Mycofumigation potential of novel endophytic fungus *Muscodor sp.* at enhancing the shelf life of fruits and vegetables

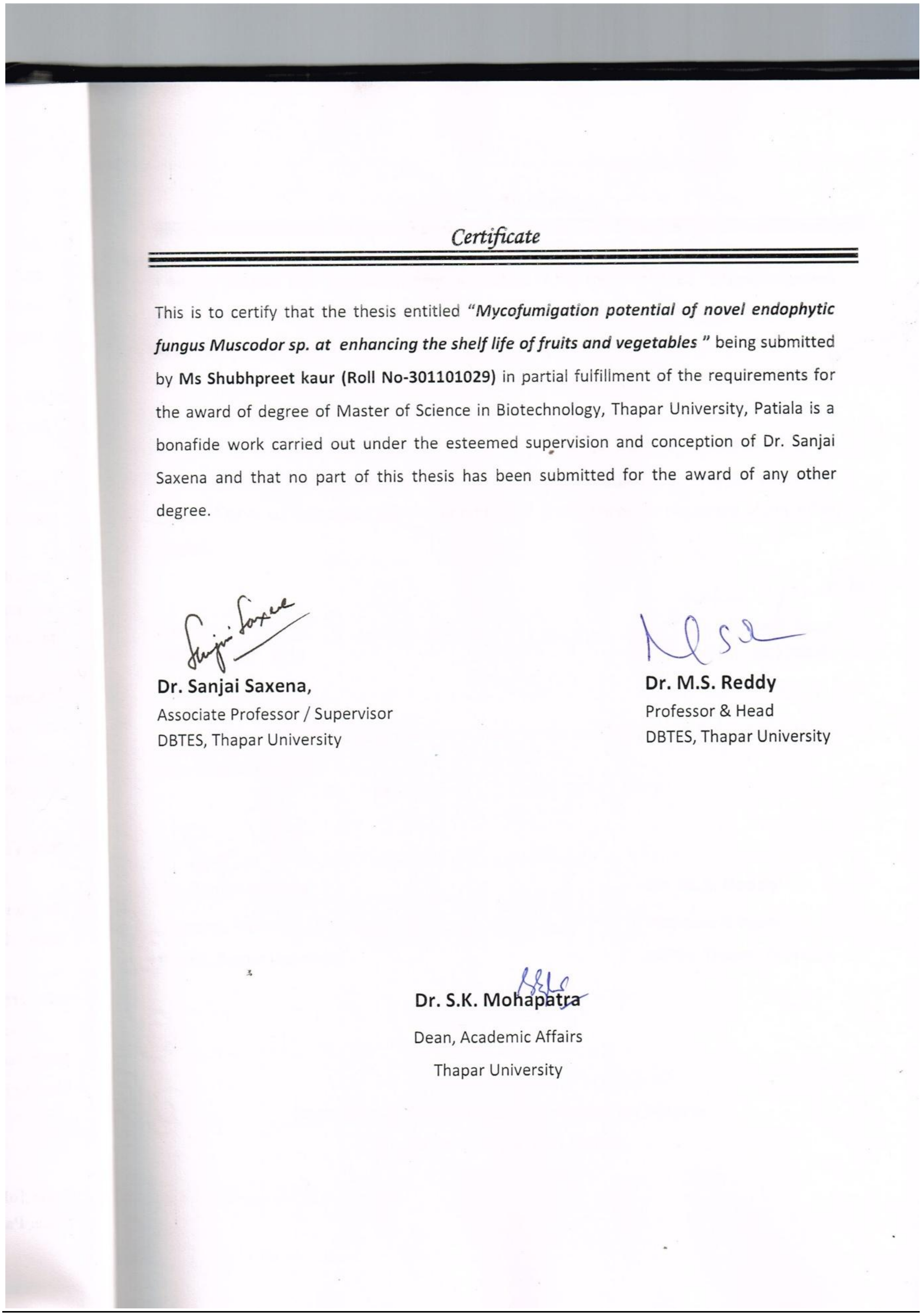
A
Thesis submitted
in partial fulfilment for the award of degree
of

Master of Science
In
Biotechnology

Submitted by
SHUBHPREET KAUR
Roll no. 301101029

Under Supervision of
Dr. SANJAI SAXENA
Associate Professor

Department of Biotechnology & Environmental Sciences
Thapar University, Patiala, Punjab
July 2013



Certificate

This is to certify that the thesis entitled ***"Mycofumigation potential of novel endophytic fungus Muscodor sp. at enhancing the shelf life of fruits and vegetables "*** being submitted by **Ms Shubhpreet kaur (Roll No-301101029)** in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.

Dr. Sanjai Saxena,
Associate Professor / Supervisor
DBTES, Thapar University

Dr. M.S. Reddy
Professor & Head
DBTES, Thapar University

Dr. S.K. Mohapatra
Dean, Academic Affairs
Thapar University

Candidate's Declaration

I hereby declare that the work being presented in the thesis entitled "***Mycofumigation potential of novel endophytic fungus Muscodor sp. at enhancing the shelf life of fruits and vegetables***" in partial fulfilment of the requirements for the award of degree of Masters in Biotechnology, Department of Biotechnology. and Environmental Sciences, Thapar University, Patiala is my own laboratory work during the period of January 2013 to June 2013, under the conception and supervision of Dr. Sanjai Saxena, Associate professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Shubhpreet kaur

Patiala
Date: July 12th, 2013

Shubhpreet kaur
Roll No. 301101029

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

Dr. Sanjai Saxena
Associate Professor / Supervisor
DBTES, Thapar University

Dr. M.S. Reddy
Professor & Head
DBTES, Thapar University

Department of Biotechnology & Environmental Sciences
Thapar University, Patiala 147004

Acknowledgement

It is said- "God has perfect plans for each one of us", earning the project I have, bears testimony to it. It has proved to be the best for me in every way, not just because of the format of work involved in its completion was an exciting task, but also because it bestowed me with the opportunity to work under the guidance of geniuses.

First and foremost, I offer my sincerest gratitude to my supervisor, Dr. Sanjai Saxena for the encouragement, guidance and support throughout the project. The patience and enthusiasm on his part was truly incredible.

My sincere thanks to Dr. K.K. Raina, Director, Thapar University and Dr. M.S. Reddy, Head, Department of Biotechnology & Environmental Sciences, Thapar University, Patiala for providing necessary infrastructure to do this project work.

I find myself searching for words when it comes to expressing my heartfelt gratitude towards Mr. Vineet Meshram, Research Scholar, his zeal, guidance, efforts, support, patience was exceptional and really heart-warming, something that has seen me through. No words of appreciation for acknowledging his role.

I warmly thank Miss Neha Kapoor and Miss Mahiti Gupta, Research Scholars, DBTES, Thapar University for sharing their knowledge and constructive advice. I am immensely indebted and owe a great many thanks to Vineet Sir & Neha Ma'am for their detailed & constructive comments on the initial draft, suggestions on their part at the effective way of data presentation were rewarding & have resulted in shaping the thesis to the present form.

I dedicate this thesis to the dedicated Research Scholars. I got the opportunity to learn from Vineet Sir, Neha & Mahiti Ma'am as a token of respect.

The co-operation offered by my friends Anjali, Rahul Rana and Sanjay Singh can no way be ignored and is deeply acknowledged. Someone who deserves special thanks is another good friend Manvir for being there in times of need.

I convey my sincere thanks to our hard-working & helpful Lab technicians & Lab-attendants for the assistance & presence which made routine work easy.

I feel a deep sense of gratitude for my parents and brother who formed part of my vision and taught me the good things that really matter in life. Above all, I'm grateful to almighty God for blessing me to complete this work successfully.

Date: July 15th, 2013

Place: Patiala **Shubhpreet Kaur**

Executive Summary

The current study exploits four *Muscodor* isolates isolated from various biodiversity hotspots of India for their mycofumigation potential. All the four isolates were resistant to the VOCs produced by native *Muscodor albus* cz 620 in VOC stress bioassay. The four isolates when subjected for antimicrobial activity using VOC stress bioassay showed potential broad spectrum antibacterial and antifungal activity against plant and human pathogens. Out of four *Muscodor* isolates #16 AMLWLS was most lethal to the plant and human pathogens. It exhibited complete inhibition of 11 pathogenic microbes whereas growth of rest of the isolates was reduced to 50-70%. #6610 CMSTITBRT and #1 CCSTITD showed antibacterial and antifungal activity whereas #2 CCSTITD showed antifungal activity. VOCs produced by #16 AMLWLS successfully act as a biofumigant. It act as a preservatives for grapes, jamun, cherry, black gram and wheat from *Botrytis cinerea, Rhizoctonia solani, Collectrotrichum gloeosporioides and cercospora beticola* infection. The inhibition was 80-100% under *in vitro* conditions. All the four *Muscodor* isolates produces sterile mycelia with coiling structures and their sequence also showed high similarity with *Muscodor* species. Thus this confirms their identity as *Muscodor* species. Thus, these *Muscodor* isolates can be taken into account to be developed as myco/biofumigant that act a biopreservative for fruits, vegetable and grains.

Chapter 1

Introduction

INTRODUCTION

Nature has always kept its basket full with the most wonderful gifts for mankind. A variety of fruits and vegetables available around the year serve not only as delicacies for many of us but also hold immense nutritional value- being a rich source of vitamins, minerals and fibre. With all its benefits fruits & vegetables also bear a disadvantage- it being their “perishable” nature, making “shelf-life” a cause of concern not just for the grower’s but also people in retail and processing businesses. Shelf-life is a period of time a food has before it becomes unsuitable for consumption or sale. Besides the physiological processes (respiration, ethylene production) and physical parameters (temperature, humidity), fruits & vegetables are attacked by a host of bacterial and fungal pathogens & suffer from a range of pre- & post-harvest diseases. After harvesting fresh fruits are washed, sanitised and handled under low temperatures in controlled or modified atmosphere, this lower’s the incidence of pathogen attacks, however, microbial pathogens are more likely to constitute a major problem in stored vegetables and fruits (Ramin *et al.*, 2007). The USDA Economic Research Service estimated that more than ninety-six billion pounds of food in the US was lost by retailers. Though food losses are a global issue, equally concerning for both industrialized & developing countries, but in developing countries more than 40% of food losses occur at post-harvest & processing levels. 42% of fruits & vegetables are lost to post-harvest, processing & distribution-the stages of food supply chain (Gustavsson *et al.*, 2011).

Bacterial and fungal infections, that occur at the time of harvest, during handling, storage, transport and marketing and after consumer purchase (Eckert and Ogawa, 1988) are a major cause of postharvest loss in horticulture crops. Several fungicides and volatile chemicals are available for postharvest treatment of fruits and vegetables, but a few of them have been removed from the market due to possible toxicological risks associated with them or because of their insufficient efficacy (Adaskaveg *et al*., 2002). Various other conventional approaches are being followed – like controlling the postharvest gray mould infecting grape berries by sulphur dioxide fumigation (Luvisi *et al*., 1992). Harvested citrus fruits are very susceptible to wound infections caused by *Penicillium digitatum* resulting in the development of green mould (Sommer *et al.*, 2002). The main methods of reducing losses caused by this pathogen include the minimization of wounds on fruit, proper temperature management, and application of postharvest fungicides (Eckert and Eaks, 1989). The main cause of pre- and post harvest decay of peaches is brown rot, caused primarily by *Monilinia fruiticola*, (Sommer *et al*., 2002).Application of de-methylation inhibitor (DMI) fungicides, and more recently, respiration inhibitor fungicides, such as boscalid, azoxystrobin, and pyraclostrobin are primarily the main methods of management of the infection (Schnabel *et al.*, 2004). Methyl bromide is another available alternative to treat fruits and vegetables postharvest. However all of these approaches suffer many drawbacks- Sulphur dioxide fumigation effectively controls gray mould, but bleaching injury to berries, particularly among those detached from the cluster rachis, and injury to the rachis itself occur among commercially stored grapes (Crisosto and Mitchell, 2002). After fumigation with sulphur dioxide, grape berries become more susceptible to subsequent infections by *B. cineria* (Taylor *et al.*, 1990). Also, sulphur dioxide is not accepted for organic grapes, under current certification rules, and some regulatory agencies do not allow the discharge of sulphur dioxide to the air after fumigation (Mlikota-Gabler *et al.*, 2006). Because of the issues associated with sulphite residues, sulphur dioxide emissions, and sulphur dioxide's negative impact on grape quality, alternative strategies to control gray mould are needed that are safe and at the same time effective, and economical (Adaskaveg *et al.*, 2002., Karabalut *et al.*, 2003., Litcher *et al.*, 2002.,Mlikota-Gabler *et al.*, 2001., Mlikota-Gabler *et al.*, 2005).

Near-harvest ethanol or biological control agent applications act as alternatives to sulphur dioxide for the control of postharvest gray mould include (Droby and Litcher, 2004, Karabulut *et al.*, 2003).

Continuous use of fungicides in citrus packing units in California has resulted in development of multiple fungicidal resistances within natural populations in *P. digitatum* isolates (Holmes and Eckert, 1999), which further complicates the management of green mould. In addition, maximum residue limits for imazalil, the most important fungicide in use, are lower in most citrus importing countries than the ones exporting them (United States Department of Agriculture-Foreign cultural Service, 2004). These constraints and a number of important advantages offered by bio-control agents over traditional approaches make their commercial outlook particularly promising.

