Assessment of volatile organic compounds from clove and endophytic fungi for their antifungal and antioxidant properties

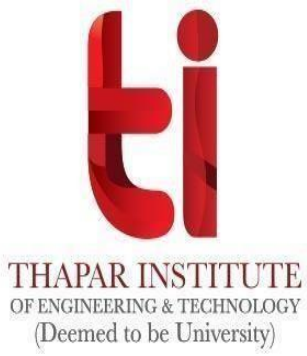
A Thesis

Submitted in partial fulfilment of the requirements for the award of the degree of

Master of Science

in

Biotechnology



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July 2023

Certificate

This is to certify that the thesis entitled **Assessment of volatile organic compounds from clove and endophytic fungi for their antifungal and antioxidant properties** being submitted by Amandeep Kaur (Reg. No. 302101002), in partial fulfilment of the requirements for the award of the degree of **Master of Science** in **Biotechnology**, **Thapar Institute of Engineering and Technology, Patiala, Punjab** is a bonafide work carried out under the guidance and conception of **Dr Sanjai Saxena** and that no part of this thesis has been submitted for the award of any other degree.

Date: 18-07-2023

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Candidate's Declaration

I hereby certify that the work, which is being presented in the thesis, entitled **Assessment of volatile organic compounds from clove and endophytic fungi for their antifungal and antioxidant properties**, in partial fulfillment of the requirements for the award of the degree of **Master of Science** and submitted to the institution is an authentic record of my own work carried out during the period **January 2023** to **June 2023** under the supervision **of Dr Sanjai Saxena, Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab India**. I have also cited the references of the text(s)/figure(s)/table(s) from where they have been taken.

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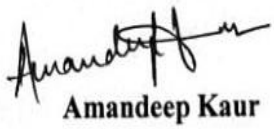

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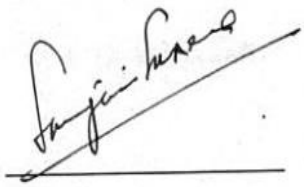

Dr Sanjai Saxena

Supervisor

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I regard this opportunity as a significant turning point in my professional progress. I will work diligently to use the knowledge and abilities I have acquired to achieve my career objective.

Amandeep Kaur

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol / Abbreviation	Meaning
$	Dollar
%	Percentage
≥	Greater Than Or Equal To
°C	Degree Celcius
µl/L	Microliter/ Litre
µl/Ml	Microliter/ Mililitre
µl/Plate	Microliter/Plate
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AFM	Atomic Force Microscopy
ATP	Adenosine Triphosphate
BCB	B-Carotene Bleaching
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
Ca	Calcium
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
FAO	Food And Agriculture Organization
FDA	Food And Drug Administration
FRAP	Ferric Reducing Antioxidant Power
GC-MS	Gas Chromatography–Mass Spectrometry
GRAS	Generally Recognized As Safe
H_2O_2	Hydrogen Peroxide
HGT	Horizontal Gene Transfer
IC_{50}	Half Maximal Inhibitory Concentration
ISR	Induced Systemic Resistance
MDA	Malondialdehyde
Mg	Milligram
Mg/L	Milligram/Liter
MIC	Minimum Inhibitory Concentration
P	Phosphorous
Ph	Power Of Hydrogen
Ppm	Parts Per Molar
RNA	Ribonucleic Acid
SAR	Systemic Acquired Resistance
SEM	Scanning Electron Microscopy
Sp.	Specie
TBHQ	Tert-Butylhydroquinone
TEM	Transmission Electron Microscopy
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
Vocs	Volatile Organic Compounds
Zn	Zinc
A	Alpha
B	Beta
1D NMR	1 Dimensional Nuclear Magnetic Resonance

Alcl3	Aluminium Chloride
Ccl4	Carbon Tetrachloride
CMA	Corn Meal Agar
ELISA	Enzyme-Linked Immunosorbent Assay
FC	Folin–Ciocâlteu
ITCC	Indian Type Culture Collection
LAF	Laminar Air Flow
MHA	Mueller-Hinton Agar
Na2CO3	Sodium Carbonate
Naoh	Sodium Hydroxide
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RBA	Rose Bengal Agar
SD	Standard Deviation
WA	Water Agar

Executive Summary

There are a variety of ecosystems where fungi may be found; they have been evolving for a billion years and are omnipresent. Out of these, several species are pathogenic and can cause significant post-harvest loss. Due to frequent use of currently existing chemical interventions for the management of these losses, the phytopathogenic fungi have developed resistance against them. Additionally, these synthetic fungi have negative effect on human health and environment as well. Hence, finding a safer and environment-friendly alternative is crucial for the control of phytopathogenic fungi. Despite the medicinal properties of *Syzygium aromaticum*, the existence of endophytic fungi within the clove plant that exhibit antifungal and antioxidant properties against phytopathogenic fungi has not been documented. Therefore, the current study examined the antifungal and antioxidant characteristics of volatile organic compounds emitted by clove and endophytic fungi isolated from the medicinal plant *S. aromaticum*. A total of 23 isolates were isolated from the stem and leaf of the clove plant, and their *in-vitro* antifungal activity was investigated against major phytopathogens of fruits and vegetables, including *Alternaria alternata*, *Aspergillus niger*, *Colletotrichum gloeosporioides*, *Fusarium lateritium*, *Botrytis cinerea*, *Fusarium moniliforme*, *Botryodiplodia theobromae*. Two endophytic fungi, #1SALB2, and #8SALB1were identified to fall under *Alternaria* sp. and *Nigrospora* sp., respectively, exhibited the highest antifungal activity in dual culture assay. After secondary screening using sandwich plate method, #1SALB2 was found to be the potent most culture; this endophytic fungus can inhibit the growth of selected phytopathogenic fungi by 52.21%-69.23%.The antioxidant activity of clove extract in 80% acetone and ethyl acetate extract of endophytic fungi was determined using DPPH and FRAP; total phenolic and flavonoid content was also evaluated. The isolate #2SASB8 identified to be a member of *Colletotrichum* sp. showed the highest antioxidant activity and phenolic content. The potent most endophytic fungi were identified using classical morphotaxonomy. This is the first study to document the antifungal and antioxidant activity of endophytic fungi isolated from *S. aromaticum*.

Keywords: *Syzygium aromaticum*, endophytic fungi, volatile organic compounds, antifungal activity, antioxidant activity

Chapter 1

Introduction

Fruits and vegetables are rich in vitamins, dietary fibre, and minerals. They provide high nutritional value and help keep our body healthy as they have antioxidants and anti-cancerous properties. They also reduce the risk of diabetes, stroke, obesity and cardiovascular disease (Mostafidi et al.,2020). India produces about 107 million metric tonnes of fruit and 204 million metric tonnes of vegetables annually (FAO, India) (Fig. 1). Studies have estimated that 30-40% of total fruits and vegetables are lost after harvest. In developing and underdeveloped countries, the loss is even more, i.e., 45-50% of food is lost during post-harvest processing (Prodhan et al., 2022; Kumar et al., 2017). Post-harvest loss is a crop's quantitative and qualitative loss, which includes interdependent events from the farm-to-fork stage (Prodhan et al., 2022). Factors that cause post-harvest losses are climatic (temperature and humidity), mechanical (poor handling during storage and transportation), and microbial action (bacterial and fungal decay) (Elik et al., 2019). Among all factors, the fungal pathogens are a prominent driver of post-harvest decay and loss. Species of fungi like *Alternaria, Aspergillus, Fusarium, Colletotricum, Botrytis, and Botryodiplodia* are the major phytopathogens that cause fungal decay in fruits and vegetables (Jiao et al., 2022) (Fig. 2).

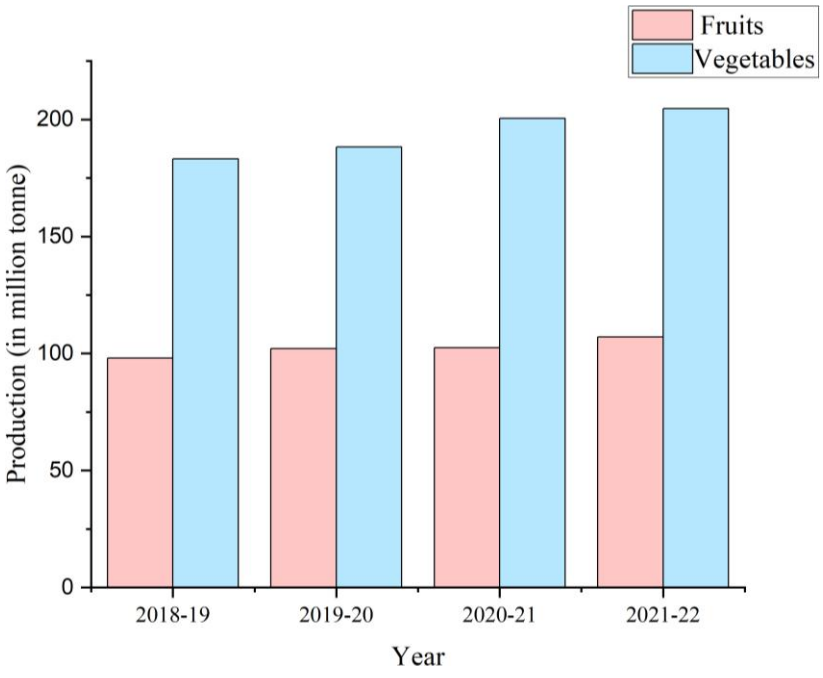


Fig. 1: Production of fruits and vegetables from 2018-2022

Synthetic antifungal agents such as sodium diacetate, calcium propionate, and chlorine dioxide are used as first line of defence to prevent these losses (Qin et al., 2023). Chemical fungicides are detrimental to the environment, humans, animals, and plants because they are non-biodegradable; furthermore, prolonged use makes them ineffective (Hassan et al., 2020). As a result, the trend is shifting towards the use of eco-friendly biological control methods. Various studies have been carried out to find antifungal properties of plant volatile organic compounds (VOCs) due to their natural origin, sustainability and biodegradability (Qin et al., 2023). Plant VOCs are hydrophobic compounds with low molecular weight and high volatility. They can easily pass through the cell membrane and cause cell wall disruption, inhibition of RNA/DNA inhibition and protein synthesis (Qin et al., 2023; Soliman et al., 2019). VOCs from various plants like clove, cumin, cinnamon, caraway and basil have been studied in recent studies for their antifungal activity. Clove has the highest antifungal activity of all tested plants (Hassan et al., 2020).

Syzygium aromaticum (Clove) is a dried flower bud that is the most valuable tropical spice and has been used in the food preservation and pharmaceutical industry for a long time (Hussain et al., 2017). *S. aromaticum* belongs to *the Myrtaceae* family. Recent studies have revealed that clove contains a good amount (14-20%) of VOCs, including Eugenol, eugenyl acetate, and β-caryophyllene (El-Saber et al., 2020; Alawiyah et al., 2019). Clove has antifungal, antioxidant, antimicrobial, and anti-inflammatory properties (El-Saber et al., 2020). Studies have reported that the pharmaceutical properties of clove are due to the presence of eugenol, a phenolic compound. The extract of *S. aromaticum* typically comprises 70-95% eugenol, and the quality of clove is contingent upon the quantity of eugenol that is present (Alawiyah et al., 2019;Radünz et al., 2019). Recent studies have revealed that clove can be used as an antifungal agent to inhibit the growth of species of *Aspergillus, Fusarium, Penicillium* and *Botrytis cinerea* (Luesuwan et al., 2021; Jahani et al., 2020). Various studies have been carried out to study the mechanism by which these VOCs show antifungal and antioxidant effects. Due to their small size and hydrophobic nature, these molecules can easily penetrate through the cell wall of fungi and disrupt the cell wall, inhibiting protein and DNA synthesis (Hou et al., 2022). VOCs also led to the inhibition of ergosterol synthesis, inhibiting fungal growth.

Ergosterol is sterol in the fungal cell wall and maintains its integrity and function (Castellanos et al., 2020).

During storage, fruits and vegetables undergo oxidation and generate reactive oxygen species (ROS) like oxygen free radicals, hydroxyl ions and superoxide ions during various metabolic pathways. These free radicals lead to DNA damage, cell damage and ultimately, depletion of nutrients. Various synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butyl hydroquinone (TBHQ) are used to stop oxidation (Ghadermazi et al., 2017). However, these antioxidants are harmful as they are carcinogenic; therefore, various studies have been done to find natural antioxidants that can inhibit the oxidation of food materials (Hou et al., 2022). According to a recent study conducted by Johari et al. (2022) clove essential oil's free radical scavenging activity was higher than the entire test compounds (basil, lemongrass, black pepper and cinnamon). The antioxidant activity of clove is higher at the flowering stage as it has a higher level of eugenol.

Apart from natural sources of fungicides and antioxidants like clove plants, there has been a growing interest in exploration of the untapped potential of endophytic fungi. Endophytic fungi reside inside the plant tissue to complete either a part of their life cycle or the whole life cycle. These fungi do not cause any harm to plants and help the plant to overcome abiotic and biotic stress by forming a mutualistic relationship (Wen et al., 2022). In general, endophytic organisms obtain nutrients and shelter from the host plant, while the host plant is likely to have advantages such as improved competitive capabilities and increased resilience to a range of environmental and biological stresses (Pavithra et al. 2020). The endophytic fungi follow two main ways to protect the host from pathogen: Direct inhibition, where the endophytic fungi itself release various bioactive compounds like terpenes, alkaloids, and flavonoids to kill the pathogen; in indirect inhibition, the endophytic fungi help in the elicitation of plant defence mechanism and leads to the production of various secondary metabolites and kills the pathogen (Segaran and Sathiavelu, 2019).

Based on several factors, including taxonomy, functional diversity, biology, transmission mechanism, evolution, and ecological functions, endophytes are divided into different categories, such as clavicipitaceous and non-clavicipitaceous, systemic and non-systemic (Wani and Hakeem, 2023).

Non-clavicipitaceous endophytes infect only the higher species of plants. They are further divided into three distinct functional categories as class 2, class 3, and class 4, whereas clavicipitaceous endophytes only infect grasses and are referred to as class 1 (Wani et al., 2015). Systemic endophytes reside inside the host plant for the entire life cycle and do not cause any harm to the plant or any part of its life cycle. With changing environmental conditions, systemic endophyte concentrations and diversity do not vary. Non-systemic endophytes reside inside the host and become parasites under unfavourable conditions. Under changing environmental conditions, non-systemic endophytes change in number and variety within their plant hosts (Orozco-Mosqueda and Santoyo, 2021). Various studies have documented the role of endophytic fungi, like *Trichoderma sp., Penicillium sp.,* and *Fusarium* sp., in the plant defence system against phytopathogens (Al-Rashdi et al., 2020). Secondary metabolites produced by these fungi have been extracted and tested for their antifungal and antioxidant activity. Recent studies have demonstrated the antifungal activity of endophytic fungi against many phytopathogenic fungi like *Fusarium sp*., *Botrytis cinerea, Cladosporium* sp.*,* and *Rhizoctonia solani* (Al-Rashdi et al., 2020; Mitra et al., 2022). Thus, the current study aims to investigate the antifungal and antioxidant potential of clove and its endophytic fungi. We intend to uncover and shed light on the remarkable properties exhibited by these natural entities by conducting a comprehensive analysis.

