. In addition to root rot, *R. solani* was also reported to cause stem canker (Wokoma, 2008) and damping-off in India. It is considered to be the most important soil-borne disease of tomato causing severe losses under favourable conditions in both nursery beds and field condition (Dutta and Dutta, 2007). This pathogen can survive in soil within diseased plant material as mycelia or sclerotia during unfavourable environmental conditions for several years. In nature, usually, *R. solani* has asexual reproduction and exists primarily as vegetative mycelium and/or sclerotia. The teleomorph of *R. solani*, *Thanatephorus cucumeris*, is classified in the phylum Basidiomycota (Roberts, 1999).

2.2 Endophytes

Every living organism harbours many beneficial microorganisms which may be helping the host in one or the other metabolic and physiological activities. Similarly, every plant on this earth do harbour endophytic microorganisms which include fungal, bacterial and also actinomycetes (Tian *et al*., 2004). Every microorganism expresses a different lifestyle with plants ranging from mutualism to parasitism. Among these, fungal endophytes have received greater attention in a wide range of areas like plant secondary metabolites, biotic and abiotic stress tolerance, pharmacy, medicine etc. Endophytic fungi

are polyphyletic; mostly belonging to ascomycetes and anamorphic fungi (Aly *et al.*, 2011 and Arnold *et al.*, 2007). It has been estimated that there may be as many as one million different endophytic fungal taxa, which were hyperdiverse in nature (Strobel and Daisy, 2003 and Petrini *et al.*, 1992).

Endophytes may be transmitted either vertically (from parent to offspring) or horizontally (from one individual to another). Vertically transmitted fungal endophytes are asexual and transmit via fungal hyphae penetrating the host's seeds. These fungi are also mutualistic because their reproductive fitness is intimately related to that of their host plant. Horizontally transmitted fungal endophytes, on the other hand, are sexual and travel by spores that can be spread by wind and/or other vectors. Horizontally transmitted endophytes are often closely related to pathogenic fungi because they spread similar to pathogens, although they are not pathogenic (Selosse *et al*., 2004).

Many of the fungi commonly reported as endophytes are considered by the forest pathologists as minor or secondary pathogens. The ambiguity of boundaries distinguishing endophytes, facultative pathogens, and latent pathogens are underlined by their common occurrences in both healthy and diseased tissues. Endophytes and several commensal saprobic and mutualistic fungi that have cryptic, non-apparent patterns of host invasion may be called pathogenic fungi, capable of symptomless occupancy of their hosts in part of the infection period, 'quiescent infections' and strains with impaired virulence (Selosse *et al.*, 2004).

2.3 Fungal endophytes

Fungal endophytes are living organisms that reside inside the plant tissues without causing any harmful effect to plants and with more beneficial effects to its host plant (Pablo *et al*., 2015). Fungal endophytes and their host plants have a variety of relationships, ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic effects. Recent studies of endophytic fungi and their interactions with host plants have shown that plant-endophyte mutualism not only plays a key role in both parties' biological functions but also directs the ecophysiology of the host plant and its symbionts to strengthen their ability to respond to environmental stresses throughout evolutionary time (Chaudhari *et al.*, 2014).

While extensive research on plant stress responses has been conducted, it is not known why so few species can colonize in high-stress habitats. However, plant stress research rarely takes into consideration a ubiquitous aspect of plant-fungi symbiosis. The fitness benefits offered by mutualistic fungi have been shown to contribute to or be responsible for plant adaptation to stress. (Schulz and Boyle, 2005; Selosse *et al.*, 2004). Collectively, mutualistic fungi may confer tolerance to drought, metals, disease, heat, and herbivory, and/or promote growth and nutrient acquisition. In the absence of fungal endophytes, it has become apparent that at least some plants are unable to tolerate habitat-imposed abiotic and biotic stresses (Schardl and An, 1993).

The ability to protect the host from diseases and to limit the damage caused by pathogenic microorganisms has been demonstrated in the fungal endophytes-pathogen interaction. Many fungal endophytes generate secondary metabolites, some of which are antifungal and antibacterial compounds that strongly inhibit the growth of other microorganisms, including plant pathogens (Arnold *et al*., 2003). It has been implied that some fungal species may switch between pathogenic and mutualistic lifestyles under certain circumstances (Clay, 1992).

Exploiting the secondary metabolites produced by the fungal endophytes was capable of producing desired bioactive compounds typically includes the screening of a plethora of different endophytes isolated from a single host plant for identifying the ‘‘competent’’ endophyte with the desired trait (Kusari *et al.,* 2012). However, recent whole-genome sequencing techniques have shown that the number of genes encoding the biosynthetic enzymes in different fungi and bacteria is certainly greater than the known secondary metabolites of these microorganisms (Kusari *et al.,* 2012).

2.4 Fungal endophytes against plant pathogens

Fungal endophytes can protect the plants from both abiotic and biotic stress, among biotic stress, it can defend many pathogenic microbes through some defined antagonistic mechanisms. The plant pathogens can be controlled either by a direct or indirect mechanism. Direct mechanisms like antibiosis, competition for space and nutrients, hyper parasitism and other lytic enzyme secretion and indirect mechanisms like induction of

systemic resistance, stimulation of secondary metabolites and promoting better growth and development of plants (Gao *et al*., 2010).

The outcome of plant-pathogen-endophyte interaction probably depends on the endophytic niche. Endophytic recognition and colonization will lead to the rapid occupation of the ecological niche and leave no space for pathogens, which may be the common and primary reason for endophytes' protective action (Petrini *et al*., 1992).

The fungal endophytes isolated from eggplant have shown complete suppression on the pathogenic effects of a post-inoculated, virulent strain of *Verticillium dahliae* in eggplant. Seven isolates from the roots of eggplant included *Heteroconium chaetospira, Phialocephala fortinii* and unidentified species of *Fusarium, Penicillium, Trichoderma,* and MRA. *P. fortinii, H. chaetospira,* a non-sporulating isolate with white mycelium (SWM) and mycelium radicis atrovirens (MRA) were easily reisolated from root segments during the colonization studies. Hyphae of *H. chaetospira, P. fortinii* and non-SWM colonized the root tissues of eggplant without causing apparent pathogenic symptoms. The mechanisms through which these endophytes confer resistance to infection by *V. dahliae* are unknown. However, the effectiveness of these fungi in a laboratory setting indicates that they have potential as biocontrol agents and merit further investigation (Narisawa *et al*., 2002).

Endophytic trichoderma species were isolated from the trunks and pods of theobroma and liana (*Banisteriopsis caapi*). The endophytic trichoderma isolates were screened against *Moniliophthora roreri* in in-*vitro* condition. Which showed their varied abilities to produce inhibitory metabolites to and in their abilities to parasitize *M. roreri* cultures. Most of the isolates studied were able to establish endophytic relationship with cacao by colonizing above-ground portions of the cacao seedlings. Further exploitation trichoderma isolates led to the development of potential biocontrol agents against *M. roreri* infecting cacao plants (Bailey *et al*., 2008).

Ayob and Simarani, (2016) isolated various filamentous endophytic fungi strains from wild grown *Catharanthus roseus*. Molecular characterization through sequencing of Internal Transcribed Spacer (ITS) region revealed these fungi as *Colletotrichum* sp.,

Macrophomina phaseolina, *Nigrospora sphaerica,* and *Fusarium solani*. All the fungal strains showed positive for hydrolytic enzyme tests by secreting cellulase. *F. solani* strain showed positive result for both amylase and protease activity, while *Colletotrichum* sp. showed protease activity.

Thirty fungal endophytes isolated from various plants were evaluated against *F. oxysporum* f. sp. *cucumerinum* causing wilt in cucumber using dual culture assay. Which were capable of inhibiting the mycelial colony growth of *F. oxysporum* f. sp. *cucumerinum* upto inhibition of 66 % as compared to control. Among thirty isolates, five isolates were highly effective *viz., Penicillium* sp., *Guignardia mangiferae*, *Hypocrea* sp., *Neurospora* sp., *Eupenicillium javanicum*, and *Lasiodiplodia theobroma.*. Among potential isolates, ten isolates with prominent inhibition in *in-vitro* conditions were selected for greenhouse studies. In greenhouse studies, three endophytic fungal isolates successfully suppressed the wilt severity when co-inoculated with pathogen *F. oxysporum* f. sp. *cucumerinum* in cucumber plants. Further, plant growth parameters of the host plants were also enhanced by the endophytes in comparison with the control plants (Abro *et al*., 2019).

Soil-borne fungal diseases of melon crops like carbonaceous rot (*M. phaseolina*), collapse (*Monosporascus cannonballus*) and the fusarium wilt (*F. oxysporum* f. sp. *niveum, F. oxysporum* f. sp. *melonis, F. solani* f. sp. *cucurbitae, Neocosmospora falciformis,* and *N. keratoplastica*) have deleterious effects on both quantity and quality of the produce. Among 350 fungal endophytic strains isolated from asymptomatic watermelon plants, seven fungal species were selected to evaluate their antagonistic potential against 14 pathogens. By performing dual culture assay, two trichoderma strains were selected based on highest per cent inhibition (93 %) further evaluation. *T. lentiforme* showed inhibition in some of the pathogens up to 67 % in melon and watermelon plants (Gonzalez *et al*., 2020).

2.4.a Antibiosis nature of fungal endophytes through diffusible compounds

Secondary metabolites of *Penicillium expansum* (R82) isolates were screened against *Botrytis cinerea, Colletotrichum acutatum,* and *Monilinia laxa* where, six isolates of *P. expansum*, revealed inhibitory activity against every pathogen tested. Culture filtrate

and double Petri dish assay were followed to confirm the antimicrobial activity of diffusible and volatile compounds, respectively. Solid-Phase Micro Extraction (SPME) – Gas Chromatography-Mass Spectrometry (GC-MS) analysis was followed for the characterization of volatile organic compounds (VOCs) produced by the R82 strain. Several compounds were detected, of which one of them identified as phenethyl alcohol (PEA). Synthetic PEA tested *in-vitro* on fungal pathogens showed strong inhibition at the concentration of 1,230 mg/ml of airspace and mycelium appeared more sensitive than conidia (Rouissi *et al*., 2013).

Pansanit and Pripdeevech, (2018) studied antibacterial and antioxidant activities of secondary metabolite extract of fungal endophyte *Arthrinium* sp. isolated from *Zingiber cassumunar*.. The ethyl acetate extract of the *Arthrinium* sp. showed activity against both gram-positive and gram-negative bacteria. Specifically, the minimum inhibition concentration against *Staphylococcus aureus* and *Escherichia coli* was 31.25 and 7.81 μg/mL, respectively. GC-MS analysis showed that the extract of the *Arthrinium* sp. contains β-cyclocitral, 3E-cembrene A, laurenan-2-one, sclareol, 2Z,6E-farnesol, cembrene, β-isocomene and γ-curcumene which were reported as antibacterial and antioxidant.

Fungal endophytes isolated from a medicinal plant *Nothapodytes nimmoniana* were screened against *S. rolfsii* for their antagonism in *in-vitro* and *in-vivo* conditions. Among 120 fungal endophytes, one isolate, *Alternaria* sp. showed 46.62 per cent inhibition of mycelium of *S. rolfsii* in dual culture assay. The Nuclear Magnetic Resonance (NMR) analysis of fraction of endophyte metabolite confirmed that, inhibition of *S. rolfssi* was found to be mediated by the mycotoxin, tenuazonic acid. Further, seed priming of chilli seeds with mycelial and spore suspension of *Alternaria* sp. reduced the seedling mortality due to *S. rolfsii* infection and also enhanced the growth and biomass of chilli seedlings compared to untreated seedlings under greenhouse conditions (Rajani *et al*., 2019).

Kamel *et al.* (2019) isolated fungal endophytes from *Euphorbia geniculata* plants which yielded 22 isolates belonging to 15 fungal genera. Among 15 genera, the genus *Aspergillus* was the most common fungus isolated from *E. geniculata*. Endophytic fungi

Isaria feline was obtained from both leaves and stem, while *Aspergillus flavus, A. ochraceus, A. terreus* var. *terreus*, *Emercilla nidulans* var. *acristata, M. phaseolina* obtained from both stem and root. All the isolated fungal endophytes showed inhibitory action against six plant pathogenic fungi *i.e*., *Eupenicillium brefeldianum*, *Penicillium echinulatum*, *Alternaria phragmospora*, *F. oxysporum*, *Fusarium verticilloides*, and *Alternaria alternata* in dual culture assay. The highest inhibitory activity by endophytic fungi was observed in case of *A. flavus, A. fumigatus,* and *Fusarium lateritium* and twining in their secondary metabolites especially terpenes and alkaloids with that of their host *E. geniculata*. Secondary metabolites extracted by ethyl acetate and *n*-butanol from these six fungal endophytes were screened against three pathogenic fungi of tomato plant (*E. brefeldianum, P. echinulatum*, and *A. phragmospora*) where these pathogens showed promising sensitivity to the secondary metabolites extracts of fungal endophytes.

The fungal endophyte *Drechslera* sp. strain 678, isolated from the roots of an Australian native grass *Neurachne alopecuroidea,* was recorded to inhibit four plant pathogens (*Pythium ultimum, R. solani, B. cinerea* and *A. alternata*). Two bioactive metabolites, monocerin, and alkynyl substituted epoxycyclohexenone derivative, were known to have antifungal activity recorded against the above listed four fungal pathogens through metabolic analysis (D'Errico *et al*., 2020).

2.4.b Antibiosis nature of fungal endophytes through volatile organic compounds

Endophytic fungi are organisms that spend most of their life cycle within plant tissues without causing any visible damage to the host plant. Many endophytes were found to secrete specialized metabolites and/or emit volatile organic compounds (VOCs), which may be biologically active and assist fungal survival inside the plant as well as benefit to their hosts. The endophytic fungi *Daldinia* cf. *concentrica* isolated from an olive tree (*Olea europaea* L.) showed prevention of molds on organic dried fruits, and eliminated *Aspergillus niger* infection in peanuts through antimicrobial volatile organic compounds. GC-MS analysis of the volatiles led to the identification of 27 VOCs that displayed a broad-spectrum antifungal activity (Liarzi *et al*., 2016).

Sornakili *et al.* (2020) isolated seven fungal endophytes from rice leaves and identified them as *Paecilomyces tenuis*, *Talaromyces pinophilus*, *Nigrospora sphaerica*, *Nigrospora oryzae*, *Trichoderma longibrachiatum*, *A. terreus* and EF7, showing anti-fungal and anti-bacterial activities. Among seven endophytes, *T. longibrachiatum* had the highest per cent inhibition activity against targeted fungal (23%–82%) and bacterial plant pathogens (13%–46%) in *in-vitro* condition. Soluble metabolites such as aliphatic organic acids, aromatic nitroamino compounds, and volatile metabolites were detected through Fourier Transform Infrared Spectroscopy (FT-IR) and GC-MS respectively. The metabolite induced antagonistic activity of *T. longibrachiatum* was much pronounced against plant pathogens such as *Magnaporthe grisea, R. solani*, and *M. phaseolina*.

Endophytic trichoderma isolates (four) were screened for their ability to produce antimicrobial volatiles against four pathogens, (*Sclerotinia sclerotiorum*-TSS, *S. rolfsii*-CSR, *Fusarium oxysporum*-CFO, and *M. phaseolina*-CMP). Four endophytic trichoderma isolates significantly inhibited the mycelial growth of three pathogens and not affected *M. phaseolina*-CMP. GC–MS analysis of VOCs captured in double plates assay, where the endophyte was grown along with either of the two plant pathogens, *F. oxysporum*-CFO or *M. phaseolina*-CMP, there was an induction of some new VOCs that were not detected in the pure cultures of either the endophyte or the pathogens. Several of these new VOCs are reported to possess antifungal and cytotoxic activity (Rajani *et al*., 2020).

Wonglom *et al*. (2020) evaluated the effects of VOCs produced by endophytic *T. asperellum* on multiple attributes in lettuce. The VOCs released by *T. asperellum* was recorded to have an antifungal action against two leaf spot fungal pathogens, *Corynespora cassiicola* and *Curvularia aeria*. Upon exposure of *T. asperellum* produced VOCs to lettuce there is increasing in the activity of the cell-wall degrading enzymes β-1,3-glucanase and chitinase in comparison with the control, which leads to morphological changes in the cell wall of fungal pathogens. Apart from regulation of cell wall degrading enzymes, VOCs emitted by *T. asperellum* significantly increased numbers of leaves and roots, plant biomass and total chlorophyll content in lettuce. GC-MS analysis of volatiles captured from *T. asperellum* revealed that 22 VOC's were generated which could be the

reason for the antifungal activity, inducing defence responses and also growth-promoting activities in lettuce.

2.4.c Mycoparasitism nature of fungal endophytes against plant pathogens

Dual culture assay was carried out by Upadhyay and Mukhopadhyay (1986) with endophyte *Trichoderma harzianum* (IMI no. 238493) isolate and pathogen *S. rolfsii.* The *T. harzianum* inhibited *S. rolfsii* by hyphal coiling, entry through haustoria-like structure and direct entry in the hyphae and sclerotia. In a glasshouse experiment, *T. harzianum* applied in the form of sorghum culture to *S. rolfsii* infested soil gave as high as 76 % and 88 % disease control in the first and second growth cycle of sugarbeet seedlings, respectively.

Endophytic *Trichoderma* species parasitize around hyphae of the plant-pathogen *R. solani* by twisting, penetrating into the hyphae, and secreting lyase to decompose the cell wall resulting in inhibition of the pathogen (Grosch *et al*., 2006).

Suebrasri *et al*. (2020) isolated the fungal endophytes from *Helianthus tuberosus*, *Zingiber officinale* and *Stemona tuberosa* and were screened against southern stem rot pathogen, *S. rolfsii* in *in-vitro* condition by dual culture assay. The dual culture assay results revealed that out of 110 fungal endophytes, *Diaporthe phaseolorum* and *M. phaseolina* isolated from Jerusalem artichoke and *Daldinia eschscholtzii* and *Trichoderma erinaceum*, isolated from Stemona root and ginger, respectively effectively inhibited *S. rolfsii* mycelium growth to the extent of 76.00, 41.20, 66.67 and 63.63 per cent, respectively. The chitinase and β-1,3 glucanase activities of all fungal endophytes were determined, which was observed maximum after seven and two days of incubation, respectively. scanning electron microscope (SEM) observation showed that *T. erinaceum* has mycoparasitic behavior by coiling and invasion of mycelium of *S. rolfsii*. Endophytic fungi *T. erinaceum* and *D. eschscholtzii* produced polyketides group as 6-*n*-pentyl-2H-pyran-2-1 (6PAP) and 2,3-dihydro-5-hydroxy-2-methyl-4H-1-benzopyran-4-1 (DHMB), respectively, for the inhibition of *S. rolfsii*. The endophytic fungi, *T. erinaceum* and *D. eschscholtzii* also showed a greater reduction of southern stem rot disease incidence to the extent of 58.14 per cent under greenhouse conditions.

2.4.d Regulation of systemic resistance in plants by fungal endophytes

Trichoderma virens (TriV_JSB100) spores or cell-free culture filtrate (CF) was employed for the regulation of growth and activation of the defence responses of tomato plants against *F. oxysporum* f. sp. *lycopersici* by the development of a biocontrol–plant-pathogen interaction system. Two-week-old tomato seedlings were primed with *T. virens* (TriV_JSB100) spores cultured on barley grains (BGS) and inoculated with *F. oxysporum* f. sp. *lycopersici* pathogen under glasshouse conditions. This resulted in a significant reduction in disease incidence in tomato plants treated with BGS than with the CF applied plants. To confirm the role of jasmonic acid (JA) and salicylic acid (SA) in defence regulation, gene expression studies were conducted. JA-deficient mutant def1 plants were susceptible to fusarium wilt causing pathogen, when they were treated with BGS. SA-deficient mutant NahG plants treated with CF were also found to be susceptible to fusarium wilt causing pathogen and displayed low SA levels. The JA and SA experimental results showed that TriV_JSB100 BGS and CF differentially induce JA and SA signalling cascades for the elicitation of resistance in tomato against *F. oxysporum* f. sp. *lycopersici* (Jogaiah *et al*., 2018).

Cheng *et al*., (2020) studied on *Serendipita indica*, a root-colonizing fungal endophyte belongs to basidiomycetous fungi against *F. oxysporum* f. sp. *cubense* (Foc) in banana plants with treatments, *S. indica* colonized (S+) and non-colonized (CK) banana plants and were inoculated with Foc tropical race 4 (TR4). Colonization by *S. indica* increased superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) activities in S+. After the pathogen inoculation, SOD activity was significantly compromised in *Foc*TR4-inoculated CK plants (F+) but not in *Foc*TR4-inoculated S+ plants (SF); POD activity was drastically increased in both F+ and SF, and that in SF was significantly higher than in F+; CAT activity was significantly improved in S+, but no obvious change was observed in F+ and SF when compared with CK. Results revealed that colonization of *S. indica* in banana plants improves defence response against FocTR4 at least partly, through regulation of antioxidant enzyme activities.

2.5 Efficacy of fungal endophytes in the *in-vivo* condition against plant pathogens

Fungal endophytes isolated from the healthy cotton root tissues were screened against verticillium wilt of cotton in greenhouse conditions. The fungal endophytes CEF-818 (*Penicillium simplicissimum*), CEF-714 (*Leptosphaeria* sp.), CEF-642 (*Talaromyces flavus*.) and CEF- 193 (*Acremonium* sp.) under greenhouse condition significantly reduced disease incidence and disease index, with the control efficacy ranging from 26 % (CEF-642) to 67 % (CEF-818) at 25 days after inoculation. Among the endophytic isolates, CEF-818 and CEF-714 provided well protection against verticillium wilt with 46.9 % and 56.6 %, respectively. Further, CEF-818 and CET-714 treatment increased transcript levels for defence related enzymes (PAL, PPO, POD) which leads to the increase in defence response of cotton plants against *V. dahliae.* These results revealed that seed treatment with potential endophytes could be a good approach in biocontrol of verticillium wilt in cotton (Yuan *et al*., 2017).

Fungal endophytes isolated from the cucurbit plants were screened against soil-borne pathogens of cucurbits (*R. solani*, *Sclerotinia sclerotiorum* and *F. oxysporum* f. sp. *cucumerinum*) in dual culture assay. Out of 1044 strains belonged to 90 genera screened against three pathogenic fungi, 47.1 per cent of the fungal endophytic strains showed antagonistic effects on at least one pathogen; 186 strains against *R. solani*, 371 strains against *S. sclerotiorum*, and 403 strains against *F. oxysporum* f. sp. *cucumerinum*. Many growth-promoting fungal endophytic strains had a good inhibitory effect on cucumber rhizoctonia root rot. Overall experiments revealed that fungal endophytes possess great inhibitory action against soil-borne pathogens of melons in both *in-vitro* and *in-vivo* conditions (Huang *et al*., 2020).

The reported literature on antimicrobial action of fungal endophytes against various plant pathogens provides insights into their potential as biocontrol agents against plant pathogens in addressing the management of plant diseases in an eco-friendly way.

III MATERIAL AND METHODS

Fungal endophytes have gained great importance for the biocontrol of plant diseases in the recent decade. From this context, the main goal of the study was to isolate and evaluate fungal endophytes against soil-borne pathogens *i.e.*, *Sclerotium*, *Fusarium* and *Rhizoctonia* infecting tomato crop. The experiments in the current study were conducted at the Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, GKVK, Bengaluru, (13.0801° N, 77.5785° E).

3.1 General laboratory procedures followed

3.1.a Glassware cleaning and sterilization

For all laboratory experimental studies, Borosil glassware's were used. Before using, the glassware's were kept for 24 hours in the cleaning solution containing 100 gm of potassium dichromate, 100 mL of concentrated sulphuric acid in 1000 mL of water and finally rinsed with distilled water. All the glassware, solid media and soil used for *in-vitro* and pot culture experiments were sterilized in an autoclave at 1.1 kg/ cm^2 pressure for 15 min at 121ºC. For *in-vitro* studies (dual culture method) disposable radiation sterile vented polystyrene Petri dishes (Tarsons) were used.

3.1.b Growth medium

For fungal endophytes isolation, evaluation, double Petri dish assay and other studies autoclaved potato dextrose agar (PDA) media (HiMedia) was used (39.0 grams in 1000 ml of water). For broth culture studies potato dextrose broth (PDB) (HiMedia) was used (24.0 grams in 1000 ml of water).

3.2 Sample collection and isolation of soil-borne plant pathogens

Soil-borne plant pathogens infecting tomato crop *viz*., *Sclerotium* sp., *Fusarium* sp. and *Rhizoctonia* sp. responsible for southern blight, wilt and dry root rot disease, respectively were targeted for management by using fungal endophytes. Tomato plants showing symptoms of fusarium wilt and dry root rot were collected from the farmer's

field in Kolar (13.1362° N, 78.1291° E) and southern blight from farmer field in Doddaballapura (13.2957° N, 77.5364° E) respectively for isolation of soil-borne fungal plant pathogens.

The three soil-borne fungal plant pathogens infecting tomato were isolated from the roots and collar region of infected tomato plant samples by standard isolation method under aseptic conditions. The infected tissues of the roots and collar region were cut into small pieces of 1-2 cm size and surface sterilized with 1% sodium hypochlorite solution for 2 min and washed thrice in sterile distilled water, placed into Petri dishes containing sterilized PDA media and incubated at 27 ºC (Al-Fadhal *et al*., 2019). The cultures obtained were purified by sub-culturing the growing hyphal tip of respective pathogenic fungi on a Petri dish containing PDA medium. Further, the isolated pure cultures of *Sclerotium, Fusarium* and *Rhizoctonia* pathogens were cultured on PDA slants and allowed to grow at 27 ºC and finally stored in a refrigerator at 4 ºC for further studies.

3.2.a Pathogenicity assay

Initially, isolated *Sclerotium, Fusarium* and *Rhizoctonia* pathogens were checked for their virulence by pathogenicity assay. The sorghum seeds were pre-soaked in water for 24 hours and then autoclaved at 121 ºC temperature, 15 kg/ cm^2 pressure for 15 min in polythene covers. The agar plugs (10 mm) of PDA media containing actively growing respective pathogens of 5-8 numbers were taken and inoculated into polythene cover containing autoclaved sorghum seeds under aseptic conditions. The inoculated substrate in polythene covers was incubated at 27 ºC (Upadhyay and Mukhopadhyay, 1986). After ten days of incubation, mass multiplied pathogens were used for conducting pathogenicity assay.

Pathogenicity of isolated *Sclerotium, Fusarium* and *Rhizoctonia* was confirmed using mass multiplied inoculum on sorghum seeds. Ten days old 3% (w/w) mass multiplied inoculum (Jinantana and Sariah, 1998) was added to the pots containing autoclaved pot mixture and allowed for seven days to multiply under glasshouse condition. After seven days, 30 days old tomato seedlings were transplanted into the pots containing mass multiplied pathogen inoculum and allowed for symptom

expression. For *Fusarium* and *Rhizoctonia*, two different methods were followed; a) healthy tomato seedlings transplanted directly and b) tertiary roots of tomato seedlings were trimmed gently with a sterile blade and subsequently transplanted. The tomato seedlings transplanted to the pots containing only autoclaved sorghum seeds served as control. For all experimental studies tomato variety, Arka Vikas procured from Indian Institute of Horticultural Research (IIHR), Hessaraghatta, Bengaluru was used.

3.3 Plant sample collection for isolation of endophytes

Weed species with traditionally well-known anti-microbial properties (Taddei and Rosas-Romero, 2000 and Gupta *et al.* 1996) growing without any pests or disease incidence were selected for fungal endophyte isolation (Plate 1). Leaf, stem and root samples from *Tridax procumbens*, *Cassia tora* and *Parthenium hysterophorus*. plants were collected from Botanical Garden, Gandhi Krishi Vigyana Kendra (GKVK, 13.0801° N, 77.5785° E), Bengaluru. The collected plant species were identified with the assistance of research team at a Botanical Garden, College of Agriculture, GKVK, Bengaluru.

3.3.a Isolation of fungal endophytes

Three weed plant samples collected for fungal endophytes isolation were initially washed through running tap water. Leaf, stem and root tissues of selected plant species were cut into 1 cm sized segments and washed with distilled water twice or thrice. These segments were then surface sterilized with 70 % (v/v) ethanol for 1 min followed by sterilization with 1 % (v/v) sodium hypochlorite for 30 seconds and again treated with 70 % ethanol for 1 min. Finally, all the segments were rinsed two to three times with sterile distilled water and were air-dried (Arnold *et al*., 2000). The processed leaf, stem and root segments were placed over a Petri dish containing PDA media supplemented with 100 ppm of streptomycin sulphate to avoid endophytic bacterial growth in aseptic condition, wrapped with saran wrap and incubated at 27 ºC. The inoculated plates were incubated for 5-7 days and observed for the growth of endophytes from the cut ends. To check the epiphyte contamination, the processed segments were imprinted on PDA medium (Schulz *et al*., 1993). After 10 days of incubation

colonization frequency of endophytic fungi was determined by a number of segments colonized by each endophyte to the total number of segments observed (Hata and Futai., 1995).

3.3.b Operational taxonomic units

The endophytic fungi grown out from the cut end tissues of inoculated plant segments were transferred to a Petri dish containing PDA media and pure cultures of fungal endophytes were obtained. Further, based on the colour of mycelia, spores, fruiting bodies and other morphological features using standard identification manuals (Barnett, 1960; Ellis, 1977; Sutton, 1980) pure cultures of fungal endophytes were categorized into different Operational Taxonomic Units (OTUs). Finally, the fungal endophytic OTUs were maintained on PDA slants by preserving at 4 ºC in the refrigerator for further use. Along with the isolated fungal endophytic OTUs, a potential OTU (HI2) previously isolated *i.e.*, *Trichoderma asperellum* from the leaf tissues of Hibiscus plant available in endophytic library of School of Ecology and Conservation laboratory was also used in the current study.

