ISOLATION, CHARACTERIZATION AND EVALUATION OF FUNGAL ENDOPHYTES AS POTENTIAL BIOCONTROL AGENTS ON *Sclerotium rolfsii* Sacc. INFECTING *Capsicum annuum* L.

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UNIVERSITY OF AGRICULTURAL SCIENCES
GKVK, BENGALURU-560065
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IN

PLANT PATHOLOGY

BENGALURU **JUNE, 2017**

Affectionately dedicated
to backbone of
our Country

DEPARTMENT OF PLANT PATHOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU 560065

CERTIFICATE

This is to certify that the thesis entitled "**Isolation, characterization and evaluation of fungal endophytes as potential biocontrol agents on *Sclerotium rolfsii* Sacc. infecting *Capsicum annum* L.**" submitted in partial fulfilment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture) in PLANT PATHOLOGY** to the University of Agricultural Sciences, Bengaluru, in a *bona fide* record of research work done by **Mr. NANDAN, M. ID No. PALB 5289** 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.

C. N.

Bengaluru

June, 2017

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(G. RAVIKANTH)

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With regardful memories,

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(NANDAN, M.)

ISOLATION, CHARACTERIZATION AND EVALUATION OF FUNGAL ENDOPHYTES AS POTENTIAL BIOCONTROL AGENTS ON *Sclerotium rolfsii* Sacc. INFECTING *Capsicum annuum* L.

NANDAN, M.

ABSTRACT

Fungal endophytes are living microorganisms present inside the plant tissues imparting tolerance against abiotic and biotic stress, in turn getting food and livelihood from plants. Exploration for fungal endophytes as potential biocontrol agents against *Sclerotium* sp. infecting chilli was made by conducting In-*vitro* studies. Fungal endophytes isolated from selected plants with antimicrobial activity *viz., Hibiscus rosa sinensis*, *Phyllanthus acidus*, *Catharanthus roseus*, *Phyllanthus amarus*, *Solanum torvum.* Total 66 fungal endophytes were obtained from 165 processed tissue segments, morphologically categorised into 25 OTU's and evaluated against *Sclerotium* sp infecting chilli by dual culture technique. Out of 25 OTU's, 5 OTU's showed greater than 50 per cent, 20 OTU's showed 1-50 per cent inhibition respectively. Endophytic OTU HI2 from *H. rosa sinensis* showed highest per cent inhibition (65) of *Sclerotium* sp. was taken for further studies. Molecular characterization of *Sclerotium* sp. infecting chilli and endophytic OTU HI2 by amplifying ITS region (ITS1 and ITS4 primers) revealed *Sclerotium* isolate as *Sclerotium rolfsii* and OTU HI2 as *Trichoderma asperellum*. To decipher the mechanism involved in inhibition of *S. rolfsii* by *T. asperellum* broth culture study and double plate assay for diffusible and volatile metabolites was conducted. The metabolite profile of HPLC analysis showed two peaks eluted at 37.5 and 39.4 min retention time in interaction. Analysis of antimicrobial VOCs produced by *T. asperellum* by GC-MS showed varied peaks and 14 tentative VOCs specific to *T. asperellum* were detected. In host colonization assay *T. asperellum* successfully colonized in chilli stem and root tissues.

June, 2017
Department of Plant Pathology
UAS, GKVK, Bengaluru

(**C. N. LAKSHMINARAYANA REDDY**)
Major advisor

ಮೆಣಸಿನಕಾಯಿ ಬೆಳೆಯನ್ನು ಬಾಧಿಸುವ *ಸ್ಕ್ಲಿ ರೋಟಿಯಮ್ ರಾಲ್ಫ್ಸೀ* (ಖಚಿಛಿಛಿ.) ಯನ್ನು ಜೈವಿಕ ನಿಯಂತ್ರಣ ಜೀವಿಯಂತೆ ಪರಿಣಾಮಕಾರಿಯಾಗಿ ನಿಯಂತ್ರಿಸುವ ಶಿಲೀಂಧ್ರ ಅಂತರ ಜೀವಿಗಳ ಪ್ರತ್ಯೇಕತೆ, ಗುಣಲಕ್ಷಣಗಳ ಅರಿಯುವಿಕೆ ಮತ್ತು ಅವುಗಳ ಮೌಲ್ಯಮಾಪನ.

ನಂದನ್, ಎಂ.

ಪ್ರಬಂಧದ ಸಾರಾಂಶ

ಸಸ್ಯ ಅಂಗಾಂಶಗಳ ಒಳಗೆ ವಾಸಿಸುವ ಶಿಲೀಂಧ್ರ ಅಂತರ ಜೀವಿಗಳು ಸಸ್ಯಗಳಿಂದ ಆಹಾರ ಮತ್ತು ಜೀವನೋಪಾಯವನ್ನು ಪಡೆಯುತ್ತವೆ, ಹಿಂತಿರುಗಿ ಜೈವಿಕ ಮತ್ತು ಅಜೈವಿಕ ಒತ್ತಡದ ವಿರುದ್ಧ ಸಹಿಷ್ಣುತೆಯನ್ನು ನೀಡುತ್ತವೆ. ಈ ಅಂತರ ಜೀವಿಗಳನ್ನು ಪರಿಣಾಮಕಾರಿ ಜೈವಿಕ ನಿಯಂತ್ರಣ ಜೀವಿಯಾಗಿ ಮೆಣಸಿನಕಾಯಿ ಬೆಳೆಗೆ ತಗುಲುವ ಸ್ಕ್ಲಿರೋಟಿಯಮ್ ಜಾತಿಯ ವಿರುದ್ಧ ಪ್ರಯೋಗಾಲಯದ ಸ್ಥಿತಿಯಲ್ಲಿ ಕೈಗೊಳ್ಳಲಾಗಿರುತ್ತದೆ. ಶಿಲೀಂಧ್ರ ಅಂತರ ಜೀವಿಗಳನ್ನು ಸೂಕ್ಷ್ಮಜೀವಿನಾಶಕ ಸಾಮರ್ಥ್ಯವುಳ್ಳ *ಹೈಬಿಸ್ಕಸ್ ರೋಸಾ ಸೈನೆನ್ಸಿಸ್, ಫಿಲ್ಲಾಂಥಸ್ ಅಸಿಡಾಸ್, ಕ್ಯಾಥರಂಥಸ್ ರೊಸೆಯಾಸ್, ಫಿಲ್ಲಾಂಥಸ್ ಅಮರಸ್* ಮತ್ತು *ಸೊಲ್ಯಾನಂ ತೊರವೂಮ್* ನಿಂದ ಪ್ರತ್ಯೇಕಿಸಲಾಗಿರುತ್ತದೆ. ಒಟ್ಟು ಸಂಸ್ಕರಿಸಿದ ೧೭೫ ವಿಭಾಗಗಳಿಂದ ೭೭ ಅಂತರ ಜೀವಿಗಳನ್ನು ಪಡೆಯಲಾಗಿದ್ದು, ರೂಪವಿಜ್ಞ್ಯಾನ ವೈಶಿಷ್ಟಗಳಿಂದ ೨೫ ಓಟಿಯು ಗಳಾಗಿ ವಿಂಗಡಿಸಿ ಅವುಗಳನ್ನು ಮೆಣಸಿನಕಾಯಿ ಬೆಳೆಗೆ ತಗುಲುವ ಸ್ಕ್ಲಿರೋಟಿಯಮ್ ವಿರುದ್ಧ ಉಭಯ ಬೆಳವಣಿಗೆ ಪದ್ದತಿಯಿಂದ ಮೌಲ್ಯೀಕರಿಸಲಾಗಿರುತ್ತದೆ. ೨೫ ಓಟಿಯು ಗಳಲ್ಲಿ ಕ್ರಮವಾಗಿ ೫ ಓಟಿಯು ಗಳು ಶೇಕಡ ೫0 ಕ್ಕಿಂತ ಹೆಚ್ಚಿನದನ್ನು ೨೦ ಓಟಿಯು ಗಳು ಶೇಕಡ ೧-೫೦ ನಿರ್ಬಂಧವನ್ನು ತೋರಿಸಿರುತ್ತವೆ. *ಹೈ. ರೋಸಾ ಸೈನೆನ್ಸಿಸ್* ನಾ ಅಂತರಜೀವಿ ಓಟಿಯು ಎಚ್ಐ೨ ಸ್ಕ್ಲಿರೋಟಿಯಮ್ ಜಾತಿಗೆ ಶೇಕಡಾವಾರು ಅತಿ ಹೆಚ್ಚಿನ (೭೫) ಪ್ರತಿಬಂಧ ತೋರಿಸಿದ ಕಾರಣ ಅದನ್ನು ಹೆಚ್ಚಿನ ಅಧ್ಯಯನಗಳಿಗೆ ತೆಗೆದುಕೊಳ್ಳಲಾಗಿರುತ್ತದೆ. ಸ್ಕ್ಲಿರೋಟಿಯಮ್ ಮತ್ತು ಓಟಿಯು ಎಚ್ಐ೨ ಶೀಲಿಂಧ್ರಗಳ ಆಣ್ವಿಕ ಗುಣಲಕ್ಷಣವನ್ನು ಐಟಿಎಸ್ ಪ್ರೈಮರ್ ಗಳನ್ನು (ಐಟಿಎಸ್೧ ಮತ್ತು ಐಟಿಎಸ್೪) ಬಳಸಿ ಸ್ಕ್ಲಿರೋಟಿಯಮ್ ರಾಲ್ಫ್ಸೀ ಹಾಗು ಟ್ರೈಕೋಡರ್ಮ ಅಸ್ಪೆರೆಲ್ಲಂ ಎಂದು ಕ್ರಮವಾಗಿ ದೃಢಪಡಿಸಲಾಗಿದೆ. ಸ್ಕ್ಲಿ. ರಾಲ್ಫ್ಸೀ ಯನ್ನು ಪ್ರತಿಬಂಧಿಸುತ್ತಿರುವ *ಟ್ರೈ. ಅಸ್ಪೆರೆಲ್ಲಂನ* ಯಾಂತ್ರಿಕ ವ್ಯವಸ್ಥೆಯನ್ನು ತಿಳಿದುಕೊಳ್ಳಲು ಬ್ರಾತ್ ಕಲ್ಚರ್ ಮತ್ತು ಡಬಲ್ ಪ್ಲೇಟ್ ಅಧ್ಯಯನವನ್ನು ಎಚ್. ಪಿ. ಎಲ್. ಸಿ ಮತ್ತು ಜಿ ಸಿ-ಎಂ ಎಸ್ ಮುಖಾಂತರ ಕ್ರಮವಾಗಿ ವಿಶ್ಲೇಷಿಸಲಾಯಿತು. ಎಚ್. ಪಿ. ಎಲ್. ಸಿ ವಿಶ್ಲೇಷಣೆಯ ಚಯಾಪಚಯಗಳ ಚಿತ್ರಗಳ ಅಧ್ಯಯನವು ೩೭.೫ ಮತ್ತು ೩೯.೪ ನಿಮಿಷಗಳ ಸಂರಕ್ಷಣೆ ಸಮಯದಲ್ಲಿ ಪರಸ್ಪರ ಕ್ರಿಯೆಯಲ್ಲಿ ಎರಡು ಶಿಖರಗಳನ್ನು ತೋರಿಸಿರುತ್ತದೆ, ಹಾಗು ಜಿಸಿ - ಎಂಎಸ್ ಅಧ್ಯಯನದಿಂದ ೧೪ ಬಾಷ್ಪಶೀಲಗಳು *ಟ್ರೈ. ಅಸ್ಪೆರೆಲ್ಲಂ* ಗೆ ನಿರ್ದಿಷ್ಟವೆಂದು ತಾತ್ಕಾಲಿಕವಾಗಿ ಗುರುತಿಸಲಾಗಿರುತ್ತದೆ. ಮೆಣಸಿನಕಾಯಿ ಗಿಡಕ್ಕೆ *ಟ್ರೈ. ಅಸ್ಪೆರೆಲ್ಲಂನ* ವಸಾಹತುಶಾಹಿ ವಿಶ್ಲೇಷಣೆಯು ಯಶಸ್ವಿಯಾಗಿ ಕಾಂಡ ಮತ್ತು ಬೇರುಗಳ ಅಂಗಾಂಶಗಳಲ್ಲಿ ವಸಾಹತುಗೊಂಡಿರುವುದು ಖಚಿತಪಡಿಸಿರುತ್ತದೆ.

ಜೂನ್, ೨೦೧೭ (ಸಿ. ಎನ್. ಲಕ್ಷ್ಮಿನಾರಾಯಣ ರೆಡ್ಡಿ)

ಸಸ್ಯ ರೋಗ ಶಾಸ್ತ್ರ ವಿಭಾಗ ಪ್ರಮುಖ ಸಲಹೆಗಾರ

ಕೃಷಿ ಮಹಾವಿದ್ಯಾಲಯ, ಗಾ. ಕೃ. ವಿ. ಕೇ. ಬೆಂಗಳೂರು.

Isolation and evaluation of fungal endophytes as a potential biocontrol agents on *Sclerotium rolfsii* infecting Tomato and Chilli

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INTRODUCTION

Tomato and Chilli belongs to the family Solanaceae are important vegetable crops grown across the world. Diseases caused by various pathogens are becoming the major constraint for their production in the country. In the recent years, disease caused by *Sclerotium rolfsi* attained the economic importance in the production of chilli and tomato. The best approach for the management of this pathogen is through use of bioagents.

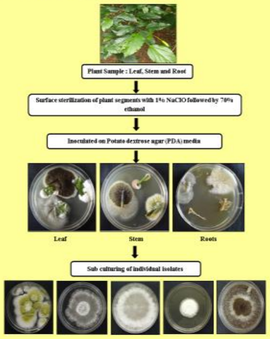
The hidden world organisms called 'Fungal endophytes' living inside the host tissue with majorly of beneficial relationship have attained greater importance in recent years by imparting resistance against both biotic and abiotic stress. Fungal endophytes have role in controlling plant pathogens through the mechanisms *viz..* antibiosis, competition for space and nutrients, hyper parasitism, and also by inducing induced systemic resistance in host plants.

OBJECTIVE

1. Isolation and characterization of fungal endophytes from selected plants.
2. Evaluation of fungal endophytes for their activity against *Sclerotium rolfsii* pathogen.

MATERIALS AND METHODS

- Isolation of fungal endophytes



Plant Sample : Leaf, Stem and Root

Surface sterilization of plant segments with 1% NaClO followed by 70% ethanol

Inoculated on Potato dextrose agar (PDA) media

Sub culturing of individual isolates

- Colonization frequency is calculated using formula,

$$CF\% = (Ncol / Nt) \times 100$$

Ncol- number of segments colonized by endophyte
Nt- total number of segments observed

- Isolated fungal endophytes were characterised morphologically into Operational Taxonomic Units (OTU's) based on colour of mycelia, colony characters and spores.

- Evaluation of isolated fungal endophytes against *Sclerotium* sps by dual culture technique.

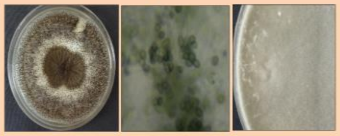
$$\%inhibition = R_1 - R_2 / R_1 \times 100$$

R1=Radial growth of pathogen in control
R2=Radial growth of pathogen in dual culture

RESULTS

- A total of 66 endophytic fungal isolates were obtained from 165 tissue segments from five plant species. Highest number of fungal endophytes were obtained from *Solanum torvum* (Table 1).

- Based on morphological characters , endophytes are categorized into 25 OTU's.



Colony character · Spores · Colour of mycelia

- The observation for inhibition of pathogen in dual culture is recorded 7 days after inoculation.

- Out of 25 OTU's five OTU's (HL5, HI2, AL2, VRL1 and VRS1) showed greater than 50% inhibition over control.

- The highest inhibition percentage (65%) and degradation of pathogen has been found in HI2 isolate, isolated from leaf and stem of Hibiscus plant (Figure 2).

Table 1: List of selected plant species, colonization frequency of endophytes and number of OTU's from respective tissue sample.

Sl. No.	Plant species	Plant part	No. of segments inoculated	No. of endophytes emerged	Colonization frequency (%)	Total number of OTU's
1.	*Hibiscus rosa sinensis*	Leaf Stem	15 15	16 4	100 100	5
2.	*Phyllanthus acidus*	Leaf	15	3	80	2
3.	*Catharanthus roseus*	Leaf Stem	15 15	14 3	100 86	5
4.	*Phyllanthus* amarus	Leaf Stem Roots	15 15 15	7 2 1	100 60 60	5
5.	*Solanum torvum*	Leaf Stem Roots	15 15 15	2 11 3	60 80 80	8

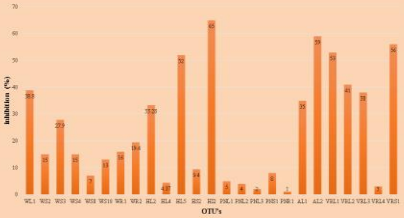


Figure 1: *In vitro* inhibition (%) of Sclerotium pathogen by endophytic fungal OTU's.

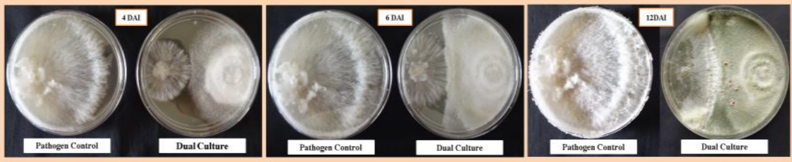


Figure 2: Inhibition and degradation of Sclerotium pathogen by HI2 isolate at different Days after inoculation (DAI).

DISCUSSION

The present study has revealed that, exploration of fungal endophytes can serve as potential source for the isolation of bioagents against fungal plant pathogens. Each endophyte has different percent inhibition on particular plant pathogen. This may be due the difference in the mechanism of suppression such as antibiosis, competition, hyper parasitism, and by inducing systemic resistance. The endophyte may have single or combination of mechanism in inhibiting the pathogen (Gao *et al*., 2010).

Further, Characterization of compounds (soluble or volatiles) involved in antibiosis will result in the discovery of lead molecules for the development of fungicides for effective management of plant diseases.

SUMMARY

- Fungal endophytes were isolated from selected plants and 25 OTU's were obtained.
- Five OTU's had shown more than 50% inhibition of Sclerotium pathogen in *in vitro*.
- Maximum percent inhibition (65%) of Sclerotium pathogen in *in vitro* was by endophytic isolate HI2.
- The mechanisms of inhibition of pathogen by endophyte and characterization of endophyte are in progress.

REFERENCE

❑ GAO, F. K., CHUAN, C. D., AND XIAO, Z. L., 2010, Mechanisms of fungal endophytes in plant protection against pathogens. *Afr. J. Microbiol. Res.* **4** (13): 1346-1351.

Advisory Committee

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I INTRODUCTION

Chilli (*Capsicum annum* L.) is one of the major vegetable and spice crop grown across the tropical and subtropical regions of the world. It is originated from Mexico and belongs to family solanaceae and genus Capsicum with several species in it. Chilli is a day neutral plant that can be grown throughout the year in well drained loamy soil with slightly acidic pH (6.5 to 7.5) (Stroehlein and Oebker, 2008). Among the chilli growing countries, India is one of the important country growing for both as vegetable and spice for having domestic consumption and export (Rao and Rao, 2014). In India, it is grown in an area of 789 million hectares with production of 1389 million tonnes (Anon, 2016). Chilli is a rich source of vitamin A, C, and E. Vitamin P, recently identified by Russian scientists in green chillies considered to protect from secondary radiation injury (Verghese, 1999).

