EVALUATION OF BIORATIONAL COMPOUNDS AND COMBINATIONS THEREOF AGAINST DECAY AND SPOILAGE MICROBES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

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CERTIFICATE

This is to certify that the thesis entitled, " **Evaluation of biorational compounds and combinations thereof against decay and spoilage microbes"**, being submitted by **Ms. Navgeet Kaur (602104008)** in partial fulfillment of the requirements for the award of the degree of Master of Technology in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a bonafide work carried out under the supervision of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.

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DECLARATION

I, hereby declare that the experimental work introduced in this dissertation entitled "**Evaluation of biorational compounds and combinations thereof against decay and spoilage microbes** "being submitted by **Ms. Navgeet Kaur (Roll no. 602104008)** for the degree of Master of Technology in Biotechnology award is an authentic record of my work. The work has been performed under the supervision of Dr. Sanjai Saxena professor at the Department of Biotechnology at Thapar Institute of Engineering and Technology Patiala. No part of the matter embodied in this report has been submitted to any other university or institute for the award of any other degree.

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This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge

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ACKNOWLEDGEMENT

First and foremost, I am grateful to God for the good health and well-being which was necessary to complete this research work.

I would like to express my sincere gratitude and appreciation to all those who have contributed to the completion of this research work. First of all, I would like to take this great opportunity to express a deep sense of gratitude to my honorable project supervisor **Dr. Sanjai Saxena, Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab** for his worthy guidance, valuable suggestions, constructive criticism, and for congenial help during the course of investigation and preparation of this manuscript. Without his help and concrete suggestions, the execution of this research work, would not have been completed on time. I respectfully thank **Ms. Ramandeep Kaur** and **Ms. Shreya Gupta** for their diligent guidance, constant encouragement, and great support during my dissertation. Their time and effort to brainstorm ideas, provide constructive feedback, and offer valuable insights that have significantly enhanced the quality of this project. I am wholeheartedly thankful to all lab attendants for their valuable help and immense support.

I would like to express my deepest gratitude to my parents **Mr. Jatinder Singh** and **Mrs. Amanjot Kaur** for their support and love throughout. Their tireless efforts and sacrifices have allowed me to pursue my academic goals and have been a constant source of inspiration. I am forever grateful for their unwavering belief in me and their unconditional love.

Last but not least, I would like to express my heartfelt gratitude to my friends Ruchika Singh, Warsha Koul and Sunny Yadav who have been invaluable part of my life. Their unwavering support, love, and companionship made my journey more vibrant, meaningful, and enjoyable.

Navgeet Kaur (602104008)

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LIST OF ABBREVIATIONS

S. NO.	ABBREVATIONS	FULL FORM
1.	PRs	Pathogenesis-Related Proteins
2.	SA	Salicylic Acid
3.	ASA	Acetyl Salicylate
4.	MeSA	Methyl Salicylate
5.	AVR gene	Avirulence gene
6.	SAR	Systemic Acquired Resistance
7.	PAL	Phenylalanine Ammonia Lyase
8.	ICS	Isochorismate Synthase
9.	TMV	Tobacco Mosaic Virus
10.	GRAS	Generally Accepted As Safe
11.	PDA	Potato Dextrose Agar
12.	NaOCl	Sodium Hypochlorite
13.	DPX	Dibutylphthalate Polystyrene Xylene
14.	ITCC	Indian Type Culture Collection
15.	$FeCl_3$	Ferric Chloride
16.	HCL	Hydrochloric acid

17.	PDB	Potato Dextrose Broth
18.	gms	Grams
19.	nm	Nanometer
20.	mm	Millimeter
21.	Cm	Centimeter
22.	µg/µl	Microgram/microliter
23.	ml/mg	Milliliter/milligrams
24.	UN-DESA	The United Nations Department of Economic and Social Affairs
25.	min	Minute
26.	SMs	Secondary Metabolites
27.	BCAs	Biocontrol agents
28.	ISR	Induced Systemic Resistanc
29.	T-CA	Trans Cinnamic Acid
30.	ROS	Reactive oxygen species
31.	SOD	Superoxide dismutase
32.	APX	Ascorbate peroxidase

33.	RPM	Revolutions per minute
34.	SDA	Sabouraud Dextrose Agar
35.	RBA	Rose Bengal Agar
36.	PLA	Pine Leave Agar
37.	CMA	Corn Meal Agar
38.	WA	Water Agar
39.	mM	Mili Molar

ABSTRACT

Salicylic acid (SA), a phytohormone, is a well-known signal molecule modulating plant immunity and plays a role in controlling plant development. Salicylic acid is a crucial plant hormone needed to develop a plant's tolerance to various diseases. SA is considered to be derived from two possible pathways; the ICS and PAL pathway, both starting from chorismite. Pre-harvest spraying, post-harvest fumigation, post-harvest dipping, and post-harvest coating are just a few ways SA and its derivatives preserve fruit. SA has been proven to be a naturally occurring plant hormone generally accepted as safe (GRAS) for use on fruit. SA and its derivatives have been shown to improve storage quality by reducing ethylene production and respiration rate, inhibiting fruit softening and color change, maintaining sugars, organic acids, and aroma, preventing chilling injury, fostering pathogen resistance, and activating the antioxidant system.

In the present study, 28 endophytic fungi were procured on PDA plates from various plants like *C. zeylanicum, C. roseus* and *S. oleracea*. These cultures were subjected to production in potato dextrose broth for the production of salicylic acid by them. After preliminary analysis, two endophytic fungi #PALAM19 and #22CRSBRT procured from *P. acerifolium* and *C. roseus* showed maximum SA production and were further analysed. It was found that #PALAM19 and #22CRSBRT showed maximum production at 0.5mg/ml cinnamic acid at 26°C. #PALAM19 and #22CRSBRT were also tested for their antifungal activity against procured pathogenic fungi and it shows maximum zone of inhibition against *Botrytis cinera*. Procured #PALAM19 displayed the most potent antifungal activity between the two cultures as it shows clearance against all the procured pathogenic fungi but #22CRSBRT shows zone of clearance only against

Aspergillus niger. Further studies on morphological and molecular identification of the potential endophytic fungi improvement in the production process need to be done.

CHAPTER-1

INTRODUCTION

Introduction

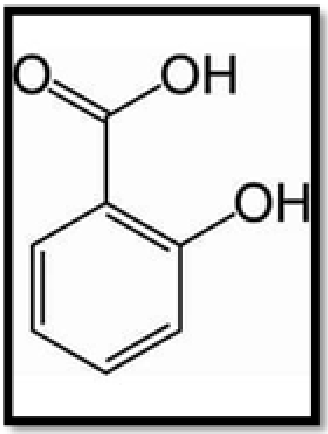
Plants endure various difficulties, from insect attacks to climatic pressures like floods, temperature changes, drought, and microbial attacks. Multiple stress factors threaten agricultural output, frequently linked to global warming, even as demand for food products rises (Zhao et al. 2017). Abiotic and biotic stress defined as external situations that negatively affect a plant's growth, development, or production. Plants respond to stress in various ways, altering their gene expression, cellular metabolism, growth rates, crop yields, etc. Plant stress usually reflects sudden environmental changes (Verma et al. 2013). A detailed study is being done on the significant abiotic stresses that impact plants and crops in the field. They include drought, salinity, heat, cold, chilling, freezing, nutrient, high light intensity, ozone (O_3), and anaerobic stresses (Gull et al. 2019). Living things, particularly viruses, bacteria, fungi, nematodes, insects, arachnids, and weeds, induce biotic stress in plants. Biotic stress can become major because of pre- and postharvest losses (Umar et al. 2021).

Plants can withstand biotic stressors even if they lack an adaptive immune system by adapting to specific sophisticated techniques. The genetic code stored in plants regulates the defense mechanisms that respond to various challenges. Various modifications to gene expression, agricultural yields, growth rates, and cellular metabolism are ways plants react to stress. Sometimes stresses to the plants injure them such that plants show various metabolic dysfunctions. The effect is transient if the stress is minor or short-term, and the plants can recover. But extreme stress can kill agricultural plants by stopping flowering, seed production and inducing senescence (Iarley et al. 2022). Plant defense against these stresses is mediated through multiple signaling pathways that produce many defensive proteins and non-protein compounds. The defense mechanisms involve physical changes such as cell wall lignification, papilla formation, or the induction of various pathogenesis-related proteins (PRs) (Kaur et al. 2022).

Activation of defense-related genes extends systemically, conferring broad-spectrum resistance to viral, bacterial, and fungal pathogens in distant, uninfected plant parts. Plant phytohormones such as salicylic acid (SA), jasmonic acid, abscisic acid, and ethylene are essential components of different signaling pathways involved in plants. A few plant hormones have been explored to enhance the quality of fruits and vegetables after harvest (Zhang et al. 2020). Salicylic acid (SA), acetylsalicylate (ASA), and methyl salicylate (MeSA) are three plant hormones that have demonstrated potential effectiveness in preserving fruit quality and extending shelf life by postponing fruit ripening and softening, reducing decay, and preserving fruit flavour (Baswal et al. 2020; Hazarika and Marak. 2021). In plants of agronomic significance, various synthetic compounds imitate SA's capacity to activate resistance to both biotic and abiotic stressors. In the site of the pathogen invasion, specific pathogen recognition mechanisms, controlled by resistance gene products that interact with matching avirulence gene products from the pathogen, typically result in a hypersensitive reaction, keeping the pathogen isolated from the rest of the plant (Saur et al. 2021). The ability of a plant to perceive pathogen infection and mount an effective response is frequently regulated by a gene-for-gene interaction (direct or indirect) between the products of the plant resistance gene and its cognate pathogen avirulence (Avr) gene (Lukan et al. 2022). To improve plant performance and reduce the loss of productivity, this can be implemented through various approaches, one of which is the application of secondary metabolites (Koza et al. 2018). Salicylic acid, chemically known as 2-

hydroxybenzoic acid (Fig. 1), is one of many secondary metabolites (phenolic chemicals) produced by plants with an aromatic ring with a hydroxyl group or its functional derivative.

