Assessment of Volatile Organic Compounds Produced By *Muscodor* for Post Harvest Storage of Fruits and Vegetables

A

Thesis submitted

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Submitted by

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CERTIFICATE

This is to certify that the thesis entitled "Assesement of volatile organic compounds produced by *Muscodor* species for post harvest shelf storage of fruits and vegetables" being submitted by Ms Tanya (Roll no. 601204028) in the partial fulfillment of the requirements for the award of degree of Masters of Technology in Biotechnology, Thapar University, Patiala is a bonafide work carried out under the supervision and conception 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 work being presented in the thesis entitled“Assesement of volatile organic compounds produced by *Muscodor* species for post harvest shelf storage of fruits and vegetables” in the partial fulfillment of requirements for the award of degree of M .Tech Biotechnology, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of July 2013 to June 2014, under the conception and supervision of Dr. Sanjai Saxena, Associate Professor, Department of Biotechnology (DBT), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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Abbreviations	Full forms
µg	Microgram
µl	Microlitrer
µmol	Micromole
APEDA	Agricultural & Processed Food Products Export Developement Authority
ASSOCHAM	Associated Chamber of Commerce and Industry of India
FDA	Food and Drug Administration
g	Gram
GAIN	Global Agriculture information network
GC	Gas Chromatography
h	Hour
Kpa	Kilo-pascals
L	Liter
M	Molar
min	Minute
ml	Milliliter
mm	Millimeter
MS	Mass spectroscopy
MT	Metric Tons
MTCC	Microbial Type Culture Collection
NHB	National Horticulture Board
NIST	National Institute of Standards and Technology
PDA	Potato Dextrose Agar

psi	Per square inch
SNA	Synthetischer Nahrstoffarmer agar
Sp.	Species
SPME	Solid phase microextraction syringe
USDA	United States department of agriculture
UV	Ultraviolet rays
V	Volt
VOC	Volatile organic compounds
YEPD	Yeast extract peptone dextrose medium

ABSTRACT

The current study explores endophytic Indian *Muscodor* isolates for their mycofumigation potential to prevent decay of various fruits by gray mold *B. cinerea*. Nutrient (1-5% dextrose) and temperature (23-30°C) conditions were optimized for each *Muscodor* isolate using one factor at a time approach. The antimicrobial potential of *Muscodor* isolates was evaluated using VOC stress bioassay where the growth of *B. cinerea* was completely inhibited by #16 AMLWLS and #130 TMDSTYEL whereas the other *Muscodor* isolates inhibited the growth of *B. cinerea* by 10 to 55%. Volatiles emanated by #1639 CCSTITD did not induce any inhibitory effect on *B. cinerea*. Based on screening results, #16 AMLWLS and #130 TMDSTYEL were chosen for evaluation of their mycofumigation potential. The volatiles produced by #16 AMLWLS and #130 TMDSTYEL over PDA medium provided complete protection to apples, grapes and strawberries till 10th days of fumigation against *B. cinerea.* 25 g of wheat grain colonized *Muscodor* isolates also showed complete inhibition *B. cinerea* thereby providing shielding effect to the tested fruits whereas the same *Muscodor* isolates when colonized over rye grain did not exhibit any mycofumigation activity. SPME/GC-MS analysis of #16 AMLWLS exhibited a mixture of 23 volatile compounds predominantly producing 3-cyclohexen-1-ol,1-(1,5-dimethyl-4-hexenyl)-4-methyl; 1,6-dioxacyclododecane-7,12-dione; 2,6-bis(1,1-dimethylethyl)-4-(1-oxopropyl) phenol; 2,4-di-tert-butylthiophenol and 4-octadecylmorpholine. These volatiles synergistically induce mycofumigation effect. Thus based on the above results it can be concluded that volatile produced by Indian *Muscodor* species possess significant mycofumigation potential which can be further applied for preventing the decay of commercially important fruits.

KEYWORDS: Mycofumigation, Volatiles, *Botrytis cineria*, Apple, Antimicrobial

Chapter 1

Introduction

1.0 Introduction

"An apple a day keeps the doctor away"

It is well said as fruit and vegetables are packed full of goodness and often contain a number of essential vitamins and minerals that cannot be found in other types of foods. They are essential source of macro and micro nutrients for example: vitamins, minerals and fibres. They also possess higher amount of antioxidant compounds which ultimately prevents from cancer, cardiovascular disorders, hypertension, diabetes etc. These compounds boost immunity level of body and protect it from oxidative stresses (Boeing *et al*., 2012). Apple, grapes, strawberry are major & nutritionally important fruits. These low glycemic fruits, contain flavanols and antioxidants which regulate blood sugar level and also possess anticancerous, anti-inflammatory & cardiovascular properties(Ellis *et al.*, 2013).

These nutritionally important fruits and vegetables production in world has been estimated 486 million and 392 million tons (Singh *et al*., 2014) and India is the second largest producer of these crops in the world, accounting for about 16% of global vegetable production and 10% of world fruit production according to report by APEDA.(FIG.1).

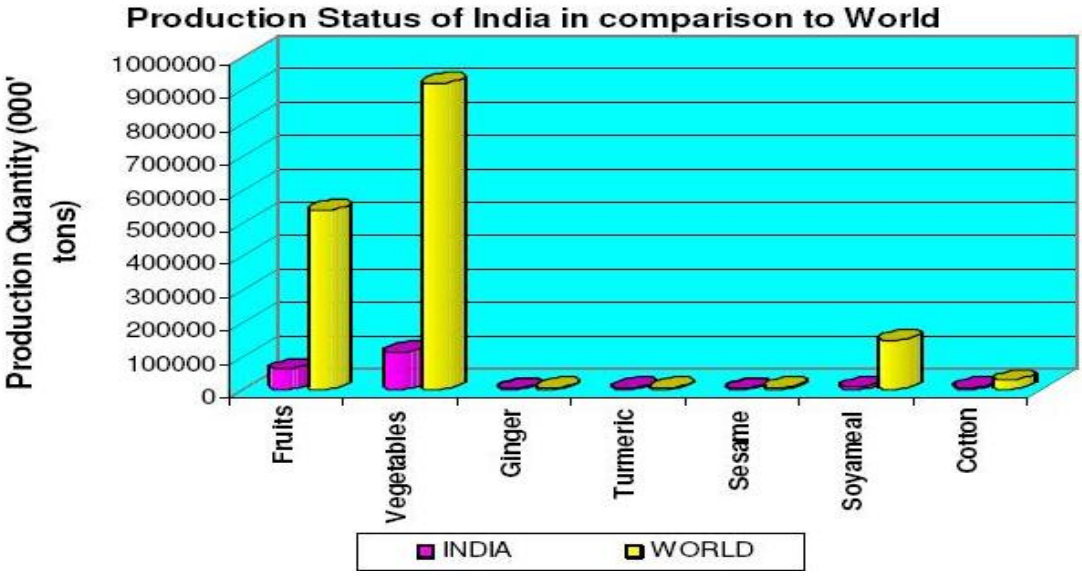
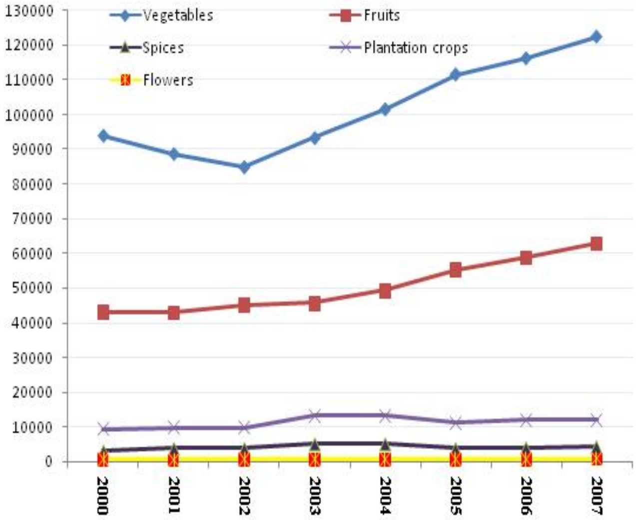


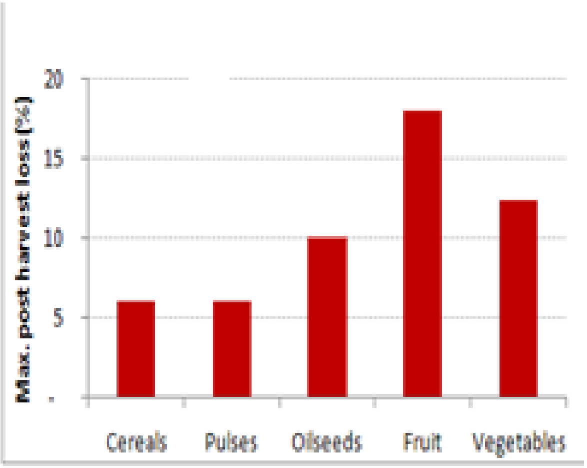
FIG 1: Proportion of production of fruits and vegetables in India in comparison to world

Presently, India produces 8441 (000' MT) of fruits and 170248 (000' MT) of vegetables (NHB, 2013-2014). Inspite of such a great produce, only 22% of this amount reach wholesale market (ASSOCHAM, 2013). The reason behind this is post harvest losses. Post harvest losses of fruits and vegetables are highest among all food groups likewise their production FIG 2 (a and b).



Source : NHB, 2006

FIG.2(a) Production of fruits and vegetables are highest among all food groups



Source: Business standard 2010

FIG.2 (b) Post harvest losses of fruits and vegetables are highest among all food groups

Postharvest losses are quantitative and qualitative losses occur in horticultural crops between harvest and consumption. 35-40% of fruits and vegetables produced in India is lost due to improper post-harvest operations like handling (15-20%), packaging (15%), transportation (30-40%), & marketing (30-40%) (European Journal of Logistics Purchasing and Supply Chain Management, March, 2014)

Post harvest losses amount to a loss of Rs 40,000 crores per year, which is not only a loss of money, but also of labour, energy and inputs involved in production. India waste fruits and vegetables every year equivalent to the annual consumption of the United Kingdom (Indian Science & Technology, 2008). It has been estimated that these postharvest losses may cross Rs. 2.50 lac crore by 2013-14. Losses do not merely reduce food available for human consumption but also cause negative externalities to society through cost of waste management, greenhouse gas production, and loss of scarce resources used in their production (Gustavasson, *et al.*, 2011; Vermeulen, *et al.*, 2012). Pathogenic infections that occur at the time of harvest, during handling, stage, transport and marketing and after consumer purchase are a major cause of postharvest loss in horticulture crops (Nadas *et al.*, 2003)

Postharvest treatments are needed to protect sites of infection. Various physical (UV, Gamma radiation, heat), Chemical (Fungicide, antibiotics), Gaseous (O_2, SO_2, CO) techniques have been applied till date. But these conventional techniques have many limitations. Use of UV, γ-radiation, heat is not practical and has great unacceptability among consumers. Ozone depletion, heavy metal pollution and toxicological risks are associated with use of antibiotics and fungicides like methyl bromide and sulphur dioxide. Although Gaseous treatments like lowering of O_2 to 1% has resulted in pathogenic growth reductions, but there is a danger that the crop will start respiring anaerobically and develop off-flavors. Facing the challenges, alternative strategies for post harvest technology is the need of hour.

Biological treatments (*Pseudomonas syringae*, *Candida* sp.) offer several advantages over traditional approaches. The bio-control agents are considered non-hazardous to humans and animals. These are biodegradable, environmental-friendly, does not require processing of fruit, attack specific target organisms without affecting the other beneficial organisms (Adaskaveg *et al.*, 2002). However, biocontrol efficacy is directly affected by the amount of pathogen inoculum present (Roberts, 1994) and lack curative activity (Janisiewicz and Korsten, 2002).Over the last two decades demand for biocontrol agents increases which could act more effectively on plant diseases. Endophytes are ubiquitous microorganisms that atleast once in their life cycle colonise the healthy and living tissue of a plant without showing any negative impact or symptoms (Bacon & White, 2002; Kusari *et al.*,2012). They are hypothesized for providing protection to host plant from the attack of herbivorous insects or vertebrate grazers by production of bioactive compounds (Tan and Zou, 2001).

Endophytic fungi are prolific producers of biologically active compounds which possesses their possible application in pharmaceutical and agriculture industries (Strobel, 2006). The discovery of a billion dollar anticancer drug paclitaxol from *Pestalotiopsis andeane,* endophytic fungus of Himalayan yew opens the new gate to the researchers across the world for exploring this hidden treasure of nature. Discovery of endophytic fungus *Muscodor albus* CZ620 from the stems of *Cinnamomum zeylanicum* by Prof. Gary Strobel during his forest forays in Honduras, Central America in 2001 (Strobel *et al*., 2001) was a novel alternative approach for controlling post harvest decay. *Muscodor albus* cz620 was able to kill or inhibit the growth of plant pathogenic fungi like *Botrytis cinerea, Penicillium digtatum, Cercospora beticola,* Pythium sp. under *in vitro* conditions. This miracle endophytic fungus also suppresses the growth of human pathogens. Thus finding and exploring the newer biotypes of *Muscodor* species is the need of hour.

Muscodor is a genus of sterile endophytic fungi which produces a blend of volatile organic compounds (e.g. amines, alcohols, esters, ketones etc) which synergistically induces antimicrobial and insecticidal effect over the plant and human pathogens (Strobel *et al,* 2001). The current trend across the globe is to exploit the myco/biofumigation potential of this biological weapon against plant pathogenic microbes involved in post harvest decay. *Muscodor albus* is being developed as a biopesticide for postharvest disease control and other agricultural applications. Rye grain culture of *M. albus* was effective as a biofumigant in controlling postharvest diseases in inoculated apples, peaches, lemons and grapes (Mercier & Jiménez , 2004) . Biofumigation with *M. albus* has been shown to control smut on grain (Strobel *et al*., 2001), soil-borne pathogens, (Stinson *et al*., 2003; Mercier and Manker, 2005) building molds (Mercier and Jimenez, 2007) and potato tuber moth (Lacey and Neven, 2006; Lacey *et a*l., 2008). 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 .

To date, 15 *Muscodor* species have been reported from various ecological niches of world viz. *Muscodor albus* (Honduras,.Central america.); *Muscodor musae*, *Muscodor. oryzae*, *Muscodor suthepensis*, *Muscodor cinnamomi* and *Muscodor equiseti* (Thailand); *Muscodor fengyangensis* ZJLQ070(southeast of china); *Muscodor sutura* CA22-D (Columbia); *Muscodor vitigenus P-1* (Amazon)*; Muscodor crispans(* Amazon*); Muscodor yucatanensis* (Mexican chakah). From India, *Muscodor* albus MOW12 has been isolated from the pepper plant dwelling in Meghalaya (Banerjee *et al*; 2014). Apart from these *M. strobelli, M. kashayum, M. Darjeelingensis* and *M. tigerii* have been reported from biodiversity hot spots of India (Meshram *et al*., 2014 ; Saxena *et al.,*2014).