Chilli is valued for its pungency imparted by an alkaloid Capsaicin and the red pigments Capsanthin, Capsorubin and Capxanthin (Satishkumar *et al*., 2015). Capsaicin has significant medicinal value and used in the preparation of balms, ointments for cold, sore throat and chest congestion. Capsanthin is extracted from the red chillies is being extensively used in the cosmetics, perfumes, paints, dyes and for colouring foods (Verghese, 1999). Further, it is also used as an ornamental plant. Due to its multipurpose uses it has demand all over the world.

Chilli crop suffers from many biotic and abiotic stresses. Biotic stresses imposed by fungi, bacteria and viruses are becoming major limiting factors for the production of chilli across the world resulting in huge crop loss (Dhandapani *et al*., 2003). Diseases caused by fungal pathogens such as anthracnose, powdery mildew, cercospora leaf spot, damping off etc. are wide spread in chilli growing areas (Thind and Jhooty, 1985). Among fungal diseases, dry root rot of chilli caused by *Sclerotium rolfsii* Sacc. attained economic importance in recent years (Madhavi and Bhattiprolu, 2011).

Sclerotium rolfsii Sacc. (teleomorph: *Athelia rolfsii* Tu & Kimbrough), is a devastating soil borne plant pathogenic fungus having wide host range and a corticioid fungus in the family Atheliaceae (Kwon *et al*., 2017). In chilli, this pathogen causes dry root rot disease with characteristic symptoms of cracks near collar region, roots were shredded with full of white mycelial growth on the surface of freshly infected area with small mustard seed like Sclerotial bodies at collar and root regions (Kalmesh and Gurjar, 2001).

Many disease management strategies such as cultural, chemical and biological measures were followed to manage soil borne fungal diseases including *sclerotium* wilt in many crops (Madhavi and Bhattiprolu, 2011). In order to overcome the problem of pesticide pollution in soil and environment, resistance development of pathogens to chemicals and reducing cost of cultivation, biological control approach of plant disease management is gaining great impetus in the recent years (Serfling *et al.*, 2006). In this back drop, exploration of the hidden world organisms called 'fungal endophytes' for potential biocontrol agents attained greater importance in recent years (Pablo *et al*., 2015).

Endophytes are living organisms may be fungi or bacteria which lives inside the plant tissues for at least a part of its life cycle without causing any apparent diseases and with more of beneficial effects to its host plant (Pablo *et al*., 2015). Fungal endophytes have wide range of functional activities in living host system like production of secondary metabolites and safeguarding host plant against many biotic and abiotic stresses, these organisms have gained interest as a promising source of new natural products that could hold the key to resolving emerging problems in medicine, agriculture, and industry, particularly those related to treatment of infectious diseases (Souvik *et al*., 2012). They also have role in controlling the plant pathogens through the mechanisms *viz.* antibiosis, competition for space and nutrients, hyper parasitism and by inducing systemic resistance in host plants (Gao *et al*., 2010).

Several studies have been reported the role of fungal endophytes in inhibiting the plant pathogens. Barley plants colonized with *Piriformospora indica,* fungal endophyte discovered from thar desert plant ecosystem imparted resistance against root pathogen *Fusarium culmorum* and bio-trophic pathogen *Blumeria graminis* f. sp. *hordei* causing powdery mildew through induced systemic resistance (Frank *et al*., 2005). Out of 225 fungal endophytes isolated from root segments of eggplant, melon, strawberry and Chinese cabbage, 11 isolates showed complete suppression of pathogenic effect on *Verticillium dahliae*, which causes wilt disease of eggplant (Kazuhiko *et al.*, 2001).

Exploration of fungal endophytes as bioagents against plant pathogens is having immense potential in addressing the management of many plant diseases. With this back drop, the current study was undertaken to isolate fungal endophytes from selected plants against chilli dry root rot caused by fungus *Sclerotium rolfsii* with the following objectives,

Objectives of investigation are

1. Isolation and morphological characterization of fungal endophytes from selected plants and *Sclerotium* sp. infecting chilli.
2. *In-vitro* evaluation of endophytic fungus for their activity against *Sclerotium* sp. infecting chilli.
3. Molecular characterization of fungal endophyte and *Sclerotium* isolate.
4. Deciphering the mechanisms of action of fungal endophyte against the *Sclerotium* sp. infecting chilli.

II REVIEW OF LITERATURE

The plant diseases are the major constraint for the food production across the world. Most often chemicals are used for the management of plant diseases which are having the harmful effect on human and animals apart from causing the environmental pollution (Margni *et al*., 2001). The limitation in the availability of resistance sources in crop plants made use of chemical obvious. In this scenario, exploitation of endophytes is hope for chemical residual free disease management strategies in the food production. In nature, every plant has its own endophytic microorganism which are having majorly beneficial effects to plants. Further, exploitation of beneficial endophytic organisms for plant disease management will have greater impact on eco-friendly farming with reduced risk of resistance development in pathogens, which is gaining momentum worldwide (Luis *et al*., 2008).

Pathogen

Sclerotium rolfsii Sacc. is the most destructive soil borne fungus, first time reported by Rolfs (1892) causing of tomato blight at Florida. Later, Saccardo (1911) named the fungus as *S. rolfsii*. In India, Shaw and Ajrekar (1915) isolated the fungus from rotted potatoes and identified as *Rhizoctonia destruens* Tassi. However, later studies showed that, the fungus involved was *S. rolfsii* (Ramakrishnan, 1930). *S. rolfsii* is generally distributed in tropical and subtropical countries wherever high temperature prevails during the rainy season (Weber, 1931). The fungus can survive in soil for years together by producing sclerotial bodies (Garret, 1956) and causing-foot rots, stem rots, collar rots and leaf blights in many economically important host crops belonging to the number of various families.

Kalmesh and Gurjar (2001) reported that, severe mortality of chilli plants was observed during March-April in chilli growing area. Mature plants of chilli crop foliage drooped and dried finally in the field. Close examination of the diseased plants showed deep cracks near collar region, roots were shredded and unhealthy with full of white mycelial growth on the surface of freshly infected area with small mustard seed like sclerotial bodies at collar and root surface.

Endophytes

In nature, since from the period of evolution of plants they are subjected to different abiotic and biotic stress conditions. Throughout their evolution plants are known to depends majorly on endophytic microorganisms for various activities such as secondary metabolites production and protection against abiotic and biotic stresses (Rusty *et al.,* 2008). Endophytes are living organisms may be fungi or bacteria which lives inside the plant tissues for at least a part of its life cycle without causing any apparent diseases and with more of beneficial effects to its host plant (Pablo *et al*., 2015).

Endophytic microbes fall into several identifiable classes often in relation to their plant organ source, with the major groups as follows: 1. Endophytic Clavicipitaceae 2. Fungal endophytes of dicots 3. Endophytic fungi 4. Other systemic fungal endophytes 5. Fungal endophytes of lichens 6. Endophytic fungi of bryophytes and ferns 7. Endophytic fungi of tree bark 8. Fungal endophytes of xylem 9. Fungal endophytes of root 10. Fungal endophytes of gall and cysts 11. Prokaryotic endophytes of plants (includes Endophytic bacteria and actinomycetes) (Bills *et al.*, 2004).

Fungal endophytes

Fungal endophytes are living organisms which lives inside the plant tissues for at least a part of its life cycle without causing any apparent diseases and with more of beneficial effects to its host plant (Pablo *et al*., 2015). The fungal endophytes are different from mycorrhizal fungi where endophytes reside entirely within host tissues (Jumpponen, 2001). These endophytic fungi are classified based on host range, colonization pattern, transmission, and ecological function. Symbiotic association of fungal endophytes with plants has beneficial effects to its host plant and in turn it obtains food and nutrients for its livelihood (Rusty *et al.*, 2008).

The fungal endophytes association with their host plants vary with respect to growth stage. Paul *et al*. (2011) reported that, *Penicillium* in seedling stage, *Fusarium* in flowering stage, *Colletotrichum* followed by *Fusarium*, *Alternaria* and *Xylaria* in fruiting stage was predominant and *Alternaria*, *Cladosporium* and *Fusarium* were common in all growth stages of *Capsicum annuum*.

Isolation and characterization of fungal endophytes

The selected healthy plant parts like leaf, stem and roots are collected and washed with running tap water for twice. Leaf, stem and root samples are cut into small segments using sterile blade, these segments are surface sterilized with 1 % Sodium hypochlorite solution for 30 seconds and rinsed with sterile distilled water, then again treated with 70 % ethanol for 1 min and thoroughly rinsed with sterile distilled water for 2 to 3 times. These segments are air dried and placed into Petri plates containing potato dextrose agar (PDA) media under aseptic condition and plates were incubated at 27 ^{0}C, after 7 to 10 days' fungal endophytes were growing from cut ends of plant segments (Arnold *et al*., 2000).

Guo *et al*. (2000) reported that, from selected plants (palm plant) all segments like leaf, petiole, veins, intervein, stem and roots were collected and cut into small discs, randomly selected discs from each segments were collected and initially washed with tap water and then surface sterilized by consecutive immersion for 1 min in 75 % ethanol, 10 min in 65 % commercial Chlorox (final concentration 3.25 % aqueous sodium hypochlorite) and 30 sec in 75 % ethanol, finally surface sterilized discs were air dried on sterile blotter paper and placed into Petri plates containing Malt Extract Agar (MEA) media supplemented with 1 mg ml^{-1} streptomycin sulphate to avoid bacterial endophytes growth. Petri plates were sealed, incubated for two months at 25 °C, and examined periodically.

Kokub *et al*. (2007) reported that, the pathogen *S. rolfsii* is morphologically characterised by taking 10 mm diameter of actively growing fungal mycelia with self-designed cutter and placed onto the centre of Petri dish containing PDA under aseptic condition and plates were incubated at 28 $^{\circ}$C for 7 days. Morphological characteristics such as, growth pattern, mycelium condition, radial colony diameter, development of sclerotia, and colour, type and location of sclerotia were noted, observations were recorded at 12 hours' interval up to 3 days and then at 24 hours' intervals for 7 days.

Mahadevakumar *et al*. (2015) isolated the genomic DNA of fungal species from the mycelial mass grown in PDB for 5 days by Cetyl trimethyl ammonium bromide (CTAB) extraction method (Zhang *et al*., 1998). Amplified the internal transcribed spacer region in rRNA using universal primers ITS1 (5 - CGGATCTCTTGGTTCTGGCA-3) and ITS4 (5 -GACGCTCGAACAGGCATGCC-3). The amplified product of approximately 600bp was sequenced. The analysed sequence data was used for identification of fungal species as *S. rolfsii*.

Evaluation of fungal endophytes for their antagonistic activity on plant pathogens

Srivastava *et al*. (2015) reported the antimicrobial ability of fungal endophytes isolated from *Prosopis juliflora* by evaluating through different antimicrobial assays as mentioned below.

Agar diffusion method

Isolated fungal endophyte was sub-cultured on Petri plates containing potato dextrose agar media. Six-millimetre diameter mycelial plugs of actively growing endophytic fungi were placed on the nutrient agar media plate inoculated with test bacteria. The plates were incubated for 24-48 hours at 37 ^{o}C. After 24 hours, and the zone of inhibition if any was measured in mm.

Dual culture method

Endophytic fungi and fungal phytopathogens were cultured separately on PDA plate and incubated at 25±2 ^{o}C for 5 days. The actively growing fungal mycelium was cut by using 6 mm diameter cork borer. Fungal discs of endophytic fungi and fungal phytopathogens were placed on the opposite side of the same PDA plate, at a distance of 5 cm between the endophyte and pathogen. A fungal disc of plant pathogenic fungi placed on one side on a PDA plate without endophytic fungi was served as control (Srivastava *et al*., 2015). These petri plates are incubated at 25±2 ^{o}C for 5 days and recorded the observations. Per cent inhibition of pathogen by endophyte was calculated by using the formula.

Per cent inhibition = [(R1-R2)/ R1] × 100

Where R1 represents radial growth of fungal phytopathogen in control plate and R2 is the radial growth of fungal phytopathogen in test sample plate.

Based on the inhibition of pathogen in dual culture method the compound and defence related enzymes were detected through various enzymatic assays and chromatographic techniques. Enzymatic activity like chitinase and cellulase were confirmed by culturing potential bioagent on media supplemented with chitin and cellulose (El-katatny *et al*., 2001). Other antimicrobial compounds were detected through liquid chromatographic techniques (Frisvad *et al*.,1987).

Disc diffusion method

Sterile discs of 6 mm diameter were impregnated with 50 μL of ethyl acetate extracts of endophytic fungi and air dried in laminar air flow for 20 min. Air-dried sterile discs impregnated with 100 mg of the ethyl acetate extracts were used to test the activity against selected pathogen. Mueller-Hinton (MH) agar plates for bacteria and Czapek Dox Agar (CDA) plates for fungi were used. The sterile discs impregnated with extract of endophytes were placed over the medium and sterile discs impregnated with only solvent were used for control. The plates were incubated at 37±2 ^{o}C for 24 hours for bacteria and 25±2 ^{o}C for 7 days for fungi and the zone of inhibition was measured after the incubation period (Srivastava *et al*., 2015).

Volatile assay

To know the antimicrobial activity of bio agent with production of any volatile organic compounds (VOCs), Rouissi *et al*. (2013) conducted double Petri dish assay. In this approach, two bottom lids of the Petri dish were taken and poured with PDA media. In the lower lid 6 mm disc of *Penicillium expansum* mycelial load was placed at the centre, and in upper lid 6 mm disc of test pathogen was placed. Another set without bio agent in bottom lid and in upper lid inoculated with 6 mm disc of test pathogen was used as control. The Petri dishes were closed and wrapped with air tight condition and incubated at 27 ^{0}C for 5-7 days. Periodic observations were carried out. Further, treatments with inhibition of test pathogen were subjected to gas chromatography mass spectrometry for analysis of VOCs (Francesco *et al*., 2014).

Mechanism of fungal endophytes against plant pathogens

Fungal endophytes have ability to safeguard the plants against both abiotic and biotic stress, among biotic stress it can defend many phytopathogens through some defined antagonistic mechanism. The phytopathogens can be controlled either by direct or indirect mechanism. Direct mechanisms like antibiosis, competition for space and nutrients, hyper parasitism and other lytic enzymes 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). They provide protection against pathogens might be due to inducing the production of phytoalexins and their endophytic ecological occupation. The enhancement of plant growth may be influenced by phytohormones produced by fungal endophytes (Gao *et al.*,2010).

Many fungal endophytes produce secondary metabolites and some of these compounds were antifungal and antibacterial strongly inhibiting the growth of other microorganisms including plant pathogens (Gunatilaka, 2006). The derivatives of cadinane sesquiterpenes, isolated from an endophytic fungus *Phomopis cassia* from *Cassia spectabilis* showed antifungal activity against *Cladosporiums phaerospermum* and *Cladosporium cladsporioides*. Among the derivatives, 3,11,12-trihydroxycadalene was found to most effective active compound (Silva *et al*., 2006).

Redman *et al*., (1999) reported that, *Citrullus lanatus* and *Cucumis sativus* exposed to a non-pathogenic mutant of *Colletotrichum magna* exhibited high levels of lignin deposition, peroxidase activity and phenylalanine ammonia lyase activity and imparted the protection against disease caused by *Colletotrichum orbiculare* and *Fusarium oxysporum*. Display of antimicrobial activity of a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mogolica*, against bacteria as well as against the fungus *Helminthosporium sativum*, colletotric acid was reported suggesting the endophytes as potential source of biogents (Zou *et al*., 2000).

Fungal endophytes isolated from leaves of *Prosopis juliflora* were evaluated against some test pathogens like *Fusarium solani, Fusarium verticillioides, Aspergillus flavus* and *Aspergillus ochraceus* isolated from maize seeds. The ethyl acetate extracts of endophytic *Colletotrichum gloeosporioides* and *Paecilomyces lilacinus* showed the presence of alkaloids, carbohydrates, sterols and coumarins which are having antimicrobial activity against fungal plant pathogens (Srivastava *et al*., 2015).

Some fungal endophytes parasitize around hyphae of pathogens by twisting, penetrating the hyphae of pathogens and secreting lyase to decompose cell wall of plant pathogens. *Trichoderma* sp are able to parasitize hyphae of plant pathogen *Rhizoctonia solani* resulting in inhibition of the pathogen (Grosch *et al*., 2006).

Amin *et al*. (2001) evaluated six isolates of *Trichoderma* spp. against *Fusarium* oxysporum, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Sclerotinia sclerotiorum*, *Colletotrichum capsici*, *Helminthosporium oryzae* and *Alternaria brassicicola* for volatile organic compound production. Out of six isolates, *T. viridae* and *T. harzianum* showed effective inhibition of test pathogens through production of volatile metabolites.

III MATERIAL AND METHODS

Endophytic fungi play an important role in modulating plants response to pathogen. From this context main goal of the study was to isolate and evaluate fungal endophytes against *Sclerotium rolfsii* infecting chilli. The experiments in the current study were conducted at School of Ecology and Conservation, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bengaluru, (13.0777° North, 77.5805° East).

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's, solid media and soil used for pot culture experiments were sterilised in an autoclave at 1.1 kg/ cm^2 pressure for 15 min at 121 $^{\circ}$C. For *in vitro* studies disposable radiation sterile vented polystyrene Petri dishes (Tarsons) were used.

3.1.b Growth medium used

For all endophytic fungal isolation and other studies potato dextrose agar media (PDA) (Hi Media) was used.

The composition of PDA was as follows,

Potatoes infusion :200 g/ L.

Dextrose: 20 g/ L.

Agar: 15 g/ L.

The media was prepared by adding 39.0 g of PDA in 1000 mL of distilled water followed by autoclaving at 1.1 kg/ cm^2 pressure for 15 min at 121 $^{\circ}$C. At the time of pouring the media into Petri dishes streptomycin sulphate (final concentration of 200 ppm) was added to medium to avoid endophytic bacterial growth.

For broth culture studies Potato Dextrose Broth (PDB) liquid media (Hi Media) was used.

The composition of PDB was as follows,

Potato infusion: 200 g/ L.

Dextrose: 20 g/ L.

The broth was prepared by adding 24.0 g of PDB in 1000 mL of distilled water followed by autoclaving at 1.1 kg/ cm^2 pressure for 15 min at 121 ^{0}C.

3.2 Sample collection for isolation of endophytes from selected plants

Leaf, stem and root samples from five selected plants (Plate 3.1) were collected from Botanical Garden, GKVK (13.0777° North, 77.5805° East), Bengaluru for fungal endophytes isolation. Plant species with traditionally well-known anti-microbial properties and wild solanaceous plant grown without any pests or disease incidence were selected. The collected plant species were identified with the assistance of research team at botanical garden, GKVK, Bengaluru.