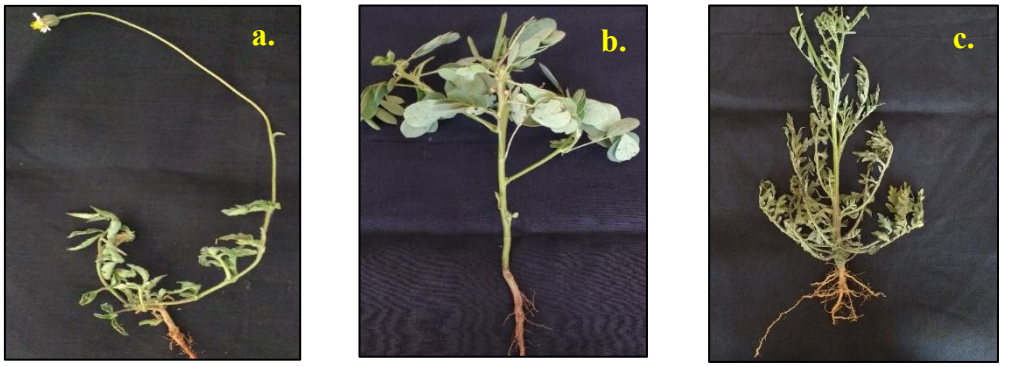


Plate 1: Plant species selected for isolation of fungal endophytes: a. *Tridax procumbens*, b. *Cassia tora* and c. *Parthenium hysterophorus*

3.4 Evaluation of fungal endophytes against selected plant pathogens

The total of thirty-six fungal endophytic OTUs isolated from three weed species were screened against *Sclerotium, Fusarium* and *Rhizoctonia* pathogens for their antagonistic activity in *in-vitro* condition by following dual culture technique.

3.4.a Dual Culture

Evaluation of fungal endophytic OTUs against selected pathogens was performed by placing the mycelial disc of eight mm diameter of actively growing endophytic fungal OTU and targeted pathogenic fungi opposite to each other in Petri dish containing PDA medium leaving one cm from the margin (Skidmore and Dickinson, 1976). The plates were incubated at 27±1 ºC. The data were recorded regularly for the growth of the pathogen and endophytic fungal isolates. Three replications were imposed. Treatment without inoculating the endophytic fungal isolate served as control in each case. The per cent inhibition was calculated after seven days of incubation by using the formula given below (Skidmore and Dickinson, 1976).

PIRG = (R1– R2) / R1 ×100
Where, PIRG – Per cent Inhibition of Radial Growth
R1- Radial growth of the pathogen in control plate
R2- Radial growth of the pathogen in dual culture with endophyte.

3.5 Molecular characterization of selected fungal endophytes and pathogens

Potential fungal endophytes (TPS2, CSR1, CSR3, PHS1, PHS3, PHR3) and the soil-borne plant pathogens of tomato (*Sclerotium* sp., *Fusarium* sp. and *Rhizoctonia* sp.) were identified by amplification of Internal Transcriber Spacer (ITS) region using ITS1 and ITS4 primers and sequencing.

3.5.a Isolation of DNA from fungal endophytes and pathogens

Fungal endophytes (TPS2, CSR1, CSR3, PHS1, PHS3, PHR3) and the soil-borne plant pathogens of tomato (*Sclerotium* sp., *Fusarium* sp. and *Rhizoctonia* sp.) were separately inoculated into the conical flask containing PDB media under aseptic condition and incubated for five days at 27±1 ºC. After five days, the mycelial mat was

harvested, air-dried and used for genomic DNA isolation by Cetyl (hexadecyl) Trimethyl Ammonium Bromide (CTAB) method (Csaikl *et al*., 1998).

3.5.b Protocol

Fungal mycelium (100 mg) was ground in a pestle and mortar using liquid nitrogen. Further, 2% Poly Vinyl Pyrrolidone (PVP) was added, mixed gently followed by 1.0 mL of CTAB buffer (Extraction buffer- 10% Cetramide, 850 mM NaCl, 100 mM Ethylene-diamine-tetra acetic acid and 50 mM Tris HCL - pH 8) was added to the extract and mixed well. The tubes were incubated at 65 ºC for 60 min in a hot water bath with stirring at every 10 min intervals. After 60 min of incubation in a water bath equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed thoroughly. Tubes were centrifuged at 10,000 rpm for 10 min, the supernatant was collected and transferred to the new tubes. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added, mixed gently and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to new tubes, 10 µL of 3 M sodium acetate was added along with 1 mL of ice-cold ethanol (absolute) and kept at -20 ºC for 45 min. After incubation, the mixture was centrifuged at 10,000 rpm for 10 min at 4 ºC. The supernatant was discarded from the tube, the pellet was washed using 200 µL of 70% (v/v) ice-cold ethanol and centrifuged. The pellet was air-dried dissolved in 20 µL of Tris-EDTA (TE) buffer. An isolated genomic DNA sample was run on 0.8% (w/w) agarose gel to confirm the presence of DNA (Csaikl *et al*., 1998).

3.5.c Polymerase Chain Reaction (PCR) amplification of Internal Transcribed Spacer (ITS) larger subunit (LSU) and smaller subunit (SSU) of rDNA region

The Internal Transcribed Spacer (ITS), larger subunit (LSU) and smaller subunit (SSU) of rDNA region in fungal endophytes and plant pathogens were PCR amplified using universal primers (Table 1). PCR amplification was carried out in 25 µL reaction volume consisted of template concentration of 100 ng/ µL, 2.5 µL of 10X Taq buffer, 1 µL of 2 mM $MgCl_2$, 2.5 µL of 1 mM dNTPs mixture, 5 pM of 1.5 µL each primer, 1.5 µL, *Taq* DNA polymerase and sterile distilled water to make full volume of reaction mixture. PCR was performed in Proflex PCR system (Carlsbad, California, United

States). The amplification was carried with initial denaturation of 94 °C for 4 min followed by 30 cycles of 94 °C for 60 s, 55 °C for ITS, SSU and LSU for 45sec, 72 °C for 90 s and final extension step at 72 °C for 10 min. The amplified products were run on 1 % (w/w) agarose gel to confirm the amplification of targeted rDNA regions and further eluted from the gel (Qiagen gel elution kit; *Cat No*./ID: *28706*) and sent for sequencing.

Table 1: The list of primers used for amplification of Internal Transcribed Spacer (ITS), small subunit (SSU) and larger subunit of rDNA region.

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
ITS1/ITS4	CTTGGTCATTTAGAGGAAGTAA	TCCTCCGCTTATTGATATGC
LSUF/LSUR	ACCCGCTGAACTTAAGC	TCC TGA GGG AAA CTT CG
SSUF/SSUR	GTAGTCATATGCTTGTCTC	CTTCCGTCAATTCCTTTAAG

3.5.d Sequence analysis for taxonomic grouping of endophytes and pathogens

The amplified PCR products were sequenced at Medauxin, Bangalore. Sequence similarity searches were performed by comparing these sequences to all available sequences in GenBank using BLASTn - NCBI. Sequences showing the highest identity scores with the present isolates were retrieved. Sequence identity matrices for potential endophyte and the pathogens were generated using the Bioedit Sequence Alignment Editor (Version 5.0.9). A phylogenetic tree was generated using MEGA X software (Kumar *et al*., 2018) and the Neighbour-Joining method (Saitou and Nei, 1987) with 1000 bootstrap replications to estimate evolutionary distances between all pairs of sequences.

3.6 Mechanisms of action of fungal endophytes against fungal plant pathogens

The biocontrol agents generally follow the competition for space and nutrients, antibiosis, hyperparasitism and induced systemic resistance as a counteraction against invading plant pathogens. To know the mechanism of action of the fungal endophytes

against the three soil-borne pathogens infecting tomato, the following assays were conducted.

3.7 Competition for space

Competition for space between endophyte and pathogen was studied under *in-vitro* condition by calculating the per cent growth inhibition of pathogens in dual culture plates in comparison with the pathogen growth in control plates (Skidmore and Dickinson, 1976).

3.8 Antibiosis

It is one of the major mechanism involved by the biocontrol agents in inhibiting the plant pathogens by producing antimicrobial substances. The antimicrobial substances can be diffusible compounds and/or volatile organic compounds. To know the antibiosis nature of fungal endophytes following studies were conducted.

3.8.a Diffusible compound

Secondary metabolites produced by fungal endophytes have a wide range of bioactive compounds involved in various biotic stress mitigating activities (Kusari *et al.*, 2012). To confirm the presence of antimicrobial diffusible compounds in the secondary metabolites produced from the potential fungal endophytes, extraction of secondary metabolites followed by antimicrobial activity assay was conducted is here under.

3.8.b Extraction of secondary metabolites

Secondary metabolites produced from biocontrol agents were known to play a significant role in the inhibition of antagonistic organisms. To check the antimicrobial diffusible compounds produced by the fungal endophytes against targeted pathogens, extraction of the secondary metabolites produced by the fungal endophytes was carried out through the ethyl acetate extraction method as described by Pansanit and Pripdeevech, (2018) with slight modifications as mentioned below.

The 10 mm mycelial disc (four to five) from PDA media of actively growing seven days old fungal endophyte was inoculated to a 1000 mL conical flask containing 200 mL of PDB media and incubated at 27±1 ºC. After 20 days of incubation, the mycelial mat was removed using sterile forceps and the filtrate was filtered through Whatman No. 1 filter paper. The 200 mL filtrate was mixed with an equal volume (200 mL) of ethyl acetate (1:1 v/v ratio) and subsequently, the mixture was partitioned in a 500 mL separating funnel. In the separating funnel, a lower fraction containing the broth was discarded and the upper solvent fraction containing the metabolites was collected into a round bottom flask. The collected metabolites in the solvent fraction were subjected to a rotary evaporation at 40 °C to evaporate ethyl acetate. Further, to get concentrated extract of secondary metabolites, it was subjected to vacuum rotary evaporator with reduced pressure at 40 °C. Finally, the concentrated ethyl acetate extract of fungal endophytes was stored in Eppendorf tubes at 4 °C for further studies.

3.8.c Antimicrobial activity assay

The concentrated ethyl acetate extract of secondary metabolites obtained from the fungal endophytes was screened for their antimicrobial activity by following disc diffusion test as described by Pansanit and Pripdeevech, (2018) with slight modifications.

The concentrated extract of secondary metabolites was diluted by ethyl acetate solvent (1:1 ratio). Sterile discs (HiMedia) were impregnated with 20 µL of diluted metabolite extract of each fungal endophyte inside the laminar airflow chamber and dried for 15 minutes. After drying, these discs were placed on PDA media along with the targeted pathogen placed 20 mm from each other. Sterile discs impregnated with 20 µL each of Carbendazim 0.1% solution and ethyl acetate solvent served as positive and negative control, respectively. The PDA plates were incubated at 27±1 ºC and observations were recorded after four days of incubation. All these experiments were carried out with four biological replicates.

3.8.d Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC/ESI-MS/MS) analysis of secondary metabolites from fungal endophytes

The ethyl acetate extracts from two fungal endophytes, *T. asperellum* isolate 1 and *T. asperellum* isolate 2 showing maximum inhibitory activity among the isolated endophytes against *Sclerotium, Fusarium* and *Rhizoctonia* were subjected to LC/ESI-MS/MS analysis. The LC/ESI-MS/MS (Bruker) system following electrospray ionization (ESI) positive ion mode for analyses of metabolomes in the ethyl acetate extracts of endophytes as described by Tang *et al*. (2020).

In positive ion mode, separation of metabolites was conducted by Agilent poroshell 120 (4.6*150mm) column. The mobile phase contained water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A syringe pump delivering 5 μL was adjusted by the direct loop injection method. Mobile phase used was 0.1% formic acid with water and acetonitrile, and operated at a flow rate of 0.3 mL/min. Mass spectrometry detections were set at, capillary temperatures of 350 °C and spray voltages of 3.5 kV. The MS resolution was set to 30000.

The operating parameters were, the cone voltage set at 3 kV and the spray was stabilized with a nitrogen sheath gas (900 L/hr). Nitrogen was used as nebulizing gas (50 L/hr), capillary source temperature and desolvation temperature were set at 135 and 350 °C, respectively. The capillary voltage of 3 kV and collision energy of 15–25 eV was maintained for MS/MS. The metabolite peaks obtained in the chromatogram were analysed through Bruker Compass Data analysis software and obtained mz/mz value of compounds were searched in MetFrag (https://msbi.ipb-halle.de/MetFragBeta/) to predict the possible elemental compositions of product ion. The observed product ions were compared with previously authenticated data available in metabolomics database *viz*., PubChem, Chemical Entities of Biological Interest (ChEBI) and Kyoto Encyclopedia of Genes and Genomes (KEGG) to identify the compounds present in the ethyl acetate extracts based on the similarity.

3.9 Prediction of interaction between beta-tubulin protein and secondary metabolites characterized from fungal endophytes

The secondary metabolites from the *T. asperellum* isolates (TA1 and TA2), characterized through LC/ESI-MS/MS analysis were used for *in-silico* docking analysis to predict the possible compound in binding the β-tubulin, a target site for binding of benzimidazole fungicides in inhibiting the plant pathogenic fungi.

3.9.a Retrieval of sequence and *ab initio* modelling of β-tubulin region of the fungus

As a prerequisite for *in-silico* docking analysis, the prediction of protein structure is an important step. The tubulins are the major components of microtubules which are ubiquitous and present in all eukaryotic organisms (McKean *et al*., 2001). They are mainly involved in many cellular processes, such as cell division, flagellar motility and intracellular transport in eukaryotic organisms. The fungal β-tubulins are the molecular targets of benomyl or MBC fungicides (Hollomon *et al*., 1998). As β-tubulin is well conserved across different fungi/eukaryotic organism, the full-length protein sequence of β-tubulin was retrieved from the NCBI repository (https://www.ncbi.nlm.nih.gov/) in the FASTA format with accession number (CUA76508.1). As a prelude to the prediction of the 3-Dimensional structure (3D), the protein sequence was subjected to Basic Alignment Search Tool (BLAST) analysis against protein data bank (PDB) to find the availability of templates for homology modelling. However, due to the unavailability of templates for β-tubulin, the sequence was uploaded to I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER) to obtain the *ab-initio* model (Yang and Zhang, 2015). Energy minimization is essential to determine the proper molecular arrangement in space since the drawn chemical structures are not energetically favourable. The side-chain refinement and energy minimization of the predicted protein was done by uploading the protein to GalaxyRefine2 web server (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE2) (Lee *et al*., 2019). Further, to validate the stereochemical quality of the refined 3D model, it was uploaded to SAVES online tool (https://servicesn.mbi.ucla.edu/SAVES/) (Daroczi, 2013) to obtain the Ramachandran plot in PROCHECK. The model with good quality was

visualized in Biovia Discovery Studio 2020 (Biovia, 2020) and PyMOL (DeLano, 2009).

3.9.b Sources of ligands and energy minimization

Based on the availability of 3D conformers in the public domain database, a total of 18 metabolites characterized from *T. asperellum* isolate 1 (9 metabolites) and *T. asperellum* isolate 2 (9 metabolites), the 3D structures of these ligands were downloaded from PubChem (https ://pubchem.ncbi.nlm.nih.gov) database in SDF format and converted to PDB format using Open Babel version 3.1.1. (O'Boyle *et al*., 2011) for further analysis. Carbendazim was used as a positive control. The ligands were optimized by minimizing their energy using MMFF94 force field type in Avogadro version 1.2.0 (Hanwell *et al*., 2012).

3.9.c Prediction of the binding pocket of protein and molecular docking

The prediction of the binding pocket in a given protein is an important step in molecular docking analysis. The active amino acid residues of the protein were determined using the Computed Atlas for Surface Topography of Proteins (CASTp) (http://sts.bioe.uic.edu/castp /index .html201l) server. Based on the information obtained from the CASTp server, the grid box was set to 60 Å × 60 Å × 60 Å centred at 64.263, 43.155, 71.908 (XYZ coordinates) in AutoDock-MGL Tools (Morris *et al*., 2009). The ligands were prepared for docking analysis by adding Gasteiger charges. The standard processes were used to convert the protein and ligands to standard PDBQT files. AutoDock Vina software (Trott and Olson, 2010) helps in finding the binding affinity of a particular protein with the ligand. Hence, the proteins and ligands were subjected to docking analysis with the exhaustiveness value of 24 (Froli *et al*., 2016). The output file with the lowest binding energy (kcal/mol) and with the least mean square deviation (RMSD) was selected. The interaction between protein and the ligand was visualized under PyMOL, similarly, the hydrogen bonds between protein and ligand were obtained from the 2D structure visualization in Biovia Discovery studio 2020.

3.10 Volatile organic compounds

The antimicrobial activity through antibiosis by fungal endophytes in inhibiting pathogens is not only confined to diffusible compounds, but also includes volatile organic compounds. To study the ability of fungal endophytes to produce antimicrobial volatile organic compounds (VOCs) following studies were carried out.

3.10.a Double Petri dish assay

The potential fungal endophytes were checked for the production of antimicrobial VOCs in *in-vitro* by following a double Petri dish assay. The 10 mm actively growing fungal endophyte mycelial disc from PDA media of seven days old culture of was inoculated into the basal lid of a Petri dish containing PDA. The upper lid of the plate was replaced with a basal lid containing PDA media inoculated with 10 mm mycelial disc of actively growing pathogen. The two plates were sealed immediately by saran wrap with air-tight condition and were incubated at 27±1 ºC. The target pathogen in the upper lid and empty base plate with PDA media only served as control. The per cent inhibition of radial growth of targeted pathogens was recorded five days after incubation (Rouissi *et al*., 2013). The per cent growth inhibition of targeted pathogens was calculated by using the formula: [(R1 - R2)/R1] ×100, where R1 is the radial growth of the target pathogen in control and R2 is the radial growth of the target pathogen exposed to endophyte (Trivedi *et al*., 2006). The assay was conducted with four biological replicates and repeated thrice.

3.10.b Gas chromatography analysis

The potential fungal endophyte *T. asperellum* isolate 1 and 2 showing greater inhibition against selected pathogens in double Petri dish assay was used to identify the composition of VOCs produced for qualitative evaluation by Headspace (HS) - Solid Phase Micro Extraction (SPME) coupled with Gas Chromatography-Mass Spectrometry (GC–MS). The SPME fiber (specification) coated with DVB/CAR/PDMS was used. Three days old cultures were used for the headspace sampling and performed in Petri dishes as described by Rouissi *et al*. (2013). Solid-Phase Micro Extraction (SPME) fiber

was exposed to adsorb the volatile compounds released from the Petri dish containing samples for 30 min at room temperature and then immediately transferred the fiber into the GC inlet. Trapped compounds were thermally desorbed into the GC injection port in a splitless mode at 270 ºC, and separated in an Agilent 7890B gas chromatograph (GC) coupled with Agilent 5977A mass spectrometer (MSD) equipped with a GC column HP-5 MS column (30 m X 0.25 mm id, 0.25 µm film thickness). The oven temperature was set at 40 ºC for 3 min hold time and then programmed to rise from 40 ºC to 90 ºC at a rate of 10 ºC/ min, from 90 ºC to 180 ºC at a rate of 5 ºC/ min and from 180 to 260 ºC at a rate of 20 ºC / min with no hold times. The transfer line was heated at 250 ºC, the ion source at 230 ºC and quadrupole temperature at 150 ºC. Helium carrier gas had a flow of 1 mL/ min.

The tentative characterization of VOCs collected from different treatments were achieved by calculating the retention index of obtained GC peaks by using the formula given below and comparing the mass spectra with the data system library (NIST 14 MS Library). Blank sample analysis (growth medium not inoculated with any microorganisms) was performed under the same conditions to exclude interfering substances. All measurements were made with two replicates, each replicate representing the analysis of a different Petri dish.

For temperature-programmed gas chromatography, the retention index is given by the equation, (Zhu, 1985).

$$I=TR(unknown) – TR(n) / TR(n+i) – TR(n) \times 100i + 100n$$

Where,

I= Retention index

TR= the retention time

n= the number of carbon atoms in the smaller n-alkane

n+i= the number of carbon atoms in the larger n-alkane.

3.11 Scanning electron microscopic studies

Hyperparasitism is one of the ancestral character of the genus *Trichoderma* where, the mycelium of trichoderma gets coiled around the mycelium of pathogenic fungi and disintegrates mycelia of the pathogen by secretion of the lytic enzymes (Kubicek *et al*., 2011). Among the potential fungal endophytes in the current study, the PHR3 OTU belongs to the taxa *Trichoderma asperellum* was selected for knowing its ability of hyperparasitism against the targeted pathogens by subjecting to scanning electron microscopic (SEM) study.

The dual culture plates containing 5 days old with different treatment of *T. asperellum* against sclerotium, rhizoctonia and fusarium were used for sampling. The small area of mycelial interaction zone between endophyte and pathogen was collected, immediately placed on the carbon adhesive tape stuck on to the aluminium stub and coated to sputter and observed in its natural state. The sputter-coated sample was taken for imaging analysis using a SEM (Hitachi TM3030, Scanning Electron Microscope) (Babu *et al*., 2018).

3.12 Host colonization assay

The potential fungal endophytes were checked for colonization in tomato plants. Seed of tomato variety Arka Vikas (Tomato variety from Vegetable Division, Indian Institute of Horticultural Research, Bangalore) were kept for pre-germination on blotter paper at 27 ºC for 4-6 days. The spore or mycelium suspension of potential fungal endophytes with the inoculum load of 2×10^6 CFU/ mL (Colony-forming unit) was prepared in sterile distilled water from 7 - 10 days old cultures grown on PDA medium using a haemocytometer (Zhang *et al*., 2014). The pre-germinated seeds were soaked for three hours in the spore or mycelial suspension. After three hours, the pre-germinated seeds were transferred to portrays containing autoclaved coir pith and watered regularly. Thirty days after sowing (DAS), the plant samples were collected and processed (3.3.a) for isolation of fungal endophytes from leaf, stem and roots to confirm the colonization of inoculated endophytes in the tomato plant.

Treatments followed: T_1= Control (without endophyte treatment) and T_2= Endophytic *T. asperellum* isolate 2 treated.

3.13 *In vivo* studies

To study the effect of potential fungal endophytes against three pathogens infecting tomato pathogens in *in-vivo*, glasshouse experiments were conducted as described below.

Soil: soil and farmyard manure mixture (1:1) was fumigated by using 4% formalin solution for one week before filling it into the plastic pots.

Endophyte treatment: The potential endophyte *T. asperellum* was treated to tomato pre-germinated seeds as described in the 3.11.

Pathogen application: The mass multiplied sclerotium pathogen was added [3% (v/v)] to the pots containing sterilized pot mixture and allowed seven days for multiplication. The 25 days old tomato seedlings raised in the protrays filled with autoclaved coir pith were transplanted to the pots containing sterilized pot mixture with different treatments as mentioned below,

Treatments:

T_1: Control – Plants without any treatment
T_2: Endophyte treated – Seeds primed with endophyte *T. asperellum* (TA2)
T_3: Pathogen treated – Plants inoculated with sclerotium pathogen inoculum
T_4: Endophyte – *T. asperellum* (TA2) primed plants inoculated with pathogen inoculum

After transplanting the tomato seedlings, it was monitored and phenotypic expression was recorded regularly. The disease incidence was recorded daily in the T_3 and T_4 treatments by using the formula,

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

3.14 Induced systemic resistance

Biocontrol agents are known to induce resistance in plants. There are few reports to claim that, endophytic *Trichoderma* isolates also known to trigger systemic resistance against plant pathogens in various plant species (Evans *et al*., 2003). In this context, to check whether endophytic *T. asperellum* isolate 2 can trigger ISR in tomato plants against selected pathogens or not was assessed by qRT-PCR was carried out for genes involved in systemic resistance pathway.

For ISR studies different treatments were followed, initially tomato seeds were primed with *T. asperellum* isolate 2 as mentioned in chapter 3.11, and all the conditions were maintained as in chapter 3.12.

T_1= Control Plants

T2 = Endophyte (TA2) primed plants

T3 = Pathogen (*S. rolfsii*) treated plants

T4 = Endophyte (TA2) primed plants exposed to pathogen (*S. rolfsii*)

3.14.a RNA isolation

Initially tomato leaf samples were harvested at different time interval (24, 48 and 72 hrs.) after imposing the different treatments (Control, Endophyte treated, Pathogen treated and Endophyte-Pathogen interaction). Then total RNA was isolated using TRI reagent (Sigma catalogue no. T9424).

The steps followed were as follows:

1. 100 mg of the tomato leaf samples was weighed.
2. The sample was ground to a fine powder by using liquid nitrogen in a pestle and mortar that was treated with diethylpyrocarbonate (DEPC) water and placed in a sterile microcentrifuge tube.
3. 100 mg ground leaf sample was homogenized with 1 ml of TRI reagent.
4. The homogenate was centrifuged at 13,000 rpm for 15 min at 4 °C.

5. The supernatant was transferred to a fresh centrifuge tube and the tube was kept for 5 minutes at room temperature.
6. A volume of 0.2 ml of chloroform was added to the microcentrifuge tubes and shaken vigorously for 30 seconds.
7. The tube was incubated for 30 minutes at room temperature.
8. The tube was centrifuged at 13,000 rpm for 10 min at 4 °C. Three separate phases were formed. Colourless upper face consisted of RNA, interphase/mid-phase consisted of DNA and bottom red organic phase consisted of protein.
9. The colourless upper aqueous phase was transferred into a fresh micro-centrifuge tube and 0.5 ml of Isopropanol was added and mixed properly.
10. The tube was kept for 30 min at room temperature and centrifuged at 13,000 rpm for 10 minutes at 4 °C.
11. The supernatant was discarded and the RNA pellet was washed with 75 per cent cold ethanol with vertexing.
12. The tube containing RNA pellet was centrifuged at 13,000 rpm for 10 minutes at 4 °C.
13. Ethanol was removed without losing the pellet and the pellet was dried briefly for 5-10 minutes.
14. Finally, the dried pellet was re-suspended in 20-30 µl of nuclease-free water or DEPC treated water.
15. The pellet was dissolved by repeated pipetting with a micro-pipette or by keeping at 50-55 °C water for 3min and stored at -80 °C for further use.

3.14.b Synthesis of cDNA

The RNA isolated from the plant samples was used for reverse transcriptase-PCR (RT-PCR). Initially, cDNA was synthesized from total RNA in a 20µl reaction using BIOSCRIPT–RT enzyme (BIOLINE catalogue no. BIO-27036) as per manufacturer's instructions

1. A volume of 8µl of template total RNA + 1µl of reverse primer were added into a 0.2ml of polypropylene tube and spin tubes gently to ensure proper mixing.

2. Then mixture was incubated at 70°C for 5 minutes in PCR machine.
3. After incubation PCR tubes were immediately chilled on ice for 2 minutes
4. All the reaction components including 4µl 5X BIOSCRIPT reaction buffer (BIOLINE), 2.5 µl 10mM dNTPs and 4µl of DEPC water were added into the tube and the tube was incubated at 37°C for 1 minute.
5. 0.5µl of Bio script – RT enzyme was added to the tube and incubated at 42°C for 60 minutes.
6. The reaction was stopped by heating the mixture at 70°C for 10 min and stored at -20°C. The cDNA obtained was used for performing PCR.

Reagents	**Volume (µl)**
Total volume of aliquot taken in PCR tube	20µl
RNA template	8µl
Reverse primer	1µl
RNase free water	4µl
RT-Buffer	4µl
10mM dNTPs mixture	2.5µl
RT enzyme	0.5µl

3.14.c Quantitative real-time (qRT)-PCR analysis of gene expression

Quantitative real-time (qRT)-PCR was performed for seven selected genes associated with plant defense along with actin on cDNA templates prepared from total RNA that was extracted from tomato tissues.

3.14.d Designing of oligonucleotide primers for gene expression studies through qRT-PCR

For studying the induced systemic resistance SA and JA pathway related genes were selected to check the regulation of these genes upon different treatments imposed to tomato plants. The targeted for expression studies were, JA upstream and downstream gene *OPR3* and *COI1* respectively. Further, *ICS1* upstream gene and *NPR1, PR1, PR2*

and *PR3* downstream genes for SA. For these seven genes, specific qRT-PCR primers were designed using Primer Quest software, and the details of the primers is presented below

Gene name	Forward primer	Reverse primer
PR1	CAAGTTGGAGTCGGTCCTATG	GTAAGGTCCACCAGAGTGTTG
PR2	TCCAGGTAGAGACAGTGGTAAA	CCTAAATATGTCGCGGTTGAGA
PR3	GTTGTGGATGACAGAACAGGA	ACCGTACCCTGGAACTCTATTA
COI1	GATAATGGTGTGCGTGCTTTAC	TATTGCCCGACATAACTGAGAC
ICS1	GCAGCTTTCCTCCGTTCTTA	ATGGTCCCAAGACGCTTTAC
NPR1	GGCTAGCATGAGGAAGAAGATAG	GCCCTAAGCCGATTCAAGT
OPR3	TCATGTAACACAGCCACGATAC	GCGTTCCTCAAAGTCCTCATTA

3.14.e Use of SYBR Green detection chemistry to perform Real time PCR amplifications in a Bio-Rad CFX96 Touch Real-Time PCR Detection System

Reactions were prepared in a total volume of 12.5µL containing the following

Reagents	**Volume (µl)**
First-strand cDNA template	0.5µl
Forward primer (10 pmol)	0.25µl
Reverse primers (10 pmol)	0.25µl
iQ™ SYBR® Green Supermix	6.2µl
Sterile de-ionized distilled water	5.25µl
Total	**12.5 µl**

The qRT-PCR was conducted with following conditions; initial denaturation at 95°C for 5 minutes, followed by 35 cycles with 30 seconds of denaturation at 95°C, 45 seconds of 60°C annealing temperature and extension at 72°C for 30 seconds. For each gene three biological replicates were used. Amplicon was subjected to melt curve

analysis to check the specificity of the amplified product. Baseline and threshold cycles (Ct) will be automatically determined using real-time PCR system software. The relative expression was calculated using comparative cycle threshold method, where delta (Δ) cycle threshold of cDNA from controls was defined as 100% transcript presence. Transcript abundance data was normalized against the average transcript abundance of endogenous control genes in each treatment was calculated using the following equation $2^{-\Delta\Delta Ct}$, where ΔΔCt = (Ct value of target gene − average Ct value of reference genes) treatment − (Ct value of target gene − average Ct value of reference genes) control.