As in general, the bio-control agents are considered non-hazardous to humans and animals, are biodegradable and environmental-friendly, attack specific target organisms without affecting the other beneficial organisms (Adaskaveg *et al.*, 2002). The main advantage of fumigation in controlling postharvest decay compared with other approaches is that it does not requires processing or manual handling of the concerned fruit, having described the negative impacts of chemical fumigants, a novel alternative for controlling post harvest decay is biological fumigation or bio-fumigation, with the fungal genus *Muscodor.*

The volatile producing fungus *Muscodor albus* has shown potential as a bio-fumigant for controlling post-harvest decay in various commodities (Mercier & Jimenez, 2004; Mercier & Smilanick, 2005).

Endophytes are micro-organisms residing in a unique and specialised biological niche- the intracellular space between the cells of higher plants without causing any overt symptoms onto the plant they are residing in (Bacon & White, 2002). Endophytes are most abundant in tropical rainforests. Novel endophytes usually have associated with them many novel secondary natural products and various new processes (Strobel, 2006). The list of natural products obtained from endophytes is quite diverse and has potential in pharmaceutical and agrochemical areas- a) microbial products act as antibiotics such as Cryptocandin A- antifungal peptide obtained from *Cryptosporiopsis quercina* b) endophytic fungal products possess potential as anticancer agents like Paclitaxel, world's first billion-dollar anticancer drug is produced by many endophytic fungus c) Endophytes are reported to produce products that can be used as antioxidants, anti-diabetic agents, immunosuppressive compounds and a few with insecticidal properties.

Isolation of volatile antibiotic producing novel endophytic fungal genus *Muscodor* is quite a promising discovery owing to its ecological implications and because of the potential practical benefits offered due to the inherent fumigation potency of the genus (Strobel & Daisy, 2003). *Muscodor* represents the genus of Xylariaceaous (non- spore producing) fungus effectively inhibiting and killing certain other fungi and bacteria by producing a mixture of volatile compounds (Strobel *et al.*, 2001). The initial fungal isolate was as an endophyte in *Cinnamomum zeylanicum* in a botanical garden in Honduras isolated in late 1990's. This endophytic fungus was named *Muscodor albus* because of its odour and white colour. VOC's of this fungus are effective against not just to a wide variety of plant and human pathogenic fungi and bacteria but also nematodes and certain insects (Strobel, 2011). This isolate has been used as a selection tool to identify other species of the genus possibly located in various hotspots of the world, till date twelve *Muscodor* species have been reported and are being exploited to kill a suite of pathogens by the synergistic action of the VOC's, which are quite distinct both qualitatively & quantitatively in all of the reported species though falling primarily in the class of ketones, esters, alcohols, acids and lipids. The aim of current project is to evaluate anti-microbial efficacy of the four novel Indian Muscodor isolates and study the bio-fumigation potential of the most efficacious culture.

Chapter 2

Review of Literature

2.1 Discovery of *Muscodor*

Fig 1 *Muscodor albus* CZ 620

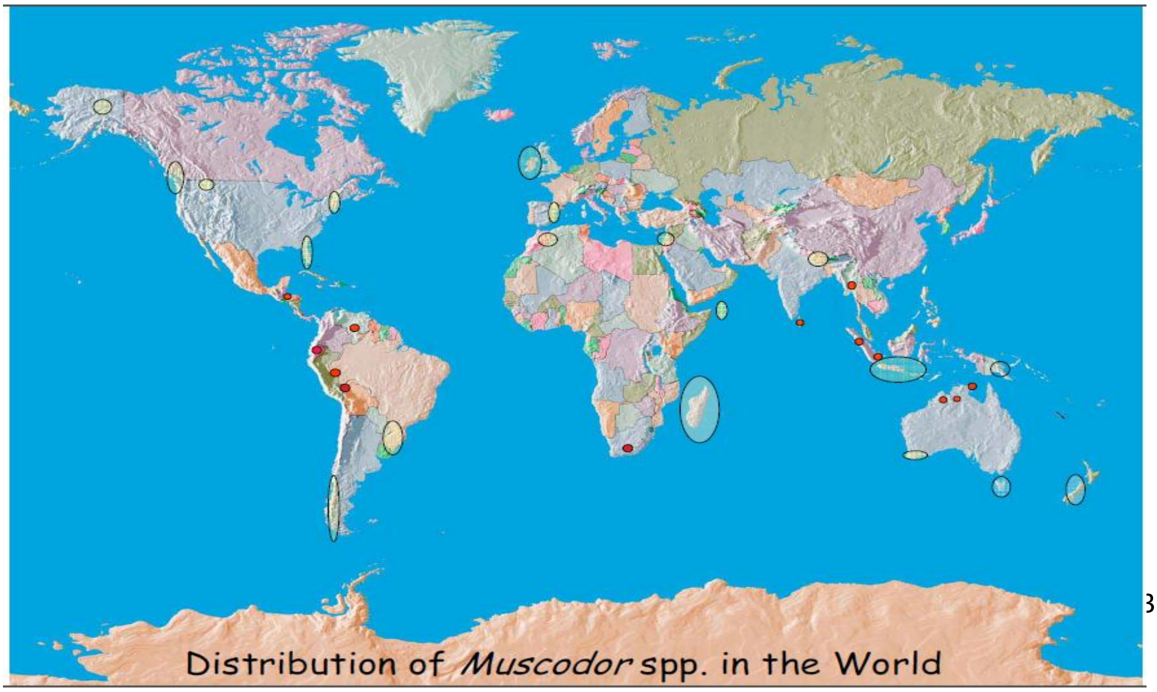
Muscodor albus is an extraordinary endophytic fungus that is renowned for producing volatile antimicrobial. *M.albus* was for the first time isolated as an endophytic fungus from the stems of *Cinnamomum zeylanicum* by Prof. Gary Strobel, Emeritus Professor, Department of Plant Sciences and Plant Pathology, Montana State University, USA during his forest forays in Honduras, Central America in 2001 (Strobel *et al.*, 2001). There is an interesting story related to the discovery of *M. albus*. Petri-plates containing plant tissues were placed in large plastic boxes with firmly fitting lids to eliminate the invasion of mites and other fastidious microbes. Endophytic fungal growth was observed in most plant parts after few days of incubation, plates were removed and the emerging fungus were transferred as individually from the hyphal tips onto fresh Potato Dextrose Agar (PDA) plates, after two days incubation only one of the transferred endophytes was observed to grow. This was due to the limited oxygen supply in the plastic box on the contrary was the production of volatile antibiotics (volatile organic compounds-VOC's) by the endophytic fungus that remained alive (designated as isolate 620), it was these VOC's that killed or inhibited the growth of other endophytes, thus out came the hypothesis, that an endophyte can produce or generate volatile antibiotic substances with a wide range of biological activities (Strobel & Daisy, 2003).

2.2 Geographical distribution of *Muscodor* species

There are 12 reported *Muscodor species* till date from different parts of the world like Central and South America, South-Eastern Asia & Australia (Worapong *et al.*, 2001, 2002; Daisy *et al.*, 2002b; Ezra *et al.*, 2004; Mitchell *et al.*, 2008; Gonzalez *et al.*, 2009; Zhang *et al.*, 2010; Kudalkar *et al.*,2012; Suwannarach *et al.*, 2013). *Muscodor sp*ecies produce a mixture of volatile organic compounds (VOC's) that consist primarily of various alcohols, esters, acids, ketones, and lipophilic substances which are lethal to a wide variety of plant & human pathogenic fungi, and bacteria as well as to nematodes and certain insects. (Daisy *et al.*, 2002a & Strobel *et al*., 2001). *Muscodor sp*. are therefore of great importance and promise for biocontrol (Strobel, 2006) therefore there is great interest among the mycologist to find and explore newer *Muscodor* isolates. *Muscodor albus* CZ 620 was used as selection tool for isolating new *Muscodor* species (Ezra *et al.,* 2004). Table no.1 represents the twelve *Muscodor* species so far.

***Muscodor* species**	**Host Plant**	**Reference**
Muscodor albus CZ-620	*Cinnamomum zeylanicum*	Worapong *et al.*,2001
Muscodor roseus A3-5	*Grivillea pteridofolia*	Worapong *et al.*, 2002
Muscodor vitigenus P-15	*Paullinia paullinoides*	Daisy *et al.*,2002
Muscodor crispans B-23	*Ananas ananassoides*	Mitchell *et al.*,2008
Muscodor yucatanensis B110	*Bursera simaruba*	Gonzalez *et al*. 2009
Muscodor fengyangensis ZJLQ070	*Actinidia chinensis*	Zhang *et al*.,2010
Muscodor sutura CA22-D	*Prestonia trifidi*	Kudalkar *et* al.,2011
Muscodor cinnamomi CMU-Cib461	*Cinnamomum bejolghota*	Suwannarach *et al.*,2010
Muscodor oryzae CMU-WR2	*Oryza rufipogon*	Suwannarach *et al.*,2013
Muscodor suthepensis CMU-Cib462	*Cinnamomum bejolghota*	Suwannarach *et al.*,2013
Muscodor musae CMU-MU3	*Musa acuminate*	Suwannarach *et al.*,2013
Muscodor equeseti CMU-M2	*Equisetum debile*	Suwannarach *et al.*,2013

Table No.1 showing different *Muscodor species r*eported till date



Distribution of *Muscodor* spp. in the World

Fig 2 Geographical distribution of *Muscodor* species in the world

Taxon	Host	Mycelium pigment production	Hyphal growth at colony front	Mycelial growth	Major compounds	Bioactivity
Muscodor albus[a, b]	*Cinnamomum zeylanicum*	White	Straight	Rope-like	2-Methyl propanoic acid, naphthalene & azulene derivatives	Antifungal and Antibacterial
Muscodor cinnamomi[c]	*Cinnamomum bejolghota*	White in dark & pale orange in light	Straight	Rope-like with cauliflower like bodies	Ethyl 2- methyl propanoate	Antifungal and Antibacterial
Muscodor crispans[d]	*Ananas ananassoides*	White in dark and pink in light	Wavy growing	Rope-like with cauliflower like bodies	2- methyl propanoic acid	Antifungal and Antibacterial
Muscodor equiseti[e]	*Equisetum debile*	White	Cottony like pattern	Rope like with coils structure & swollen cells	2- methyl propanoic acid	Antifungal and Antibacterial
Muscodor fengyangensis[f]	*Actinidia chinensis*	White	Straight	Rope like with coils structure	2- methyl propanoic acid	Antifungal and Antibacterial
Muscodor musae[e]	*Musa acuminata*	White	Straight & hairy like mycelium	Rope like with coils structure	2- methyl propanoic acid	Antifungal and Antibacterial
Muscodor oryzae[e]	*Oryza rufipogen*	Pale orange	Straight	Rope like with coils structure	3- Methylbutan-1-ol	Antifungal and Antibacterial
Muscodor roseus[g]	*Grivillea pteridifolia*	Lightly rose	Felt like mycelium	Forming rope like strands & coils structure	Ethyl 2- butanoate & 1,2,4- trimethylbenzene	Antifungal
Muscodor suthepensis[e]	*Cinnamomum bejolghota*	White in dark & pale pink in light	Straight	Forming rope like strands & coils structure	2- methyl propanoic acid	Antifungal and Antibacterial
Muscodor sutura[h]	*Prestonia trifidi*	White in light & reddish in dark	Suture like pattern	Rope-like nondescript extracellular	2- methyl propanoic acid	Antifungal and Antibacterial
Muscodor vitigenus[i]	*Paullinnia paullinioides*	White	Straight	Rope like	Naphthalene	Anti-insect
Muscodor yucatanensis[j]	*Bursera simaruba*	White	Flocculose pattern	Rope like	1R, 4S, 7S, 11R-2,2,4,8-Tetramethyltricyclo (5.3.1.0(4,11)) undec- 8-ene	Phytoinihibitory activity

[a] Worapong et al. (2001), [b] Ezra et al. (2004), [c] Suwannarach et al. (2010), [d] Mitchell et al. (2008), [e] present study, [f] Zhang et al. (2010), [g] Worapong et al. (2002), [h] Kudalkar et al. (2012), [i] Daisy et al. (2002), [j] González et al. (2009)

Table no.2 Biological and culture characteristic comparison of *Muscodor* species

These type strains have been described on the basis of morphological, physiological, biological and genetic characters (Kudalkar *et al*., 2012, Suwannarach *et al*., 2013). These fungi are classified in the family Xylariaceae and have unique molecular identity as compared to other genera in the family (Ezra *et al*., 2004, Zhang *et al*., 2010). They are sterile, non-sporulating, slow-growing, flat, undulating, hyaline, septate mycelia possessing characteristic hyphal coiling, ropyness, right-angle branching.