Chapter 2

Objectives

1. Isolation and identification of endophytic fungi from *Syzygium aromaticum* (clove)
2. Assessment of antifungal and antioxidant activity of clove VOCs and endophytic fungi

Chapter 3

Review of literature

Post-harvest losses in fruits and vegetables:

Fruits and vegetables are energy-rich food which plays an important role in maintaining a healthy body as they provide vitamins (A, B, C, E), minerals (Mg, Zn, P, Ca) and dietary fibre. Recent research has revealed that fruits are also rich in phytochemicals and antioxidants (Dhandevi et al., 2015). India is the second-largest producer of fruits and vegetables, after China (Kaur et al., 2019). The top ten countries with the highest production of fruits and vegetables are China, India, Brazil, the USA, Turkey, Mexico, Indonesia, Spain, Iran, and Italy. In 2022, around 109 and 200 million metric tonnes of vegetables and fruits were produced, respectively (FAO, 2022). Studies have documented that in the last few years, the production of fruits and vegetables has extensively increased as there is an increase in population globally (Balali et al., 2020). Approximately 14% of the total food produced is lost as post-harvest loss. Post-harvest loss measures the quantitative loss of food (fruits, vegetables and grains) between and marketing (Sil et al., 2020). FAO estimates that around 17% of food is lost and wasted during retail and by a consumer (2% in retail, 5% in transportation and 11% in household), equal to $400 billion per year (FAO, 2021). Various factors are responsible for post-harvest loss, such as environmental factors like humidity and temperature, microbial action (fungi, bacteria) and mechanical factors like storage, handling and transportation (Elik et al., 2020).

Recent studies have reported that the major post-harvest loss in fruits and vegetables is due to fungal decay. There are over 19,000 fungi which come in the category of phytopathogens, which can be found on dead and alive plants. Many fungi produce spores which may remain dormant under unfavourable conditions but start germinating as soon as the conditions become favourable (Jain et al., 2019). Some common diseases caused by fungi are reported as green mould and blue mould caused by *Penicillium digitatum*, and *P. italicum,* respectively, in citrus fruits, grey mould caused by *Botrytis cinerea* in table grapes, *Colletotrichum gloeosporioides* in banana and avocado caused anthracnose, Alternaria rot in apple is caused by *Alternaria alternata*, *Monilinia fructicola* causes brown rot in peaches*, Aspergillus niger* causes black mould in onion, and Vascular wilt is caused by *Fusarium*

oxysporum in tomato (Papoutsis et al., 2019; Bi et al., 2022; Zakaria and 2021; Oyom et al., 2022; Xu et al., 2021; Borkar et al., 2020; Srinivas et al., 2019). Various chemical fungicides such as pyrimethanil, fludioxonil, and imazali are being used to prevent post-harvest loss (Papoutsis et al., 2019). Due to the widespread use of chemical fungicides, these phytopathogens have acquired resistance thereby, reducing the efficacy of these treatments (Baibakova et al., 2019). Also, chemical fungicides are non-biodegradable, negatively impacting the environment (Sánchez-Torres, 2021). As a result of these1 side effects, the demand for natural drugs is rising worldwide. These circumstances led researchers to look into various natural sources to address these contemporary problems. Therefore, numerous studies are being conducted to develop natural, biodegradable fungicides that have no harmful effects on plants, animals, humans and the environment (Gupta et al., 2020).

Clove

Syzygium aromaticum, a member of the *Myrtaceace* family of dicotyledon plants, is the second most valuable and significant spice traded worldwide and has been utilised for more than 2000 years in Ayurveda and traditional Chinese medicine (Haro-González et al. 2021). Synonyms include *Caryophyllus aromaticus, Eugenia caryophyllus, Jambosa caryophyllus,* and *Myrtus caryophyllus* are all used to refer to the clove (Kaur and Kaushal, 2021). *S. aromaticum*, commonly known as clove (flower buds), is endemic to the Moluccas Islands of Indonesia and produces highly aromatic flower buds. Indonesia, India, Pakistan, Sri Lanka, the Comoro Islands, Madagascar, Seychelles, and Tanzania are some countries that commercially cultivate the plant (Mbaveng et al., 2017) (Table 1). *S. aromaticum* tree grows to 10-12 meters in height and begins to produce flower buds four years after the tree is planted. In coastal areas, clove cultivation occurs at high altitudes of roughly 200 m above sea level (Ayushi et al., 2020). It grows well in rich loamy soil with high humus content. It is also discovered to grow well in the loose sandy loam and laterite soil of southern Kerala and Tamil Nadu. The soil should have excellent moisture, good drainage and a pH of 4.0 to 5.6 (Yadav et al., 2020). The clove trade in India amounts to approximately 500-100 million metric tons annually (Saroja et al., 2022). Clove is categorized by the FDA as generally recognized as safe (GRAS), and as a result, it is used in foods, pharmaceuticals, sanitary goods, fragrances, and

cosmetics (Kumar Pandey et al., 2022). Due to various phenolic compounds, clove exhibits various properties like antifungal, anticancer, antiviral, antioxidant, antidiabetic, anti-inflammatory, antibacterial, and antithrombotic properties (El-Saber Batiha et al., 2020) (Fig. 2).

Table 1: Common names of clove in various countries (Dey and Mukherjee, 2021).

Country	Names used
Algeria	Alqaranful
Bulgaria	Karanfil
China	Ding xiang
Denmark	Kryddernellike
France	Clou de girofle
German	Nelke
Greece	Garifalo
Georgia	Mixaki
Hungary	Szegfuszeg
India	Clove, laung, lavang, devakusuma
Indonesia	Cengkeh
Itay	Chiodo di garofano
Japan	Choji
Korea	Jeonghyang
Latvia	Krustnaglinas
Nepal	Lwaang
Netherlands	Kruindnagel
Sweden	Kryddnejlika
Spain	Clavo

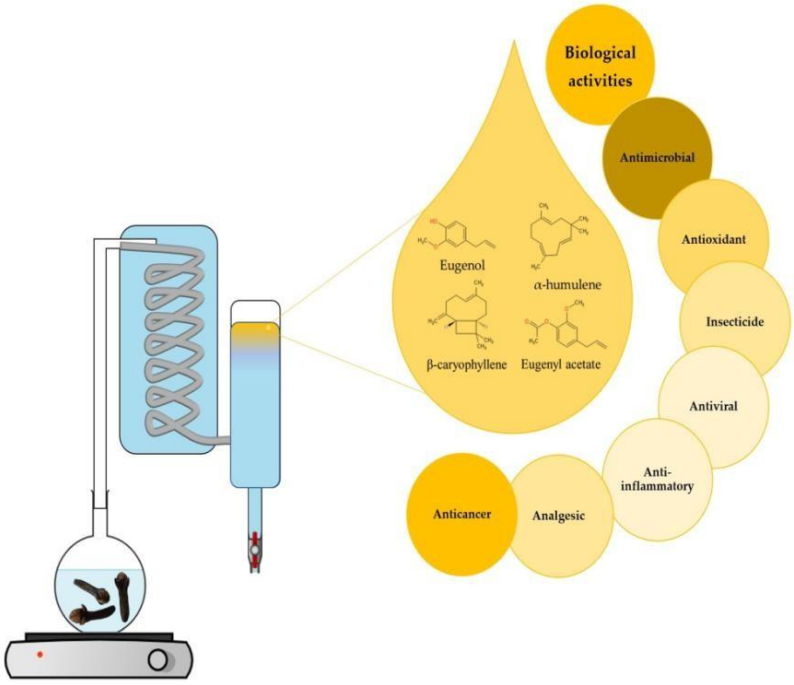
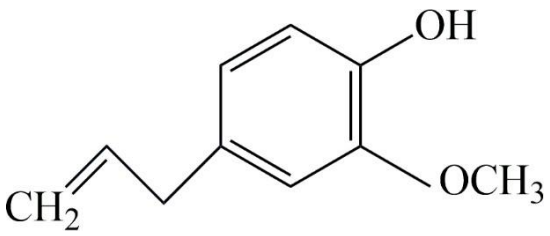
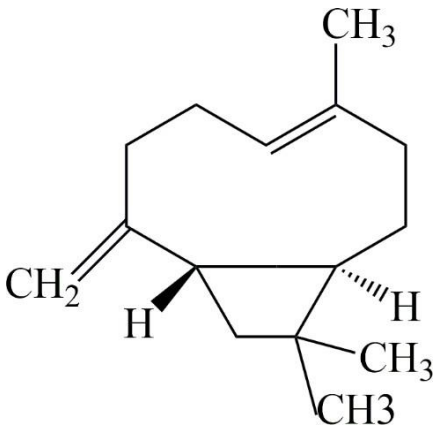
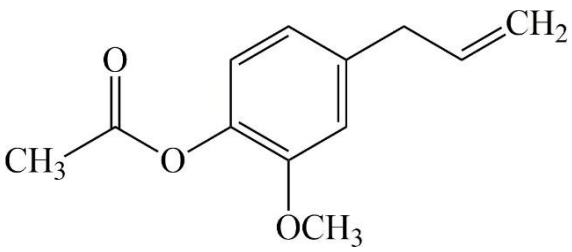
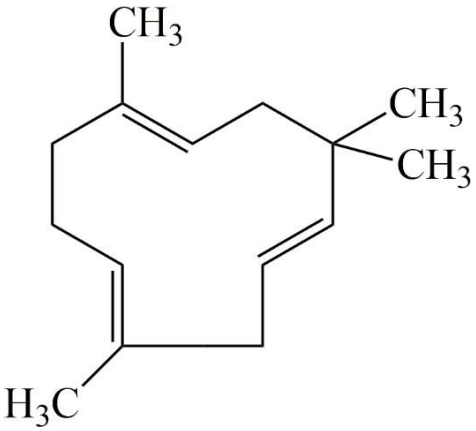


Fig. 2: Various properties of clove VOCs (Haro-González et al., 2021)

Young clove trees produce a significant amount of oil due to the shape of the vacuole structures. As the plants mature, little vacuoles combine to form a single, substantial structure. Young trees often contain a dense arrangement of tiny vacuoles, but adult plants have a single, larger vacuole with higher water content. The quantity of clove oil can be reduced due to large vacuoles with high water content (Alfikri et al., 2020). Numerous studies have been conducted to document the primary constituent of clove. The volatile organic compounds present in the clove primarily consist ofeugenol (70-90%), along with caryophyllene, eugenyl acetate, and humulene, as reported by Hiwandika et al. (2021) (Table 2). Cloves contain a volatile substance called eugenol, which can be colourless or pale yellow. It has a strong odour and flavour and poor water solubility (about 2460 mg/L at 25°C). Insecticidal, antibacterial, anti-inflammatory, wound-healing, antiviral, antioxidant, and anticancer activity are a few of the biological properties of eugenol (Banerjee et al., 2020).

Table 2: Major VOCs of clove (Boughendjioua, 2018)

Sr. No.	Compound	Concentration	Structure
1.	Eugenol	70-90%	
2.	β-caryophyllene	10-15%	
3.	Eugenyl acetate	5-7%	
4.	α-humulene	2-4%	

Antifungal activity of clove volatile organic compounds (VOCs)

Fruits and vegetables serve as important sources of essential nutrients, including minerals, vitamins, fibres, carbohydrates, proteins, and organic acids, within the human diet (Porat et al., 2018). Nonetheless, a significant issue regarding these crops pertains to their perishable nature, as they are prone to respiration and transpiration subsequent to harvesting. This phenomenon leads to an overabundance of ripening-related softening during storage after harvest, rendering them susceptible to fungal contamination (Kumari et al., 2022). A range of synthetic fungicides have been used to impede the growth of pathogenic fungi. Synthetic fungicides are made up of chemicals that harm the environment, animals, humans and plants. These fungicides are non-biodegradable and deplete some of the nutrients of fruits. Additionally, the fungi can develop resistance to these fungicides (Qin et al., 2023). Due to these reasons, various studies have been done to find a natural compound which can be used as a fungicide.

Several plant species, including clove, cinnamon, basil, caraway, cardamom, and pepper, are currently undergoing antifungal activity evaluations. The clove extract is composed of multiple constituents. GC-MS an analytical technique used to analysis revealed that the primary volatile organic compounds (VOCs) in clove extract are eugenol (69.7%), β-caryophyllene (12.2%), and eugenyl acetate (14.4%).Eugenol, a phenolic compound with weak acidic properties, is the primary constituent of clove, comprising 30-90% of the clove extract depending on the particular part of plant. It has a boiling point of 254°C (Khalil et al., 2017). The highest quality of clove essential oil is present in the bud, and the lowest is in leaves (Luesuwan et al., 2021 and Nilmini et al., 2021). These phenolic compounds in the clove are responsible for its antifungal activity.

Recent studies have documented that clove essential oil shows good antifungal activity. In a study conducted by Nilmini et al. (2021), it was revealed that in the disc volatilisation method, clove essential oil was most effective against 3 test phytopathogens—*Lasiodiplodia theobromae, Diaporthe nelumbonis,* and *Fusarium oxysporum* which were isolated from *Persea americana* (avocado). Clove essential oil inhibited the growth of *D. nelumbonis* at a concentration of 4 µL/plate and the growth of the other two fungi (*L. theobromae* and *F. oxysporum)* at a concentration of 5 µL/plate. Mehalaine S

and Chenchouni, (2021)reported that environmental factors such as light, temperature, rainfall, and the nature of the soil in which the plant is growing affect the chemical properties of essential oil. It was concluded that stem end rot in avocados could be controlled by treating them with clove essential oil, and storage at 15 °C can further increase the shelf life by seven days (Nilmini et al., 2021).

Davy et al. (2020) reported that clove essential oil had significant antifungal activity against anthracnose caused by *Colletotrichum gloeosporoides* in *Carica papaya* L. GC-MS results indicated that the presence of eugenol (87.62%) as a major compound of clove essential oil similar to other studies. *In-vitro*, studies showed that clove essential oil inhibited mycelial growth and conidia germination at 375 µL/L and 100 µL/L MIC. *In-vitro*, results revealed that clove essential oil could inhibit the necrosis caused *by C. gloeosporoides* in papaya at a concentration of 3000 µL/L. It was summarized that clove essential oil could be used to inhibit the fruit rot in papaya caused by *C. gloeosporoides,* as it can significantly inhibit spore germination and mycelia growth. A study reported that clove essential oil could significantly inhibit the growth of *Penicillium italicum*, which cause blue mould in citrus fruits. *In vivo*, studies showed that a concentration of 0.4% clove essential oil could significantly decrease the disease severity and lesion diameter.

The mechanism of action of clove essential oil was also studied by analysing the levels of H_2O_2 (Hydrogen Peroxide) plays a role in the host defence mechanism) and Malondialdehyde (MDA), a marker for lipid oxidation and cell membrane damage, in citrus fruits (Lin et al., 2020). It was found that 0.4% of clove essential oil-treated fruits have higher levels of H_2O_2 than clove essential oil-untreated citrus fruits. The presence of higher levels of H_2O_2 helped in the activation of defence-related enzymes in the host and hence, inhibited the growth of fungus. Levels of MDA were also analysed in clove essential oil-treated and untreated citrus fruits. Levels of MDA were lower in clove essential oil-treated citrus fruits, which indicated the activation of defence-related enzymes. It was concluded that the shelf life of citrus fruits could be increased by treating them with clove essential oil and further storage at 25°C (Chen et al., 2019).

Thabet et al. (2018) documented that the rot and wilting of tomatoes can be inhibited by clove essential oil. Four different fungi, including *Fusarium oxysporum, F. solani, F.*

semitectum and *Rhizoctonia solani* were isolated from infected tomato and treated with different concentrations of clove essential oil. Results indicated that 4% concentration of clove essential oil could inhibit the mycelia growth and conidia formation of all the test phytopathogens. The Minimum Inhibitory Concentration (MIC), which refers to the lowest concentration of a compound that can inhibit the visible growth of microbes, was observed to completely inhibit the growth of *R. solani* and *F. oxysporum* at a concentration of 4%. On the other hand, the growth of *F. semitectum* and *F. solani* was reduced by 88.5% and 91.8%, respectively, at the same concentration. The concentration of phenolic compounds is higher in clove essential oil, which is responsible for its antifungal activity. Comparative microscopic analysis of fungi treated with clove essential oil and untreated revealed significant variations in surface morphology and structure. It was noted that the fungus treated with clove essential oil has fewer conidia than the untreated one; furthermore, the hyphae were disrupted and had irregular morphology.