IV RESULTS AND DISCUSSION

Tomato crop suffers from soil-borne fungal pathogens *viz., Sclerotium, Fusarium* and *Rhizoctonia* which are responsible for reduction in production and productivity. Due to the ill effects of chemical management of plant diseases, exploitation of fungal endophytes against soil-borne plant pathogens are gaining importance in the present scenario (Abro *et al*., 2019). Fungal endophytes were known to confer tolerance against various plant pathogenic fungi by multiple modes of action. In the current study mode of actions of potential fungal endophytes against soil-borne fungal pathogens infecting tomato*, viz., Sclerotium, Fusarium* and *Rhizoctonia* were deciphered and results obtained were presented in this chapter.

4.1 Isolation of soil-borne plant pathogens

Tomato plants showing symptoms typical to southern blight, fusarium wilt and dry root rot caused by *Sclerotium* sp., *Fusarium* sp. and *Rhizoctonia* sp., respectively were collected separately from the farmer's field. The samples were processed, inoculated into Petri plates containing PDA media and incubated at 27 ºC. After four days of incubation, mycelial growth was observed from the cut ends and surface of the inoculated tissues on PDA media. The individual mycelial colony was sub-cultured and pure cultures of the pathogens were obtained (Plate 2). The morphological features like white silky mycelial growth with sclerotial bodies in *Sclerotium*, micro and macroconidia in *Fusarium* and right-angle branching of mycelium with a constricted region at the base in *Rhizoctonia* were observed under a microscope (Plate 2). Initial identity of the pathogens in the current study was confirmed based on the morphological features and colony characters in comparison with earlier reports (Hsieh 1992; Sharma *et al*., 2005 and Matuo and Snyder, 1973). Based on this the pathogens isolated from southern blight, fusarium wilt and dry root rot samples were identified as *Sclerotium* sp., *Fusarium* sp. and *Rhizoctonia* sp. After morphological confirmation, the pure cultures were grown on PDA slants and stored at 4°C for further studies.

4.1.a Pathogenicity assay

Tomato seedlings (variety Arka Vikas) raised in the portrays were transplanted to the pots containing mass multiplied *Sclerotium, Fusarium* and *Rhizoctonia*

pathogens and maintained under glasshouse condition. The symptoms produced by each pathogen were described, a) *Sclerotium*; After 4 days of transplanting, the tomato plants transplanted to the pots containing mass multiplied *Sclerotium* pathogen showed drooping of leaves and at the collar region white silky mycelial growth was observed. After 5 days, entire foliage starts showing drooping symptom with drying of leaves. Finally, after 8 days, entire plant gets wilted with white mycelial growth and small brown to black coloured sclerotial bodies on the stem, collar region and root tissues of tomato plants were observed (Plate 3a). b) *Fusarium*; *Fusarium* infected plants start showing symptoms after 12 days in case of directly planted seedlings and 4 days in case of tertiary roots trimmed seedlings before transplanting. Initially, lower leaves start showing yellowing and gradually starts drooping. After 8 days from initial symptom expression, yellowing spreads to entire foliage with drying and wilting of plants. Upon longitudinal splitting of stem and roots of infected plants, brown discoloration of vascular tissues was observed (Plate 3b). c) *Rhizoctonia*: Directly transplanted and root trimmed tomato seedlings expressed symptoms after 10 and 3 days of transplanting, respectively. In case of both *Fusarium* and *Rhizoctonia* infected tomato plants initially produced yellowing of lower leaves and later resulted in complete drying of foliage. However, necrotization at the collar region leading to rotting was observed only in *Rhizoctonia* infected plants (Plate 3b). The symptoms observed in the current study on tomato plant upon infection with *Sclerotium, Fusarium* and *Rhizoctonia* were similar to the earlier reports of symptoms produced by *S. rolfsii, F. solani and R. solani* isolates, respectively (Young and Ashford, 1995; Jones *et al*., 1997 and Dutta and Dutta, 2007).

4.2 Isolation of fungal endophytes

Fungal endophytes were reported to regulate secondary metabolites production in their host plants and are also known to produce many antimicrobial compounds under biotic stress conditions to safeguard the plants (Kusari *et al*., 2012). By considering these well-proven concepts, weed species *viz*., *Tridax procumbens, Cassia tora* and *Parthenium hysterophorus* possessing antimicrobial nature against various plant pathogens were selected for isolation of fungal

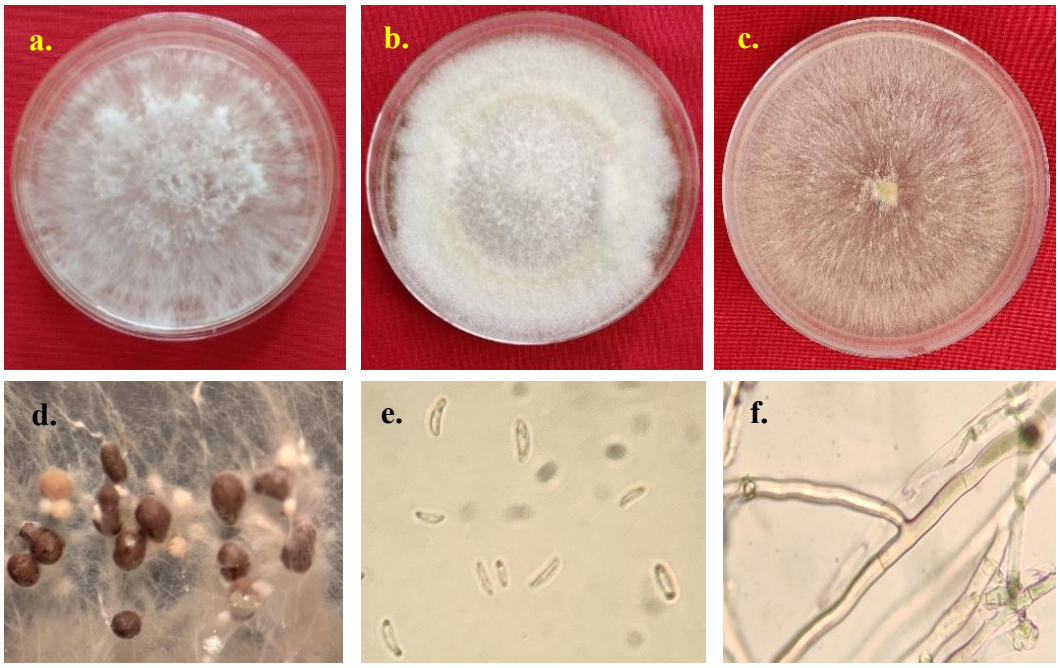


Plate 2: Pure culture of a) *Sclerotium* sp., b) *Fusarium* sp. and c) *Rhizoctonia* sp. on potato dextrose agar (PDA) media, with their morphological features, d) Sclerotial bodies e) Macroconidia and f) Right angle branching of mycelium, respectively

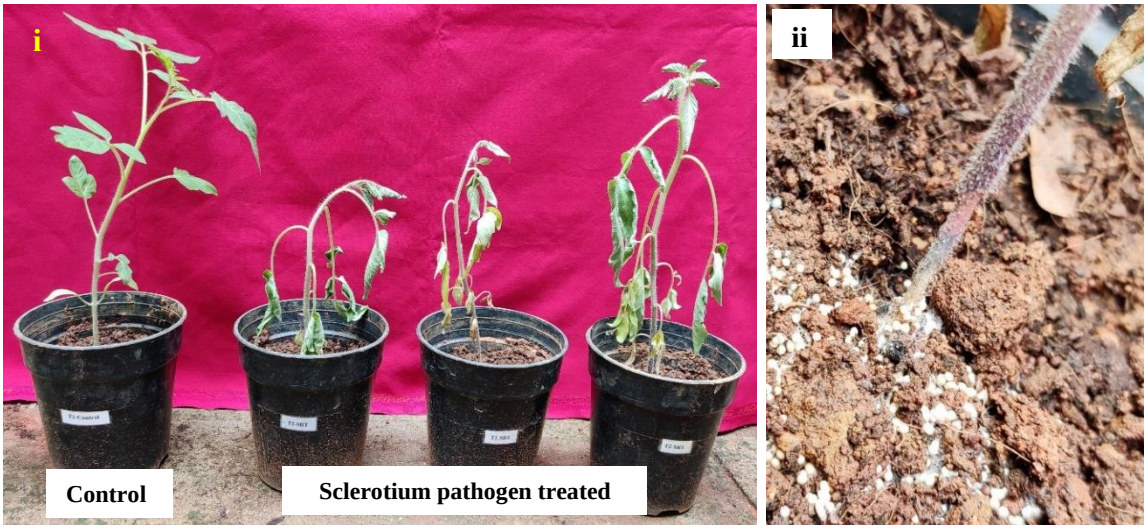
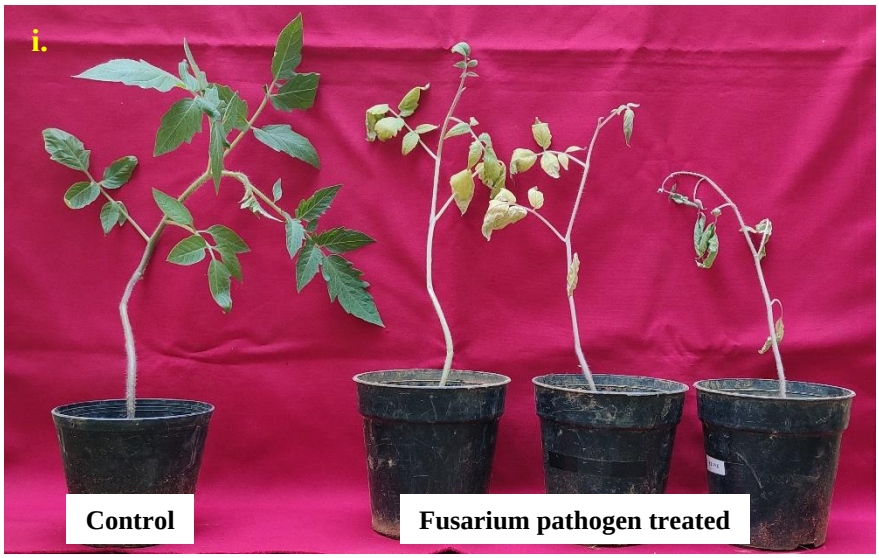


Plate 3a: Pathogenicity assay; i) *Sclerotium* sp. inoculated tomato plants showing wilting symptoms similar to field infected plants. ii) Sclerotial bodies at collar region observed in *Sclerotium* sp. inoculated tomato plants



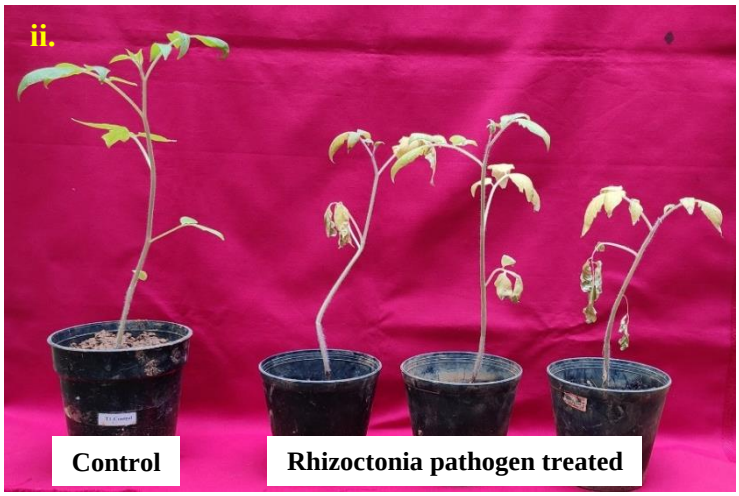


Plate 3b: Pathogenicity assay; i) *Fusarium* sp. inoculated tomato plants showing wilting symptoms and ii) *Rhizoctonia* sp. inoculated tomato plants showing root rot symptoms similar to field infected plants. and iii) Rotting at the collar region caused by *Rhizoctonia* sp.

endophytes (Taddei and Rosas-Romero, 2000; Pandya *et al*., 2017 and Gupta *et al.,* 1996).

Leaf, stem and root segments of *Tridax procumbens*., *Cassia tora* and *Parthenium hysterophorus* were processed for fungal endophytes isolation by inoculating onto the Petri dishes containing PDA media. After 4 to 7 days of inoculation mycelial growth was observed from cut end tissues of inoculated plant segments. The colonization frequencies of the endophytic fungi from the five plant species was ranged from 66.66% to 100%. The highest colonization frequency was observed with *T. procumbens,* while *P. hysterophorus* showed the lowest colonization frequency (Table 2). Among the different sample segments, leaf has the highest colonization frequency followed by stem and root segments in the plant samples processed. After ten days of incubation, individual fungal colony was sub-cultured and purified based on their morphological features. Totally, 45 endophytic fungal isolates were isolated from 108 tissue segments processed from the leaf, stem and root samples collected from three weed species (36 from leaf, 36 from stem and 36 from root segments).

Table 2: Isolation of fungal endophytes from selected plant species, their categorization into Operational Taxonomic Units (OTUs) and calculation of colonization frequency based on number of segments colonized by the fungal endophytes

Sl. No.	Plant species	Taxonomic Family	Plant part	No. of segments placed in PDA media	No. of endophytes emerged	No. of OTUs categorized	Colonization frequency (%)
1.	*Tridax procumbens*	Asteraceae	Leaf	12	6	5	100
			Stem	12	6	5	91.66
			Roots	12	7	4	100
2.	*Parthenium hysterophorus*	Asteraceae	Leaf	12	8	5	91.66
			Stem	12	4	3	66.66
			Roots	12	3	3	66.66
3.	*Cassia tora.*	Fabaceae	Leaf	12	5	3	100
			Stem	12	5	4	91.66
			Roots	12	3	3	75

4.2.a Categorization of fungal endophytic isolates into Operational Taxonomic Units (OTUs)

Based on the morphological characters such as colour of mycelia, spores, fruiting bodies, etc., 45 fungal endophytic isolates were categorized into 35 OTUs (Plate 4). Totally, 35 fungal endophytic OTUs from the current study and one potential fungal endophyte isolated from hibiscus leaf tissue, *Trichoderma asperellum* (H12 OTU) in the previous study were used for further experiments.

The fungal endophytes diversity in a particular plant species depends on habitat, season, geographical location etc. In the current study, the results revealed that, even though the three weed species were collected from the same habitat, location and time, they varied in per cent colonization and a number of endophytes obtained, which is in accordance with the earlier reports (Schulz and Boyle, 2005; Kim *et al*., 2013 and Selosse *et al.*, 2004).

The diverse population of fungal endophytes are expected from the different tissue segments of the same host plants used for endophytes isolation (Carroll, 1988). In the current study, same trend was observed in the root segments. However, the fungal endophytes obtained from the leaf and stem were almost the same with few exceptions. The present results indicating that, the diversity of endophytes was higher in root compared to leaf and stem. The possible reason might be the influence of rhizosphere environment on the root ecology as well the physiological and biochemical difference among the different tissues of host plants. Further, study into this may provide new insights into the species diversity of fungal endophytes in different parts of the plant.

4.3 Evaluation of fungal endophytes against selected plant pathogens

All the 36 fungal endophytic OTUs were evaluated against *Sclerotium, Fusarium* and *Rhizoctonia* pathogens in dual culture to check their efficacy under *in-vitro* condition by following dual culture technique (Plate 5a-5c). Per cent inhibition of individual pathogens by the fungal endophytic OTUs was recorded seven days after inoculation and represented in the Fig 1. Out of 36 fungal endophytic OTUs, six and seven OTUs showed more than 50% inhibition against *Fusarium* and *Rhizoctonia* pathogens, respectively. Two OTUs showed more than

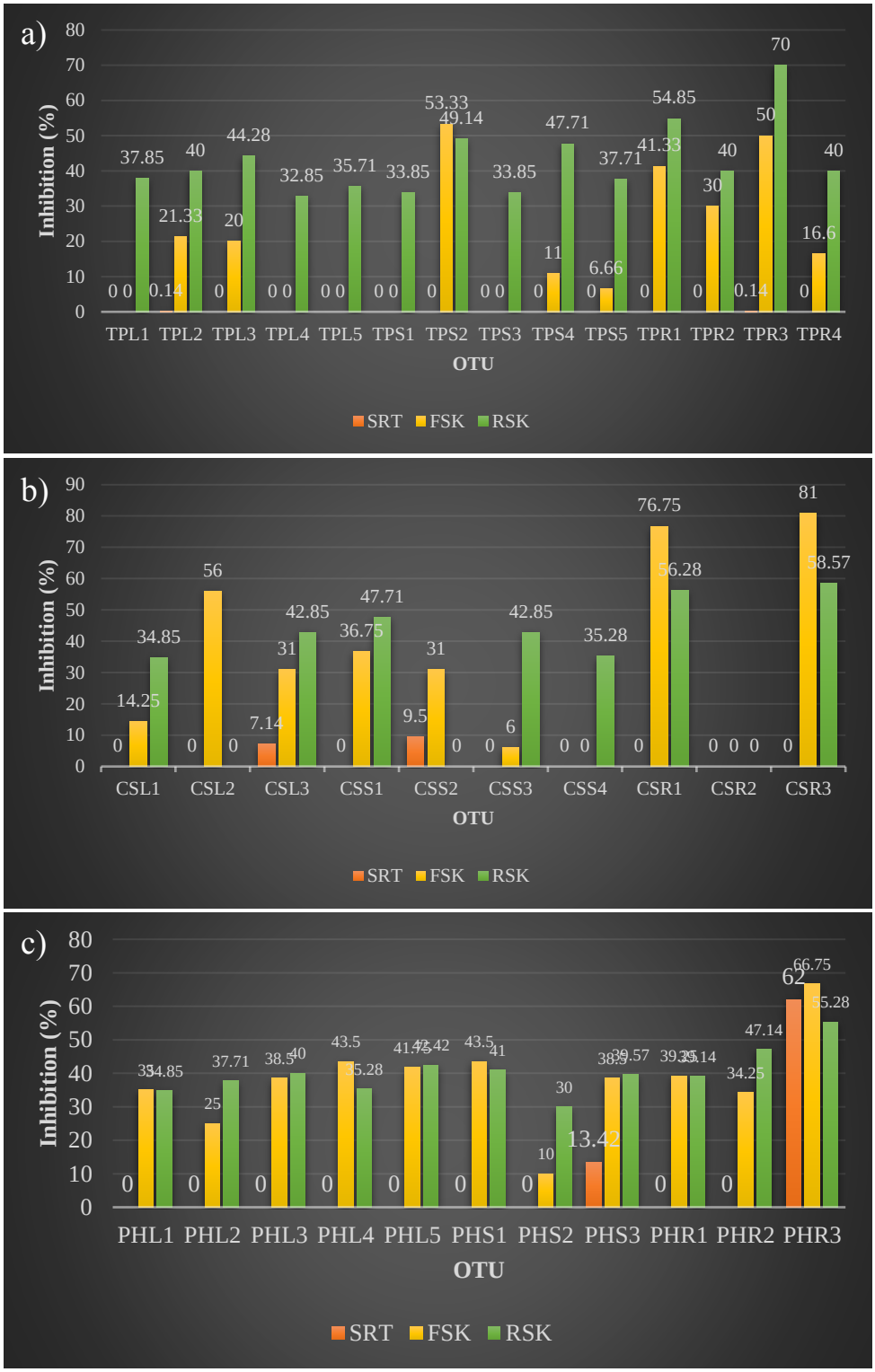


Figure 1: Per cent inhibition of *Sclerotium* (SRT), *Fusarium* (FSK) and *Rhizoctonia* (RSK) pathogens by a) *Tridax procumbens* b) *Cassia tora* and c) *Parthenium hysterophorus* fungal endophytic OTUs in dual culture assay

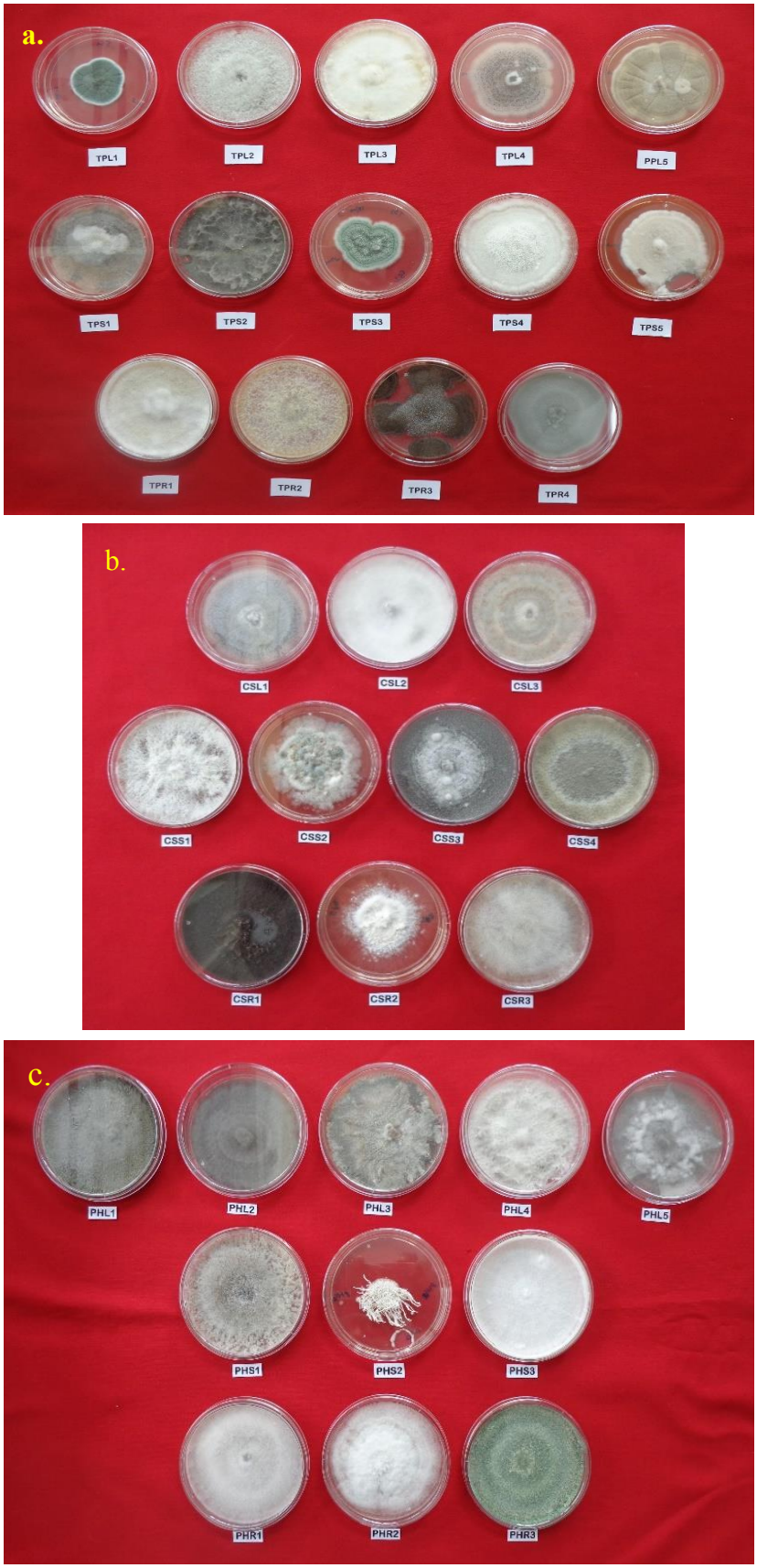


Plate 4: Pure culture of fungal endophytic Operational Taxonomic Units (OTUs) obtained from a) *Tridax procumbens,* b) *Cassia tora* and c) *Parthenium hysterophorus*

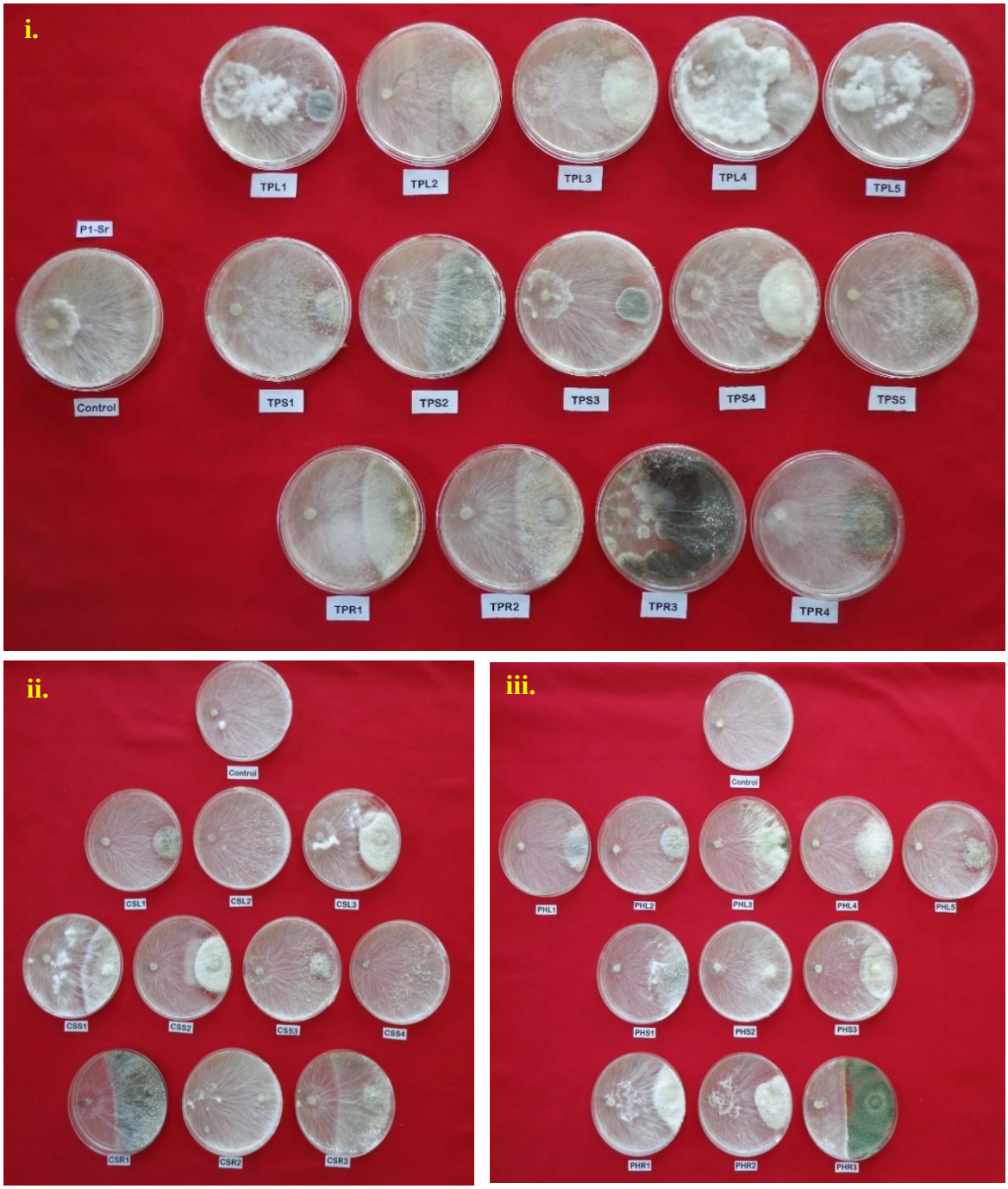


Plate 5a: Dual culture assay for screening fungal endophytic OTUs against *Sclerotium* pathogen isolated from i) *T. procumbens*, ii) *C. tora* and iii) *P. hysterophorus*