3.2.a Isolation of fungal endophytes

Leaf, stem and root samples collected from selected plant species for fungal endophytes isolation were cut into 1 cm long segments and washed with running tap 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 washed 2-3 times with sterile distilled water and air dried (Arnold *et al*., 2001). The processed segments are placed over petri dish containing PDA media in aseptic condition and wrapped with saran wrap and incubated at 27 °C for the growth of fungal endophytes. To check the epiphyte contamination, the processed segments were imprinted on PDA medium (Sculz *et al*., 1993). The inoculated plates were incubated for 5-7 days and

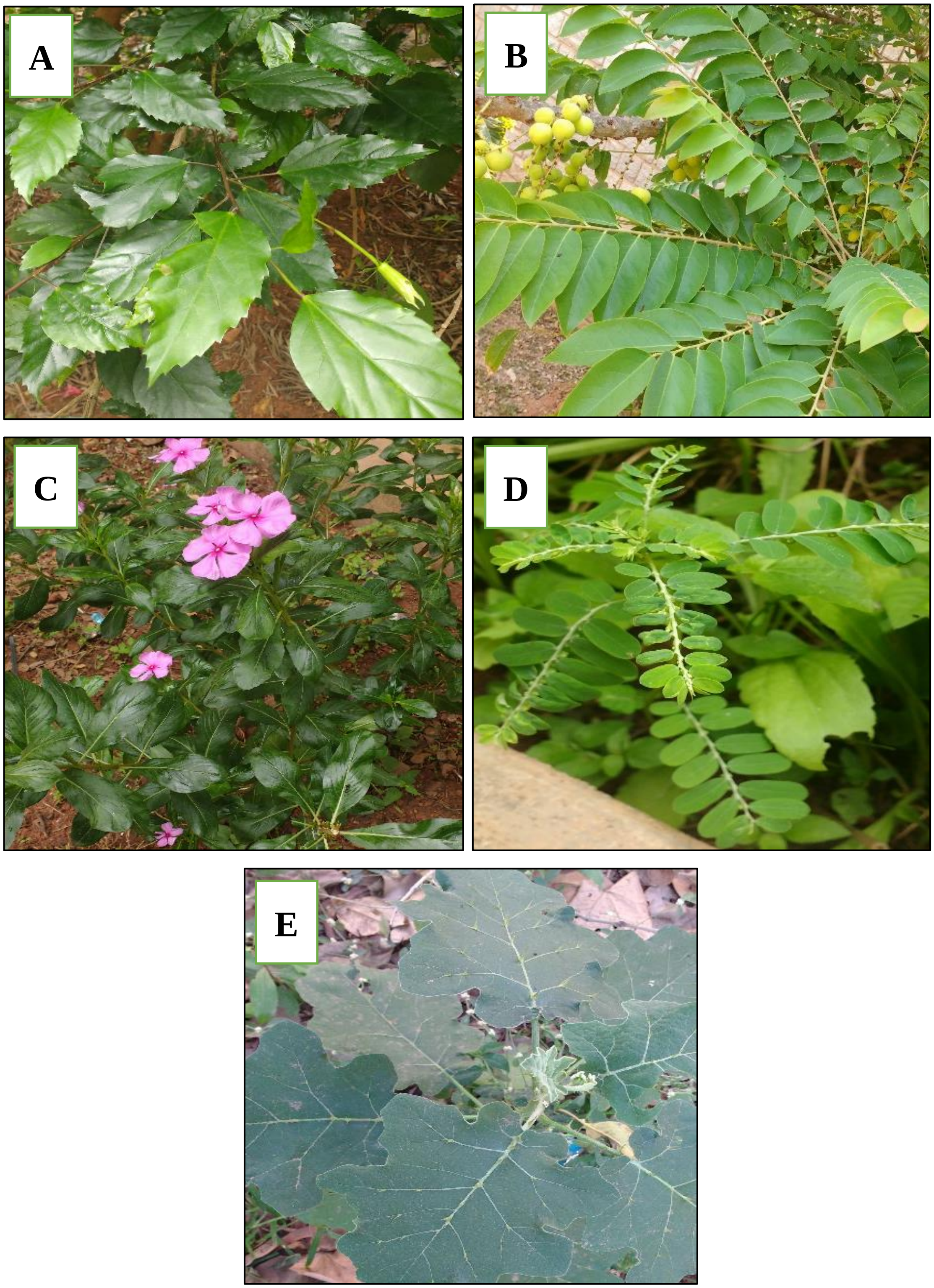

Plate 3.1: Plant species selected for isolation of fungal endophytes: A. *Hibiscus rosa sinensis*, B. *Phyllanthus acidus*, C. *Catharanthus roseus*, D. *Phyllanthus amarus* E. *Solanum torvum.*

observed for the growth of endophytes from the cut ends (Plate 3.2). Colonization frequency of endophytic fungi was determined by number of segments colonized by each endophyte to the total number of segments observed (Hata and Futai., 1995).

3.2.b Purification and maintenance of individual fungal endophytic isolates

The fungi grown out from the inoculated plant segments were transferred to petri dish containing PDA media and pure cultures of endophytes were obtained. Purified fungal endophytes were maintained on PDA slants by preserving at 4 °C in refrigerator for further use.

3.2.c Morphological characterization of fungal endophytes

Pure cultures of fungal endophytes were identified based on colour of mycelia, spores, fruiting bodies and other morphological features using standard identification manuals (Barnett, 1972; Ellis, 1971; Sutton, 1980) and finally categorised into different Operational taxonomic units (OTU's).

3.3. Sample collection and isolation of *Sclerotium* sp. from chilli

Chilli plant showing symptoms of dry root rot characterized with white mycelia on the collar region and associated with sclerotial bodies of the fungus was collected from the farmer's field of Mudachinthalahalli, Chintamani Taluk, Chikkaballapur Dist., Karnataka (13.4020° North and 78.0551° East) for isolation of the pathogen.

The fungal pathogen was isolated from the roots of infected chilli plant by standard tissue isolation method under aseptic conditions. The infected tissues of the roots were cut into small pieces of 1-2 cm in size and surface sterilized with 1 % mercuric chloride solution for 1 min and washed thrice in sterile distilled water, placed into petri dishes containing sterilized PDA media and incubated at 27 °C (Madhavi and Bhattiprolu, 2011). The culture obtained was purified by sub culturing the growing hyphal tip of Sclerotium fungi on Petri dish containing PDA medium. Based on the microscopic studies and morphological descriptions given by Hsieh (1992) it was identified as *Sclerotium* sp. The *Sclerotium* fungus was cultured on PDA slants and allowed to grow at 27±1 °C. The culture obtained was designated as Chitntamani isolate of *Sclerotium* sp. and stored in refrigerator at 4 °C for further studies.

3.3.a Mass multiplication of pathogen and pathogenicity assay

Sorghum seeds were pre-soaked in water for 24 hours and then autoclaved at 121 °C temperature, 15 kg/ cm^2 pressure for 15 min in autoclavable polythene covers. The discs (6-8 mm) from PDA media containing actively growing *Sclerotium* isolate fungus of 5 - 8 numbers were taken and inoculated into polythene cover containing autoclaved sorghum seeds under aseptic condition. The inoculated substrate in polythene covers were incubated at 27±1 °C. After 8 days of incubation the mass multiplied pathogen was used for further studies (Upadhyay and Mukhopadhyay, 1986).

To confirm the pathogenicity of isolated *Sclerotium* pathogen, it was inoculated to the chilli plants. The eight days old mass multiplied *Sclerotium* pathogen of 3 % (w/w) (Jinantana and Sariah, 1998) was added to pots containing 30 days old chilli plants and allowed for symptom expression. For all experimental studies chilli variety Arka Lohit procured from Division of Vegetable Crops, Indian Institute of Horticultural Research (IIHR), Hessaraghatta Lake Post, Bengaluru was used.

Treatments followed: Five plants for each treatment

T_1= Inoculated with Chintamani isolate of *Sclerotium*

T_2= Control (without inoculating pathogen)

3.4 Evaluation of fungal endophytes as a potential biocontrol agent (*In-vitro*)

Each OTU of endophyte isolated was screened against *Sclerotium* pathogen for their antagonistic activity in *In-vitro* condition. The most common screening method, dual culture technique was followed for every endophyte and pathogen interaction.

3.4.a Dual Culture

The mycelial disc of 8 mm diameter of actively growing endophytic and *Sclerotium* fungi were placed opposite to each other in PDA medium containing Petri

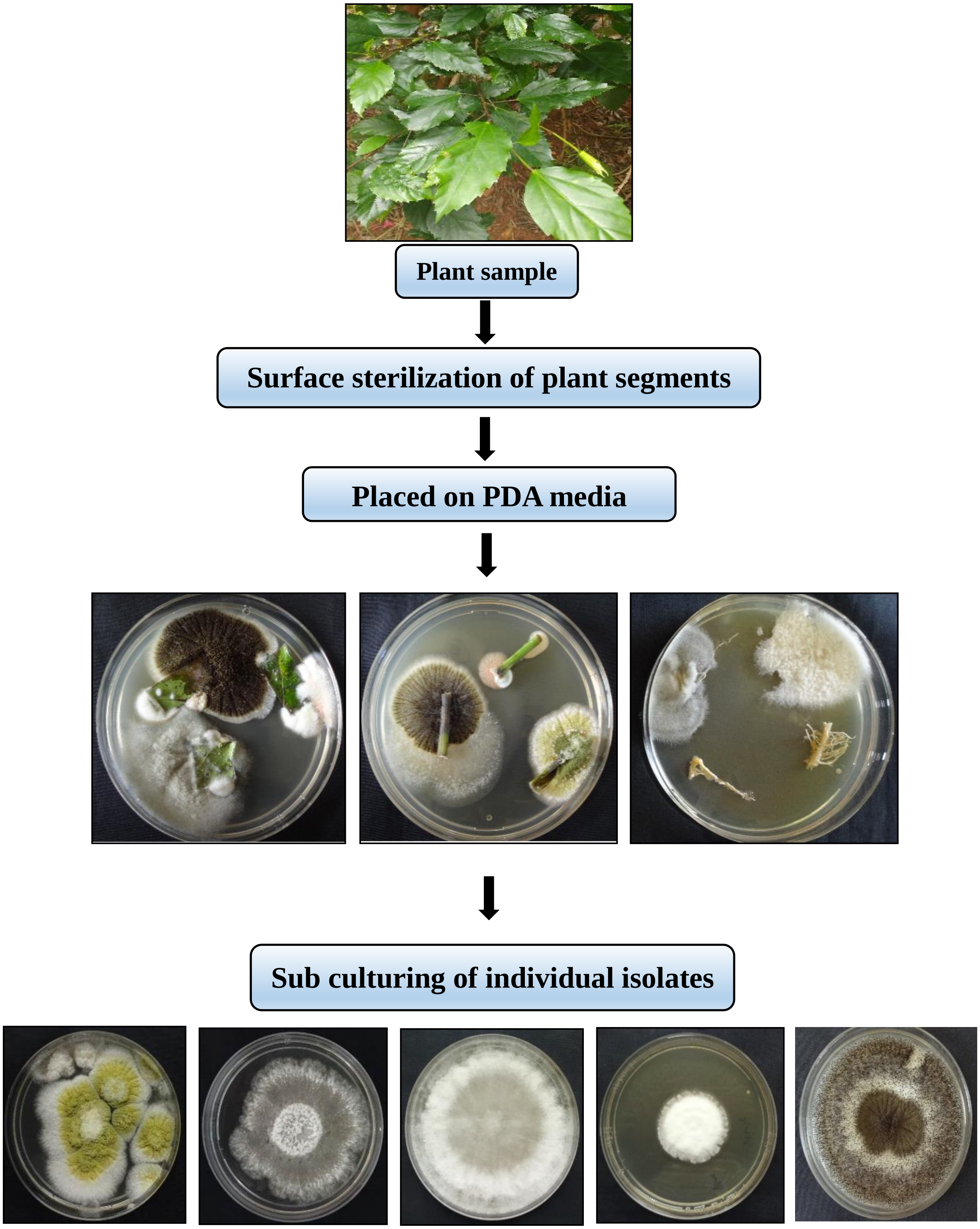

Plate 3.2: Schematic illustration of isolation and purification of fungal endophytes

dishes leaving 1 cm from the margin. Triplicates each case with one set of control of *Sclerotium* pathogen without inoculating the endophytic fungal isolate was included. The plates were incubated at 27±1 ºC. The data were recorded regularly for the growth of the pathogen and endophytic fungal isolate. The percent inhibition was calculated at seven days after inoculation by using formula given below (Skidmore and Dickinson, 1976).

$$\text{PIRG} = R_1 - R_2 / R_1 \times 100$$

Where, PIRG -Percent Inhibition of Radial Growth.

R_1- Radial growth of *Sclerotium* pathogen in control plate.

R_2- Radial growth of *Sclerotium* pathogen in dual culture with endophyte.

3.5 Molecular characterization fungal endophyte and pathogen

Both the dry root rot causing pathogen in chilli and potential endophyte HI2 were characterized by amplification of internal transcriber spacer region (ITS) using ITS1 and ITS4 primers.

3.5.a Preparation of fungal samples

Fungal endophyte HI2 and foot rot causing pathogen were separately inoculated into conical flask containing PDB under aseptic condition and incubated for 7 days at 28 °C. After seven days, 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 Isolation of DNA from fungal endophyte and pathogen

About 100 mg of fungal mycelium was ground in a pestle and mortar using liquid nitrogen. 2 % Poly Vinyl Pyrrolidone (PVP) and Sodium Dodecyl Sulfate (SDS) were added, mixed gently and further 1.5 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. Tubes were incubated at 65 °C for

60 min in a water bath with stirring at every 10 min intervals. After 60 min of incubation in water bath 1 mL of Phenol: Chloroform: Iso-amyl alcohol (25:24:1) was added and mixed thoroughly. Tubes were centrifuged at 10,000 rpm for 10 min, supernatant was collected and transferred to the new tubes. Then added 1ml of isopropyl alcohol and mixed well, the tubes were incubated at -20^0C for 30 minutes. Then tubes were centrifuged at 10,000 rpm for 10 min, pellet was retained and supernatant was discarded. Then pellets were air dried and dissolved in Tris-EDTA (TE) buffer (pH 8.0) by adding 1µl RNAse (10µg/ml) and incubated at 37 ^{o}C for 30 min. Then tubes were removed from the incubation and added with 1 ml of Chloroform: Iso-amyl alcohol (24:1) and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to new tubes, 10 µL of 3 M sodium acetate (pH 5.0) was added along with two times of ice cold ethanol (absolute) and kept at -20 °C for 45 min. After incubation, the tubes were centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatant was discarded from the tube, the pellet was washed using 200 µL of 70 % ice cold ethanol and centrifuged. The pellet was air dried dissolved in 20 µL of Tris-EDTA (TE) buffer and incubated at 37 °C in a water bath for 15 min. 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 PCR amplification of Internal transcribed spacer (ITS) region using ITS primers

The ITS region in endophyte and pathogen were PCR amplified using ITS1 forward primer and ITS4 reverse primer. PCR amplification was carried out in 25 µL reaction volume which consisted of template concentration of 100 ng/ µL, 2.5 µL of 10X Taq buffer, 1 µL of 2 mM MgCl2, 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. PCR was performed in an Eppendorf Master Cycler Gradient (Hamburg, Germany). The amplification profile was 94 °C for 4 min followed by 30 cycles of 94 °C for 60 s, 55 °C for 45 s, 72 °C for 90 s with a final extension step at 72 °C for 10 min. The amplified products were eluted from the gel (Qiagen gel elution kit; *Cat No*./ID: *28706*) and sent for sequencing.

3.5.d Sequence analysis for taxonomic grouping of endophyte and pathogen

The amplified PCR products were sequenced at Scigenome, Cochin. Sequence similarity searches were performed by comparing these sequences to all available sequences in GenBank using BLASTN. Sequences showing the highest identity scores with the present isolates were retrieved. Sequence identity matrices for endophyte and pathogen were generated using the Bioedit Sequence Alignment Editor (Version 5.0.9; Hall, 1999). A phylogenetic tree was generated using MEGA 6.01 software (Tamura *et* al., 2013) and the Neighbour-Joining method (Saitou and Nei, 1987) with 1000 bootstrap replications in order to estimate evolutionary distances between all pairs of sequences.

3.6 Mechanisms of inhibition of pathogen by fungal endophyte

To know the mechanisms of action of fungal endophyte through which it inhibits the pathogen the following assays were conducted.

3.6.a High Performance Liquid Chromatography (HPLC)

To extract the secondary metabolites produced during the fungal endophyte HI2 and *Sclerotium* sp. interaction, endophyte was co-cultured with *Sclerotium* sp. into conical flask containing 100 mL PDB and incubated at 29 °C. Two other conical flasks with100 mL PDB was inoculated endophyte and *Sclerotium* sp. separately to serve as control. After 7 days of incubation the mycelial mat of colonies were removed using sterile forceps and the broth was filtered through filter paper. Metabolite was extracted from the broth by adding equal volume (100 mL) of ethyl acetate (1:1 v/v) after mixing for 30 min using a separating funnel and then kept for separation. Upper solvent layer was collected and concentrated using rotary evaporator, and the dried extract was re-dissolved in 500 µl of methanol (HPLC grade). 20 µl sample was injected into HPLC system with a total run time of 45 min. The condition used for the HPLC analysis was as follows: Column: Luna C18 (250 mm 94.6 mm), 5 lm pore size (Phenomenex, USA), Detector: SPD-M 20A photodiode array, oven temperature 35 ºC, wave length was set as 254 nm. The mobile phase consists of 0.1 % Trifluoroacetic acid (TFA) (Pump B) and acetonitrile (Pump A) in binary gradient mode with following ratio of solvents table 5.1 (Smedsgaard, 1997).

Table 5.1: The gradient solvent system conditions for HPLC.

Pump A (%)	Pump B (%)	Time (min)
100	0	0.20
85	15	1
0	100	40
0	100	43
85	15	45
100	0	45.01
	Stop	45.02

3.6.b Volatile assay

To study the ability of fungal endophyte to produce volatile organic compounds, double Petri dish assay was followed. In this assay, the antifungal activities of volatile organic compounds (VOCs) produced by the endophyte were tested against the mycelial growth and Sclerotial bodies production in pathogen *Sclerotium* sp. Actively growing 7 days old 6 mm agar plug of endophyte was inoculated in petri dish containing PDA. The lid of the plate was replaced with a base plate of PDA inoculated with 6 mm agar plug of actively growing pathogen, and the two base plates were sealed immediately with saran wrap and plate was incubated at 29 ºC. The control treatment with pathogen in the upper lid and empty base plate with PDA only sealed with saran wrap. The percentage of growth inhibition and sclerotial bodies production were recorded after 7 days of incubation. (Rouissi *et al.*, 2013). The percentage of growth inhibition was calculated by the formula: [(T1 - T2)/T1] ×100, where T1 is the diameter of growth of the target pathogen in control and T2 is the diameter of growth of the target pathogen exposed to endophyte (Trivedi *et al*., 2006). The assay was conducted in six replicates and repeated thrice.

3.6.c Gas chromatography analysis

To identify the composition of VOCs, the fungal cultures were qualitatively evaluated by Headspace - Solid Phase Micro extraction (HS-SPME) coupled with gas chromatography - mass spectrometry (GC–MS). The SPME fiber (specification) coated with DVB/CAR/PDMS was used. Five 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 chemicals released from 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 (30m 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 identification of VOCs was achieved by comparing the GC retention times and mass spectra with those of pure standard compounds. All mass spectra were also compared with the data system library (NIST 14 MS Library). Blank sample analysis (growth medium not inoculated with antagonists) was performed under the same conditions in order to exclude interfering substance. All measurements were made with three replicates, each replicate representing the analysis of a different Petri dish. GC peaks were used to identify each volatile compound. For temperature programmed gas chromatography, the Retention index is given by the equation, (Zhu, 1985).

$$I = TR(\mathrm{unknown}) - TR(n) / TR(n+i) - TR(n) \times 100i + 100n.$$

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.

3.7 Host colonization assay

The potential fungal endophyte was checked for colonization in chilli plants. Seed material of chilli variety Arka Lohit (Procured from Vegetable Division, Indian Institute of Horticultural Research, Bengaluru) was kept for pre-germination on blotter paper at 29 °C for 4 -7 days. The concentration of spore suspension (2×10^6 CFU/ mL) of fungal endophyte was adjusted using haemocytometer as described by Zhang *et al*. (2014). The pre-germinated seeds were soaked for three hours in the spore suspension. After three hours, the seedlings were transferred to portrays containing autoclaved coir pith and watered regularly. Thirty days after sowing (DAS), the plant samples were collected and processed (as described in 3.2.a) for isolation of fungal endophyte from leaf, stem and roots to confirm the colonization of endophyte in chilli plant.

Treatments: T1= Control (without endophyte treatment)

T2= Endophyte treatment.