Fig. 1: Chemical structure of salicylic acid



To improve plant performance and reduce the loss of productivity, this can be implemented through various approaches, one of which is the application of secondary metabolites (Koza et al. 2018). Salicylic acid, chemically known as 2-hydroxybenzoic acid (Fig. 1), is one of many secondary metabolites (phenolic chemicals) produced by plants with an aromatic ring with a hydroxyl group or its functional derivative. Salicylic acid is the best-known “antistress compound” used by human beings. SA can also be produced directly from phenylalanine and, depending on the plant species, from free benzoic acid, benzoyl glucose, or o-coumaric acid. The therapeutic property of the phenolic compound salicylic acid and its derivatives, collectively known as salicylates, has been known since the early 4th century B.C. when Hippocrates prescribed willow bark, which is rich in salicylates, for pain relief during childbirth (Lefevere et al. 2020). On detecting pathogens such as viruses, fungi, and bacteria plants accumulate SA and activate immune responses. While plants fully fight pathogens, the accumulated SA actively suppresses growth and developmental processes, interrupting biomass increase (Van et al. 2020). Salicylic acid is a crucial plant hormone to develop a plant's disease tolerance. SA mediates the phenylpropanoid pathway and plays an important role against pathogens and some insect pests and abiotic stresses. At the same time, the latter is mostly meant to defend against insect pests and some pathogens (Guedes et al. 2023). SA first emerged as an endogenous signal capable of inducing plant defense responses both at the site of infection and in the systemic tissue of the plant. Since then, genetic and biochemical methods have been used to characterize and deconstruct SA-mediated signalling networks. The phenolic compound activates plant defense, especially systemic acquired resistance (SAR) (Vlot et al. 2009). Recently, many studies have reported that SA could be used as a commercial application for maintaining postharvest quality to prolong the shelf-life of fruits, vegeTables, and ornamental products. Both pre-and postharvest SA applications in extending shelf-life and maintaining the quality of postharvest products have been investigated and developed for commercial use (Perumal et al. 2021). SA plays a part in numerous physiological processes, including seed germination, flowering, and fruit ripening, and it can protect plants from environmental stresses such as low temperature, salinity, and water stress (Hadjipieri et al. 2021). SA has a significant potential for reducing ethylene production and preventing fungal rot in harvested fruits. Fruit and vegetables have abundant nutrient substances and delicious flavors (Zhang et al. 2020). However, inaccurate preservation techniques and hindered storage circumstances can lead to fruit senescence and disease infection, leading to a loss in quality and a decline in the economy (Zhang and Jiang, 2020).

Several studies have shown that plants can synthesize salicylic acid. SA is derived from two PAL and ICS pathways starting from chorismite. The chemical analysis of food products has proven that salicylates are also found in food, and their main sources are vegetables, fruits, herbs, and spices (Akbari et al. 2022). About 20 years ago, White and coworkers found that tobacco leaves treated with SA or acetylsalicylic acid (aspirin) had increased protein accumulation and TMV resistance (Antoniw et al. 1980). Using modern analytical technics, it was found that salicylates are distributed in many important agricultural plant species. The main method by which many bacteria and fungi synthesize SA is through chorismic acid, a crucial intermediary in shikimic acid pathways. The SA production and excretion rate can be high in plants (Sambyal. 2021). Microorganisms associated with crop plants are also capable of synthesizing and exerting SA. In particular, SA has been proven to be a naturally occurring plant hormone that is generally accepted as safe (GRAS) for use on fruit (Fan et al. 2021; Kumar et al. 2021), which can replace chemical synthetic preservatives to lessen their adverse effects (Hanif et al. 2020).

The present study is based on the screened of endophytic fungi isolated from various plants, were done for the production of salicylic acid. Potent endophytic fungi were analysed at different temperatures, and substrate concentrations i.e., cinnamic acid which initiates PAL pathway for SA synthesis. The antifungal activity of procured potent fungal isolates is also determined.

CHAPTER-2

REVIEW OF LITERATURE

Review of Literature

2.1.Overview

The average global crop loss caused by pests and pathogens ranges from 11 to 30 percent (Savary et al. 2019). It is predicted that there will be about 10 billion people on the planet by 2050 (UN-DESA Population Division 2017), and even now, it is estimated that 821 million people lack access to wholesome food (FAO 2018). An estimated 10–15% of the world's major cash crops are lost to plant diseases each year, and 70–80% of these illnesses are caused by pathogenic fungi, resulting in direct economic losses of hundreds of billions of dollars (Santra et al. 2023). As per the agricultural reports, plant pathogens, and pests reduce the global annual crop yield by an estimated 30 to 50%, and this loss must be combatted to ensure food security for an ever-increasing human population (Jat et al. 2016).

Due to decreased yield and quality of food, fiber, and biofuel crops, plant diseases have been a severe problem to agriculture since the beginning (Visković et al. 2023). Worldwide, more than 19,000 fungi are known to infect crop plants with illnesses. They may survive on living and dead plant tissues while dormant until the conditions are right for their multiplication (Lazarovits et al. 2020). As fungal diseases constitute a significant threat to crop production, applying fungicides to control fungal infestations is often considered indispensable to securing global food supply (Fones et al. 2020). Plant diseases include anthracnose, leaf spot, rust, wilt, blight, coiled, scab, gall, canker, damping-off, root rot, mildew, and dieback that are carried on by pathogenic fungi (Iqbal et al. 2018).

On chestnut trees, Cryphonectria parasitica outbreaks have caused nearly 100% defoliation. Phytophthora cinnamomic, which causes root rot or dieback in many crops, was the cause of another more recent blight (Neto et al. 2018). Fungicides can be used multiple times during the growing or even over several seasons because most fungi cause plant illnesses and are challenging to treat. Using fungicides frequently might encourage the formation and transfer of toxic chemicals in ecosystems, endangering the environment, particularly soil. Fungicides exert a negative effect on soil-dwelling microorganisms and biochemical processes in soil (Baćmaga et al. 2019). Several reports also suggest that phytopathogens are mutating and acquiring resistance due to the widespread and negligent use of pesticides and antifungals (Geiger et al. 2017). Currently, most techniques used to protect plants from diseases primarily include antibiotics and pesticides (Nazarov et al. 2020). A 2020 study found that 44% of an estimated 860 million farmers worldwide are affected by pesticide poisoning yearly. With regard to non-fatal UAPP, Southern Asia has the highest estimated number of cases, followed by South-Eastern Asia and East Africa (Boedeker et al. 2020). Even though these shotgun approaches offer immediate protection, they eventually cause resistance and the bioaccumulation of dangerous substances in agricultural systems. These adverse effects highlight the significance of using environmentally friendly crop management techniques to combat diseases (Rigano et al. 2018). Utilizing beneficial endophytes as biological control agents for crop protection is one under-researched but potential alternative strategy that is gaining popularity (Cocq et al. 2017).

2.2. Endophyte as biological control agent

In recent times, a world population index by 2050 has recently been projected, focusing on using natural ways to tackle the food demand, food insecurity, and impending food scarcity

(Sahu et al. 2021). Farmers have used agrochemicals to increase food production since ancient times, but they are not sustainable since they pose risks to the ecology (Teklu et al. 2022). According to this assumption, reducing these eco-threats and investigating potential endophytic microbes will aid in achieving an ecosystem that is stable and will enable the cultivation of pathogen-free plants for increased crop output (Akanmu et al. 2021).

Endophytes are microorganisms that usually have no effect on their host or produce any signs and symptoms of the disease while spending all or a portion of their life cycle inside the plant (Vermiglio et al. 2021). They have been studied for their potential use as biocontrol agents, which means they can protect plants from pests and diseases. Endophytes have drawn attention for their roles as biological pest controllers and promoters of the plant's response to biotic and abiotic challenges. Due to their capacity to secrete secondary metabolites, act as biocontrol agents, antimicrobial agents, antitumor agents, and immunosuppressants, as well as secrete antiviral compounds and develop natural antioxidants, antidiabetic agents, antibiotics, and insecticidal products, endophytes are becoming more and more relevant in biotechnology and industry (Gouda et al. 2016; Yadav. 2018). Endophytic microbes support plant growth through several processes and offer defense against pathogens and pests. Endophytes generate and secrete secondary metabolites, including volatile substances that can inhibit pathogen growth and reduce plant disease's adverse effects (Busby et al. 2016). The by-products of environmental interactions during plant growth and development are known as secondary metabolites (SMs). Alkaloids, flavonoids, terpenoids, peptides, phenols, sterols, and other small molecular organic substances are the primary components of SMs (Xie et al. 2018).

Organic substances known as secondary metabolites are not directly connected to an organism's regular growth, development, or reproduction (Jan et al. 2021). They are synthesized by plants, fungi, and bacteria and play essential roles in plant survival and in creating ecological connections between other species. Besides controlling plant development and biological defence, SMs also play a role in how plants react to abiotic challenges such as drought, low temperatures, salinity, and metals (Jin et al. 2017).

An in-depth understanding of the general biology of the tripartite interaction between endophyte, host plant, and the pathogen is required as of the underlying physiological processes involved to optimize the selection and utilization of endophytes (Lefevere et al. 2020). For BCAs, thus for endophytes, the four different types of control principles are commonly accepted: Competition for resources and space, antibiosis-mediated direct suppression, mycoparasitism, and plant-induced resistance brought on by activation of its defensive mechanisms (Heydari et al. 2010). Consequently, endophytic fungi are being researched more and more due to their potential to benefit plant health (Cheng et al. 2020).

According to studies, endophytes primarily have two effects on plants. On the one hand, endophytes cause the host to produce systemic resistance (ISR). ISR differs from conventionally system-acquired resistance (SAR) in that its phenotype is comparable to pathogen-induced SAR, which can result in broad-spectrum pathogen resistance in plants. Systemic acquired and induced systemic resistance are distinct processes, but both are active plant defense reactions to phytopathogen infection. IS is similar to hypersensitive response, while SAR is similar to the inherent immunity of the plant system. The terms were first coined in 1961 by Ross during his research on interactions between tobacco and its mosaic virus (TV).

Non-pathogenic plant growth-promoting rhizobacteria (PGPR) are responsible for ISR induction. The infection of a pathogen triggers SAR (Bentham et al. 2020). Despite being two distinct processes, systemic and systemic acquired resistance were induced to signify active plant defense reactions to phytopathogen invasion. Like the SAR, a plant can develop a defense against an invader, such as a pathogen or parasite, if an infection occurs. In contrast to SAR which is triggered by the accumulation of salicylic acid, ISR instead relies on signal transduction pathways activated by jasmonate and ethylene (Yu et al. 2022).