A recent investigation has reported that among clove, cinnamon, lemongrass, and *eucalyptus lycopersici* 1322, clove oil has exhibited the most potent inhibitory impact on *Fusarium oxysporum*. At a concentration of 125 ppm, the application of clove essential oil resulted in complete inhibition of mycelial growth and spore formation of the tested fungus. The concentration at which clove essential oil exerts half of its maximum inhibitory action, also known as the IC_{50} value, was determined to be 18.22 ppm for the inhibition of mycelia growth. In contrast, the IC_{50} value pertaining to the inhibition of spore formation was 0.3ppm. The clove essential oil was subjected to GC-MS analysis to determine its various components. The findings revealed the existence of thirty-one distinct components, with eugenol being the predominant constituent, accounting for 75.41% of the total components. Scanning Electron Microscope (SEM), results indicated the difference in clove essential oil-treated and untreated fungus morphology. It was documented that the clove essential oil-treated fungus had distorted and shrunken hyphae devoid of cytoplasm and a distorted cell wall. Atomic Force Microscope (AFM) showed that the spores of clove essential oil-treated fungus were shrunken and distorted with a rough and fragmented surface. In contrast, untreated spores were spherical and had uniform structures (Sharma et al., 2017).

Few studies have been conducted to examine the VOCs of clove as a fungicide. Qin et al. (2023), documented that VOCs of clove can be used to inhibit the growth of *Aspergillus flavus* on wheat with 20% moisture. In GC-MS analysis, twenty-seven different VOCs were found, out of which, eugenol (49.8%), and caryophyllene (36.68%) were identified as the main component of the spectra. This study also indicated that eugenol was the major component responsible for the antifungal activity of clove. *In-vitro* studies showed that MIC of 0.12µL/mL eugenol inhibited the growth of *A. flavus*. In *in-vivo* testing, eugenol fumes inhibited the growth of test phytopathogens at MIC of 600µL/L. Metabolomic analysis and biochemical index analysis were done to study the mechanism of action of eugenol; studies indicated that the fungus treated with eugenol had rough hyphae and damaged cell walls. Additionally, ergosterol concentration was decreased in eugenol-treated fungus, indicating that cell membrane integrity was disrupted. The results of the metabolomic analysis indicated a reduction in the levels of L-carnitine, a crucial component in the production of ATP, in the presence of eugenol which led to a disruption in the β-oxidation of fatty acids. This study provided the experimental basis for utilization of clove VOCs as natural bio-fumigants to control *A. flavus* growth on wheat grains.

Another study conducted by Soliman et al. (2019) highlighted the effect of clove ethanolic extract on the growth of four different test fungi, i.e., *Geotrichum candidum, Alternaria alternata, Fusarium oxysporum*, and *Mucor hiemalis* which cause fungal decay of potatoes and tomatoes. Phytochemical analysis showed the presence of phenolic compounds like flavonoids and alkaloids. GC-MS analysis was done to analyse the biochemical profile of clove extract, and results revealed the presence of phenolic compounds like eugenol, caryophyllene and humulene. This study also suggested the mechanism behind the fungicidal activity of clove. Clove extract leads to inhibition of cell wall formation, fungal mitochondria dysfunction, cell wall disruption, and inhibition of DNA/RNA and proteins (Nazzaro et al., 2017). Clove extract inhibited the growth of all test fungi except *F. oxysporum.* This study suggested that clove extract could be used as a biological fungicide to prevent the post-harvest loss of potatoes and tomatoes (Soliman et al., 2019).

Another study found that clove essential oil and mustard essential oil vapours can significantly inhibit *Botrytis cinerea's* growth, which causes grey mould

in *Fragaria* x *ananassa* (strawberries). Eugenol (75.4%), caryophyllene oxide (11.2%), and eugenyl acetate (5.2%) were recorded as the main components of clove essential oil on GC-MS analysis. The study revealed that a concentration of ≥92.56µL/Lair clove essential oil could inhibit the growth of test phytopathogen. In contrast, only ≥15.42µL/Lair concentration of mustard essential oil was required to inhibit the growth of test fungus in *in-vitro* and *in-vivo* testing. A combination of 46.28µL/Lair (i.e., ½ MIC) of clove essential oil with 3.86µL/Lair (i.e., ¼ MIC) of mustard essential oil was able to inhibit the growth of *B. cinerea* during *in- vitro* testing. The combination testing in *in- vivo* conditions showed that 1/8 MIC of both clove essential oil (i.e., 11.57µL/Lair) and mustard essential oil (i.e., 1.93µL/Lair) was required to inhibit the growth of test phytopathogen. The study concluded that combining VOCs of essential oils can be more effective than individual oil (González, et al. 2015).

Boukaew et al. (2017) reported the effect of fumigation of four essential oils, such as clove, cinnamon, capsicum, and vatica oil, on the growth of ten different strains of *A. flavus* which cause post-harvest losses of maize seeds. The study documented that clove essential oil has wholly inhibited the growth of all test strains except *A. flavus* PSRDC-2, which showed an inhibition percentage of 14.9%. After five days of fumigation, a viability test showed that all strains were dead except *A. flavus* PSRDC-2. At a concentration of 50µL/L (MIC), clove essential oil can completely inhibit the growth of *the A. flavus* PSRDC-2 strain. Clove essential oil inhibited that sporulation at MIC of 10µL/L, whereas conidia germination was completely inhibited by vatica oil at a concentration of 100µL/L. Sporulation of *A. flavus* PSRDC-2 strain on maize seeds was completely inhibited by fumigation of clove essential oil at a concentration of 10µL/L. *In-vivo* assay revealed that vatica oil had total (100%) protective and curative benefits on maize seeds infected by *A. flavus* PSRDC-2 after being fumigated for at least six hours. In contrast, clove oil requires twenty-four hours to have the same protective effect. In conclusion, clove and vatica oil can be bio-fumigants to protect maize from post-harvest losses.

Antioxidant activity of clove VOCs

Fruits and vegetables generate ROS during various physiological pathwaysduring postharvest storage. ROS are oxygen-containing molecules with unpaired electrons capable ofreacting with other molecules (Das and Roychoudhury, 2022).Even though these ROS have an important role as secondary messengers in various intracellular activities, overproduction of ROS canbe harmful as it can deplete various important nutrients of plants. These ROS can be neutralised by specialised compounds called antioxidants (Meitha et al., 2020). Nowadays, people are more investedin finding a natural antioxidant, as synthetic antioxidants have various side effects, and are non- biodegradable. Various studies have been done on clove, cinnamon, caraway, basil, lemon grass, and black pepper to investigate their antioxidant activity (Johari et al., 2022).

A study by Alawiyah et al. (2019) evaluated the antioxidant activity of essential oil of *S. aromaticum* (L.) leaf. GC-MS analysis revealed 15 different VOCs of clove extract; eugenol, caryophyllene, and humulene were the main components of clove extract with a concentration of 73.25%, 19.43%, and 2.32%, respectively. DPPH assay analysis revealed that clove leaf extract exhibited significant antioxidant potential. The *S. aromaticum* (L.) essential oil test had an IC_{50}value of 8.224µg/mL, while the standard Ascorbic acid had an IC_{50}value of 2,216µg/ml. It was deciphered that the strong antioxidant activity of clove extract is due to eugenol.

Another study investigated the antioxidant activity of different plants such as clove, lemongrass, cinnamon, black pepper, and basil using 3 different antioxidant tests (Johari et al., 2022). DPPH, FRAP, and ABTS assays are commonly employed chemical-based tests to evaluate the free radical scavenging activity and antioxidant potential of a given compound (Rumpf et al., 2023). DPPH (1,1-diphenyl-2-picrylhydrazyl) measures the ability of a compound to scavenge free radicals, while FRAP (Ferric Reducing Antioxidant Potential) is based on the reduction of Ferrous ions to ferric ions by the sample antioxidants. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay, on the other hand, assesses the capacity of antioxidants to scavenge the ABTS-generated radicals (Shah and Modi,2015). The FRAP assay results revealed that clove essential oil significantly reduced ferrous ions to ferric ions at a concentration of 7.66 ±1.12μmol Fe (II)/mg. GC-MS results

showed that eugenol was the main component responsible for the antioxidant activity of clove essential oil. Similarly, significant ABTS generated scavenging activity at the concentration of 500µg/ml clove essential oil extract, and clove methanol extract. In the DPPH assay, clove oil extract had higher antioxidant activity at 500µg/ml due to presence of eugenol. In comparison to earlier stages, flowering-stage buds had more eugenol than the others, therefore, had higher antioxidant activity (Johari et al., 2022).

A previous study investigated the antioxidant activity of clove and lemon essential oils. GC-MS analysis was carried out to analyse the compounds of essential oils, and results revealed that eugenol was the main compound in both clove essential oil and lemon essential oil. The total phenolic content (TPC) of clove essential oil, and lemon essential oil was measured using the Folin- Ciocalteu test (antioxidant assay based on electron transfer, which measures the reductive capacity of an antioxidant), and it was reported that the number of phenolic compounds present in lemon (816.07±46.81) was higher than clove (128.06±6.78). Phenolic compounds are a class of natural antioxidants that can scavenge free radicals due to the presence of hydroxyl groups (Al-Mamary and Moussa, 2021). As the total phenolic content was higher in lemon, it in turn indicated that it has significantly higher antioxidant potential than clove. The IC_{50} required for scavenging DPPH free radicals in lemon was 4.38±0.11mg/ml, whereas in clove, it was 7.84±0.21mg/ml. It was concluded that these essential oils could be used as natural preservatives and help extend food's shelf life (Hosseini et al., 2019).

Ghadermazi et al. (2017) investigated the antioxidant potential of clove, sage, and oregano essential oils using DPPH, FRAP, and β-carotene bleaching (BCB) assay. It was reported that clove essential oil has significantly higher antioxidant activity out of all test essential oils. Clove essential oil has stronger antioxidant properties as it contains eugenol and eugenyl acetate, two phenolic compounds which neutralize the free radicals (El Ghallab et al., 2020). At a 200µg/mL concentration, clove essential oil neutralized all the free radicals of DPPH, i.e., showed 100% antioxidant activity. In BCB assay clove hindered the bleaching of β carotene at a concentration of 1000µg/ml by 96.3%. Clove showed the highest ferric-reducing ability at all the concentrations due to the presence of

eugenol as the main component. The study concluded that clove could be used as a natural antioxidant, and can prevent food from oxidation, increasing its shelf life (Ghadermazi et al., 2017). Another study investigated the effect of solvent (water, methanol and acetone) on clove extract yield, quality, and antioxidant activity. Acetone (80%) extract of clove bud gave the highest yield of 18.33%, followed by methanol (80%) extract, which yielded 16.05%. Water extract of clove bud gave the lowest yield of 15.1%. This difference in yield indicated that most of the compounds of clove bud are non-polar. The results suggested that the organic solvents incurred in higher yield (Adaramola et al., 2016). Total flavonoids and phenolic content were also measured using Aluminium chloride and the Folin-Ciocalteu methods, respectively (Asem et al., 2020). It was documented that acetone extract has higher flavonoid and phenolic content than methanol and water extract. Results also showed that clove bud has more flavonoid compounds than phenolic compounds. The 80% acetone extraction showed the strongest DPPH free radical scavenging activity and overall reducing power, followed by the 80% methanol extraction, while the water extract showed the least. This suggests that the semi-polar components of clove buds are more potent antioxidants than the polar ones. The study also suggested the relationship between flavonoids and phenolics with antioxidant activity. Therefore, it was concluded that aqueous acetone (80%) is the most efficient and acceptable solvent for recovering a greater amount of phenolics and flavonoids from the clove bud (Adaramola et al., 2016).

Endophytic fungi

In recent years, fungal endophytes have gained popularity as a potential replacement for plant secondary metabolite sources. Endophytic fungi exist in all healthy plant tissues without exhibiting any symptoms of disease or morphological changes for at least a portion of the entire life cycle (Abo Nouh FA, 2019). In exchange for food and shelter from plants, endophytic fungi help their host survive biotic (damage from pathogens) and abiotic stress (such as excessive salt, temperature, and drought) by producing a variety of bioactive compounds (Segaran and Sathiavelu, 2019). Most endophytic fungi are Ascomycota members or their mitosporic fungi, and a few species belong to Basidiomycota, Zygomycota, and Oomycota. These endophytic fungi form symbiotic or pathogenic associations with the host's living tissues (Caruso et al., 2020).

According to their taxonomy, evolution, ecological purpose, and host specialisation, endophytic fungi were previously divided into Clavicipitaceous and non-Clavicipitaceous (Wani et al., 2015). The existence of four distinct groups based on six characteristics—tissue(s) colonised, plant colonisation pattern, host range, plant biodiversity levels, ecological roles, and host generational transmission mechanism—was then revealed. Clavicipitaceous endophytes are classified into Class 1, while non-Clavicipitaceous endophytes are divided into Classes 2, 3, and 4. Most are classified as non-clavicipitaceous endophytes, class 2 (Suresh and Sona, 2020). According to Lugtenberg et al. (2016), endophytes can be transmitted either vertically through seeds from one generation to the next or horizontally acquired from the environment with every subsequent generation. The acquisition of novel adaptation characteristics by endophytes is the focus of current studies. One important mechanism that has been identified is Horizontal Gene Transfer (HGT), which involves the transfer of genetic material between species that are phylogenetically distinct. This process has been found to play a significant role in the evolutionary adaptability of endophytes within their host plants. (Tiwari and Bae, 2020). Many advantageous traits, such as prokaryotic tolerance to environmental changes, acquisition of novel characteristics or capabilities, and evolutionary adaptation in eukaryotes, can be achieved by transmitting, and integrating transferred genes (Filip and Skuza, 2021).

Endophytes have two main biocontrol mechanisms: Direct and Indirect (Fig. 3). In direct inhibition, the endophyte produces various bioactive compounds, such as alkaloids, flavonoids and phenolic compounds, which kill the invading pathogen (Fadiji and Babalola, 2020). In contrast, stimulating plant defense mechanisms, such as synthesising secondary metabolites, is frequently associated with indirect endophyte influences on plant resistance to pathogens (Filip and Skuza, 2021). In direct inhibition, the endophyte follows three different ways: Mycoparasitism or hyperparasitism, Competition and Antibiosis; indirect inhibition includes induced resistance (Segaran and Sathiavelu, 2019). In induced resistance, the host plant's physical and chemical barriers actively stimulate induced resistance to plant pathogens, which is a preventive mechanism elicited by both abiotic and biotic stimuli (Akram et al., 2023). Endophytes may act by increasing the production of phytoalexins and pathogenesis-related-proteins, thickening the cell wall by glucan and lignin

deposition, and thickening the cuticle that may prevent pathogen penetration and growth in the host plant (Kaur et al., 2022; Fontana et al. 2021).