India shares a special position amongst twelve world biodiversity hotspots. These biodiversity hotspots are reservoir of diversity of flora and fauna. The eight *Muscodor* isolates were isolated from the forest areas of the two major biodiversity hotspots in India; The Western Ghats (a World Heritage Site) and The North eastern Himalayas. Preliminary studies suggested that the Indian *Muscodor* isolates possesses antimicrobial property , (Meshram *et al*., 2014; Saxena *et al.,*2014). None of these isolates have been evaluated/ assessed for their mycofumigation potential and their ability to prevent postharvest losses of fruits and vegetables. So the present study aims at assessment of mycofumigation potential of these Indian *Muscodor* isolates to possibly prevent post harvest decay

Chapter 2

Review of Literature

2.1 Fruits and vegetables

Fruits and vegetables are important component of a healthy diet. They play a significant role in human nutrition, especially as sources of vitamin C, vitamin A , thiamine (B_1), niacin (B_3), pyridoxine (B_6), folacin (B_9), vitamin E, minerals, and dietary fibers (Boeing *et al.*, 2012). There is a great association between fruit and vegetable intake and reduced risk of major chronic diseases (Dauchet *et al.*, 2006). Fruit and vegetable consumption was associated with reduced cancer and cardiovascular mortality (Oyebode *et al*, 2014). Grapes, apples and strawberries are also categorised in list of major fruits and vegetables (Coolong *et al.*, 2010). Grapes are an outstanding source of phenols, carotenoids and phenolic acids. Resveratrol, stilbene phytonutrients present in skin and seeds of grapes increase the expression of genes related to longevity (Lekli *et al.*, 2010). Grapes also have antioxidant and anti-inflammatory properties which make them a natural agent for protection against cancer (Zhou K and Raffoul J.,2012) . Similarly apples (*Malus pumila*) also contain antioxidants including quercetin, catechin, phloridzin and chlorogenic acid which inhibit cancer cell proliferation, decrease lipid oxidation and reduces cholesterol (Boyer and Liu., 2004). Strawberries also have anti-inflammatory and antioxidant properties. Amongst all worlds' healthiest foods, strawberries are the best source of vitamin C and contain polyphenols which help in regulating blood sugar response (Giampieri *et al.*, 2014).

2.2 Post harvest losses

One-quarter of these nutritionally significant fruits and vegetables never reaches the consumer for whom it was grown. According to a survey conducted by McKinsey and Co, it was found that at least 50% of the production of fruits and vegetables in the country is lost and in US more than ninety-six billion pounds of food is lost by retailers (USDA Economic Research Service) which result in price rise, hunger and malnutrition. The reason behind this is post harvest losses. Losses which occur after harvesting, starts first from the field, after harvest, in grading and packing areas, in storage, during transportation and in the wholesale and retail markets, are termed post harvest losses . According to Chadha (2009) these post harvest handling losses is around 35 -40% which lead to a loss of Rs. 40,000 crores per year. More than three-quarters of a billion people suffer from malnutrition, therefore to adequately feed the world's expected 10 billion people within the next 40 to 50 years, food production efficiency and distribution needs to be improved immensely (Campbell , 1998).

There is an utmost need of reducing these post harvest losses for ensuring the future global food security and before going on for treatments cause of these losses must be known. The main cause of post harvest losses are physiological (wilting, shriveling, chilling, injury etc), pathological (decay due to fungi and bacteria), developmental (sprouting, rooting, seed germination) and physical (mechanical injury). Origin of pathogens in the field is one of important cause of postharvest losses of these fruits (Nadas *et al.*, 2003). *Penicillium expansum* and *Botrytis cinerea* cause wound infection in Pome fruits and make them susceptible to blue and gray mold (Blanpied and Purnasiri, 1968; Spotts *et al.*, 1998; Sommer *et al.*, 2002). Similarly, *Monilinia fructicola* infect peaches which results in brown rot (Eckert and Sommer,1967). Although there are many pathogens that cause decay in fruits, but pathogenic fungus *Botrytis cineria* is common which attack over 200 crop hosts worldwide. *Botrytis cinerea* (teleomorph*: Botryotinia fuckeliana*) is an airborne plant pathogen with a necrotrophic lifestyle, produces a range of cell-wall-degrading enzymes, toxins and other low-molecular-weight compounds such as oxalic acid. It cause soft rotting of all aerial plant parts, vegetables, fruits and flowers post-harvest to produce prolific grey conidiophores and (macro) conidia typical of the disease (Williamson *et al.*, 2007).

2.3 Strategies to prevent post harvest losses

Postharvest treatments are needed to protect sites of infection. Various physical chemical, gaseous and biological treatments have been applied till date.

Physical treatment: Use of UV radiation, gamma radiation, heat, etc. Low doses of ultraviolet light irradiation (254 nm UV-C) controlled postharvest brown rot of peaches (Stevens *et al.*, 1998) by reducing the inoculum of the pathogen and creating resistance in host. However, it has not become a practical postharvest treatment and requires more research. Gamma radiation has been studied for controlling disinfestation, and extending the storage and shelf-life of fruits and vegetables but due to the cost effectiveness, size of equipment and uncertainty among consumer about the acceptability of irradiated foods, application of gamma radiation is limited. High temperature may be used to control postharvest decay on crops that are injured by low temperatures such as mango, papaya, pepper, and tomato (Spotts, 1984) but the primary obstacle to the widespread use of heat is the sensitivity of many fruit to the temperature required for effective treatment.

Chemical Treatments: involve use of fungicides, antibiotics, etc. Application of de-methylation inhibitor (DMI) fungicides, and more recently, respiration inhibitor fungicides, such as boscalid, azoxystrobin, and pyraclostrobin have been used as main methods of management of the infection (Schnabel *et al.*, 2004) but resistance has been reported in many other crops to several different fungicides with different modes of action (Delp, 1988). *P. expansum,* become widely resistant to the fungicide thiabendazole (Baraldi *et al* ,2003) . Other fungicides are not used because of concerns with residues and possible toxic effects. Antibiotics produced by various species of *Trichoderma* have potential antifungal activity against *Botrytis cinerea*, *Sclerotinta sclerotiorum* and other important plant pathogens.

Gaseous Treatments: Lowering of O_2 to 1% has resulted in pathogenic growth reductions, but there is a danger that the crop will start respiring anaerobically and develop off-flavors (Spotts, 1984;).Use of carbon monoxide has also been tested for lowering postharvest decay (Spotts, 1984;). Sulphur dioxide fumigation can control the postharvest gray mould infecting grape berries (Luvisi *et al.*, 1992).

Biological Treatments: These agents alleviate need for new fungicide treatments (Wisniewskim and Wilson, 1992;) and offer several advantages over conventional biological control (Wilson and Pusey, 1985;) *Pseudomonas syringae* control blue and gray mold of pome fruit (Janisiewicz and Marchi, 1992). This agent is now commercially available for postharvest disease control (Janisiewicz and Jeffers, 1997). Yeast species like *Candida saka* and *Cryptococcus infirmominiatus* has been successfully employed for control of brown rot and blue mold on sweet cherry (Spotts *et al.*, 1998), and three diseases of apple (Vinas *et al.*, 1998). Although there is no doubt that biocontrol agents are effective but they do not always give consistent results. *Bacillus subtilis* controlled peach brown rot disease, but when a commercial formulation of the bacterium was made, adequate disease control was not obtained (Pusey, 1989). Biocontrol efficacy is directly affected by the amount of pathogen inoculum present (Roberts, 1994). Thus finding and exploring the newer biotypes which could act more affectively on plant diseases is of utmost importance.

2.4 Discovery of *Muscodor albus*

Muscodor is a genus of sterile endophytic fungi which produces a blend of volatile organic compounds (e.g. amines, alcohols, esters, ketones etc) which synergistically induces antimicrobial and insecticidal effect over plant and human pathogens (Strobel *et al.*, 2001). *M. albus* CZ620 was isolated 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).

M. albus was discovered when 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. After few days endophytic fungal growth was observed in these plant tissues and after few days of incubation plates were removed and the emerging colonies of fungus were transferred as individually from the hyphal tips onto fresh Potato Dextrose Agar (PDA) plates, after two days incubation all the transferred fungus were not growing, 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 a hypothesis, was made that an endophyte has the ability to produce or generate volatile organic compounds with a wide range of biological activities (Strobel and Daisy, 2003). Later on, it was found that this *Muscodor* albus CZ620 is a sterile endophytic fungus having interesting hyphal characteristics including coiling, ropyness, and angle branching. The mycelia of the fungus on most media are whitish and suppressed.

2.5 Geographical distribution of *Muscodor* species: To date 15 *Muscodor* species have been discovered from different ecological niches of the world. They are, *Muscodor albus CZ-620 , Muscodor roseus A3-5, Muscodor vitigenus P-15, Muscodor crispans B-23, Muscodor yucatanensis* B110, *Muscodor fengyangensis* ZJLQ070, *Muscodor sutura* CA22-D, *Muscodor cinnamomi* CMU-Cib461, *Muscodor oryzae* CMU-WR2, *Muscodor suthepensis* CMU-Cib462, *Muscodor musae* CMU-MU3, *Muscodor equeseti, M. strobelii, M. kashayum,* *M. darjeelingensis* and *M. tigerii* from different parts of the world.(Worapong *et al*., 2001; Worapong *et al*., 2002; Daisy *et al*., 2002; Ezra *et al*., 2004; Mitchell *et al*., 2008; Gonzalez *et al*., 2009; Zhang *et al*.,2010; Kudalkar *et al*.,2012; Suwannarach *et al*.,2013; Meshram *et al*.,., 2014; Saxena *et al*.,2014) (FIG.3.1).

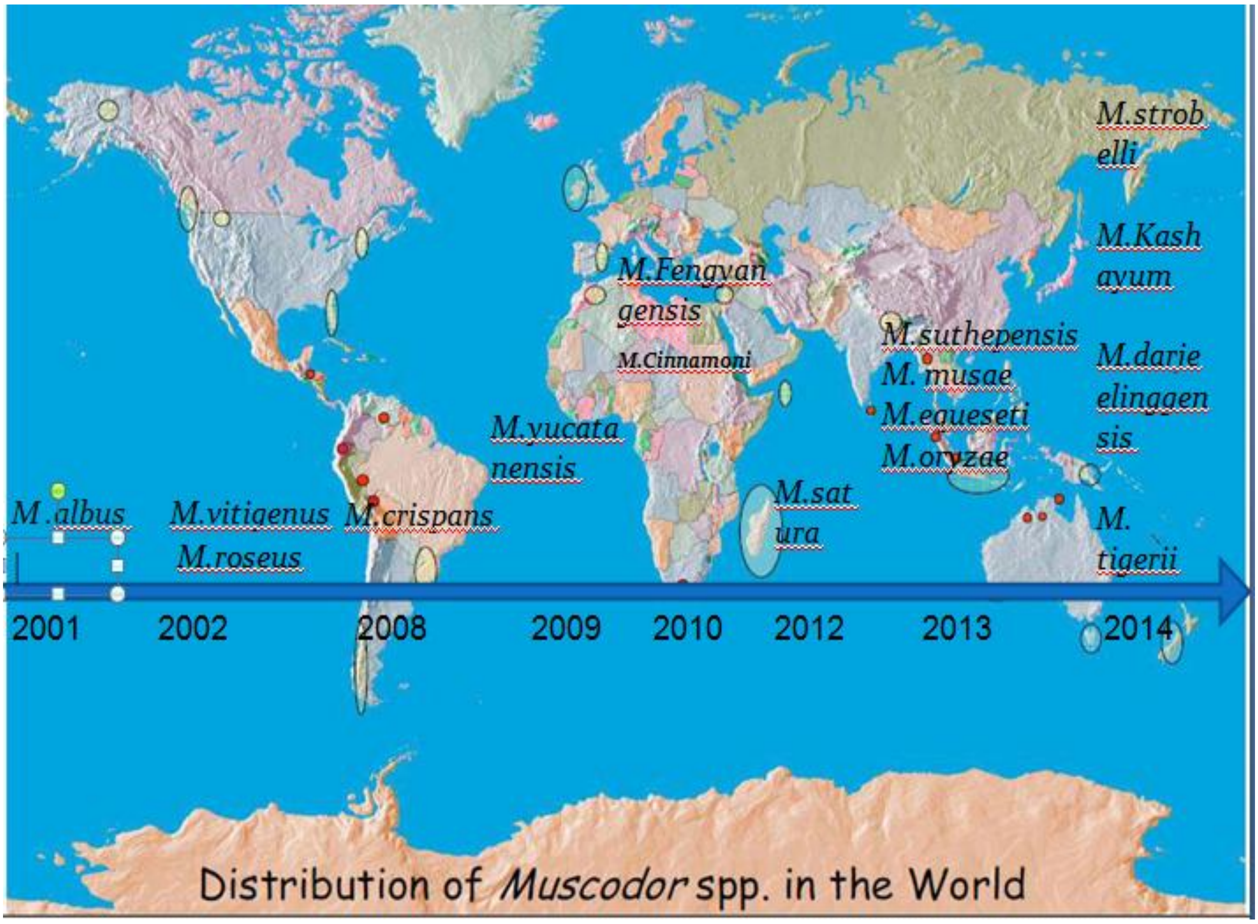


FIG.3: Different *Muscodor* species discovered till date and their distribution

Major volatiles produced by different Muscodor along with their biological activities are mentioned in Table1:

Muscodor sp.	Volatile compounds	Biological activity	Reference
Muscodor albus CZ-620	2-methyl proponic acid	Antifungal Antibacterial	Worapong *et al*., (2001)
Muscodor roseus A3-5	Ethyl 2- butanoate & 1,2,4- trimethylbenzene	Antifungal	Worapong *et al*., (2002)

*Muscodor vitigenus*P-15	Naphthlene	Anti-insect	Daisy *et al.,* (2002)
Muscodor crispans B-23	2-methylpropanoic acid	Antifungal Antibacterial	Mitchell *et al.*, (2008),
Muscodor yucatanensis B110	1R,4S,7S,11R-2,2,4,8Tetramethyltricyclo (5.3.1.0(4,11)) undec- 8-ene	Phytoinihibitory activity	González *et al.,* (2009)
Muscodor fengyangensis ZJLQ070	2- methyl propanoic acid	Antifungal Antibacterial	Zhang *et al.*, (2010)
Muscodor sutura CA22-D	2- methyl propanoic acid	Antifungal Antibacterial	Kudalkar *et al*., (2012)
Muscodor cinnamomi CMU-Cib461	Ethyl 2- methyl propanoate	Antifungal Antibacterial	Suwannarach *et al.,* (2010)
Muscodor oryzae CMU-WR2	3- Methylbutan-1-ol	Antifungal Antibacterial	Suwannarach *et al.,* (2013)
Muscodor suthepensis CMU-Cib462	2- methyl propanoic acid	Antifungal Antibacterial	Suwannarach *et al.,* (2013)
Muscodor musae CMU-MU3	2- methyl propanoic acid	Antifungal Antibacterial	Suwannarach *et al.,* (2013)
Muscodor equeseti	2- methyl propanoic acid	Antifungal Antibacterial	Suwannarach *et al.,* (2013)

Table1: VOC’s produced by various *Muscodor* species.

2.6 VOCs 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 (Saxena *et al.,* 2014). Compartments in PDA plate were made by removing the agar strip from the centre to inhibit the movement of any diffusible metabolite. One side of the petri plate was inoculated with mycelial plug of actively growing culture of *Muscodor.* The plates were sealed and incubated at 24 ºC for 5 days for production of VOC's. Test fungus or bacterium was inoculated on opposite side of the plate (Strobel, 2001). Plates were incubated at 23 °C. The antimicrobial action of VOCs was determined by comparing the growth of test organism in the control plates as to test plates (Strobel *et al*., 2001; Mitchell *et al.,* 2010).

2.7 Antimicrobial activity of *Muscodor* species

Volatile gases produced by *Muscodor* inhibit pathogenic fungi and bacteria like *Aspergillus fumigatus* and *Candida albicans* (Strobel *et al*., 2001). *M.albus* has ability to control soil borne plant diseases caused by *Rhizoctonia solani, Pythium ultimum* and *Verticillum dahlia* (Strobel *et al*., 2001). *Muscodor* isolate CZ-620 and KN-205 inhibited growth of *Bacillus subtilis* (Ezra *et al.,* 2004). *C. acutatum, C. coccodes* and *Rhizopus* sp., were also inhibited in presence of *M. albus* (Mercier and Jimenez, 2004). Different *Muscodor* species inhibit plant pathogens (Table2)

Pathogens	*M. albus*	*M. fengyangensis*	*M. satura*	*M. musae*	*M. oryzae*	*M. suthepensis*	*M. equiseti*
Aspergillus clavatus		+					
Botrytis cinerea	+	+		+	+	+	+
Colletotrichum musae	+	+		+	+	+	+
Colletotrichum acutatum	+						
Colletotrichum fragariae		+				+	
Erwinia carotovora	+						
Escherichia coli		+		+	+	+	+
Geotrichum candidum	+						
Penicillium digitatum	+	+		+	+	+	+
Rhizoctonia solani		+	+	+	+	+	+
Sclerotium rolfsii		+		+	+	+	+
Sclerotinia sclerotiorum	+		+				
Verticillum dahlia		+					
Saccharomyces cerevisiae		+	+	+	+	+	+

Table2: Postharvest pathogens inhibited or killed by the volatiles produced by different *Muscodor* species.