2.3. Salicylic acid as a Secondary metabolite

2.3.1 History

Beta hydroxy phenolic acid, the secondary metabolite SA, is frequently generated by prokaryotes and plants. SA has long been more well-known for its uses in medicine than for its role in plants. One of the causes was the discovery of SA as a chemical messenger in plants, where they were found to regulate biological processes at relatively low concentrations. The sixth primary plant hormone (phytohormone), SA, was thus recognized in the early 1990s (Raskin. 1992). When (White. 1979) observed that acetylsalicylic acid (aspirin) increased resistance to tobacco mosaic virus (TMV), boosting PR protein accumulation and reducing lesion numbers, he first recognized salicylic acid as an endogenous signal in the resistance response in tobacco. Malamy et al. (1990) then noted that after TMV inoculation, the endogenous salicylic acid levels in resistant cultivars increased in infected and uninfected leaves but not in susceptible cultivars. In addition, SA at low concentrations facilitates "priming," a process that speeds up and intensifies the activation of callose deposition and gene expression in response to pathogen or microbial elicitors and aids in the induction of defense mechanisms (Kohler et al. 2002).

2.3.2. Role of salicylic acid in plant defense

Salicylic acid, a plant hormone, plays an essential role in the induction of plant defense against various biotic and abiotic stresses through morphological, physiological, and biochemical mechanisms (Wahab et al. 2022). Salicylic acid is not required to induce systemic resistance (ISR), a property of plant growth-promoting microorganisms (PGPR). In contrast, SA or SA-like substances are necessary to induce SAR. More than 30 groups of di- and monocotyledonous plants have been found to produce SAR Plant protection using pesticides offers resistance against a particular pathogen or a group of pathogens. In contrast, the multistep process involved in a SAR response generated by a pathogen or another method makes it difficult for pathogens to build resistance (Faize et al. 2018). Stress situations, therapy with pathogen elicitors, and diverse pathogen infections can all cause SA formation. SA is a plant hormone involved in defense essential for resistance to various microbial diseases, including bacteria, fungi, viruses, and oomycetes. Plants can develop disease resistance by applying SA and its synthetic equivalents exogenously (Zehra et al. 2021). Additionally, SA is crucial for controlling thermogenesis, abiotic stress tolerance, plant growth, and maintaining plant disease resistance (Koo et al. 2020).

2.3.3. Synthesis of salicylic acid

According to Pérez-Llorca et al. (2019), the shikimate pathway produces SA. By chorismate synthase, shikimate is transformed into chorismate in the first step. Chorismate is further converted into isochorismate for SA biosynthesis. Two distinct processes, isochorismate synthase- and phenylalanine ammonia-lyase-dependent pathways, are used to biosynthesize SA from chorismate (Dempsey et al. 2017). The main pathway, ICS, synthesizes more than 90%

of SA. The ICS enzyme converts chorismate to its isomer, isochorismate, in the first stage of the ICS pathway, and this process is shown to occur often in both bacteria and plants. Additionally, plants synthesize a small portion (10%) of SA via the PAL pathway, which occurs exclusively in the cytosol. The PAL enzyme is responsible for converting phenylalanine to trans-cinnamic acid (t-CA), and plants have numerous copies of the gene that codes for this enzyme (Guo et al. 2020). Later, ortho-coumaric acid and benzaldehyde, two potential intermediates, convert t-CA to SA.

2.3.4. SA in plant resistance to biotic stresses

A variety of biotic stress conditions can affect plants. Plants have evolved complex sensing mechanisms to detect biotic invasion and prevent the harm it causes to growth, yield, and survival (Lamers et al. 2020). Various pests, parasites, and diseases cause plant infections and biotic stress. According to Sobiczewski et al. (2017), fungal parasites can either be necrotrophic (destroy host cells by toxin release) or biotrophic (feed on living host cells). SAR creates systemic resistance throughout the entire plant against a variety of diseases. Through intraplant communication, a local encounter in SAR stimulates resistance to the other plant organs (Riedlmeier et al. 2017). Various studies (Table 3.1) have reported that SA could be used as a commercial application for maintaining postharvest quality to prolong the shelf-life of fruits, vegeTables, and ornamental products to increase shelf life and preserve the quality of postharvest product pre-and postharvest SA treatments have been researched and developed for commercial usage. The methods utilized for pre-harvest treatment include spraying and adding to the growth medium. Spraying and adding to the growth medium are the approaches used for pre-harvest treatment. The foliar spray of SA (10-6 and 10-4 M) could improve the postharvest quality of pepper fruit by increasing fruit weight, the level of biologically active compounds, and regulating sugar content (Oliveira et al. 2023). Niazmand et al. (2020) reported that SA pre-harvest treatment three weeks before harvest improved the quality and reduced fungal infection of 'Mashhad' sweet cherry fruit.

Table 2.1: Enhanced biotic stress upon exogenous SA application in different plants (Koo et al. 2020)

Host plant	Pathogen (infection style)	Sa conc. and treatment method	Effect	References
Tomato *(Lycopersicon esculentum)*	*Fusarium oxysporum* (hemibiotrophic) *Botrytis cinerea*	0.2mM 2 mM	55%reduction in disease incidence 62% reduction in disease severity	Jendoubi et al. (2017) Li and Zou (2017)
Pepper *(Capsicum annuum)*	*Ralstonia solanacearum* (hemibiotrophic)	0.5 mM	Induced seedling growth inhibition is recovered. Notably, 0.5 mM SA increased seedling growth by almost 150%.	Chandrasekhar et al. (2017)

Rice (*Oryza sativa*)	*Oebalus pugnax* (piercing and sucking insect)	16 mM	35% reduction in number of bugs found in plots; retarded nymph development to adult insect	Stella et al. (2019)
Potato (*Solanum tuberosum)*	*Fusarium oxysporum*	15 mM	Hyphal cell death causes deficient growth of Spore production of *F. oxysporum* was also significantly decreased in a SA dose-dependent manner	Li et al. (2022)

2.3.5. SA in plant resistance to abiotic stresses

Abiotic stresses cover various stresses, and it is crucial to understand their importance and relevance to agricultural output. The application of anthropogenic activities, which has led to abiotic stresses such as high metal content in the soil, nutrient depletion, salinity, and changes to the physicochemical structure of the soil, is one of the leading causes of the degradation of our agricultural systems (Hasanuzzaman et al. 2020). Abiotic stresses affect plant biochemistry and physiology, and this effect transcends all developmental phases, from seed germination to maturity, with direct effects on growth, development, and yield. Abiotic stress can severely impact rice yield component, with losses occasionally reaching 70% of predicted output (Nadarajah et al. 2021). The studies listed below (Table 3.2) show how SA can protect the crop from abiotic stresses.

Table 2.2: Enhanced abiotic stress upon exogenous SA application in different plants (Koo et al. 2020)

Host plant	**Stress**	**SA conc. and treatment method**	**Effects**	**References**
Wheat *(Triticum aestivum)*	Freezing	0.01, 0.1-, and 1-mM SA sprayed on wheat leaves at the four-leaf stage three times, with an interval of 12 h	Inhibited freezing stress-induced PS II quantum yield reduction and cell death. SA enhanced production of ABA and H_20_2	Wang et al. (2018)
Alfalfa *(Medicago sativa)*	Freezing	Pretreatment with 200 µM and 0 µM SA, which were exposed to freezing	SA induce *MPK3* to regulate *WRKY22* to participate in freezing stress to induced gene	Miao et al. (2020)

		stress (-10°C) for 0, 0.5, 1, and 2h	expression related to SA signaling pathway	
Olive *(Olea europaea)*	Drought	SA treatment ($p \leq 0.05$)	SA improves SOD and APX activities and leaf gas exchange	González et al. (2022)
Rice *(Oryza sativa)*	High temperature	SA (SA1-SA2: 0.5 and 1.5 mmol L−1), and their combinations were applied to leaves	SA improve the osmotic adjustment, antioxidant ability and reduce lipid peroxidation and the production of ROS to promote rice growth under the high temperature stress conditions	Yang et al. (2022)

2.3.6. Other applications of Salicylic acid

The numerous pathogenic risks plants have to deal with, particularly after post-harvesting, ultimately lower output and leave plants unfit economically. Many synthetic agents, including fungicides and insecticides, combat this post-harvest pathogenic threat. However, these substances are bad for the environment and hazardous to human health. Hence several researchers have switched them out for environmentally friendlier SA (Gupta et al. 2023).

According to Islam et al. (2022), treating stored pomegranate fruit with SA decreased ascorbic acid loss, suppressed PAL activity, and decreased chilling injury and electrolyte leakage. SA (2 mM) increased infection resistance and decreased ethylene production associated with fruit degradation.

The relationship of concentration (0.8 mol L^{-1} to 5.0 mmol L^{-1}) and time (2 min to 6 h.) assesses the treatment impact of SA on fruit, which varies depending on the fruit's kind. Compared to directly edible fruits without peeling, such as peach, grape, cherry, and so on, fruits with inedible peel, including bananas, chestnuts, and pineapple, may tolerate higher concentrations and longer treatment times. Coating involves drenching fruit in a solution of SA and coating substance, much as dipping (Ehteshami et al. 2020). Studies by various researchers (Table 3.4) show that specific conc. of SA protects the crop from different harmful effects. Though SA also helps crops to fight pathogenic fungi.