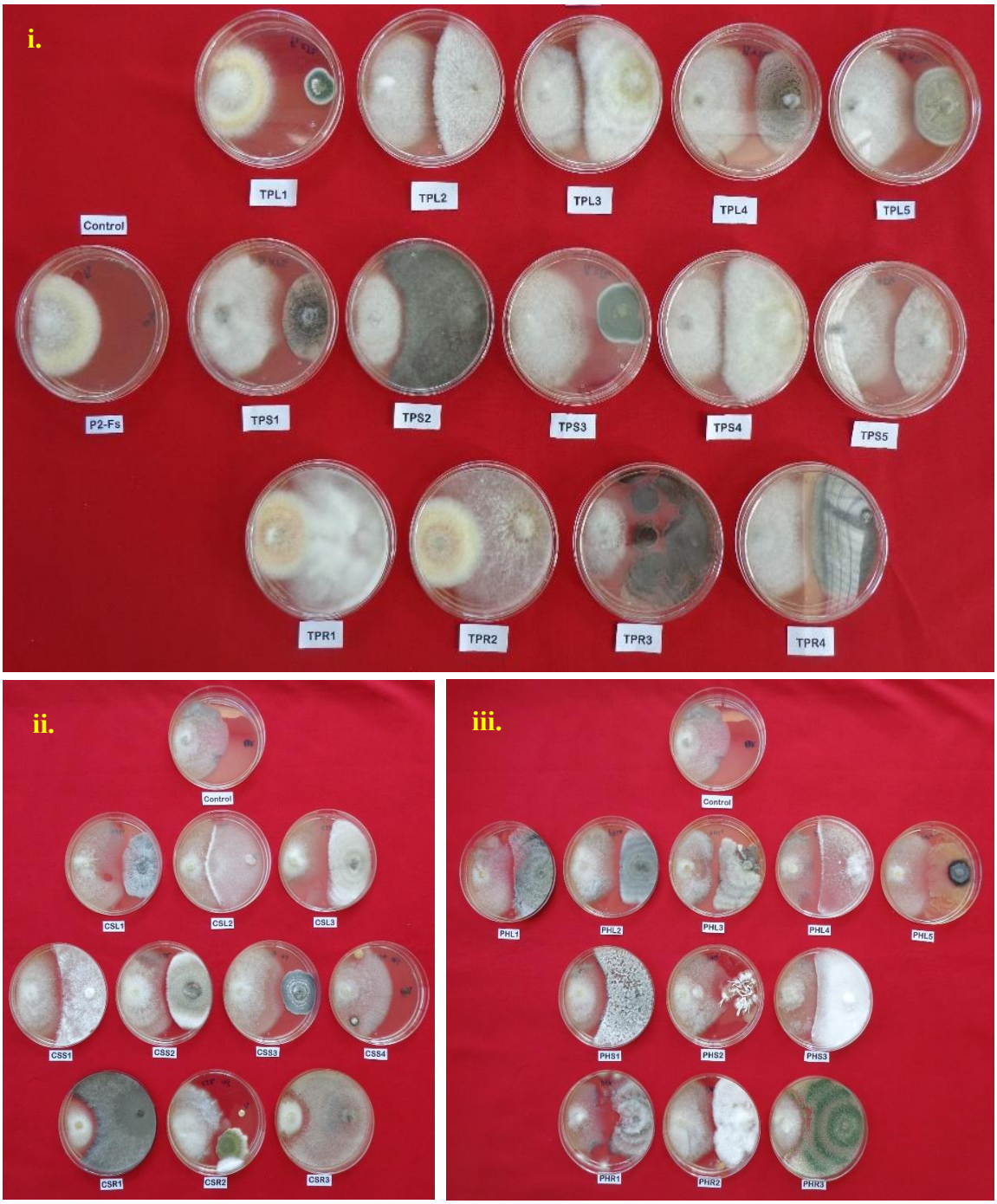


Plate 5b: Dual culture assay for screening fungal endophytic OTUs against *Fusarium* pathogen isolated from i) *T. procumbens*, ii) *C. tora* and iii) *P. hysterophorus*

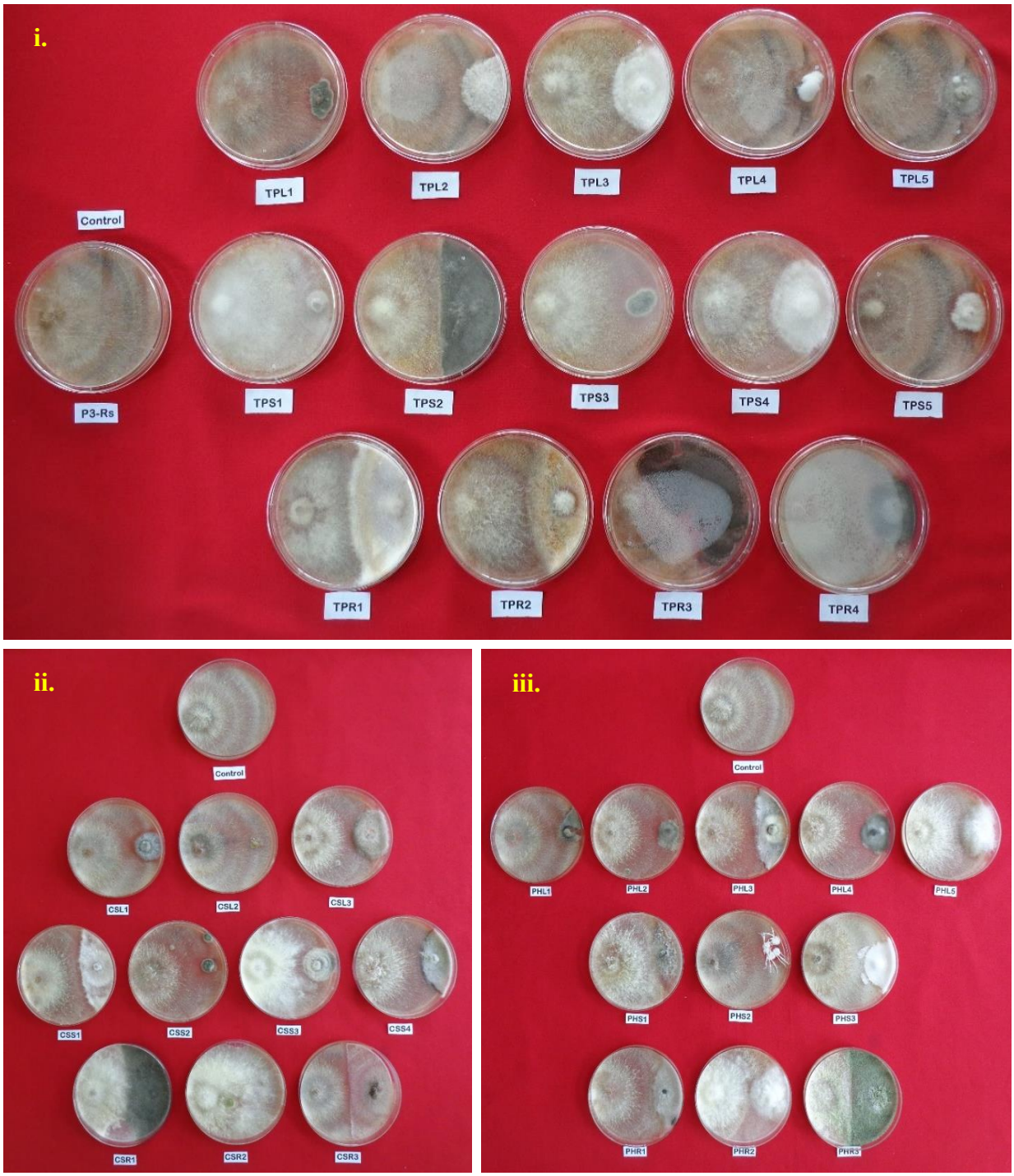


Plate 5c: Dual culture assay for screening fungal endophytic OTUs against *Rhizoctonia* pathogen isolated from i) *T. procumbens*, ii) *C. tora* and iii) *P. hysterophorus*

50 per cent inhibition of *Sclerotium*. The results showed that, number of OTUs from three plant species inhibiting the *Rhizoctonia* was maximum followed by *Fusarium* and the least number of OTUs inhibited the *Sclerotium*.

Maximum per cent inhibition of *Sclerotium, Fusarium* and *Rhizoctonia* was 62% (PHR3), 81% (CSR3) and 70% (TPR3), respectively. Among all the fungal endophytic OTUs, only two OTUs, PHR3 (from the roots of Parthenium) and *T. asperellum* (selected from previous studies) showed more than 50% of inhibition against all the three pathogens.

Each fungal endophyte may possess a different mechanism of action against different plant pathogens or maybe a combination of different mechanisms against the individual pathogen (Gao *et al*., 2010). The results obtained in the current study under *in-vitro* condition showed that, varied per cent inhibition of each pathogen which might be due to, a) different mechanism of action against individual pathogens, b) growth rate of endophytes, which play crucial role in competition for space and nutrients and c) growth rate of the pathogens, with fast growing pathogens such as *Sclerotium* can compete with endophyte for space and nutrition when compared to slow growing pathogen like *Fusarium* (Gao *et al*., 2010 and Scott, 1956).

4.4 Molecular characterization of selected fungal endophytes and pathogens

The genomic DNA was isolated from selected potential fungal endophytic OTUs (TPS2, CSR1, CSR3, PHS1, PHS3, PHR3) and the soil-borne plant pathogens infecting tomato (*Sclerotium* sp., *Fusarium* sp. and *Rhizoctonia* sp.). The isolated DNA was subjected to PCR amplification of Internal Transcriber Spacer (ITS) region (White *et al.*, 1990). This resulted in the expected amplification product of approximately 650 base pairs (Fig. 2) (Gardes and Bruns, 1996). The products were eluted and sequenced. The obtained sequences were queried in BLASTN available in NCBI GenBank. The identity matrices derived from the comparison of these sequences with the retrieved sequences from the GenBank revealed that the fungal endophytic OTUs TPS2, CSR1, CSR3, PHS1, PHS3 and PHR3 showed maximum identity with *Macrophomina phaseolina, Macrophomina pseudophaseolina, Fusarium falciforme, Nigrospora* sp., *Polyporales* sp., and *Trichoderma asperellum,*

respectively. Further, the pathogens *Sclerotium*, *Fusarium* and *Rhizoctonia* showed maximum identity with *Athelia rolfsii*, *Fusarium solani* and *Rhizoctonia solani*, respectively (Table 3). The DNA of pathogenic *S. rolfsi*, *F. solani*, *R. solani* and potential endophytes *T. asperellum* isolate 1 and *T. asperellum* isolate 2 were was subjected to PCR amplification of larger subunit (LSU) and smaller subunit (SSU) resulted in the expected amplification product of approximately 900 and 1200 base pairs, respectively (Fig 3). The potential endophyte procured from the previous study was named as *Trichoderma asperellum* isolate 1 (TA1) and the OTU obtained from Parthenium root was named as *Trichoderma asperellum* isolate 2 (TA2).

Fungal endophytes derived from the same genera or species might behave in contrast such as pathogen or endophyte beneficial to host. In the current study also some of the endophytes identified based on morphological features and ITS region sequence analysis are sharing maximum phylogenetic relationship with their pathogenic members.

Fungal endophytes, *M. phaseolina*, *M. pseudophaseolina*, *F. falciforme* and *Nigrospora* sp identified in the study have inhibitory effect on the *Sclerotium*, *Fusarium* and *Rhizoctonia*. These species reported to have pathogenic nature, however, there are substantial reports which have claimed them as fungal endophytes in various plant species (Ayob and Simarani, 2016; Mastan *et al*., 2019 and Song *et al*., 2016). Currently, comparison of ITS region sequence is the widely followed across the world for taxonomic profiling of fungi up to species level (Schoch *et al*., 2012). However, there may be huge variation within the species with respect to pathogenicity, habitat niche and parasitism driven by selection pressure to adopt different ecological niches (Price *et al*., 1986). The complete genome sequence will provide comprehensive insights into the pathogenicity related genes confirming them as endophytic or pathogenic in nature (Brader *et al*., 2017).

In addition to this, the isolated DNA of three pathogens and one fungal endophyte having high potential in inhibiting the pathogens were subjected to PCR amplification of larger subunit (LSU) and smaller subunit (SSU) of rDNA region, which resulted in the expected amplicons of 1200 bp and 900 bp, respectively.

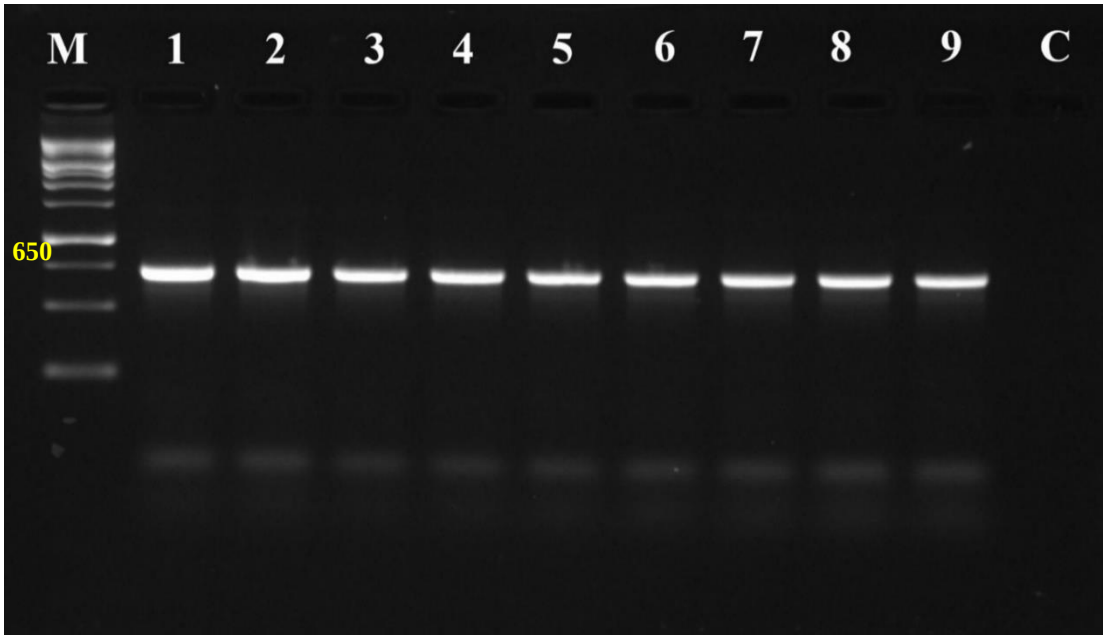


Fig. 2: Ethidium bromide stained agarose gel showing amplification product of approximately 650 bp PCR amplicon specific to Internal Transcribed Spacer (ITS) region of fungal endophytes and pathogens for the primer pair ITS1 and 4, 1) TPS2, 2) CSR1, 3) CSR3, 4) PHS1, 5) PHS3, 6) PHR3 and 7) *Sclerotium* sp., 8) *Fusarium* sp. and 9) *Rhizoctonia* sp. (M= 1kb Ladder and C=Sterile water as negative check)

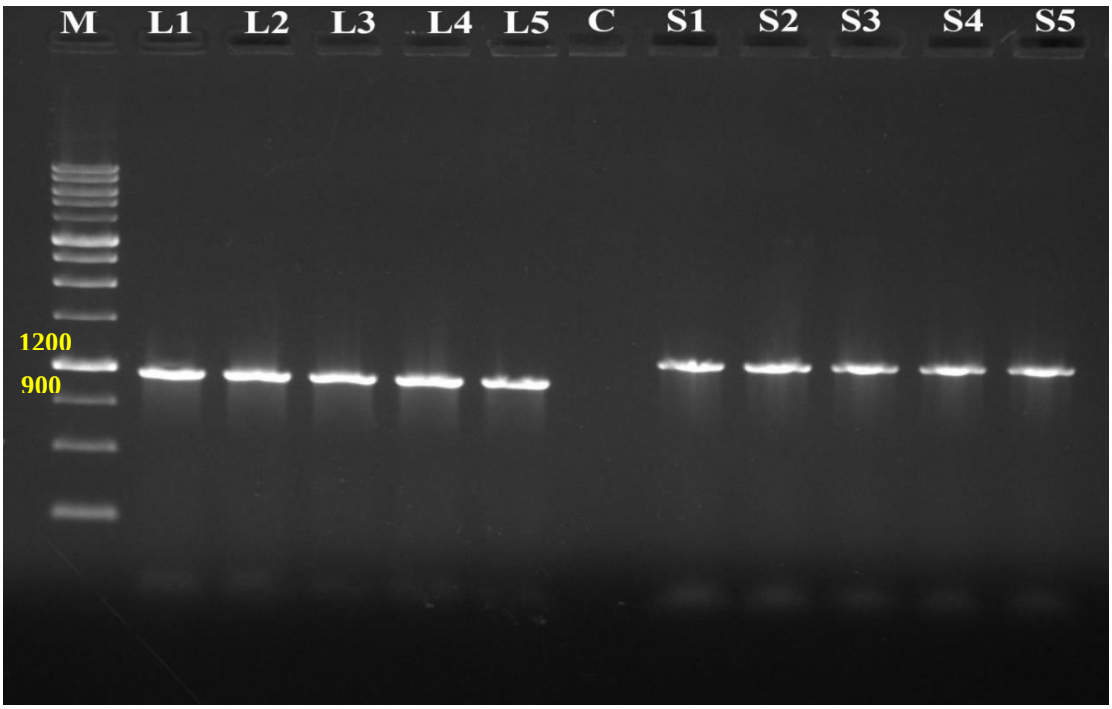


Fig 3: Ethidium bromide stained agarose gel showing amplification product of approximately 900 bp and 1200 bp PCR amplicon specific to larger subunit (LSU) and smaller subunit (SSU) region, respectively of fungal endophytes and pathogens for the primer pair LSU and SSU, L1&S1. *Sclerotium* sp., L2&S2. *Fusarium* sp., L3&S3. *Rhizoctonia* sp., L4&S4. *Trichoderma* sp. 2, and L5&S5. *Trichoderma asperellum* isolate 1.
(M= 1kb Ladder and C=Sterile water as negative check)

Table 3: Identification of potential fungal endophytic OTUs and pathogens used in the study based on the Internal Transcribed Spacer (ITS) region sequence comparison and phylogenetic analysis

Sl.no.	Endophytic OTU	Confirmed taxa	Source
1.	TPS2	*Macrophomina phaseolina*	*Tridax procumbens* stem
2.	CSR1	*Macrophomina pseudophaseolina*	*Cassia tora* roots
3.	CSR3	*Fusarium falciforme*	*Cassia tora* roots
4.	PHS1	*Nigrospora* sp.	*Parthenium hysterophorus* stem
5.	PHS3	*Polyporales* sp.	*Parthenium hysterophorus* stem
6.	PHR3/TA2	*Trichoderma asperellum*	*Parthenium hysterophorus* roots
Sl.no.	**Pathogenic isolate**	**Confirmed taxa**	**Source**
1.	SRT	*Athelia rolfsii*	Tomato roots
2.	FSK	*Fusarium solani*	Tomato roots
3.	RSK	*Rhizoctonia solani*	Tomato roots

Potential fungal endophyte *T. asperellum* and the pathogens *Athelia rolfsii, F. solani* and *R. solani* sequences were utilized for phylogenetic analysis. This was further confirmed with the phylogentic analysis showing close clustering of potential endophytes and pathogens with already reported isolates of *T. asperellum* and *A. rolfsii, F. solani* and *R. solani,* respectively (Fig 4a-4e).

4.5 Mechanisms of action of fungal endophytes against fungal plant pathogens

To study the different mechanisms of action of fungal endophytes against *S. rolfsii, F. solani* and *R. solani* infecting tomato, various assays and analysis was carried out and the results obtained were presented here.

4.6 Competition for space

Competition for space in *in-vitro* conditions was assessed through space occupancy by the fungal endophytes against targeted pathogens in dual culture assay. The number of fungal endophytic OTUs showing more than fifty per cent space occupancy against *Sclerotium, Fusarium* and *Rhizoctonia* pathogens were two (TA1 and TA2), eight (TPS2, TPR1, TPR3, CSL2, CSR1, CSR3, TA1 and TA2) and six (TPR1, TPR3, CSR1, CSR3, TA1 and TA2), respectively.

The space occupancy by fungal endophytic OTUs against *Sclerotium, Fusarium* and *Rhizoctonia* pathogens revealed that, fungal endophytic OTUs

obtained from root tissues were known to play a major role in competition for space in dual culture assay. Generally, roots are most interactive tissues with microorganisms where it comes in contact with various rhizospheric microbes that could be beneficial or pathogenic (Bowen and Rovira, 1976). To thrive in this extreme environment and to safeguard the plants against many harmful microbes the root endophytes might have evolved to counter the root infecting pathogens through continuous interaction when compared to stem and leaf colonizing endophytes.

4.7.a Antimicrobial activity assay

The diffusible antimicrobial compounds produced by potential fungal endophytic OTUs TA1 and TA2 was studied by disc diffusion assay. Secondary metabolites produced by TA1 and TA2 was extracted by ethyl acetate extraction method followed by the dissolving the obtained crude extract in ethyl acetate. The extracts were impregnated on to the sterile discs and used for screening against *S. rolfsii*, *F. solani* and *R. solani* pathogens under *in-vitro* conditions. The crude extract of both TA1 and TA2 inhibited *S. rolfsii, F. solani* and *R. solani.* The zone of inhibition of *S. rolfsii, F. solani* and *R. solani* by the crude extract from TA1 and TA2 fungal endophytes in disc diffusion assay is represented in the plate 6. Crude extracts from fungal endophytes TA1 and TA2 showed greater inhibition against all the three pathogens when compared with the positive control (0.1% Carbendazim). The extracts from TA1 and TA2 fungal endophytes showed maximum zone of inhibition against *R. solani* followed by *S. rolfsii* then *F. solani*. The negative control (ethyl acetate) didn't show any inhibition against these pathogens (Fig. 5a-5b). Between the crude extracts of TA1 and TA2, extract from TA2 has shown a greater inhibition zone against all three pathogens compared to extract from TA1. The zone of inhibition formed by the crude extract in disc diffusion assay confirmed the presence of antimicrobial diffusible compounds in the secondary metabolites of TA1 and TA2 fungal endophytic OTUs.

Ethyl acetate extracts of various endophytic fungi showed the presence of antimicrobial diffusible compounds against various plant pathogenic fungi and bacteria (Hermosa *et al*., 2014). Among fungal genera, *Trichoderma* is known to produce many antimicrobial diffusible compounds against various plant pathogenic microbes (Hermosa *et al*., 2014). Crude extract from the *Trichoderma koningii*

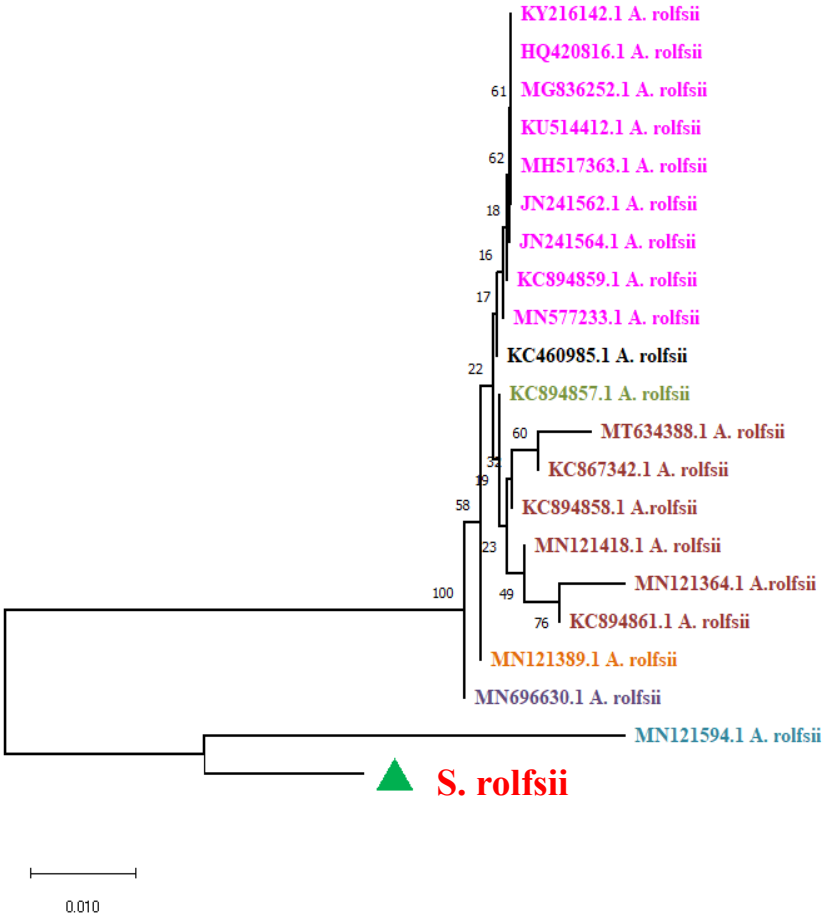


Figure 4a: Phylogenetic tree constructed from sequences of internal transcribed spacer (ITS) region of *Sclerotium rolfsii* isolate infecting tomato with sequences of *Sclerotium* spp. retrieved from NCBI GenBank using Neighbor-joining method available in MEGA X (*S. rolfsii* GKVK – isolate used in the current study)

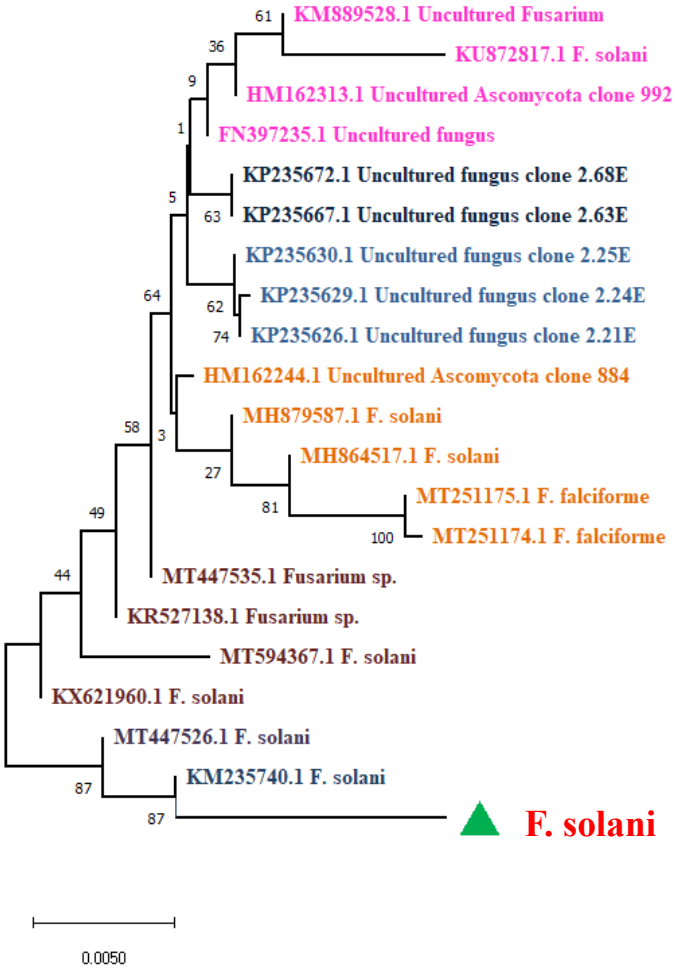


Figure 4b: Phylogenetic tree constructed from sequences of internal transcribed spacer (ITS) region of *Fusarium solani* isolate infecting tomato with sequences of *Fusarium* spp. retrieved from NCBI GenBank using Neighbor-joining method available in MEGA X (*F. solani* GKVK – isolate used in the current study)

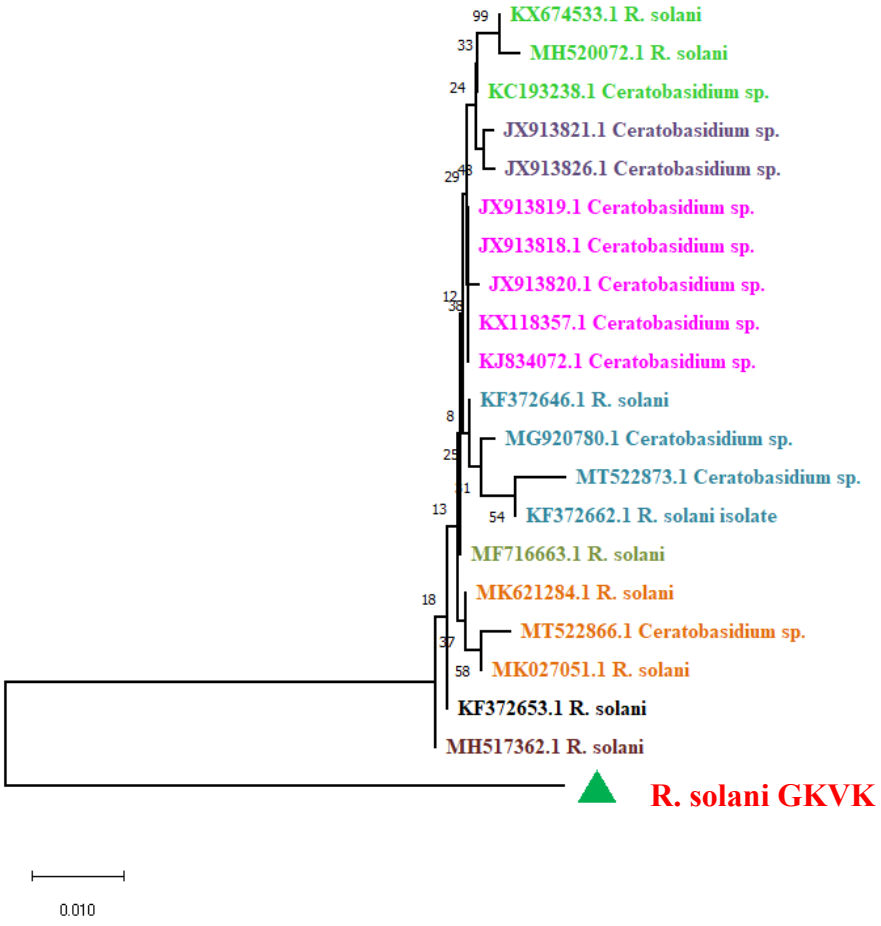


Figure 4c: Phylogenetic tree constructed from sequences of internal transcribed spacer (ITS) region of *Rhizoctonia solani* isolate infecting tomato with sequences of *Rhizoctonia* spp. retrieved from NCBI GenBank using Neighbor-joining method available in MEGA X (*R. solani* GKVK – isolate used in the current study)