2.8 Volatiles produced by *Muscodor albus*

Volatiles produced which inhibit pathogens were mostly esters, alcohols, acids, lipids and ketones. On the basis of total area of SPME/GC-MS analysis 1-butanol, 3-methyl-acetate was most abundant volatile organic compounds and these are selective in microbes they affect. Lipids and ketones were least biologically active compounds followed by acids, alcohols and esters (Strobel, 2001). However, methyl-1-butanol (48.5%) was the major component in the headspace when *M. albus* was grown on autoclaved rye along with isobutyric acid (14.9%) and ethyl propionate (9.63%) as second and third major components (Mercier and Jimenez, 2004). KN-26, GP-100 and many new isolates of *Muscodor* produce naphthlene/azulene derivatives that possess biological activity both *in vivo* and *in vitro* mixtures. (Ezra *et al.,* 2004) .Concentration of volatiles is an important factor for inhibition of test organism. 2-methyl-1-butanol(MB) from *M. albus* inhibit *B. cineria ,P.expansum , S.sclerotiorum , E. coli* at 100µl/l and *Erwinia carotovora* at 75µl/l and Isobutyric acid (IBA) at conc. of <45 µl/l inhibit *B.cineria, P. expansum.(* Ramin *et al.,* 2005).

2.9 Rye grain formulation

Rye-grain formulation was prepared by transferring the contents of liquid mycelia suspension (in PDB), or plugs of actively growing culture to autoclaved rye-grain (150 g of rye grain was placed in 250 ml of water and autoclaved twice for 30 min on two consecutive days.). The colonised grain culture is ready for use after 10-14 days (Mercier and Jimenez, 2004). Amount of grains to be inoculated is important factor for inhibition of pathogens on fruit. 1 to 13 g/l of *M. albus* colonised rye grain controlled blue and grey mold of apple (Mercier and Jimenez, 2004) whereas 0.25g/l of colonised rye grain was enough to control fungal and bacterial pathogens (Ramin *et al.*, 2005). The culture so prepared was evaluated for its bio-fumigant potential at controlling the fungal decay of apples and peaches. 7days long exposure to VOC’s of grain culture completely controlled blue mould and gray mould in wound-inoculated apples. 72 hr fumigation in case of peaches completely controlled brown rot caused by *Monilinia fruiticola* (Mercier and Jimenez, 2004).

2.10 Mycofumigation with *Muscodor:* Biofumigantion potential of *Muscodor* species are being tested on different fruits and vegetables.

2.10.1 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 and “Thompsons grapes” respectively when used in combination with Colony fumigation (Gabler, 2010). In ‘Red Seedless’ grapes, decay incidence was the highest, the combination of the *M. albus* sachet and the modified atmosphere liner proved to be the most effective treatment, regardless of the rate of *M. albus* used.(Mercier *et al*; 2009)

2.10.2 Control of brown rot of peaches

Bio-fumigation of peaches proved to be 100% effective in controlling the incidence of infection of brown rot caused by *Monilinia fruiticola* from fumigation time of 24 h to 5 days (Mercier and Jimenez, 2004). Mycofumigation was done by adding 30 g of *Muscodor.albus*-colonized grain in a plastic cup and nearby peaches free from injuries were placed, wounded on one side by piercing the skin with a 2 mm long nail tip and inoculated with 10 µl of *M. fructicola* suspension at 5 × 10^3 conidia/ml in 11.4 l airtight plastic boxes (three boxes per treatment) and incubated for period of 24, 48, and 72 h for 5 days.

2.10.3 Control of green mould and sour rot of stored lemon

In vitro exposure to rye-grain culture of *Muscodor albus* controlled the incidence of green mould infection to 26.2% when incubated for 3 days and was an effective treatment for reducing sour-rot of lemons, but the efficiency declines if the treatment is applied after a gap of 24 hr (Mercier and Smilanick, 2005).

2.10.4 Control of blue and gray mold of apple

Exposure to volatiles produced by rye grain culture growing in sealed glass jars of capacity 4 litre completely controlled blue mould (*Penicillium expansum*), gray mould (*Botrytis cinerea*) and *Sclerotinia sclerotiorum* after 24 h long fumigation (Ramin *et al*., 2007). Fumigation with *Muscodor* volatiles was done by adding a measured (12.5, 25, 50, 75 or 150 g) amount of rye grain colonized with *M. albus* in a plastic cup and nearby apples (nine per box) free from injuries were placed and wounded with 15 µl of conidial suspension of *P. expansum* (1×10^4 ml^{-1}) or *B. cinerea* (1×10^4 or 5×10^4 ml^{-1}) in 11.4 l of airtight and autoclavable plastic boxes and incubated at 23 °C for 7 days, control consisted of inoculated fruits in boxes with no colonized grain. Independent of fumigation time and amount of *M. albus*, treated fruits do not develop any lesion for two weeks while control developed complete infection (Mercier and Jimenez, 2004).

2.10.5 Control of smut disease on barley seeds ,seedling diseases of sugar beet and root-knot nematode on tomato

Barley seeds infested with *Ustilago hordei* were completely controlled by *M. albus* (Strobel, 2001**).** Root-knot nematode, *Meloidogyne incognita* on tomato, and 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)

Chapter 3

AIM& OBJECTIVE

3.0 Aim of Study

The current study aims at “Assessment of volatile organic compounds produced from *Muscodor* species for post harvest storage of fruits and vegetables”.

The objectives of the current study are:

1. Nutrient and temperature optimisation of *Muscodor* isolates for maximum volatile production
2. Evaluation of *Muscodor* species for their mycofumigation potential
3. Identification of the volatiles produced by the *Muscodor* isolates using SPME/GC-MS analysis.

Chapter 4

Materials and Methods

4.1 Re-culturing/ activation of *Muscodor* isolates

Endophytic *Muscodor isolates* under study in present study were isolated from different parts of plant growing in biodiversity hotspot regions of Indian subcontinent. The stock cultures were inoculated on Potato Dextrose Agar (PDA) plates, sealed with double layer of parafilm and incubated at 24±2°C for 7-8 days. The *Muscodor* isolates under study are represented in Table 4:

Culture Code	**Plant part**	**Host plant**	**Location**
# 2CCSTITD[a] (*M. tigerii*)	Stem	*Cinnamomum camphora*	Tiger hills, Darjeeling, West Bengal
#1CCSTITD[b] (*M. darjeelingensis*)	Stem	*Cinnamomum camphora*	Tiger hills, Darjeeling, West Bengal
#6CCSTITD[c] (*M. ghoomensis*)	Stem internal tissue	*Cinnamomum camphora*	Ghoom Monastery, West Bengal
#16AMLWS[d] (*M. kashayam*)	Leaf	*Aegle marmelos*	Wayanad Wildlife sanctuary, Kerala
#6(b) CCSTITD[e] (*M. indica*)	Stem internal tissue	*Cinnamomum camphora*	Tiger hills, Darjeeling, West Bengal
#6610CCSTITD[f] (*M. strobelii*)	Stem internal tissue	*Cinnamomum zeylanicum*	BRT wildlife sanctuary, Karnataka
#1639CCSTID[g] (*M. camphora*)	Stem internal tissue	*Cinnamomum camphora*	Darjeeling, West Bengal
#130TMDSTYEL[h]	Stem	*Tabernaemontana divaricata*	Kerala

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

[a, b,] Meshram *et al*; 2014 , [d,f] Saxena *et al.,*2014, [c, e] (in press); [g, h] (unpublished).

4.2 Long term preservation of *Muscodor* isolates

Muscodor isolates were preserved by placing agar plugs (diameter 5 mm) supporting *Muscodor* mycelium onto slants of PDA containing 10% (v/v) glycerol. Slants were incubated at 24°C for 10 days after which they were stored at -20ºC (Ezra *et al*., 2004).

4.3 Procurement & Activation of test pathogen

Botrytis cinerea (MTCC 359) was procured in lyophilized form from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. Lyophilised powder was inoculated onto PDA plates and incubated at 28 °C for 7 days. The cultures thus obtained were maintained as pure culture on PDA slants supplemented with 10% glycerol. Slants were incubated at 24°C for 10 days after which they were stored at -20ºC.

4.4 Temperature Optimisation of Indian *Muscodor* isolates

Each *Muscodo*r was inoculated on PDA plate by placing agar pug of 5mm and incubated at varying temperatures (20°C, 23°C, 25°C ,28°C , 30°C) respectively (Zhang *et al*; 2010). Two replica plates were used for each isolate. Colony diameter and production of volatiles was noted for next 6 days based on which the optimum temperature for growth of each *Muscodor* isolates were finalised for further experimentation.

4.5. Nutrient Optimisation of Indian *Muscodor* isolates

Each *Muscodo*r isolate was inoculated with agar plug of diameter 5 mm on PDA plate with varying dextrose concentration 1%, 1.5%, 2%, 2.5% and 3%, respectively and incubated at their optimised temperature respectively. Two replica plates were used for each isolate.

Colony diameter, amount of volatiles produced was noted for next 6 days based on which the optimum dextrose concentration was finalised for future testing.

4.6 Bioassay of VOC's produced by Indian *Muscodor* isolates

Antimicrobial activity of the volatiles produced by eight *Muscodor* isolates were tested using dual culture volatile bioassay technique against pathogenic fungus *B. cinerea*. 20 ml of PDA was poured in a 90 mm plate and the plate was allowed to solidify. After which agar strips were removed to create compartments in the plate and thereby prohibiting the movement of any diffusible inhibitory compound from the *Muscodor* sp. to the test fungus. One compartment of the plate was inoculated with an agar plug of actively growing *Muscodor* species. The plates were sealed and incubated at 24±2 °C for five days for VOC production. Test fungus was inoculated by placing a 3 mm plug of seven days old culture on the remaining Compartments. Correspondingly, the control plates were comprised of only inoculated test fungi and were devoid of *Muscodor* isolate allowing it to grow normally. Antimicrobial action of VOCs was determined by monitoring the difference in growth of microorganisms in test and control plates (Mitchell *et al.*, 2010; Suwannarach *et al.*, 2013). All the tests were performed in triplicates and values calculated as mean±SD.

4.7 Mycofumigation potential of *Muscodor* to prevent decay in Fruits

4.7.1. Control of gray mold decay of grapes by #16AMLWLS and #130TMDSTYEL

4.7.1.1 Preparation of *Muscodor* inoculum

#16AMLWLS, #130 TMDSTYEL were individually grown on 35 mm PDA plate under optimised nutrient and temperature conditions.

4.7.1.2 Processing of test fruits

Fresh and healthy green grapes (devoid of lesion) available in the market were selected for the study. Grapes were washed in running water for 10 mins to remove debris. Grapes were sterilised with 10% formaldehyde to minimise obtrusion from natural infections, air dried and then placed in a 90 mm petri plate with 15 grapes in each plate in 11.4 L boxes.

4.7.1.3 Preparation of conidial suspension of *Botrytis cinerea*

Botrytis cinerea (MTCC 359) was grown on SNA (Synthetischer Nahrstoffarmer agar) medium for 6-7 days at 26 °C. For formulation of conidial suspension 5-6 plugs (diameter 5mm) of *Botrytis cinerea* were excised from growing front of the colony and added in physiological saline. The saline solution was vigorously vortexed to recover the conidia. Concentration of conidia was adjusted to (1×10^4 ml^{-1}) using haemocytometer (Mercier & Jimenez, 2004).

4.7.1.4 Infection of *B. cinerea*

Fruits were infected with *B. cinerea* by creating lesion on one location of fruit at meridian with a 4 mm metal nail tip and then 10 µL of spore suspension of *B. cinerea* was inoculated into the wound created by metal tip.

4.7.1.5 Mycofumigation

Volatile producing #16AMLWLS was placed along with the wounded fruit inside a 11×20 cm box. The boxes were covered and placed at 26 °C for next 7-10 days. Mycofumigation potential of #16AMLWLS was checked after 7 days by comparing the fruit with infections in test box with infected grapes in the control box. Control boxes consist of infected fruits but with no fumigant. The experiment was performed in triplicates with 15 grapes in each box.

Similar procedure was adopted to evaluate the mycofumigation potential of #130 TMDSTYEL (Mercier & Jimenez, 2004).

4.7.2 Control of fungal (*Botrytis cinerea*) decay of Strawberries by #16AMLWLS, #130 TMDSTYEL

4.7.2.1 Preparation of *Muscodor* inoculum

#16AMLWLS**,** #130 TMDSTYEL were individually grown on 35 mm PDA plate under appropriate nutrient and temperature conditions.

4.7.2.2 Processing of test fruits

Healthy strawberries *(Fragaria vesca)* were purchased from the local market (Easy day, Wall Mart India) of Patiala, Punjab. Strawberries were free from any kind of visible wounds (to reduce obtrusion from innate infections). Strawberries were washed under running tap water for 10 mins followed by washing with 10% formaldehyde. Finally the fruits were washed with sterile distilled water and to remove interference of pungent smell of formaldehyde. Then fruits were parched over blotting sheet aseptically and placed in 90 mm petriplate to immobilize fruit and subsequently placed in 11 ×20 cm plastic boxes.

4.7.2.3 Preparation of conidial suspension of *Botrytis cinerea*

Conidial suspension of *Botrytis cinerea* was prepared using the same procedure described in section 4.6.1.3 (Mercier & Jimenez, 2004).

4.7.1.4 Infection of *Botrytis cinerea*

Fruits were infected with *B. cinerea* by creating cut on one part of fruit at meridian with a 4 mm metal nail tip and then 10 µL of spore suspension of *B. cinerea* was inoculated into the wound created by metal tip.

4.7.2.5 Mycofumigation

Fumigation with *Muscodor* #16AMLWLS was accomplished by placing the volatile producing actively growing *Muscodor* isolate (#16 AMLWLS) inside a 11 × 20 cm box along with infected strawberries. The Muscodor isolate and Strawberries were kept 10 cm apart. Control box was devoid of *Muscodor* isolate. The boxes were kept closed with a tight fitting and incubated at 26 °C for next 7-8 days. Fumigation potential of #16 AMLWLS was checked after 7 days by counting infected strawberries in test boxes with control boxes. The experiment was performed in triplicates with 15 grapes in each box.

Similar procedure was adopted to evaluate the mycofumigation potential of #130 TMDSTYEL against *B. cinerea* infection (Mercier and Jimenez, 2004).

4.7.3. Control of fungal (*Botrytis cinerea*) decay of apples by *Muscodor* #16AMLWLS and #130 TMDSTYEL grown on wheat grains

4.7.3.1 *Muscodor* sp. formulation on wheat grains

A two litre flask containing 150 g of wheat and 250 ml of distilled water was autoclaved twice for 30 min on 2 consecutive days (Mercier and Jimenez, 2004). Five agar plugs (10 mm diameter) of test *Muscodor* isolate were transferred into the flasks containing autoclaved wheat grains. The flask was incubated at 23 °C for 10-15 days. The colonised wheat grains were further tested for their mycofumigation potential.

4.7.3.2 Processing of test fruits

Fresh and healthy apples were purchased from the local market (Easy day, Wall Mart India) of Patiala, Punjab. Apples were free from any kind of visible wounds or infection. Apples were washed under running tap water for 10 mins followed by washing with 10% formaldehyde. Finally the fruits were washed with sterile distilled water and to remove interference of pungent smell of formaldehyde. Then fruits were placed over blotting sheet over the laminar hood and then placed over a 90 mm petri plate to immobilize and subsequently placed in 11 × 20 cm plastic boxes.

4.7.3.3 Preparation of conidial suspension of *Botrytis cinerea*

Conidial suspension of *B. Cinerea* was prepared using the same procedure described in section 4.6.1.3 (Mercier and Jimenez, 2004).