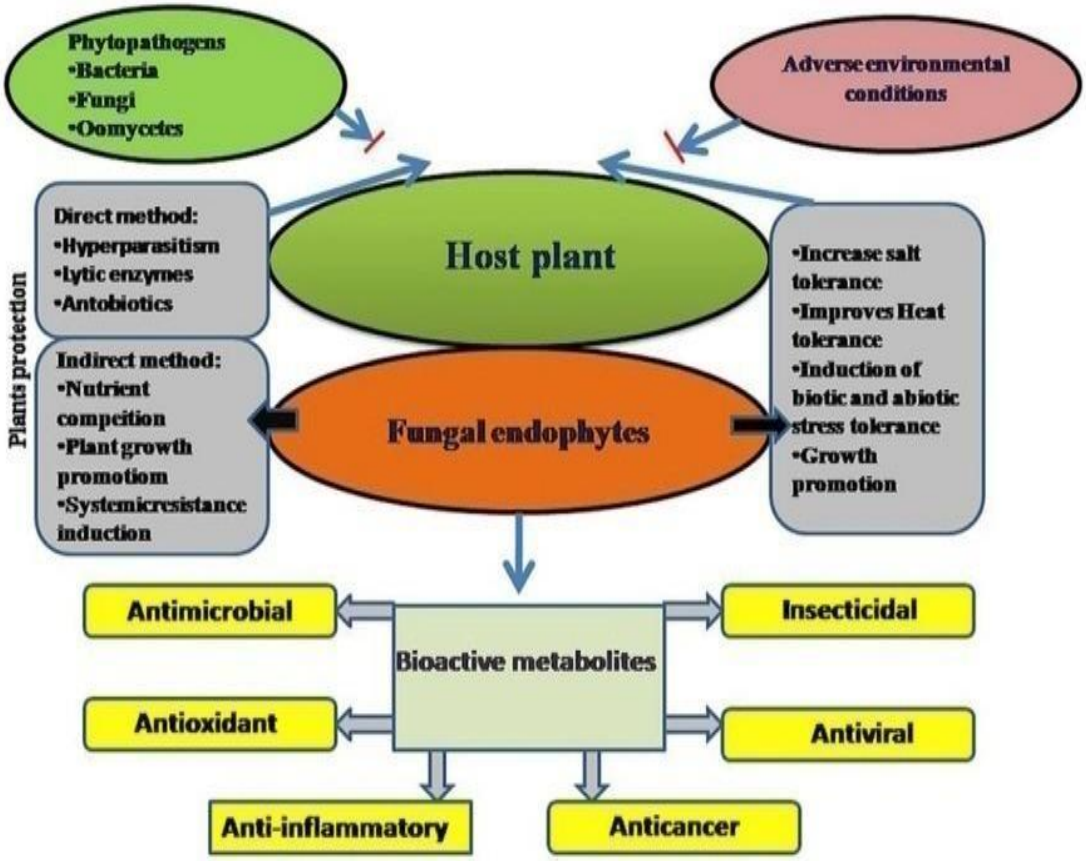


Fig. 3: Overview of interactions among host, pathogens and endophytes (Segaran and Sathiavelu, 2019)

Induced systemic resistance (ISR) and systemically acquired resistance (SAR) are two categories of induced plant resistance. The most significant secretions produced during ISR are ethylene and hormonal jasmonic acid; salicylic acid is crucial for developing systemically acquired resistance (Kamle et al., 2020; Latz et al., 2018). Endophytic fungi exhibit an antagonistic relationship for pathogen inhibition known as antibiosis and produce secondary metabolites, which help to control potential plant pathogens (Latz et al., 2018). According to Adeleke et al. (2022), fungal endophytes colonise various plant tissues locally or systemically, intracellularly or intercellularly in competitive inhibition. They rapidly colonise the area and scavenge nearby resources, taking up the space that a

pathogenic organism would otherwise occupy. In addition, the endophytic fungi colonise intracellular plant parts where the pathogen may attack (Akram et al., 2023).

Studies have reported that the competitive exclusion method works along with other mechanisms. As a direct biological control strategy for plant pathogenic fungi, mycoparasitism or hyperparasitism is the parasitic relationship between two or more fungi. Based on the parasitism mechanism and how it affects the host fungus, mycoparasitism is divided into Biotrophic and necrotrophic parasitism (Kim and Vujanovic, 2016).Moreover, the presence of endophytes leads to the production of numerous secondary metabolites resulting from the emergence of microbial interactions with pathogenic organisms (Devi et al., 2023).

Antifungal activity of endophytic fungi

A numerous studies have been conducted to exploit the antifungal activity of various endophytic fungi. A recent study has shown the fungicidal effect of endophytic fungi isolated from *Aloe dhufarensis* Lavranos. against phytopathogenic fungi, i.e., *Fusarium* sp., and *Cladosporium* sp. A total of three endophytic fungi were isolated from leaves of *A. dhufarensis*, out of which two showed the antagonistic effect against test phytopathogens and were identified as *Sarocladium kiliense*, and *Penicillium oxalicum.* SEM analysis revealed that shrivelling, pit development, loss of turgidity, and hyphae disintegration were among the endophytes' morphological alterations. GC-MS results showed that 2,3-Butanediol and dodecanoic acid were the most prevalent substances in the extract of *S. kiliense*, and 2-furan-methanol and dodecanoic acid was abundantly present in the *P. oxalicum* extract. This study also suggests that geographical location, environmental factors, the type of plant tissues, the host's genetic makeup, and the host plants' age significantly affects the population structure of endophytes found in plants. The study concluded that *S. kiliense* and *P. oxalicum* could control the growth of *Fusarium* sp. and *Cladosporium* sp. (Al-Rashdi et al., 2022).

Similarly, Samapti et al. (2022) evaluated the antifungal and antioxidant properties of endophytic fungi from *Syzygium cumini*. Eight different fungal strains were isolated from the bark and leaves and then identified using morphological and molecular methods. Three strains are associated with *Colletotrichum* sp., while others are associated with *Diaporthe* sp*., Fusarium* sp*., Pestalotiopsis*

sp., *Penicillium* sp., *and Phyllosistica* sp. Out of all, *Penicillium* sp. showed the highest antifungal activity. The phytochemical screening demonstrated the existence of many substances, including flavonoids, terpenoids, steroids, coumarins, iso-coumarins and anthraquinones. The DPPH test was used to measure antioxidant activity. The result revealed that *Penicillium* has the highest antioxidant activity of all the isolates with IC_{50} of 2.43µg/ml. During comparative studies of leave and bark extract, leaves extract showed higher antioxidant activity than bark extract.

In a study by Yang et al. (2021) observed the effect of VOCs produced by *Sarocladium brachiariae* HND5 was investigated on phytopathogens, including *Fusarium oxysporum* f. sp. *cubense* (FOC) which cause vascular wilt in bananas. VOCs released by *S. brachiariae* were analysed by GC-MS; the result showed 17 different compounds present in VOCs which were alkenes, alkyls, ketones, esters and aromatic hydrocarbons. Out of which, only 3 showed the fungicidal effect on (FOC) namely 2-methoxy-4-vinylphenol (2M4V), 3,4-dimethoxy styrol (34D), and caryophyllene. At a concentration of 36µL/L, 60µL/L and 2900µL/L 2M4V, 34D, and caryophyllene, respectively, showed an inhibitory effect. The results revealed that these VOCs could inhibit the mycelia and conidia growth of the test pathogen. The selected VOCs' impact on the hyphae structure was assessed using Transmission Electron Microscope (TEM). The 2M4V and 34D-treated FOC hyphae displayed distinct, abnormal forms that lacked intact plasma membranes, smeared cytoplasm, and leaked cell content. Whereas the EC_{50} concentration of caryophyllene did not significantly alter the hypha structure, the hyphae treated with caryophyllene had defined cell walls, intact plasma membranes, and visible cell organelles. Cell death was analysed by double fluorescent staining with FDA/PI. Fluorescein diacetate (FDA) is an enzyme activity probe that non-specific esterase can detect (Krasnow et al., 2008). When FDA penetrates living cells, this recognition releases green fluorescence, which can be used as a live cell indicator. Propidium iodide (PI) fluoresces red when a membrane is damaged and serves as a marker for dead cells. The results were as per TEM analysis; 2M4V and 34D-treated FOC hyphae were red, indicating more dead cells, whereas β-C-treated FOC hyphae were green, indicating more live cells (Yang et al., 2021).

Another study documented the fungicidal effect of VOCs of endophytic fungi isolated from Plants Growing in the Central *Andean Precordillera* of Chile against *Botrytis cinerea*. Three fungi,

Ac1, Lc1 and Ec1, showed antifungal activity against *B. cinerea*. It was reported that the *B. cinerea* mycelium displayed grey due to conidia without treatment with VOCs. In contrast, the mycelia of *B. cinerea* remained white when treated with volatile chemicals produced by isolates Lc1, Ec1, or Ec1. This indicated that VOCs produced by test endophytic fungi inhibit the production of spores. It was also revealed that Ec1 inhibited the production of spores on test pathogens by 98.4% (Vidal et al., 2020).

Wonglom et al. (2020) reported that EF *Trichoderma asperellum* T1 could inhibit the growth of *Corynespora cassiicola* and *Curvularia aeria,* which cause lettuce leaf spots. The volatiles from *T. asperellum* T1 inhibited *C. cassiicola* and *C. aeria* by 61.31% and 41.46%, respectively. According to a GC/MS analysis, *T. asperellum* T1 was found to release 22 volatile chemicals, which are involved in antifungal activity, eliciting defence responses, and stimulating lettuce development. Results revealed three main VOCs, namely 2-ethyl-1-hexanol, 1- nonanoland 6-pentyl-2H-pyran-2-one. It was observed that the activity of the cell wall degrading enzymes chitinase and β-1,3-glucanase in lettuce increased in response to VOCs released by endophytic fungi, reaching 1.26 U/mL and 4.45 U/mL, respectively.

Antioxidant activity of Endophytic fungi

Fewer studies have been conducted to investigate the antioxidant activity of endophytic fungi isolated from clove. In a study, four different endophytic fungi were isolated from *Syzygium jambos*. According to macroscopic and microscopic identification, the endophytic fungi were characterized as *Lasiodiplodia, Bipolaris, Aureobasidium*, and *Cladophialophora.* The antioxidant activity was measured using DPPH, and results reported that the endophytic fungus *L. theobromae* has maximum antioxidant activity with an IC_{50}value of 29.29mg/ml. The spectroscopic analysis combined with 1D NMR showed that the compound released by *L. theobromae* is 3,5-dihydroxy-4-(4-hydroxyphenyl)tetrahydro-2H-pyran-2-one which is a phenolic compound (Aini et al., 2022). Hapida et al. (2022) documented the antioxidant activity of endophytic fungi isolated from *Syzygium malaccense*. Four different endophytic fungi were isolated from *S. malaccense*, of which three belong to Ascomycota and one to Basidiomycota. The ethyl acetate extract was used to

measure the antioxidant activity using DPPH (Jadid et al., 2017). Two endophytic fungi showed significant free radical scavenging activity identified as *Tritirachium oryzae* and *Rhizopus* sp. with IC_{50} 381.04 ± 24.54 g/mL 482.83 ± 64.85 g/mL, respectively. The bioactive compound responsible for the antioxidant activity of *T. oryzae* was identified as s 2-(4-hydroxyphenyl)-4-methoxytetrahydrofuran- 3-ol. The study concluded that further study is required, namely a semi-synthetic procedure to add a hydroxyl group at the C-3 and C-5 locations to increase the antioxidant activity of compound 1 (Hapida et al., 2022).

Similarly, the antioxidant activity of endophytic fungi isolated from *Syzygium aqueum* was investigated in a study. Four endophytic fungi were isolated from the leaves stalk of *S. aqueum*. Molecular characterization identified these as *Cochliobolus* sp., *Penicillium* sp., *Fusarium* sp., and *Beltrania rhombic.* To evaluate the free radical scavenging activity, the DPPH assay was performed, and the results demonstrated that *B. rhombic* has the highest antioxidant activity, with an IC_{50} of 59.2 µg/ml. In contrast, other fungi including, *Cochliobolus* sp., *Penicillium* sp., and *Fusarium* sp. had IC_{50} of 98.26 g/mL, 92.3 g/mL, and 64.33 g/mL, respectively. The spectrophotometric analysis identified the main compound produced by *B. rhombic* as 3-(hydroxyl (3,4,5-trihydroxylphenyl) methyl)-3,4-dihydro-2H-pyran- 4,5,6-triol which is a white-yellowish powder with IC_{50} 44.2 µg/ml. The study concluded that this new compound could potentially source new antioxidants (Habisukan et al., 2021).

Recent *in-vitro* studies revealed that *Syzygium samarangense* leaves and its endophytic fungi have significant antioxidant activity in DDPH assay. Leaf extract prepared in ethyl acetate showed the highest antioxidant activity with an IC_{50}value of 74.37µg/mL. After spectroscopic analysis using NMR, the compound responsible for antioxidant activity was identified as 5,7-dihydroxy-6,8-dimethyl flavanone. Four endophytic fungi were isolated from the leaves of *S. samarangense.* Out of which one showed significant free radical scavenging activity. The endophytic fungi were identified as *Lasiodiplodia venezuelensis* using a molecular identification approach. More research is required to isolate and purify the bioactive components that are antioxidants in specified endophytic fungus. These results suggest that endophytic fungi from plants that produce antioxidants may have the potential to be a source of antioxidant chemicals (Budiono et al., 2019).

Chapter 4

Material and method

Procurement of fungal phytopathogens

The phytopathogenic fungi used in the current study including, *Alternaria alternata* (6343), *Fusarium lateritium* (4533), *Colletotrichum gloeosporioides* (6152), *Botrytis cinerea* (6011), *Fusarium moniliforme* (6435), *Botryodiplo diatheobromae* (5597), and *Aspergillus niger* (6354) were procured from Indian Type Culture Collection (ITCC), ICAR, New Delhi.

Preparation of Potato Dextrose Agar (PDA)

For media preparation, 39g of PDA (Hi-media, India) was mixed in 1L of distilled water in a flask, and the pH was maintained at 5.2. Then the flask was covered with a cotton plug before autoclaving. The media prepared was autoclaved at 121°C, 15 psi for 15 minutes. The autoclaved media was aseptically poured into sterile Petri dishes of dimensions 90 x 15 mm in Laminar air flow (LAF) (Thermodyne Pvt. Ltd., India). The Petri dishes were left undisturbed till the media solidified and then used.

Revival of procured fungal pathogen

Under sterile conditions, the phytopathogens procured from ITCC were picked from slants with the help of a sterile inoculation loop and were inoculated on prepared PDA plates. The entire procedure was performed under aseptic conditions in LAF. Petri plates inoculated with pathogen were then incubated at 28 ± 2 °C for seven days.

Collection of plant sample

Healthy and mature leaves and stems of *Syzygium aromaticum* L. (clove) were collected from Bangalore (12°89’43.53 “N, 77°42’11.61 “E) in January 2023. The collected plant samples were labelled and packed in a sterile zip lock bag and stored at 4°C till further use.

Isolation of endophytic fungi

The leaves and stems collected were washed thoroughly under running tap water. The samples were further surface-disinfected using 0.5 % sodium hypochlorite solution for 1 minute, followed by 70%,

50% and 30% ethanol for 30s each, and finally rinsed with sterile distilled water and air-dried under aseptic conditions in LAF.

The disinfected stems and leaves were cut into small segments of 5mm using a sterile scalpel and blade. Eight such segments were picked with the help of sterile forceps and placed on freshly prepared petri plates containing Water Agar (WA). The plates were incubated at 25 ± 2 °C and were regularly monitored for growth for 14 days (Kuo et al., 2021).