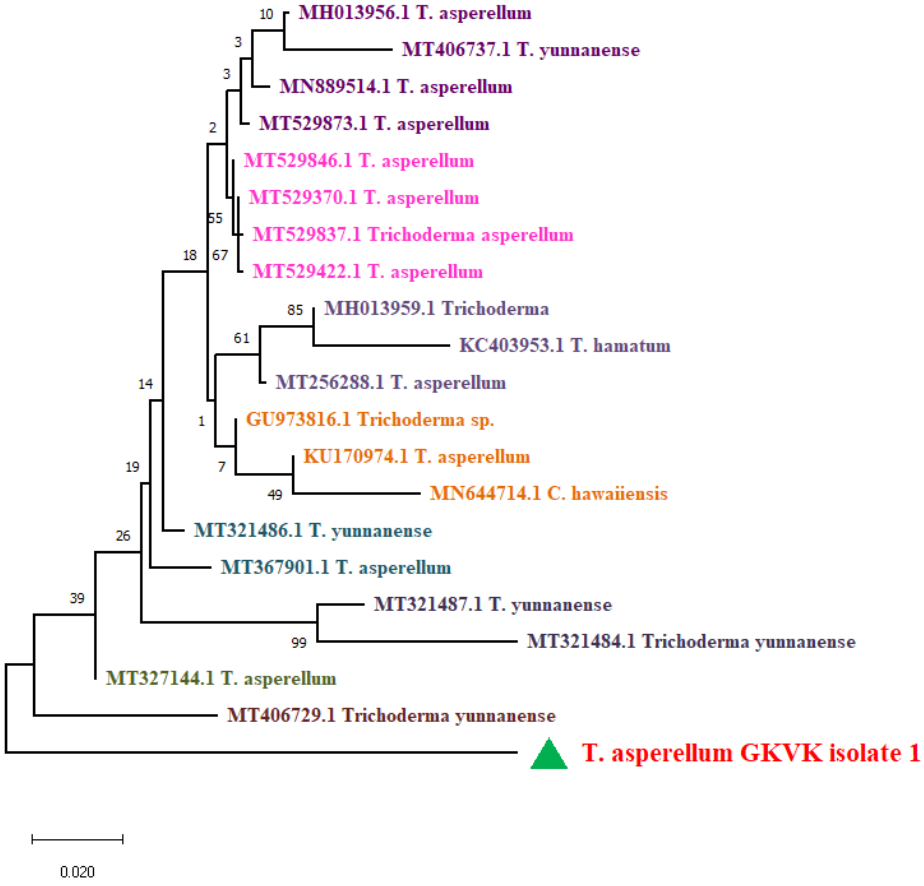


Fig. 4d: Phylogenetic tree constructed from sequences of internal transcribed spacer (ITS) region of *Trichoderma asperellum* isolate 1 from hibiscus leaf tissue with sequences of *Trichoderma* spp. retrieved from NCBI GenBank using Neighbor-joining method available in MEGA X. (*T. asperellum* isolate 1 GKVK – isolate used in the current study)

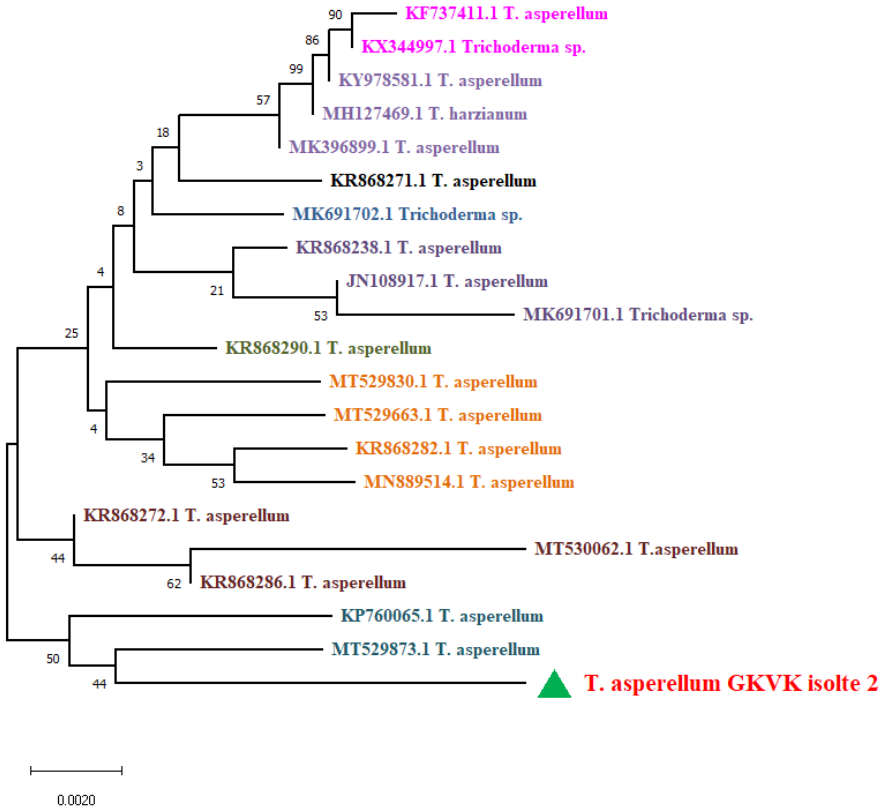


Fig. 4e: Phylogenetic tree constructed from sequences of internal transcribed spacer (ITS) region of *Trichoderma asperellum* isolate 2 from parthenium root tissue with sequences of *Trichoderma* spp. retrieved from NCBI GenBank using Neighbor-joining method available in MEGA X. (*T. asperellum* isolate 2 GKVK – isolate used in the current study)

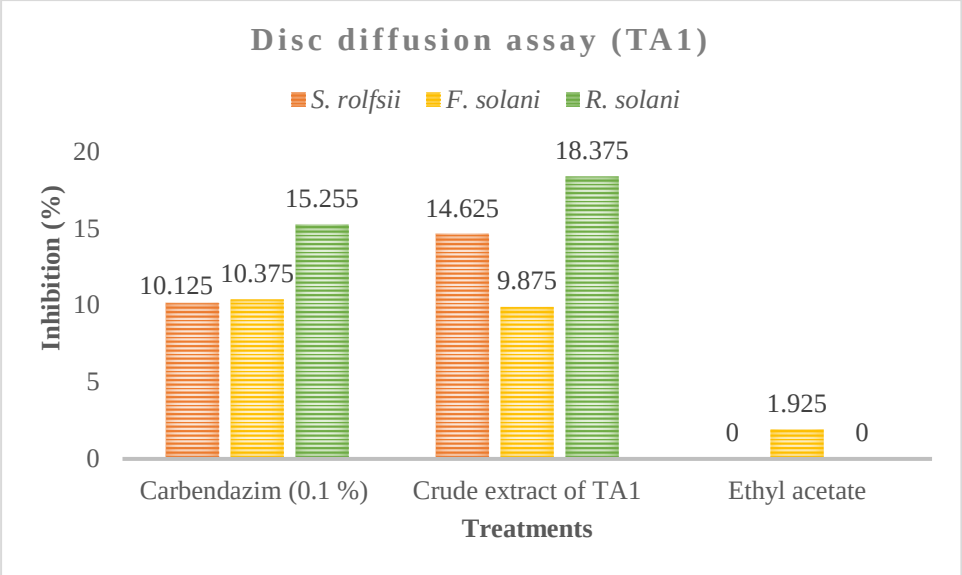


Fig. 5a: Per cent inhibition of *S. rolfsii, F. solani*, and *R. solani* by the crude extract of *Trichoderma asperellum* isolate (TA1) in disc diffusion assay. (Carbendazim 0.1 % - Positive check; Ethyl acetate - Negative Check)

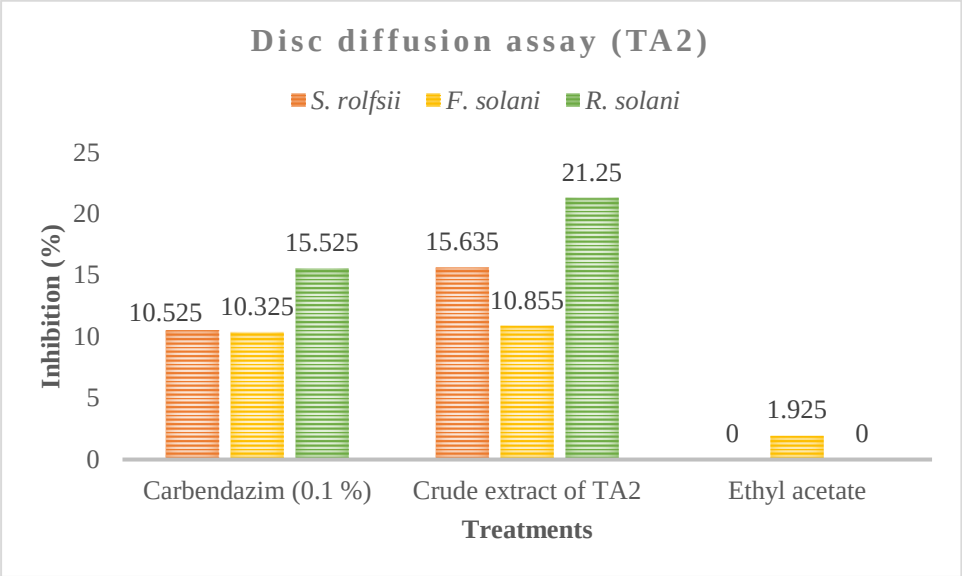


Fig. 5b: Per cent inhibition of *S. rolfsii, F. solani*, and *R. solani* by the crude extract of *Trichoderma asperellum* isolate 2(TA2) through disc diffusion assay. (Carbendazim 0.1 % - Positive check; Ethyl acetate - Negative Check)

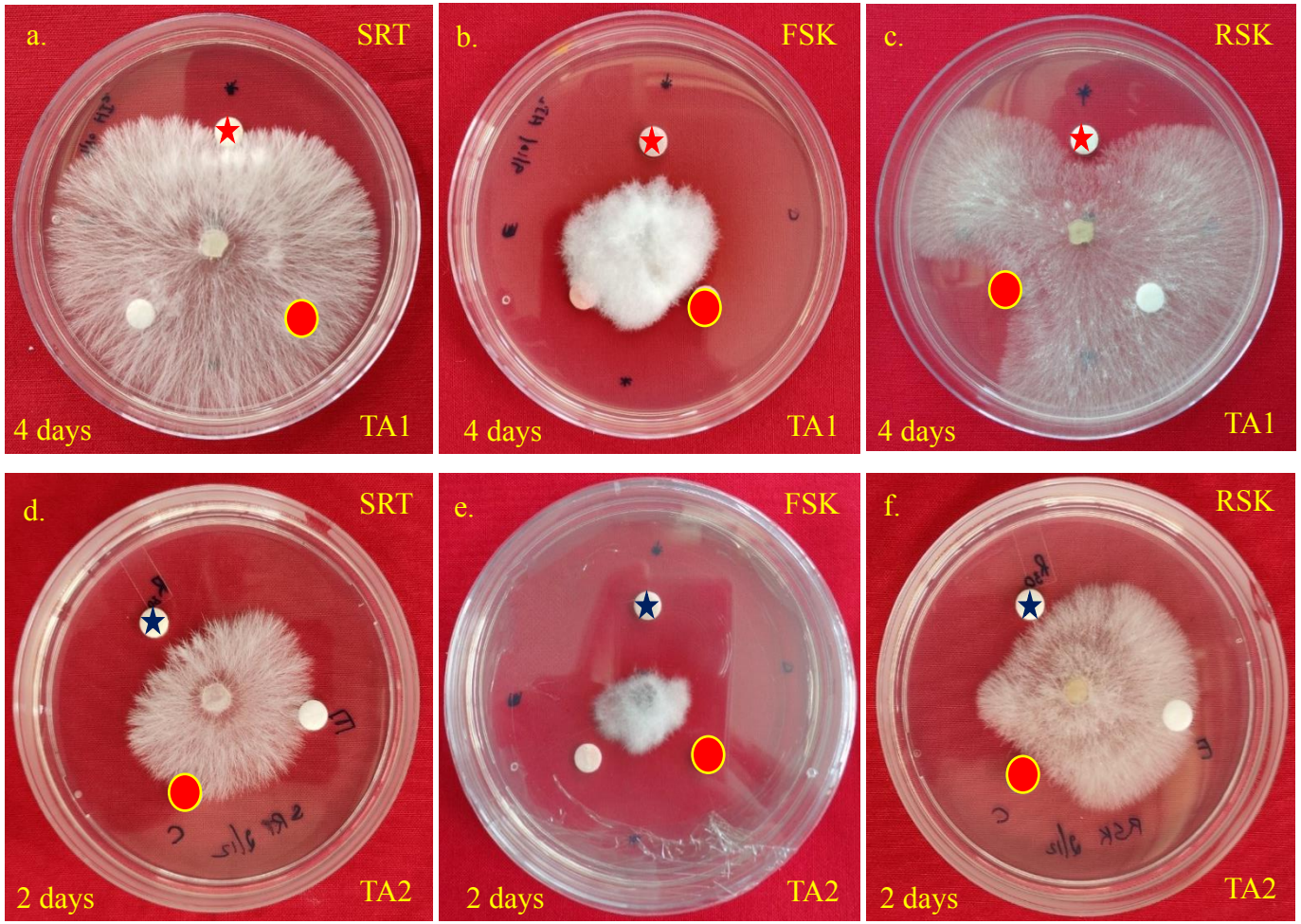


Plate 6: Disc diffusion assay to confirm the presence of antimicrobial diffusible compounds in the crude extract of fungal endophytes, *T. asperellum* isolate 1 (TA1) and *T. asperellum* isolate (TA2) against plant pathogens *S. rolfsii* (SRT), *F. solani* (FSK) and and *R. solani* (RSK). a, b &c represents inhibition of pathogens by extracts from TA1 and d, e & f represents inhibition of pathogens by extracts from TA2

★ indicates sterile disc impregnated with 20 µl crude extract of *T. asperellum* isolate 1 (TA1)
★ indicates sterile disc impregnated with 20 µl crude extract of *T. asperellum* isolate 2 (TA2)
● indicates sterile disc impregnated with 20 µl of 0.1% Carbendazim as positive check
○ indicates sterile disc impregnated with 20 µl of Ethyl acetate as negative check

isolates showed inhibition of wheat root rot causing pathogen *R. solani* in dialysis membrane overlay technique, where the isolates producing pyrones were known to reduce the growth of *R. solani* in both *in-vitro* and *in-vivo* condition (Worasatit *et al*., 1994). *T. asperellum* produced various secondary metabolites belongs to polyketides, alkanes and antimicrobial peptides which inhibited the fusarium wilt causing pathogens in cucumber (*Fusarium oxysporum* f. sp. *cucumerinum* and *Fusarium graminearum*) (Wu *et al*., 2017).

The ability of crude extracts from fungal endophytic OTUs TA1 and TA2 in inhibiting *S. rolfsii, F. solani* and *R. solani* in disc diffusion assay indicate them as a potential biocontrol agent against these soil-borne fungal pathogens of tomato.

4.7.b Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC/ESI-MS/MS) analysis of secondary metabolites from fungal endophytes

The crude extract from two fungal endophytes, *T. asperellum* isolate 1 (TA1) and *T. asperellum* isolate 2 (TA2) showing maximum inhibitory action against *S. rolfsii, F. solani* and *R. solani* pathogens in disc diffusion assay was subjected to LC/ESI-MS/MS analysis. This resulted in the chromatogram profiles of crude extracts from TA1 and TA2 showing the retention time for different compounds produced by them are shown in figure 6a and 6b. The resultant chromatographs were analysed using Compass Data analysis software, which revealed the m/z and mz/mz values for all the secondary metabolites present in the two fungal endophytes. Among these 16 and 14 prominent peaks from TA1 and TA2, respectively were selected for identification through MetFrag similarity search in chemical structure databases and considered for knowing their biological activity based on the previously available information. The results obtained were tabulated along with retention time, mass and chemical formula of the compounds (Table 4 & 5). The significant limit of less than 5 ppm mass error was observed for all the identified compounds. Five compounds identified from both the ethyl acetate extracts in the current study were similar to the compounds identified from fungi and plant extracts earlier, which were reported to possess antimicrobial, antifungal, antibacterial and antioxidant action (Marín-Loaiza *et al.,* 2008; Tuenter *et al*., 2017 and Zarina and Nanda, 2014). The secondary metabolite compounds produced by both TA1 (Table

4) and TA2 (Table 5) endophytic *Trichoderma* isolates were almost similar except for the few metabolites, which are unique in the individual isolates.

In the current study, the ethyl acetate extract of TA1 and TA2 was screened only against *S. rolfsii*, *F. solani* and *R. solani* pathogens. Evaluating the ethyl acetate extract of TA1 and TA2 against various foliar and soil-borne fungal and bacterial pathogens will lead to understand the specificity or broad-spectrum nature.

Many of the microbial, algal extracts were using as potential molecules to manage pathogens and also to induce systemic defence response against various plant pathogens (Chowdhury *et al*., 2015 and Mukherjee and Patel, 2020). Similarly, utilizing the extract/culture filtrate of TA1 and TA2 for triggering defence against plant pathogens in crop model system has great importance in countering the plant diseases through novel eco-friendly disease management approach. Further, the validation of the uncharacterized compounds obtained in the current study might lead to identification of novel compounds having application in plant disease management.

Agrochemicals market at present is dominated by strobilurins isolated and extracted from the fungus *(Strobilurus tenacellus*), used for wide range of plant fungal disease management. These molecules were known to induce defence response in plants apart from directly inhibiting the fungal pathogens (Clough, 2000). Similarly, exploiting the antimicrobial compounds of TA1 and TA2 might result in the lead compound for commercial fungicide development against many pathogens.

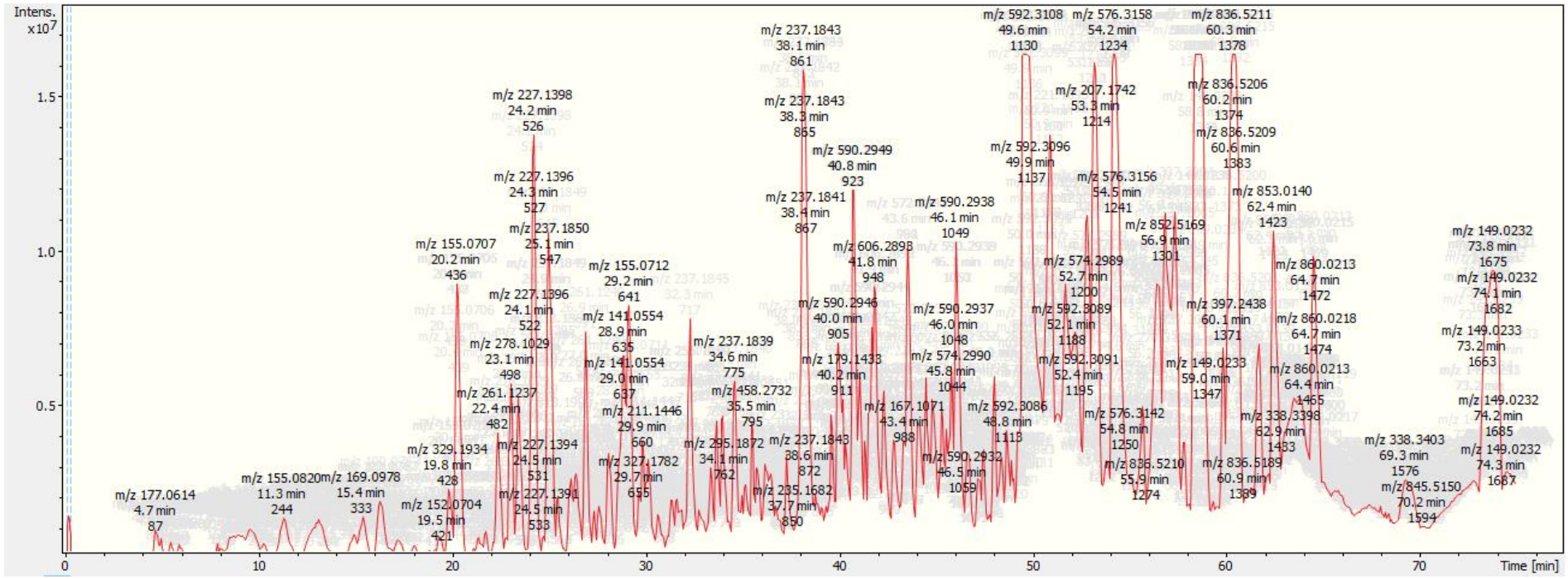


Fig. 6a: Total chromatogram of secondary metabolites from *T. asperellum* isolate 1 (TA1) by LC/MS-ESI/MS analysis

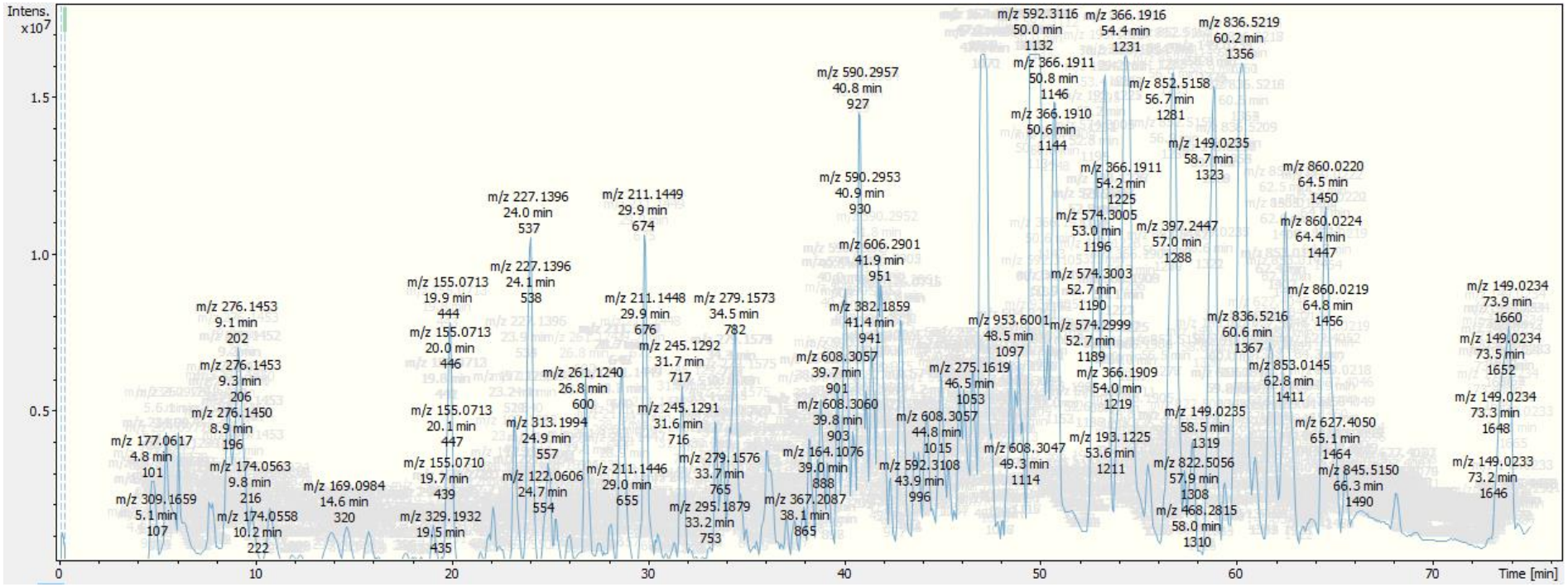


Fig. 6b: Total chromatogram of secondary metabolites from *T. asperellum* isolate 2 (TA2) by LC/MS-ESI/MS analysis

Table 4: Nature of compounds representing major peaks obtained from the secondary metabolites profile of *T. asperellum* isolate 1 (TA1) by LC/MS-ESI/MS analysis

Sl. No	Retention time (min.)	Mass	Compound	Chemical formula	Biological activity	Reference
1.	11.5	154.07	2-acetyl-3,4-dihydropyrazol-5-yl)imino-imino-ammonium	$C_5H_8N_5O$	Unknown	---
2.	15.2	168.08	Pyridoxamine	$C_8H_{12}N_2O_2$	Antioxidant	Ramis *et al*., 2019
3.	20.1	154.06	Vanillyl alcohol	$C_8H_{10}O_3$	Flavoring Agent	JECFA
4.	24.1	226.13	Cyclopropylmethyl-(2-oxoazepan-3-yl)carbamic acid	$C_{11}H_{18}N_2O_3$	Unknown	---
5.	43.7	571.27	2-[[2-[2-[[4-(3-azidopropylamino)-4-oxo-1-phenyl-butoxy]carbonylamino]pent-4-ynoylamino]-4-methyl-pentanoyl]amino]acetic acid	$C_{27}H_{37}N_7O_7$	Unknown	---
6.	38.0	236.17	Humulene-8-hydroperoxide	$C_{15}H_{24}O_2$	Antioxidant	Afzal *et al*., 2013
7.	40.7	589.28	Apetaline C	$C_{32}H_{39}N_5O_6$	Antimicrobial	Tuenter *et al*., 2017
8.	43.7	573.29	Apetaline B	$C_{32}H_{39}N_5O_5$	Antimicrobial	Tuenter *et al*., 2017
9.	49.4	591.30	Nummularine B	$C_{32}H_{41}N_5O_6$	Antiplasmodial	Panseeta *et al*.,2011
10.	50.8	220.18	alpha-Santalol	$C_{15}H_{24}O$	Anti-inflammatory	Bommareddy *et al*., 2017
11.	53.4	206.16	4-Octylphenol	$C_{14}H_{22}O$	Environmental pollutant	Zhao *et al*., 2009
12.	54.0	575.30	Mauritine A	$C_{32}H_{41}N_5O_5$	Antimicrobial	Goyal *et al*., 2012
13.	57.1	336.19	Smardaesidin G	$C_{19}H_{28}O_5$	Cytotoxic activity	Wang *et al*., 2011
14.	58.3	148.01	Phthalic anhydride	$C_8H_4O_3$	Plasticizer	Guo *et al*., 2015
15.	60.1	835.51	1,2-di-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphoethanolamine 43/748	$C_{49}H_{74}NO_8P$	Unknown	---
16.	62.4	852.00	(2S)-1-[2,6-dibromo-4-[1-[3,5-dibromo-4-[(2S)-2-hydroxy-3-(4-methylpiperazin-1-yl)propoxy]phenyl]-1-methyl-ethyl]phenoxy]-3-(4-methylpiperazin-1-yl)propan-2-ol	$C_{31}H_{44}Br_4N_4O_4$	Unknown	---

Table 5: Nature of compounds representing major peaks obtained from the secondary metabolites profile of *T. asperellum* isolate 2 (TA2) by LC/MS-ESI/MS analysis

Sl. No	Retention time (min.)	Mass	Compound	Chemical formula	Biological activity	Reference
1.	19.8	154.06	Vanillyl alcohol	$C_8H_{10}O_3$	Flavoring Agent	JECFA
2.	24.3	226.12	1-[[1-(2-fluoroethyl)pyrrolidin-2-yl]methyl]triazole-4-carbaldehyde	$C_{10}H_{15}FN_4O$	Unknown	---
3.	29.7	210.13	L,L-Cyclo(leucylprolyl)	$C_{11}H_{18}N_2O_2$	Antibacterial	Zaher *et al*., 2015
4.	34.2	278.15	2-{1-[1-(4-Fluorophenyl) cyclopentyl]-N-methylformamido}acetamide	$C_{15}H_{19}FN_2O_2$	Unknown	---
5.	37.4	276.13	Saccharopine	$C_{11}H_{20}N_2O_6$	Precursor of lysine	Mukherjee *et al*., 2012
6.	40.7	589.28	1-[3-[4-chloro-3-(1-piperidyl)benzoyl]-6-(2-pyridylmethyl)-3,6,10-triazabicyclo[9.4.0]pentadeca-1(15),11,13-trien-10-yl]-2-methoxy-ethanone	$C_{33}H_{40}ClN_5O_3$	Unknown	---
7.	47.2	166.10	(3S)-3-ethoxy-4-fluoro-pentane-1,2-diol	$C_7H_{15}FO_3$	Unknown	---
8.	49.9	591.30	Nummularine B	$C_{32}H_{41}N_5O_6$	Antiplasmodial	Panseeta *et al.*,2011
9.	54.3	365.18	Senkirkine	$C_{19}H_{27}NO_6$	Antifungal and Antibacterial	Marín-Loaiza *et al.*, 2008
10.	52.8	573.29	Apetaline B	$C_{32}H_{39}N_5O_5$	Antimicrobial	Tuenter *et al*., 2017
11.	53.1	192.11	2-cyclopentyl-1-hydroperoxy-3-methyl-benzene	$C_{12}H_{16}O_2$	Unknown	---
12.	56.6	851.50	Palau"amide	$C_{46}H_{69}N_5O_{10}$	Antioxidant	Zarina and Nanda, 2014
13.	59.0	148.01	Phthalic anhydride	$C_8H_4O_3$	Plasticizer	Guo *et al*., 2015
14.	62.4	852.00	(2S)-1-[2,6-dibromo-4-[1-[3,5-dibromo-4-[(2S)-2-hydroxy-3-(4-methylpiperazin-1-yl)propoxy]phenyl]-1-methyl-ethyl]phenoxy]-3-(4-methylpiperazin-1-yl)propan-2-ol	$C_{31}H_{44}Br_4N_4O_4$	Unknown	---

4.8 Prediction of interaction between beta-tubulin protein and secondary metabolites characterized from fungal endophytes

4.8.a *ab initio* modelling of beta-tubulin of *R. solani*

The retrieved sequence was used for *ab initio* modelling of the beta-tubulin gene of *R. solani,* five models were predicted by the I-TASSER server (Yang and Zhang, 2015). Based on the C-score, the model with the least C-score of 0.58 was selected for further analysis (Figure 7). Side chain refinement and energy minimization were carried out by GalaxyRefine2 web server (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE2) (Lee *et al*., 2019). The Ramachandran plot displayed 89.7 per cent amino acids in the favourable region, 8.5 per cent in allowed region, one per cent in the generously allowed region and 0.8 per cent amino acids in the disallowed region (Figure 8). The refined model was used in the docking analysis. The binding pocket and amino acid residues involved in interaction have been represented in figure 9.