4.7.3.4 Infection of *Botrytis cinerea*

Botrytis cinerea (10 µl) was inoculated at the wound site of apple created using a meridian with a 4 mm metal nail tip.

4.7.3.5 Mycofumigation: Fumigation was done by adding a known amount of colonised wheat grain (12.5 g or 25 g) of #16AMLWLS in 35 mm petridish. He petri plate is then placed nearby wounded fruit such that fumigant is never in direct contact of apple. The boxes were then closed with a tight fitting lid .Control boxes consisted of infected fruits but with no fumigant. The boxes are kept at 26 °C for 7-10 days. Effect of mycofumigation was checked after 7 days by counting uninfected apples in the test box with subsequent comparison with the control box. This experiment was conducted with 2 apples in each box.

Mycofumigation potential of #130 TMDSTYEL against *B. Cinerea* infection was also evaluated following the same procedure.

4.7.4 Control of fungal (*Botrytis cinerea*) decay of apples by biofumigant fungus #16AMLWLS, #130 TMDSTYEL and #1CCSTITD grown on rye grains:

4.7.4.1 *Muscodor* sp. formulation on rye grains

150 g of rye grain was placed in a 2l flask with 250 ml of water and autoclaved twice for 30 min on 2 consecutive days for production of rye grain culture (Mercier & Jimenez, 2004). 5 agar plugs (10 mm diameter) or 25 ml of a liquid mycelial suspension of fresh *Muscodor* isolate was transferred into 2l flasks containing autoclaved rye grains. In case of liquid mycelia, suspension was grown on autoclaved rye grain by adding solid culture to a 1l flask containing 100 ml of potato dextrose broth and placed on a rotary shaker. The colonized rye grain culture was ready to use in 10–14 days at 26 °C,

Processing of apples, conidial formulation and infection of *B. cinerea* was done as described earlier in section 4.7.3.2.

4.7.4.5 Mycofumigation: Fumigation was done by adding a measured amount of colonised rye grain (12.5 g, 25 g, 50g) of *Muscodor* #16AMLWLS, in 35 mm petridish and then placed nearby the wounded site of the apple. The boxes were then closed and incubated at 26 °C for next 7-10 days. Control boxes consisted of infected fruits but with no fumigant. Effect of mycofumigation was evaluated after 7 days of incubation by counting the apples with infections in test boxes to that of infected apples in the control box. This experiment was conducted with three apples in each box.

Mycofumigation potential of #130 TMDSTYEL and #1 CCSTITD against *B. cinerea* infection was also evaluated following the same procedure.

4.7.5 Control of fungal (*Botrytis cinerea*) decay of grapes by biofumigant fungus #1CCSTITD grown on rye grains:

Rye grain formulation, processing and infection of fruits were done as described in the previous section 4.7.4.1.

Mycofumigation: Measured amount of rye grain inoculum (12.5-25 g) of #1CCSTITD was placed in a small perti pate and kept nearby the wounded fruit. The boxes were then closed with a tight fitting lid and kept at 26 °C for 7-10 days. Control boxes contain infected fruits and were devoid of *Muscodor* isolate. Effect of mycofumigation was analysed after 7 days by counting fruit with infections in test with infected fruits in the control box. This experiment was conducted in triplicates with 15 grapes in each petri plate.

4.8 SPME-GC/MS analysis Volatile produced by #16 AMLWLS

Solid phase microextraction (SPME) syringe having a stable flex fiber made up of 50/30 divinylbenzene/carboxen on polydimethylsiloxane (Supelco, Sigma Aldrich) was used to trap the VOCs produced by a 10 day old culture of *#16* AMLWLS following the method of (Ezra *et al*.,2004). The fibre was exposed for 45 min by placing the SPME syringe through a small bore made using sterile needle over the headspace of culture in the petri dish. Subsequently the fibre was injected for 30 s in the Shimadzu QP 2010 plus Gas chromatograph with thermal desorption system TD 20. An RTX column (diphenyl 95%, dimethyl polysiloxane 5%) with 30 m x 0.25 mm ID and 0.25 mm DF was used for separating of the fungal volatiles. The column was programmed at 100 °C for 2 min and the temperature was then raised to 250 °C for 2 min and then finally to 300 °C for 13 min. The carrier gas was helium and the initial column head pressure was 94.4 KPa. Data acquisition and processing was done on GC-MS solution software. The compounds obtained after

GC/MS analysis was then subtracted from the control plate consisting only PDA medium. The obtained compounds were then tentatively identified based on their high quality matching (above 70% similarity) with database of National institute of Standard and Technology compounds (NIST05) and compared with all reported species of *Muscodor* to date (Ezra *et al*, 2004; Kudalkar *et al*, 2012)

Chapter 5

Results &Discussions

5.1 Re-culturing and storage of fungal culture

Eight Indian *Muscodor* species were maintained as pure culture over PDA slants supplemented with 10% glycerol (Table no.1, FIG no.4). *Muscodor* isolates were viable for period of six months over PDA slants. Muscodor species under study are listed below in Table no 4.

Culture Code	Plant part	Host plant	Location
# 2CCSTITD (*M. tigerii*)	Stem	*Cinnamomum camphora*	Tiger hills, Darjeeling, West Bengal
#1CCSTITD[b] (*M. darjeelingenis*)	Stem	*Cinnamomum camphora*	Tiger hills, Darjeeling, West Bengal
#6CCSTITD[c] (*M. ghoomensis*)	Stem internal tissue	*Cinnamomum camphora*	Darjeeling
#16AMLWS[d] (*M. kashayam*)	Leaf	*Aegle marmelos*	Wayanand Wildlife sanctuary, Kerala
#6(b)CCSTID[e] (*M. indica*)	Stem internal tissue	*Cinnamomum camphora*	Darjeeling, West Bengal
#6610CCSTID[f] (*M. strobelli*)	Stem internal tissue	*Cinnamomum zeylanicium*	BRT wildlife sanctuary, Karnataka

#1639CCSTID[g] (*M. camphora*)	Stem internal tissue	*Cinnamomum camphora*	Darjeeling, West Bengal
#130TMDSTYEL[h]	Stem	*Tabernaemontana divaricata*	Kerala

Table no.4: Indian *Muscodor* isolates used in study

[a,b,] Meshram *et al*., 2014; [d,f] Saxena *et al*., 2014; [ceg] (in press); [h] (unpublished).

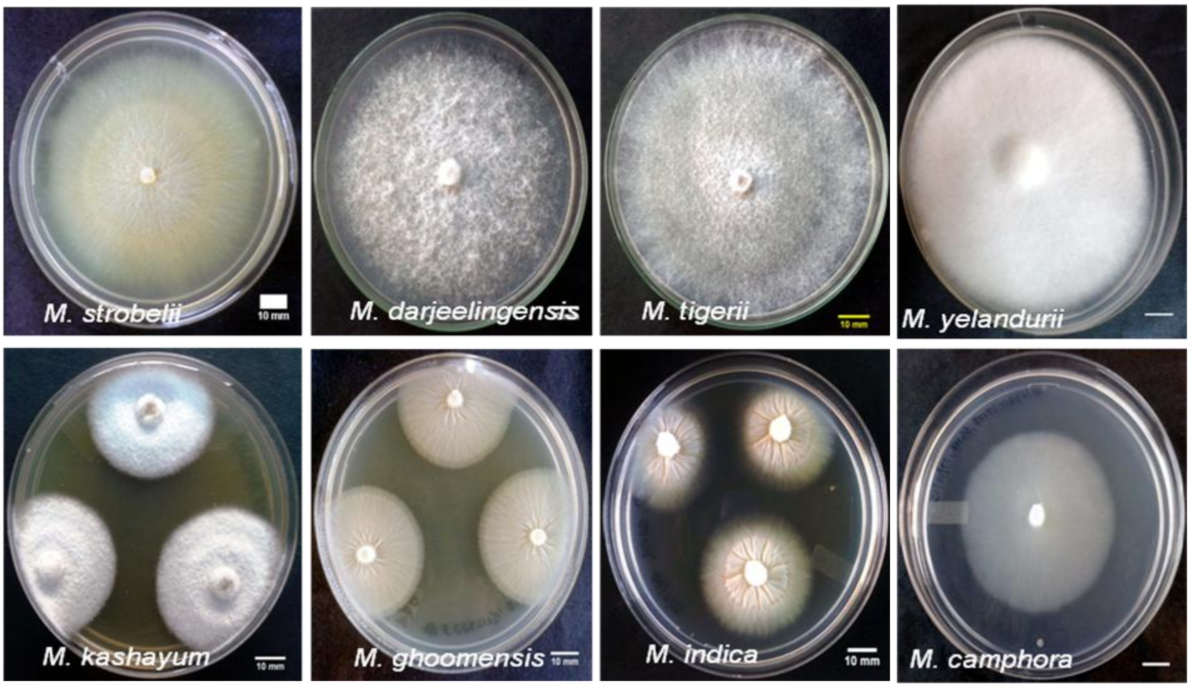


FIG 4: Morphological features of *Muscodor* species on PDA plates.

5.2 Activation of test-pathogens

Botrytis cinerea (MTCC 359), obtained from MTCC, IMTECH, Chandigarh was successfully recultured onto PDA plates & maintained for a longer period over PDA slants containing 10% glycerol.

5.3 Temperature Optimisation of *Muscodor* isolates

Temperature-dependence of growth is a very important factor for the production of volatile organic compounds. Colony growth is directly proportional to the VOC production. Based on observance of this parameters it was found that # 2CCSTITD and #1639 CCSTITD grew best at 28° C whereas the optimum temperature for #130 TMDSTYEL, #1CCSTITD, #6 CCSTITD and #6(b) CCSTITD was 23°C. Other *Muscodor* isolates like #6610 CZSTITBRT and #16 AMLWLS grew best at 25°C, respectively. Production of volatile organic compounds decreased significantly when temperature rose above 28 °C. *Muscodor* isolates did not grow above 30 °C.. Colony diameter of each isolate differed at different temperature (Graph 1). All the *Muscodor* isolates produced volatiles in different proportions but *M. kashayum* and *M. yelandurii* emanated volatiles with strong pungent smell .*M. tigerii* and *M. darjeelingensis* were fast growing in contrast to *M. indica*, *M. ghoomensis* and *M. strobelii* which were slow growing. However *M. albus* and *Muscodor* sp.1, in contrast, displayed a slightly higher optimum temperature than these Indian isolates . Also their colony diameter was larger, and they were able to grow at 30° C (Zhang *et al* ,2010).

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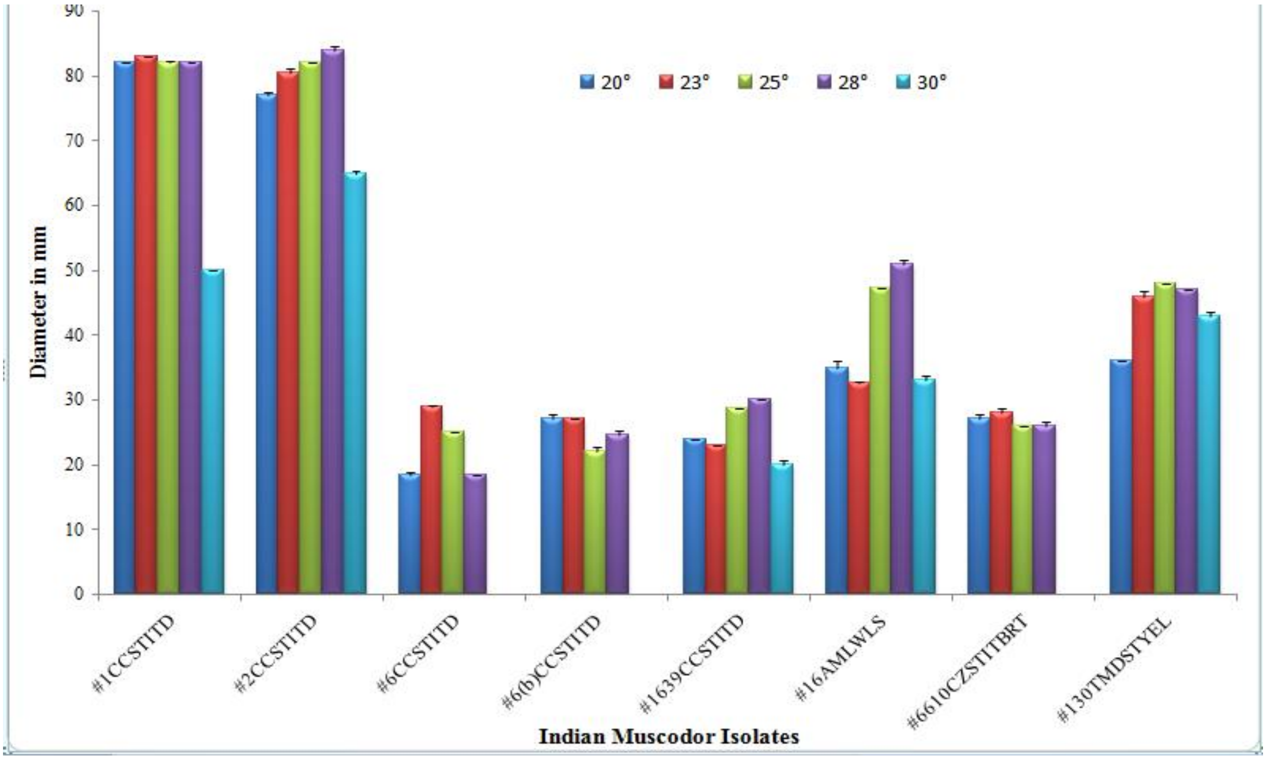


GRAPH 1: Colony diameter of Indian *Muscodor* isolates at different temperatures

5.3.1 Temperature Optimisation of *Muscodor* #2CCSTITD

#2 CCSTITD was fast growing *Muscodor* species which covers 90 mm perti plate in 6-7 days. It formed white mycelium that turned brown color after 15 days. The optimum temperature for maximum volatile production was 28 °C (Table 5; FIG.5)

Time	Temperature									
	20 °C		23 °C		25 °C		28 °C		30 °C	
	Dia	smell	Dia	smell	Dia	Smell	Dia	smell	Dia	Smell
24	0	-	0	-	0	-	0	-	0	-
48	13	-	6	-	17	-	1	-	3	-
72	35	-	32	-	42	-	43	-	2	-
96	42	-	39.5	-	57	-	62	++	2	-
120	68	+	63	+	62	+	83	+++	3.5	-
144	77	+	80.5	+	82	+	84	+++	6.5	-

Table 5: Colony diameter and odour intensity of #2CCSTITD at different temperatures

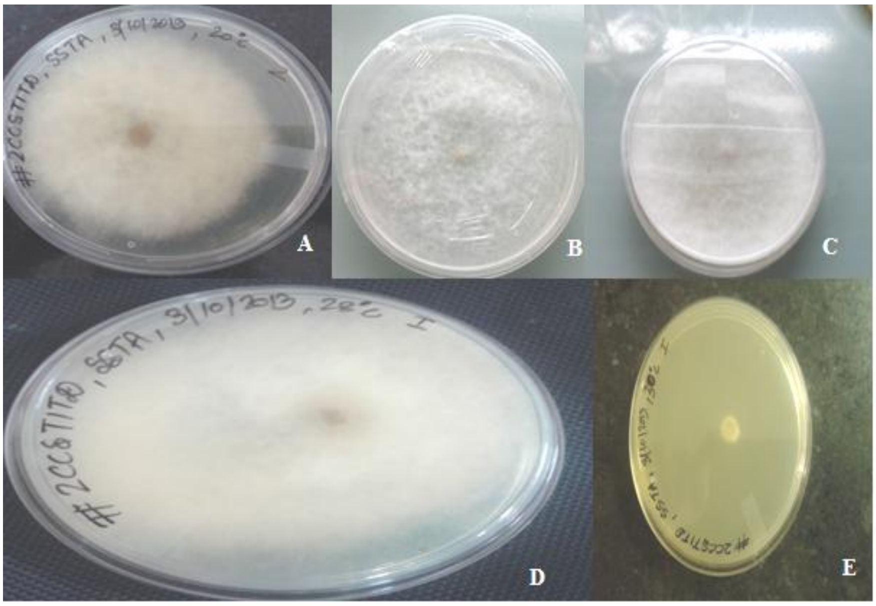


FIG.5: Colony diameter of *Muscodor* #2CCSTITD at varying temperatures A) 20° C B) 23°C C)25°C D) 28°C E) 30°C

5.3.2: Temperature Optimisation of *Muscodor* #1CCSTITD

Based on colony diameter and odour the optimum temperature for #1CCSTITD was 23℃ (FIG.6). It formed 83 mm colony diameter with white sterile mycelium on 6 days of incubation.