IV RESULTS

In nature, the association of plants and microorganisms leading to their mutual benefit were well documented in several cases. However, documentation regarding the endophytes (fungal/bacterial) present with in the plants were recent revelation. These discoveries provided a great impetus for exploration of fungal endophytes for the management of biotic and abiotic stress in the crop plants. In this backdrop, attempts were made in the current study to explore fungal endophytes against *Sclerotium rofsii* infecting chilli. The results obtained are presented in this chapter.

4.1 Isolation and purification of fungal endophytes

Leaf, stem and root samples from five selected plant species viz., *Hibiscus rosa sinensis*, *Phyllanthus acidus*, *Catharanthus roseus, Phyllanthus amarus* and *Solanum torvum* were collected from the botanical garden, GKVK, Bengaluru for isolation of fungal endophytes.

Total sixty-six endophytic fungal isolates were isolated from 165 tissue segments processed from the five different plant species (75 from leaf, 60 from stem and 30 from root segments). The colonization frequencies of the endophytic fungi from the five different plant species ranged from 60 % to 100 %. The highest colonization frequency was observed with *Hibiscus rosa sinensis,* while *Phyllanthus amarus* and *Solanum torvum* showed the lowest colonization frequency. Among the different sample segments, leaf has highest colonization frequency followed by stem and root segments (Table 5.2). The fungal endophytes emerging from the tissue segments were purified and sub-cultured on PDA medium.

4.1.a Morphological characterization of fungal endophytes

The endophytic fungal isolates were classified into different Operational taxonomic units (OTU's) based on morphological characters like colour of mycelia, growth pattern, spores and fruiting bodies. Sixty-six isolates from five plant species were grouped into 25 OTU's. Highest number of OTU's was obtained from *Solanum torvum* (8) and least number of OTU's (2) was obtained from *Phyllanthus acidus* (Table 5.3). All the plant species used in this study yielded both sporulating and non-sporulating endophytic fungi. However, the diversity of sporulating fungi was less compared to that of non-sporulating fungi.

4.2 Isolation of plant pathogen

The *Sclerotium* pathogen was isolated from root and collar portion of diseased chilli plant showing dry root rot symptoms collected from the farmer field, Mudachintalahalli Chintamani Taluk, Chikkaballpur Dist., Karnataka. Morphological characters of the fungus grown on PDA were studied. The fungal mycelium was initially silky white in colour later turned to dull white and had vigorous growth, when fungus attained maturity small mycelial knots were formed which later turned to mustard seed like sclerotia which were deep brown or brownish black and spherical to irregular in shape (Plate 3.3). The pure culture of the fungus isolated from the disease sample was cultured on PDA slants and stored in refrigerator at 4 °C for further studies and designated as Chintamani chilli 1 (CC1) isolate of *Sclerotium*.

4.2.a Pathogenicity assay

The pathogenicity was proved by inoculating the CC1 isolate of *Slcerotium* on Chilli variety Arka Lohit. After five to seven days of inoculation symptom expression was recorded in pathogen treated plants, which starts at collar and root region. Leaves of infected plants became initially pale green followed by yellowing. Later, plants showed death of growing tip, drooping of leaves and white mycelial growth around the collar and root region similar to the symptoms observed in the diseased plant sample collected from farmer’s field. Further, the collar region was sunken and rotted resulting in root rot symptom and drying of the infected plants. The sclerotial bodies were formed on collar and root region. No symptoms were observed in the control plants where the pathogen was not inoculated (Plate 3.4). Fungal pathogen was re-isolated from diseased plant tissue and it was identical with original culture of *Sclerotium* fungus isolated from chilli.

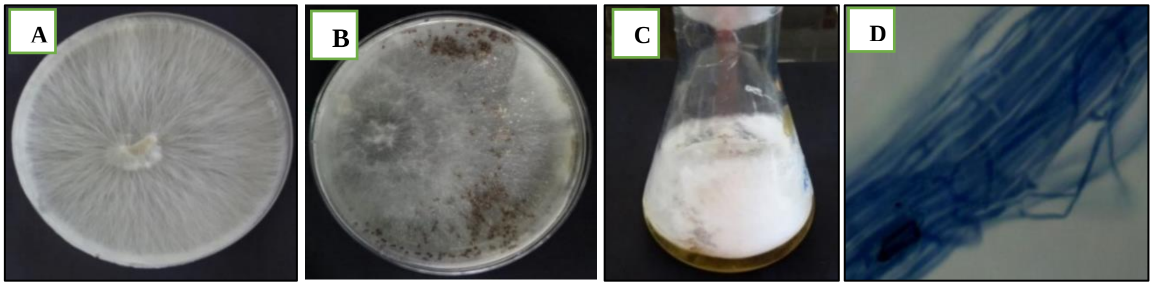

Plate 3.3: Morphological features of CC1 isolate of *Sclerotium*: A. *Sclerotium* fungus in PDA media. B. Sclerotial bodies production. C. *Sclerotium* fungus in PDB. D. Microscopic view of *Sclerotium* mycelia stained with Lactophenol cotton blue.

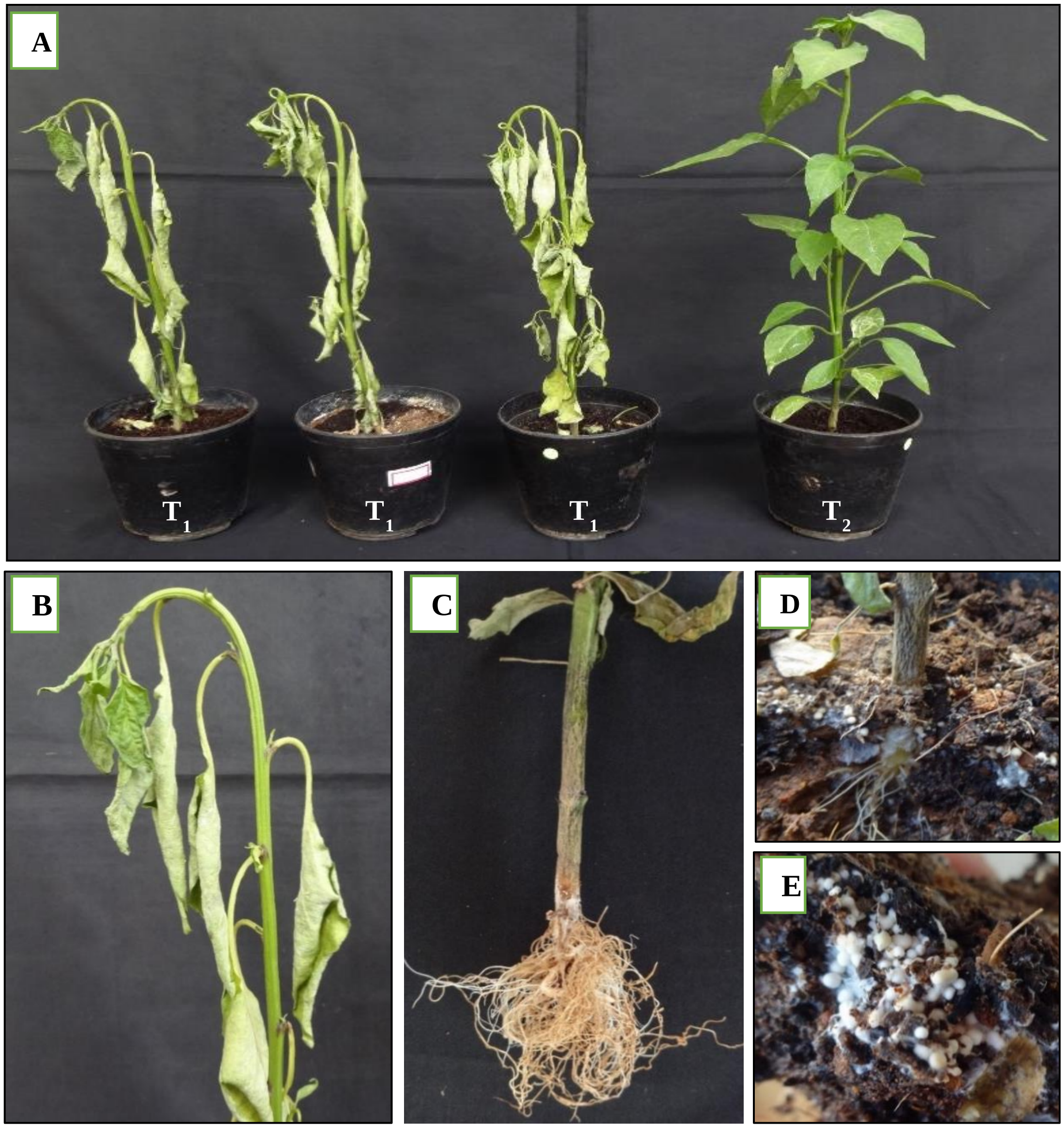

Plate 3.4: Pathogenicity assay and symptomatology for CC1 isolate of *Sclerotium*: A. T_1= Plants inoculated with CC1 isolate of *Sclerotium* T_2= Control. B. Drooping of foliage. C. Sunken area with rotted tissue at collar region. D. Mycelial growth on root and collar region. E. Sclerotial bodies formed in root zone.

Table 5.2: Number of endophytes emerged and their colonization frequency in selected plant tissue segments collected from botanical garden, GKVK, Bengaluru.

Sl. no.	Plant species	Taxonomic Family	Plant part	No. of segments placed in PDA media	No. of endophytes emerged	Colonization frequency (%)
1.	*Hibiscus rosa sinensis*	Malvaceae	Leaf	15	16	100
			Stem	15	4	100
2.	*Phyllanthus acidus*	Phyllanthaceae	Leaf	15	3	80
3.	*Catharanthus roseus*	Apocynaceae	Leaf	15	14	100
			Stem	15	3	86
4.	*Phyllanthus amarus*	Phyllanthaceae	Leaf	15	7	100
			Stem	15	2	60
			Roots	15	1	60
5.	*Solanum torvum*	Solanaceae	Leaf	15	1	60
			Stem	15	11	80
			Roots	15	3	80

4.3 *In-vitro* evaluation of fungal endophytes as potential biocontrol agents:

Twenty-five OTU's obtained from the five different plant species were screened against CC1 isolate of *Sclerotium* by dual culture technique (Plate 3.5.a – 3.5.e). Inhibition percentage was recorded seven days after inoculation. Out of twenty-five OTU's, five OTU's showed greater than 50 per cent, six OTU's showed 25-50 per cent and fourteen OTU's showed 0-25 per cent inhibition, respectively (Figure 4.1). Among these, endophyte (OTU HI2) isolated from *H. rosa sinensis* showed maximum inhibition of sixty-five per cent with complete degradation of pathogen (Plate 3.6).

Table 5.3: Categorization of fungal endophytes into operational taxonomic units (OTU's) based on morphological features.

Plant species	**OTU Code**	**Segment**	**Colony Character**	**Total number of OTU's**
Hibiscus rosa sinensis	HL2	Leaf	White with greenish	5
	HL4	Leaf	Whitish bold	
	HL5	Leaf and Stem	Brown	
	HS2	Stem	Dull white sticks	
	HI2	Leaf and Stem	Greenish	
Phyllanthus acidus	AL1	Leaf	White with greenish	2
	AL2	Leaf	Dark green	
Catharanthus roseus	VRL1	Leaf	Dark green	5
	VRL2	Leaf	Grey	
	VRL3	Leaf and Stem	Whitish bold	
	VRL4	Leaf	Pink	
	VRS1	Stem	Black	
Phyllanthus amarus	PNL1	Leaf	Brown	5
	PNL2	Leaf and Stem	Dark green	
	PNL3	Leaf	Dull white sticks	
	PNS1	Stem	Dull white	
	PNR1	Root	Whitish bold	
Solanum torvum	WL1	Leaf	White with greenish	8
	WS2	Stem	Greyish bold	
	WS3	Stem	White sticks	
	WS4	Stem	Pink	
	WS8	Leaf and Stem	Whitish bold	
	WS10	Stem	Black	
	WR1	Root	Dark green	
	WR2	Root	Cottony White	

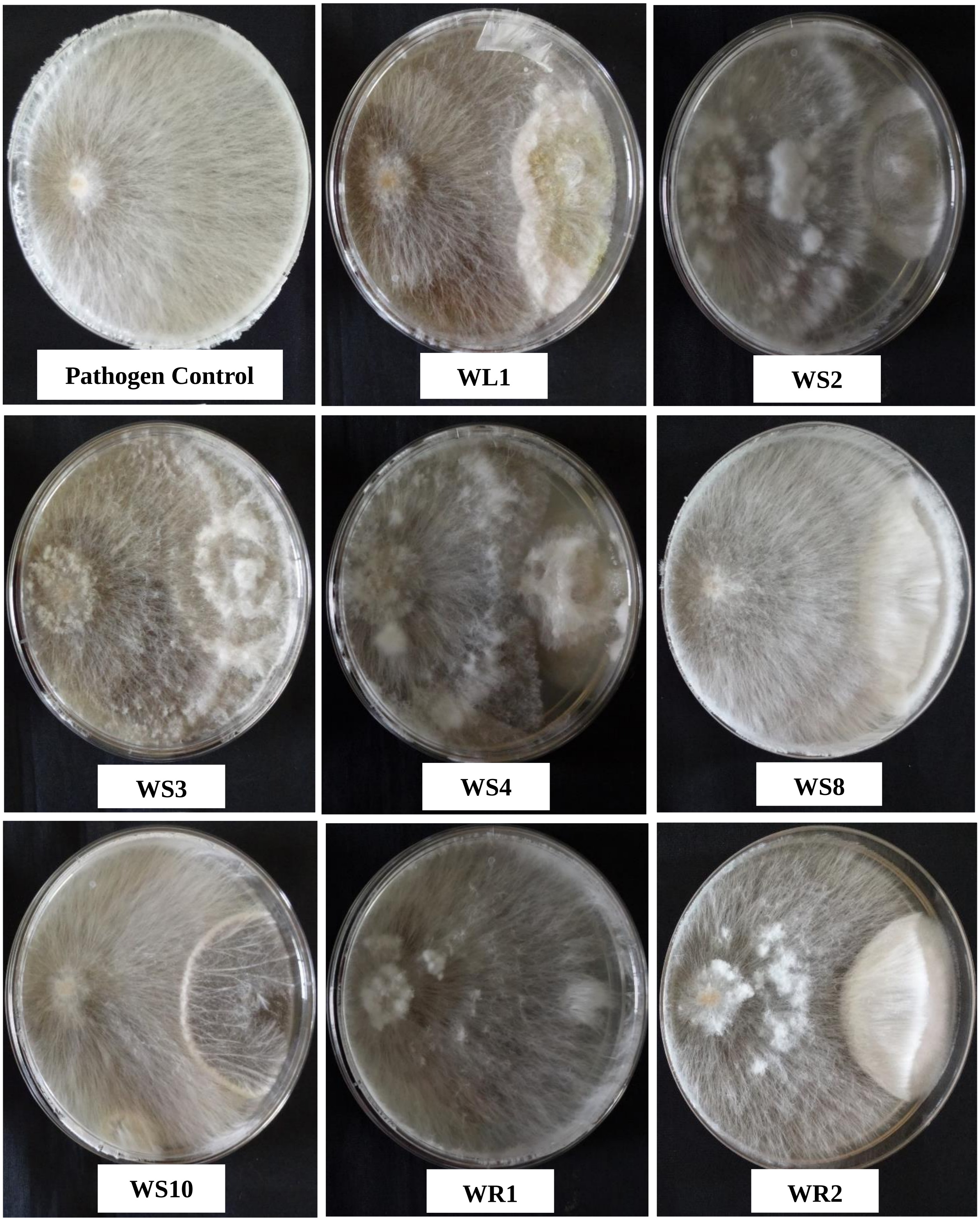

Plate 3.5.a: *In-vitro* evaluation of fungal endophytic OTU's isolated from *Solanum torvum* against CC1 isolate of *Sclerotium*.

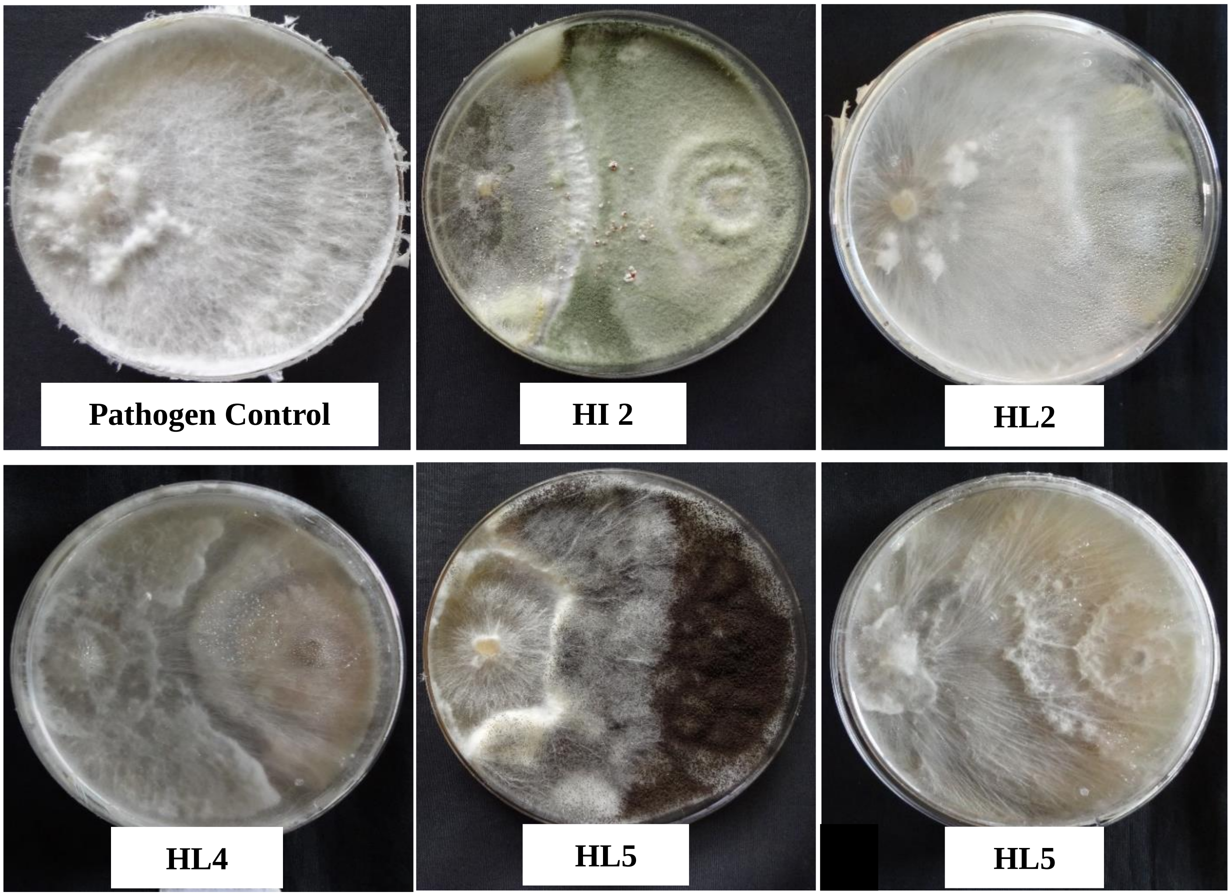

Plate 3.5.b: *In-vitro* evaluation of fungal endophytic OTU's isolated from *Hibiscus rosa sinensis* against CC1 isolate of *Sclerotium*.