4.8.b Prediction of interaction between beta-tubulin protein and metabolites obtained from *T. asperellum* isolates

The dock score can be utilized to evaluate a large number of potential metabolites *in-silico*. In the process of molecular docking, generally, the binding affinity lesser than the upper threshold (− 6 kcal/mol) is regarded as a cut off value for determining good binding affinity between ligand and protein (Shityakov *et al*., 2014). The widely used systemic, broad-spectrum fungicide carbendazim was used as a positive control as it targets the fungal tubulin protein. The binding affinity between the beta-tubulin gene and carbendazim was shown to be -5.40 kcal/mol, which is more than the upper threshold value (-6 kcal/mol). However, as it is a well-proven fungicide *in vitro* as well as in the field condition, in the current study the metabolites dock score showing less than -5.40 kcal/mol can be considered as potential molecules binding with the tubulin protein.

The metabolites obtained from *T. asperellum* isolate 1 have shown binding affinity ranging from -5.90 kcal/mol (for Cyclopropylmethyl-(2-oxoazepan-3-yl) carbamic acid) to -7.5 kcal/mol (for Mauritine A) (Table 6). Among eight metabolites present in *T. asperellum* isolate 1, five metabolites, viz., cyclopropylmethyl-(2-oxoazepan-3-yl) carbamic acid (-5.90 kcal/mol), humulene-

8-hydroperoxide (-6.60 kcal/mol), smardaesidin G (7.40 kcal/mol), Mauritine A (-7.50 kcal/mol) and Apetaline C (-8.30 kcal/mol) exhibited very good binding affinity in comparison to the positive control carbendazim (-5.40 kcal/mol). Apetaline C (-8.30 kcal/mol) showed the highest binding affinity and formed two hydrogen bonds with ARG276 and ARG359 amino acid residues of the beta-tubulin protein (Table 7).

The binding affinity in case of metabolites present in *T. asperellum* isolate 2 ranged from -5.50 kcal/mol (1-[[1-(2-fluoroethyl) pyrrolidin-2-yl] methyl] triazole-4-carbaldehyde) to -8.90 kcal/mol (Apetaline B). Among ten metabolites obtained from *T. asperellum* isolate 2, eight metabolites, 1-[[1-(2-fluoroethyl) pyrrolidin-2-yl] methyl] triazole-4-carbaldehyde (-5.50 kcal/mol), 2-cyclopentyl-1-hydroperoxy-3-methyl-benzene (-6.00 kcal/mol), L,L-Cyclo(leucylprolyl) (-6.10 kcal/mol), Phthalic anhydride (-6.20 kcal/mol), 2-{1-[1-(4-Fluorophenyl) cyclopentyl]-N-methylformamido}acetamide (-6.20 kcal/mol), Senkirkine (-6.90 kcal/mol), Nummularine B (-8.70 kcal/mol) and Apetaline B (-8.90 kcal/mol) showed better binding affinity than the positive control carbendazim (-5.40 kcal/mol) (Table 6). Apetaline B with the highest dock score of -8.90 kcal/mol interacted with ARG359 amino acid through one hydrogen bond (Table 7). The 3D and 2D pictures are depicted in figure 10, 11, 12 and 13, respectively.

4.9.a Double Petri dish assay

Double Petri dish assay was conducted to check the production of antimicrobial volatile organic compounds (VOCs) by endophytic isolates (TPS2, TPS3, CSR1, CSR3, PHS1, PHS3, TA1 and TA2) during their interaction with the pathogens, *S. rolfsii, F. solani,* and *R. solani*. Eight fungal endophytic OTUs showed varied per cent inhibition of radial growth of *Sclerotium, Fusarium* and *Rhizoctonia* in double Petri dish assay (Table 8). Inhibition of radial growth of *F. solani* was ranging from 4.0% to 12.0% in all fungal endophytes tested. However, inhibition of *S. rolfsii* and *R. solani* observed only in case of TA1 and TA2 OTU. Further, TA1 and TA2 showed the maximum per cent inhibition (43.0% and 41.0% respectively) of radial growth of pathogenic *Sclerotium* and were considered for characterization of VOCs produced them against *Sclerotium* pathogen (Plate 7).

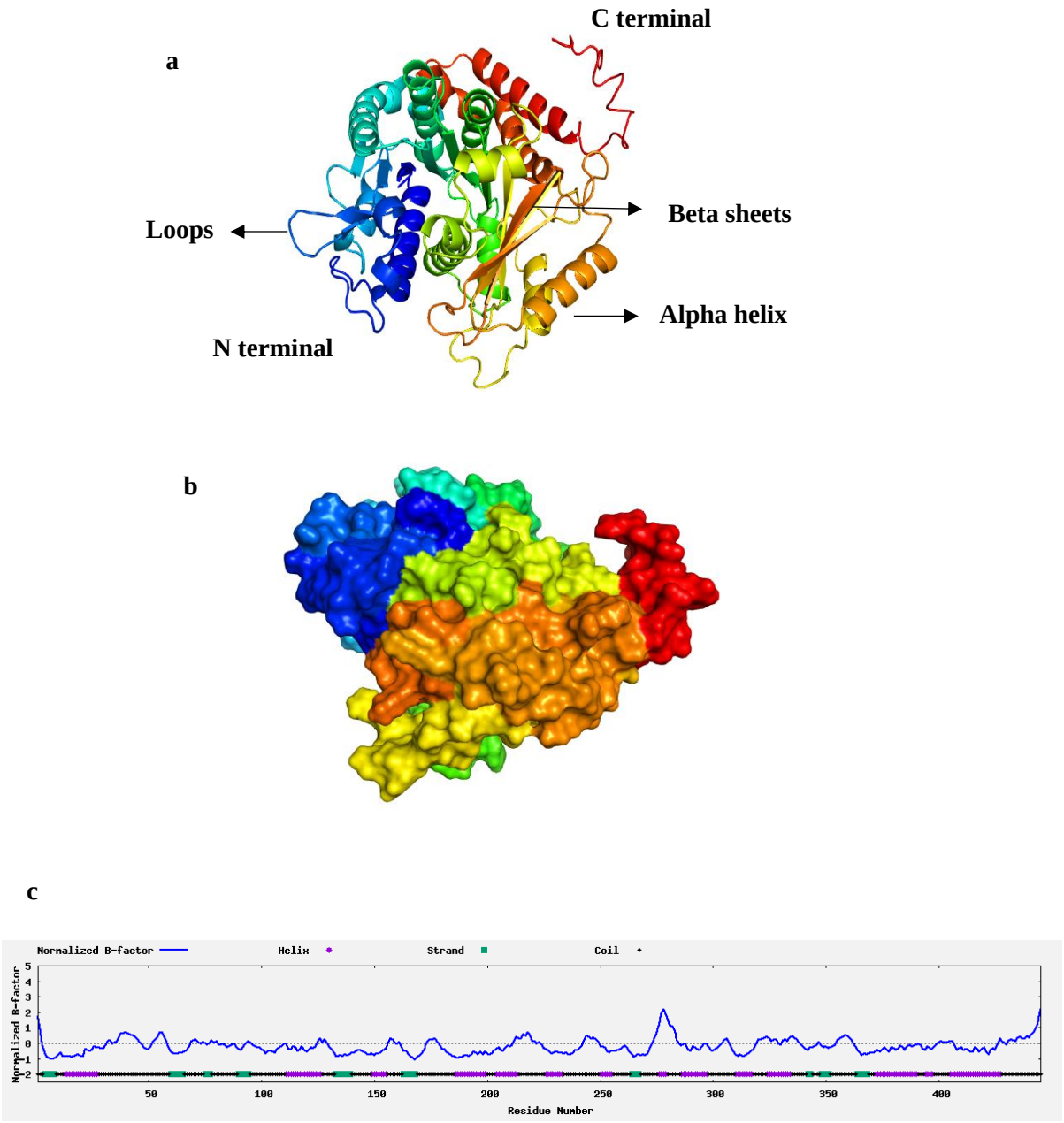


Figure 7: a) The 3D visualization of tubulin protein in cartoon format. It represents the tertiary structure of a protein. The 3D conformer was obtained from I-TASSER and the protein structure with low C-score was selected **b)** The representation of tubulin protein in surface format. **c)** B-factor is a value to indicate the extent of the inherent thermal mobility of residues/atoms in proteins. It also represents the secondary structure of the protein

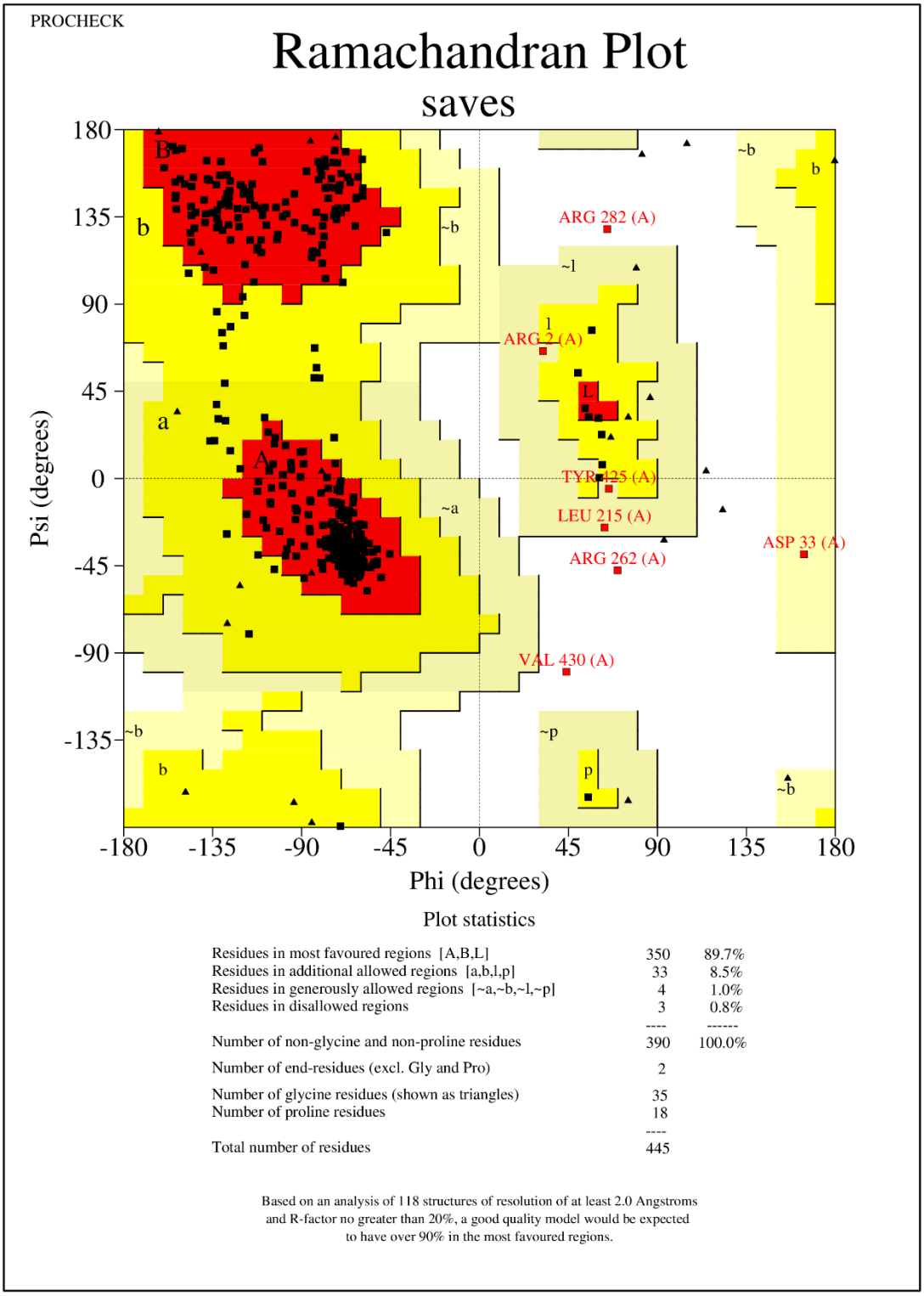
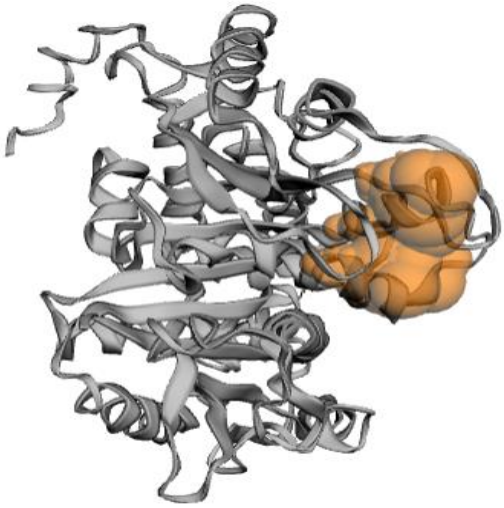


Figure 8: Ramachandran plot: It shows the statistical distribution of the combinations of the backbone dihedral angles ϕ and ψ. It also represents the arrangement of amino acids in different regions such as, favored, allowed, generously allowed and disallowed region

a



b

Figure 9: Binding protein of tubulin protein with its amino acid sequences **a)** The orange pocket on the protein surface is the binding pocket of the tubulin protein. The amino acids present in this region are considered to be active residues. **b)** It represents the stretch of an amino acid sequence of tubulin protein. Besides, the amino acids highlighted in light blue colour represents the active amino acid residues present in the binding pocket

Table 6: The binding affinity between tubulin and metabolites from *T. asperellum* isolates in kcal/mol. The binding affinities higher than positive control carbendazim have been represented in bold form.

Sl. No	Source	Mass	Compound	Chemical formula	PubChemID	Binding energy
1	Pos Cntrl	191.19	Carbendazim	$C_9H_9N_3O_2$	25429	**-5.40**
2	TA1	168.08	Pyridoxamine	$C_8H_{12}N_2O_2$	1052	-5.30
3	TA1	226.13	Cyclopropylmethyl-(2-oxoazepan-3-yl)carbamic acid	$C_{11}H_{18}N_2O_3$	69515095	**-5.90**
4	TA1	236.17	Humulene-8-hydroperoxide	$C_{15}H_{24}O_2$	131752029	**-6.60**
5	TA1	589.28	Apetaline C	$C_{32}H_{39}N_5O_6$	56926935	**-8.30**
6	TA1	220.18	alpha-Santalol	$C_{15}H_{24}O$	11085337	-5.20
7	TA1	206.16	4-Octylphenol	$C_{14}H_{22}O$	15730	-4.30
8	TA1	575.3	Mauritine A	$C_{32}H_{41}N_5O_5$	11353668	**-7.50**
9	TA1	336.19	Smardaesidin G	$C_{19}H_{28}O_5$	56599466	**-7.40**
10	TA2	154.06	Vanillyl alcohol	$C_8H_{10}O_3$	62348	-5.20
11	TA2	210.13	L,L-Cyclo(leucylprolyl)	$C_{11}H_{18}N_2O_2$	102892	**-6.10**
12	TA2	278.15	2-{1-[1-(4-Fluorophenyl) cyclopentyl]-N-methylformamido}acetamide	$C_{15}H_{19}FN_2O_2$	53507080	**-6.20**
13	TA2	591.3	Nummularine B	$C_{32}H_{41}N_5O_6$	101798848	**-8.70**
14	TA2	365.18	Senkirkine	$C_{19}H_{27}NO_6$	5281752	**-6.90**
15	TA2	573.29	Apetaline B	$C_{32}H_{39}N_5O_5$	56926827	**-8.90**
16	TA2	192.11	2-cyclopentyl-1-hydroperoxy-3-methyl-benzene	$C_{12}H_{16}O_2$	121282030	**-6.00**
17	TA2	148.01	Phthalic anhydride	$C_8H_4O_3$	6811	**-6.20**
18	TA2	226.12	1-[[1-(2-fluoroethyl)pyrrolidin-2-yl]methyl]triazole-4-carbaldehyde	$C_{10}H_{15}FN_4O$	121208663	**-5.50**
19	TA2	276.13	Saccharopine	$C_{11}H_{20}N_2O_6$	160556	-5.10

***Pos contrl-** Positive control, **TA1-** *T. asperellum* isolate 1 and **TA2-** *T. asperellum* isolate 2

Table 7: Number of hydrogen bonds and amino acid residues involved in the process of interaction between β tubulin protein and metabolites from *T. asperellum* isolates

Sl. No	Source	Compound	Chemical formula	No. of hydrogen bonds	Amino acid residues of β-tubulin protein in hydrogen bond with metabolites
1	Pos Cntrl	Carbendazim	$C_9H_9N_3O_2$	2	THR274, ARG276
2	TA1	Cyclopropylmethyl-(2-oxoazepan-3-yl)carbamic acid	$C_{11}H_{18}N_2O_3$	0	
3	TA1	Humulene-8-hydroperoxide	$C_{15}H_{24}O_2$	1	ARG359
4	TA1	Apetaline C	$C_{32}H_{39}N_5O_6$	2	ARG276, ARG359
5	TA1	Mauritine A	$C_{32}H_{41}N_5O_5$	1	THR351
6	TA1	Smardaesidin G	$C_{19}H_{28}O_5$		
7	TA2	L,L-Cyclo(leucylprolyl)	$C_{11}H_{18}N_2O_2$	2	THR274, ARG276
8	TA2	2-{1-[1-(4-Fluorophenyl) cyclopentyl]-N-methylformamido}acetamide	$C_{15}H_{19}FN_2O_2$	1	ARG276
9	TA2	Nummularine B	$C_{32}H_{41}N_5O_6$	1	ARG359
10	TA2	Senkirkine	$C_{19}H_{27}NO_6$	0	
11	TA2	Apetaline B	$C_{32}H_{39}N_5O_5$	1	ARG359
12	TA2	2-cyclopentyl-1-hydroperoxy-3-methyl-benzene	$C_{12}H_{16}O_2$	0	
13	TA2	Phthalic anhydride	$C_8H_4O_3$	0	
14	TA2	1-[[1-(2-fluoroethyl)pyrrolidin-2-yl]methyl]triazole-4-carbaldehyde	$C_{10}H_{15}FN_4O$	2	GLY142, THR143

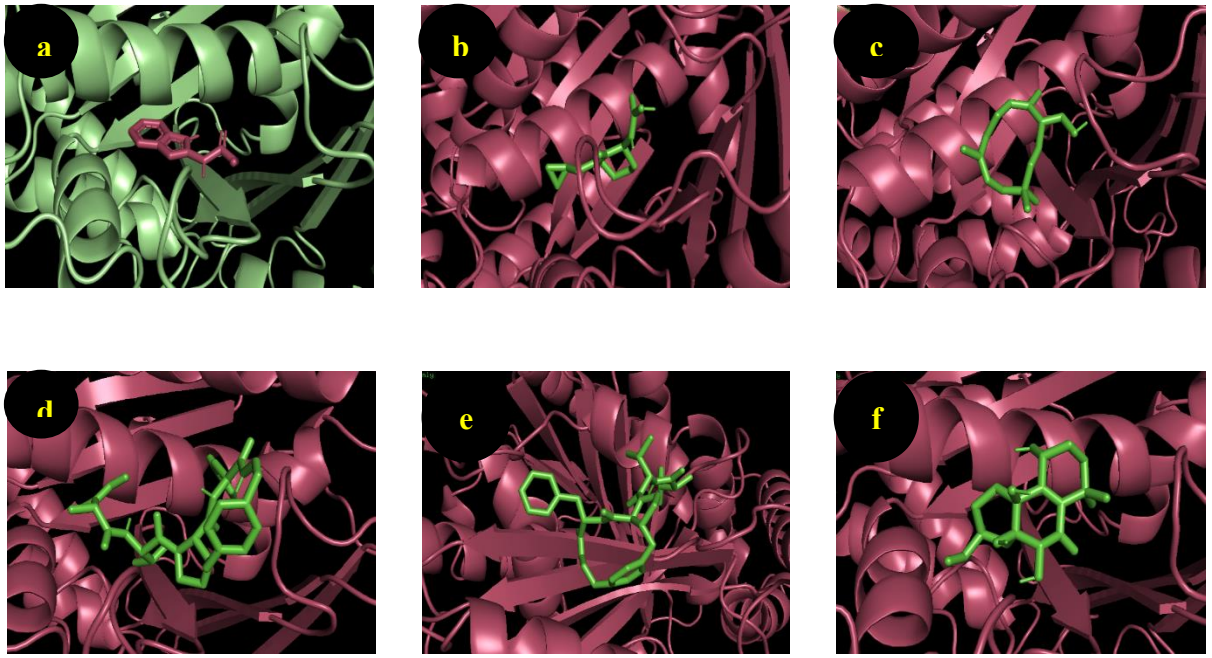


Figure 10: 3D visualization of interaction between tubulin protein with metabolites from *T. asperillum* isolate 1 showing higher binding affinity than positive control carbendazim: **a)** carbendazim (-5.40 kcal/mol) **b)** cyclopropylmethyl-(2-oxoazepan-3-yl) carbamic acid (-5.90 kcal/mol), **c)** humulene-8-hydroperoxide (-6.60 kcal/mol), **d)** smardaesidin G (7.40 kcal/mol), **e)** Mauritine A (-7.50 kcal/mol) **f)** Apetaline C (-8.30 kcal/mol)

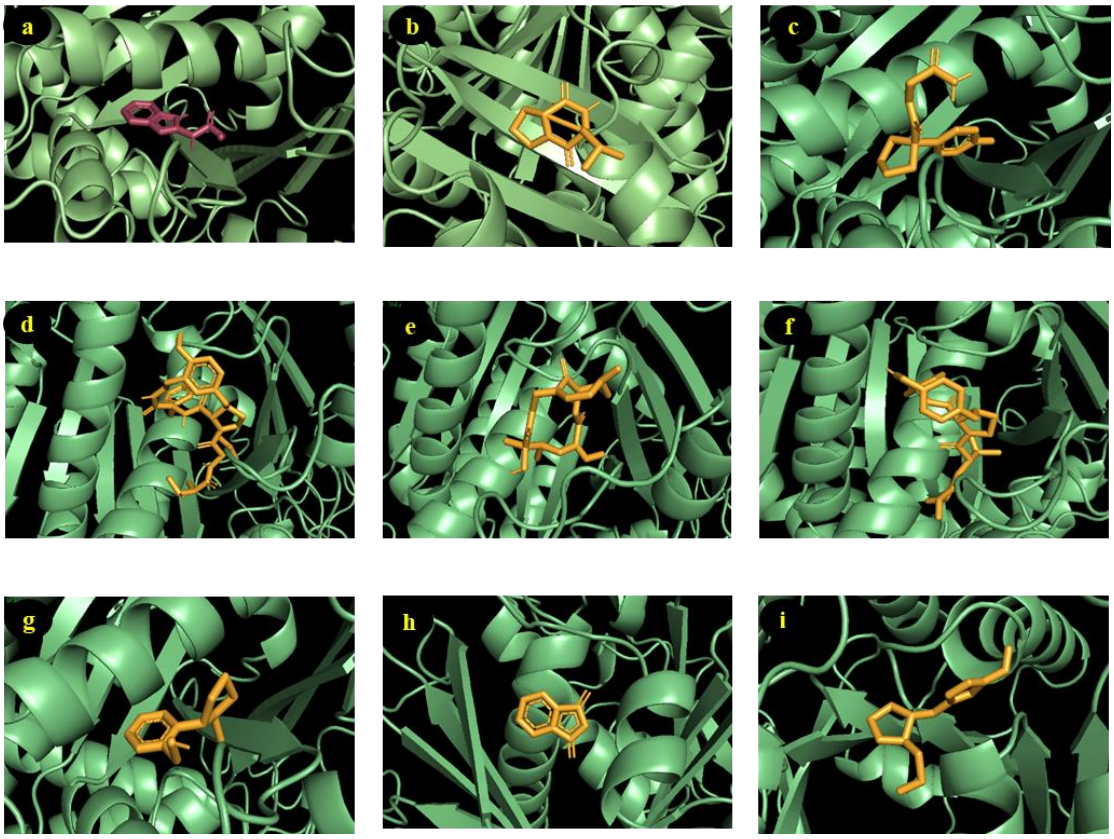


Figure 11: 3D visualization of interaction between tubulin protein with metabolites from *T. asperillum* isolate 2 showing higher binding affinity than positive control carbendazim: **a)** carbendazim (-5.40 kcal/mol) **b)** -[[1-(2-fluoroethyl) pyrrolidin-2-yl] methyl] triazole-4-carbaldehyde (-5.50 kcal/mol), **c)** 2-cyclopentyl-1-hydroperoxy-3-methyl-benzene (-6.00 kcal/mol), **d)** L,L-Cyclo(leucylprolyl) (-6.10 kcal/mol), **e)** Phthalic anhydride (-6.20 kcal/mol), **f)** 2-{1-[1-(4-Fluorophenyl) cyclopentyl]-N-methylformamido}acetamide (-6.20 kcal/mol), **g)** Senkirkine (-6.90 kcal/mol), **h)** Nummularine B (-8.70 kcal/mol) **i)** Apetaline B (-8.90 kcal/mol)

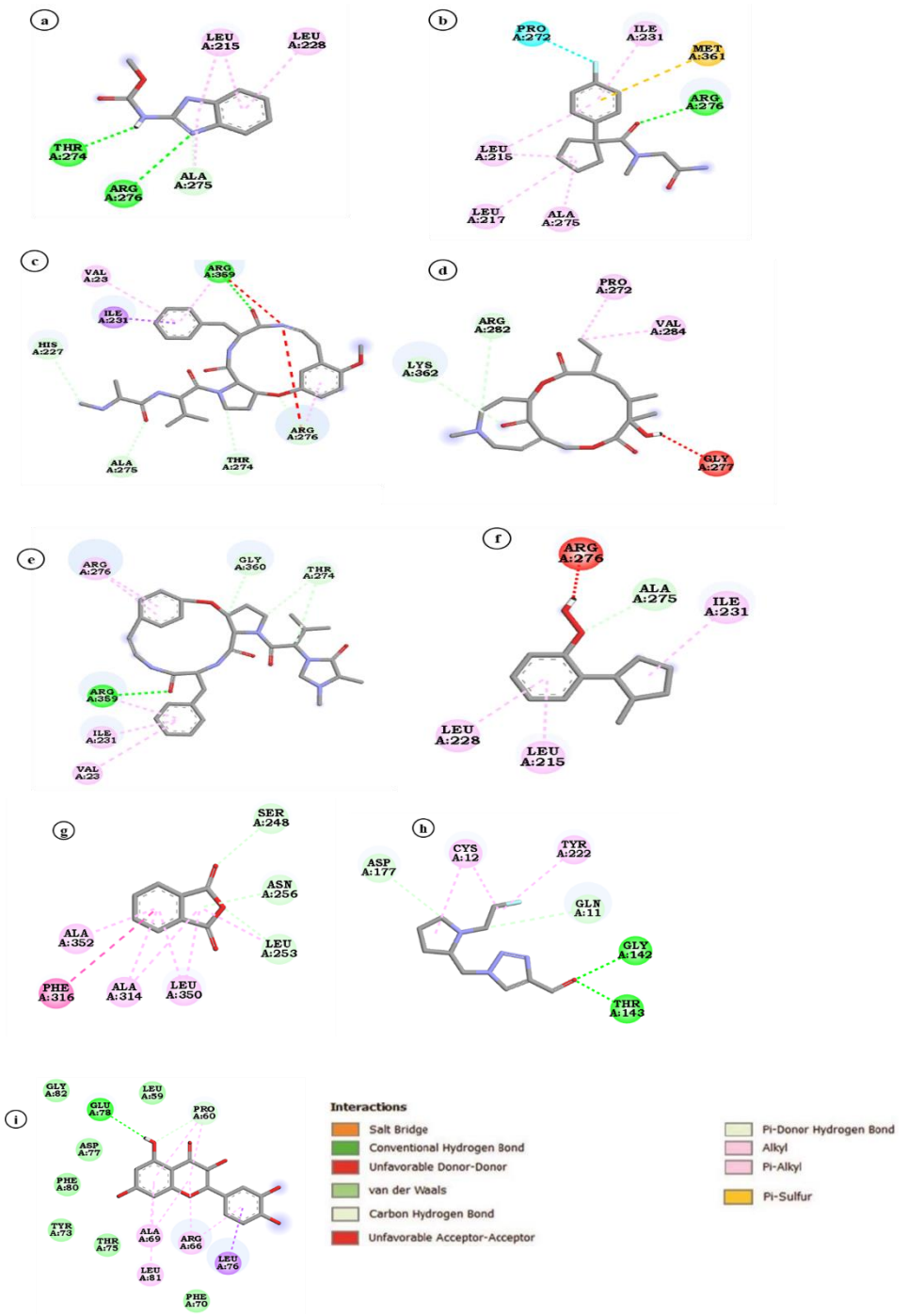
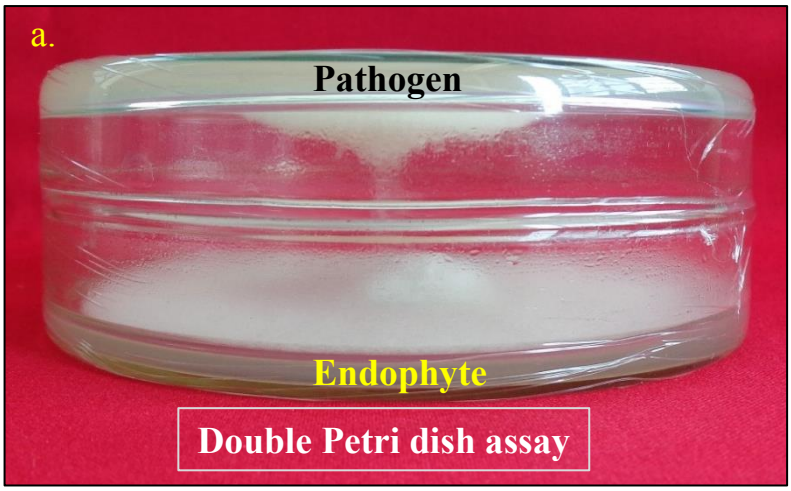
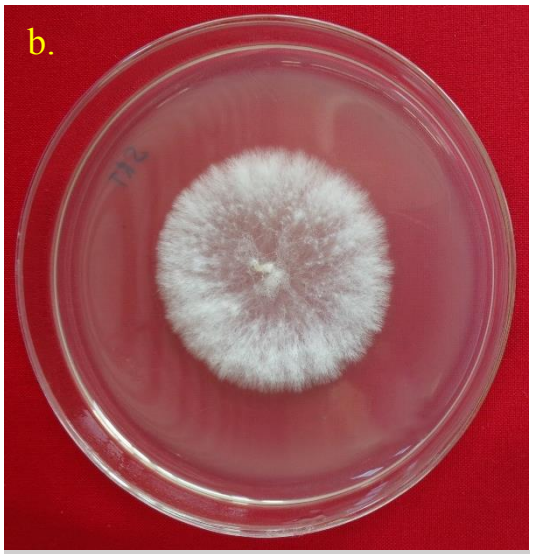
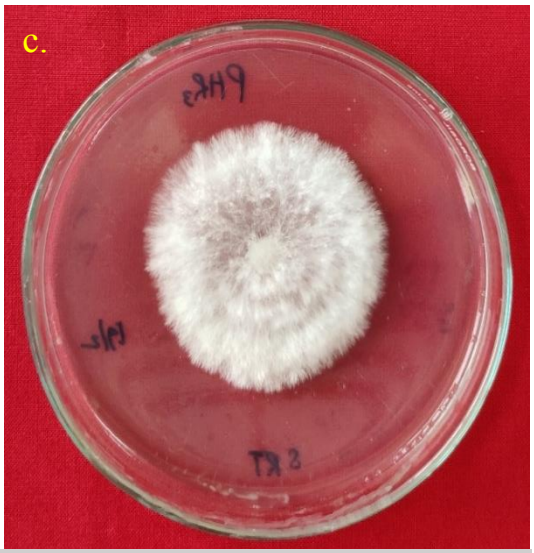


Figure 13: 2D visualization of interaction between tubulin protein with metabolites from *T. asperillum* isolate 2 showing higher binding affinity than positive control carbendazim. The 2D images represents the different bonds formed between the protein and the ligand: **a)** carbendazim (-5.40 kcal/mol) **b)** -[[1-(2-fluoroethyl) pyrrolidin-2-yl] methyl] triazole-4-carbaldehyde (-5.50 kcal/mol), **c)** 2-cyclopentyl-1-hydroperoxy-3-methyl-benzene (-6.00 kcal/mol), **d)** L,L-Cyclo(leucylprolyl) (-6.10 kcal/mol), **e)** Phthalic anhydride (-6.20 kcal/mol), **f)** 2-{1-[1-(4-Fluorophenyl) cyclopentyl]-N-methylformamido}acetamide (-6.20 kcal/mol), **g)** Senkirkine (-6.90 kcal/mol), **h)** Nummularine B (-8.70 kcal/mol) **i)** Apetaline B (-8.90 kcal/mol).