Hours	Temperature									
	20°C		23 °C		25 °C		28 °C		30°C	
	Dia	Odour	dia	Odour	Dia	Odour	dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	19	-	15	-	16	-	17	-	3	-
72	39	-	34	-	49	-	38	-	4	-
96	72	-	72	+	82	-	82	+	4.2	-
120	81	-	83	++	82	+	82	++	4.5	-
144	82	-	83	+++	82	+	82	++	5.0	-

Table6: Colony diameter and odour intensity of #1CCSTITD at different temperatures

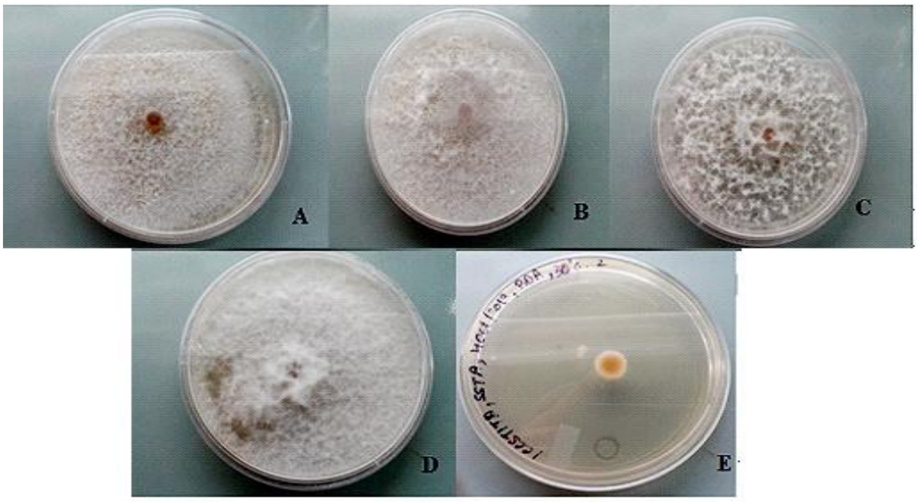


FIG6: Colony diameter of *Muscodor* #1CCSTITD at varying temperatures A) 20°C B) 23°C C)25° C D) 28°C E) 30°C

5.3.3 Temperature Optimisation of *Muscodor* #6 CCSTITD

The optimum temperature for #6 CCSTITD was 23 °C. It is a slow growing *Muscodor* species which formed pale yellow colored colony. The intensity of VOC production was less in comparison to other *Muscodor* species.

Time in hr.	Temperature									
	20°C		23C		25°C		28°C		30°C	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	0	-	18	-	17	-	0	-	0	-
72	8	-	13	-	13	-	9	-	0	-
96	13.5	-	15.7	-	14.2	-	12.5	-	0	-
120	16	-	19	+	18	+	14.5	-	0	-
144	18.5	+	29	+++	25	+	18.5	+	0	-

Table7: Colony diameter and odour observations of #6CCSTITD at different temperatures after every 24 hours**.**

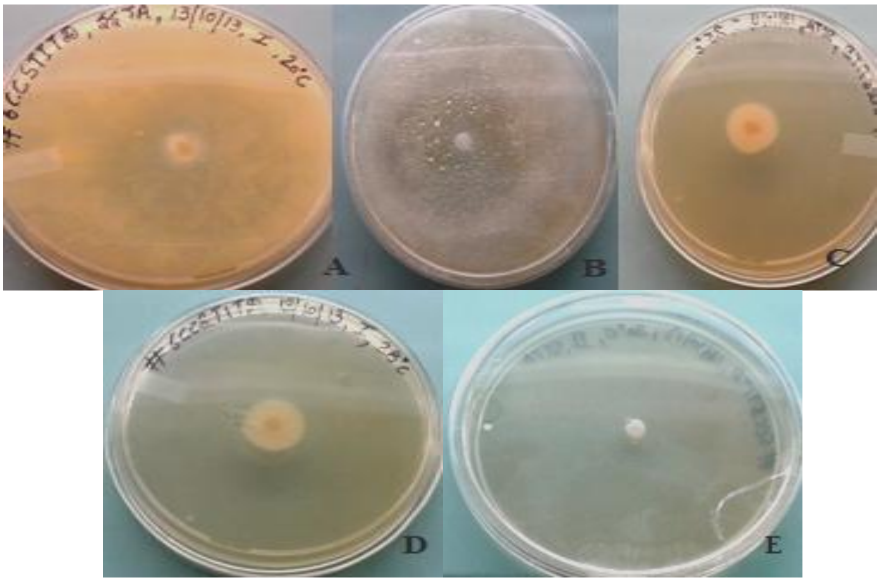


FIG.7: Colony diameter of *Muscodor* #6CCSTITD at various temperatures. A) 20°C B) 23°C C)25° C D)28°C E)30°C

5.3.4) Temperature Optimisation of *Muscodor* #6(b) CCSTITD

The optimum temperature for #6(b) CCSTITD was 23 °C. It is a slow growing *Muscodor* species which formed pale yellow colored colony. The intensity of VOC production was less in comparison to other *Muscodor* species.

Time in hours	Temperature									
	25 °C		23 °C		25 °C		28 °C		30 °C	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	9.7	-	13.5	-	13.2	-	8.7	-	0	-
72	16.7	-	18.5	-	15.2	-	16	-	0	-
96	19	-	19	-	17	-	17	-	0	-
120	22.2	-	22.5	+	18.7	-	20.5	-	0	-
144	27	-	27.2	++	22	+	24.5	+	0	-

Table8: Colony diameter and odour observations of #6(b) CCSTITD at different temperatures after every 24 hours.

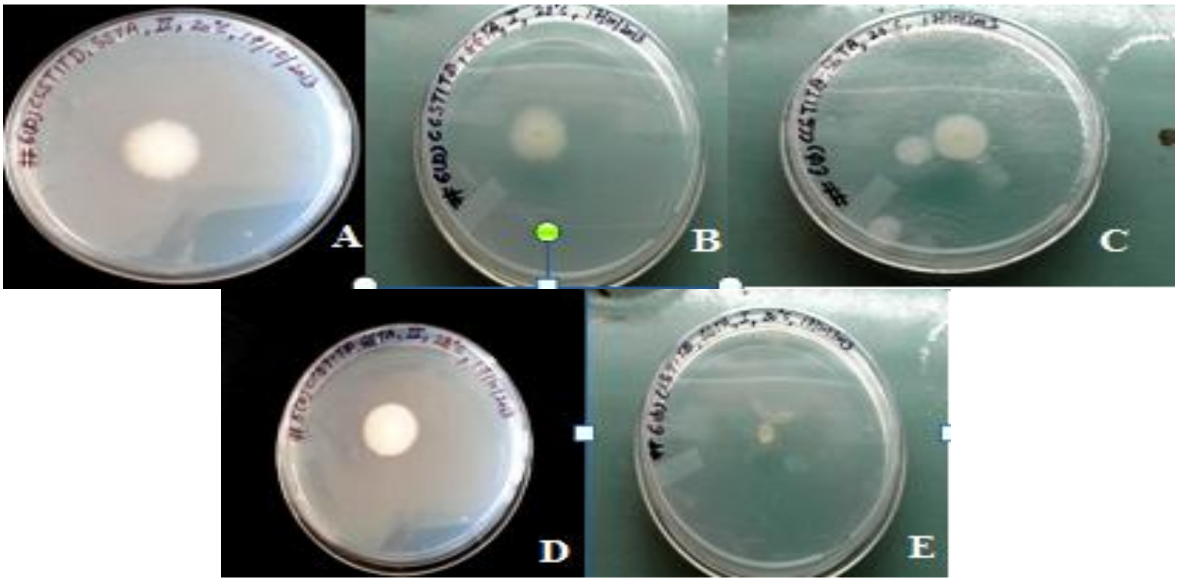


FIG.8: Colony diameter of *Muscodor* #6(b) CCSTITD at various temperatures. A) 20°C B) 23°C C)25° C D)28°C E)30°C.

5.3.5 Temperature Optimisation of #16AMLWLS

#16 AMLWLS produces whitish sterile colonies over PDA medium. It produces volatiles with pungent smell. The optimum temperature for its growth was 25° C. It formed a 47 mm colony at 25 °C (Table7, FIG.7).

Time in hour	Temperature									
	20°C		23°C		25°C		28°C		30°C	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	1	-	11.2	-	12.5	-	14.5	-	8.6	-
72	21.5	-	21.7	-	22	+	23.75	-	13	-
96	29	-	30.5	+	37	+	40.5	+	14.5	-
120	30	+	32	+	43	+++	40.7	++	15	-
144	35	+	32.5	+	47	+++	41	++	21	-

Table 9: Colony diameter and odour observations of #16AMLWLS at different temperatures after every 24 hours.

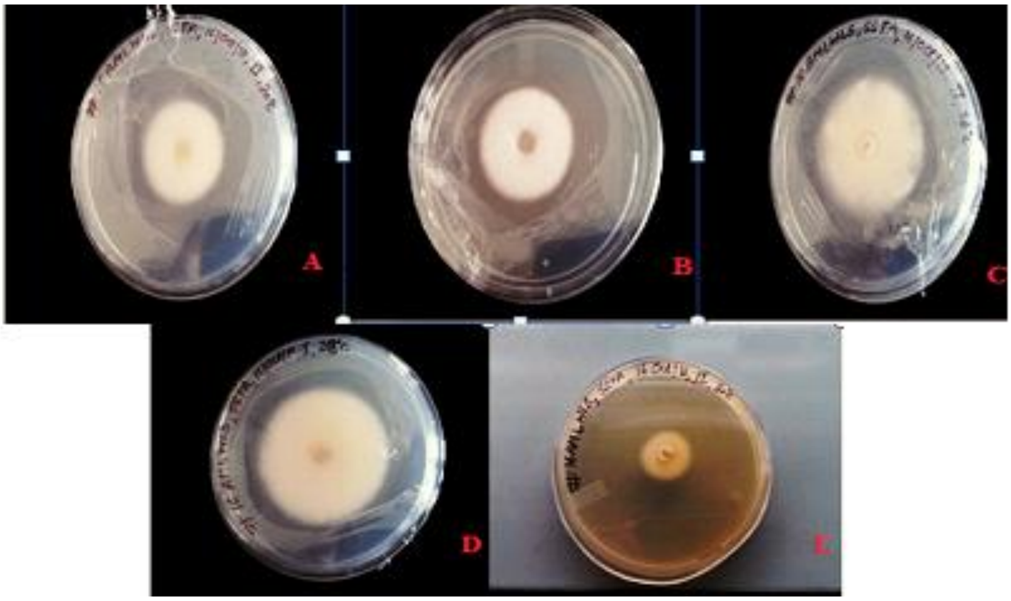


FIG.9: Colony diameter of *Muscodor* #16AMLWLS at varying temperatures. A) 20°C B) 23°C C) 25° C D)28°C E)30° C

5.3.6: Temperature Optimisation of #6610 CZSTITBRT

#6610 CZSTITBRT exhibited maximum volatile production at 23° C. It formed a slow growing pale colored colony over PDA medium. (Table10, FIG.10).

Time	Temperature									
	20°		23°		25°		28°		30°	
In hrs	Dia	Odour	Dia	Odour	dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	9.5	-	10	-	10	-	10	-	0	-
72	16	-	16.2	-	15.5	+	14.5	-	0	-
96	19.2	-	20.2	+	18.7	+	18.2	+	0	-
120	24.2	+	25.2	+	22.5	+++	21.7	++	0	-
144	27	+	28	+	26.3	+++	26	++	0	-

Table10: Colony diameter and odour observations of #6610CCSTITD at different temperatures after every 24 hours.

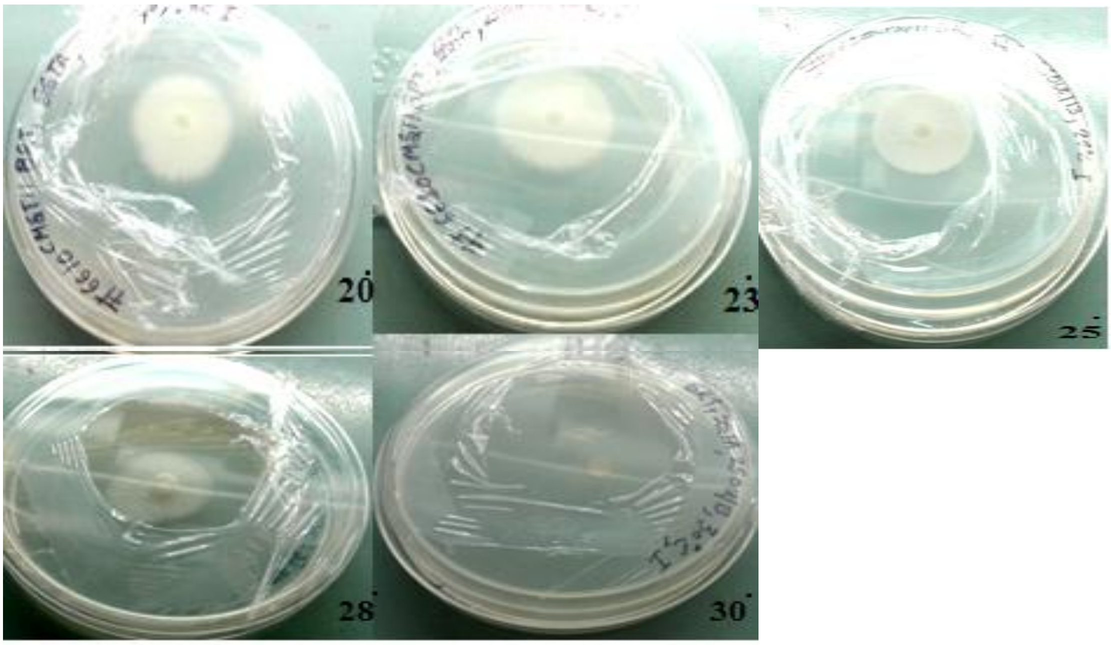


FIG.10: Colony diameter of *Muscodor* #6610CCSTITD at varying temperatures A) 20°C B) 23°C, C) 25 °C D) 28°C E)30°C

5.3.7: Temperature Optimisation of #1639CCSTITD :

Based on colony diameter and odour the optimum temperature for #1639CCSTITD was 28 ℃ where it produces maximum volatiles (FIG.11).