Table 2.3: Various other applications of salicylic acid

Concentration and treatment time	Storage temperature and time	Storage effect	References
Postharvest dipped in 0.8 mM for 6 h 20	± 2°C for 8 days	Enhanced antioxidant capacity. Maintained soluble sugar and soluble solids. Decreased weight loss and decay percentage of banana	Xu et al. (2019)

Postharvest dipped in 4 mM for 10 min	0°C for 30 days	Enhanced antioxidant capacity and total phenolics. Alleviated CI incidence and decay percentage. Maintained soluble solid, titraTable acid contents, firmness and color of apricot	Batool et al. (2021)
Postharvest dipped in 0.5, 1, 1.5 and 2 mM for 5 min	Ambient temperatures for 16 days	Maintained soluble solid and sugar contents. Reduced browning and decay percentage of grape	Hazarika and Marak (2021)
Postharvest dipped in 0.75 mM for 20 min	25 ± 1°C for 15 days	Maintained the integrity of cell wall composition and firmness of tomato	Kumar et al. (2021)
Postharvest dipped in 4 mM	5 ± 1°C for 90 days	Enhanced antioxidant capacity of mandarin	Haider et al. (2021)
Preharvest sprayed 0.5 mmol L − 1 four times at 21 d	8°C for 35 days	Enhanced antioxidant capacity and total phenolics. Decreased weight and firmness loss of lemon	Serna-Escolano et al. (2021)
Either pre-harvest or post-harvest 1.5 mM	24±0.5°C for 28 days	The most firmness (52.10±0.11)	Minh et al. (2022)

The common dipping of salicylic acid technique is used to treat post-harvest fruits and vegeTables, such as that of dates (Atia et al. 2018), sweet cherry (Giménez et al 2017), blood orange (Habibi et al. 2020), orange fruit (Amiri et al. 2021) and brinjal (Bahadur et al. 2022) and various other. Kavya et al. (2022) suggested that the use of pre-harvest treatment followed by postharvest treatment was the most effective strategy for preventing fungal decay and to maintain the overall quality of Selva strawberry fruits. In a similar vein Erogul et al. (2020) reported that SA pre-harvest spray (2 mM) and/or postharvest dip (0.5 mM SA) could maintain the fruit quality and enhance the resistance to internal browning. A technique used on trees at critical times before fruit harvest is preharvest spraying (Valverde et al., 2015). It's worth noting that the salicylate solution for spraying is always combined with 0.5% of Tween 20, which leads to the better dissolution of salicylates in water and better absorption by plants (Fan et al. 2022). It should be emphasised that although increasing endogenous SA accumulation improves plant immunity, it typically inhibits growth (Butselaar et al. 2020). However, under some circumstances, SA controls plant immunity and development separately using various receptors or pathways.

CHAPTER-3

OBJECTIVES

Objectives of the study

3.1.Preliminary analysis of procured fungal endophytes for the production of salicylic acid

3.2.Analysing the effect of substrate concentration, temperature for the optimisation of salicylic acid production

3.3.Genomic DNA isolation of selected endophytic fungus

CHAPTER-4

MATERIAL AND METHODS

Material and Methods

4.1. Preparation of Potato Dextrose Agar plates

39.0 grams of Potato Dextrose Agar (Hi-Media) was suspended in 1lt of distilled water in a flask. The solution was heated up so that each particle dissolved completely. The pH of the media was set at 5.6. The flask was sealed by a cotton plug autoclaved at 121°C, 15 psi, for 15 minutes. Before pouring, the media was cooled to 45° to 50°C. Cooled PDA media was poured into the sterilized plates. In order to drain the moisture, plates were turned upside down after being allowed to cool at ambient temperature. Moisture was removed from the plates, and the plates were kept at 26±2°C overnight for quality check.

4.2. Culture revival from repository

28 endophytic fungi were procured from existing repository of Dr. Sanjai Saxena, DBT, TIET Patiala. Mycelia was transferred from stored vials to a fresh potato dextrose agar media plate to obtain the culture of fungal endophytes. The plate was incubated for 7-14 days at 25°C. The loop full of the culture was aseptically inoculated onto PDA slants containing 10% glycerol for long-term preservation

4.3. Identification of retrieved fungal isolates

4.3.1. Culture morphology

To identify the endophytic fungi culture morphology of the samples was done. procured fungi were characterized on the basis of various aspects like front and back color, form, elevation, and margin, and then the diameter of the fungal growth was measured. The culture was grown in different media plates namely Sabouraud Dextrose agar for 7 days at 26 ± 2 °C for growth optimization.

4.3.2. Microscopic morphology

The glass slide was cleaned with alcohol and air dried. A drop of water was put onto glass slide, upon which the mycelial mass was placed and teased properly with the help of sterile. The slide was covered with 18 x 10 mm coverslip avoiding the formation of air bubbles and mounted with DPX. The microscopic features were observed at 10X, 40X and 100X using Nikon binocular microscope. Morphological features such as colony spores, color, and appearance were critically observed and noted

4.4. Revival of pathogenic fungi from repository

Different pathogenic culture collected from ITCC *Alternaria alternata* (ITCC- 6129 and 6343), *Aspergillus niger* (ITCC-6354), *Botrytis cinera* (ITCC-6011), *Botryodiplodia theobromae* (ITCC-5597), *Colletotrichum gloeosporioides* (ITCC-3801 and 6152), *Fusarium lateritium* (ITCC-4533) and *Fusarium moniliforme* (ITCC-6240 and 6435) were procured from existing repository of Dr. Sanjai Saxena, DBT, TIET Patiala (Table 4.1). These pathogens were revived on the PDA plate and were kept at 26°C. Further, the activity of salicylic acid was tested against these pathogens.

Table 4.1: ITCC cultures, code, name and source used to determine the antifungal activity

S.no.	Itcc code	Culture	Source
1.	6343	*Alternania alternata*	Tomato, Arti Yadav, Jaipur,
2.	6129	*Alternania alternata*	Kinnow, R.B. Gaur, Sriganganagar

3.	6354	*Aspergillus niger*	Apple, Alemayehu Gateachew, IARI
4.	6011	*Botrytis cinera*	Strawberry, PN Chowdry, New Delhi
5.	5597	*B. theobromae*	Citrus, SK Ahmad, Tirupati
6.	3801	*C. gloeosporioides*	Orange, JN Kapoor, New Delhi
7.	6152	*C. gloeosporioides*	Orange, M.S. Josh, Rahuri
8.	4533	*Fusarium lateritium*	Kinnow, R.B. Gaur, Jammu
9.	6240	*Fusarium moniliforme*	Onion and Garlic, KE Lanearde, Pune

Spores of 7-day-old revived ITCC pathogens were collected by pouring 5ml of 0.8% saline water on the plates and then collecting the spores in the sterilized centrifuge tube under aseptic condition. Spores were collected, centrifuged for 10 minutes at 10,000 rpm, the supernatant was removed, and saline water was added to the spore pallet. Using a hemocytometer, density of each spore suspension was adjusted to $1x10^5$ spores/ml (Choi et al. 1999). Various dilutions (1-5 mg/ml) of salicylic acid (Loba) were prepared in acetone and used as a stock to determine their effect on the growth of various pathogens.

4.5. Analysis effect of pure salicylic acid on the growth of pathogenic fungi

100 µl of spore suspense on was spread onto the PDA plate. Then the 6 bores of 5mm each were punched aseptically on the same PDA plate with the help of a borer and 60µl of each dilution of salicylic acid was added into each bore and control was prepared by adding 60µl of acetone into it. The plates were correctly wrapped and were kept in an incubator at 28°C for 7 days, and the zone of inhibition was measured (Luangtongkum, 2007).

4.6. Quantitative analysis of SA by endophytic fungi

4.6.1. Standard of salicylic acid

Ferric chloride reagent is prepared by adding 1 gm of $FeCl_3$ to 100 ml of 1% HCl by dissolving 100 mg of salicylic acid in a few ml of methanol and bringing the volume up to (1mg/ml) with distilled water in a volumetric flask, a stock solution of salicylic acid (1 mg/ml) was made up. 10 ml of this stock solution was diluted with 100 ml of distilled water to get 100 µg/ml salicylic acid solution. Respective samples (1ml, 2ml, 3ml, 4ml, 5ml, 6ml) were taken in each test tube reagent, and distilled water was added to make a total volume of 10 ml to make different conc. (10-60µg/ml) and then the absorbance of the prepared sample was taken in a UV-Visible spectrophotometer at a wavelength of 525 nm against a blank sample (Venkataswamy, 2018).

4.6.2. Estimation the SA production using culture filtrate

Potato Dextrose Broth production bottles were prepared by mixing 24 gm of PDB (Hi-Media) and 1mg/ml of cinnamic acid in 1000 ml of distilled water. 30 ml of this solution was added to each production bottle and were autoclaved at 15 lbs pressure (121°C). 5mm plugs of procured fungi were inoculated under the laminar airflow and were incubated in an orbital shaker at 120rpm at 27°C for 9 days. The fungal biomass was separated using Whatmann No. 4 filter paper. Wet and dried weight of the mycelia is noted and Mycelia-free liquid was frozen at 4°C till further use (Prasanna et al. 2022). By measuring the absorbance or optical density at 525 nm, the ELISA test can quantify the amount of the target substance present in the sample to check the presence of salicylic acid in procured cultures the ELISA (Enzyme-Linked Immunosorbent Assay) test was performed and absorbance was noted at 525 nm (Venkataswamy, 2018).

4.7. Optimization of substrate concentrations

Maximum SA producing fungal endophytes were used to determine the effect of substrate concentration on the production of SA. Cinnamic acid act as a substrate in salicylic acid production by inducing PAL pathways; so different concentrations ranging from 0.1 mg/ml to 1mg/ml of cinnamic acid were added to the production flasks containing growth medium and incubated at 25°C for 7 days. After completion of incubation, culture filtrates were used to determine the amount of SA production.

4.8. Optimization of temperature for SA production

Temperature is an important factor that has a vital effect on the yield of the SA production. The selected cultures were subjected to the production of culture filtrate at various temperatures 25°C, 36°C, 50°C and 55°C in PDB media supplemented with selected concentration of cinnamic acid to find out the optimum temperature at which maximum SA can observed.

4.9. Antifungal activity of potent endophytic fungi against Pathogens

To check the antifungal activity of procured endophytic fungi 5mm plug of endophytic fungi and pathogens was placed on the edge of the PDA plate. Control plates were prepared by adding a 5mm plug of each pathogen. All the plates were kept in an incubator at 25°C for 7 days. The diameter of each pathogen culture was measured, and reduction was noted (Erhonyota et al. 2023).

4.10. Identification of potent endophytic fungi

4.10.1. Plate morphology

The potent endophytic fungi were cultured on different growth medium such as potato dextrose agar (PDA), SDA, RBA, PLA, CMA and water agar (WA) medium. The petri plates were incubated for 7-8 days at 26± 2° C (Chow and Ting., 2015).