Mycelial growth inhibition of *S. rolfsii* by TA1



Mycelial growth inhibition of *S. rolfsii* by TA2

Plate 7: a. Double Petri dish assay for knowing the effect of volatile organic compounds (VOCs) produced by fungal endophytes against *S. rolfsii* by b) *T. asperellum* isolate 1 (TA1) c) *T. asperellum* isolate 2 (TA2)

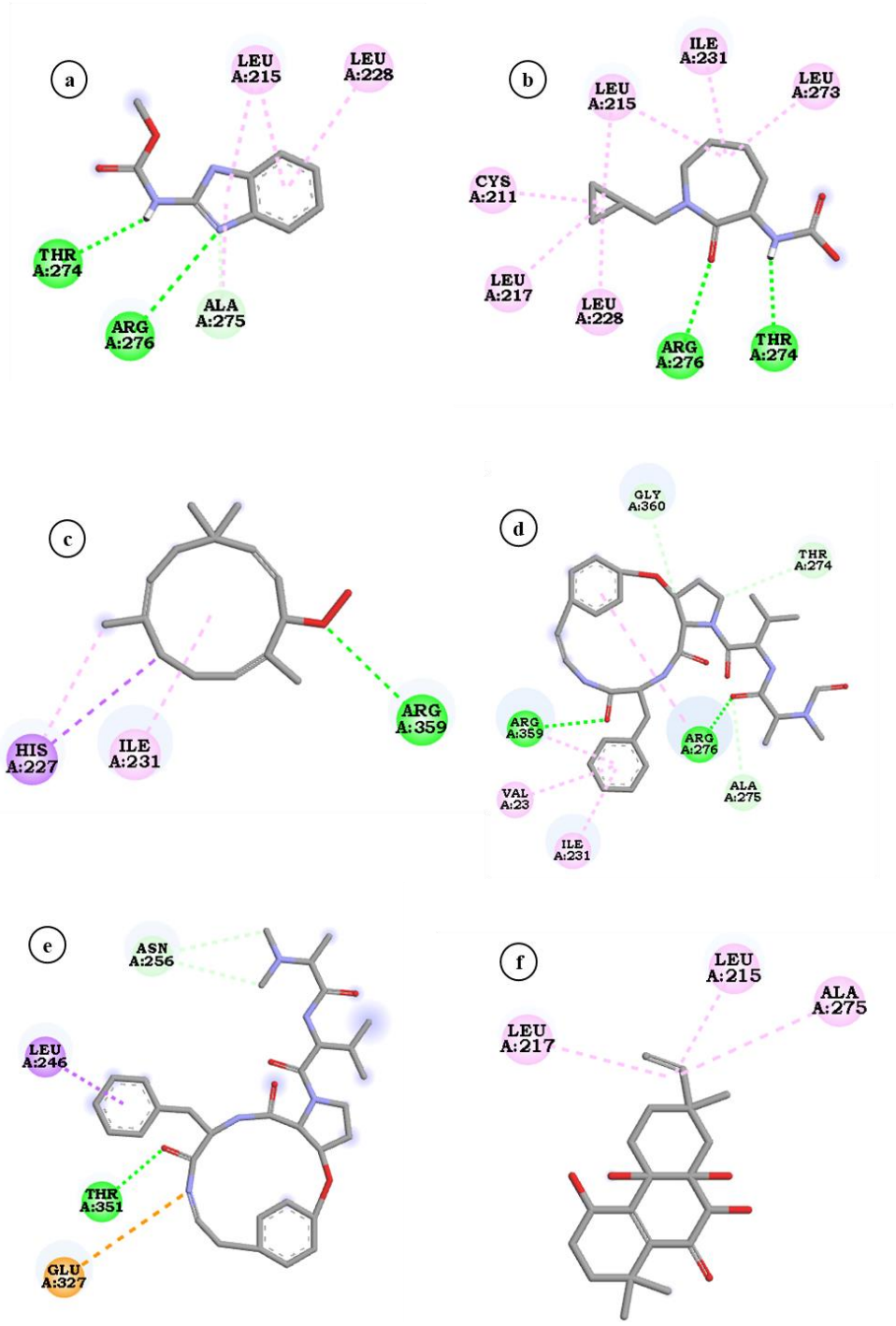


Figure 12: 2D visualization of interaction between tubulin protein with metabolites from *T. asperillum* isolate 1 showing higher binding affinity than positive control carbendazim. The 2D images represents the different bonds formed between the protein and the ligand: **a)** carbendazim (-5.40 kcal/mol) **b)** cyclopropylmethyl-(2-oxoazepan-3-yl) carbamic acid (-5.90 kcal/mol), **c)** humulene-8-hydroperoxide (-6.60 kcal/mol), **d)** smardaesidin G (7.40 kcal/mol), **e)** Mauritine A (-7.50 kcal/mol) **f)** Apetaline C (-8.30 kcal/mol)

Table 8: Inhibition of radial growth of pathogens in double Petri dish assay by endophytic OTU's through production of antimicrobial volatile organic compounds (VOCs)

	Per cent inhibition by endophytic OTUs							
Pathogen	TA1	TPS2	TPS3	CSR1	CSR3	PHS1	PHS3	TA2
S. rolfsii	43.0	0.0	0.0	0.0	0.0	0.0	0.0	41.0
F. solani	12.0	16.0	3.0	6.16	29.0	4.0	12.0	12.0
R. solani	24.0	0.0	0.0	0.0	0.0	0.0	0.0	24.0

Many fungal and bacterial endophytes were reported to produce antimicrobial VOCs against various plant pathogenic fungi and bacteria (Pansanit and Pripdeevech, 2018; Rajani *et al*., 2019 and Wonglom *et al*., 2020). Among the different genera, *Trichoderma* was known to produce many VOCs which are having potential antimicrobial activity against different plant pathogens (Hermosa *et al*., 2014). In the present study, the antimicrobial activity of VOCs produced by *T. asperellum* isolates was in accordance with many previous reports of antimicrobial VOCs production by *T. asperellum* strains against different plant pathogenic fungi (Wonglom *et al*., 2020). Apart from counteracting the plant pathogens by antimicrobial VOCs in the field condition, exploiting the antimicrobial VOCs activity of these fungal endophytes against post-harvest plant pathogens have a huge importance in the present scenario because of the deleterious effects of fungicidal residual toxicity in food products.

4.9.b Gas chromatography analysis

The fungal endophytic OTUs, TA1 and TA2 showing greater inhibitory activity against *S. rolfsii* was subjected to GC-MS analysis for tentative characterization of volatile metabolites. VOC's produced by TA1 and TA2 were qualitatively evaluated by headspace (HS)-SPME coupled with gas chromatography-mass spectrometry (GC–MS). Preliminary evaluations were performed by exposing for 30 min SPME fiber to VOCs produced by endophyte, pathogen and endophyte-pathogen interaction samples in Petri dishes. After the exposition of sample, the obtained chromatograms (Figure 14a – 14b) were analysed through software (Agilent MassHunter Qualitative Analysis B.07.00) and the VOCs representing peaks were detected and tentatively identified using Retention index formula (nearby value) in access through National Institute of Standards and Technology (NIST) 14 version. Total twenty-two VOCs were obtained from all the PDA (control), *S. rolfsii* and endophytic OTUs (TA1 and TA2) in double Petri dish

assay treatments. The obtained VOCs from different samples is presented in table 9. Excluding the VOCs from PDA, endophytic OTUs produced fifteen unique VOCs. The VOCs obtained from TA1 and TA2 are similar, but the intensity of each compound was more in case of TA1 compared with the TA2 endophyte. Most of the obtained VOCs belongs to the terpenes group which have already been reported from various microorganism, plants and others possessing antimicrobial nature. Apart from antimicrobial volatiles, few VOCs with flavouring agent, insect repellent and anti-inflammatory action were also produced by TA1 and TA2 endophytic OTUs (Table 10).

The VOC's produced by different *Trichoderma* isolates was well documented by GC-MS analysis from different part of the world (Rajani *et al*., 2020 and Wonglom *et al*.,2020). These studies revealed that there is difference in the profile of VOCs produced by different isolates of *Trichoderma* isolate. Possible reason from this may be difference in the source of origin influenced by ecosystem leading to divergent evolution with respect to VOCs production. Tentative characterization of VOCs from two fungal OTUs (TA1: *Trichoderma asperellum* isolate 1 and TA2: *Trichoderma asperellum* isolate 2) in the current study also showed the difference in their VOC's profile compared to previously reported isolates. However, between them there is no much difference in their profile of VOCs. Two isolates in the current study were collected from the same location, even though they were isolated from two different host plants may be the reason for having common VOCs profile giving insight into habitat based evolution with respect to VOCs production with in the same species of endophytic fungi.

4.10 Scanning electron microscope studies

The interactive zone between endophytic *T. asperellum* (TA2) and the selected pathogens in dual culture plate were collected and observed under the scanning electron microscope (SEM). In the *Fusarium* - TA2 interaction, the mycelium of TA2 surrounded over the mycelium of *Fusarium* and single-celled spores of TA2 gets colonized over the mycelium (Fig. 15a). Similarly, the single-celled spores of TA2 get colonized on the mycelium of *Rhizoctoina* (Fig. 15a). Further, stereo microscopic observation of sclerotial bodies of *S. rolfsii* showed that the mycelium of TA2 gets parasitized on the sclerotial bodies of *Sclerotium*

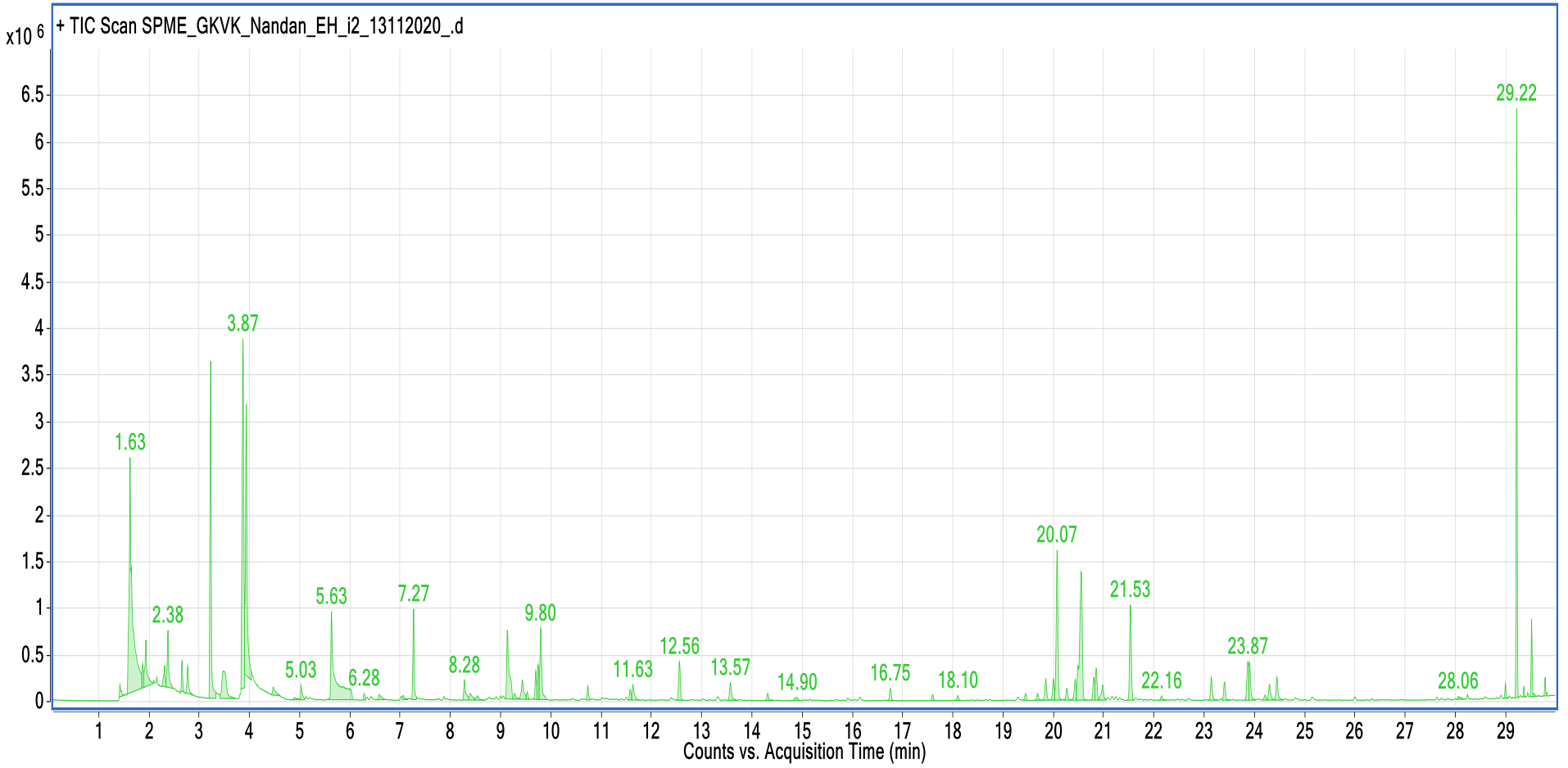


Fig. 14a: Total chromatogram of VOCs released from *T. asperellum* (TA1) SPME-GC-MS analysis

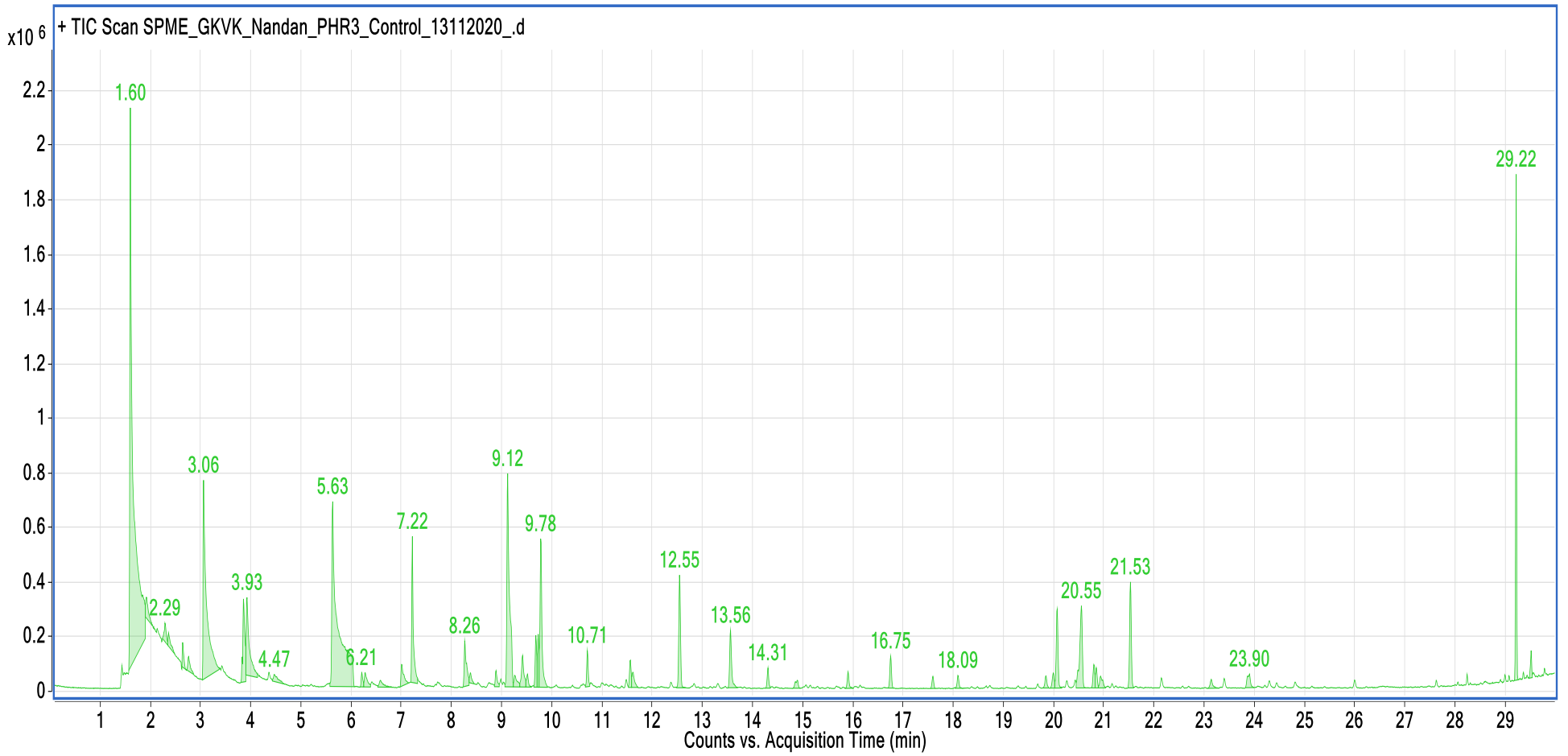


Fig. 14b: Total chromatogram of VOCs released from *T. asperellum* isolate 2 (TA2) by SPME-GC-MS analysis

disintegrating sclerotial bodies (Fig. 15b). All these obtained results show that the TA2 isolate can parasitize these three fungal pathogens in *in-vitro* conditions.

The mycoparasitic behaviour of *Trichoderma* against *Sclerotium, Fusarium* and *Rhizoctonia* pathogens observed in the current study was in accordance with various earlier reports (Upadhyay and Mukhopadhyay, 1986; Akrami *et al*., 2011 and Halifu *et al*., 2020). The *Trichoderma* fungi was known to produce lytic enzymes from the single-celled spores, which are responsible for the disintegration and degradation of mycelium of plant pathogenic fungi (Markovich and Kononova, 2003).

4.11 Host colonization assay

Endophytes have been shown to colonize and confer tolerance against many diseases in not only the host plants from which they are isolated but also in the genetically divergent crop plants (Rusty and Regina, 2008). Considering these reports pre-germinated tomato seeds were soaked in the spore suspension of endophyte TA2 and sowed in portrays. After thirty days of sowing plant samples were collected and processed for isolation of fungal endophytes. Five days after inoculation the treated TA2 endophyte was emerged from the stem and root segments and not emerged from leaf segments of endophyte-treated tomato plants. In the control treatment (samples from plants without endophyte treatment) there was absence of endophyte TA2 in all tissue segments (Plate 8).

Table 9: Tentative characterization of volatile organic compounds (VOCs) produced by *T. asperellum* isolates TA1 and TA2 by solid-phase micro extraction (SPME) gas chromatography – mass spectrometry (GC-MS) analysis

Sl. No.	Retention time	Calculated Retention index	Retention index (Reference)	Volatile compound predicted	Treatments			
					PDA	Pathogen	TA1	TA2
01.	1.60	-	427	Ethanol	+	+	+	+
02.	1.80	-	518	Methane, iodo-	+	+	+	+
03.	2.20	-	606	Furan, 2-methyl-	+	+	+	+
04.	2.31	-	615	Trichloromethane	+	+	+	+
05.	3.06	-	-	Benzoic acid, 2-fluoro-	+	+	+	+
06.	3.88	-	736	1-Butanol, 3-methyl-	+	+	+	+
07.	3.93	-	739	1-Butanol, 2-methyl-	+	+	+	+
08.	6.21	851.44	854	Butanoic acid, 3-methyl-, ethyl ester	-	-	+	+
09.	8.89	984	986	3-Octanone	-	-	+	+
10.	9.42	1011.85	1018	Benzene, 1,4-dichloro-	-	+	+	+
11.	9.73	1027.83	1030	1-Hexanol, 2-ethyl-	-	-	+	+
12.	9.78	1030.92	1031	β-Phellandrene	-	-	+	+
13.	15.90	1293	1294	2-Undecanone	-	-	+	+
14.	19.85	1456.66	1458	(1R,4R,4aS,8aR)-4,7-Dimethyl-1-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene	-	-	+	+
15.	20.00	1462.91	1461	Alloaromadendrene	-	-	+	+
16.	20.07	1465.83	1467	Patchoulene	-	-	+	+
17.	20.55	1485.83	1483	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	-	-	+	+
18.	20.80	1496.25	1500	1H-Benzocycloheptene, 2,4a,5,6,7,8-hexahydro-3,5,5,9-tetramethyl-, (R)-	-	-	+	+
19.	20.85	1498.33	1495	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-	-	-	+	+
20.	21.53	1527.58	1524	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-	-	-	+	+
21.	29.22	2004.41	1939	1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-	-	-	+	+
22.	29.52	2048.52	2027	8,12,15,15-Tetramethyl-4-methylenebicyclo[9.3.1]pentadeca-7,11-diene	-	-	+	+

"+" indicates presence and "-" indicates absence

Table 10: Nature of volatile organic compounds (VOCs) produced by *T. asperellum* isolates TA1 and TA2

Sl. No	Volatile compound	Retention time (min.)	Chemical formula	Molecular weight (g/mol)	Metabolite group	Reported biological activity	Reference
01.	Butanoic acid, 3-methyl-, ethyl ester	6.21	$C_7H_{14}O_2$	130.09	Alcohol	Flavouring agent	JECFA*
02.	3-Octanone	8.89	$C_8H_{16}O$	128.12	Ketone	Flavouring agent	JECFA*
03.	Benzene, 1,4-dichloro-	9.42	$C_6H_4Cl_2$	145.96	Aromatic hydrocarbons	Insecticide	Eastmond and Balakrishnan (2010)
04.	1-Hexanol, 2-ethyl-	9.73	$C_8H_{18}O$	130.13	Alcohol	Flavouring agent	JECFA*
05.	β-Phellandrene	9.78	$C_{10}H_{16}$	136.12	Terpene	Antimicrobial	Dai *et al*., 2013
06.	2-Undecanone	15.90	$C_{11}H_{22}O$	170.16	Ketone	Insect repellent	Bohbot and Dickens, 2010
07.	(1R,4R,4aS,8aR)-4,7-Dimethyl-1-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene	19.85	$C_{15}H_{24}$	204.18	Terpenes	Precursor to artemisinin	Tsuruta *et al*., 2009
08.	Alloaromadendrene	20.00	$C_{15}H_{24}$	204.18	Terpene	Anti-inflammatory, antioxidant and antimicrobial	Su *et al*., 2015
09.	Patchoulene	20.07	$C_{15}H_{24}$	204.18	Terpene	Antimicrobial	Tilocca *et al*., 2020
10.	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	20.55	$C_{15}H_{22}$	202.17	Terpene	Antimicrobial	Al-Rahmah *et al*., 2013
11.	1H-Benzocycloheptene, 2,4a,5,6,7,8-hexahydro-3,5,5,9-tetramethyl-, (R)-	20.80	$C_{15}H_{24}$	204.18	Terpene	Antimicrobial	Daoubi *et al*., 2005
12.	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-	20.85	$C_{15}H_{24}$	204.18	Terpene	Antimicrobial	Al-Rahmah *et al*., 2013
13.	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-	21.53	$C_{15}H_{24}$	204.18	Terpene	Antimicrobial	Al-Marzoqi *et al*., 2016
14.	1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-	29.22	$C_{20}H_{32}$	272.25	Terpene	Antimicrobial	Chen *et al*., 2009
15.	8,12,15,15-Tetramethyl-4-methylenebicyclo[9.3.1]pentadeca-7,11-diene	29.52	$C_{20}H_{32}$	272.25	Terpene	Anti-inflammatory	Zhang *et al*., 2020

*JECFA – The Joint FAO/WHO Expert Committee on Food Additives

Finally, the endophytic TA2 was confirmed to get colonized in the stem and root tissues of tomato plants. Confirming the colonization of fungal endophyte in genetically divergent plants can lead to the exploitation of fungal endophytes from diversified plant species for the management of crop diseases.

The colonization of endophytic *Trichoderma* from diversified host plants was well documented in various crop plants leaf, stem and root tissues (Bailey *et al*., 2008; Bae *et al*., 2011). Colonization of endophytes other than its host is the challenging aspect in endophytes study for utilizing them in our desired crop plant. Even though the endophyte gets colonized in other host plants upon inoculation, a) how much duration it will remain inside the plant tissues b) whether it colonizes in all the tissue segments of the plant c) whether it affects the fitness of plants upon colonization d) the parasitic relationship gets shift from endophytic to pathogenic or not, are questions that need to be answered before considering them for commercial exploitation.

4.12 *In-vivo* studies

The TA2 endophyte primed tomato seedlings were screened against *S. rolfsii* in a pot culture experiment. The symptom expression in pathogen treated plants was similar to the results obtained in pathogenicity assay for *S. rolfsii* and incidence in T_3 (Plants inoculated with *Sclerotium* pathogen inoculum) and T_4 (*T. asperellum* (TA2) primed plants inoculated with pathogen inoculum) treatments on each day after transplantation was presented in table 11. The data on per cent disease incidence showed that there is a delay in the onset of disease in endophyte primed tomato seedlings compared with control plants (Plate 9). It took six days for complete death of plant in case of control plants in contrast to eight days in case of endophyte primed plants. There is twenty-four hours' delay in the onset of disease and forty-eight hours' delay in the complete death of infected plants in endophyte primed tomato plants compared to control. These results indicated that there could be a delay in the colonization process of *S. rolfsii* in TA2 primed tomato seedlings, which can be attributed to the ability of TA2 in delaying the pathogenesis process.