Time in hrs	Temperature									
	25 °C		23 °C		25 °C		28 °C		30 °C	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	9.5	-	10	-	10	-	10	-	0	-
72	16	-	16.2	-	15.5	+	14.5	-	0	-
96	19.2	-	20.2	+	18.7	+	18.2	+	0	-
120	24.2	+	25.2	+	22.5	+	21.7	++	0	-
144	27	+	28	+	26.3	+	26	+++	0	-

Table 11: Colony diameter and odour observations of #1639CCSTITD at different temperature after every 24 hour

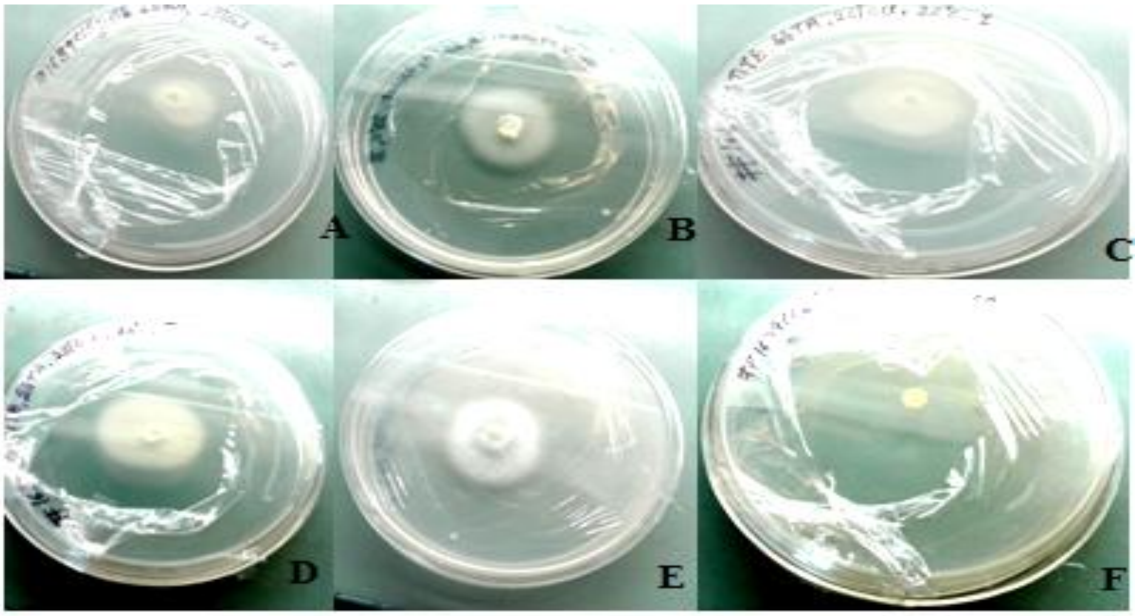


FIG.11: Colony diameter of *Muscodor* #1639CCSTITDat varying temperatures. A) 20˚C B) 23˚C C) 25˚C D)28˚C E)28˚C F)30˚C

5.3.10: Temperature Optimisation of #130TMDSTYEL

This *Muscodor* produce maximum colony diameter (64mm) at 30° C but maximum volatiles is produced at 23° C. This is an exception in the optimization study. (Table12). (FIG.8)

Time in hr	Temperature									
	20°		23°		25°		28°		30°	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	0	-	5	-	7	-	7	-	8	-
48	11	-	12.5	-	17	-	12.5	-	22	-
72	17	-	21.7	-	22	+	20	-	32	-
96	22	-	31	+	36	+	34	+	51	-
120	26	+	34	+	43	+++	40.7	++	54	-
144	30	+	43	+	50	+++	52	++	64	-

Table 12: Colony diameter and odour observations of #130TMDSTYEL at different temperatures after every 24 hours

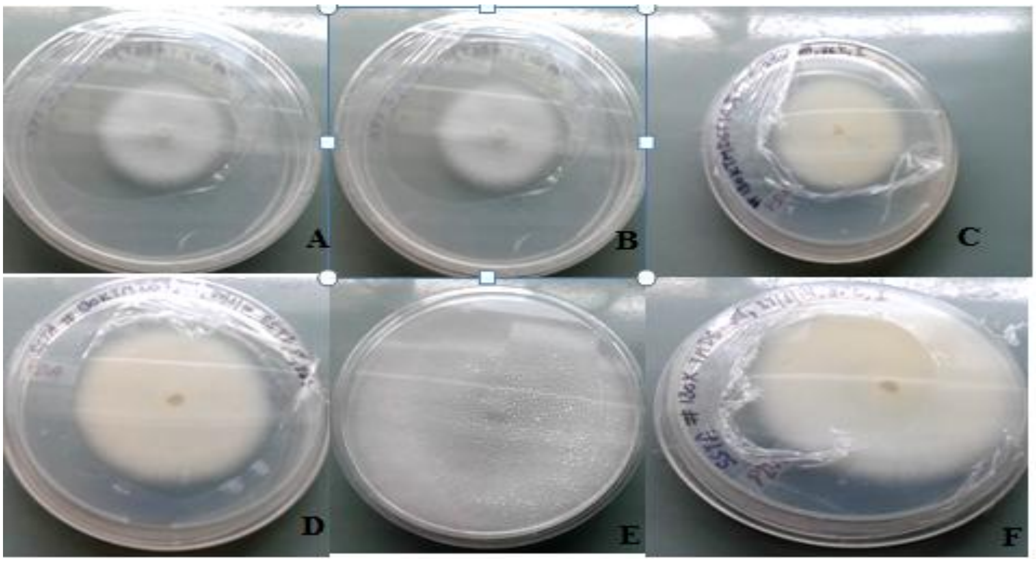
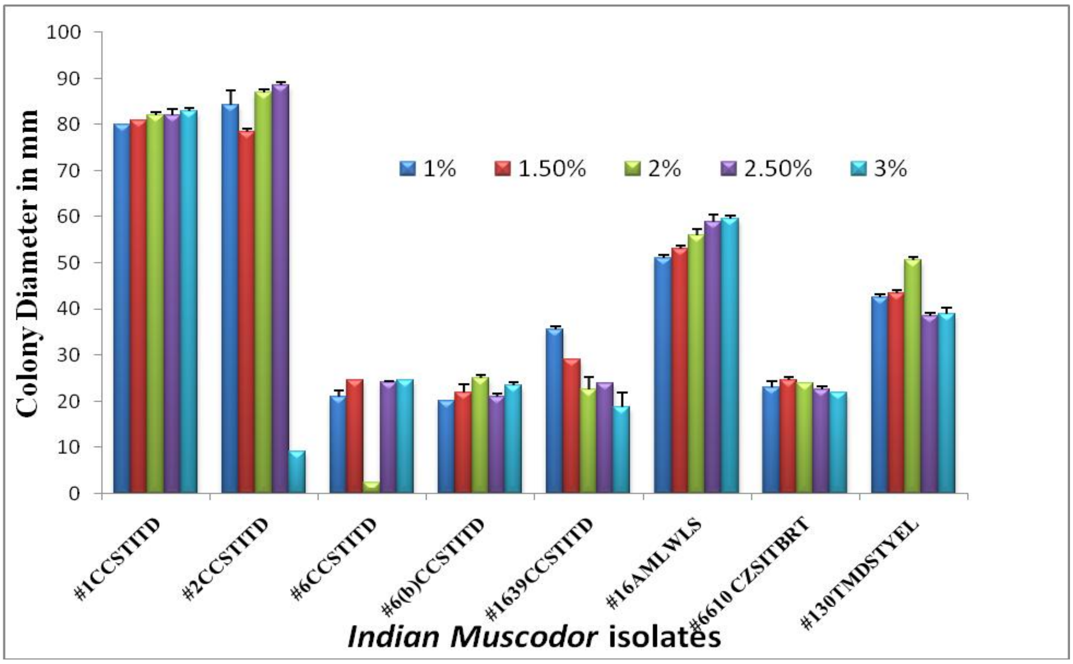


FIG.12: Colony diameter of *Muscodor* #130TMDSTYEL at varying temperatures. A) 20°C B) 23°C C) 25 °C D)28°C E)23°C F)30°C

5.4 Nutrient Optimisation of *Muscodor* isolates

Nutrient dependence of growth is a crucial factor for the production of volatile organic compounds. Colony growth is directly related to VOC production. 6CCSTITD, #6(b) CCSTITD, 1639 CCSTITD and #130 TMDSTYEL grew best at 2% dextrose concentration of PDA whereas the optimum nutrition for #16 AMLWLS, #1CCSTITD ,#2CCSTITD was 3% dextrose. Other *Muscodor* isolates like #6610CZSTITBRT produced maximum volatiles at1.5%. Production of volatile organic compounds decreased significantly when dextrose concentration rose above 4.5%. *Muscodor* isolates did not grow above 5%. However, *M. darjeelingensis* (#1CCSTITD) displayed a slightly higher optimum concentration (4.5%) than other isolates. Colony diameter of each isolate differed at different dextrose concentration (Graph 2). *M. tigerii* and *M. darjeelingensis* were fast growing in contrast to *M. indica*, *M. ghoomensis* and *M. strobelii* which were slow growing. All the *Muscodor* isolates produced volatiles in different proportions but *M. kashayum* and *M. yelandurii* emanated volatiles with strong pungent smell.



Graph2: Colony diameter of Indian *Muscodor* isolates at different dextrose concentration.

5.4.1 Nutrient Optimisation of *Muscodor* #2CCSTITD

#2CCSTITD was fast growing *Muscodor* species which covers 90 mm perti plate in 6-7 days. It formed white mycelium that turned brown color after 15 days. The optimum dextrose concentration for maximum volatile production was 3% (Table12; FIG.12)

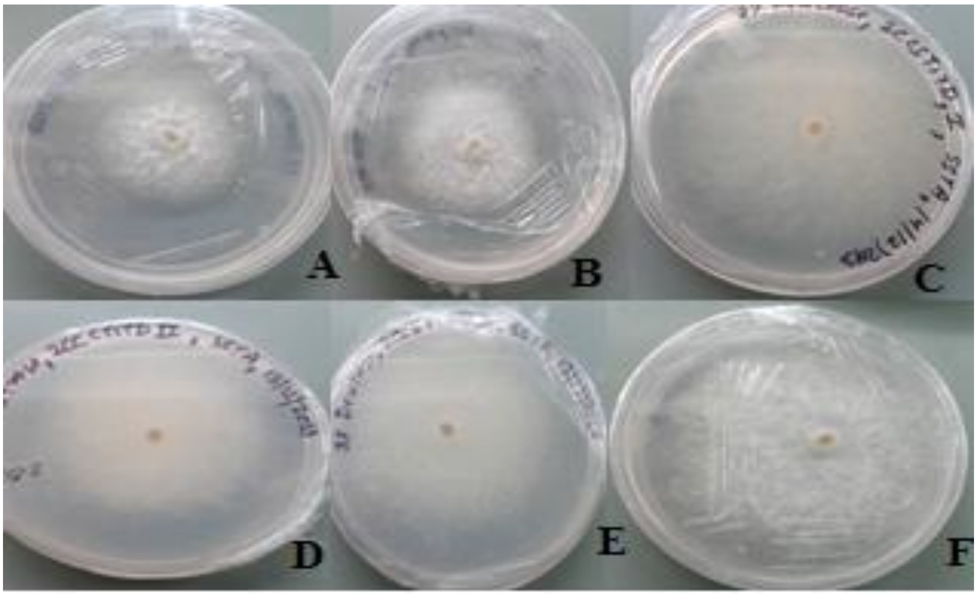


FIG.13: Colony diameter of *Muscodor* #2CCSTITD at varying temperatures A) 1% B) 1.5% C)2% D) 2.5% E) 3%.

Time	Dextrose Concentration									
	1%		1.5%		2%		2.5%		3%	
	Dia	smell	Dia	smell	Dia	smell	Dia	smell	Dia	Smell
24	0	-	0	-	0	-	0	-	0	-
48	31	-	32.5	-	42	-	48.7	-	51	-
72	48	-	49.5	-	69	-	74	-	81	-
96	64.5	-	65	-	90	-	90	-	90	-
120	84	-	78	-	90	-	90	-	90	++
144	90	-	87	-	90	+	90	+	90	+++

Table 13: Colony diameter and odour observations of #2CCSTITD at different dextrose concentration after every 24 hours

5.4.2 Nutrient Optimisation of *Muscodor* #1CCSTITD

Based on colony diameter and odour the optimum dextrose concentration for #1CCSTITD was 3% **(**Table13; FIG13). It formed a 90 mm colony diameter with white sterile mycelium on 6 days of incubation.

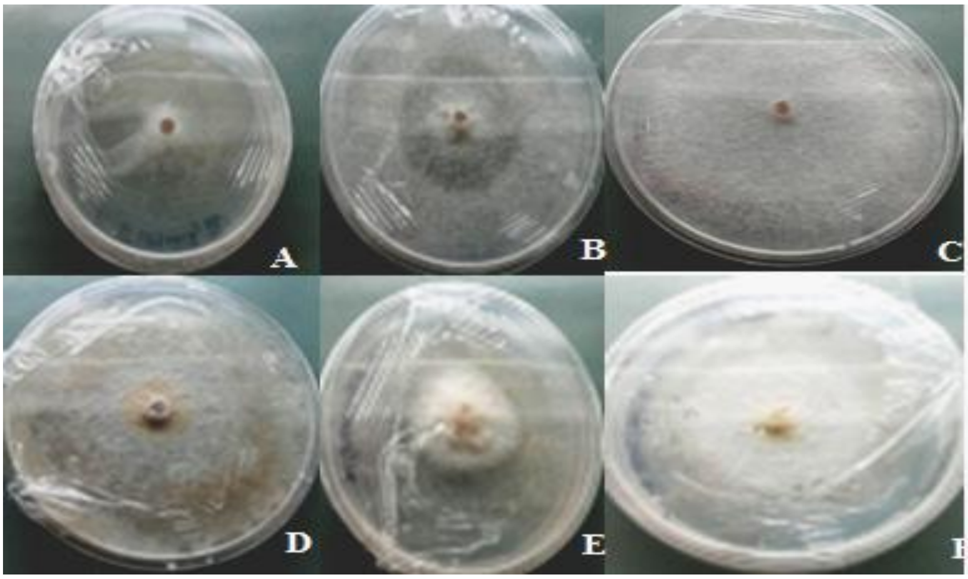


FIG.14: Colony diameter of *Muscodor* #1CCSTITD at varying temperatures A) 1% B) 1.5% C)2% D) 2.5% E) 3%.

Time	Dextrose Concentration									
	1%		1.5%		2%		2.5%		3%	
	Dia	smell	Dia	smell	Dia	smell	Dia	smell	Dia	Smell
24	0	-	0	-	0	-	0	-	0	-
48	17.5	-	15.5	-	22	-	13.7	-	20.7	-
72	45	-	45	-	54.2	-	50.7	-	56.5	-
96	52	-	49	-	56	-	46	-	58	-
120	64.5	-	60	-	65	+	57	-	74	++
144	90	-	90	+	90	+	90	+	90	+++

Table 15: Colony diameter and odour observations of #1CCSTITD at different dextrose concentrations after every 24 hours

5.3.3 Nutrient Optimisation of *Muscodor* #6 CCSTITD

The optimum dextrose concentration for #6 CCSTITD was 2% (Table14; FIG14). It is a slow growing *Muscodor* species which formed pale yellow colored colony. The intensity of VOC production was less in comparison to other *Muscodor* species.

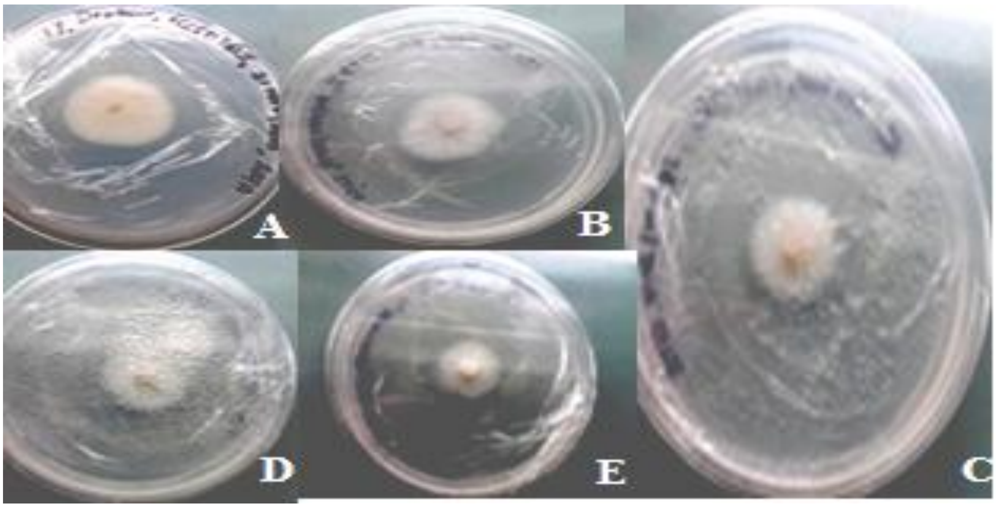


FIG.15: Colony diameter of *Muscodor* #6CCSTITD at various dextrose concentrations. A) 1% B) 1.5% C) 2% D) 2.5% E) 3%

Time in hr.	Dextrose concentration									
	1%		1.5%		2%		2.5%		3%	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	9	-	10	-	9	-	9	-	8	-
72	10.5	-	12	-	12	-	12	-	11.5	-
96	14	-	13	-	15.4	-	15	-	14	-
120	16	-	17	-	18.9	+	18	-	17	-
144	21	-	24	+	28	++	27	+	26.5	-

Table15: Colony diameter and odour observations of #6CCSTITD at different temperatures after every 24 hours

5.3 Nutrient Optimisation of *Muscodor* #6(b) CCSTITD

Based on colony diameter the optimized dextrose concentration for #6(b) CCSTITD was 2% (Table15; FIG15). It formed a 23 mm colony diameter with white sterile mycelium on 6 days of incubation.