4.10.2. Genomic DNA isolation of potent endophytic fungi

The fungal genomic DNA was isolated from 5-7 days old culture grown on PDA plate using CTAB Genomic DNA isolation method. 6-7 mycelial plugs of 5 mm diameter were scooped out. The mycelia plugs were crushed in mortar-pestle using liquid nitrogen (N_2). 1ml cell lysis (CTAB) solution and 600µl of nuclei lysis solution was added and crushed again in mortar-pestle. The solution was transferred to the fresh eppendorf and incubated at 65°C for 15 min with intermittent mixing. After incubation the eppendorfs were kept at room temperature (RT) for 5 min. The eppendorfs were then centrifuged at 12,000 rpm for 3 min to remove the cell debris. Then 5µl of RNAse was added and incubated at 37°C for 5 min followed by addition of 200µl of protein precipitation solution. The eppendorfs were centrifuged at 12,000 rpm for 3 min to remove contaminating proteins. The aqueous phase containing DNA was transferred to 600-700µl of chilled iso-propanol in the eppendorfs and centrifuged at 12,000 rpm for 3 min. The DNA pellet was rinsed with 70% ethanol followed by centrifugation at 13,000 rpm for 1 min. Then pellet was air dried and dissolved in 50µl of DNA dehydration buffer (Tris EDTA buffer (pH 8)). The qualitative estimation of the DNA isolated was done by agarose gel electrophoresis (Surzycki et al. 2020)

CHAPTER-5

RESULTS AND DISCUSSION

Results and discussion

5.1. Procurement of endophytic fungi

In the present work, 28 endophytic fungi procured from leaves and stems of *Cinnamomum zeylanicum*, *Catharanthus roseus, Spinacia oleracea, and Pterospermum Acerifolium* (L.) (Table 5.1) were procured from existing repository of Dr. Sanjai Saxena, DBT, TIET Patiala. They were regularly maintained at 28 °C by cultivating on PDA plates (fig 5.1). For long-term storage, all the procured fungi were kept on PDA-glycerol slants. Over the long period of co-evolution, a symbiotic relationship between the endophyte and its host plant evolved. Some endophytic fungi can produce salicylic acid independently, while others can stimulate the host plant to produce SA. Since the production of SA by endophytes can strengthen the plant's defenses against pathogens, the current study focuses on selecting the best salicylic acid concentration for testing against particular phytopathogenic fungi and their activity in various environmental conditions.

Fig 5.1: Endophytic fungi procured from *Cinnamomum zeylanicum*, *Catharanthus roseus, Spinacia oleracea, and Pterospermum Acerifolium (L)*

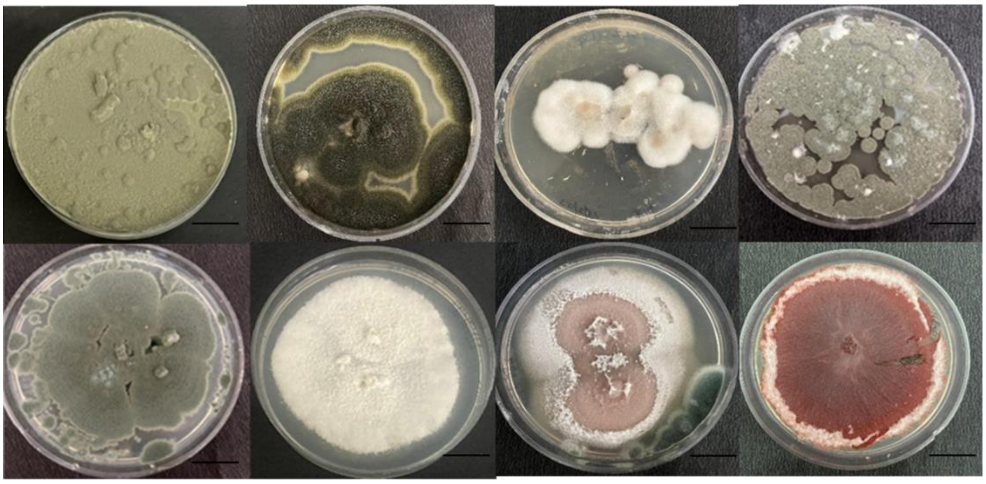


Table 5.1: List of endophytic fungi procured from *C. zeylanicum, C. roseus, Spinacia oleracea, P. acerifolium* and *T. aestivum.*

Culture code	**Plant name**	**Plant part**	**Location**
23CZSTITG	*C. zeylanicum*	Stem	Guwahati, Assam
2106CZSTITG	*C. zeylanicum*	Stem	Guwahati, Assam
53CZSTITBRT	*C. zeylanicum*	Stem	BRT Wildlife Sanctuary, Karnataka
2164CZSTITG	*C. zeylanicum*	Stem	Guwahat Assam
41CZTITBRT	*C. zeylanicum*	Stem	BRT Wildlife Sanctuary, Karnataka
34CZSTITBRT	*C. zeylanicum*	Stem	BRT Wildlife Sanctuary, Karnataka
2107CZSTITBRT	*C. zeylanicum*	Stem	BRT Wildlife Sanctuary, Karnataka
2107CZSTITG	*C. zeylanicum*	Stem	Guwahat, Assam
5CZBAWLS	*C. zeylanicum*	Stem	Kerela
31CZBRT	*C. zeylanicum*	Stem	BRT Wildlife Sanctuary, Karnataka
29CRSTNEY	*C. roseus*	Stem	Neyyar, Kerela
22CRSBRT	*C. roseus*	Stem	BRT Wildlife Sanctuary, Karnataka
6CRSTBRT	*C. roseus*	Stem	Neyyar, Kerela
2SOL1PTL	*S. oleracea*	Leaves	Moti Bir, Patiala
5SOL2PTL	*S. oleracea*	Leaves	Moti Bir, Patiala
H4SOSPTL	*S. oleracea*	Stem	Urban estate, Patiala
H2SOSPTL	*S. oleracea*	Stem	Urban estate, Patiala

3SOLPTL	*S. oleracea*	Leave	Patiala
4SOSPTL	*S. oleracea*	Stem	Moti Bir, Patiala
7SOLPTL	*S. oleracea*	Leave	Moti Bir, Patiala
PALPTL12	*P. acerifolium*	Leaves	Patiala
PALAM19	*P. acerifolium*	Leaves	Amritsar
PALAS20	*P. acerifolium*	Leaves	Amritsar
PALAS21	*P. acerifolium*	Leaves	Amritsar
105TICSTITPLM	*T. cordifolia*	Leaves	Palampur
7TALRP	*T. aestivum*	Leave	Ropar
12TALRP	*T. aestivum*	Leave	Ropar
9TALRP	*T. aestivum*	Leave	Ropar
5TALRP	*T. aestivum*	Leave	Ropar

5.2.Preservation of endophytic fungi

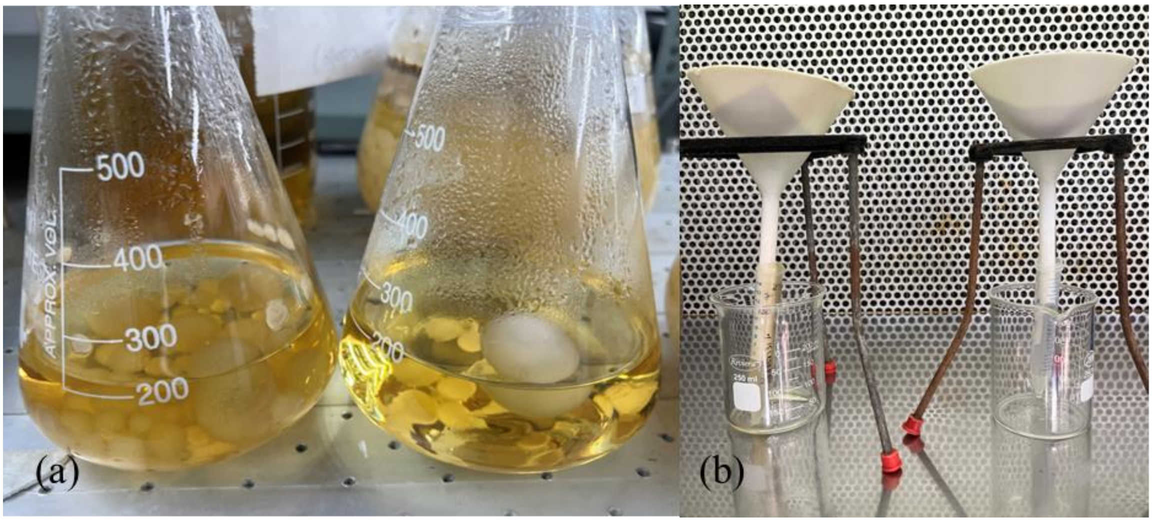
The endophytes were aseptically kept in the PDA slants and vials (Fig.5.2) including 10% glycerol for storing them for a long time as it is not feasible to save cultures in plates for a long-term duration. And were stored at 4°C till further required.

Fig 5.2: PDA slants of endophytic fungi preserved for long-term storage

5.3.Production of procured endophytic fungi

Each procured fungi were incubated in a PDB with a 1 mg/ml dose of cinnamic acid as salicylic acid. Its derivatives are one of the plant hormones produced by the plant naturally and belong to the group of phenolic, the starting ingredient to form is the cinnamic acid, so a compound that helps in the production of salicylic acid by activating the PAL pathway is added to the broth and kept on an orbital shaker at 120 rpm and 27 °C for 9 days. Following incubation, the mycelia fully develop (fig. 5.3 a), and the broth is filtered (fig. 5.3 b). The fungal mycelia are separated from the broth using Whatmann No. 4 filter paper. Filtrates were stored at 4°C for further testing.

Fig 5.3: (a) Production of mycelia in broth (b) Broth is filtered using Whatmann No. 4 filter paper.



5.4.Standard curve of salicylic acid

Different dilutions of 1µg/ml, 2µg/ml, 3µg/ml, 4µg/ml, and 5µg /ml of standard salicylic acid (Loba chemi) with distilled water were prepared and tested via ELISA test (Table 5.2). An increase in color intensity (Fig 5.4) indicates salicylic acid content at different concentrations. Absorbance at 525 nm was noted and the standard curve (fig 5.5) appeared to be a straight line as an increase in the concentration of salicylic acid.