Purification and preservation of isolated endophytes

For pure culturing of isolated endophytic fungi, each fungus was picked up from the colony's edge and aseptically sub-cultured onto a fresh PDA plate and incubated at 25 ± 2 °C for seven days. For long-term preservation, the isolates were subsequently preserved on PDA slants containing 10% (v/v) glycerol (Moubasher et al., 2022)

In-vitro antifungal activity

a. Preliminary screening

The dual confrontation assay was used for preliminary screening of the antifungal activity of VOCs secreted by endophytic fungi and clove extract as described by Saxena et al. 2015. Briefly, an agar strip of 2 cm was removed using a sterile scalpel and blade from the middle to form compartments in the plate to prevent the transfer of any diffusible substance of endophytic fungi to the test microorganism(s). On one compartment of the PDA plate, a 5mm mycelium disc of the 7-day-old culture of the endophytic fungus was inoculated. Then the plates were sealed with parafilm and incubated at 25 ± 2°C for seven days. On the seventh day, a 5mm disc of 7-day-old cultures of a fungal pathogen was inoculated on the other compartment, and plates were again sealed and kept at 25 ± 2°C. The procedure was repeated to test the antifungal property of each fungal endophyte against all the selected phytopathogens. The growth of the test pathogen was measured on the10^{th} day. A petri plate containing only test phytopathogenic fungus devoid of endophytic fungi served as control.

Similarly, the antifungal activity of clove's VOCs was tested by using clove extract. To prepare clove extract, dried clove buds were collected from the local market of Patiala and pulverised in a grinder to make a fine powder. Using a mortar pestle, 100 mg of clove powder was macerated in different

solvents (CCl_4 and 80% Acetone). The extract was stored at 0°C for further use (Adaramola et al., 2016).

A dual-culture assay was performed with slight modifications to test the antifungal potential of prepared clove extracts. 100µL of clove extract was placed in a sterilised micro-cup located on one side of the Petri plate containing PDA. 5 mm plug of the seven-day-old culture of the pathogen was placed on the other side of the same petri plate. A petri dish inoculated with only the test pathogen served as a control. Plates were sealed with parafilm and kept at 25 ± 2°C. The growth of the test pathogen was measured on 10thday (Strobel et al., 2001).

$$Percentage\ inhibition\ (\%) = \frac{\mathrm{Dc} - \mathrm{Dt}}{\mathrm{Dc}}\ \mathrm{x}\,100$$

Where, Dc = mycelial growth of test phytopathogens on control plate, and Dt = mycelial growth of test phytopathogens on the test plate. Each test was performed in triplicates, and the results are presented as the mean ± standard error.

b. Secondary screening

Potent cultures selected after preliminary screening were subject to secondary testing. The sandwich plate method, as described by Ebadzadsahrai et al. (2020), was used for secondary screening with slight modifications. Briefly, a 5 mm diameter plug of 7 days old cultures of potent endophytic fungi was placed in the centre of a petri plate filled with PDA media. The plates were wrapped with parafilm and kept at 25 ± 2°C for 5 days; then, on another plate containing PDA, the selected set of phytopathogen was inoculated as described above. The plate lid inoculated with endophytic fungi was replaced with the bottom of the Petri plate containing the pathogen inoculated on PDA. This arrangement, called the "sandwich plate", was secured carefully with parafilm and kept at 25 ± 2°C and results were recorded on the 5th day. The plates inoculated with phytopathogens only were used as a control so that the pathogen could grow normally.

$$Percentage\ inhibition\ (\%) = \frac{\mathrm{Dc} - \mathrm{Dt}}{\mathrm{Dc}}\ \mathrm{x}\,100$$

Where, Dc = mycelial growth of test phytopathogens on control plate, and Dt = mycelial growth of test phytopathogens on the test plate. Each test was performed in triplicates, and the results are presented as the mean ± standard error.

Identification of endophytic fungi

Microscopic identification and plate morphology were used to identify the isolated fungal endophytes. The morphological and microscopic characteristics of the potent culture was observed on five different media, including PDA (Hi-media, India), Mueller Hinton Agar (MHA) (Hi-media, India), Corn Meal Agar (CMA) (Hi-media, India), Water Agar (WA) (Hi-media, India), and Rose Bengal Agar (RBA) (Hi-media, India). Morphological features like colony development patterns, including form, elevation, texture, margins, colour, and other characteristics, were observed for identification. The mycelia mass was picked with a sterile needle for microscopic characterization and placed on a clean slide. The culture was then teased with the help of a needle and stained using lactophenol cotton blue. Then the culture was carefully covered with a cover slip (18 x 10 mm). The prepared slide was viewed under a Nikon binocular microscope at 10X, 40X and 100X (Meshram et al., 2013).

In-vitro antioxidant activity

Production of endophytic fungi

Submerged fermentation of isolated endophytic fungi was done in 100ml flasks containing 30ml of PDB. For each 30 mL of culture medium, 5 mm diameter discs of 7-day-old fungal cultures were added as the inoculum. The inoculated bottles were kept in an orbital shaker to maintain the submerged cultures for seven days at 120 rpm and 25°C. After fermentation, mycelium and supernatant were separated using Whatman filter paper (Druzian et al., 2020).

Solvent-solvent extraction of bioactive compounds

Solvent-solvent extraction of culture filtrates was performed using ethyl acetate (RANKEM, India). The culture filtrate was mixed with ethyl acetate in a ratio of 2:1 and vigorously shaken for 1-2 minutes. The organic layer was taken in a crucible and dehydrated using anhydrous sodium sulphate, and the ethyl acetate was evaporated with a rotator evaporator. After complete evaporation, the dried crude fraction was mixed with methanol (RANKEM, India), and stored at 4°C till further use.

DPPH (2,2-diphenyl-1-picrylhydrazyl)

The antioxidant activity of the culture filtrate/solvent fractions and their ability to scavenge free radicals were assessed using DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals, with slight modifications to a method previously described by Kaur and Saxena (2023).In a nutshell, 230 µl of DPPH (100 µM in methanol) was added to 20 µl of the 1 mg/ml test sample (200-1000 mg/ml dilution) and thoroughly mixed. The mixture was allowed to stand at room temperature in the dark for 30 minutes. Then ELISA reader (Biotek, USA) was used to measure the absorbance at 517 nm. Working DPPH was used as the control, while quercetin (10–50 µg/ml) was used as the standard. The DPPH radical scavenging capacity was expressed as micrograms of quercetin equivalents per milligram of extract. The test was performed in triplicate, and the data was reported as mean ± SD. The fraction of the fungal extract that can scavenge free radicals was estimated as follows:

$$\% \quad = \frac{\text{Absorbance(Control)} - \text{Absorbance(Sample)}}{\text{Absorbance(Control)}} \text{ x } 100$$

FRAP (Ferric ion reducing antioxidant power)

To prepare FRAP reagent, 0.3M sodium acetate buffer, 10 mM 2, 4, 6-tripyridyl-s-triazine, and 20 mM Iron (III) chloride hexahydrate were mixed in the following proportions: 10:1:1 (Benzie and Strain, 1996). Then 1 ml of FRAP reagent was added to 10 µl of 1 mg/ml extract. The reaction mixture was incubated for 30 minutes at room temperature then the absorbance at 595 nm was measured using ELISA reader. Ascorbic acid was used as a standard with concentrations ranging from 10 to 50 g/ml, and a working solution of FRAP in deionized water was used as a blank. In order to determine the concentration of the sample, a linear regression between the standard concentrations and their absorbance was plotted. The ferric reducing antioxidant power was measured as µg ascorbic acid equivalent per mg of extract. The test was performed in triplicate and the data was reported as mean ± SD.

Total phenolic content (TPC)

Using the Folin-Ciocalteu (FC) reagent, the total phenolic content was calculated (Mashkor, 2015). 1.5 ml of deionized water and 100 µl of FC reagent were added to 100 µl of 1 mg/ml fungal extract. The mixture was incubated at room temperature for 10 minutes. Then, 200 µl of Na_2CO_3 (6% w/v) was added to reaction mixture and left to stand for an hour at room temperature. Gallic acid with

concentration ranging from 10–100 µg/ml was used as standard. After incubation, the absorbance was measured at 760 nm using an ELISA reader. The concentration of the sample was determined by plotting a linear regression between the standard values and their absorbance. Total phenolic content was expressed as µg of Gallic acid equivalent per mg of sample. The test was performed in triplicate and the data was reported as mean ± SD.

Total flavonoid content (TFC)

The total flavonoid content of the fungal extracts was determined using Aluminum chloride colorimetric assay (Mashkor, 2015). 800 µl of deionized water, 60 µl of NaNO2 (5% w/v), and 200 µl of 1 mg/ml fungal extract were added. The reaction mixture was incubated at room temperature for 5 minutes. After the addition of 60 µl of $AlCl_3$ (10% w/v), the reaction mixture was again incubated for 1 minute at room temperature. Then, 400 µl of 1N NaOH was added to reaction mixture, and the volume was made up to 2 ml using distilled water. For standard, quercetin at various concentrations (50–250 g/ml) was used then at 510 nm the absorption was measured. The concentration of the sample was determined by plotting a linear regression between the standard values and their absorbance. Total flavonoid content was expressed as µg of Quercetin equivalent per mg of sample. The test was performed in triplicate and the data was reported as mean ± SD.

Statistical analysis

The results of each experiment were presented as mean ± standard deviation, and each experiment was carried out in triplicate. The data were examined in Graph Pad Prism 9 using one-way ANOVA, followed by a Tukey's post hoc test ($P < 0.05$). Along with this, correlation between antifungal and antioxidant activity was calculated using Pearson correlation coefficient using SPSS software.

Chapter 5

Results

Isolation and maintenance of endophytic fungi

A total of 23 fungal endophytes were isolated from the leaves and stem of *S. aromaticum* plant where 4 endophytes were isolated from stem and 19 from leaf (Fig. 5). Based on the name of the plant, the part of the plant, the location where the sample was collected, and the isolation number, each isolated fungal endophytes were given a special code mentioned in Table 3. To preserve the cultures, they were regularly sub-cultured on new PDA plates and kept at 25 ± 2°C for 7-8 days. Fig. 4 shows the clove plant and some of the endophytic fungi grown for this study are shown in Fig. 6a. Fig. 6b shows the microscopic view of some of the isolated endophytic fungi.

Fig. 4: Clove plant collected from site

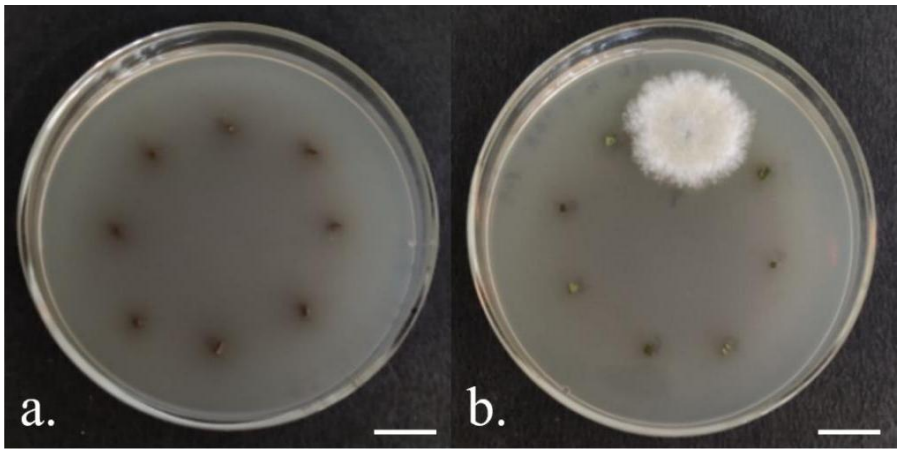


Fig. 5: a. leaf/stem segments placed on PDA plate for isolation of endophytic fungi; b. endophytic fungi emerging out of segments (Bar- 10mm)

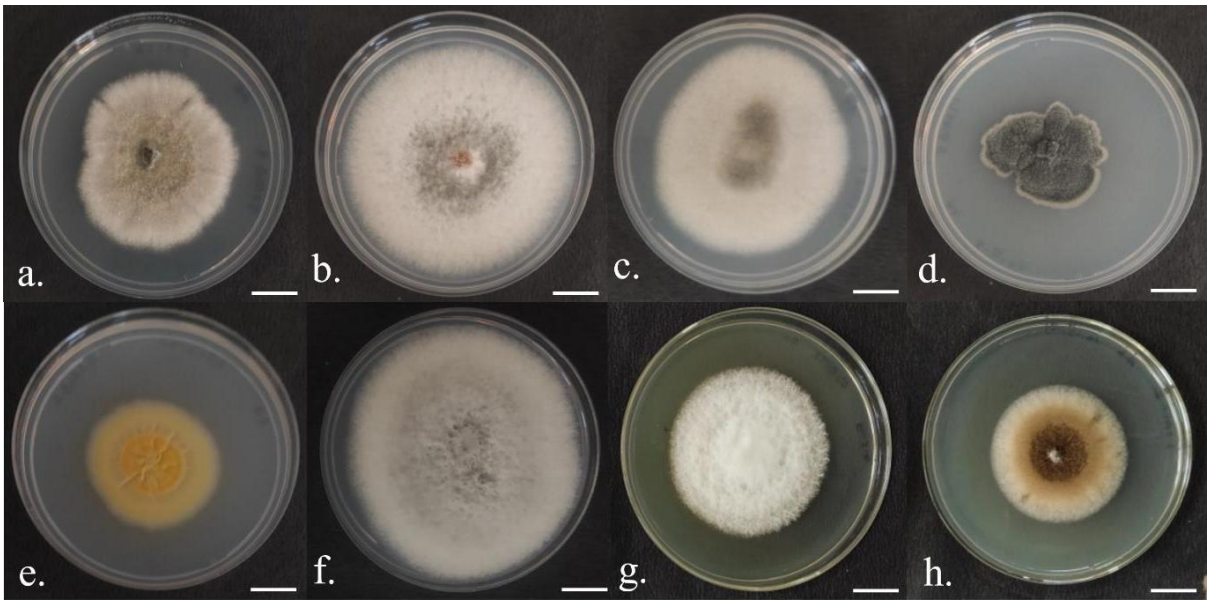


Fig. 6(a): Some of the pure culture of endophytic fungi isolated during the study a) #9SALB8 b) #18SALB6 c) #15SALB6 d) #17SALB5 e) #7SALB3 f) #17SALB2 g) #8SALB1 h) #1SALB2

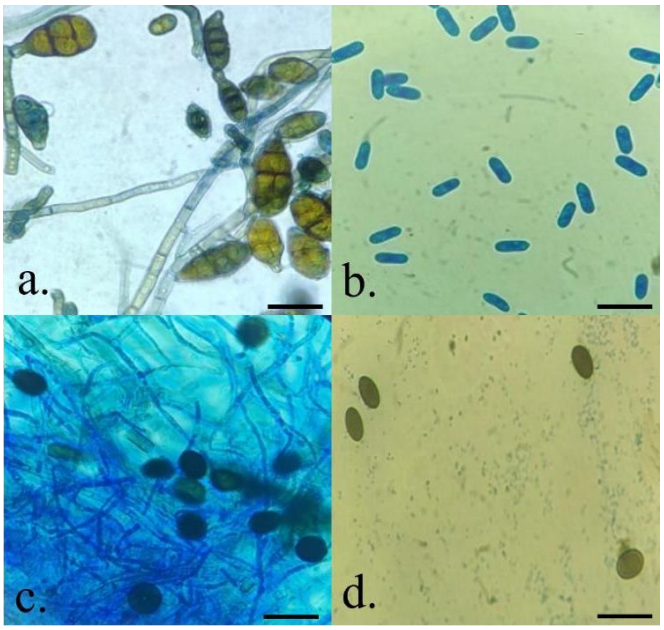


Fig. 6(b): Microscopic view of some of the isolated endophytic fungi from *S. aromaticum*. a. *Alternaria* sp., b. *Colletotrichum* sp., c. *Paraconiothyriumn* sp., d. *Nigrospora* sp. (Bar- 10µm)

Table 3: Morphological features of endophytic fungi isolated from *S. aromaticum*