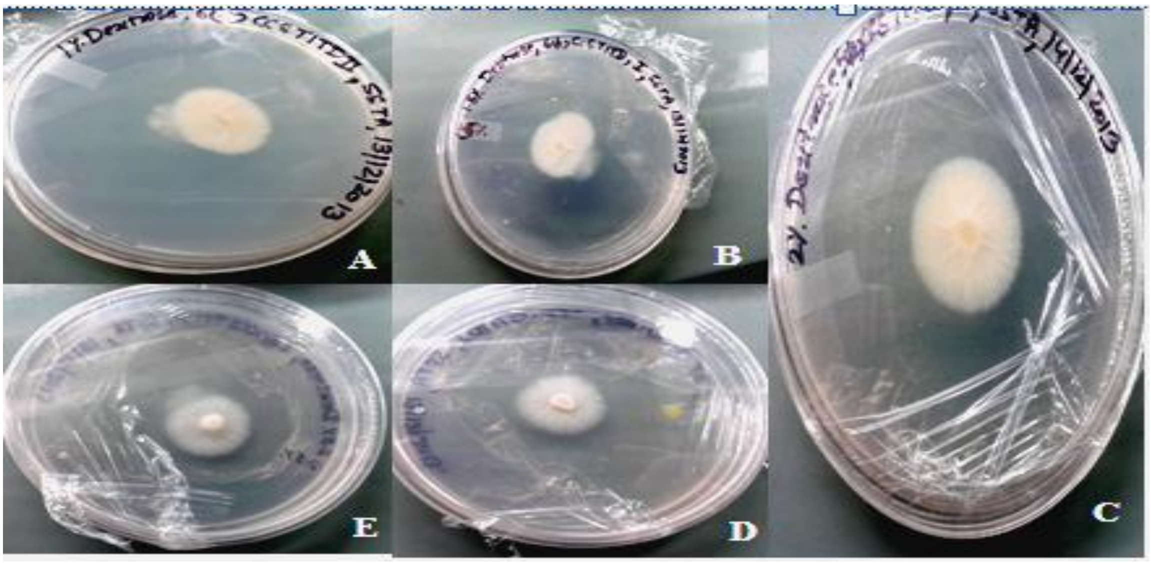


FIG.16: Colony diameter of *Muscodor* #6(b) CCSTITD at various dextrose concentrations.

A) 1% B) 1.5% C) 2% D) 2.5% E) 3%

Time in hours	Dextrose Concentration									
	1%		1.5%		2%		2.5%		3%	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	8.5	-	17.5	-	10.5	-	9.5	-	10	-
72	12.5	-	12.5	-	14.5	-	12	-	12	-
96	16.5	-	16	-	18	-	15.5	-	17	-
120	18.5	-	18	-	19.5	++	19.5	-	21	-
144	20.7	-	22.2	+	25.5	+++	21.5	+	23	-

Table16: Colony diameter and odour observations of #6(b) CCSTITD at different concentration after every 24 hours

5.3.5 Nutrient Optimisation of #16AMLWLS

#16 AMLWLS produces whitish sterile colonies over PDA medium. It produces volatiles with pungent smell. The optimum dextrose concentration for its growth was 3%. It formed a 60 mm colony at 3% (Table16; FIG.16).

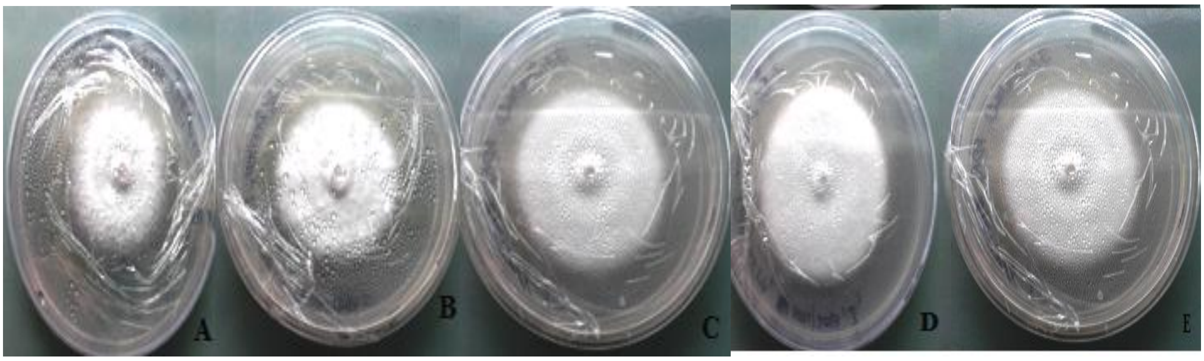


FIG.17: Colony diameter of *Muscodor* #16AMLWLS at various dextrose concentrations. A) 1% B) 1.5% C) 2% D) 2.5% E) 3%

Time in hour	Dextrose Concentration									
	1%		1.5%		2%		2.5%		3%	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	2	-	10	-	12.5	-	12	-	16	-
72	29	-	30	-	35	-	30	-	35	-
96	35	-	35	-	40	-	41.5	-	42.5	++
120	47	-	48	-	55	+	53	+	52	+++
144	51	-	53	+	56	+	59.5	+	60	+++

Table 17: Colony diameter and odour observations of #16AMLWLS at different dextrose concentration after every 24 hours

5.3.6 Nutrient optimisation of #6610 CZSTITBRT

#6610 CZSTITBRT exhibited maximum volatile production at 1.5%dextrose concentration It formed a slow growing pale colored colony over PDA medium (Table17; FIG.17)

	Dextrose concentration									
Time	1%		1.5%		2%		2.5%		3%	
In hrs	Dia	Odour	Dia	Odour	dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	9.7	-	9.5	-	9	-	8.5	-	8	-
72	13.2	-	13.5	-	13.2	-	13.2	-	12	-
96	17	-	17.7	-	17.2	-	16.2	-	16	-
120	21	-	19.7	-	21.2	++	20.5	-	20	-
144	23	-	24.5	+	24	++	22.5	+	22	-

Table18: Colony diameter and odour observations of #6610CZSTITBRT at different dextrose concentration after every 24 hours

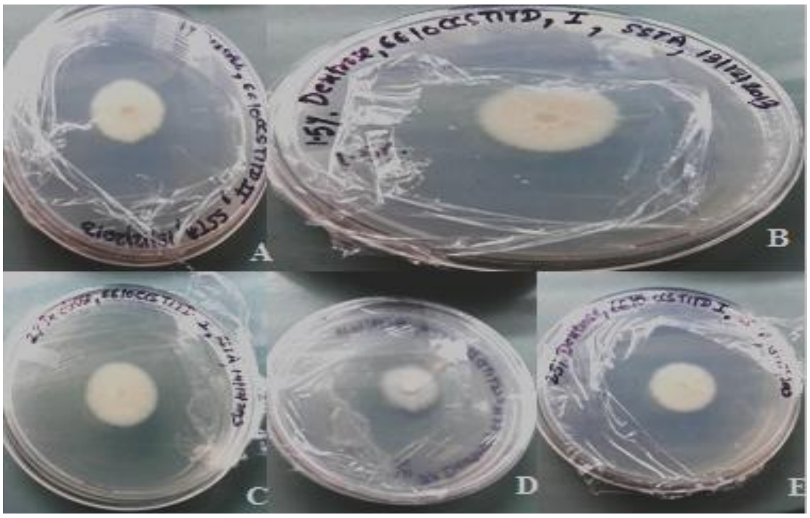


FIG.18: Colony diameter of *Muscodor* #6610CZSTITBRT at various dextrose concentrations. A) 1% B) 1.5% C) 2% D) 2.5% E) 3%

5.4.7 Nutrient Optimisation of #1639CCSTITD

Based on colony diameter the optimum dextrose concentration for #1639CCSTITD was 2%where it produces maximum volatiles (Table18; FIG.18).

Time in hrs	Dextrose Concentration									
	1%		1.5%		2%		2.5%		3%	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	0	-	0	-	0	-	0	-	0	-
48	0	-	0	-	0	-	0	-	0	-
72	9	-	7.2	-	10	-	8	-	8.5	-
96	19	-	15	-	10.7	+	14	-	10	-
120	28.5	-	24	-	18.2	+++	19.7	++	15	-
144	35.5	-	29	+	18.7	+++	24	++	18	-

Table19: Colony diameter and odour observations of #1639CCSTITD at different dextrose concentrations after every 24 hour.

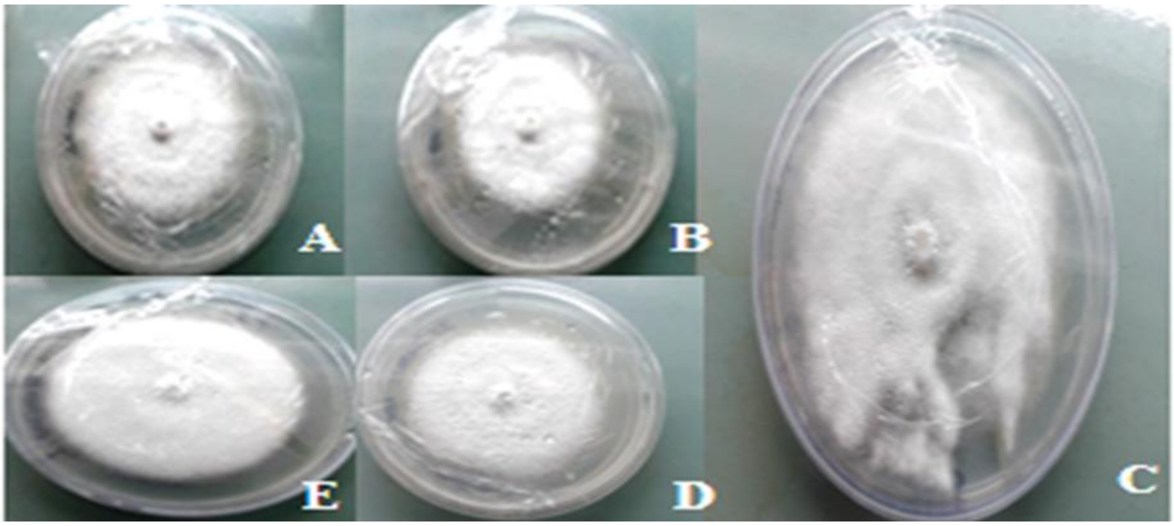


FIG.19: Colony diameter of *Muscodor* #1639CCSTITD at varying dextrose concentration. A) 1% B)1.5% C)2% D) 2.5% E) 3%

5.4.10 Nutrient Optimisation of #130TMDSTYEL

Based on colony diameter and odour optimum dextrose concentration for #130TMDSTYEL has 2% dextrose concentration. It formed a 50 mm colony diameter with white sterile mycelium on 6 days of incubation.

Time in hr	Dextrose Concentration									
	1%		1.5%		2%		2.5%		3%	
	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour	Dia	Odour
24	7	-	6.5	-	7	-	8	-	7.5	-
48	11	-	10	-	17	-	14	-	14.5	-
72	22	-	22	-	22	-	20	-	32	-
96	27	-	27	-	36	+	37	+	28	-
120	37	-	36	-	43	+++	42	++	35	-
144	42	-	44	+	50	+++	49	++	47	-

Table 20: Colony diameter and odour observations of #130TMDSTYEL at different dextrose concentrations after every 24 hours.

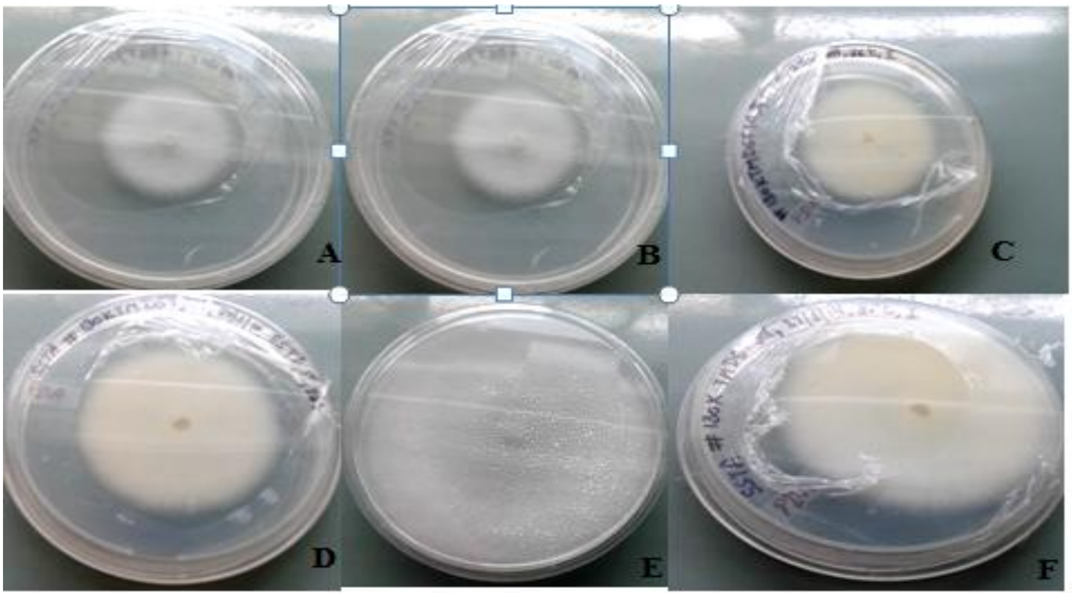


FIG.20: Colony diameter of *Muscodor* #130TMDSTYEL at varying dextrose concentration.

A) 1% B)1.5% C)2% D) 2.5% E) 3%

5.5 VOC stress bioassay

Out of eight *Muscodor* species, two isolates #16 AMLWLS and #130 TMDSTYEL displayed complete inhibition of *B. cinerea* on 3 days exposure. The other isolates like #6610 CZSTITBRT, #1 CCSTITD, #2 CCSTITD, #6 CCSTITD and # 6(b) CCSTITD did not completely kill the test fungus but exhibited slight inhibitory activity which ranges from 4 to 57%. *B. cinerea* was completely resistant to the volatiles produced by #1639 CCSTITD. Based on the above results the two most potential isolate against *B. cinerea* was chosen for further assessment of mycofumigation potential.