Fig 5.4: Change in colour intensity as the concentration of salicylic acid increases

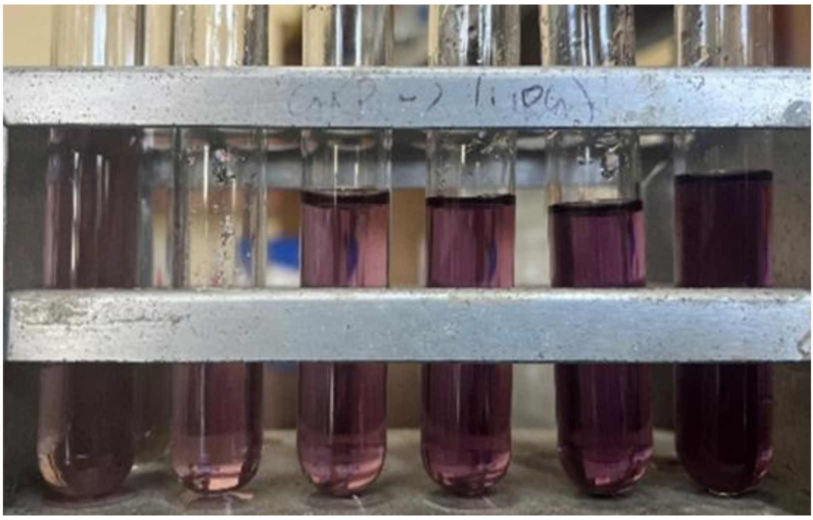
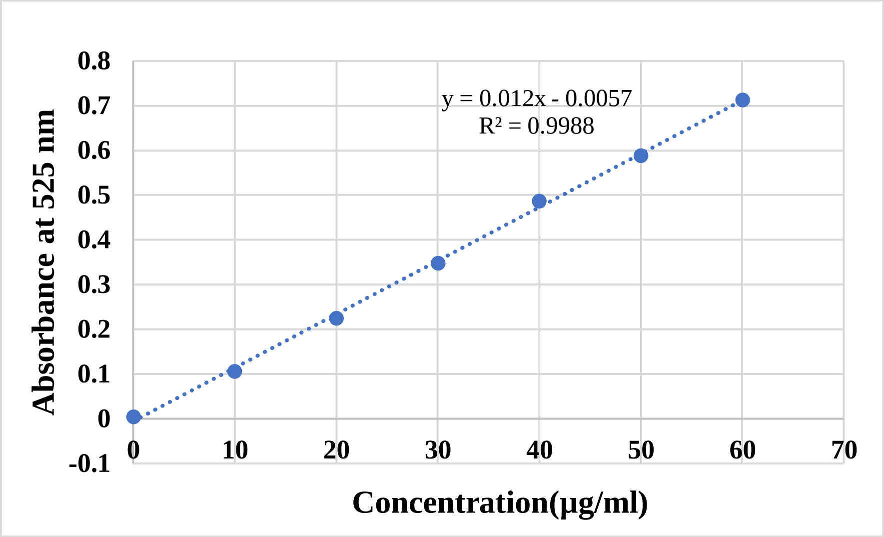


Table 5.2 Measurement of absorbance for prepared samples

Volume of stock solution (ml)	Volume of reagent (ml)	Distilled water to make 10 ml	Concentration of salicylic acid (µg/ml)	Absorbance At 525 nm
0 (blank)	1	9	0	0.0048
1	1	8	10	0.1063
2	1	7	20	0.2256
3	1	6	30	0.3477
4	1	5	40	0.4863
5	1	4	50	0.5880
6	1	3	60	0.7130

Fig 5.5: Standard curve of salicylic acid



The same procedure was used to evaluate the presence of salicylic acid in all the stored filtrates (fig 5.6), and outcomes suggest that #22CRSBRT and #PALAM19 have the highest salicylic acid content, i.e., 101.70 mg/ml and 102.06 mg/ml respectively (Table 5.3). Therefore, only these two samples were used in the follow-up study

Fig 5.6: ELISA plate showing concentration of salicylic acid

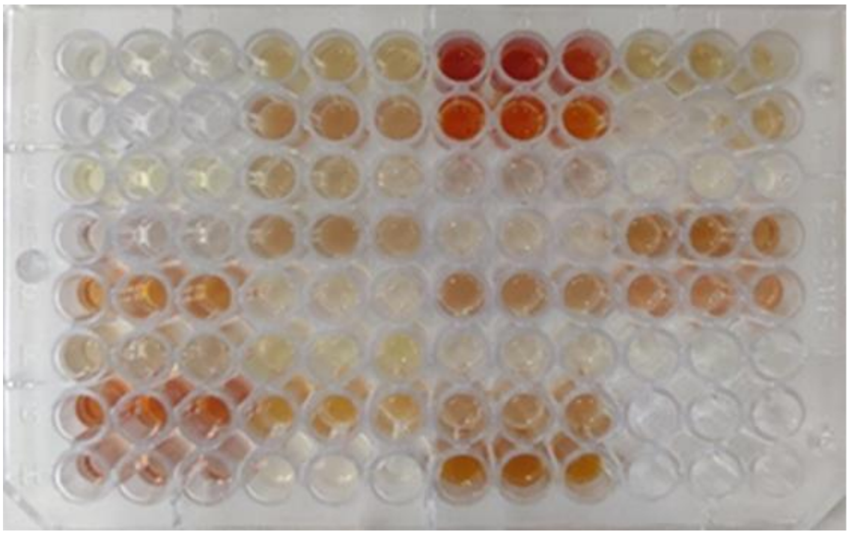


Table 5.3: Concentration of salicylic acid produced by procured endophytic fungi

S.no.	Culture code	A1	A2	A3	Average	Conc.
1	PDB+R	0.233	0.27	0.241	0.25	21.31
2	DW+R	0.052	0.056	0.046	0.05	4.64
3	PAL/PTL/12	0.28	0.214	0.168	0.22	18.81
4	12TALRP	0.161	0.17	0.146	0.16	13.81
5	2107CTITGO	0.464	0.484	0.63	0.53	44.64
6	HSOL9,2	0.472	0.411	0.499	0.46	38.81
7	105TICTPLM	0.909	0.928	0.94	0.93	77.97
8	PTA/AS/21a	0.334	0.363	0.289	0.33	27.97
9	4TICSITICM	0484	0.489	0.447	0.47	39.64
10	7TALRP	1.291	1.287	0.997	1.19	99.64
11	5SOL2	0.238	0.223	0.138	0.20	17.11
12	**22CRSBRT**	**1.242**	**1.200**	**1.202**	**1.21**	**101.70**
13	9TALRP	0.394	0.414	0.27	0.36	30.42
14	2107CSTITGN	0.451	0.302	0.557	0.44	36.86
15	PAL/AS/20a	0.668	0.629	0.409	0.57	47.86
16	34CSTBRT	0.305	0.299	0.239	0.28	23.89
17	23CZSTITG	1.28	1.331	0.873	1.16	97.25
18	53CZSTIBRT	1.094	1.063	0.724	0.96	80.50
19	SOL7,9	0.204	0.198	0.184	0.20	16.75
20	5CZBAWLS	0.431	0.478	0.308	0.41	34.28
21	10571STPLM	1.25	1.119	1.047	1.14	95.36
22	5SOL2	0.301	0.317	0.307	0.31	26.17
23	2106CSTITG	0.453	0.434	0.573	0.49	41.03
24	**PALAM19**	**1.245**	**1.285**	**1.127**	**1.22**	**102.06**

25	5TALRP	0.256	0.306	0.194	0.25	21.48
26	41CZTITBRT	0.17	0.157	0.806	0.38	31.95
27	2SOL4	0.124	0.481	0.198	0.27	22.78
28	31CBAG	0.428	0.601	0.445	0.49	41.42
29	3SOL	0.459	0.499	0.533	0.50	41.89
30	2HSOL	0.034	0.033	0.031	0.03	3.20

To determine the minimum concentration at which the maximum salicylic acid can be produced, those endophytic fungi that have the highest amounts of salicylic acid, according to previous tests, were fermented the same way as previously in section 5.3 at different concentrations (Table 5.4). The results reveal that the highest salicylic acid produced in both samples is at a concentration of 0.5 mg/ml.

Table 5.4: Amount of salicylic acid produced in #PALAM19 and #22CRSBRT at different concentrations

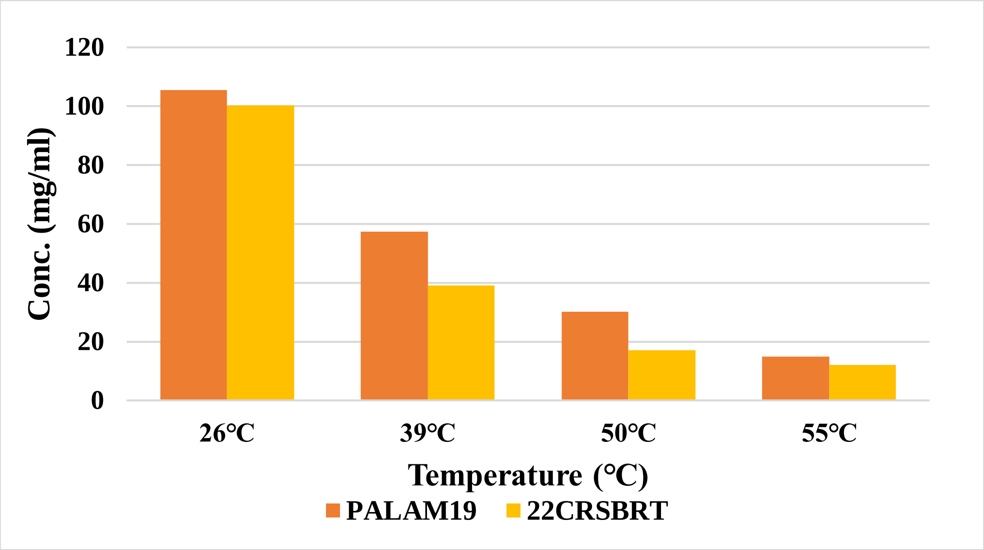
Conc. Of cinnamic acid (mg/ml)	**Conc. Of salicylic acid produced in #PALAM19 (mg/ml)**	**Conc. Of salicylic acid produced in #22CRSBRT (mg/ml)**
0.1	30.97	23.99
0.2	57.26	39.07
0.3	62.83	50.66
0.4	79.97	69.01
0.5	**103.86**	**99.36**

5.5.Optimum temperature

The endophytic fungi #PALAM19 and #22CRSBRT were assayed at 4 different temperatures

26°C,39°C, 50°C and 55°C following the same procedure as above for culture filtrates given in section 5.3. Based on the observations (fig 5.7), maximum salicylic acid production existed at 26°C

Fig 5.7: Production of SA at different temperatures



5.6. Morphotaxonomy of selected endophytic fungi

Morphotaxonomy of the endophytic fungi #PALAM19 and #22CRSBRT was done (Table 5.8 and 5.9) and it revealed that #PALAM19 produced white, slow-growing, readily cultivated fluffy, white aerial mycelium on PDA, RBA, and SDA media after 7 days of incubation. On RBA, growth was not regular, but white mycelia were grown. No growth was there on WA, PLA, and CMA (fig 5.8). At the same time, endophytic fungi #22CRSBRT was produced green, fast-growing, and densely packed on PDA and SDA. RBA Media shows the scattered growth of different colonies. Scattered growth was visible on PLA and CMA, and no colony was seen on WA after 7 days of incubation at 27°C (fig 5.9).