2.3 Myco/Bio-fumigation potential of *Muscodor albus*

Volatiles produced by *M.albus* kill most of the pathogens exposed *in vitro*. This opens up new possibilities to develop bio-fumigant as a post-harvest treatment for a range of products (Mercier *et al*., 2007). Bio-fumigation with *M. albus* controlled blue mould & gray mould of apple, brown rot of peaches (Mercier and Jiménez, 2004), green mould & sour rot of lemons (Mercier & Smilanick, 2005), gray mould of grapes (Mlikota-Gabler *et al*., 2006), bacterial soft rot (Corcuff *et al*., 2006). The potential of *Muscodor albus* as a microbial control agent of potato tuber moth in stored potatoes was also explored (Lacey & Neven, 2006), efficacy of *Muscodor albus* for control of phytophthora blight of sweet pepper & butternut squash was examined in greenhouse study (Camp *et al*., 2008), pupal mortality & adult emergence of western cherry fruit fly exposed to *M. albus* has also been evaluated (Yee *et al*., 2009), seedling diseases on sugar beet & root-knot nematode on tomato have been controlled during *in vitro* experiments using *M. albus* (Grimme *et al*., 2007). Mycofumigation has been demonstrated as a new alternative to methyl bromide fumigation for control of soil-borne pathogens, many more applications of this genus are being explored each passing day.

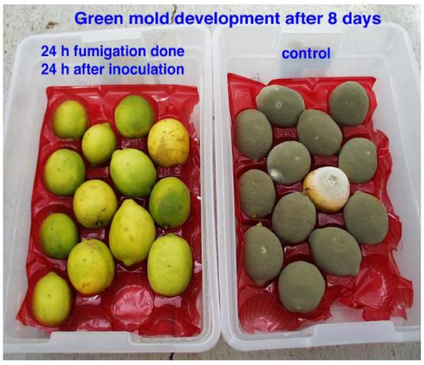


Fig 3 Mycofuimigation of lemon with *M.albus*

2.4 Need for new fumigant/ preservative

Use of chemical fumigants, pesticides, fungicides, insecticides though account for conventional approaches employed to control decay in food crops but the inherent drawbacks of all these approaches compel to look for an alternative.

There is increasing concern about the environmental effects and safety of chemical pesticides and fungicides all over the world. Regulatory agencies have reacted to public pressure and introduced comprehensive legislation to reduce pesticide use. Some of fungicides registered for postharvest use, particularly Benzimidazol, are becoming ineffective due to the development of fungicide-resistant strains of postharvest pathogens (Irtwange, 2006). Soil fumigation in United States is highly dependent on the use of methyl bromide-chloropicrin mixtures (MBC). Methyl bromide containing products are currently being phased out due to their role in depletion of ozone layer (EPA, 2004).

Sulphur dioxide fumigation effectively controls gray mould, but bleaching injury to berries, particularly among those detached from the cluster rachis, and injury to the rachis itself occur among commercially stored grapes (Crisosto and Mitchell, 2002). After fumigation with sulphur dioxide, grape berries become more susceptible to subsequent infections by *B. cineria* (Taylor *et al.*, 1990) that can occur during transportation and marketing. Also, sulphur dioxide is not accepted for organic grapes, under current certification rules, and some regulatory agencies do not allow the discharge of sulphur dioxide to the air after fumigation (Mlikota-Gabler *et al.*, 2006). Alternatives to sulphur dioxide for control of postharvest gray mould include near-harvest ethanol or biological control agent applications (Droby and Litcher 2004; Karabulut *et al.*, 2003). However methods requiring additional postharvest processing and handling, increase costs and could alter the appearance of the berries or could cause detachment of berries from cluster rachis (Mlikota-Gabler *et al.,* 2006).

Continuous use of fungicides such as Imizalil, Thiabendazole, and o-phenylphenol in citrus packing facilities in California has resulted in appearance of *P.digitatum* isolates with multiple fungicide resistances within natural populations (Holmes and Eckert, 1999) which further complicates the management of green mould. In addition, maximum residue limits for Imazalil, the most important fungicide in use, are lower in most citrus importing countries than in the United States (United States Department of Agriculture-Foreign cultural Service, 2004).

2.5 Advantages of bio-fumigation with *Muscodor albus*-

Bio-control agents possess a number of important advantages over traditional chemical pesticides which make their commercial outlook particularly promising, as in general, they are considered non-hazardous to humans and animals, biodegradable and environmentally friendly, attack specific target organisms, leaving other beneficial organisms unaffected (Adaskaveg *et al.*, 2002). *Muscodor* effectively fumigates the soil without the need for taping. The main advantage of fumigation with *Muscodor* in controlling postharvest decay compared with other approaches is that it does not require processing or manual handling of the concerned fruit.

2.6 *In vitro* VOC Stress bioassay

It is a simple bioassay which is used for both isolation of newer *Muscodor* species and determination of the antimicrobial activity of the VOCs. The PDA plate was partioned into two halves. 1cm agar strip was removed from the centre to limit the movement of any diffusible metabolite. One quadrant of the petri plate was inoculated with mycelial plug of actively growing culture of *Muscodor sp*. The plates were sealed and incubated at 24°C for 5 days for production of VOC's. The bacteria were streaked in rest of the columns while in case of test fungi 3 mm mycelial plugs were inoculated. The antimicrobial action of VOC's was determined by monitoring the growth of test organism in the control plates as compared to test plates. To check the inhibitory or killing effect of the VOC's the inoculum plugs to fresh solid media (PDA, YEPD or MHA) after 3 days exposure to the VOCs (Mitchell *et al.*, 2010)

2.7 Rye grain formulation

Rye- grain formulation is prepared by transferring the contents of liquid mycelia suspension (in PDB), or plugs of actively growing culture to previously autoclaved rye-grain. The colonised grain culture is ready for use after 10-14 days. The culture so prepared was evaluated for its bio-fumigant potential at controlling the fungal decay of apples & peaches, 7day long exposure to VOC's of grain culture completely controlled blue mould & gray mould in wound-inoculated apples. 72 hr fumigation in case of peaches completely controlled brown rot caused by *Monilinia fruiticola* (Mercier & Jimenez, 2004).

2.8. Mycofumigation with *Muscodor*

The potential uses of *Muscodor* as a biofumigant explored till date are briefly described below

Control of post-harvest gray mould of table grapes

Grain culture of *Muscodor albus* reduces the incidence of infection to 10% and 3.4% in "Autumn seedless" grapes & "Thompsons grapes" respectively when used in combination with ozone fumigation (Mlikota-Gabler, 2009).

Control of brown rot of peaches

Bio-fumigation of plastic bagged cartons of peaches proved to be effective in controlling the incidence of infection caused by *Monilinia fruiticola* (Schnabel & Mercier, 2006).

Control of green mould & sour rot of stored lemon

3 day long *in vitro* exposure to rye-grain culture of *M. albus* controlled the incidence of green mould infection to 26.2% & was an effective treatment for reducing sour-rot of lemons as well, though efficiency declines if the treatment is applied after a gap of 24 hr (Mercier & Smilanick, 2005).

Control of post-harvest fungal decay of apple

Exposure to volatiles produced by rye grain culture growing in sealed glass jars of capacity 4l completely controlled blue mould *(Penicillium expansum)*, gray mould (*Botrytis cinerea*) & *Sclerotinia sclerotiorum* after 24 hr long fumigation (Ramin *et al*., 2007).

Control of seedling diseases of sugar beet & root-knot nematode on tomato

Root-knot nematode, *Meloidogyne incognita* on tomato, & pathogens causing seedling diseases of sugar beet (*Pythium ultimum, Rhizoctonia solani* AG 2-2, *Aphanomyces cochliodes)* were significantly controlled by synthetic bio-rational mixture of gases mimicking key antimicrobial gases produced by *M. albus* (Grimme *et al*., 2007).

Some other important applications of *Muscodor albus*

Phytophthora blight of sweet-pepper & butternut squash, tuber moth in stored potatoes, emergence of adults from pupae of western fruit fly, pathogens contaminating seeds of barley, many building moulds are also susceptible to the VOC's produced by *M. albus* (Camp *et al*., 2008, Lacey & Neven, 2006, Yee *et al*., 2009, Strobel *et al*., 2001, Mercier & Jimenez, 2007).

Fumigation with *Muscodor sp.* seems a safer, effective, economical alternative approach of preventing post-harvest decay in food crops inhibiting the growth of spoilage causing organisms by the activity volatile antibiotics it generates, the species of this novel genus can also be exploited for various other purposes like decay of human wastes etc. and it can be a boon for mankind.

Chapter 3

Aim of the study

Aim and objectives of the present study include the following:

- Screening 4 different Indian *Muscodor* isolates for their antimicrobial efficacy.
- Production and formulation of bio-fumigant preparations of *Muscodor* species

Chapter 4

Materials & Methods

4.1. Re-culturing/ Activation of *Muscodor* isolates

Muscodor isolates, isolated from different plant sources growing in biodiversity hotspot regions of Indian subcontinent was used as test cultures for the present study. The cultures were inoculated on Potato Dextrose Agar (PDA) plates, sealed with double layer of parafilm and incubated at 24±2°C for next 7-10 days. Table no.3 represents different *Muscodor* isolates used for the study.

Culture Code	Plant part	Host plant	Location
#6610 CMSTITBRT	Stem internal tissue	*Cinnamomum zeylanicium*	BRT wildlife sanctuary, Karnataka
#16 AMLWLS	Leaf	*Aegle marmelos*	Wayanand Wildlife sanctuary, Kerala
#1 CCSTITD	Stem	*Cinnamomum camphora*	Tiger hills, Darjeeling, West Bengal
#2 CCSTITD	Stem	*Cinnamomum camphora*	Tiger hills, Darjeeling, West Bengal

Table no.3 Different *Muscodor* species used in the study

4.2 Long term preservation of *Muscodor* isolates

The actively growing *Muscodor sp.* cultures were preserved by placing small pieces of agar supporting fungal growth on PDA slants containing 10% (v/v) glycerol. The plates were incubated at 24°C for next 10 days after which they were stored at -80°C (Ezra *et al.*, 2004).

4.3 Procurement of test-pathogens

Plant and Human pathogenic microorganisms including bacteria, fungi and yeasts were obtained in lyophilized or living form from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. *Cercospora beticola* was procured from Montana State University, USA and a few other test pathogens were also availed from Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab, India.

4.3.1 Activation of pathogenic cultures

Activation of the lyophilized cultures was done by adding 200µl of saline to lyophilized culture and a drop of which was placed onto specific (PDA, Muller Hinton Agar (MHA), Yeast Extract Peptone Dextrose Agar (YEPDA)) culture plates. The plates were incubated at specific conditions. Live cultures obtained from DBTES, Thapar University, Patiala and Montana State University, Bozeman, USA were either inoculated or streaked over specific media plates and incubated at specific growth temperatures. The cultures thus obtained were maintained as pure culture on PDA, MHA and YEPDA slants supplemented with 10% (v/v) glycerol.

4.4 VOCs stress test against *Muscodor albus* CZ620

Muscodor albus CZ620 was employed as a selection tool for finding out other *Muscodor species*. Briefly describing the procedure developed by Ezra *et al.*, 2004, the PDA plate was divided into four quadrants with the help of a sterile scalpel blade or using a partitioned petriplates. In one of the quadrant, *Muscodor albus* CZ620 was inoculated. The plates were sealed with parafilm and incubated at 24°C for next 4-5 days for the production of VOCs. After the incubation period the test endophytic fungi (#6610 CMTITBRT, #16 AMLWLS, #1 CCSTITD and #2 CCSTITD) were inoculated into the remaining quadrants. *Arthrinium phaeospermum* was taken as control on which the effect of VOCs produced by *M. albus* was checked. The plates were sealed with double layer of parafilm and incubated at 24°C for 7 days. The growth was observed at regular interval of 24 hrs. Growth of test endophytic fungal isolates was checked for next 3 days for confirmation of *Muscodor* species (Ezra *et al.*, 2004).

4.5 Bioassay of VOC's produced by *Muscodor species*

A simplified bioassay was conducted to evaluate the antimicrobial potential of the VOCs produced by various test *Muscodor* isolates. 90 mm plate was divided into three quadrants. In one of the quadrant, agar plug of 5mm actively growing *Muscodor* isolate (#6610 CMTITBRT, #16 AMLWLS, #1 CCSTITD and #2 CCSTITD) was inoculated and incubated at 24°C for next 5-7 days for production of VOCs. Thereafter test fungi were inoculated by inoculating 3 mm plug of 7 days old culture on the rest of the quadrants. Bacteria and yeasts were tested by streaking in remaining quadrants. Control plates comprised of only inoculated test bacteria or fungi and were devoid of *Muscodor* isolate allowing it to grow normally. Antimicrobial efficacy in terms of percentage inhibition due to VOCs of each *Muscodor* specie against test organism was determined by visually observing the difference in growth of test organism on test and control plates. All the tests were performed in triplicates and values calculated as Mean ± SD.

4.6 Screening for mycofumigation potential of #16 AMLWLS

4.6.1 Inoculum preparation

Actively growing 3mm mycelial plug of test fungi was inoculated into Potato dextrose broth (PDB) and incubated at 26 ± 2ºC over orbital shaker for 5 days. Spore suspension of the test culture was aseptically adjusted to 1x10^6 conidia/ml in physiological saline. *Botrytis cinerea, Rhizoctonia solani, Collectrotrichum gloeosporioides and Cercospora beticola* was used as test culture for the study (Mlikota-Gabler *et al.*, 2010).