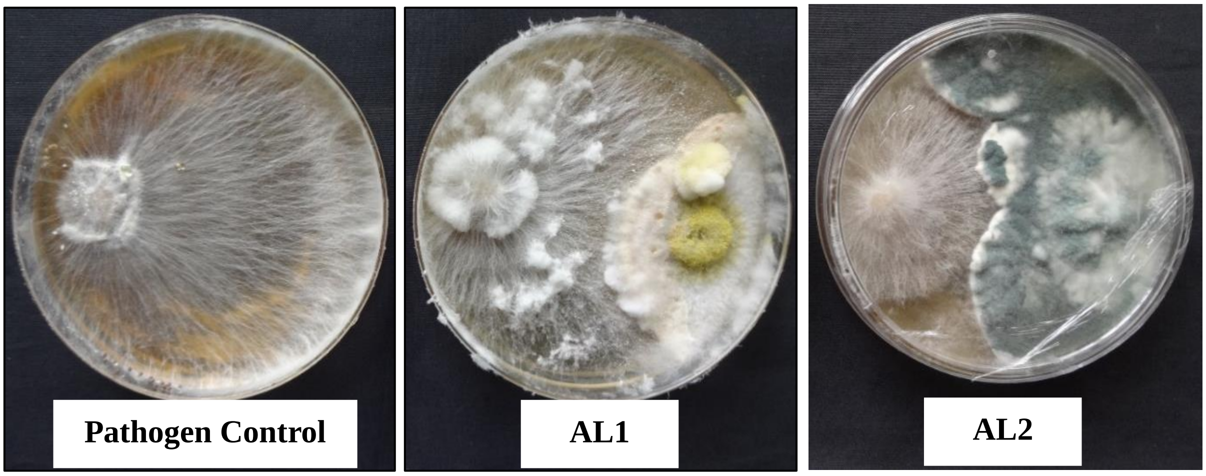

Plate 3.5.c: *In-vitro* evaluation of fungal endophytic OTU's isolated from *Phyllanthus acidus* against CC1 isolate of *Sclerotium*.

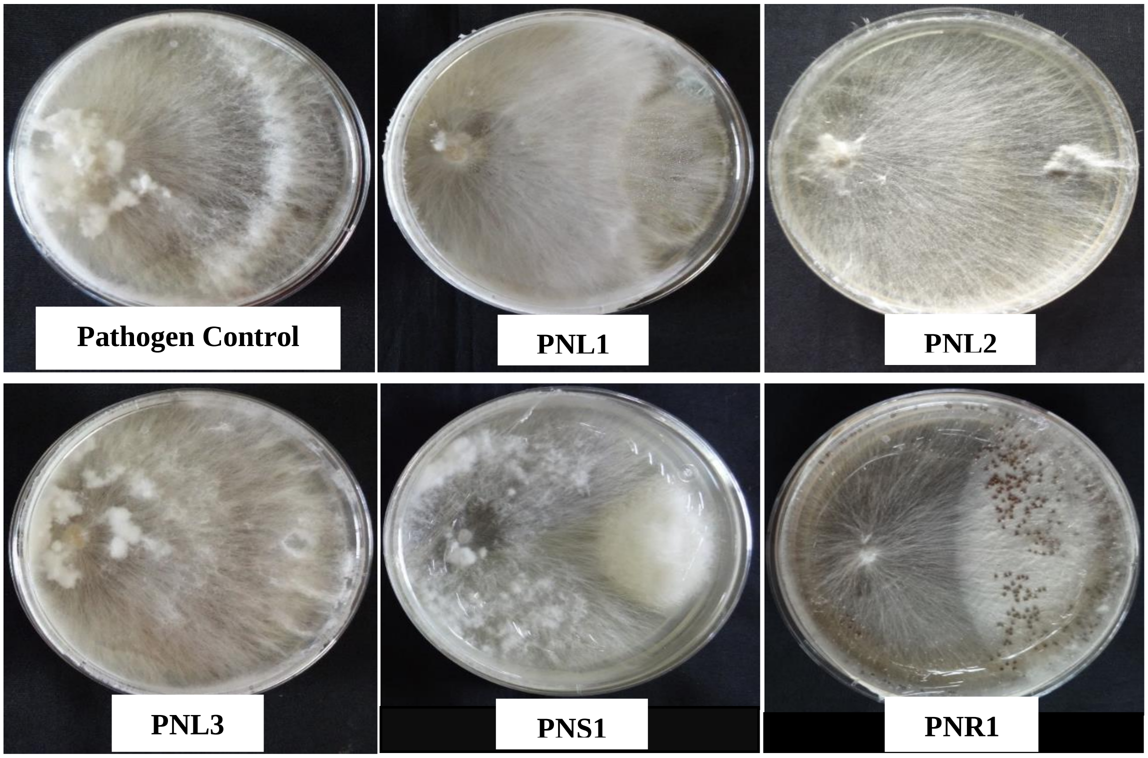

Plate 3.5.d: *In-vitro* evaluation of fungal endophytic OTU’s isolated from *Phyllanthus amarus* against CC1 isolate of *Sclerotium*.

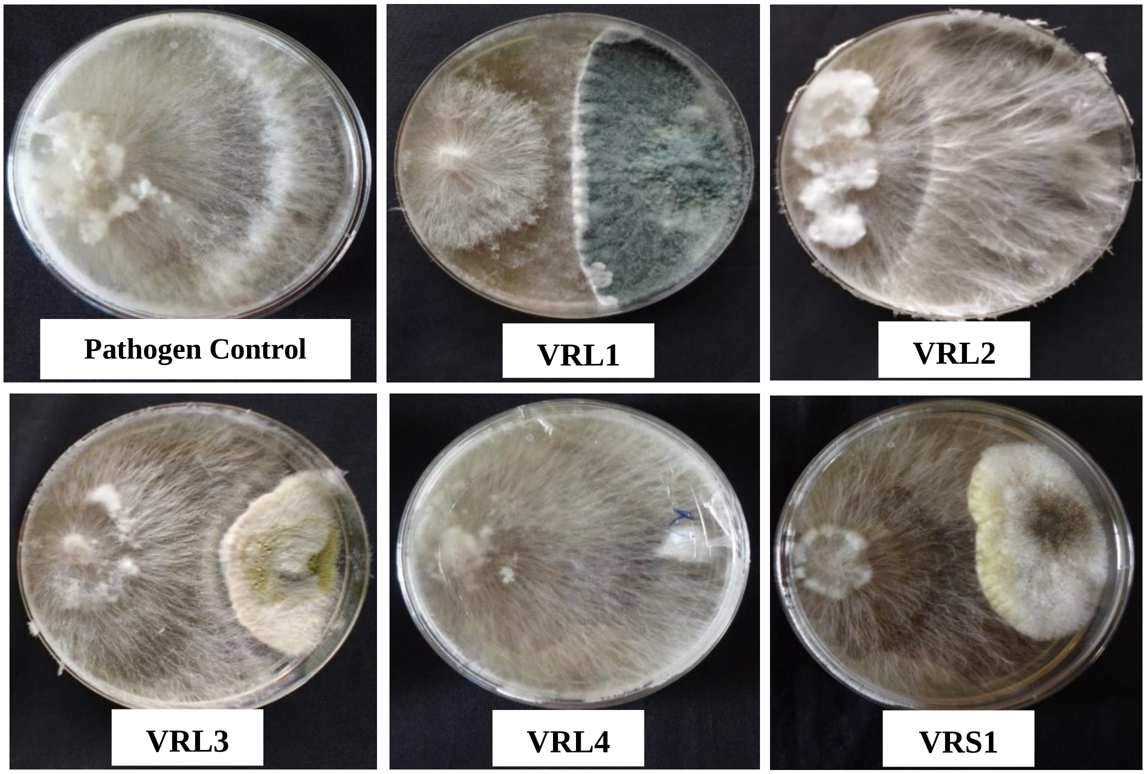

Plate 3.5.e *In-vitro* evaluation of fungal endophytic OTU’s isolated from *Catharanthus roseus* against CC1 isolate of *Sclerotium*.

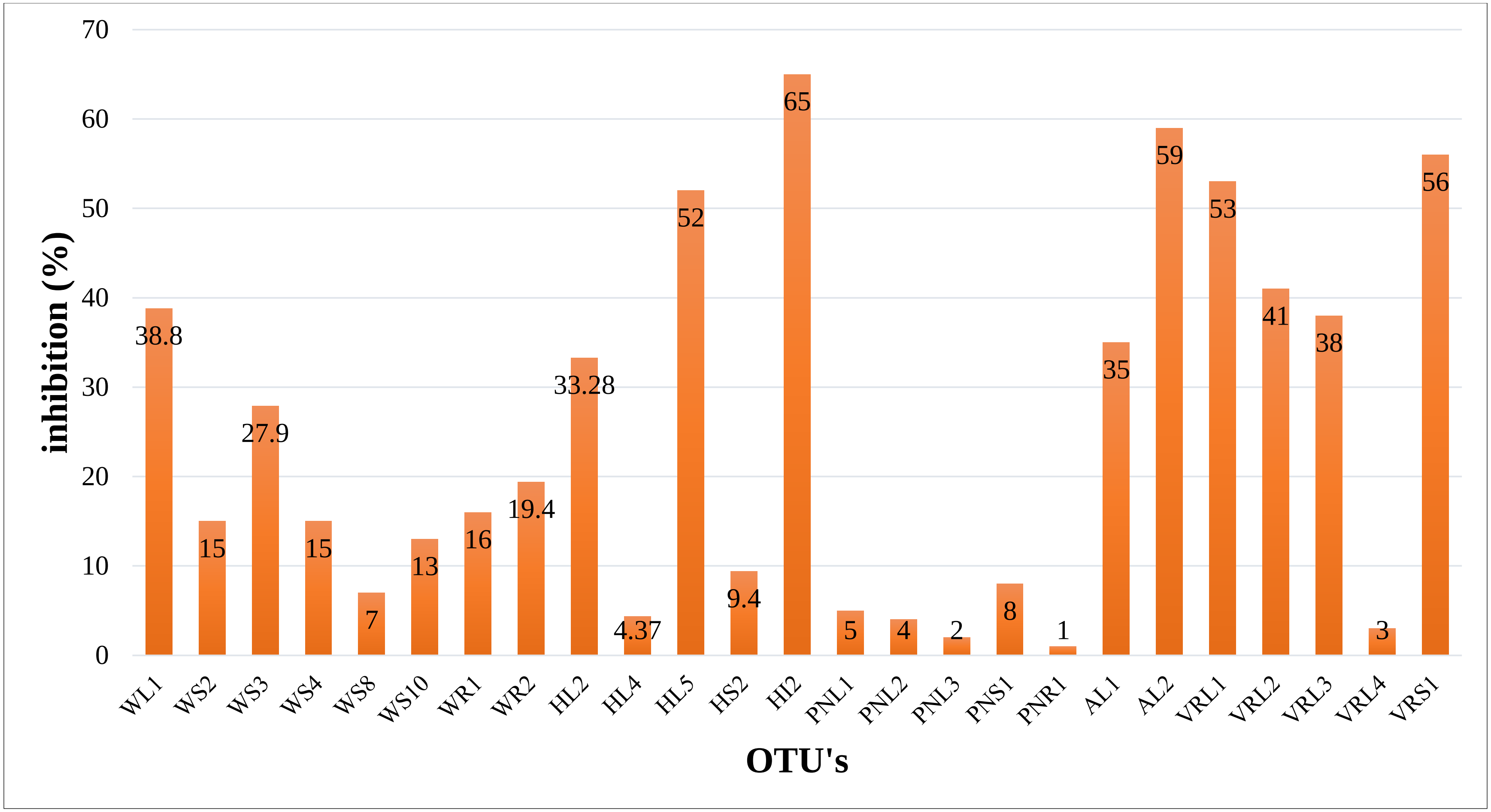

Figure 4.1: Inhibition percentage of CC1 isolate of *Sclerotium* by different fungal endophytic OTU's in dual culture (All the values represent mean of three replicates; x-axis= respective fungal endophytic OTU code and y-axis= per cent inhibition).

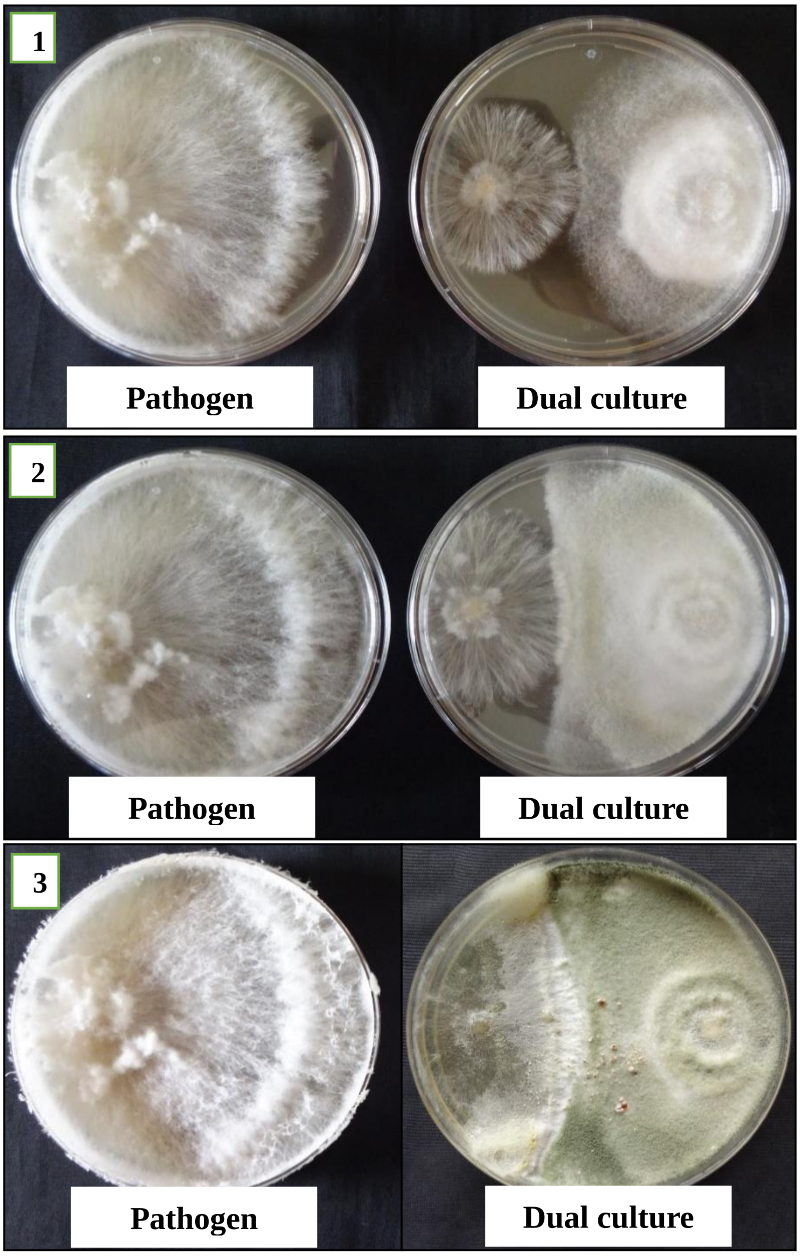

Plate 3.6: Dual culture of endophytic OTU HI2 and *Sclerotium*- CC1 isolate at different Days after inoculation (DAI): 1) 4DAI; 2) 6DAI; 3) 12DAI.

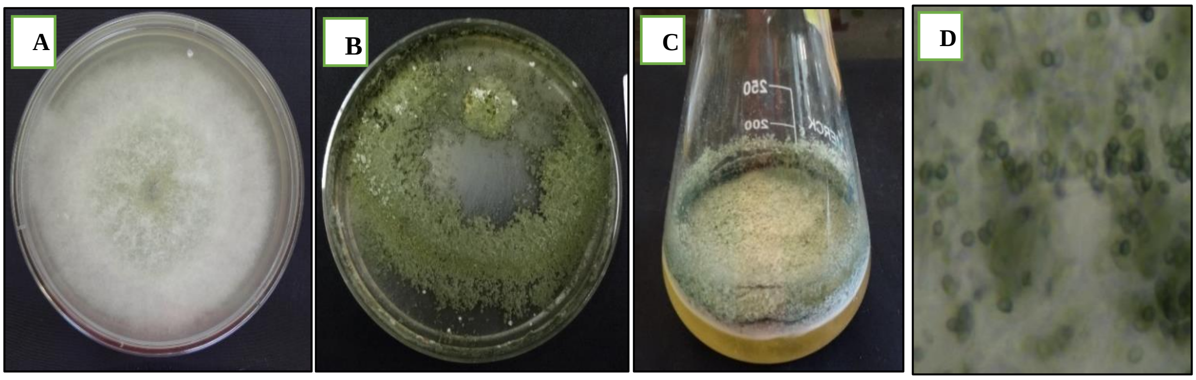

Plate 3.7: Morphological feature of fungal endophytic OTU HI2: A. In PDA media. B. Sporulated culture. C. In Potato dextrose broth. D. Microscopic view (Single celled conidia).

Fungal endophyte (OTU HI2) isolated from *Hibiscus rosa sinensis* showing highest percent inhibition was selected for all further studies The morphological features of OTU HI2 given in Plate 3.7.

4.4 PCR amplification, sequencing and characterization of fungal endophyte and *Sclerotium* isolate

The total DNA isolated from the endophytic fungal isolate HI2 and *Sclerotium* isolate CC1 was subjected PCR amplification using universal primer pair ITS-1 and ITS-4 (White *et al.,* 1990). The resulted PCR amplicon of 550 bp in size is obtained, which is corresponds to Internal transcribed spacer region (ITS) of fungi (Figure 4.2). The PCR amplified products were eluted and sequenced. The obtained sequences were queried in BLASTN available in NCBI GenBank. The GenBank accessions showing highest identity were retrieved, multiple aligned and identity matrices were obtained for both the endophyte and pathogen separately (Table 5.4 – 5.6). The identity matrices clearly revealed the pathogen sequence was queried and accessions showing maximum homology were retrieved from NCBI, GenBank, multiple aligned and identity matrices were obtained. The pathogen isolate in the current study shared maximum identity with the *Athelia rolfsii* (teleomorphic stage of *Sclerotium rolfsii* Sacc:) reported from different parts of the world. This was further confirmed with the phylogentic analysis showing close clustering of CC1 isolate of *Sclerotium* with already reported isolates of *Athelia rolfsii* indicating it as *Sclerotium rolfsii* (Figure 4.3). Similarly, the endophyte OUT HI2 from *H. rosa sinensis* shared highest homology of 99 per cent with *Trichoderma asperellum* reported from different parts of the world. This was well supported by the phylogenetic analysis confirming the endophytic isolate in the current study was *Trichoderma asperellum* (Figure 4.4).

4.5 High Performance Liquid Chromatography (HPLC)

To extract the secondary metabolites produced during the fungal endophyte *Trichoderma asperellum* –HI2 and *Sclerotium rolfsii* isolate CC1 interaction, the endophyte was co-cultured with *Sclerotium* sp. The secondary metabolites were extracted with ethyl acetate, re-dissolved in methanol and injected into HPLC for analysis. The metabolite profile study revealed that the metabolite content of the endophyte was eluted between 21-39 min retention time. Major peaks were observed between 21 to 25 min and 36 to 39 min. In case of pathogen the metabolite peak was eluted at 24 min of retention time. Few metabolite peaks observed individually for pathogen and endophyte were absent in the samples processed from interaction between pathogen and endophyte. However, two peaks were obtained at 37.5 and 39.4 min retention time, which were not observed in the samples with pathogen or endophyte alone (Figure 4.5).

Table 5.4: Pairwise percent of nucleotide identities between the Internal transcribed spacer (ITS) region of *Sclerotium rolfsii* –CC1 isolate with the selected other fungal species sequences available in the GenBank, NCBI (www.ncbi.nlm.nih.gov).