Similar results were documented in a few reports, where endophytic *Trichoderma* colonized in the roots of hot pepper delayed the disease development

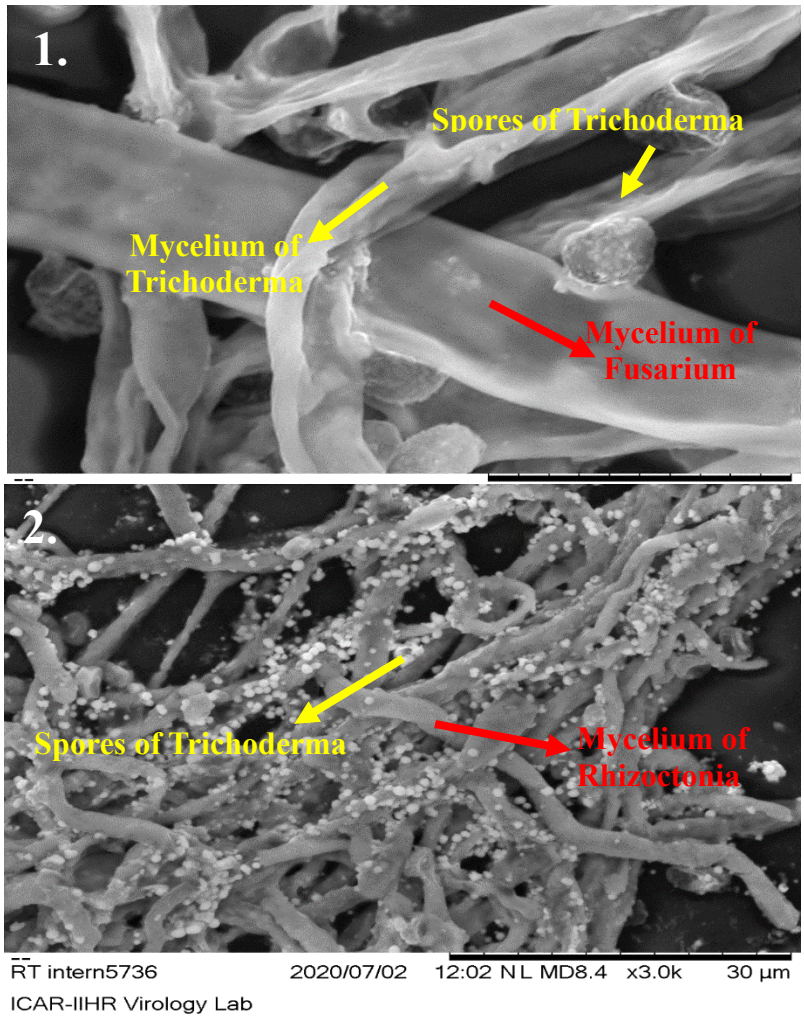


Fig. 15a: Scanning electron microscopic pictures showing of interaction between *T. asperellum* isolate 2 (TA2) with 1) *F. solani* and colonization of TA2 spores on mycelium of 2) *R. solani* in *in-vitro* condition

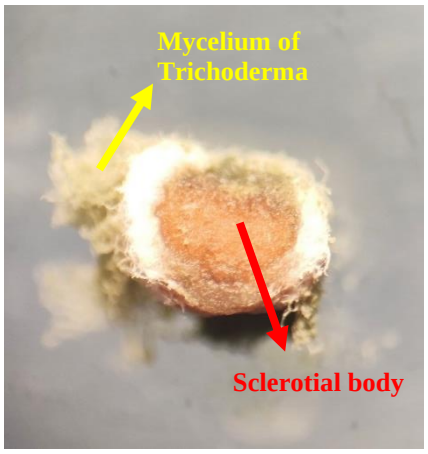


Fig. 15b: Stereo microscopic observation of mycelium of *T. asperellum* isolate 2 (TA2) parasitizing the sclerotial body of *S. rolfsii* in *in-vitro* condition

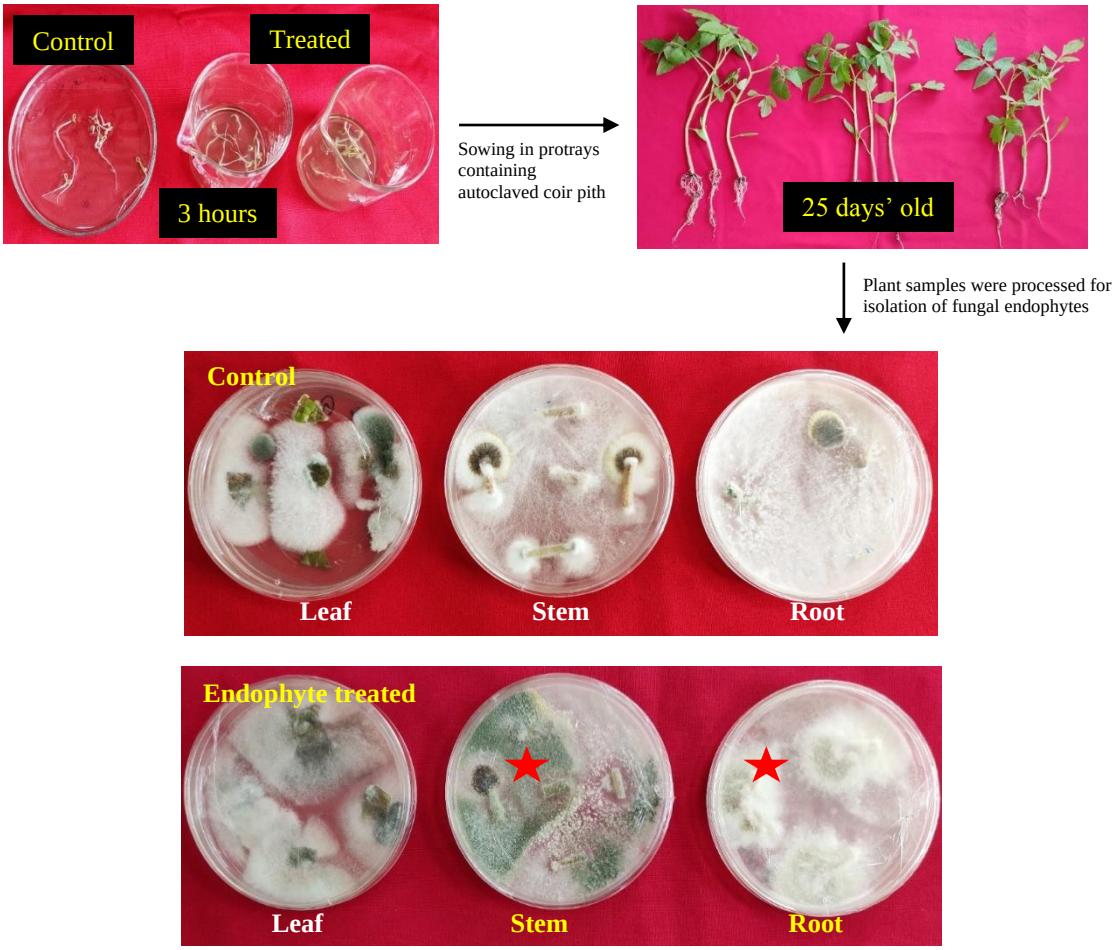


Plate 8: Pictorial representation of colonization studies for *T. asperellum* (TA2) in tomato seedlings

★ - indicates treated endophyte

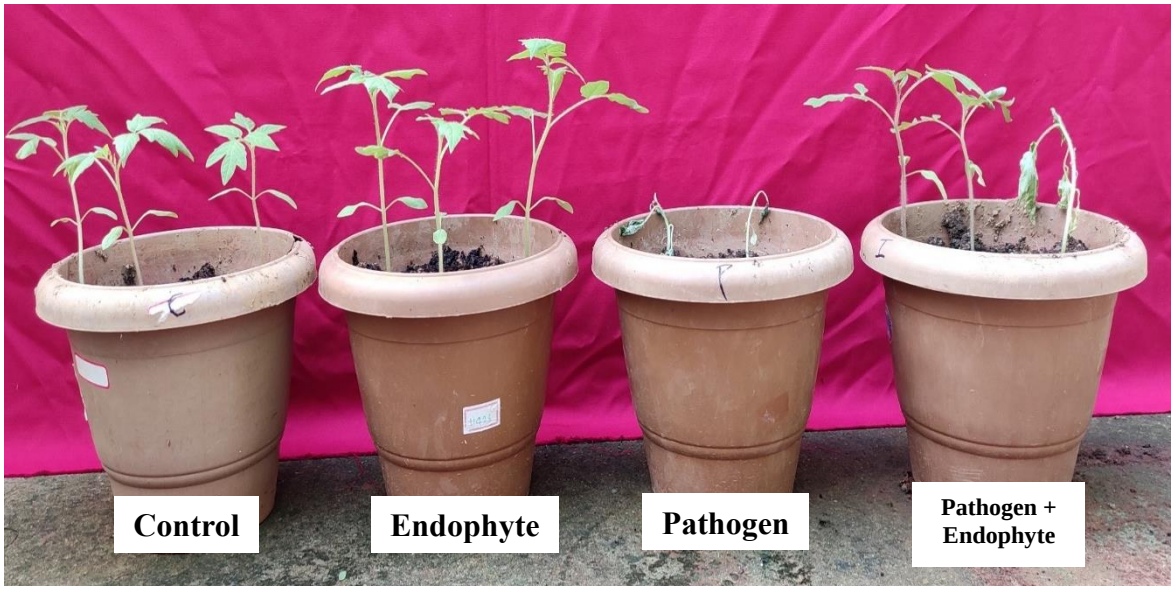


Plate 9: *In-vivo* screening of *T. asperellum* (TA2) colonized tomato plants against *S. rolfsii* in pot culture experiments

by *Phytophthora capsici* by inducing divergent defence reactions (Bae *et al*., 2011). This argument is further supported by endophytic *Trichoderma* colonized in the roots of coffee plants delayed the disease development by *Fusarium* sp. (Mulaw *et al*., 2013).

Table 11: Response of endophytic *T. asperellum* isolate 2 (TA2) treated tomato seedlings to *S. rolfsii* causing southern blight/wilt

Sl. No	Days after transplantation	Pathogen treated (% Disease incidence)	TA2 endophyte primed (% Disease incidence)
1.	01	0.0	0.0
2.	02	0.0	0.0
3.	03	20.0	0.0
4.	04	37.5	22.5
5.	05	80.0	50.0
6.	06	100	75.0
7.	07	-	92.5
8.	08	-	100

Note: In each treatment twenty-five days old 40 tomato seedlings were used

However, the colonization frequency, amount of fungal endophyte inoculum colonized in the ecological niche of roots and stem tissues decides the fate of interaction and conferring tolerance against the invading pathogen (Adame-Alvarez *et al*., 2014). The possible reason for lack of efficient control of TA2 endophyte in tomato against *S. rolfsii* could be a) colonization frequency of endophyte in primed plants b) fast-growing nature of the pathogens c) toxins production by pathogens and d) lack of host-factors in the endophyte introduced crop plant which are pre-requisite for endophyte for anti-pathogenic activity.

4.13 Induced systemic resistance

Induced systemic resistance (ISR) is one of the mechanisms of action by many biocontrol agents to counteract against invading plant pathogens. Several studies have well documented regarding involvement of many plant growth promoting rhizobacteria's (PGPR's) and biocontrol agents in elevating the plant defence response against plant pathogens by inducing systemic resistance through jasmonic acid (JA) or ethylene (ET) or salicylic acid (SA) mediated pathways. Apart from these microorganisms, many species of *Trichoderma* were known to trigger

systemic defence response in various plants and conferring resistance against different plant pathogens (Calderon *et al.,* 1993; Mukherjee *et al*., 2012 and Zhang *et al*., 2013).

The TA2 endophyte primed tomato plants upon challenge inoculated with *S. rolfsii* showed delay in symptom expression was selected to check the induction of systemic defence response in tomato plants. The RNA from different treatments was used for gene expression studies for seven genes through RT-PCR. Expression levels of these genes were determined at 24, 48 and 72 hrs time series after challenge inoculation with *S. rolfsii*. Total RNA was isolated from different tomato (Arka Vikas) plants imposed with treatments [T_1= Control Plants; T2 = Endophyte (TA2) primed plants; T3 = Pathogen (*S. rolfsii*) treated plants and T4 = Endophyte (TA2) primed plants exposed to pathogen (*S. rolfsii*)] and cDNA was synthesized. Quality of the cDNA was confirmed gel electrophoresis.

Differential expression of JA and SA responsive genes was observed across the different treatments in tomato plants primed with endophyte and subsequently inoculated with *Sclerotium rolfsii,* wilt causing pathogen. Among the seven genes analysed, three genes *PR1, PR3, NPR1* related to SA pathway and two genes, *ICS1* and *COI1* related to JA pathway shown increased expression level starting from 24 hours post inoculation (hpi) of pathogen and same trend was observed at 48 to 72hr time points. Among seven genes, expression of JA upstream gene *OPR3* was increased by 0.8 fold, downstream gene *COI1* increased by 0.5 fold in T4 in comparison with control treatment at 24 hpi. In case of SA pathway genes, expression of SA upstream gene *ICS1* increased by 0.25 fold and downstream genes N*PR1*, *PR1* and *PR3* was increased by 0.5, 0.8 and 1.0 folds, respectively in T4 treatment in comparison with control treatment at 24 hpi (Figure 16).

Similarly, in 48 hpi samples, JA upstream gene *OPR3* was increased by 1.8 fold, downstream gene *COI1* increased by 5.0 fold in T4 treatment in comparison with control treatment. In case of SA pathway genes, expression of SA upstream gene *ICS1* increased by 2.7 fold and downstream genes *NPR1*, *PR1* and *PR3* was increased by 1.0, 2.0 and 4.4 folds, respectively in T4 treatment in comparison with control treatment (Figure 16).

Finally, in 72 hpi samples, JA upstream gene *OPR3* was increased by 1.0 fold, downstream gene *COI1* increased by 5.4 fold in T4 treatment in comparison with control treatment. In case of SA pathway genes, expression of SA upstream gene *ICS1* increased by 10.6 fold and downstream genes *NPR1*, *PR1* and *PR3* was increased by 3.5, 7.0 and 20.4 folds, respectively in T4 treatment in comparison with control treatment (Figure 16).

The expression of SA pathway (*ICS1*, *NPR1*, *PR1* and *PR3*) and JA pathway (*OPR3* and *COI1*) related genes increased by several folds in pathogen inoculated TA2 endophyte primed tomato plants led to delay in the *Sclerotium* wilt incidence and a significant increase in expression of SA and JA related genes was observed relative to *Sclerotium* inoculated control plants.

This result showed that, expression level of SA and JA pathway related genes upregulated in the endophyte primed plant indicating the role of endophyte in inducing systemic resistance in tomato against *S. rolfsii.* The expression level of these genes was in increased trend with the elapsed time of inoculation of pathogen. Similar findings were recorded in various biocontrol agents mediating systemic resistance response (Pieterse *et al*., 2014 and Jogaiah *et al*., 2018).

In TA2 endophyte primed plants disease development was delayed by 24 hours and there was delay in complete death of all plants by 24 hrs in comparison with control. Even though, the potential endophyte TA2 showed better inhibition against *S. rolfsii* in *in-vitro* studies and induced expression of genes involved in defence mechanisms of the plants, still there is death plants primed with TA2 endophyte. The possible reasons may be a) Colonization success and durability of endophyte in tomato plants, b) Pathogenesis nature of *S. rolfsii,* c) Lack of tritrophic interaction between plant, endophyte and pathogen and d) Hijacking the defence related pathways by pathogen. Further, extensive research work is required to study the durability of endophyte mediated resistance in tomato plants and screening for other soil-borne and foliar pathogens infecting tomato.

These results indicate delay in the colonization and symptom expression in TA2 primed tomato plants inoculated *S. rolfsii* might be due to the induction of defence genes in tomato by the endophyte.

The exploration of fungal endophytes for plant disease management in the current study has provided insights in harnessing them as potential biocontrol agents against soil-borne fungal plant pathogens in tomato. However, further investigation is essential to understand the antimicrobial compounds from endophytes, which are responsible for the inhibition of pathogens. Characterization of actual diffusible compounds involved in antibiosis would result in the discovery of lead molecules for development of novel fungicides for effective management of plant diseases. Further, understanding the biochemical pathways involved in defence mechanisms, changes in the transcriptome and metabolome upon endophyte colonization and endophyte-pathogen interaction in the plants will help in understanding the molecular mechanisms underlying the endophyte induced defence in plants against pathogens and might lead design novel plant disease management strategies.

Future line of work

- ✓ Evaluating the potential endophytes against different foliar and soil-borne fungal and bacterial plant pathogens
- ✓ Identifying the lead compounds from the secondary metabolite extract of potential endophytes which are responsible for inhibition of soil-borne fungal pathogens of tomato
- ✓ Studying the transcriptome, metabolome and proteome changes during plant-pathogen-endophyte interaction to understand the mechanisms of endophytes in imparting the resistance
- ✓ Evaluating the efficacy of potential endophytes against soil-borne fungal pathogens of tomato under field condition

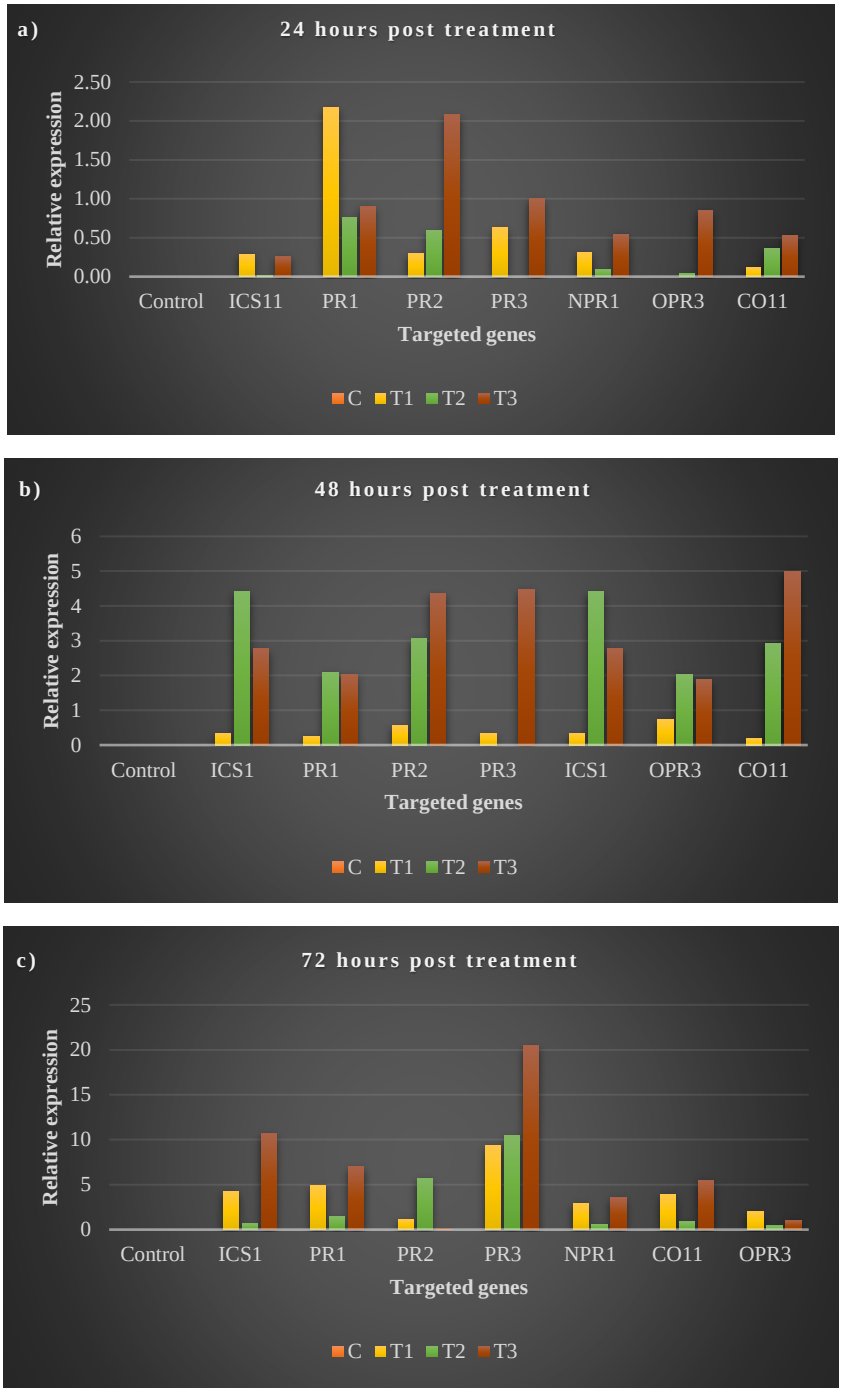


Figure 16: Gene expression studies through quantitative real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) of jasmonic acid (JA) and salicylic acid (SA) responsive genes in tomato plants, after a) 24 hours b) 48 hours and c) 72 hours of post inoculation with *S. rolfsii* (C=Control; T1= Endophyte primed tomato plants; T2=Control plants inoculated with *S. rolfsii* alone and T3=TA2 endophyte primed tomato plants inoculated with *S. rolfsii*).

V. SUMMARY

Tomato (*Solanum lycopersicum* L.) belongs to the family *Solanaceae,* is one of the major vegetable crops grown across the world. Diseases caused by various pathogens are becoming the major constraints for its production in the country. In the recent years, soil-borne fungal pathogens *viz.*, southern blight, fusarium wilt and dry root rot caused by *Sclerotium rolfsii*, *Fusarium solani* and *Rhizoctonia solani,* respectively are becoming limiting factors in the production of tomato. Currently, various management approaches are being employed to mitigate these diseases. However, the best approach for the management of these pathogens is through the use of bioagents. Among the biological management approaches, fungal endophytes are attaining greater importance in recent years due to their characteristic feature of imparting resistance against invading plant pathogens.

Fungal endophytes are the living organisms that reside inside the plant tissues with majorly beneficial relationship and confers resistance against various biotic and abiotic stress. They are reported to inhibit the invading plant pathogens through the mechanisms *viz.,* competition for space and nutrients, antibiosis, hyperparasitism, and also by inducing induced systemic resistance (ISR) in host plants.

In the present study an attempt was made for isolation and screening endophytes against the *S. rolfsii, F. solani* and *R. solani* infecting tomato and to decipher the mechanisms employed by fungal endophytes in inhibiting these pathogens.

Tomato plants showing symptoms typical to southern blight/wilt, fusarium wilt and dry root rot caused by *S. rolfsii, F. solani* and *R. solani,* respectively were collected from farmer's fields. *Sclerotium, Fusarium* and *Rhizoctonia* pathogens isolated from the infected plants, pure cultures were obtained and pathogenicity assay on tomato was carried out.

Totally, 45 fungal endophytic isolates were isolated from leaf, stem and root tissues of three weed species *Tridax procumbens*., *Cassia tora* and *Parthenium hysterophorus*. Based on the morphological characters 45 fungal endophytic isolates were categorized into 35 Operational Taxonomic Units (OTUs) and endophytic

Trichoderma asperellum OTU isolated from hibiscus leaf tissue in the previous study was used for further studies.

Thirty-six fungal endophytic OTUs were screened against *Sclerotium, Fusarium* and *Rhizoctonia* in dual culture to check their efficacy in *in-vitro* conditions by following dual culture technique. Out of 36 fungal endophytic OTUs, six and seven OTUs showed more than 50 per cent inhibition against *Fusarium* and *Rhizoctonia* pathogens, respectively and two OTUs showed more than 50 per cent inhibition of *Sclerotium* in dual culture.

The soil-borne fungal pathogens *S. rolfsii, F. solani* and *R. solani* and the fungal endophytic OTUs showing more than 50 per cent inhibition against these three pathogens were characterized by amplification and sequencing of Internal Transcribed Spacer region (ITS). The sequence results revealed the pathogens as *Sclerotium rolfsii*, *Fusarium solani*, *Rhizoctonia solani* and the fungal endophytic OTUs TPS2, CSR1, CSR3, PHS1, PHS3 and PHR3 as *Macrophomina phaseolina, Macrophomina pseudophaseolina, Fusarium falciforme, Nigrospora* sp., *Polyporales* sp., and *Trichoderma asperellum*, respectively. Further, amplification and sequencing of ribosomal Small Sub Unit (SSU) and ribosomal Larger Sub Unit region was done for *S. rolfsii, F. solani, R. solani* and one potential endophytic isolate *T. asperellum.* The ruslts revealed the further evidence for the identification these pathogens and endophyte, which are already identified by ITS region sequence comparison.

To decipher the mechanisms of action of potential fungal endophytic OTUs against *S. rolfsii, F. solani* and *R. solani* pathogens, various assays and analysis was done. Among 36 fungal endophytic OTUs, two (TA1 and TA2), eight (TPS2, TPR1, TPR3, CSL2, CSR1, CSR3, TA1 and TA2) and six (TPR1, TPR3, CSR1, CSR3, TA1 and TA2) OTUs showed more than 50 per cent of space occupancy against *S. rolfsii, F. solani* and *R. solani*, respectively in dual culture assay indicating the ability of fungal endophytic OTUs in competition for space against these pathogens.

Among the potential endophytic OTUs, TA1 and TA2 have showed greater inhibition against *S. rolfsii, F. solani* and *R. solani* pathogens and selected for for disc diffusion assay by extracting the secondary metabolites from them. The crude

extract of both TA1 and TA2 isolates showed inhibition zone against all the three pathogens in disc diffusion assay.

The crude extracts of potential endophytic OTUs, TA1 and TA2 showing inhibition against *S. rolfsii, F. solani* and *R. solani* pathogens were subjected to Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC/ESI-MS/MS) analysis. The LC/ESI-MS/MS analysis revealed that, five compounds identified in the current study were reported to possess antimicrobial, antifungal, antibacterial and antioxidant action which is similar to the compounds identified from fungi and plant extracts from the earlier reports.

In-silico docking analysis revealed, the diffusible compounds characterized from *T. asperellum* isolates having potentiality in binding to β-tubulin which was responsible for inhibition of *S. rolfsii, F. solani* and *R. solani*.

Inhibition of *S. rolfsii, F. solani* and *R. solani* pathogens by antimicrobial volatile organic compounds (VOCs) of fungal endophytes was studied by following double Petri dish assay. Evaluation of potential endophytic OTUs, TPS2, CSR1, CSR3, PHS1, PHS3, TA1 and TA2 against *S. rolfsii, F. solani* and *R. solani* showed that, each OTU showed varied per cent inhibition against individual pathogens. Among the OTUs, TA1 (43 %) and TA2 (41 %) has showed greater inhibition of radial growth of *Sclerotium* mycelium and confirmed the production of antimicrobial VOCs.

Fungal endophytic OTUs, TA1 and TA2 showing greater inhibition against *S. rolfsii* in double Petri dish assay were subjected for qualitative evaluation and tentative characterization of VOCs produced through headspace (HS)-SPME coupled with gas chromatography-mass spectrometry (GC–MS) analysis. The GC-MS results revealed that totally fifteen VOCs were obtained from endophytic OTUs (TA1 and TA2) in double Petri dish assay and the VOCs produced by both the TA1 and TA2 were similar, but the intensity of each volatile compound was more in case of TA1 in comparison with the TA2 endophytic OTU. Among the fifteen VOCs, majority of them were reported as antimicrobial volatiles, some as flavoring agents, insect repellent and anti-inflammatory action.

To study on the mycoparasitic behaviour of TA2 against *S. rolfsii, F. solani* and *R. solani* pathogens, the interactive zone between TA2 and respective pathogen in dual culture plate was selected and observed under the scanning electron microscope (SEM). The SEM results showed that the mycelium of TA2 gets surrounded over the mycelium of *Fusarium* along with the colonization of spores of TA2. Similarly, TA2 spores get colonized on the mycelium of *Rhizoctonia* was observed. The stereo microscope observation of sclerotial bodies in the dual culture plate with TA2 showed the parasitation and disintegration of sclerotial bodies by the mycelium of TA2. These results confirmed the mycoparasitic ability of TA2 against *S. rolfsii, F. solani* and *R. solani* pathogens.

Further, to study the colonization ability of TA2 in tomato seedlings colonization assay was carried out. The pre-germinated tomato seeds were treated with the spore suspension of TA2 and seeds treated with distilled water served as control. The seeds were sowed in autoclaved coir-pith. Twenty-five days old seedlings (TA2 endophyte treated and control plants) were harvested and processed for endophyte isolation. The results revealed the colonization of treated TA2 endophyte in the stem and root tissues of tomato seedlings.

The tomato seedlings primed with TA2 endophyte were evaluated in glasshouse condition against *S. rolfsii.* The endophyte TA2 primed tomato seedlings showed delay in the onset of disease and complete death of seedlings compared to tomato seedlings without endophyte priming screened against *S. rolfsii*.

The gene expression studies using qRT-PCR analysis for seven genes involved in resistance pathways showed increase in six genes (*ICS1, NPR1*, *PR1*, *PR3, OPR3* and *COI1*) relative expression, indicated the role of *T. asperellum* in inducing the plant systemic resistance.

Salient findings

1. Five potential fungal endophytes isolated from three weed species having greater inhibition against *S. rolfsii, F. solani* and *R. solani* infecting tomato were identified.
2. Five diffusible compounds having antimicrobial activity were identified in the metabolome of TA1 and TA2 fungal isolates against *S. rolfsii, F. solani* and *R. solani* infecting tomato.
3. Seven volatile organic compounds having antimicrobial activity against *S. rolfsii* infecting tomato from TA1 and TA2 fungal isolates were tentatively characterized.
4. Induction of systemic resistance through elicitation of salicylic acid and jasmonic acid pathway was revealed.

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Dear Mr. M. Nandan

The manuscript entitled "Morphological and molecular characterization of *Fusarium solani* (Mart.) Sacc. infecting tomato" by M. Nandan, Shridhar Hiremath, H. D. Vinay Kumar, M. Mantesh, C, R. Jahir Basha, C. N. Lakshminarayana Reddy has been accepted and will be published in the forthcoming issue of "Plant Disease Research".

Regards,

(Vineet K. Sharma)
Editor-in-Chief,
Plant Disease Research

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The research article entitled **"Molecular Characterization and Phylogenetic Analysis of *Rhizoctonia Solani* Kuhn. Infecting Tomato"** submitted by M. Nandan, H. D. Vinay Kumar, Shridhar Hiremath, M., Mantesh, C. R., C. Jahir Basha and N. Lakshminarayan Reddy is accepted for publication in 55 (2) 2021 issue of '*The Mysore Journal of Agricultural Sciences*'.

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