4.6.2 Preparation of fruits and grains under study

Fruits and grains namely *Vitis vinifera* (Grapes), *Prunus avium* (Cherry), *Syzygium acumini* (Jamun), *Triticum aestivum* (Wheat), *Vigna mungo* (Black gram) were surface sterilized by dipping into 10% formaldehyde for 30 min and subsequent washing under running tap water after formaldehyde treatment. Fruits were dried over blotting sheet under aseptic conditions.

4.6.3 Mycofumigation potency test

Sterile PDA plates were cut into half-moon using sterile scalpel blade on solidification. Autoclaved moist filter papers were placed. In test plates actively growing *Muscodor* isolate, #16 AMLWLS was inoculated using sterilized cork-borer. The plates were sealed with parafilm and incubated at 23±2ºC for 7 days for VOC's production. The plates in which *Muscodor sp.* was not inoculated served as control plates. Surface sterilized fruits & grains were sprayed with conidial suspensions of test pathogens and the treated fruits and grains were placed over the moist filter paper of test as well as control plates and incubated at 26± 2ºC for 72 hrs. The plates were regularly observed after every 24 hrs for signs of infection on fruits and grains in test as well as control plates. The percentage inhibition of the test pathogen was calculated by comparing the severity of infection in test plate as compared to control plate.

4.7 Identification of potential *Muscodor* specie

4.7.1 Morphotaxonomy

The *Muscodor* sp. showing highest antimicrobial efficacy was examined under microscope to characterize the isolate on the basis of their microscopic characters and morphology. The culture was grown over PDA for 10 days after which it was critically observed under microscope. Briefly, the glass slide was cleaned with spirit and a drop of water was put over the glass slide, upon which the mycelial mass was placed and teased properly. It was then stained with Lactophenol cotton blue (Hi Media). The slide was covered with 18 x 10 mm cover slip avoiding the formation of air bubble and mounted with DPX. The slide was observed at 10X, 40X and 100X using Nikon binocular microscope. The fungi were identified based upon their morphological characteristics.

4.7.2 DNA isolation

The fungal genomic DNA was isolated from 3-4 days old fungal culture grown on PDA plates. 3 discs of 10mm mycelial plugs was scooped out of the actively growing culture plate and crushed to very fine powder by using liquid nitrogen in pestle and mortar. 660-750µl of the extraction buffer was added to it and crushed again. The contents were transferred to a 1.5 ml micro centrifuge tube and 10µl of β-mercaptoethanol and 4µl of Proteinase K was added and vortexed followed by incubation at 65°C in water bath for 1 hour (intermittent mixing after every 15 min). After 1h incubation is over, the micro centrifuge tubes were centrifuged at 10,000 rpm for 15 minutes to remove cell debris. Further 6µl of RNase was added to remove RNA contamination and incubated at 37°C for 30 minutes. Equal volume of Phenol: Chloroform (1:1) was added to each tube to precipitate the contaminating protein and mixed properly for 15 min followed by centrifugation at 12,000 rpm for 10 min, this step was repeated thrice. Transfer the aqueous layer containing DNA to the fresh micro centrifuge tube carefully and add 20µl of 3M sodium acetate and contents of each micro centrifuge was topped up with absolute ethanol and incubated at 4°C overnight. On the next day, the micro centrifuge tubes were centrifuged at 12,000 rpm for 10 min and the pellet was washed with 70% ethanol followed by centrifugation at 12,000 rpm for 5 minutes. Then pellet was air dried and dissolved in 50µl of Tris EDTA buffer (pH=8). The qualitative estimation of extracted DNA was done by agarose gel electrophoresis.

4.7.3 Agarose gel electrophoresis

0.8% agarose gel was prepared in 1X Tris Acetate EDTA and 0.5µg /ml of ethidium bromide (EtBr) was added and then gel was casted in the electrophoretic apparatus. The gel was allowed to solidify and the comb was carefully removed. The running buffer (1 X TAE) was poured into the electrophoretic tank so that the gel is fully immersed into the buffer. The DNA samples were mixed with the 5X loading buffer. The samples were loaded into wells and allowed to run at 50 volts for 1h. The gel was observed under UV transilluminator to visualize the presence of DNA. Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software.

Quantitative Estimation of the Genomic DNA was done by spectrophotometric analysis of the sample. The absorbance of the sample was taken at 260nm, to determine the concentration of the sample. 1 OD is equivalent to 50µg/ml DNA sample. So, accordingly the concentration of the sample was calculated using following formula.

$$\text{Concentration } (\mu g/ml) = O.D_{260nm} \times 50\mu g/ml \times \text{Dilution factor}$$

The purity of the DNA sample was determined by taking the ratio of its absorbance at 260nm and at 280nm. If the ratio is less than 1.6, then there is RNA contamination, if the ratio lies between 1.6-1.8 then DNA sample is free from RNA and protein. If the ratio is more than 1.8, the DNA might be contaminated with proteins or some other particulate matter.

4.7.4 PCR Amplification

PCR is a rapid process for *in vitro* amplification of desired DNA sequence by using specific primer so as to produce a large amount of desired DNA fragment of defined sequence length.

The *M. albus* specific primers were used to amplify the dissimilar regions located at the 3’ and 5’ ends of the *M. albus* strains ITS and 5.8S region (Ezra *et al.*, 2010). Primers *M. albus* F (5’GGGAGGCTACCCTATAGGGGATAC3’) and *M. albus* R (5’CAGGGGCCGGAACCACTACAGAGG3’) were synthesized from IDT, Inc.

S.No	Reagents	Stock concentration	Quantity	Final concentration in 25µl
1.	Autoclaved double distilled water	_	15µl	_
2.	Taq buffer	10X	2.5µl	1X
3.	dNTPs	2.5mM	2.0µl	0.2mM
4.	Primers	10µM	2.0µl	0.8µM
5.	Taq DNA Polymerase	3U/µl	0.5µl	1U
6.	Template DNA	25ng/µl	1.0µl	25ng

Table no.4- Different reagents used during PCR reactions.

Amplification was carried out in 25µl reaction mixture (Table no.4) containing: 1µl of extracted fungal genomic DNA, 10µM of each primer, 2.5mM of dNTP (Bangalore GeNei), 25mM $MgCl_2$ (Bangalore GeNei), 1.5U of Taq DNA Polymerase (Bangalore GeNei) in 10X Taq buffer (Bangalore GeNei) in a Thermocycler (My Cycler, Bio-Rad Laboratories, Inc). The PCR cyclic conditions ***consisted of initial denaturation at 96ºC for 5 min followed by 39 cycles of 95ºC for 45 sec, 60ºC for 45 sec, 72ºC for 45 sec followed by final extension at 72ºC for 5 min (Table no.5, Figure 4). The PCR products were resolved by using agarose gel electrophoresis (1.5 % agarose gel dissolved in 1X TAE buffer) at 50V for 1.30 hr. Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software. An approximate 450-500bp PCR product was purified by using the Wizard® SV Gel and PCR clean up system kit (Promega, USA).***

STEP	TEMPERATURE	TIME
STEP I : Initial Denaturation	96°C	5 min
STEP II : Denaturation	95°C	45 sec
STEP III : Annealing	60°C	45 sec
STEP IV: Extension	72°C	45 sec
STEP V	Step II to Step IV repeated 39 times	
STEP VI : Final extension	72°C	5min
STEP VII: Store	4°C	∞

Table no.5- Temperature profile of PCR reaction

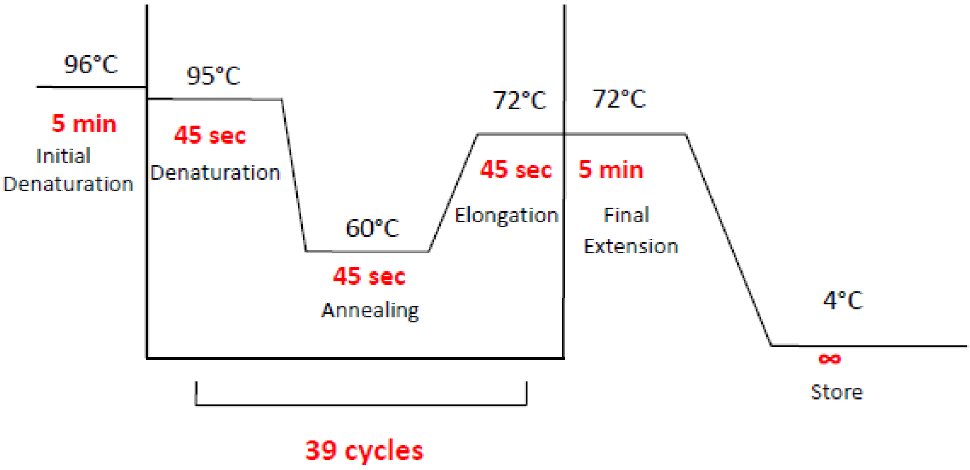


Figure 4- showing ray diagram of temperature profile of PCR

Chapter 5

Results & Discussion

5.1 Re-culturing and storage of fungal culture

The *Muscodor* species were obtained as pure culture over PDA plate (Table no.6, Figure no.5). *Muscodor* isolates were successfully maintained for a longer period over PDA slants containing 10% glycerol.

Culture Code	Plant part	Host plant	Location
#6610 CMSTITBRT	Stem internal tissue	*Cinnamomum zeylanicium*	BRT wildlife sanctuary, Karnataka
#16 AMLWLS	Leaf	*Aegle marmelos*	Wayanand Wildlife sanctuary, Kerala
#1 CCSTITD	Stem	*Cinnamomum camphora*	Tiger hills, Darjeeling, West Bengal
#2 CCSTITD	Stem	*Cinnamomum camphora*	Tiger hills, Darjeeling, West Bengal

Table no.6: Different *Muscodor* species used in the study

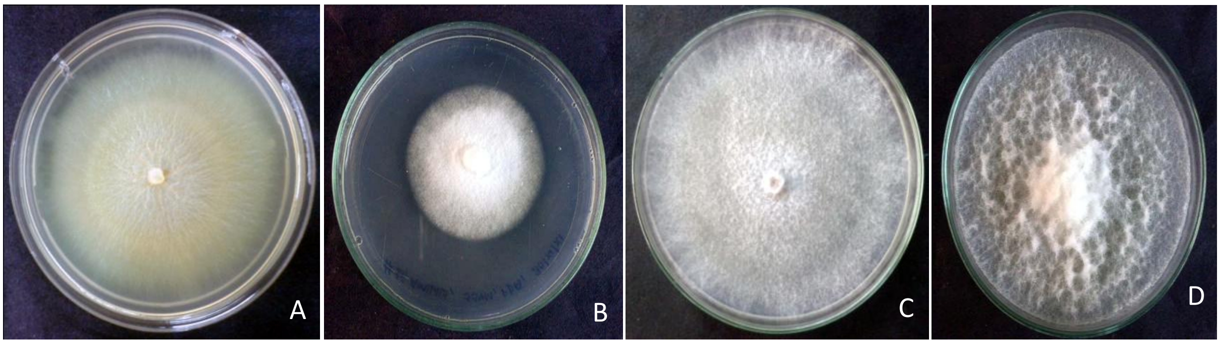


Figure 5 Pure culture of *Muscodor* species on PDA plates; A) #6610 CMSTITBRT, B) #16 AMLWLS, C) #1 CCSTITD and D) #2 CCSTITD

5.2 Procurement of test-pathogens

A total of 30 pathogenic microorganisms were procured from various repositories which were successfully maintained as pure cultures over their respective medium**.** Table no.7 lists the procured pahtogenic isolates .

S.No	Test pathogen	Repository
FUNGI		
1.	*Alternaria alternate*	DBTES, Thapar University, Patiala
2.	*Arthrinium phaeospermum*	DBTES, Thapar University, Patiala
3.	*Aspergillus flavus*	DBTES, Thapar University, Patiala
4.	*Aspergillus japonicus* (MTCC 1975)	MTCC, IMTECH, Chandigarh
5.	*Aspergillus niger*	DBTES, Thapar University, Patiala
6.	*Bionecteria ochroleuca*	DBTES, Thapar University, Patiala
7.	*Botrytis cinerea* (MTCC 359)	MTCC, IMTECH, Chandigarh
8.	*Cercospora beticola*	Montana State University, USA
9.	*Collectrotrichum gloeosporioides* (MTCC 9623)	MTCC, IMTECH, Chandigarh
10.	*Fusarium solani*	DBTES, Thapar University, Patiala
11.	*Fusarium oxysporum*	DBTES, Thapar University, Patiala
12.	*Lasodiplodia pseudotheobromae.*	DBTES, Thapar University, Patiala
13.	*Lasodiplodia theobromae*	DBTES, Thapar University, Patiala
14.	*Penicillium marneffei*	DBTES, Thapar University, Patiala
15.	*Penicillium chrysogenum*	DBTES, Thapar University, Patiala
16.	*Phomopsis theiocola* (MTCC 373)	MTCC, IMTECH, Chandigarh
17.	*Rhizoctonia solani* (MTCC 4634)	MTCC, IMTECH, Chandigarh
YEASTS		
18.	*Candida albicans* (MTCC 183)	MTCC, IMTECH, Chandigarh
19.	*Candida glabrata* (MTCC 3019)	MTCC, IMTECH, Chandigarh
20.	*Candida vishwanathii* (MTCC 1629)	MTCC, IMTECH, Chandigarh
21.	*Candida albicans* (MTCC 854)	MTCC, IMTECH, Chandigarh
22.	*Saccharomyeces cerevisiae* (MTCC 36)	MTCC, IMTECH, Chandigarh
BACTERIA		
23.	*Agrobacterium tumefaciens* (MTCC 2251)	MTCC, IMTECH, Chandigarh
24.	*Escherichia coli* (MTCC 1302)	MTCC, IMTECH, Chandigarh
25.	*Pseudomonas aeruginosa* (MTCC 3541)	MTCC, IMTECH, Chandigarh
26.	*Pseudomonas aeruginosa* (MTCC 647)	MTCC, IMTECH, Chandigarh
27.	*Pseudomonas aeruginosa* (MTCC 424)	MTCC, IMTECH, Chandigarh
28.	*Staphylococcus epidermidis* (MTCC 2639)	MTCC, IMTECH, Chandigarh
29.	*Staphylococcus aureus* (Sau 902)	DBTES, Thapar University, Patiala
30.	*Staphylococcus aureus* (MTCC 96)	MTCC, IMTECH, Chandigarh

Table no.7 Showing repository of test pathogenic cultures.