Table 5.5: Colony morphology of #PALAM19 on different media after 10 days at 28°C

Medium	Colony Color		Margin	Odour
	Front	**Back**		
PDA	White	White	Regular and flat	No odour
SDA	White	White	Regular and flat	No odour
RBA	White	White	Regular and flat	No odour
PLA	No growth		-	-
CMA	No growth		-	-
WA	No growth		-	-

Fig 5.8: #PALAM19 culture grown on different media plate a) PDA b) SDA c) RBA d) PLA e) CMA and f) WA

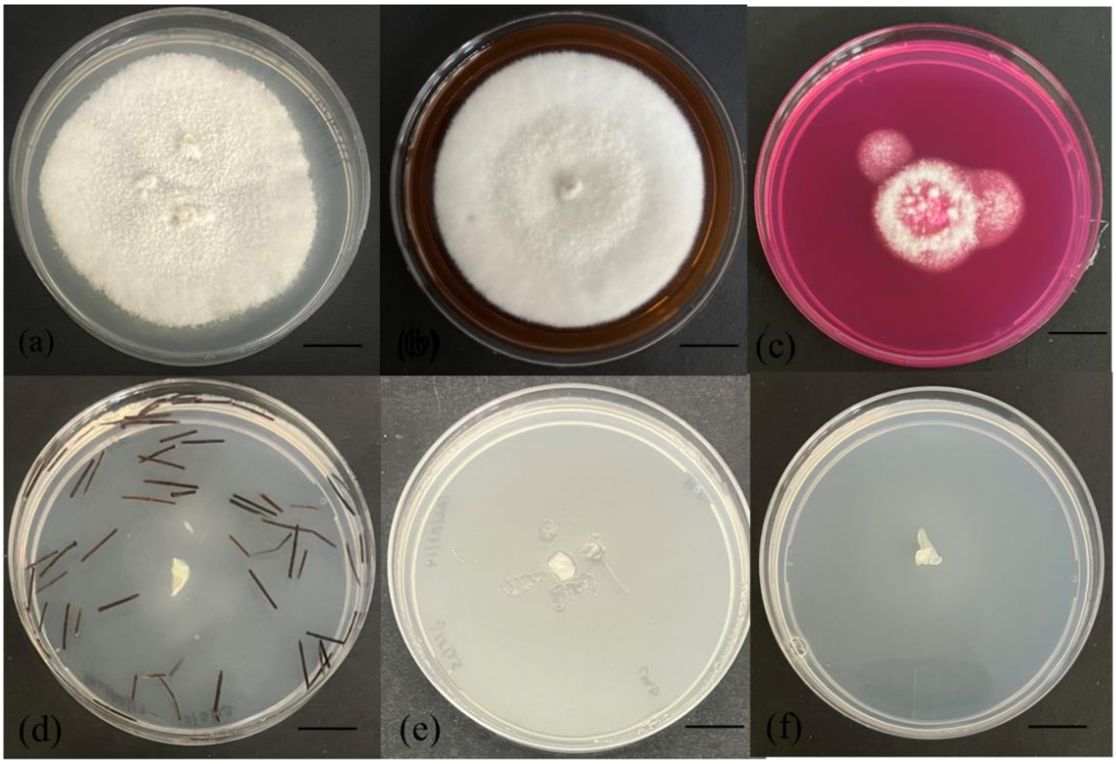
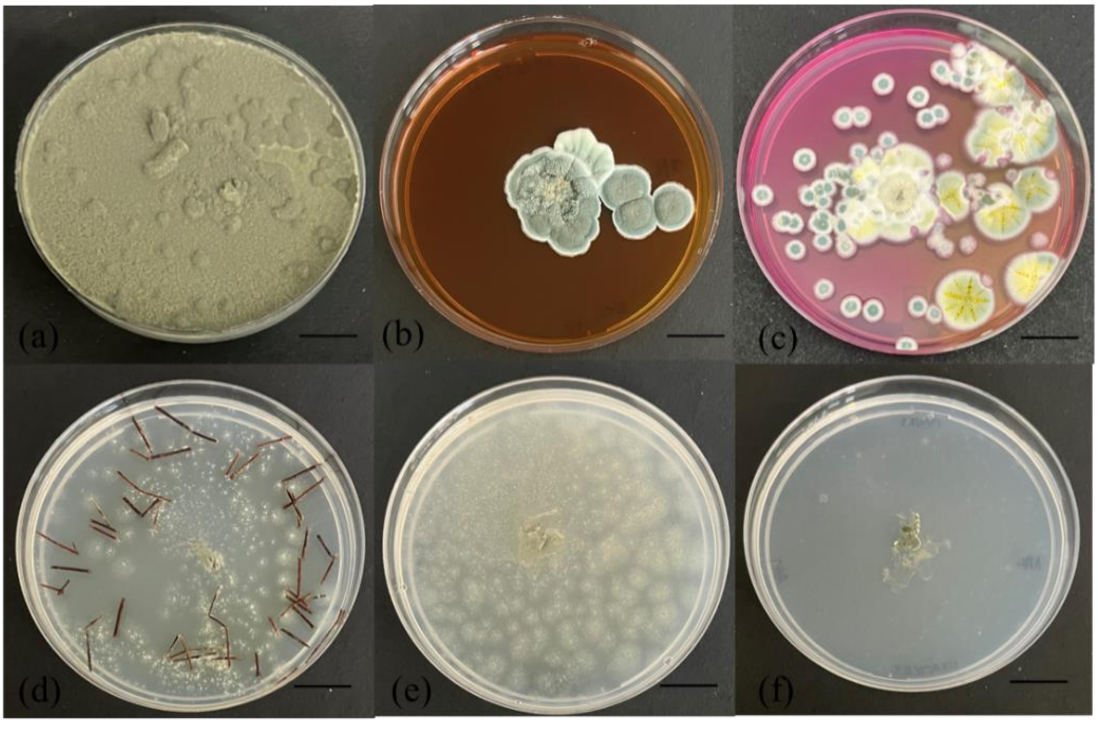


Table 5.6: Colony morphology of #22CRSBRT on different medias after 10 days, 28°C

Medium	Colony color		Margin	Odour
	Front	back		
PDA	Green	White	Entire	No odour
SDA	Green	White	Irregular	No odour
RBA	Green	White	Irregular	No odour
PLA	Green	White	Irregular	No odour
CMA	Green	Green	Irregular	No odour
WA	No growth		-	-

Fig 5.9: #22CRSBRT culture grown on different media plate a) PDA b) SDA c) RBA d) PLA e) CMA and f) WA



5.7.Genomic DNA Isolation:

The genomic DNA of #PALAM19 and #22CRSBRT were isolated (fig. 5.10) and the size of genomic DNA was found to be 10 kb approximately. The concentration of the genomic DNA isolated viz. #PALAM19 and #22CRSBRT was 32ng/µl and absorbance at 260nm/280nm was taken which was 1.67 to determine the quantity and purity of the DNA isolated. Further PCR amplification can also be carried out.

Fig 5.10: a) Genomic DNA isolation of #PALAM19 and #22CRSBRT

5.8.Optimum concentration of salicylic acid

The results of the present study are in line with research carried out by different researchers. The zone of inhibitions was measured by the agar well diffusion method to find out the antifungal activity of the salicylic acid (loba chemi) against procured pathogens.

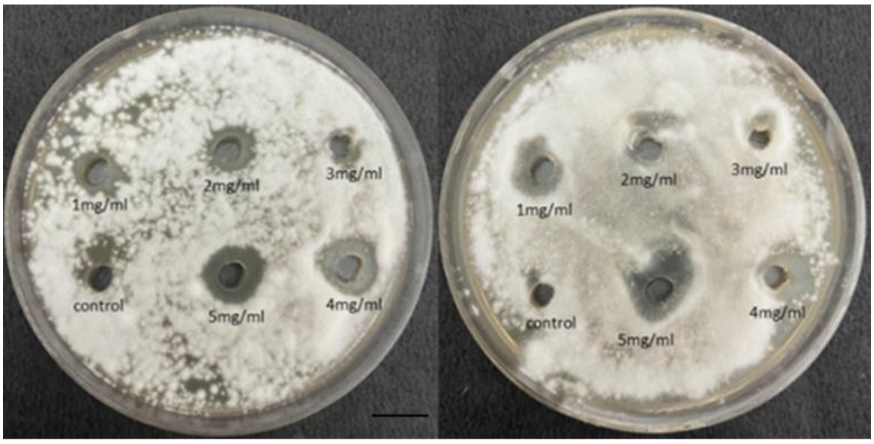
Table 5.7: Zone of inhibition of pathogens at various concentrations of SA