S. No	Culture code	Front colour	Back colour	Form	Elevation	Margin	Diameter(In cm)
1.	3SALB1	Core- Greenish Yellow Borders- White	Core- Yellow Borders- White	Filamentous	Flat	Curled	7.1
2.	7SALB3	Core- Yellow Borders- Off White	Yellow	Circular	Flat	Curled	4.5
3.	9SALB8	Core- Olive Green Borders-White	Core- Blackish Green Borders- Off White	Irregular	Flat	Undulate	5.8
4.	12SALB7	Black	Black	Irregular	Raised	Undulate	3.65
5.	12SALB8	Brown and White	Core- Brown Border- White	Irregular	Raised	Undulate	3.5
6.	13SALB1	Grey	Blackish Grey	Irregular	Umbonate	Undulate	1.5
7.	13SALB2	Core- Grey Borders- White	Off White	Filamentous	Convex	Filiform	2.5
8.	14SALB6	Brownish White	Brown	Circular	Flat	Entire	8
9.	15SALB6	Core- Brown Borders- Whitish	Off White	Circular	Raised	Entire	6.3
10.	15SALB7	Brownish- Grey	Grey	Circular	Raised	Entire	6

11.	17SALB2	White	Core- Greenish Grey Borders- Off White	Circular	Flat	Entire	8.5
12.	17SALB4	White	Off White	Irregular	Umbonate	Undulate	1.6
13.	17SALB5	Grey	Black- Grey	Irregular	Raised	Undulate	3.8
14.	18SALB6	Core- Greyish Borders- White	Off White	Circular	Raised	Entire	8
15.	19SALB2	Yellowish White	Brown	Rhizoid	Flat	Filiform	6.5
16.	19SALB3	Olive Green	Olive Green	Circular	Flat	Undulate	6.4
17.	19SALB7	White	Core- Yellow Borders- Off White	Irregular	Raised	Undulate	1.6
18.	1SASB5	White	Borders- Off White Core- Brown	Filamentous	Flat	Entire	5.8
19.	2SASB6	White	Off-White	Circular	Flat	Entire	7.25
20.	1SASB1	Off- White	Off White	Rhizoid	Flat	Lobate	4.25
21.	2SASB8	Grey-White	Black	Filamentous	Raised	Filiform	7.5
22.	8SALB1	White	Off-White	Filamentous	Raised	Filiform	5.8
23.	1SALB2	White Core- Brown	Core- Brown Borders- Off White	Circular	Raised	Entire	6.5

Preservation of endophytic fungi

PDA vials or slants containing 10% (v/v) glycerol were used to maintain the isolates. Under aseptic conditions, the 7–8-day-old culture of isolates was point inoculated on the prepared PDA slants, and incubation at 25 ± 2°C for 10–12 days (Fig. 7). The vials were then stored at 4°C.

Fig. 7: Preserved endophytic fungi on slants

In-vitro antifungal assay

Preliminary screening

In preliminary test the antifungal activity of VOCs produced by all the 23 isolates were studied against the selected set of phytopathogens namely *A. alternata* (6343 ITCC), *F. lateritium* (4533 ITCC), *C. gloeosporioides* (6152 ITCC), *B. cinerea* (6011 ITCC), *F. moniliforme* (6435 ITCC), *B. theobromae* (5597 ITCC), and *A. niger* (6354 ITCC). Out of 23 isolated endophytes 2 endophytes, i.e., #8SALB1 and #1SALB2 showed the highest antifungal activity (Fig. 8). Based on the dual-culture assay performed during primary screening the endophytic fungi with code #8SALB1and #1SALB2 suppressed the growth of the selected group of phytopathogens on PDA plates with percentage inhibition range from 45% to 61% (Table 4). The results also showed that the VOCs produced by endophytic fungi exhibited fungi-static properties.

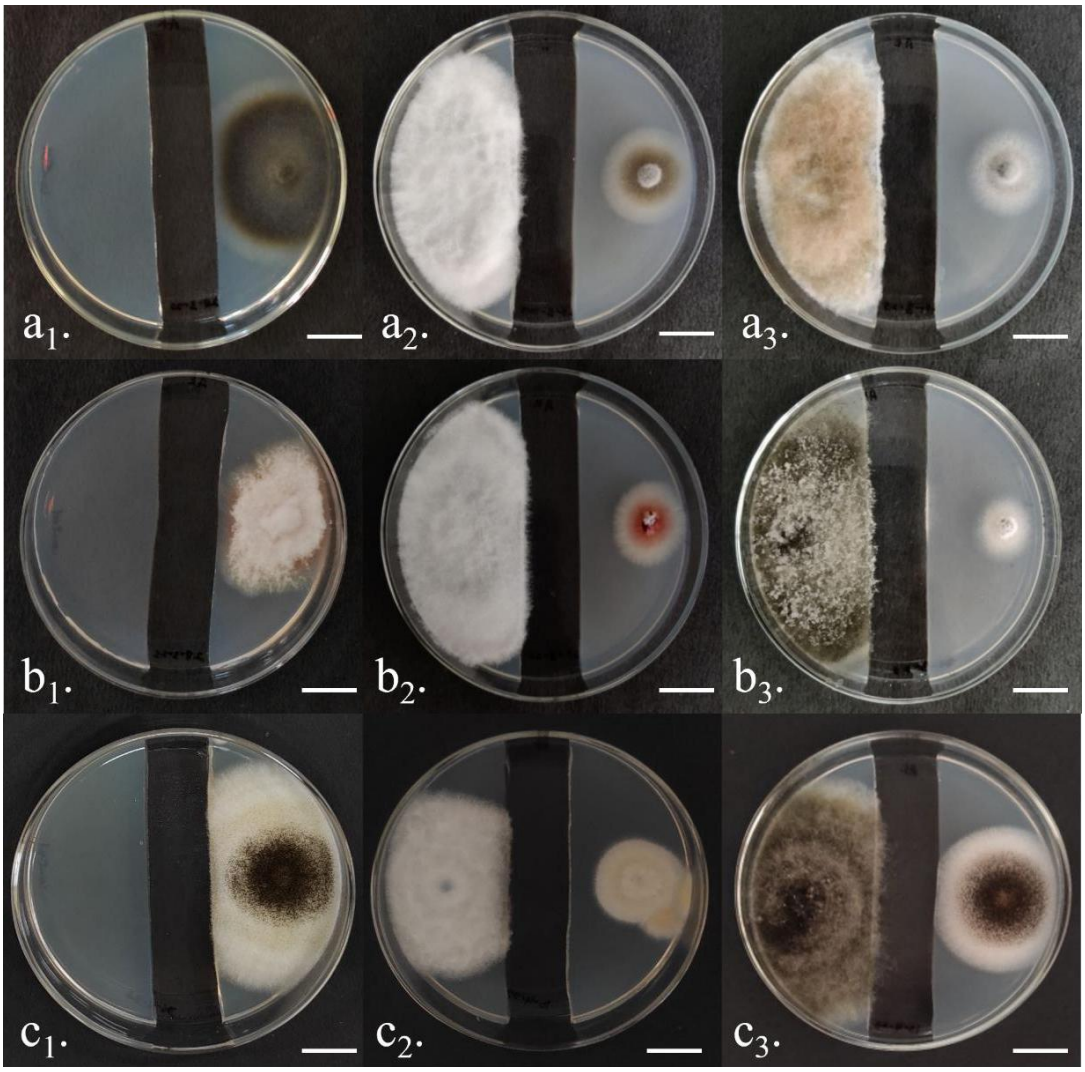


Fig. 8: Dual culture assay of potent endophytic fungi against phytopathogenic fungi. a1, b1, and c1: control plate of *A. alternata*, *F. moniliforme*, and *A. niger*, respectively; a2, b2, and c2: test plate of #8SALB1 against *A. alternata*, *F. moniliforme*, and *A. niger*; a3, b3, and c3: test plate of #1SALB2 against *A. alternata*, *F. moniliforme*, and *A. niger* (Bar- 10mm)

Table 4: % inhibition shown by endophytic fungi against selected pathogens

Endophytic fungi	Percentage inhibition (%)						
	6343ITCC	**6435ITCC**	**4533ITCC**	**6354ITCC**	**5597ITCC**	**6152ITCC**	**6011 ITCC**
17SALB4	$4.09^{fgh} \pm 0.18$	$10.76^{defghi} \pm 0.64$	$9.63^{ef} \pm 0.68$	$2.46^{jk} \pm 0.36$	$21.49^{cdefg} \pm 0.74$	$1.82^{i} \pm 0.59$	$1.59^{i} \pm 0.61$
17SALB2	$28.67^{b} \pm 0.22$	$24.52^{cd} \pm 0.62$	$38.23^{b} \pm 0.53$	$43.54^{ab} \pm 0.70$	$30.58^{bc} \pm 0.91$	$41.03^{b} \pm 1.01$	$14.29^{efgh} \pm 0.51$
3SALB1	$12.90^{cdefgh} \pm 0.26$	$38.70^{b} \pm 0.75$	$7.34^{jkl} \pm 0.85$	$0.90^{k} \pm 0.37$	$0.83^{ij} \pm 0.50$	$1.52^{i} \pm 0.65$	$31.43^{bc} \pm 0.80$
15SALB6	$20.79^{bcd} \pm 0.78$	$14.93^{defgh} \pm 0.35$	$27.83^{cd} \pm 0.66$	$10.81g^{hijk} \pm 0.90$	$24.79^{cde} \pm 0.65$	$9.70^{ghi} \pm 0.97$	$21.27^{de} \pm 0.11$
13SALB2	$18.42^{bcde} \pm 0.71$	$10.34^{efghi} \pm 0.41$	$11.31^{hij} \pm 0.48$	$6.01^{hijk} \pm 1.33$	$19.83^{cdefg} \pm 1.23$	$5.15^{hi} \pm 0.28$	$1.59^{i} \pm 0.42$
19SALB3	$1.79^{h} \pm 0.32$	$15.76^{defg} \pm 0.63$	$1.83^{l} \pm 0.42$	$34.35^{bc} \pm 0.64$	$26.45^{bcd} \pm 0.14$	$36.36^{bc} \pm 1.14$	$22.86^{cde} \pm 0.27$
19SALB7	$16.13^{bcdefg} \pm 0.42$	$22.44^{de} \pm 0.93$	$28.44^{cd} \pm 0.56$	$21.32^{def} \pm 0.47$	$5.21^{hij} \pm 0.79$	$20.00^{defg} \pm 1.01$	$8.57^{fghi} \pm 0.68$
17SALB5	$3.66^{gh} \pm 0.71$	$1.58^{hi} \pm 0.23$	$17.80^{efgh} \pm 0.47$	$19.82^{defg} \pm 0.59$	$13.22^{efghi} \pm 0.48$	$22.12^{def} \pm 1.15$	$21.59^{cde} \pm 0.53$
13SALB1	$8.96^{defgh} \pm 0.99$	$3.67^{ghi} \pm 0.69$	$9.48^{ijk} \pm 0.91$	$23.96^{de} \pm 1.15$	$21.90^{cdef} \pm 0.02$	$37.52^{bc} \pm 0.80$	$6.35^{ghi} \pm 0.84$
7SALB3	$18.28^{bcde} \pm 0.59$	$13.26^{defghi} \pm 0.35$	$011.93^{ghij} \pm 0.37$	$7.51^{hijk} \pm 0.89$	$11.98^{fghij} \pm 0.61$	$7.58^{hi} \pm 0.40$	$21.90^{cde} \pm 0.62$
18SALB6	$9.32^{defgh} \pm 0.18$	$0.33^{i} \pm 0.52$	$19.27^{efg} \pm 0.76$	$28.53^{cd} \pm 0.47$	$3.31^{hij} \pm 0.54$	$28.79^{bcd} \pm 0.45$	$38.10^{b} \pm 0.36$
9SALB8	$17.85^{bcde} \pm 0.50$	$18.27^{def} \pm 0.98$	$12.78^{fghij} \pm 0.92$	$15.44^{efgh} \pm 0.48$	$0.91^{ij} \pm 0.49$	$15.27^{efgh} \pm 0.74$	$0.95^{i} \pm 0.21$
12SALB8	$20.79^{bcd} \pm 0.53$	$14.60^{defgh} \pm 0.83$	$32.42^{bc} \pm 0.40$	$12.91^{fghi} \pm 0.68$	$11.16^{fghij} \pm 1.21$	$11.82^{fghi} \pm 0.67$	$40.00^{b} \pm 0.08$
1SALB2	$54.84^{a} \pm 0.43$	$61.30^{a} \pm 0.65$	$50.46^{a} \pm 0.84$	$51.05^{a} \pm 0.76$	$56.69^{ab} \pm 0.56$	$56.97^{a} \pm 1.17$	$55.24^{a} \pm 1.55$

8SALB1	52.33^{a} ±1.04	56.13^{a} ± 0.26	52.29^{a} ±1.04	46.25^{a} ± 0.64	51.57^{a} ± 0.96	54.55^{a} ± 0.70	59.05^{a} ± 1.05
1SASB5	6.81^{efgh} ± 0.59	37.86^{bc} ± 0.40	17.43^{efgh} ± 1.02	8.71^{hijk} ± 0.46	0.58^{j} ± 0.35	26.06^{cde} ± 1.05	3.43^{i} ± 0.65
1SASB2	21.94^{bc} ± 0.44	8.51^{efghi} ± 0.88	15.05^{fghi} ± 0.74	2.70^{ijk} ± 0.51	29.59^{bcd} ± 0.75	1.82^{i} ± 0.36	4.76^{hi} ± 0.64
2SASB8	16.63^{bddef} ± 0.83	10.09^{fghi} ± 0.54	23.36^{de} ± 0.43	22.52^{def} ± 0.68	17.93^{defg} ± 0.27	23.03^{def} ± 0.67	15.24^{efg} ± 0.86
3SALB8	2.15^{h} ± 0.59	19.02^{def} ± 1.15	3.18^{kl} ± 0.41	11.11^{ghij} ± 1.31	13.64^{efgh} ± 0.54	26.06^{cde} ± 1.36	3.49^{i} ± 0.82
2SASB6	18.64^{bcde} ± 0.16	5.92^{fghi} ± 0.75	0.31^{l} ± 0.48	3.30^{ijk} ± 0.40	38.02^{b} ± 0.18	4.55^{hi} ± 0.70	0.63^{i} ± 0.24
15SALB7	0.93^{h} ± 0.19	0.33^{i} ± 0.56	15.47^{fghi} ± 0.91	25.83^{cd} ± 0.68	9.09^{ghij} ± 1.34	27.88^{cd} ± 0.95	16.83^{ef} ± 0.48
12SALB7	8.24^{defgh} ± 0.68	4.09^{ghi} ± 0.77	3.36^{kl} ± 0.36	7.81^{hijk} ± 0.51	13.22^{efghi} ± 0.46	6.97^{hi} ± 0.40	0.95^{i} ± 0.43
14SALB6	22.80^{bc} ± 0.88	40.78^{b} ± 0.73	34.13^{bc} ± 0.78	2.10^{jk} ± 0.72	26.86^{bcd} ± 0.79	0.30^{i} ± 0.55	26.98^{cd} ± 0.34

The ability of clove extract volatiles to suppress the growth of selected set of pathogenic fungi was ascertained. Antifungal potential of clove extract in two different solvents i.e., 80% acetone and CCl_4 was tested. Clove extract prepared in 80% acetone exhibited better antifungal activity with percentage inhibition ranging from 40% to 60% (Fig. 9). On the other hand, clove extract prepared in CCl_4 evinced no antifungal activity. Therefore, this could imply that the bioactive compounds present in clove are more semi-polar than non-polar. It can also be concluded that antifungal activity of isolated endophytic fungi is comparable to that of clove extract (80% acetone).