Sl. No.	**Fungal species**	**GenBank Accession number**	**Per cent identity with *S. rolfsii* –CC1**	**SL. No.**	**Fungal species**	**GenBank Accession number**	**Per cent identity with *S. rolfsii* – CC1**
1	*Athelia rolfsii*	KT337426	75.3	14	*Athelia rolfsii*	AY684917	91.5
2	*Athelia rolfsii*	KX139196	92.1	15	*Athelia rolfsii*	EU338381	90.6
3	*Athelia rolfsii*	KU128903	92.1	16	*S.delphinii*	JN241551	84.1
4	*Athelia rolfsii*	GU080230	91.8	17	*A.solani*	JQ625580	34.5
5	*Athelia rolfsii*	JN017199	91.6	18	*N.haematococca*	DQ535186	25.6
6	*Athelia rolfsii*	KJ546416	91.9	19	*M.phaseolina*	DQ314733	35
7	*Athelia rolfsii*	JN081867	91.9	20	*A.triticina*	DQ489293	33
8	*Athelia rolfsii*	AF499018	91.8	21	*C.kikuchii*	JF747035	32.6
9	*Athelia rolfsii*	KY216142	91.5	22	*F.solani*	JN235284	24.3
10	*Athelia rolfsii*	HQ420816	91.5	23	*F.oxysporum*	JN020659	27.2
11	*Athelia rolfsii*	JF819727	92.1	24	*C.gloeosporioides*	JX010147	35.8
12	*Athelia rolfsii*	KX186998	91.3	25	*C. acutatum*	AJ301936	29.5
13	*Athelia rolfsii*	KU760984	91.3	26	*P.palmivora*	KP183963	30.8

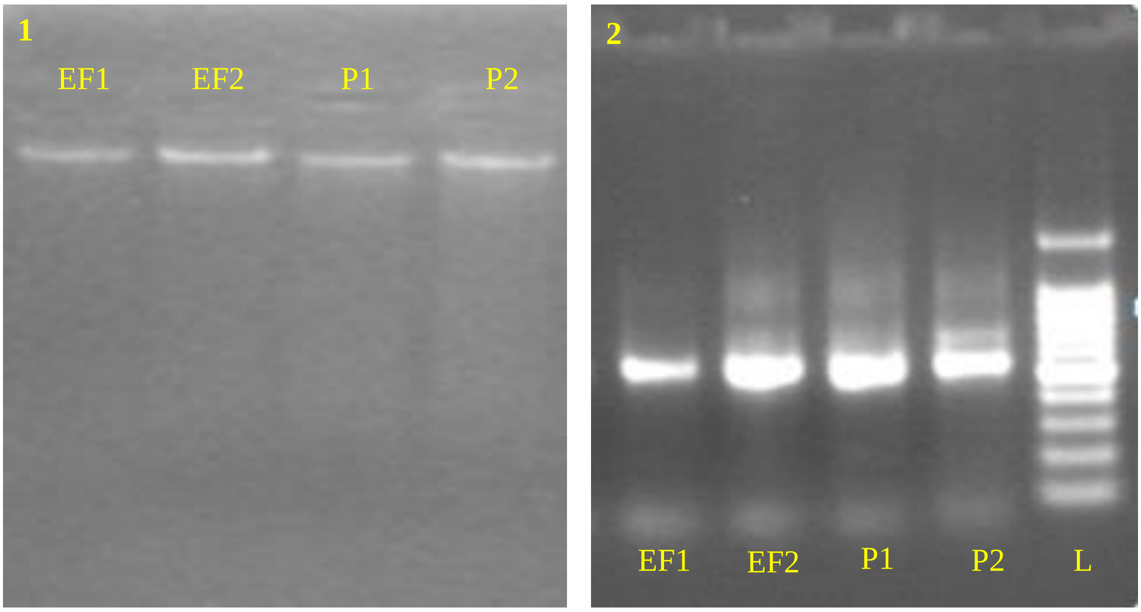

Figure 4.2: 1. Quality analysis of fungal genomic DNA on agarose gel electrophoresis and 2. PCR amplification of Internal transcribed spacer (ITS) region using ITS1 and ITS4 primers: EF1 and EF2= Endophytic fungal OTU HI2; P1 and P2= CC1 isolate of Sclerotium and L= Ladder (1000bp).

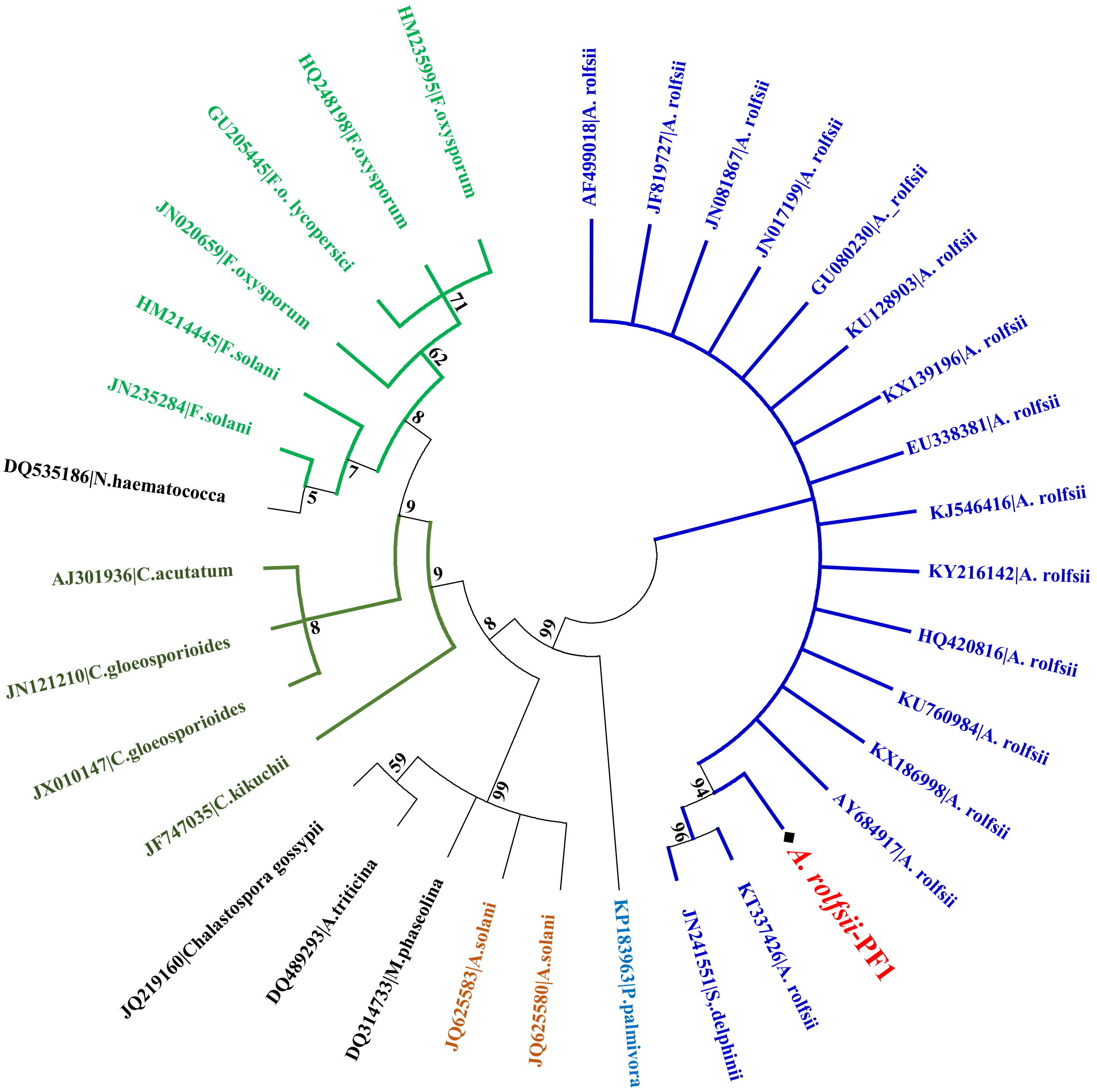

Figure 4.3: Phylogeny of Internal transcribed spacer region of *Sclerotium rolfsii*. The tree was generated using the Neighbor-joining method in MEGA 6.01. Horizontal distances are proportional to sequence distances; vertical distances are arbitrary (PF2=*Sclerotium* isolate of Chintamani).

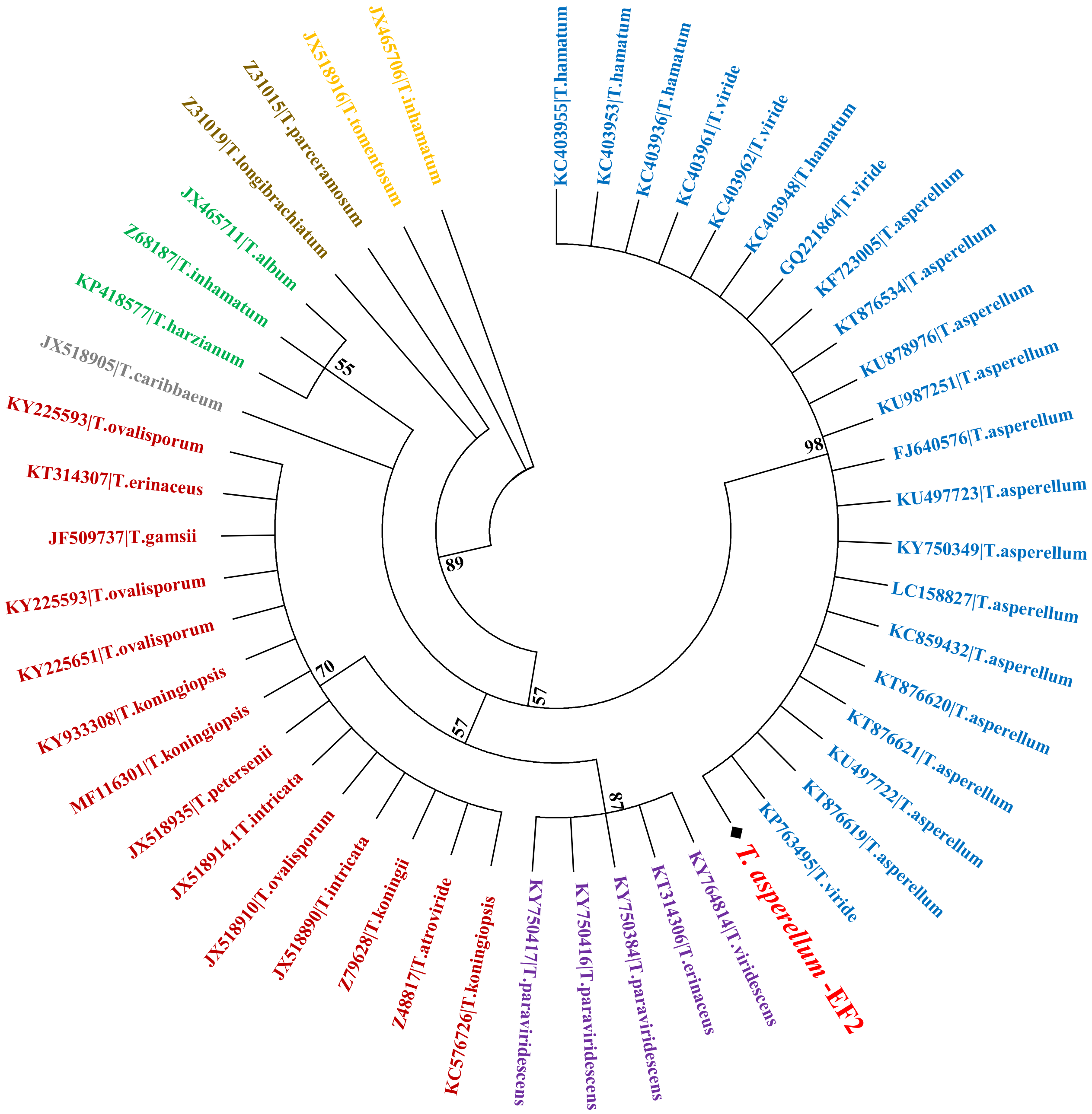

Figure 4.4: Phylogeny of Internal transcribed spacer region of *Trichoderma asperellum*. The tree was generated using the Neighbor-joining method in MEGA 6.01. Horizontal distances are proportional to sequence distances; vertical distances are arbitrary (EF2= Fungal endophytic OTU HI2).

Table 5.5: Sequence of fungal species retrieved from NCBI for phylogenetic and identity matrices studies of *Trichoderma asperellum* (OTU HI2).

Sl. No.	Fungal species	GenBank Accession number	Sl. No.	Fungal species	GenBank Accession number
1	*T.asperellum*	KT876619	25	*T.inhamatum*	Z68187
2	*T.asperellum*	KU497722	26	*T.koningii*	Z79628
3	*T.asperellum*	KT876621	27	*T.longibrachiatum*	Z31019
4	*T.asperellum*	KT876620	28	*T.parceramosum*	Z31015
5	*T.asperellum*	KC859432	29	*T.intricata*	JX518890
6	*T.asperellum*	LC158827	30	*T.album*	JX465711
7	*T.asperellum*	KY750349	31	*T.inhamatum*	JX465706
8	*T.asperellum*	KU497723	32	*T.caribbaeum*	JX518905
9	*T.asperellum*	FJ640576	32	*T.ovalisporum*	JX518910
10	*T.asperellum*	KU987251	33	*T.intricata*	JX518914
11	*T.asperellum*	KU878976	34	*T.tomentosum*	JX518916
12	*T.asperellum*	KT876534	35	*T.petersenii*	JX518935
13	*T.asperellum*	KF723005	36	*T.koningiopsis*	MF116301
14	*T.viride*	GQ221864	37	*T.koningiopsis*	KY933308
15	*T.viride*	KP763495	38	*T.ovalisporum*	KY225651
16	*T.hamatum*	KC403948	39	*T.ovalisporum*	KY225593
17	*T.viride*	KC403962	40	*T.gamsii*	JF509737
18	*T.viride*	KC403961	41	*T.paraviridescens*	KY750417
19	*T.hamatum*	KC403936	42	*T.paraviridescens*	KY750416
20	*T.hamatum*	KC403953	43	*T.paraviridescens*	KY750384
21	*T.hamatum*	KC403955	44	*T.erinaceus*	KT314307
22	*T.koningiopsis*	KC576726	45	*T.viridescens*	KY764814
23	*T.harzianum*	KP418577	46	*T.ovalisporum*	KY225593
24	*T.atroviride*	Z48817	47	*T.erinaceus*	KT314306

Table 5.6: Pairwise percent of nucleotide identities between the internal transcribed spacer (ITS) region of *Trichoderma asperellum* (OTU HI2) with the selected other fungal species sequences available in the GenBank, NCBI (www.ncbi.nlm.nih.gov).

Sl. No.	**Fungal species**	**GenBank Accession number**	**Per cent identity with *Trichoderma asperellum* (OTU HI2)**
1	*T.asperellum*	KT876619	97.5
2	*T.viride*	KP763495	96.1
3	*T.hamatum*	KC403948	85.2
4	*T.harzianum*	KP418577	67.7
5	*T.atroviride*	Z48817	80.3
6	*T.inhamatum*	Z68187	66.6
7	*T.koningii*	Z79628	80.1
8	*T.longibrachiatum*	Z31019	65.4
9	*T.parceramosum*	Z31015	65.5
10	*T.intricata*	JX518890	85.7
11	*T.album*	JX465711	50.7
12	*T.caribbaeum*	JX518905	54.2
13	*T.intricata*	JX518914	71.1
14	*T.tomentosum*	JX518916	60.8
15	*T.petersenii*	JX518935	85.3
16	*T.koningiopsis*	MF116301	83.4
17	*T.ovalisporum*	KY225651	78.7
18	*T.ovalisporum*	KY225593	79.9
19	*T.gamsii*	JF509737	80.0
20	*T.paraviridescens*	KY750417	83.2
21	*T.erinaceus*	KT314307	82.7
22	*T.viridescens*	KY764814	83.6
23	*T.ovalisporum*	KY225593	79.9
24	*T.erinaceus*	KT314306	83.3

4.6 Volatile assay

Double Petri dish assay was conducted to capture volatile organic compounds (VOCs) produced by the endophytic OTU (HI2) *T. asperellum* during its interaction with pathogen *S. rolfsii* isolate CC1 to know their probable role in inhibiting the pathogen. The VOCs generated by the endophyte inhibited the mycelial growth 56.46 % of pathogen significantly over control (Plate 3.8).

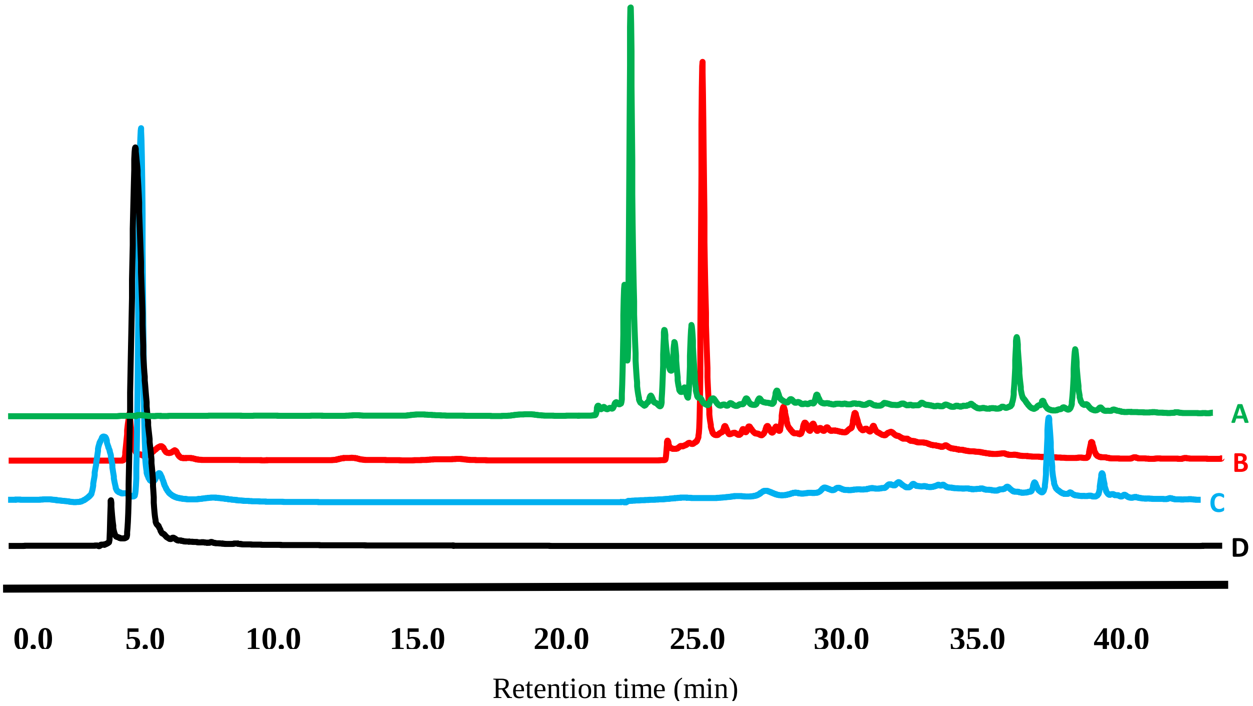

Figure 4.5: HPLC chromatogram of secondary metabolites from broth extracts of *Trichoderma asperellum* and *Sclerotium rolfsii* interaction: A. Endophyte; B. Pathogen; C. Interaction of endophyte and pathogen; D. Broth.