5.3 VOCs stress test against *Muscodor albus* cz620

The test *Muscodor* isolates was found resistant to the VOCs produced by *M. albus* CZ620. The VOCs produced by *M .albus* did not induce any inhibitory effect over the growth of *Muscodor* isolates whereas there was complete inhibition of the other fungal cultures that were tested as control. Thus this test can be applied as a selection tool for finding and selecting novel *Muscodor* isolates. Ezra *et al.,* 2004 isolated four *M. albus* strain by following the similar approach.

Culture Code	**Resistance to VOCs**
#6610 CMSTITBRT	+++
#16 AMLWLS	+++
#1 CCSTITD	+++
#2 CCSTITD	+++
Arthenium phaeospermum	-

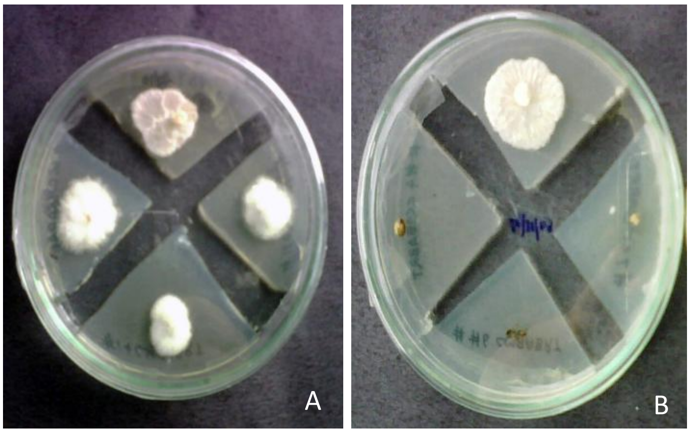


Table no.8 and Figure no.6: VOCs stress test of the *Muscodor* isolates against *Muscodor albus* CZ 620

5.4 *in vitro* Antimicrobial activity of *Muscodor species*

Antimicrobial activity of the VOCs produced by the four *Muscodor* isolates #16 AMLWLS, #6610 CMSTITBRT, #1CCSTITD & #2CCSTITD was evaluated against a battery of 28 pathogenic microorganisms which comprises 16 fungal, 8 bacterial and 5 yeast cultures. (Table no.7)

#6610 CMSTITBRT showed 20-60% growth inhibition of the test cultures. Maximum growth inhibition was observed in *Phomopsis theiocola* (MTCC 373*)* whose growth was hindered by 59.21 ± 4.69% followed by *Aspergillus niger* and *Penicillium marneffei* which was inhibited by 46 and 30 %. Among the yeast isolates *C. vishwanathii* and *Candida glabrata* (MTCC 3019) showed 30-50% inhibition whereas there was no suppression observed in case of *Candida albicans* (MTCC 183). The bacterial pathogen *Pseudomonas aeruginosa* (MTCC 424) showed 70% inhibition whereas other tested bacterial cultures were found resistant to the VOCs produced by #6610 CMSTITBRT (Table no.9, Figure no.7).

Test microorganisms	**Antimicrobial efficacy of # 6610CMSTITBRT**		
	Mean Diameter Test	**Mean Diameter Control**	**% age inhibition**
A. alternata	8.25±1.5	9.5±3.53	13.15±2.03
A. phaeospermum	12.75±1.5	17.5±0.71	27.14±0.79
A. flavus	34±3.46	50±7.07	32±3.61
A. japonicus	15.75±2.5	15.75±2.5	0.00
A. niger	12.5±9.95	23±4.24	45.65±5.71
B. ochroleuca	23±6.97	26±12.73	11.53±5.76

B. cinerea	28.5±3.11	29.5±0.71	3.39±2.4
C. beticola	14±5.23	14.5±0.71	3.45±4.52
C. gloeosporioides	25.45±3.5	25.45±3.5	0.00
F. solani	18.66±2.42	21±2.82	11.14±0.40
F. oxysporum	22.83±1.16	24±1.41	4.87±0.25
L. pseudotheobromae	50±1.41	51±1.41	1.96±0.00
L. theobromae	42.5±2.88	45±0	5.55±2.88
P. marneffei	10.25±3.40	15.5±0.70	33.87±2.70
P. chrysogenum.	19.25±5.5	20±4.24	3.75±1.26
P. theiocola	7.75±0.96	19±5.65	59.21±4.69
R. solani	43.75±5.85	54±1.41	18.98±4.44
A. tumefaciens	6.5±1.2	6.5±1.2	0.00
E. coli	7.75±1.25	7.75±1.25	0.00
P. aeruginosa (MTCC 424)	3.45±1.35	11.5±1.35	70
P. aeruginosa (MTCC 647)	9.5±3.2	9.5±3.2	0.00
P. aeruginosa (MTCC 3541)	10.25±1.72	10.25±1.72	0.00
S. aureus (Sau 902)	6.75±1.32	6.75±1.32	0.00
S. aureus (MTCC 96)	8.95±2.67	8.95±2.67	0.00
S. epidermidis (MTCC 2639)	14.78±3.25	14.78±3.25	0.00
C. albicans (MTCC 183)	4.77±2.23	4.77±2.23	0
C. albicans (MTCC 854)	4.9±1.8	7±1.8	30
C. glabrata (MTCC 3019)	3.32±1.34	6.64±1.34	50
C. vishwanathii	13.95±1.45	6.975±1.45	50.00
S. cerevisae	11.5±1.5	11.5±1.5	0

Table no. 9 Showing antimicrobial activity (% age inhibition) of VOCs produced by #6610CMSTITBRT

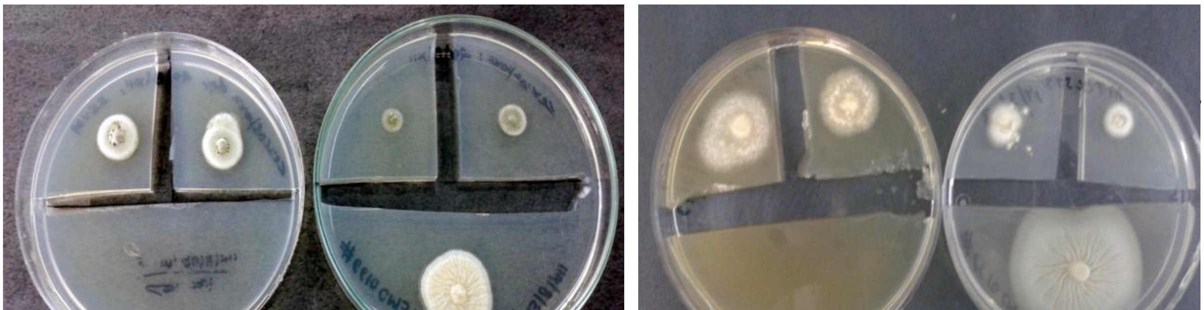


Figure no.7: *in vitro* antimicrobial efficacy of VOCs produced by #6610CMSTITBRT with A) *Cercospora beticola*, B) *Rhizoctonia solani*

The VOCs produced by #16 AMLWLS were most lethal against the tested spectrum of microbes. It exhibits complete inhibition of 11 pathogens. The fungal pathogens whose growth was totally hindered by the VOCs were *Alternaria alternata, Cercospora beticola, Fusarium solani, Fusarium oxysporum, Rhizoctonia solani* (MTCC 4634), *Penicillium chrysogenum* (MTCC 160), *Arthrinium pheosporium and Botrytis cinerea* (MTCC 359). The other tested fungal isolates: *Penicillium marneffei, Aspergillus flavus, Phomopsis theiocola* also exhibited 50-60% inhibition whereas amongst the tested yeast isolates only *Candida albicans* (MTCC 183) showed complete inhibition to the VOCs whereas *Saccharomyeces cerevisiae* exhibited 50% inhibition but there was no effect on *Candida glabrata* (MTCC 3019) and *Candida vishwanathii* (MTCC 1629). Amongst the bacterial pathogens, *Staphylococcus aureus* (MTCC 96), *Agrobacterium tumefaciens* (MTCC 2251), showed complete 100% inhibition whereas other test microbes like *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* showed 50-70% inhibition in their growth. (Table no 10, Figure no.6).

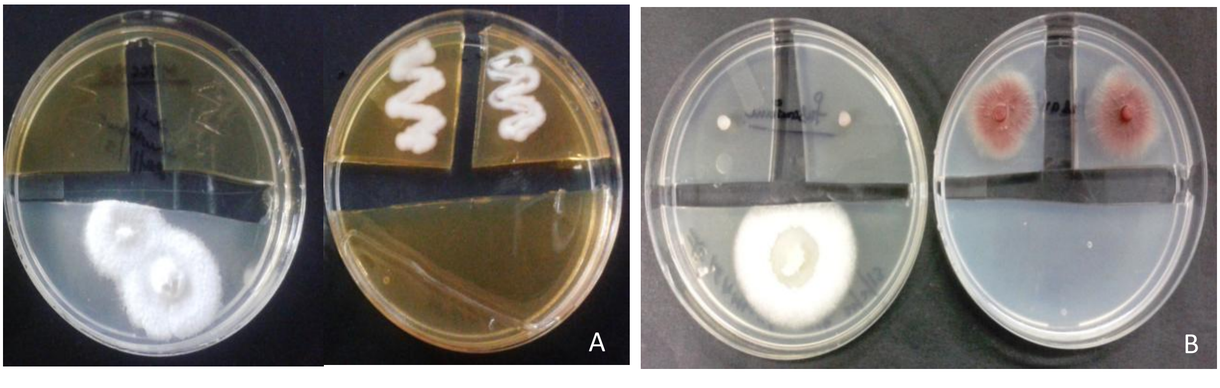


Figure no.8: Showing *in vitro* antimicrobial efficacy of VOCs produced by 16AMLWLS with A) *Candida albicans* (MTCC 183) B) *Fusarium oxysporum*

Test microorganism	**Antimicrobial efficacy of # 16AMLWLS**		
	Mean Diameter Test	**Mean Diameter Control**	**% age inhibition**
A. alternata	0.00	24.5±2.12	100%
A. phaeospermum	0.00	17.5±0.71	100%
A. flavus	17±1.82	33±5.65	48.5±3.83
A. japonicus	29.25±0.5	34±1.41	13.97±0.91
A. niger	15±10	27±2.83	44.44±7.17
B. ochroleuca	19.75±0.96	33.5±4.95	38.05±3.99
B. cinerea	0.00	23.5±0.70	100.00
C. beticola	0.00	17±4.24	100.00
C. gloeosporioides	17.5±0.55	17.5±0.55	0.00
F. solani	0.00	31±1.41	100.00
F .oxysporum	0.00	12.5±4.95	100.00
L. pseudotheobromae.	50.75±0.96	77±9.90	34.09±8.94
L. theobromae	54.5±2.64	80.5±0.70	32.29±1.94
P. marneffei	15.75±4.42	32±2.83	50.78±1.59
P. chrysogenum	0.00	45±0	100.00
P. theiocola	2.5±2.88	7.5±0.71	66.66±2.17
R. solani	0.00	45±0	100.00
A. tumefaciens	0.00	7.12±1.11	100.00
E. coli	3.5±1.2	5.00±1.2	30
P. aeruginosa (MTCC 424)	2.1±1.5	7.00±1.5	70
P. aeruginosa (MTCC 647)	6.2±1.34	6.2±1.34	0.00
P. aeruginosa (MTCC 3541)	8.50±1.2	8.50±1.2	0.00

S. aureus (Sau 902)	3.6±1.5	9.00±1.5	60
S. aureus	0.00	8.5±1.7	100
S. epidermidis	3.6±1.00	7.2±1.00	50
C. albicans (MTCC 183)	0.00	4.5±1.34	100
C. albicans (MTCC 854)	1.6	3.2	50
C. glabrata	2.75±1.2	5.5±1.2	50
C. vishwanathii	1.75	3.5	50
S. cerevisiae	3.75±1.00	7.5±1.00	50