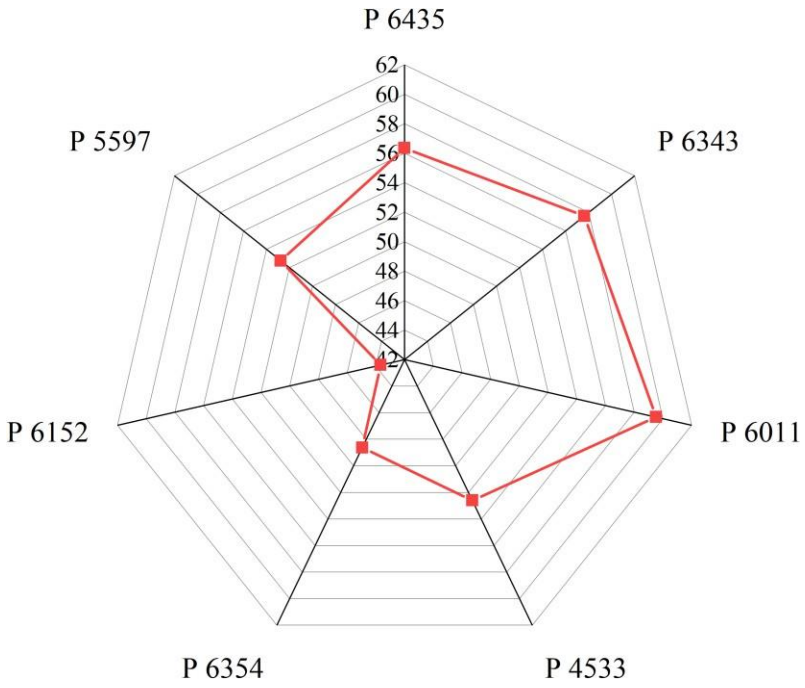


Fig. 9: Percentage inhibition shown by clove extract (80% acetone) against selected set of pathogens

Secondary screening

The sandwich plate method was used to examine the effect of VOCs released by potent cultures #1SALB2 and #8SALB1 on the growth of the test phytopathogens. After five days of incubation the colony diameter of each test pathogen was smaller in test plates than those in the control plates (Fig. 10). The results showed that endophytic fungi #1SALB2 has higher antifungal activity ranging from 52.21%-69.23% whereas endophytic fungi #8SALB1 has antifungal activity ranging from 50.44%-66.43% (Table 5), which confirmed that the VOCs released by #1SALB2 have higher bioactivity.

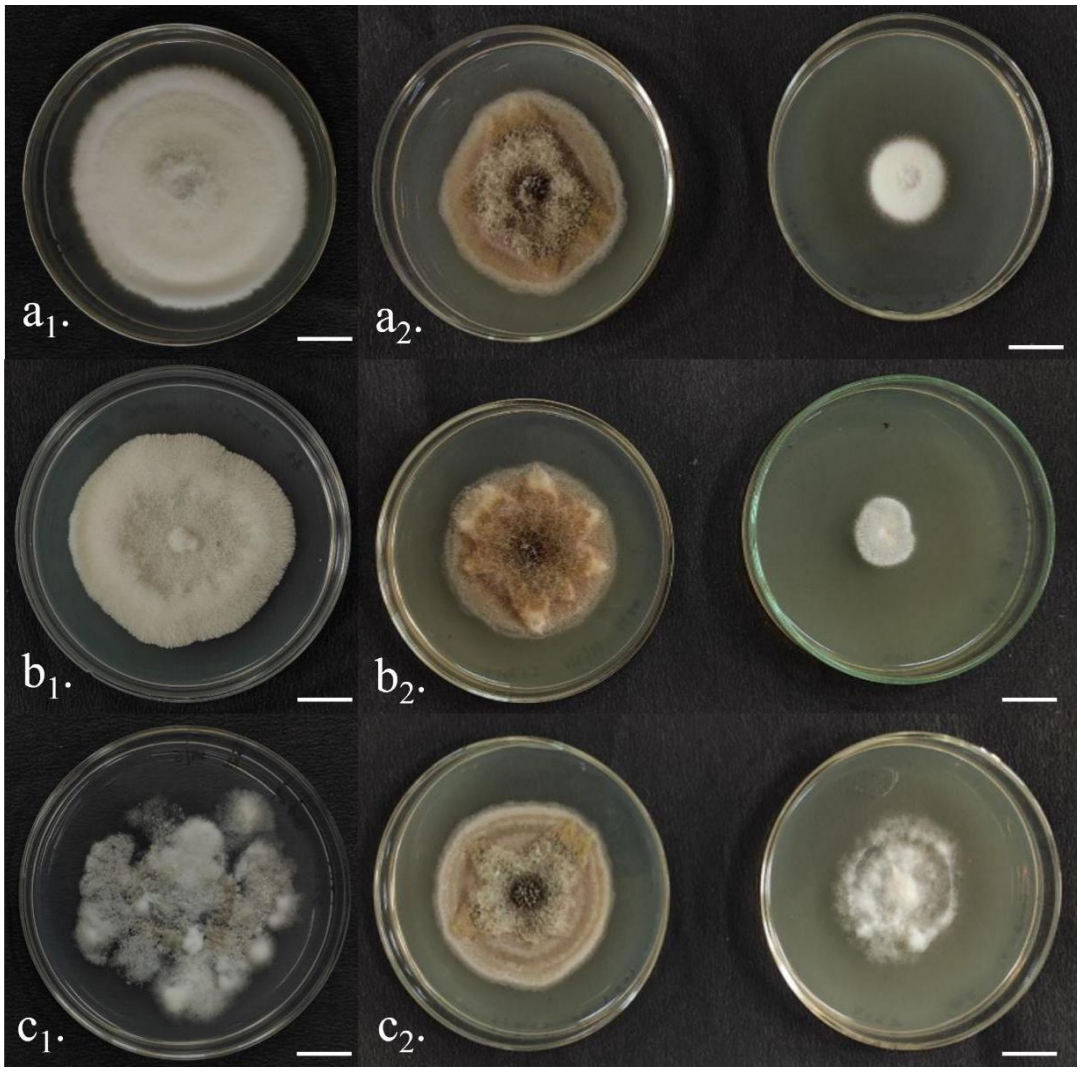


Fig. 10: Inhibition shown by phytopathogenic fungiagainst#1SALB2. a1, b1, c1: Control plates of *B. cinerea*, *F. lateritium*, and *B. theobromae*, respectively; a2, b2, and c2: #1SALB2; a3, b3, and c3: test plates of *B. cinerea*, *F. lateritium*, and *B. theobromae*, respectively (Bar- 10mm)

Table 5: % inhibition shown by endophytic fungi against selected pathogens

Pathogen	Percentage inhibition (%)	
(ITCC)	8SALT1	1SALT2
6343	56.10± 0.79	68.78± 1.43
6435	55.08± 0.68	62.72± 0.54
4533	50.96± 0.64	52.21± 0.72
6354	50.44± 1.09	65.59± 0.77

5597	53± 0.69	55.68± 0.50
6152	66.43± 0.62	69.23± 0.78
6011	60.85± 1.62	68.63± 0.84

Production of secondary metabolites

In the potato dextrose broth medium, the fungal endophytes isolated from *S. aromaticum* were subjected to the synthesis of secondary metabolites.

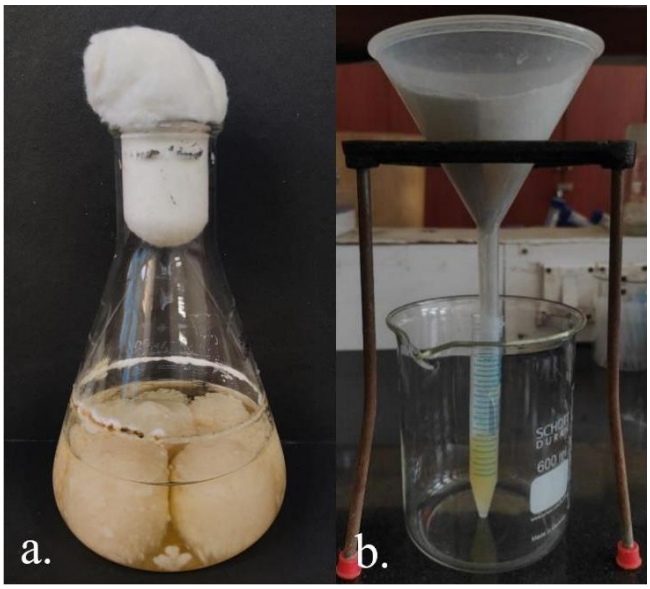


Fig.11:a) Culture filtrate produced by endophytic fungi #1SASB2. b) Filtration of culture filtrate

Identification of potent cultures

Morphotaxonomy was used to identify the isolate #1SALB2 which is the potent cultures evaluated in the study.

1. **#1SALB2:** The endophytic fungus #1SALB2 produced brown-white colony (90 ± 0mm) mostly consisting of a dense felt of erect and aerial conidiophores over PDA. Colonies are woolly, due to the presence of numerous hyphae which are brown from core and white from edges initially and later becomes brown to black with a white apron on the top over PDA. The reverse side is white to tan which later become brown. Conidiophores are erect and short with swollen apex and has terminal scar which indicate the attachment of conidia. Conidia are olive green, multi-celled, smooth walled, obcalavate to obpyriform, simple and branched and possess 2-3 longitudinal

septa and 4-8 transverse septa with short conical beak. Morphological and microscopic features of the isolate can be seen in Fig. 12 Based on these morphological features the fungus was identified as *Alternaria* sp. Fig. 13 shows the morphology of endophytic fungi on different media including ½PDA, MHA, Water Agar, CMA, and RBA.

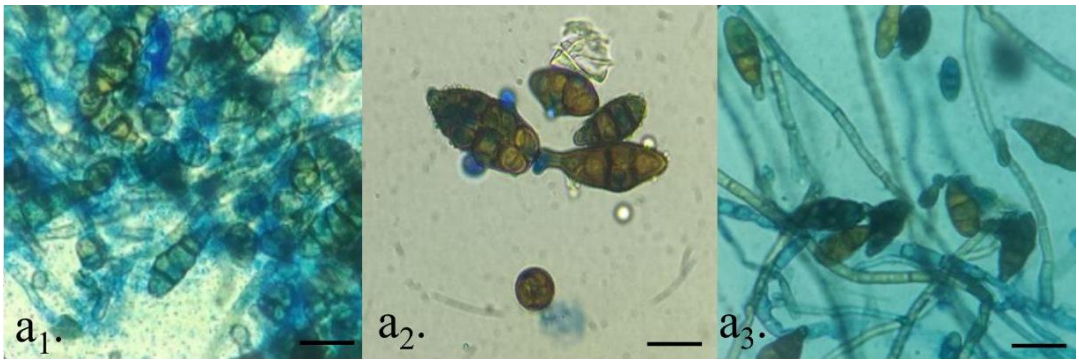


Fig. 12: Microscopic feature of #1SALB2. a_1-a_3: structure of spores and hyphae (Bar 10µm)

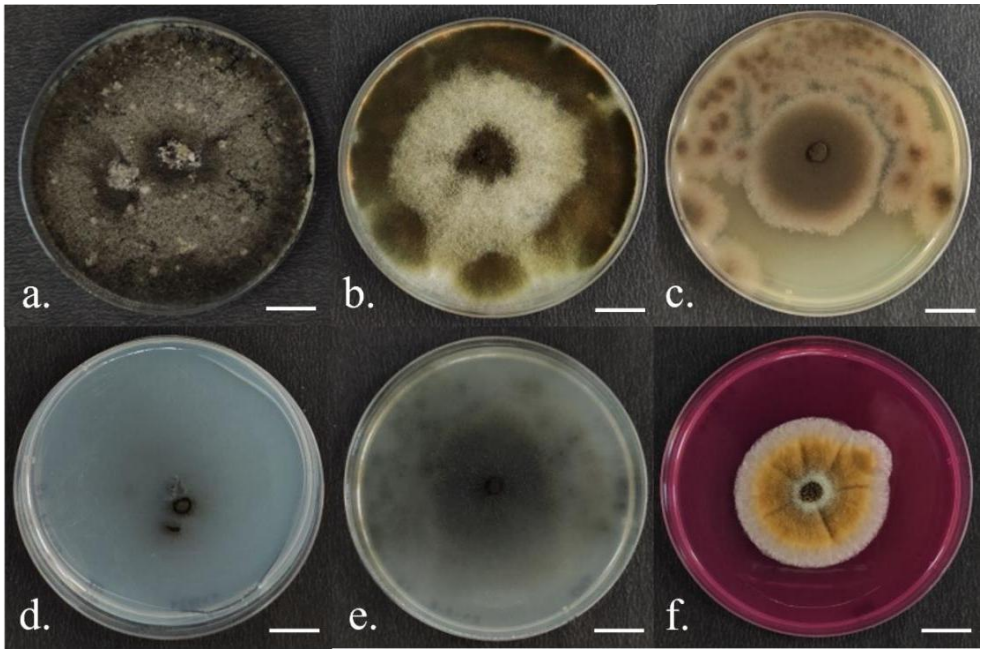


Fig. 13:a. 10-day-old culture of #1SALB2 on PDA; Morphology of endophytic fungi #1SALB2 on different media. b. ½ PDA, c. MHA, d. WA, e. CMA, f. RBA (Bar- 10mm)

2. **8SALB1:** The endophytic fungus #8SALB1 displayed a white-coloured colony with a diameter of 90 ± 0mm, predominantly circular in shape, and possessing a smooth margin. The colony displayed a floccose texture and showed rapid growth on potato dextrose agar. The formation of woolly colonies was attributed to the presence of multiple smooth, branched, septate, and

hyaline hyphae. Conidiophores often exhibit unbranched, erect, and originating. The conidia observed on the aerial mycelia were characterized by their brownish black colour, oblate spheroid shape, single-celled structure, and smooth surface. Fig. 14 displays the morphological and microscopic characteristics. The identification of the fungus as a member of *Nigrospora* sp. was made based on microscopic characteristics.

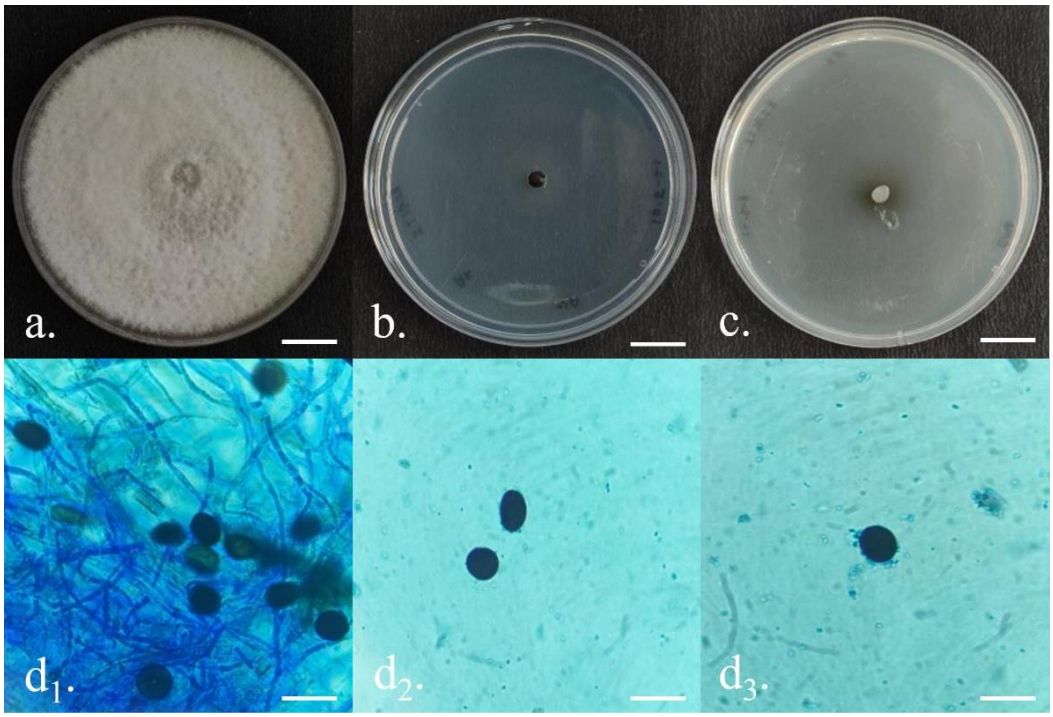


Fig. 14: Morphology of endophytic fungi #8SALB1on different media. a. ½ PDA, b. WA, c. CMA(Bar- 10mm); Microscopic feature of #8SALB1.d_1 -d_3: structure of spores and hyphae (Bar 10µm)

In-vitro antioxidant activity

The following assays were used to evaluate the antioxidant capacity of the endophytic fungi isolated during this study and clove extract.