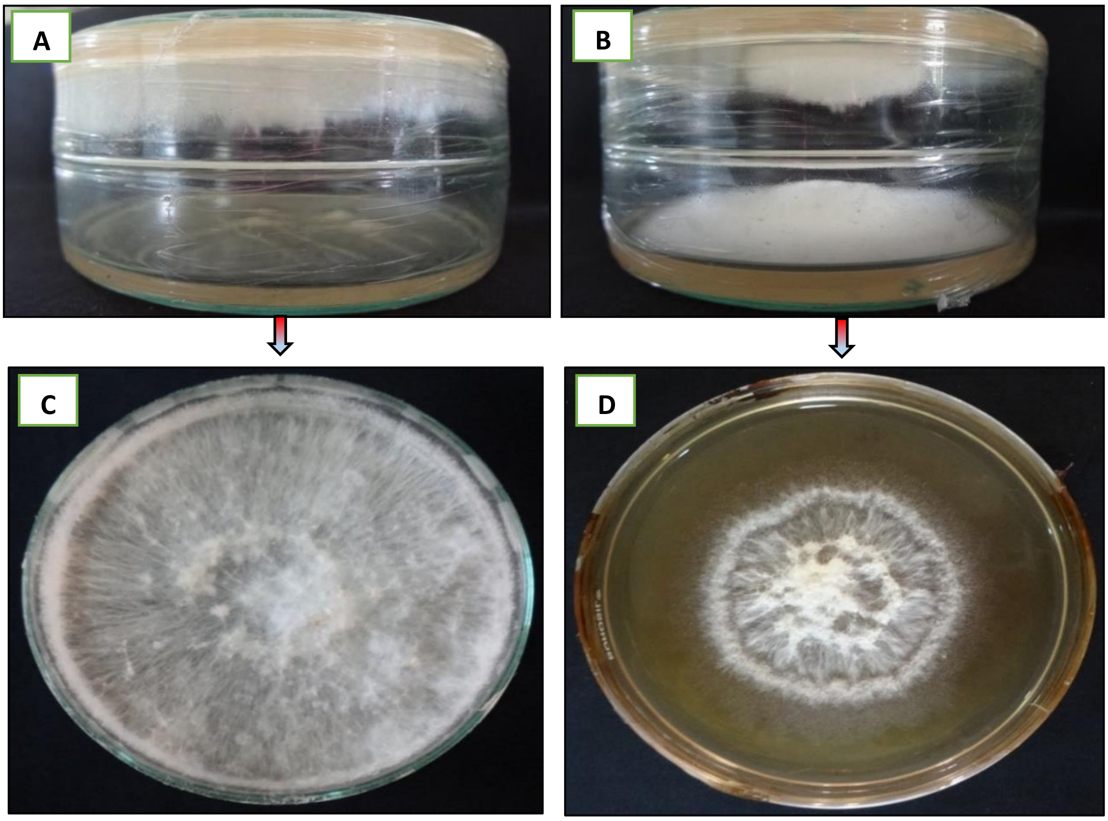

Plate 3.8: Volatile assay for endophytic *Trichoderma asperellum* against *Sclerotium rolfsi*: A. Control (Pathogen in upper lid without endophyte). B. Pathogen in upper lid and endophyte in lower lid. C. Growth of pathogen in control. D. Growth of pathogen in treatment with endophyte in lower lid.

4.6.a Gas chromatography analysis

The headspace profile of endophyte, pathogen and endophyte-pathogen interaction for VOCs was investigated using SPME combined with GC–MS technique. 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 exposition of sample, the obtained chromatograms (Figure 4.6.a - 4.6.d) 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 14 version (NIST). Total thirty-four VOCs were obtained from all the PDA(control), endophyte, pathogen and interaction (endophyte and pathogen) in double Petri dish assay treatments. Including the common VOCs from PDA control in all treatments, the number of VOCs are, thirteen from pathogen, twenty-two from endophyte and twenty from interaction. The obtained VOCs in different samples is presented in table 5.7. The VOCs common in all treatments, PDA and pathogen; PDA and endophyte; PDA and interaction; pathogen and endophyte; pathogen and interaction; endophyte and interaction were presented in figure 4.7.

4.7 Host colonization assay

The segments of leaf, stem and root samples collected from thirty days old pre-germinated seeds of chilli was soaked in endophyte *T. asperellum* –HI2 suspension for three hours. Three plants from each treatment was selected randomly and processed for isolation of *T. asperellum* – isolate HI2. Fifteen segments each from leaf, stem, and roots were kept for isolation of endophyte on PDA medium. Five days after inoculation endophyte was emerged from stem and root segments and not emerged from leaf segments of endophyte treated plants. In the control treatment (samples from plants without endophyte treatment) there was absence of endophyte *T. asperellum* – isolate HI2 (Plate 3.9).

Table 5.7: Tentative characterization of VOCs from *Trichoderma asperellum* and *Sclerotium rolfsii* interaction detected by HS-SPME-GC-MS from a culture grown for five days.

Sl. No.	**Retention time**	**Calculated Retention index**	**Volatile metabolite**	**Retention index (Literature)**	**Treatment**			
					PDA	**Pathogen (Control)**	**Endophyte (Control)**	**Interaction**
1.	1.33	--	Ethanol	427	+	+	+	+
2.	1.73	--	n-hexane	600	+	+	+	+
3.	1.77	619.8	Furan, 2- methyl	614	-	+	-	-
4.	1.86	629.6	Trichloromethane	615	+	+	+	+
5.	2.13	659.1	Butanal, 3- methyl	652	+	+	+	+
6.	2.20	666.6	Butanal, 2- methyl	662	+	+	+	+
7.	2.71	711.4	Propane, 2-bromo-1-chloro	747	-	-	+	-
8.	2.74	712.6	Furan, 2, 5-dimethyl	707	-	+	-	-
9.	3.16	735	1-Butanol, 3-methyl	736	-	+	+	+
10.	3.23	738.4	1-Butanol, 2-methyl	739	-	-	+	+
11.	3.631	759.5	Propanoic acid, 2-methyl-ethyl ester	755	-	-	+	+
12.	3.73	765	Toulene	763	-	+	-	-
13.	5.53	856.5	Butanoic acid, 3-methyl-ethyl ester	854	-	-	+	+
14.	6.26	893.8	Styrene	893	-	+	-	-
15.	8.13	986.8	3-octanone	986	-	-	+	-
16.	8.92	1026	Cyclopentene, 3-isopropenyl -5, 5 –dimethyl	1028	-	-	+	-
17.	8.96	1028.1	1-Hexanol, 2-ethyl	1030	-	-	+	+

18.	11.83	1165	Cyclohexanol, 5 methyl-2 (-1-methyl ethyl)	1169	-	-	+	-
19.	12.23	1183.2	Naphthalene	1182	+	+	+	+
20.	12.55	1197	Dodecane	1200	-	+	-	-
21.	14.36	1276.7	Cyclohexanol, 5-methyl-2-(1-methylethyl)-acetate	1274	-	-	+	-
22.	17.29	1399.9	Tetradecane	1400	+	+	-	-
23.	18.79	1464.7	Patchoulene	1467	-	-	+	+
24.	19.23	1484.3	γ-Selinene	1481	-	-	+	+
25.	19.31	1487.4	Benzene, 1-(1, 5-dimethyl-hexemyl)-4-methyl	1483	-	-	+	+
26.	19.52	1496.8	1-methyl - 4 -(6 methylhept-5-en-2yl) cyclohexa-1, 3-diene	1480	-	-	+	-
27.	19.68	1503.7	Epizonarene	1501	-	-	-	+
28.	20.25	1528.5	Cyclohexane, 3-(1, 5-dimethyl-4-hexenyl)-6-methylene	1524	-	-	+	+
29.	21.94	1601	Hexadecane	1600	+	-	-	-
30.	22.06	1607.68	Guaiol	1596	-	-	-	+
31.	22.57	1630.63	α-Eudesmol	1631	-	-	-	+
32.	24.13	1701	Heptadecane	1700	+	-	-	-
33.	28.51	1991.4	8,15-Pimaradiene	1994	-	-	+	+
34.	28.84	2024.3	Verticilla-4(20),7,11-triene	2027	-	-	-	+

(+ indicates presence of compound; – indicates absence of compound)

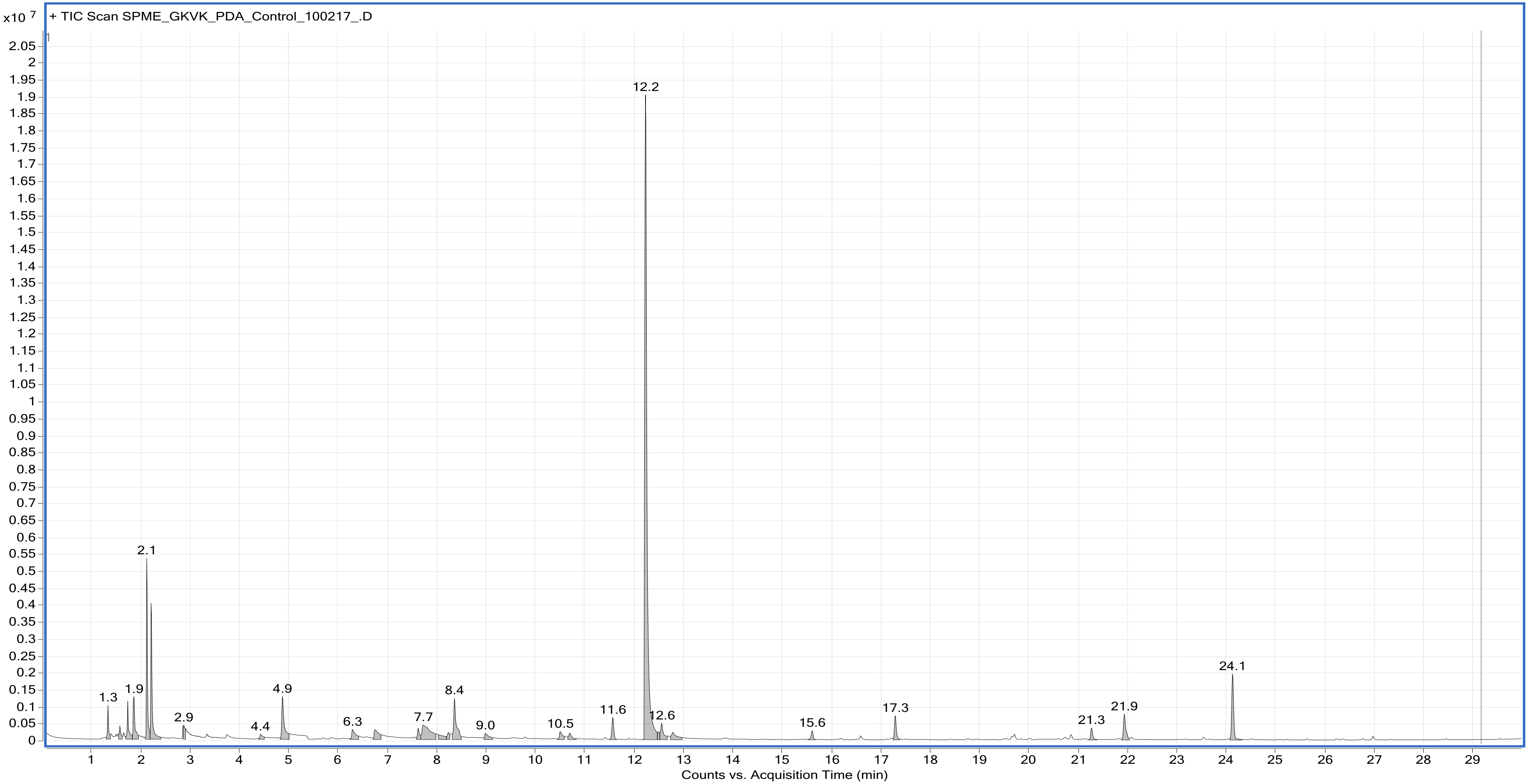

Figure 4.6.a: HS-SPME-GC-MS total chromatogram of VOCs from potato dextrose agar media (Control).

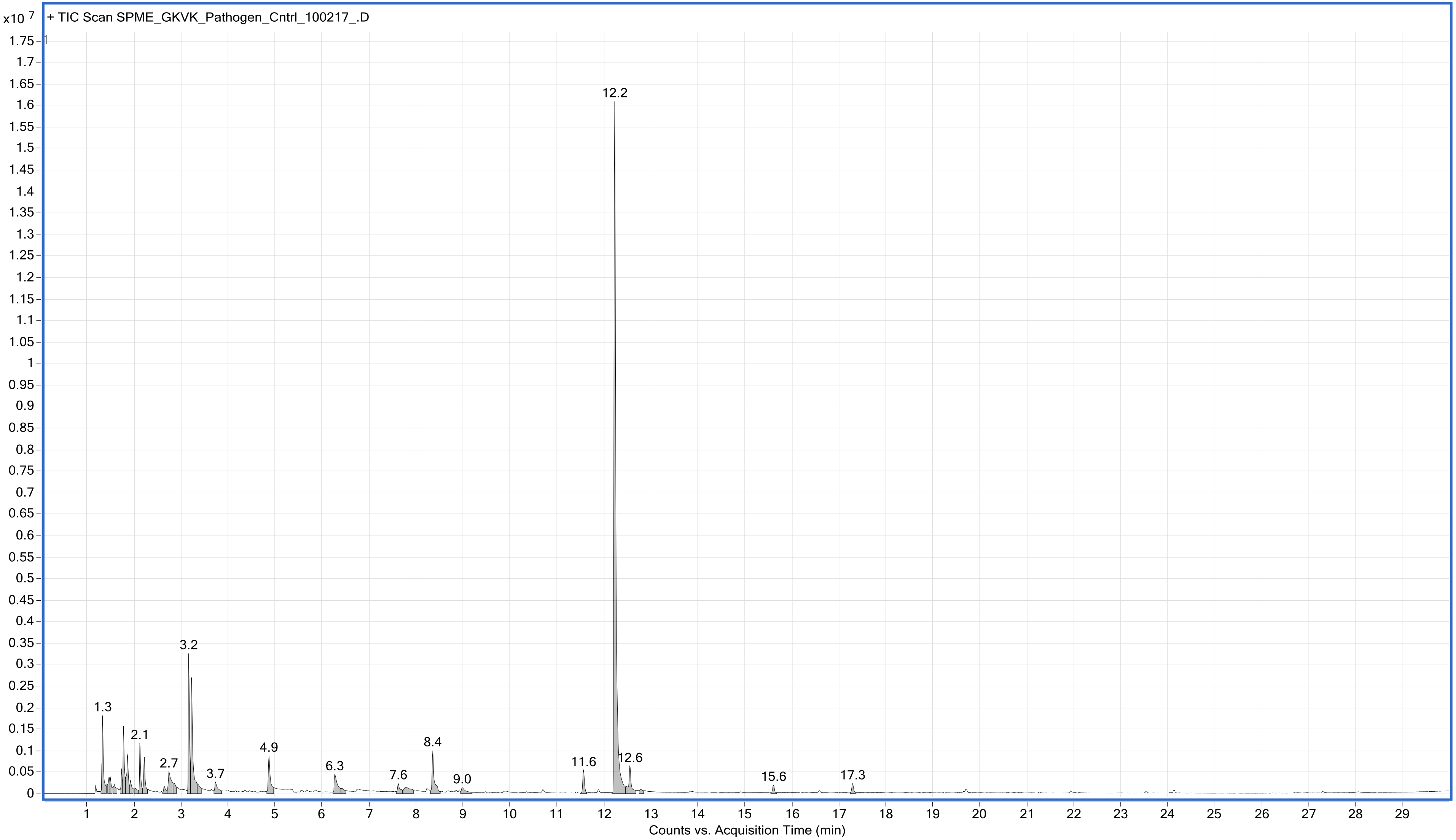

Figure 4.6.b: HS-SPME-GC-MS total chromatogram of VOCs from *Sclerotium rolfsi*.

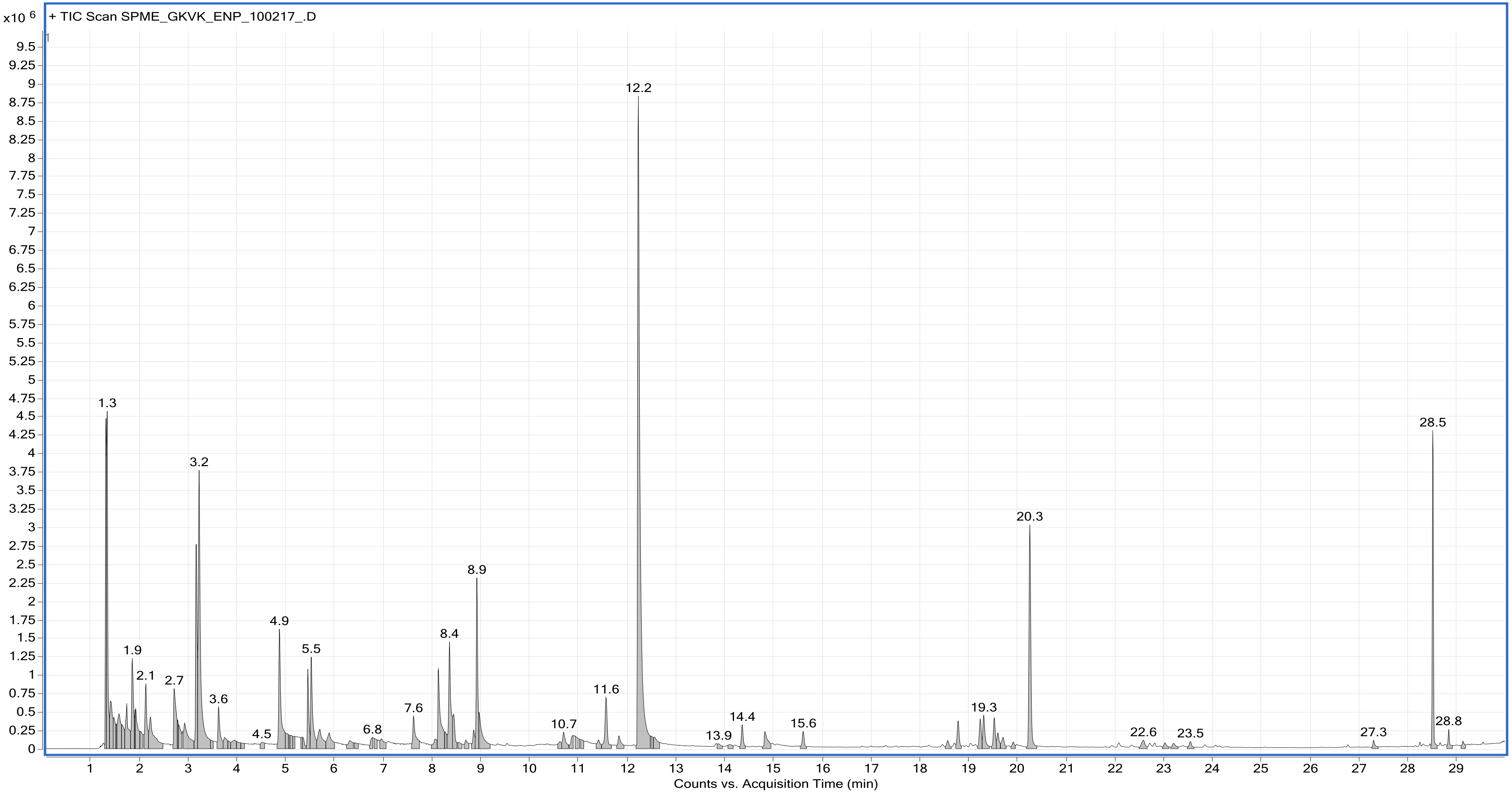

Figure 4.6.c: HS-SPME-GC-MS total chromatogram of VOCs from *Trichoderma asperellum*.