Sr.no.	Pathogen	Concentration of sa (mg/ml)					
		C	1	2	3	4	5
		And					

		Zone of inhibition (in cm)					
1	3801 *(Collectotrichum gloeosporiodes)*	0	0.4	0.4	0.4	0.4	0.7
2	6152 *(Collectotrichum gloeosporiodes)*	0	0.1	0.1	0.2	0.3	0.5
3	4533 *(Fusarium lateritium)*	0	0.2	0.3	0.4	0.5	0.6
4	6129 *(Alternaria alternata)*	0	0	0	0.1	0.1	0.1
5	6343 *(Alternaria alternata)*	0	0	0.1	0.1	0.1	0.1
6	6011 *(Botrytis cinera)*	0	0	0.1	0.1	0.1	0.1
7	6240 *(Fusarium moniliforme)*	0	0	0.1	0.3	0.3	0.5
8	6435 *(Fusarium moniliforme)*	0	0	0.1	0.2	0.4	0.9
9	6354 *(Aspergillus niger)*	0	0.2	0.3	0.5	0.6	0.6

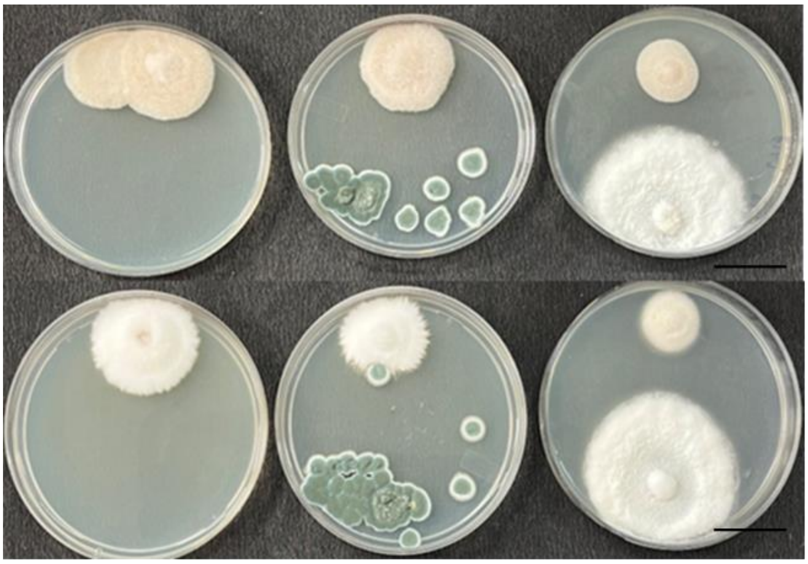
The results demonstrated an inhibitory effect at different conc. i.e., 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml, and 5mg/ml concentration of SA against different procured pathogens (Table 5.7). A significant zone of inhibition is visible at a concentration of 5 mg/ml (fig 5.11). 5mg/ml conc. of Salicylic acid shows a maximum zone of clearance, i.e., 0.9 cm against *Fusarium moniliforme*.

Fig 5.11: Antifungal activity of different delusions of salicylic acid



Procured endophytic fungus #PALAM19 and #22CRSBRT were evaluated for antifungal effect against procured ITCC pathogens. As seen from (fig 5.12), our samples exhibit decreased growth in response to all pathogens obtained. So we further tested antifungal activity by agar well diffusion method for more precise examination.

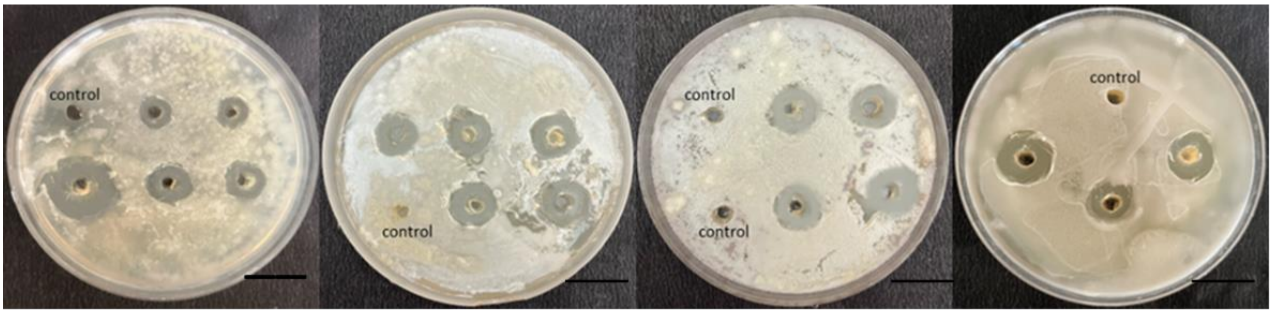
Fig 5.12: #PALAM19 and #22CRSBRT shows positive antifungal activity



As #PALAM19 shows the best antifungal activity and produces the highest amount of salicylic acid, so agar well diffusion test was carried out to check the antifungal activity and appropriate zone of inhibitions against procured ITCC pathogens as mentioned in Table 2.1 (fig 5.13).

Salicylic acid produced by #PALAM19 at 26°C was tested against procured pathogens. And the results illustrated that against most of the pathogens, #PALAM19 shows 0.9cm to 1.2 cm of the zone of inhibition but mostly 1cm, which is better than chemically obtained salicylic acid.

Fig 5.13: #PALAM19 showing zone of inhibition against various pathogen



Discussion

Salicylic acid (SA) has been widely studied for its potential applications in post-harvest treatments to enhance the quality and shelf life of various fruits and vegetables. Several studies have shown that treating fruits and vegetables with salicylic acid can delay senescence, the natural aging process leading to deterioration and loss of quality. SA treatment has been found to maintain firmness, reduce weight loss, and delay the onset of rotting and decay, thereby extending the shelf life of produce. Salicylic acid has been shown to inhibit the growth of various fungal pathogens. The inhibitory effect of SA on fungal growth is attributed to its ability to disrupt fungal cell membranes, interfere with vital metabolic pathways, and induce programmed cell death in fungi. Various studies have demonstrated that salicylic acid has efficacy in controlling post-harvest fungal pathogens, which are responsible for the decay and spoilage of harvested fruits and vegetables (Simone et al. 2020).

Martín in 2019 studied the effect of SA on the growth of the *Ophiostoma novo* on *Ulmus minor*. 1X 10^5 spore suspension was absorbed on the plate and concentrations of SA in the wells (control wells), 0.2, 1, 10, 50, 100, and 500 µg/ml were poured and plates were incubated at 20 ºC. Rangel in 2020 prepared fungal spores of *P. cinnamomic* (1×10^6 spores/mL) in a liquid culture from fungal cultures grown on PDA plates. The spore-containing suspensions were incubated at 24 °C and were checked for zone of inhibition against different concentrations ranging from 0.1 to 0.5 mg/ml of salicylic acid. In this current study the antifungal activity of salicylic acid against *Collectotrichum gloeosporiodes*, *Fusarium lateritium, Alternaria alternata, Botrytis cinera, Fusarium moniliforme* and *Aspergillus niger* was studied. We collected 1X10^5 spores suspension of all the pathogens and tested their antifungal activity by agar well diffusion method at different concentration of SA i.e., 0.1mg/ml to 0.5 mg/ml.

Huang (2007) illustrated that fruit treated with high SA concentrations (1.0 and 2.0 mmol L^{-1}) have higher resistance towards the pathogens which cause loss of orange (*Citrus sinenisis* L. Osbeck). Gacnik (2021) found that 2 mM solution of SA stimulate the activity of the phenylalanine ammonia lyase (PAL) which led to an increase in certain proven anti fungicidal phenolics. Applications of 4 mM SA conserved strawberry from ascorbic acid (AsA) loss compared to control at the end of the storage period as noted by Darvish in 2021. Our results are also in line of these studies as SA present in our procured endophytic fungi #PALAM19 stimulates antifungal effects at 0.5 mg/ml concentration.

The involvement of SA in the priming of plant defense by *Trichoderma* strains against *Botrytis cinerea* was described in *Arabidopsis* and *Solanum lycopersicum.* Kou and colleagues showed that *Epichloë* endophytes are capable of inducing SA-dependent defence responses in the host plants to provide significant resistance against the pathogen *Blumeria graminis*. Previously as noted by Chitra et al (2008), the effect of salicylic acid in inducing resistance in groundnut plants against *Alternaria alternata* was investigated. Simone in 2020 noted that Fungi such as *Botrytis cinerea, Alternaria spp, Penicillium spp*, and *Rhizopus spp*. are sensitive to SA treatment.The role of salicylic acid (SA) was investigated in basal defense and induced resistance to powdery mildew (*Oidium neolycopersici*) and grey mould (*Botrytis cinerea*) in tomato (*Lycopersicon esculentum*) by Achuo in 2019.

Under glasshouse circumstances, foliar application of SA at a dosage of 1 mM dramatically decreased the leaf blight disease intensity and enhanced pod output. In 2021 Gomaa noted that a 2.5 mM concentration of salicylic acid shows inhibitory results against *Aspergillus niger* on wheat plants. Our study describes the potential role of SA produced by procured fungi #PALAM19 in defense against *Collectotrichum gloeosporiodes*, *Fusarium lateritium,* *Alternaria alternata, Botrytis cinera, Fusarium moniliforme,* and *Aspergillus niger.* SA extracted from #PALAM19 shows 1cm of zone of clearance at 0.5mg/ml concentration against above-mentioned pathogens. We compared the activity of chemically obtained salicylic acid (Loba chemi) and salicylic acid produced by procured endophytic fungi. Results illustrated that 5mg/ml of chemically obtained SA acid shows effective antifungal properties against pathogens like *Collectotrichum gloeosporiodes*, *Fusarium lateritium, Alternaria alternata, Botrytis cinera, Fusarium moniliforme* and *Aspergillus niger*.

CHAPTER-6

CONCLUSION

CONCLUSION

1. A total of 28 endophytic fungi procured from stems and leaves of *C. zeylanicum, C. roseus, Spinacia oleracea, P. acerifolium and T. aestivum.* The fungi #PALAM19 and #22CRBRT exhibited best result on the two factors i.e., maximum salicylic acid production at 0.5% concentration of cinnamic acid and antifungal activity.
2. Further these cultures were tested for production of SA at different temperatures and their zone of inhibition against pathogenic fungi. #PALAM19 shows the best results.
3. Then the results were compared to the chemically obtained SA and it revealed that naturally produced SA is better than industrial salicylic acid.

CHAPTER-7

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