Table no.10 Showing antimicrobial activity (% age inhibition) of VOCs produced by #16AMLWLS

#1 CCSTITD possess inhibitory effect over the tested pathogenic microorganisms. It inhibits the growth of fungal pathogens by 30-70%. *Lasodiplodia theobromae, Cercospora beticola and Alternaria alternata* were the most susceptible isolates whereas 40-55 % hindrance was observed in case of *Candida albicans*, *Candida glabrata* and *Candida vishwanathii*. The bacterial pathogen *Pseudomonas aeruginosa* (MTCC 424) showed 70% inhibition where as other bacterial cultures were resistant to the VOCs produced (Table no.11, Figure no.9)

Test microbe	**Antimicrobial efficacy of # 1CCSTITD**		
	Mean Diameter Test	**Mean Diameter Control**	**% age inhibition**
A. alternata	4.25±0.5	11±1.41	61.36±0.91
A. phaeospermum	14±0.82	20.5±6.36	31.70±5.54
A. flavus	11.75±1.5	16±0.00	26.56±1.5
A. japonicus	29.78±3.46	29.78±3.46	0.00
A. niger	30.5±7.14	40.5±13.43	24.69±6.29
B. ochroleuca	14.24±2.21	16±1.41	11±0.80

B. cinerea	14.25±1.26	20.5±0.71	30.49±0.55
C. beticola	7±1.82	16.5±2.12	57.57±0.30
C. gloeosporioides	9.75±0.5	16.5±0.70	40.90±0.20
F. solani	13.25±4.35	22.5±2.12	41.11±2.23
F. oxysporum	5.5±1.00	11.5±2.12	52.17±1.12
L. pseudotheobromae	15.75±5.7	15.75±5.7	0.00
L. theobromae	10.5±1.00	36±0.00	70.83±1.00
P. marneffei	5.75±0.50	10±0.50	42.5±0.00
P. chrysogenum	11.5±1.00	22.5±2.12	48.89±1.12
P. theiocola	10.5±0.58	13±4.24	19.23±3.66
R. solani	30.5±10.97	45±0.00	32.22±10.97
A. tumefaciens	8.78±1.50	8.78±1.50	0.00
E. coli	27.5±0.71	27.5±0.71	0.00
P. aeruginosa (MTCC 424)	27±0.00	27±0.00	0.00
P. aeruginosa (MTCC 647)	27.25±0.96	30±0.00	9.16±0.96
P. aeruginosa (MTCC 3541)	6.25±0.96	23.5±2.12	73.40±1.16
S. aureus (Sau 902)	9.5±1.2	9.5±1.2	0.00
S. aureus	12.75±5.78	12.75±5.78	0.00
S. epidermidis	24.75±0.5	31±1.41	20.16±0.91
C. albicans (MTCC 183)	7.75±1.5	13.5±0.70	42.6±0.80
C. albicans (MTCC 854)	5.5±1.00	11.5±2.12	52.17±1.12
C. glabrata	6.5±0.58	13±0	50±0.58
C. vishvanathi	6.75±0.96	11.5±0.70	41.3±0.26
S. cerevisae	8.00±1.45	8.00±1.45	0.00

Table no. 11Showing antimicrobial activity of VOCs produced by #1CCSTITD

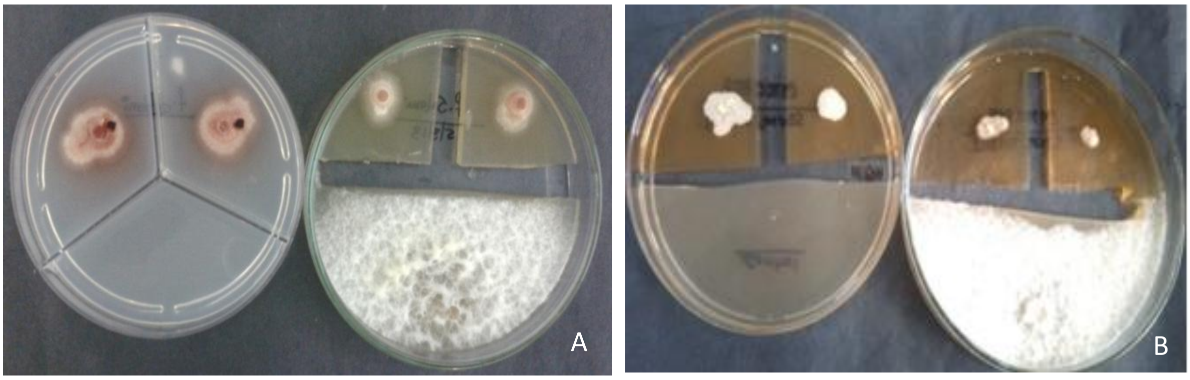


Figure no. 9 showing antimicrobial efficacy of VOCs produced by 1CCSTITD with A) *Fusarium solani* B) *Candida albicans* (MTCC 854)

The VOCs emanted by #2CCSTITD showed 100% inhibition of two fungal pathogens *Alternaria alternata* and *Cercospora beticola* whereas the other fungal isolates like *Fusarium oxysporum, P. marneffei, Fusarium solani* showed 10-70% retardation in their growth when exposed to VOCs for 72 hrs. The tested *Candida* pathogens exhibited 50-65% inhibition. The bacterial isolates were resistant against the VOCs produced.

Test microbe	**Antimicrobial efficacy of # 2CCSTITD**		
	Mean Diameter Test	**Mean Diameter Control**	**% age inhibition**
F. oxysporum	15.75± 0.50	25.5± 0.71	38.23
A .alternata	0	11±1.41	100
P. marneffei	6± 1.41	21± 1.41	71.42
A. phaeospermum	16.75± 2.63	24±1.41	30.2
P. theiocola	10.5± 1.29	16.5±0.71	36.36
R. solani	12.75± 0.50	45±0.00	71.67
B. ochroleuca	12.25±1.50	16 ±1.41	23.43
C. beticola	0±0.00	16.5±2.12	100
F. solani	19.75±0.50	22.5±2.12	12.22
B. cinerea	18.75±1.50	20.5±0.71	8.53
C. gloeosporioides	16.5±1.73	16.5±0.71	0
L. theobromae	32.75±2.50	36 ±0.00	9.02

M. albus	8.75±0.96	9.5±0.71	7.8
A. flavus	9.5±2.08	16 ±0.00	40.62
C .albicans	6.25±0.5	18.5 ±3.54	62.21
C .albicans	8.5±1.73	18.5 ±0.71	54.04
C. albicans	8.5±1.91	20.5 ±3.54	58.53
C. vishwanathii	7.5±1.91	20.5 ±0.71	63.41

Table no.12: Showing antimicrobial activity of VOCs produced by #2CCSTITD

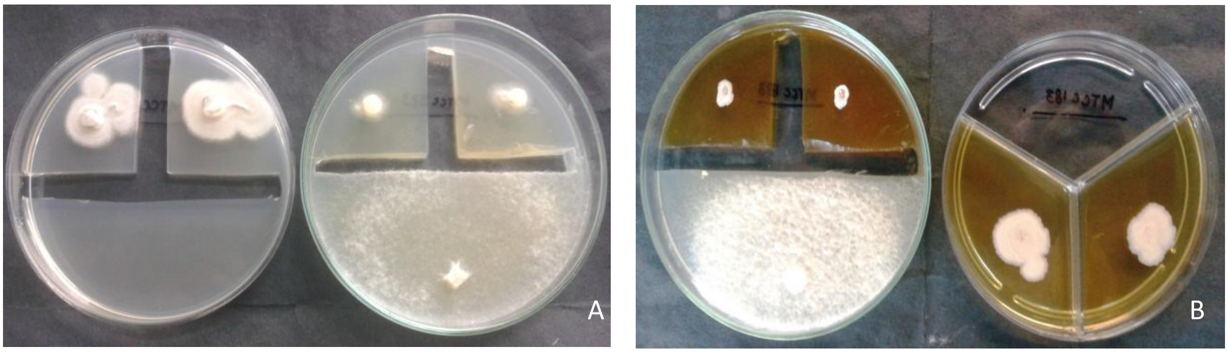


Figure 10: Showing antimicrobial efficacy of VOCs produced by #2CCSTITD with A) MTCC 359 B) MTCC 183

Thus it can be inferred from the above results that out of four tested *Muscodor* isolates, VOCs produced by #16 AMLWLS were the most lethal ones which completely inhibits most of the tested pathogens whereas the two other *Muscodor* isolates #6610 CMSTITBRT and # 1CCSTITD possess antibacterial and antifungal activity where as #2 CCSTITD possess only antifungal potential (Table no.13, summarized table).

Test microbe	**Antimicrobial efficacy (% age growth inhibition)**			
	#6610CMSTITBRT	**#16AMLWLS**	**#1CCSTITD**	**#2CCSTITD**
Alternaria alternata	13.15 ± 2.03	100 %	61.36 ± 0.91	100
Arthrinium phaeospermum	0	100%	0	30.2
Aspergillus flavus	32 ± 3.6	48.5 ± 3.83	26.56 ± 1.5	40.62
Aspergillus japonicus	0	13.97 ± 0.97	0	0
Aspergillus niger	45.65 ± 5.71	44.44 ± 7.17	24.69 ± 6.29	0
Bionecteria ochroleuca	11.53 ± 5.76	38.05 ± 3.99	11.00 ± 0.80	23.43
Botrytis cinerea	3.39 ± 2.4	100	30.49 ± 0.55	8.53
Cercospora beticola	3.45 ± 4.52	100 %	57.57 ± 0.30	100

Colletotrichum gloeosporioides	0	0	40.90 ± 0.20	0
Fusarium solani	11.14 ± 0.40	100%	41.11 ± 2.23	12.22
Fusarium oxysporum	4.87 ± 0.25	100%	17.30 ± 0.91	38.23
Lasodiplodia pseudotheobromae	1.96	34.09 ± 8.94	0	0
Lasodiplodia theobromae	5.55 ± 2.88	32.29 ± 1.94	70.83 ± 1.00	9.02
Penicillium marneffei	33.87 ± 2.70	50.78 ±1.59%	42.50	71.42
Penicillium chrysogenum.	3.75 ± 1.26	100%	48.89 ± 1.12	0
Phomopsis theiocola	59.21 ± 4.69	66.66 ± 2.17	19.23 ± 3.66	36.36
Rhizoctonia solani	18.98 ± 4.44	100%	32.22 ± 10.97	71.67
Candida albicans (MTCC 183)	0	100%	42.60 ± 0.80	23.68±0.20
Candida glabrata	30	50	50 ± 0.58	26.47±0.20
Candida vishwanathii	50	50	41.3 ± 0.26	63.41
Candida albicans (MTCC 854)	50	50	52.17 ± 1.12	62.21
Saccharomyeces cerevisiae	0	50	0	0
Agrobacterium tumefaciens	0	100%	0	0
Escherichia coli	0	30	0	0
P. aeruginosa (MTCC 3541)	0	0	73.40 ± 1.16	0
P. aeruginosa (MTCC 647)	0	0	9.16 ± 0.96	0
P. aeruginosa (MTCC424)	70	70	0	0
Staphylococcus epidermidis	0	50	20.16 ± 0.91	0
Staphylococcus aureus (Sau 902)	0	60	0	0
Staphylococcus aureus	0	100%	0	0

Table no.13: Summarized table showing Antimicrobial activity of VOCs produced by four Indian *Muscodor* species

The variation in inhibition potential against same pathogens by four test *Muscodor* isolates & already reported *Muscodor* species can be attributed to the different & specific volatile organic compounds (VOC's) produced by each *Muscodor* species & also to the amount of the VOC's produced in each case, it also indicates that the source of pathogen might also have a role in determining the potential of the isolates, it can be concluded that *Muscodor* isolates possess marked specificity towards different pathogens.

M. albus, M. roseus, M. crispans and M. sutura (Strobel *et al.*, 2001, Worapong *et al.*, 2002, Mitchell *et al.*, 2007 and Kudalkar *et al.*, 2012) possess antibacterial and antifungal properties whereas *M. yucatanensis* (Gonzalez *et al.*, 2009) and *M. vitigenus* (Daisy *et al.*, 2002) posses antifungal and anti-insecticidal property respectively by the virtue of VOCs produced by it .

From the above results, #16 AMLWLS emerges as a more potent bio-control agent & it was hence selected for exploring the bio-fumigation potential to act as bio preservative for fruits and grains.

5.5 Bio-fumigant potential of #16 AMLWLS

Bio-fumigation potential of the #16AMLWLS was investigated to control three post harvest fungal pathogens- *Botrytis cinerea, Colletotrichum gloeosporioides* and *Rhizoctonia solani* in selected fruits & grains (Grapes, Cherry, Jamun, Wheat, Black gram).