DPPH

The culture #2SASB8 showed highest antioxidant capacity as compared to the other samples and the standard quercetin. The IC_{50} value of #2SASB8 came out to be 14.88 ± 0.11 µg/ml followed by #12SALB7, exhibiting IC_{50} value of 32.52 ± 0.10 µg//ml and clove extract has IC_{50} of 8.51 ± 0.42 µg/ml

(As shown in Table 5b and Fig. 15). Whereas the IC_{50} value of standard Quercetin came out to be 0.375 ± 10.5 which was higher than all the samples. #14SALB6 exhibited least IC_{50} value of 50.36 ± 0.20 µg/ml.

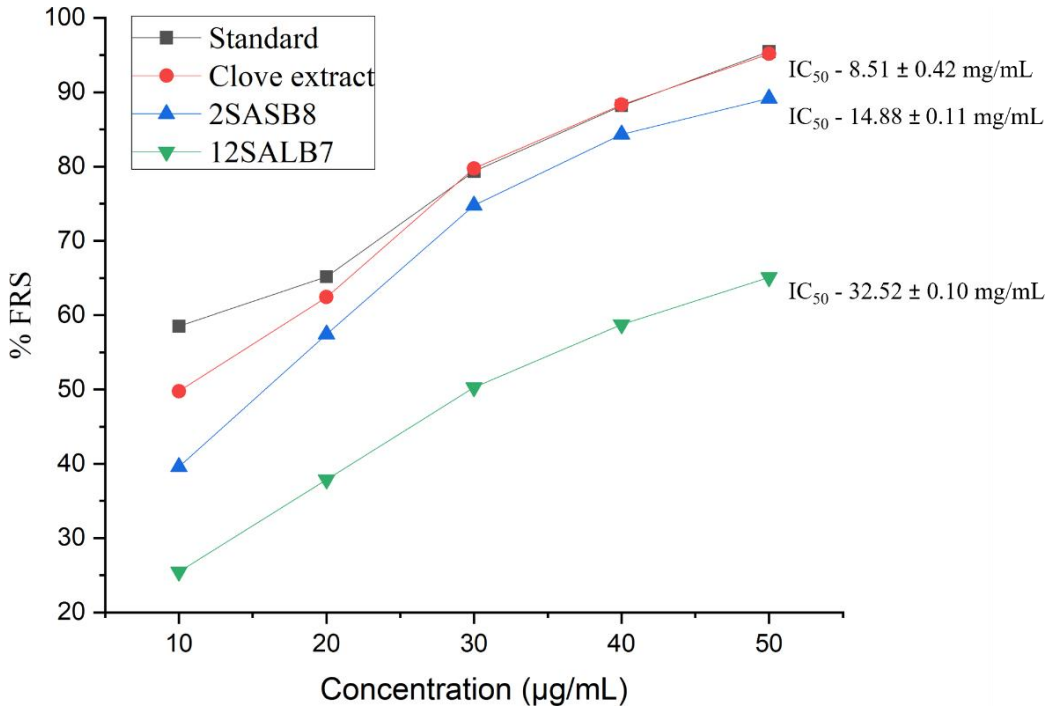


Fig. 15:% FRS of potent cultures and clove extract at different concentration (mg/ml) analysed using DPPH assay

FRAP

The maximum Fe (II) ion reducing capacity was that of #2SASB8 at 49.16± 0.10 µg Fe (II) equivalent per mg of sample, followed by #12SALB7 at 44.18± 0.06 µg Fe (II) equivalent per mg of sample. The Fe (II) ion reducing capacity of clove extract was 58.12± 0.05 µg Fe (II) equivalent per mg of sample (As shown in Table 5a and 5b) which was evaluated from the equation (y= 0.002x + 0.472). The least reducing capacity was that of #14SALB6 at 15.94 ± 0.035 µg Fe (II) equivalent per mg of sample.

TPC

The total phenolic content was highest in #2SASB8 at 243.8 ± 0.005µg Gallic acid equivalent per mg of sample followed by #12SALB7 at 191.8 ± 0.071µg Gallic acid equivalent per mg of sample. The TPC in clove extract was 196.3 ± 0.009µg Gallic acid equivalent per mg of sample. The least

phenolic content was seen in #14SALB6 at 23 ± 0.002 µg Gallic acid equivalent per mg of sample (As shown in Table 5a and 5b). The concentration was evaluated from the equation (y=0.004x + 0.02).

TFC

Total flavonoid content was seen maximum in the isolate #12SALB7 at 108 ± 1.00 µg Quercetin equivalent per mg of sample followed by #2SASB8 at 93.23 ± 0.28µg Quercetin equivalent per mg of sample. Clove extract has flavonoid content at 46.71 ± 0.97µg Quercetin equivalent per mg of sample (As shown in Table 6a and 6b). The concentration was evaluated from the equation y = 0.002x + 0.031.

Table 6a: TFC, TPC, and FRAP value of isolated endophytic fungi

Culture	TFC	TPC	FRAP
Clove extract	$46.71^{n}\pm 0.81$	$196.13^{c}\pm 0.49$	$58.12^{a}\pm 0.11$
19SALB3	$57.57^{mn}\pm 0.80$	$7.96^{o}\pm 0.53$	$27.47^{c}\pm 0.52$
3SALB1	$58.15^{mn}\pm 0.20$	$29.38^{mn}\pm 0.41$	$18.80^{ef}\pm 0.45$
7SALB3	$64.68^{m}\pm 0.92$	$63.55^{hi}\pm 0.69$	$24.82^{cde}\pm 0.66$
1SALB8	$69.31^{lm}\pm 0.55$	$39.55^{klm}\pm 0.85$	$16.70^{f}\pm 0.28$
19SALB7	$80.91^{kl}\pm 0.67$	$78.3^{gh}\pm 0.33$	$16.52^{f}\pm 0.33$
18SALB6	$91.78^{jk}\pm 0.88$	$32.63^{lmn}\pm 0.53$	$16.64^{f}\pm 0.56$
2SASB8	$93.23^{ijk}\pm 0.41$	$243.8^{b}\pm 0.52$	$49.16^{b}\pm 0.36$
14SALB6	$95.40^{ijk}\pm 0.43$	$22.8^{no}\pm 0.71$	$15.94^{f}\pm 0.64$
1SALB2	$101.05^{ij}\pm 0.13$	$65.46^{hi}\pm 0.58$	$18.15^{f}\pm 0.59$
12SALB7	$108.449^{hi}\pm 0.89$	$191.8^{c}\pm 0.69$	$44.18^{b}\pm 0.57$
2SASB6	$117.43^{gh}\pm 0.39$	$96.46^{f}\pm 0.47$	$18.02^{f}\pm 0.18$
17SALB4	$121.92^{gh}\pm 0.58$	$53.63^{ijk}\pm 0.67$	$18.63^{f}\pm 0.39$
1SASB2	$126.71^{fg}\pm 0.71$	$61.3^{ij}\pm 0.55$	$18.54^{f}\pm 0.40$
8SALT1	$137.72^{ef}\pm 0.49$	$45.96^{jkl}\pm 0.69$	$15.74^{f}\pm 0.33$
17SALB2	$137.72^{ef}\pm 0.60$	$189.13^{c}\pm 0.88$	$27.38^{c}\pm 0.43$
9SALB8	$143.52^{de}\pm 0.62$	$266.63^{a}\pm 0.25$	$27.47^{c}\pm 0.56$
18SALB2	$152.50^{de}\pm 0.96$	$32.63^{ij}\pm 0.87$	$16.766^{f}\pm 0.47$
13SALB1	$153.52^{d}\pm 0.29$	$62.05^{i}\pm 0.35$	$17.40^{f}\pm 0.38$

15SALB7	154.10^{d}± 0.39	100.63^{f}± 0.65	17.72^{def}± 0.49
12SALB8	169.46^{c}± 0.74	92.3^{fg}± 0.70	18.36^{f}± 0.44
2SASB3	169.89^{c}± 0.13	156.05^{d}± 0.72	17.46^{f}± 0.46
1SASB5	206.42^{b}± 0.41	125.05^{e}± 0.60	25.52^{cd}± 0.42
17SALB5	289.02^{a}± 0.68	126.63^{e}± 0.58	19.65^{def}± 0.63

Table 6b: TPC, TFC, FRAP and IC_{50} value of top cultures and clove extract

S. No	Culture	TPC Conc.(µg Gallic acid equivalent / mg of sample)	TFC Conc. (µg Quercetin equivalent / mg of sample)	FRAP Conc.(µg Fe (II) equivalent / mg of sample)	DPPH IC_{50} (mg/ml)
1.	**#2SASB8**	$243.8^{b} \pm 0.005$	$93.23^{ijk} \pm 0.28$	$49.16^{b} \pm 0.10$	$14.88^{b} \pm 0.11$
2.	**#12SALB7**	$191.8^{c} \pm 0.071$	$108^{hi} \pm 1.00$	$44.18^{b} \pm 0.06$	$32.52^{a} \pm 0.10$
3.	**Clove extract**	$196.3^{c} \pm 0.009$	$46.71^{n} \pm 0.97$	$58.12^{a} \pm 0.051$	$8.51^{c} \pm 0.42$

Chapter 6

Discussion

Fruit and vegetables are an essential source of nutrients and secondary metabolites that are good for human health. However, due to their short post-harvest life and extreme perishability, these products undergo significant losses on a global scale (Oliveira et al., 2022). Recently, VOCs emitted by plants and endophytic fungi have received widespread attention and have suggested a viable solution for controlling fungal contamination during post-harvest. This study analyzed the antifungal and antioxidant properties of VOCs released by clove and endophytic fungi isolated from *S. aromaticum.*

Few studies have been conducted to isolate the endophytic fungi from different species of *Syzygium*. Endophytic fungi from different genera, such as *Botryosphaeria* sp., *Trichothecium* sp., *Aspergillus* sp., *Penicillium* sp., *Colletotrichum* sp., *Diaporthe* sp., and *Fusarium* sp., have been isolated from various species of *Syzygium* (Samapti et al., 2022; Aini et al., 2022; Hapida et al., 2022; Habisukan et al., 2021; Widjajanti et al., 2022; Yenn and Ibrahim, 2019; Yang et al., 2021). In this study total, twenty-three endophytes were isolated. However, this is the first study which documented the isolation of endophytic fungi from the leaf and stem of *S. aromaticum*. Microscopic identification of isolated endophytic fungi has been made, and five different genres have been identified: *Alternaria*, *Colletotrichum*, *Paraconiothyrium*, *Nigrospora*, and *Fusarium.*

Few studies have shown the antifungal activity of the VOCs released by endophytic fungi. The antifungal activity of VOCs documented by Aini et al. (2022) showed that the endophytic fungi isolated from *S. jambos* inhibited the growth of *B. cinerea*.

In this present study, the antifungal activity of VOCs emitted by endophytic fungi and clove was analyzed. Out of twenty-three isolates, only two isolates showed significant antifungal activity during dual culture assay. The antifungal potential of endophytic fungi isolated from *S. aromaticum* has yet to be analyzed. Hence, it can be proposed that *S. aromaticum* can be a potent source of endophytes which can be further used as biocontrol agents.

A recent study has documented the antifungal activity of VOCs emitted by clove. The study reported that eugenol, a main component of clove VOCs, can completely inhibit the growth of *A. flavus* on wheat containing 20% moisture (Qin et al., 2023). Another study conducted by Chen et al. (2019) showed that CEO can inhibit the growth of *Penicillium italicum* on citrus fruits.

The present investigation showed that VOCs of clove extract prepared in acetone (80%) could inhibit the growth of significant phytopathogens, which was in accordance with previous studies (Adaramola and Onigbinde, 2016). The identification of the potent isolate has been made using conventional morphtaxonomy and microscopic studies. Various characteristics including structure, form, colony size, and pigment production, have been investigated and used for identification. The endophytic fungus was identified as *Alternaria* sp.

According to previous studies, endophytic fungi isolated from various species of genera *Syzygium* has antioxidants which help neutralize the ROS formed during the post-harvest period, as ROS are known to deplete the nutrients present in fruits and vegetables. *Lasiodiplodia venezuelensis* strain CBS 129753, an endophytic fungus isolated from *S. samarangense* L., was evaluated for the production of antioxidant compounds. The IC_{50} value was calculated as 49.96µg/ml in ethyl acetate extract (Budiono et al., 2019). In another study conducted by Widjajanti et al. (2023), the antioxidant potential of endophytic fungi isolated from *S. polyanthum* was evaluated. In the DPPH assay, out of all isolated endophytes, *Clonostachys rosea* exhibited the highest antioxidant activity with IC_{50} of 11.40 µg/ml.

In the current study, the potent cultures were subjected to various antioxidant assays, including DPPH, FRAP, TPC and TFC, to assess their antioxidant activity. It was deciphered that endophytic fungi coded as #2SASB8 showed the highest antioxidant activity in every assay, as mentioned above. The same culture that evinced the highest antioxidant potential also exhibited the highest phenolic content, proposing that these parameters are correlated. Endophytic fungi isolated from *S. aromaticum* have yet to be studied for their antifungal properties. As a result, *S. aromaticum* may be a potent source of endophytes that could be used as antioxidants.

Various studies have been conducted to evaluate the antioxidant activity of clove extract. In a study by Adaramola et al. (2016), the antioxidant potential, TPC, and TFC of clove extract prepared in 80%

acetone were analyzed. The IC_{50}value, ferric reducing power, TPC, and TFC were recorded as 7.82±0.06 µg/ml, 1.68±0.04µg/ml, 200.20±0.09 mgGAE/g, and 501±0.58mgQE/g, respectively. A similar investigation was done in the current study, and comparable results were obtained for 80% acetone extract. However, the DPPH scavenging potential, ferric reducing power, TPC, and TFC were comparatively lesser in the case of CCl4 extract. The results suggested that the semi-polar constituents of clove bud have higher antioxidative capacity than the non-polar ones.

Chapter 7

Conclusion

This study investigated the antifungal and antioxidant activity of VOCs released by clove and endophytic fungi isolated from *S. aromaticum* against the main phytopathogens.

1. A total of 23 endophytic fungi were isolated from the stem and leaf of *S. aromaticum* and further subjected to analyze antifungal activity. Endophytic fungi with code #1SALB2 showed the highest antifungal activity. Additionally, it may be said that isolated endophytic fungi have antifungal activity similar to clove extract prepared with 80% acetone. Since the endophytic fungi and clove VOCs inhibited pathogenic fungi growth, they can be used as bio-fungi statics.
2. The isolates were also examined for their phenolic and flavonoid content and antioxidant activity. The isolate #2SASB8 has the highest phenolic content and antioxidant activity levels.

To the best of our knowledge, this study is the first to show the presence of endophytic fungi in S. *aromaticum*. Therefore, further studies are required to determine the biological activity and mode of action of VOCs produced by endophytes on phytopathogenic fungi.

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