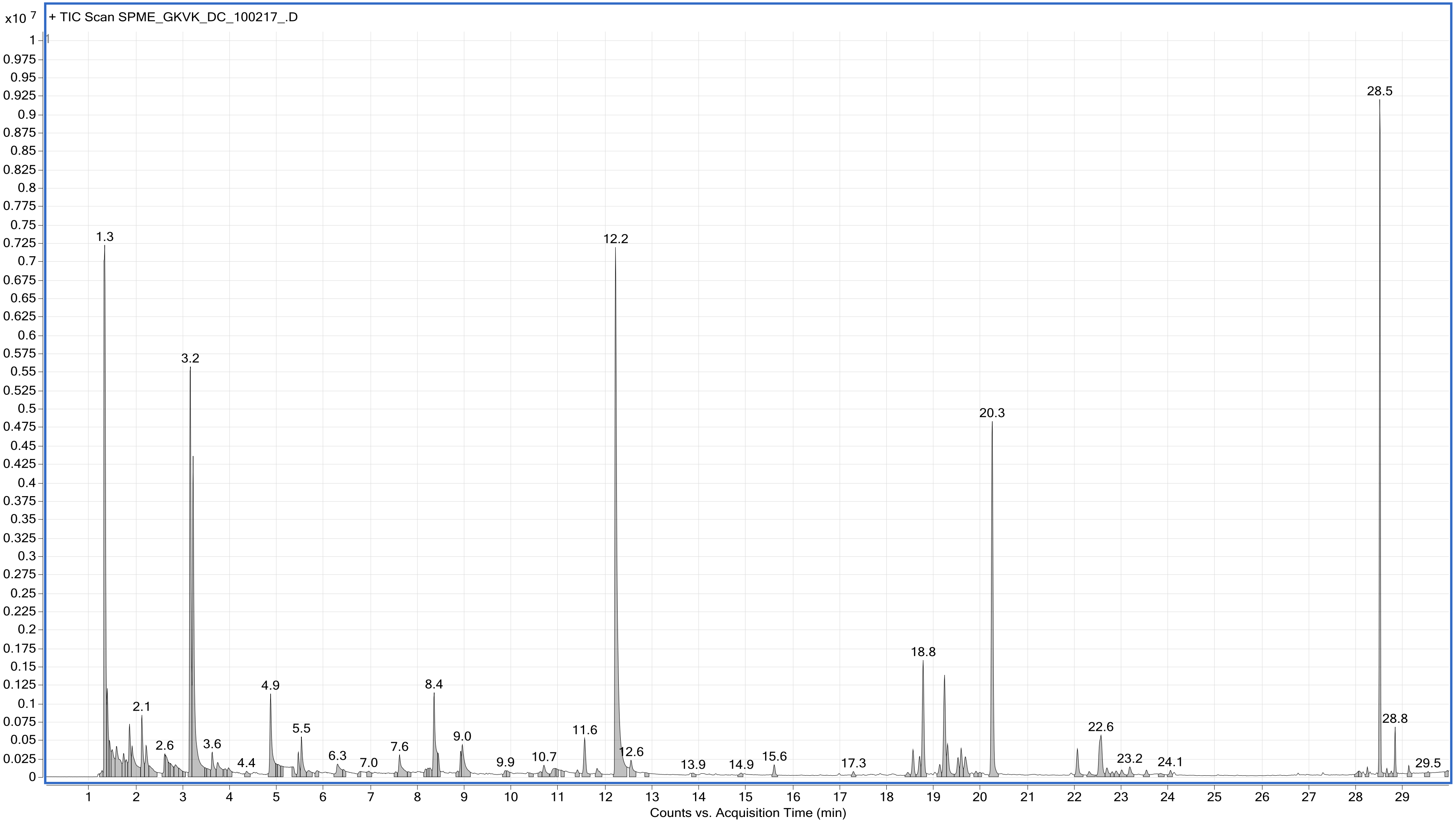

Figure 4.6.d: HS-SPME-GC-MS total chromatogram of VOCs from *Trichoderma asperellum* and *Sclerotium rolfsi*.

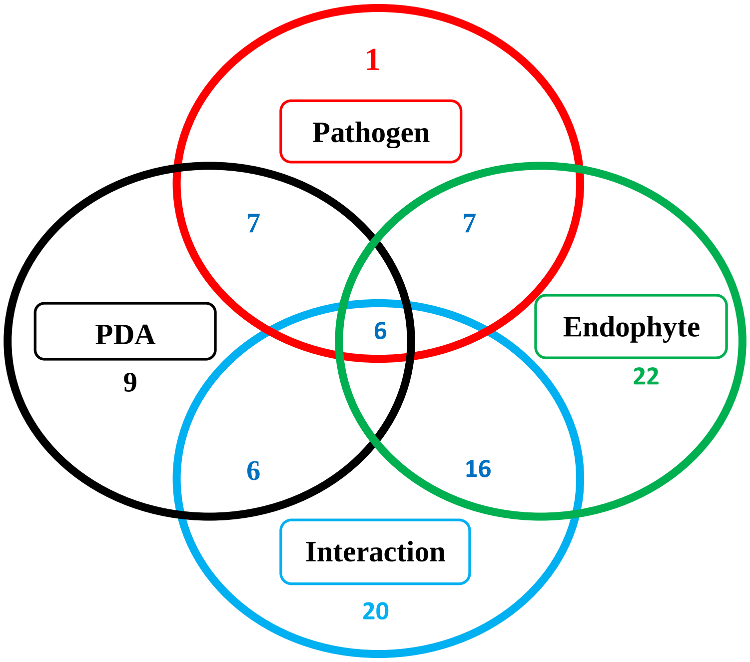

Figure 4.7: Venn diagram showing total number and common VOCs produced by endophytic *Trichoderma asperellum, Sclerotium rolfsii* and interaction between them.

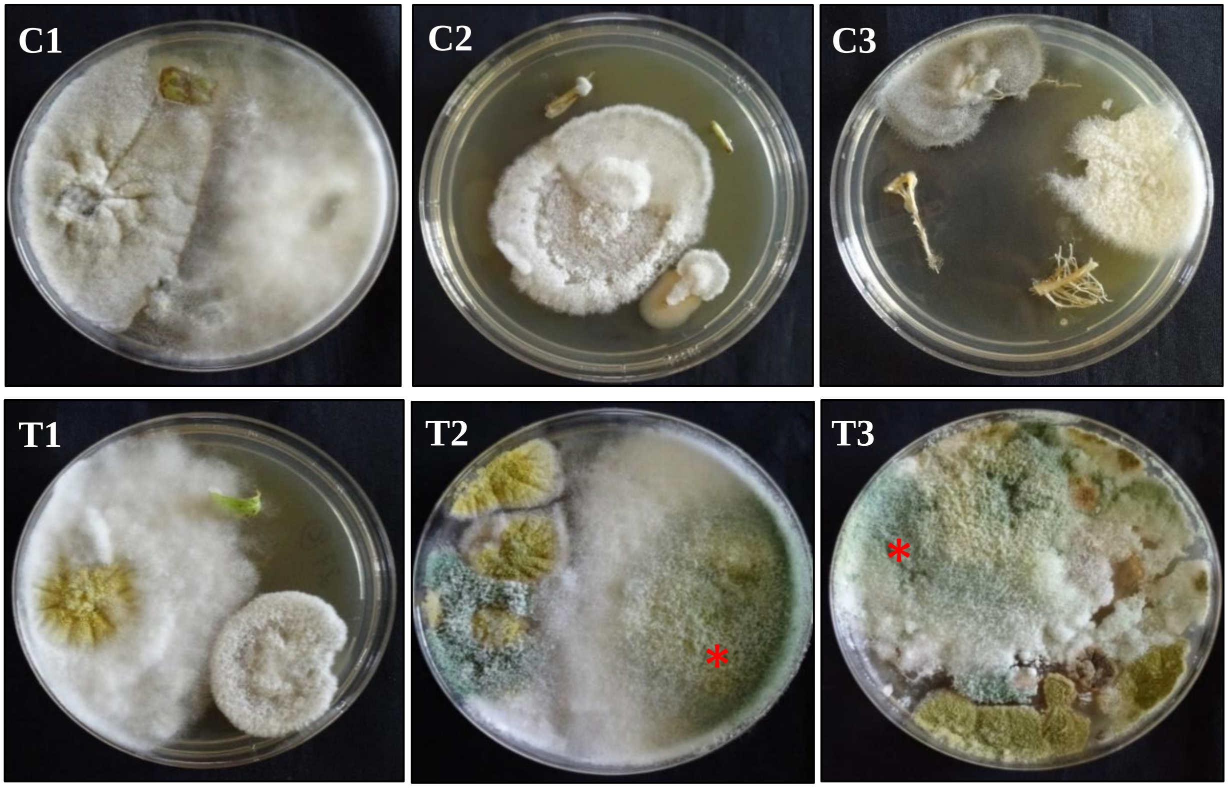

Plate 3.9: Colonization assay for endophytic *T. asperellum* in chilli plants: C1, C2 and C3 = Leaf, Stem and Root segments in control; T1, T2 and T3 = Endophyte treated Leaf, Stem and Root segments.

* indicates treated fungal endophyte.

V DISCUSSION

Plant diseases are the major constraint for the food production across the world. Most often chemicals are used for management of plant diseases which are having the harmful effect on human and animals apart from causing the environmental pollution (Margni *et al*., 2001). The use of microorganisms for biological control of plant pathogens can move significantly beyond the current successes. In this scenario, exploitation of microorganisms is hope for chemical residual free disease management strategies in the food production (Luis*et al*., 2008).

In natural ecosystem all plants are symbiotic with fungi that either reside entirely (endophytes) or partially (mycorrhizae) within plants. Fungal endophytes are reported to be providing both biotic and abiotic stress tolerance and may be responsible for the survival of both host and fungal symbionts in high stress habitats (Jumpponen, 2001; Pablo *et al.* 2015). Throughout their evolution, majority of the plants are depending on endophytic microorganisms for various activities such as secondary metabolites production and enduring against both abiotic and biotic stresses (Rusty *et al.*, 2007).

The findings of this study indicate that certain endophytic fungi have the ability to inhibit the growth of *Sclerotium* pathogen through different mechanisms in *In-vitro*, confirming the promise these fungi can be used for the biological control of *Sclerotium rolfsii.*

Isolation of plant pathogen and pathogenicity assay

Sclerotium pathogen infecting chilli crop was isolated and pathogenicity test was carried out in pot culture under glasshouse conditions by inoculating chilli plants with culture of *S. rolfsii* isolate CC1. Leaves of infected plants became initially pale green followed by yellowing. Later, plants showed drooping of leaves and white mycelial growth around the collar and root region. The collar and root region was sunken and rotted resulting in dry root rot symptom and drying of the infected plants. The symptoms of root rot observed in the current study were typical to the symptoms produced by *S. rolfsii* isolates reported earlier (Kalmesh and Gurjar, 2001). The wide host range and report of dry root rot disease in chilli caused by *S. rolfsii* was first reported by Kalmesh and Gurjar (2001).

Amplification and sequence analysis of Internal Transcribed Spacer (ITS) region is used for bar-coding and identification of different fungi using ITS region specific primers (Mahadevakumar *et al*., 2015). The identity matrices obtained from amplified ITS region of *Sclerotium* isolate CC1 in the current study and its subsequent phylogenetic analysis clearly indicated it is an isolate of *Sclerotium rolfsii* Sacc. (teleomorph: *Athelia rolfsii* Tu & Kimbrough).

Isolation and *In-vitro* evaluation of fungal endophytes against *Sclerotium* sp.

Fungal endophytes were isolated from the selected host plants to know their possible role in imparting disease resistance against the fungal diseases. In order to increase the chances of getting fungal endophytes having antagonist properties against the plant pathogens the host plants viz., *Phyllanthus acidus, Hibiscus rosa sinensis, Catharanthus roseus, Pyllanthus amarus* and *Solanum torvum* having antimicrobial compounds and volatiles in plant extracts (Jagajothi *et al.*, 2013; Kobaisy *et al*., 2001; Svoboda *et al*., 1962; Mazumder *et al*., 2006; Bari *et al*., 2010) were selected. The fungal endophytes obtained from the leaf and stem were almost same with few exceptions. Contrary to this, endophytes from root were different from leaf and stem in majority of cases with few exceptions indicates the diverse of endophytes are associated with different parts of plants, which is well documented in several plant species (Carroll, 1988). Based on their morphological similarities the fungal endophytes were grouped into operational taxonomic units (OTU's). The screening of fungal OTU's resulted in yielding fungal endophytes having antifungal activity against *S. rolfsii* –CC1 isolate in the dual culture. However, the per cent inhibition of *S. rolfsii* –CC1 isolate by these endophytes varied fromone to sixty-five per cent. Which is similar to previous reports of fungal endophytes inhibiting soil borne plant pathogens *Verticillium dahliae, V. longisporum* and *Rhizoctonia solani* under *In-vitro* studies (Narisawa *et al*., 2001; Lahlali and Hijri, 2003; Narisawa *et al.*, 2003). The varied per cent inhibition may be due to difference in the mechanism of suppression such as competition for space and nutrients, antibiosis, and hyper parasitism (Gao *et al*., 2010).

The sequence analysis of amplified ITS region of fungal endophytic OTU HI2 isolated from *Hibiscus rosa sinensis* showing maximum inhibition (65 %) growth of *S. rolfsii* –CC1 isolate with selected sequences of fungi retrieved from NCBI, GenBank revealed that OTU HI2 is an isolate of *T. asperellum*. The existence of *T. asperellum* in endophytic lifestyle having antagonistic effect on fungal pathogens is well documented earlier in few plants (Bailey *et al*., 2008; Lilian *et al.*, 2004). However, this is the first report of endophytic *T. asperellum* from *H. rosa sinensis*.

Mechanisms of inhibition of pathogen by fungal endophyte

The mechanism of action of fungal endophytes in inhibition of plant pathogens in *In-vitro* conditions are competition for space and nutrients, hyperparasitism, diffusible antifungal compounds and antimicrobial VOC's.

The antagonistic assay for *T. asperellum*-HI2 and *S. rolfsii* - CC1 isolate showed that there is competition for space in *In-vitro* studies. Endophyte occupied more space, thereby reducing the area availability for pathogen and inhibiting up to sixty-five percent. Similar findings were also reported by Talapatra *et al.* (2017) in interaction of pathogens *Alternaia solani* and *Pythium aphanidermatum* with *Trichoderma viridae.*

The HPLC analysis for the antimicrobial diffusible compounds produced by *T. asperellum*-HI2 and *S. rolfsii* - CC1 isolate interaction in PDB through ethyl acetate extraction, revealed prominent eluted peaks of diffusible compounds predicting as accountable for antifungal activity (Bailey *et al*., 2008). Further characterization and screening these compounds against other plant pathogens may lead to development of novel chemicals for managing fungal diseases in crop plants.

Based on the reports of antimicrobial VOCs produced by *Trichoderma* spp. (Amin *et al*., 2010, Stoppacher *et al.*,2010), VOCs analysis carried out for the samples obtained with *T. asperellum*-HI2 and *S. rolfsii* - CC1 isolate interaction through double

Petri dish assay. GC-MS analysis revealed the presence of VOCs inhibiting the pathogen growth.

Host colonization assay

Endophytes have been shown to infect, colonize and provide tolerance against diseases in not only the host plants from which they are isolated but also to genetically divergent crop plants (Rusty *et al*., 2008). Based on these reports endophytic *T. asperellum* HI2 isolate was treated to pre-emerged chilli seeds, grown in pot culture and re-isolated after thirty days. It is revealed that colonization of endophyte, *T. asperellum* HI2 isolate in stem and roots of chilli plants, further confirming the colonization of fungal endophytes in genetically divergent plants and possibility of exploitation of endophytes from diversified plant species for the management of crop diseases.

The present study has revealed that, exploration of fungal endophytes can serve as potential source for the isolation of bioagents against fungal plant pathogens. Much more work is essential to understand endophytes physiology, biochemical pathways, defensive role and secondary metabolites production for enduring the plants against biotic stress. Further, Characterization of compounds (soluble and volatiles) involved in antibiosis would result in the discovery of lead molecules for the development of fungicides for effective management of plant diseases.

VI SUMMARY

Chilli (*Capsicum annuum* L.) belongs to the family solanaceae is one of the major vegetable and spice crop grown across the world. Diseases caused by various pathogens are becoming the major constraint for their production in the country. In the recent years, dry root rot/ foot rot disease caused by *Sclerotium rolfsi* become the major constraint for the production of chilli. The best approach to manage of this pathogen is through use of bioagents. The hidden world organisms called ‘fungal endophytes’ living inside the host tissue with majorly of beneficial relationship have attained greater importance in recent years by imparting resistance against both biotic and abiotic stress. Fungal endophytes have role in controlling plant pathogens through the mechanisms *viz.,* antibiosis, competition for space and nutrients, hyper parasitism, and also by inducing induced systemic resistance in host plants.

In the present study assessment of the fungal endophytes for *In-vitro* evaluation of *Sclerotium rolfsii* infecting chilli has been carried out.

Total sixty-six fungal endophytes were isolated from the *Hibiscus rosa sinensis, Phyllanthus acidus, Catharanthus roseus, Phyllanthus amarus* and *Solanum torvum,* which are known have antimicrobial compounds. Based on colour of mycelia, spores and colony characters endophytic isolates were categorised into twenty-five OTU’s.

The dry root rot disease infected chilli sample was collected from farmer’s field and the pathogen was isolated under aseptic condition using standard tissue isolation protocol and identified as *Sclerotium,* based on morphological characters and designated as *Sclerotium* isolate CC1.

Twenty-five OTU’s obtained from the five different plant species were screened against CC1 isolate of *Sclerotium* by dual culture technique. Out of twenty-five OTU’s, five OTU’s showed greater than 50 per cent of inhibition, six OTU’s showed 25-50 per cent and fourteen OTU’s showed 25 per cent inhibition of pathogen. Among these, endophytic OTU HI2 from *H. rosa sinensis* showed maximum inhibition of sixty-five per cent with complete degradation of pathogen and was selected for all further studies.

The genomic DNA isolated from the endophytic fungal isolate HI2 and Sclerotium isolate CC1 was subjected to PCR amplification of ITS region using ITS-1 forward and ITS-4 reverse primers. Approximately 550bp in size fragments was amplified in both pathogen and endophyte, the products were eluted and sequenced. The obtained sequences identity matrices revealed that endophytic OUT HI2 is isolate from *H. rosa sinensis* shared highest homology of 99 per cent with *Trichoderma asperellum* and the pathogen isolate CC1 showing maximum homology of 100 per cent with the *Athelia rolfsii*. The phylogenetic analysis indicated that the endophyte OTU HI2 in the curent study is isolate of *T. asperellum.* Similarly, The Sclerotium isolate CC1 is an isolate of *S. rolfsii* (teleomorph: *Athelia rolfsii* Tu & Kimbrough).

To decipher the mechanism of action of *T. asperellum* on *S. rolfsii* analysis of diffusible antimicrobial compounds responsible for inhibition of pathogen was done by HPLC analysis of broth cultures. The metabolite profile study showed disappearance of few pathogen and endophyte metabolite in interaction and two peaks were eluted at 37.5 and 39.4 min retention time. To study the ability of fungal endophyte to produce volatile organic compounds, double Petri dish assay was followed and analysis of antimicrobial VOCs produced by *T. asperellum*-HI2 isolate was by GC-MS analysis. The VOCs metabolite profile showed varied peaks and fourteen tentative VOCs specific to *T. asperellum* HI2 isolate were detected. This revealed the competition and antibiosis modes of actions of *T. asperellum* isolate HI2 in inhibiting *S. rolfsii* CC1 isolate.

The endopyhtic *T. asperellum* isolate HI2 from *H. rosa sinensis* colonized in chilli stem and roots revealing the colonization potential of fungal endophytes isolated from diverse ecosystems to different crop species and their subsequent exploitation for the management of plant diseases.

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