Botrytis cinerea was not able to infect the fumigated grapes, Jamun and cherry whereas it infects the non fumigated grapes. There was complete inhibition in growth of the fungal pathogen in presence of VOCs produced by #16AMLWLS. #16 AMLWLS also provides shielding effect to grapes and jamun against *Collectrotrichum gloeosporioides* and *Rhizoctonia solani*. There was 80-100% inhibition in the growth of fungal pathogens in test plates as compared to control plates (Table no.14, Figure no.11).

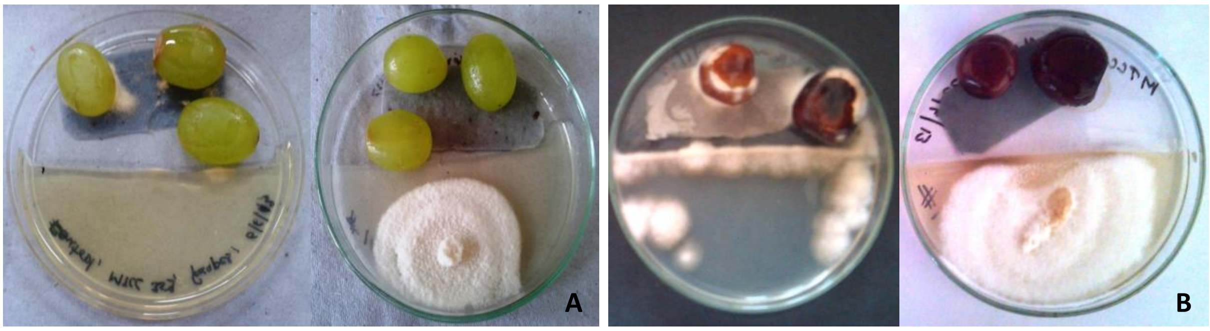


Figure 11 Showing bio-fumigant potential of 16AMLWLS against *Botrytis cinerea* (MTCC 359) infected A) Grapes B) Cherry

Test pathogen	**Percentage fungal growth inhibition**		
	Grapes	**Jamun**	**Cherry**
Botrytis cinerea (MTCC 359)	100%	100%	100%
Rhizoctonia solani (MTCC 4634)	100%	90%	ND
Colletotrichum gloeosporioides (MTCC 9623)	80%	100%	ND

Table no.14: Showing % age inhibition of fungal growth on chosen fruits by #16AMLWLS

(*ND- Not Done)

The experiment has further resulted in identification of actual spoilage organisms (storage pathogens) ruling out the possibility of decay of seasonal fruit jamun by *Rhizoctonia solani* & *Colletotrichum gloeosporioides*

80-100% reduction in infection incidence was observed in case of bio-fumigated black gram and wheat treated with *B. cinerea*, *C. beticola* & *Colletotrichum gloeosporioides.* (Table no. 15, figure no.12)

S.No	**Test**	**Percentage fungal growth inhibition**	
		Black gram	**Wheat**
1.	*Botrytis cinerea* (MTCC 359)	80%	100%
2.	*Colletotrichum gloeosporioides* (MTCC 9623)	100%	100%
3.	*Cercospora beticola*	100%	90%

Table 15Showing % age inhibition of fungal growth on chosen grains by #16AMLWLS

#16 AMLWLS was found to be more potent than *Muscodor albus* in terms of the bio-control efficiency. *Muscodor albus* is reported to reduce the gray mould incidence of grapes to 4.4% (Gabler *et al.*, 2009) but #16 AMLWLS was found capable of completely diminishing the grey mould infection of grapes. Therefore, #16 AMLWLS, a novel Indian *Muscodor* specie can well be touted as a reasonable preservative. It could be further exploited as an alternative to chemical pesticides and fumigants in near future.

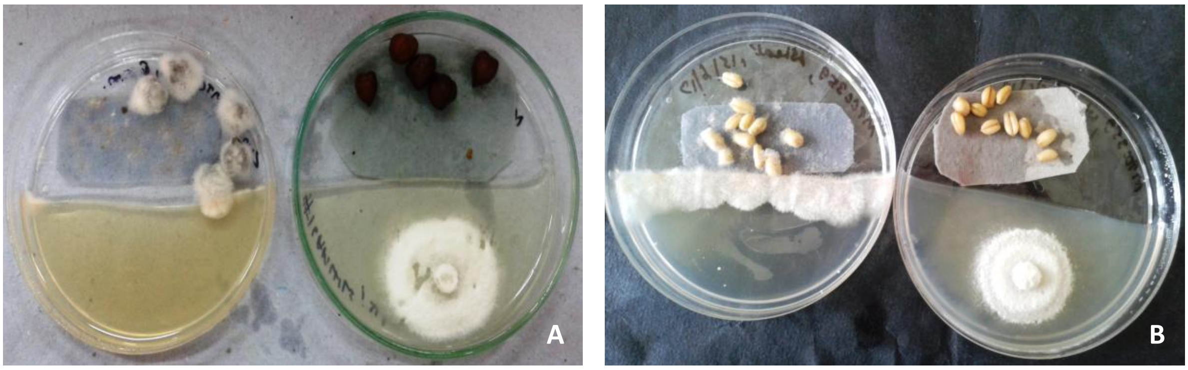


Figure no.12 Showing bio-fumigant potential of #16AMLWLS on A) *Colletotrichum gloeosporioides* (MTCC 9623) treated Black gram B) *Botrytis cinerea* (MTCC 359) treated Wheat.

5.6 Morphotaxonomy

#6610 CMSTITBRT forms yellow orange colored colony over PDA with slow growth rate and formation of VOCs with fruity smell. The colony diameter was 26 mm after 7 days of incubation. It forms rope like thick sterile mycelium. No fruiting bodies were observed (Figure no.13A-C). #16 AMLWLS produces floccose, moderate growing, white colored colony over PDA medium. The average colony diameter was 45-47 mm. It produces sterile, septate, branched ropy mycelium with certain coiling structure. Hyphae and coils measure 2.4-5.41µm and 47.38-50.28 µm respectively. The fungus did not produce any pigment but produces VOCs with strong pungent smell. It is a sterile endophytic fungus that does not produce any sexual or sporulating bodies (Figure no. 13 D-F)

#1 CCSTITD and #2 CCSTITD both were fast growing endophytes which were initially white later turns to brown in color. The colonies were floccose with fruity smell and VOCs formation. It covers the 90 mm plate within 5 days of incubation. Mycelium was thick with coiling structures. No fruiting bodies were observed (Figure no.13G-L)

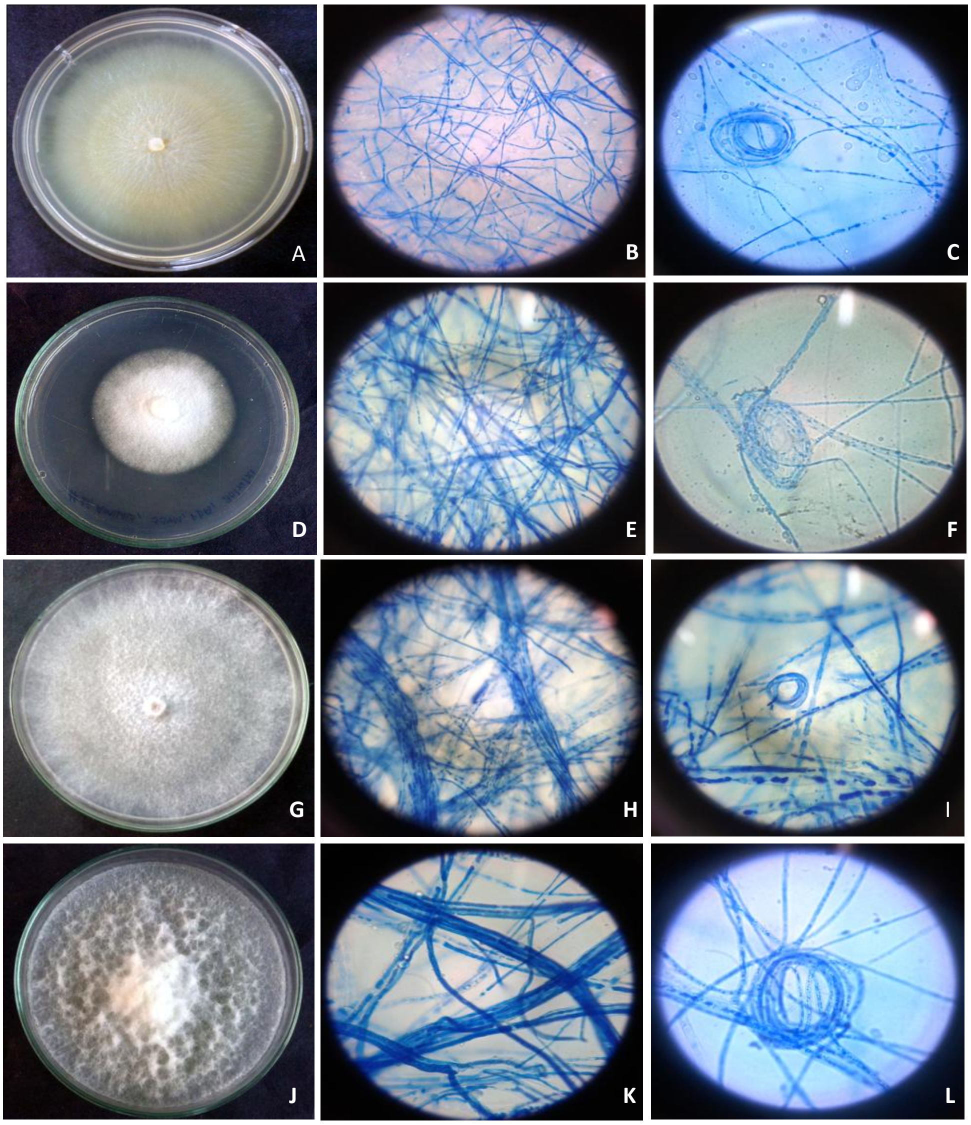


Figure 13 Morphotaxonomy of the four Muscodor isolates (A-C: #6610 CMSTITBRT, D-F: #16 AMLWLS, G-I: #1 CCSTITD and J-L: #2 CCSTITD)

Thus all the four endophytic isolates fulfil the primary criterion of *Muscodor* species by producing sterile mycelium with VOCs production.

All the previously reported Muscodor species are sterile in nature. They from thick sterile mycelium with or without coiling structures.

M. roseus and *M. sutura* also produces red colored soluble pigment where as few *Muscodor* species also produces cauliflower like structures (Strobel *et al*., 2001; Worapong *et al.,* 2002; Daisy *et al*., 2002; Mitchell *et al*., 2008; Gonzalez *et al*., 2009; Zhang *et al*., 2010; Kudalkar *et al*., 2012 and Suwannarach *et al.,* 2013). These are critical characters that help in identification of *Muscodor* species

5.7 Genomic DNA Isolation and PCR Amplification

The genomic DNA isolation of #16 AMLWLS, #6610 CMSTITBRT, #1CCSTITD & #2CCSTITD was qualitatively estimated using agarose gel electrophoresis (Figure no.14). No smear or RNA bands were seen. The purity and concentration of genomic DNA was quantified by taking the absorbance at 260 nm and 280 nm (Table No.16).

Culture	A_{260}/A_{280}	Concentration (µg/ml)
#16 AMLWLS	1.78	0.294
#6610 CMSTITBRT	1.72	0.500
#1 CCSTITD	1.62	0.652
#2 CCSTITD	1.65	0.750

Table no.16: Showing concentration and purity of genomic DNA of *Muscodor sp.*

The *Muscodor* specific region amplification of all four *Muscodor* species were carried out with *Muscodor* specific primers and band of approximate size 450-500bp was observed (Figure no.15). This amplified and characterized region reconfirms the placement of four isolates in *Muscodor* lineage. The BLAST hit table of four *Muscodor* species with already reported species is shown in Table no. 17.

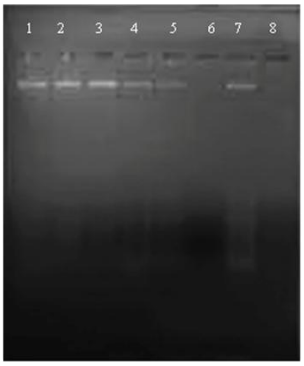


Figure 14: Agarose gel electrophoresis of the genomic DNA isolated from four *Muscodor* isolates. Well 1-3: #6610CMSTITBRT, Well 4: #16AMLWLS, Well 5: #1CCSTITD, Well 6-7: #2CCSTITD, Well 8: Blank

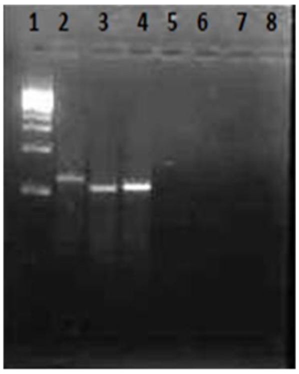


Figure no.15: PCR amplicons of the *Muscodor* isolates using *Muscodor* specific primers. Well 1: 500bp ladder, Well 2: #6610CMSTITBRT, Well 3: #16AMLWLS, Well 4: #1CCSTITD

Culture	**#16AMLWLS (KC481680)**		**#6610CMSTITBRT (JQ409999)**		**#1CCSTITD (JQ409997)**		**#2CCSTITD (JQ409998)**	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**
M. albus (AF324336)	98%	99%	98%	94%	98%	97%	99%	98%
M. roseus (AF034665)	98%	99%	98%	94%	98%	97%	99%	98%
M. vitigenus (AY100022)	91%	95%	91%	91%	91%	92%	91%	93%
M. crispans (EU195297)	98%	99%	98%	94%	98%	97%	99%	98%
M. yucatanensis (FJ917287)	98%	88%	90%	92%	98%	86%	99%	86%
M. fengyangensis (HM034853)	81%	95%	90%	90%	82%	95%	81%	95%
M. sutura (JF938595)	91%	95%	91%	91%	91%	92%	91%	93%
M. oryzae (JX089321)	98%	99%	98%	94%	98%	97%	99%	98%
M. cinnamomi (GQ848369)	98%	99%	98%	94%	98%	97%	99%	98%
M. suthepensis (JN558830)	98%	97%	98%	92%	98%	94%	99%	94%
M. musae (JX089323)	98%	99%	98%	94%	98%	97%	99%	98%
M. equeseti (JX089322)	91%	95%	91%	91%	91%	92%	91%	93%

*1: Query coverage, 2: Sequence similarity

Table no.17 Showing sequence similarity of four Indian *Muscodor* species with already reported *Muscodor* species.

Chapter 6

Conclusion

Conclusion

The current study showed that the four *Muscodor* isolates possess broad spectrum antimicrobial potential. Out of these four isolates, #16 AMLWLS possess maximum potential to be exploited as a bio fumigant. It act as a preservative for grapes, jamun, cherry, black gram and wheat. Thus #16 AMLLWLS stands as a potential candidate to be developed as bio fumigant to act as bio preservative for fruit and grains.

Chapter 7

References

1. Adaskaveg JE, Forester H and Sommer NF (2002). Principles of postharvest pathology and management of decays of edible horticulture crops. In:AA Kader (Ed.). *Postharvest technology of horticulture crops.*

1. Atmosukarto I, Castillo U, Hess WM, Sears J and Strobel G (2005). Isolation and characterization of *Muscodor albus* I-41.3s, a volatile antibiotic producing fungus. *Plant Science*. (169): 854-861.

2. Bacon CW and WhiteJF (2002). Microbial Endophytes. Marcel Dekker, New York.

3. Camp AR, Dillard HR and Smart CD (2008). Efficacy of *Muscodor albus* for the control of phytophthora blight of sweet pepper & butternut squash. *Plant Disease*. (92): 1488-1492.

4. Corcuff R, Mercier J, Marquet X and Arul J (2006). Bio-fumigation potential of *Muscodor albus* in storage of potato tubers. *Phytopathology*. (96): S26

5. Crisosto CH, Mitchell FG (2002). Postharvest handling systems: small fruits. I.Table grapes. In: Kader, A.A. (Ed.), *Postharvest Technology of Horticulture Crops.* 357–363.

6. Daisy B, Strobel G, Ezra D, Castillo U, Baird G and Hess WM (2002). *Muscodor vitigenus* anam. sp. nov., an endophyte from *Paullinia paullinioides*. *Mycotaxon*. (84): 39-50.

7. Daisy BH, Strobel GA, Castillo U, Ezra D, Sears J, Weaver DK and Runyon JB (2002). Naphthalene, an insect repellent, is produced by *Muscodor vitigenus*, a novel endophytic fungus. *Microbiology*. (148): 3737-3741.

8. Eckert JW, Eaks IL (1989). Postharvest disorders and diseases of citrus fruits. In: Reuther, W., Calavan, E., C., Carman, G., E., (Eds.), *The Citrus Industry*. (4): 179-260.

9. Eckert JW and Ogawa (1988). The chemical control of postharvest diseases: Deciduous fruit, berries, vegetables and root/tuber crops. *Annual Review Phytopathol*ogy. (26): 433-460.
10. Ezra D, Hess WM and Strobel GA (2004). New endophytic isolates of *Muscodor albus*, a volatile-antibiotic-producing fungus. *Microbiology.*(150): 4023-4031.

11. Ezra D, Skovorodnikova J, Kroitor KT, Denisov Y, Liarzi O (2010). Development of methods for detection and *Agrobacterium*-mediated transformation of the sterile, endophytic fungus *Muscodor albus*. *Biocontrol Science and Technology*. (20): 83-97.

12. Gonzalez MC, Anaya AL, Glenn AE, Macıas-Rubalcava ML, Hernandez-Bautista BE, and Hanlin RT (2009). *Muscodor yucatanensis,* a new endophytic ascomycete from Mexican chakah, *Bursera simaruba*. *Mycotaxon.*(110): 363-372.

13. Grimme E, Zidack NK, Sikora RA, Strobel GA and Jacobsen BJ (2007). Comparison of *Muscodor albus* volatiles with a biorational mixture for control of seedling diseases of sugar beet & root-knot nematode on tomato. *Plant Disease*. (91): 220-225.

14. Gustavsson J ,Cederberg C, Sonesson U, Otterdijk RV, Meybeck A, (2011). Global food losses and food waste. *Save Food*: 1-29.

15. Holmes GJ, Eckert JW (1999). Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. *Phytopathology*. (89): 716-721.

16. Irtwange SV (2006). Application of biological control agents in pre-and postharvest operations. *Agricultural Engineering International.* Volume VIII.

17. Jacobsen BJ, Zidack NK, Strobel GA, Ezra D, Grimme E and Stinson AM (2004). Mycofumigation with *Muscodor albus* for control of soil-borne microorganisms. *IOBC/WPRS Bull*. (27): 103–113.

18. Karabulut OA, Smilanick JL, Mlikota-Gabler, Mansour M, and Droby S (2003). Near-harvest applications of *Metschnikowia fruiticola*, ethanol, and sodium bicarbonate to

control postharvest diseases of grape in central California. *Plant Disease*. (87): 1384-1389.

19. Kudalkar P, Strobel G, Hassan S, Geary B and Sears J (2011). *Muscodor sutura*, a novel endophytic fungus with volatile antibiotic activities. *Mycoscience*. DOI:10.1007/s10267-011-0165-9.

20. Lacey LA and Neven LG (2006). The potential of the fungus, *Muscodor albus*, as a microbial control of potato tuber moth (Lepidoptera: Gelechiidae) in stored potatoes. *Journal of invertebrate pathology*. (91): 195-198.

21. Li DW and Yang CS (2004). Fungal contamination as major contributor to sick building syndrome. *Advances in Applied Microbiology*. (55): 31-112.

22. Litcher A, Zutchy Y, Sonego L, Dvir O, Kaplunov T, Sarig P, and Ben-Arie R (2002). Ethanol controls postharvest decay of table grapes. *Postharvest Biol. Technol.* (24): 301-308.

23. Luvisi DA, Shorey HH, Smilanick JL, Thompson JF, Gump BH, and Knuston J (1992). Sulfur dioxide fumigation of table grapes.

24. Mercier J and Jimenez JI (2004). Control of fungal decay of apples and peaches by the bio-fumigant fungus *Muscodor albus*. *Postharvest Biology and Technology*. (31): 1–8.

25. Mercier J and Manker D (2005). Bio-control of soil-borne disease and plant growth enhancement in green house soilless mix by the volatile-producing fungus *Muscodor albus*. *Crop Protection*. (24): 355–362.

26. Mercier J, Santamaria JIJ and Guerra PT (2007). Development of the volatile producing fungus *Muscodor albus* Worapong, Strobel, and Hess as a novel antimicrobial bio-fumigant. *Revista Mexicana de Fitopatologia*, Julio-diciembre, año/vol. 25, número 002 Sociedad Mexicana de Fitopatologia, A. C. Ciudad Obregón, Mexico: 173-179.

27. Mercier J and Smilanick JL (2005). Control of green mold and sour rot of stored lemon by bio-fumigation with *Muscodor albus*. *Biological Control.* (32): 401–407.

28. Mitchell AM, Strobel GA, Hess WM, Vargas PN, and Ezra D (2008). *Muscodor crispans*, a novel endophyte from *Ananas ananassoides* in the Bolivian Amazon. *Fungal Diversity.* (31): 37-43.

29. Mittermeier RA, Meyers N, Gil PR and Mittermeier CG, (1999). Hotspots: Earth's Biologically Richest and Most Endangered Eco Regions. Toppan Printing Co.

30. Mlikota-Gabler FM, Mercier J, Jimenez JI, Smilanick JL, (2010). Integration of continuous bio-fumigation with *Muscodor albus* with pre-cooling fumigation with ozone or sulphur dioxide to control postharvest gray mould of table grapes. *Postharvest Biology and Technology.* (55): 78–84.

31. Mlikota-Gabler F, Fassel R, Mercier J and Smilanick JL, (2006). Influence of temperature, inoculation interval, and dosage on bio-fumigation with *Muscodor albus* to control postharvest gray mould on grapes. *Plant Disease.* (90): 1019-1025.

32. Mlikota-Gabler F and Smilanick JL (2001). Postharvest control of table grape gray mould on detached berries with carbonate and bicarbonate salts and disinfectants. *American Journal of Enology and Viticulture.* (52): 12-20.

33. Mlikota-Gabler F, Smilanick JL, Ghosoph JM, and Margosan DA (2005). Impact of postharvest hot water or ethanol treatment of table grapes on gray mould incidence, quality, and ethanol content. *Plant Disease.* (89): 309-316.

34. Ramin AA, Prange RK, Braun PG, and Delong JM (2007). Bio-fumigation of postharvest fungal apple decay with *Muscodor albus* volatiles. *Journal of Applied Sciences Research.* 3(4): 307-310.

35. Schnabel G, Bryson PK, Bridges WC and Brannen PM (2004). Reduced sensitivity in *Monilinia fruiticola* to propiconazole in Georgia and implications for disease management. *Plant Disease.* (88): 1000-1004.

36. Sommer NF, Fortlage RJ and Edwards DC (2002). Post harvest diseases of selected commodities. In: Kader, A.A. (Ed.), *Postharvest technology of horticultural crops. pp:* 197-249.

37. Sopalun K, Strobel GA, Hess WM and Worapong J (2003). A record of *Muscodor albus*, an endophyte from *Myristica fragrans* in Thailand. *Mycotaxon.* (88): 239e247.

38. Stinson M, Zidack NK, Strobel GA and Jacobsen B (2003). Mycofumigation with *Muscodor albus* and *Muscodor roseus* for control of seedling diseases of sugar beet and *Verticillum* wilt of eggplant. *Plant Disease.* (87):1349–1354.

39. Strobel G (2006). Harnessing endophytes for industrial microbiology. *Current opinion in microbiology.* (9): 240-244.

40. Strobel G (2011). *Muscodor* species- endophytes with biological promise. *Phytochemical Review.* (10): 165-172.

41. Strobel G and Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular biology Reviews. pp:* 491-502.

42. Strobel GA, Dirksie E, Sears J and Marksworth C (2001). Volatile antimicrobials from a novel endophytic fungus. *Microbiology.* (147): 2943-2950.

43. Strobel GA, Kluck K, Hess WM, Sears J, Ezra D and Vargas PN (2007). *Muscodor albus* E-6, an endophyte of *Guazuma ulmifolia* making volatile antibiotics: isolation, characterization and experimental establishment in the host plant. *Microbiology.* (153): 2613-2620.

44. Suwannarach N, Bussaban B, Hyde KD and Lumyong S (2010). *Muscodor cinnamoni*, a new endophytic species from *Cinnamomum bejolghota*. *Mycotaxon.* (114): 15-23.

45. Suwannarach N, Kumla J, Bussaban B, Hyde KD, Matsui K and Lumyong S (2013). Molecular and Morphological evidence support four new species in the genus *Muscodor* from north Thailand. *Annals of microbiology.* (ISSN): 1590-4261.

46. Taylor MA, Watts JE, and Chambers KR (1990). Sulphur dioxide damage as a predisposing factor to botrytis rot of table grape berries. *Deciduous Fruit Grow.* (40):35-41.

47. U.S. Environmental Protection Agency (EPA) (2004). Questions & Answers about methyl bromide phase-out. Online resources.

48. Worapong J, Strobel GA, Daisy B, Castillo UF, Baird G and Hess WM (2002). *Muscodor roseus* anam. sp. nov., an endophyte from *Grevillea pteridifolia*. *Mycotaxon.* (81): 463-475.

49. Worapong J, Strobel GA, Ford EJ, Li JY, Baird G and Hess WM (2001). *Muscodor albus* anam. nov., an endophyte from *Cinnamomum zeylanicum*. *Mycotaxon.* (79): 67-79.

50. Yee WL, Lacey LA and Bishop BJB (2009). Pupal Mortality and Adult Emergence of Western Cherry Fruit Fly (Diptera: Tephritidae) Exposed to the Fungus *Muscodor albus* (Xylariales: Xylariaceae). *Journal of Economic Entomology*. *102*(6): 2041-2047.

51. Zhang CL, Wang GP, Mao LJ, Komon-Zelazowska M, Yuan ZL, Lin FC, Druzhinina IS and Kubicek CP (2010). Muscodor fengyangensis sp. nov. from southeast China: morphology, physiology and production of volatile compounds. *Fungal biology.* (114): 797-808.