Muscodor isolate	Mean diameter in mm		Growth inhibition in %
	Test	Control	
#6610 CZSTITBRT	9 ± 2	21 ± 0. 71	57.1
#16 AMLWLS	0		100
# 130 TMDSTYEL	0		100
#1 CCSTITD	14.25±1.26		32.1
#2 CCSTITD	18.75±1.50		10.7
#6 CCSTITD	11 ±0.57		47.6
#6 (B) CCSTITD	14 ± 2.64		33.3
#1639 CCSTITD	20.13±1.5		4.2

Table 21: Inhibition of *B. cinerea* in VOC stress assay by *Muscodor* isolates**.**

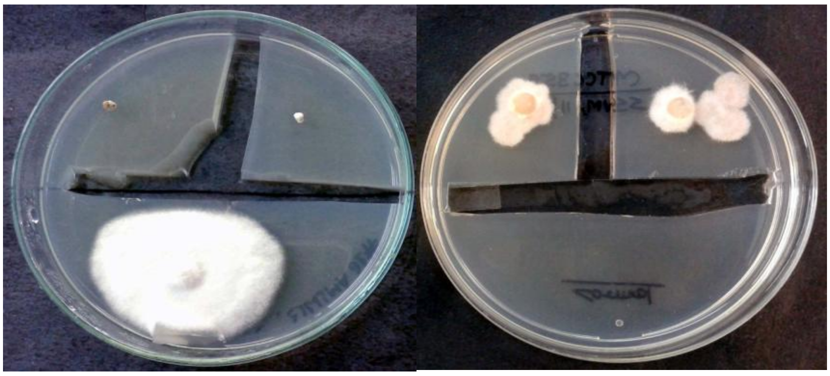


FIG 21 Inhibition of *B. cinerea* (MTCC 359) in VOC stress assay by #16 AMLWLS

5.5 Mycofumigation potential of *Muscodor* to prevent fruit decay

5.5.1 Control of gray mold decay of grapes by #16AMLWLS, #130 TMDSTYEL

Muscodor sp. #16 AMLWLS exhibited excellent mycofumigation potential when tested against *B. cinerea* to control decay of grapes for 7-10 days. Brown colored spots developed over infected grapes in the test box whereas the grapes in control box were fresh and healthy (free from any infection) even after 10 days of incubation. Recovery of the healthy grapes was 100% (FIG.2).

	Percentage infection	
	#16AMLWLS	#130TMDSTYEL
TEST 1	0	0
TEST 2	0	0
TEST 3	0	0
Control	100	100

Table 22: Effect of mycofumigation of two *Muscodor* isolates on gray mold of grapes

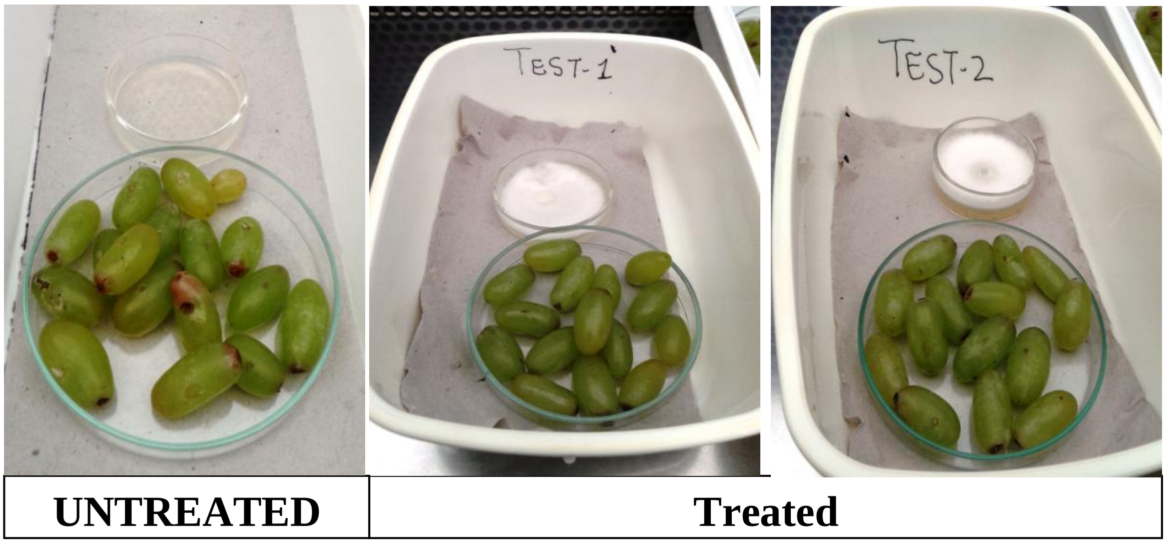


FIG.22: Treated and untreated grapes inside boxes

The volatiles produced by #130TMDSTYEL showed 100% curing of grapes against *Botrytis cinerea* infection. Similar brown spots developed at the wound site in control boxes where as in test boxes the grapes were shielded by the mycofumigation potential of #130 TMDSTYEL (Table 23) Grapes remained uninfected till tenth day of incubation.

FIG 23: Treated grapes by #130TMDSTYEL and untreated grapes in boxes

5.5.2 Control of fungal (*Botrytis cinerea*) decay of Strawberries by biofumigant fu3gus #16AMLWLS, #130 TMDSTYEL

Mycofumigation potential of #16 AMLWLS and #130 TMDSTYEL was also evaluated to check the decay of strawberries infected by *B. cinerea*. #16 AMLWLS provided complete shielding to the strawberries against *B. cinerea* infection in *in vitro* conditions for 7-10 days, win control box strawberries were largely infected by the pathogen. Thus by comparing the two boxes it can be concluded that #16 AMLWLS possess strong mycofumigation potential against *B. cinerea* (FIG.23).

UNTREATED TREATED

FIG. 24: Treated and untreated strawberries inside boxes

Similarly when #130 TMDSTYEL was evaluated for its mycofumigation potential, it was found that strawberries were totaly protected by the volatiles of #130 TMDSTYEL. They remain fresh and healthy for a period of 7-10 days on fumigation with the *Muscodor* species where as complete decay was observed in the strawberries infected with *B. cinerea* and devoid of fumigation (Table.23.)

	Percentage infection	
	#16AMLWLS	#130TMDSTYEL
TEST 1	0	0
TEST 2	0	0
TEST 3	0	0
Control	100	100

Table: 23: Effect of *Muscodor* isolates on gray mold of strawberries

5.5.3 Control of fungal (*Botrytis cinerea*) decay of apples by biofumigant *Muscodor* #16AMLWLS and #130 TMDSTYEL grown on wheat grains

Two different amounts of *Muscodor* colonised wheat grain (12.5 g and 25 g) cultures were tested for its potential to control *B. cinerea* infection in apples. 12.5 g of colonised culture was not able to control the infection caused by *B. cinerea* whereas 25 g of wheat colonised culture completely check the growth of pathogen. The fruits remain uninfected for a period of 7-10 days on fumigation. Thus it can be concluded that at least 25 g of colonised culture is required to the growth of *B. cinerea* (Table 24, FIG.24).

Wheat grain colonised *Muscodor* (g/ container)	Percentage infection	
	#16AMLWLS	#130 TMDSTYEL
12.5 g	100	100

25 g	0	0
Control	100	100

Table24: Effect of Muscodor isolates on grey mold of apples.

Untreated	Colonised *Muscodor g*rain culture 25g

FIG 24: Treated and untreated apples inside boxes.

UNTREATED	**TREATED**

FIG 25: Treated and untreated apples inside boxes.

5.5.4 Control of *Botrytis cinerea* by biofumigant fungus #16AMLWLS, #130 TMDSTYEL grown on rye grains

Three different concentrations of rye grain (12.5 g, 25 g and 50 g) colonised *Muscodor* isolates were evaluated for their mycofumigation potential. When the volatiles of *Muscodor* isolates #16 AMLWLS and #130 TMDSTYEL were exposed to the apples for 7 days it was found that none of the *Muscodor* culture inhibits the growth of *B. cinerea* in apples. Growth in test as well as in control box was almost similar. This might be attributable to substrate and moisture content of the medium. Thus further optimisation is required for evaluating the rye grain colonised *Muscodo*r culture for their mycofumigation efficacy (Table 25).(FIG.25).

Rye grain colonised *Muscodor* (g per container)	Percentage infection	
	#16AMLWLS	#130 TMDSTYEL
12.5 g	100	100
25g	100	100
50g	100	100
Control	100	100

Table 25: Effect of *Muscodor* Isolates on grey mold of apples

FIG.26: Treated and untreated apples by two *Muscodor* isolates inside boxes.

5.6 SPME GC-MS based volatile analysis of #16 AMLWLS

#16 AMLWLS produces a mixture of 23 volatile compounds which were identified by comparing the GC/MS spectra with the authentic standards obtained from commercial sources as well as by organic synthesis (Table 26). Primarily the compounds were identified on the basis of their mass spectral properties when compared to the NIST database. Of all the compounds produced, 3-Cyclohexen-1-ol, 1-(1, 5-dimethyl-4-hexenyl)-4-methyl- was the most abundant compound with percentage peak area of 16.152. Other major VOCs produced by *M. kashayum* are 2, 6-Bis (1, 1-dimethylethyl)-4-(1-oxopropyl) phenol, 1, 6-Dioxacyclododecane-7, 12-dione, Triamylbenzenes, 2,4-Di-tert-butylthiophenol, 4-Octadecylmorpholine. It also produces some unknown volatile moieties which cannot be identified on the basis NIST data. #16 AMLWLS posses an entirely different gas chemistry from the earlier reported *Muscodor species* which dominantly produces propanoic acid, methyl esters, azuelene, naphthalene derivatives and thujopsene. The volatiles produced by #16 AMLWLS are unique belonging to ketones, amines, phenols and alcohols and have not been reported by any other *Muscodor species* so far.

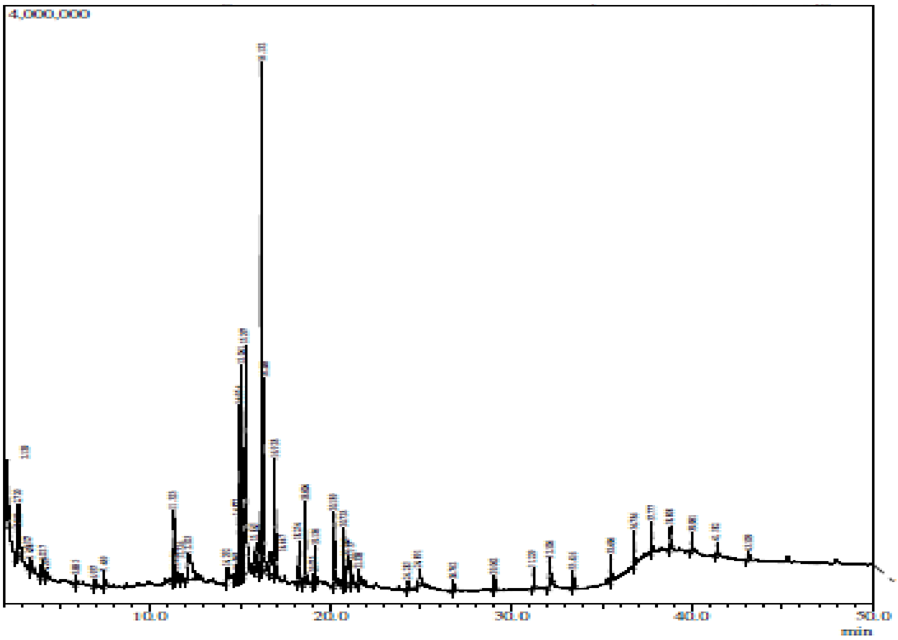


FIG 27: SPME-GC spectra of volatiles produced by #16 AMLWLS

RT	Possible name	Relative %	Quality	Molecular Formula	Mass(Da)
2.129	Cyclohexene, 1-methyl-4-(1-methylethylidene) -	1.69	96	$C_{10}H_{16}$	136
2.618	1-Hexanol, 2-Ethyl-	1.23	95	$C_8H_{18}O$	130
2.720	Cyclohexene, 1-methyl-4- (1-methylethenyl)-,	2.35	96	$C_{10}H_{16}$	136
4.027	2-Cyclohexen-1-one, 3,5,5- trimethyl-	0.80	89	$C_9H_{14}O$	138
4.293	Acetic acid, 2-ethylhexyl ester	0.19	87	$C_{10}H_{20}O_2$	172
6.937	Unknown	0.26	76		278
11.323	2,6-Di-butyl-2,5-cyclohexadiene - 1,4-dione (or p-benzoquinone)	2.02	93	$C_{14}H_{20}O_2$	220
11.726	1-chlorohexadecane	0.14	78	$C_{16}H_{33}Cl$	260
12.103	2-(4-Morpholinyl)ethanamine	4.40	92	$C_6H_{14}N_2O$	130
14.292	Unknown	0.72	62		222
14.767	Farnesene epoxide, E-	0.35	73	$C_{15}H_{24}O$	220
14.887	2-Furanmethanol, tetrahydro-.alpha.,.alpha.,5-trimethyl-5-(4-methyl-3-cyclohexen-1-yl)-,	1.76	79	$C_{15}H_{26}O_2$	238
14.954	2,6-Bis(1,1-dimethylethyl) -4-(1-oxopropyl)phenol	13.03	91	$C_{17}H_{26}O_2$	262
15.297	1,6-Dioxacyclododecane-7,12-dione	12.37	78	$C_{10}H_{16}O_4$	200
15.843	2,3-Dihydro-1,1-dimethyl-6-tert-butyl-1H-indene-4-acetic acid	10.81	74	$C_{17}H_{24}O_2$	260
16.152	3-Cyclohexen-1-ol, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-, synonym:β-Bisabolol	20.67	84	$C_{15}H_{26}O$	222
16.288	2,4-Di-tert-butylthiophenol	6.36	73	$C_{14}H_{22}S$	222
16.667	4-Octadecylmorpholine	4.65	92	$C_{22}H_{45}NO$	339

18.254	3,5-di-tert-Butyl-4-hydroxyacetophenone	1.74	70	$C_{16}H_{24}O_2$	248
19.156	Tri-T-Butyl-Phenol	1.25	80	$C_{19}H_{30}O_2$	290
21.137	Unknown	1.23	57	$C_{18}H_{26}O_2$	274
21.558	1H-Purin-6-Amine, [(2-Fluorophenyl)Methyl]-	0.63	69	$C_{12}H_{10}FN_5$	243
24.891	9-Octadecenoic acid, methyl ester, (E)-	1.25	90	$C_{19}H_{36}O_2$	296

Table 26: Composition of the volatiles produced by #16 AMLWLS after 10 days incubation at 24 ± 2°C on potato dextrose agar (PDA) entrapped using a solid-phase micro-extraction (SPME) fibre and GC/MS analysis.

Conclusion

The volatiles produced by Indian *Muscodor* species exhibited significant mycofumigation potential against gray mold *B. cinerea* (MTCC 359). #16 AMLWLS and #130 TMDSTYEL possesses maximum potential to be exploited as a mycofumigation agent. It acts as a preservative for grapes, apples and strawberries. Thus #16 AMLLWLS and #130 TMDSTYEL stands as a potential candidate to be developed as mycofumigant to act as bio preservative for commercially important fruits. Future studies needs to be done on mass production and purification of volatile compounds. Through studies on host parasite-relationship also needs to be done to unveil the mystery of symbiosis between plant and microbe. The life cycle of *Muscodor* is still an enigma.

References:

1. Adaskaveg JE, Forster H and Sommer NF (2002). Principles of postharvest pathology and management of decays of edible horticulture crops. *Postharvest technology of horticulture crops* 38:127–145.
2. Bacon CW and White JF (2002). Microbial Endophytes. (New York. Marcel Deker In
3. Banerjee D,Pandey A.,JanaM, and Strobel G,(2014). *Muscodor albus* MOW12 an Endophyte of Piper nigrum L. (Piperaceae) Collected from North East India Produces Volatile Antimicrobials**.***Indian journal of microbiology*.54:27-32
4. Baraldi E., Mari M, Chierici E , Pondrelli M, Bertolini P. and . Pratella .C.(2003) Studies on thiabendazole resistance of Penicillium expansum of pears: pathogenic fitness and genetic characterization *Plant Pathology* 52: 362–370
5. Blanpied, GD and A Purnasiri. (1968). Penicillium and Botrytis rot of McIntosh apples handled in water. *Plant Disease* . 52:865-867.
6. Boeing H, Bechthold A, Bub A, Ellinger S , Haller D, Kroke A,Leschik Bonnet E, Muller M, Oberritter H Schulze, Stehle P and Watz B (2012). Vegetables and fruit in the prevention of chronic diseases. *European journal of nutrition* 51(6):637-603
7. BoyerJ and Liu RH (2004): Apple phytochemicals and their benefits *Nutrition journal* DOI:10.1186/1475-2891-3-5
8. Campbell, C L 1998. Food security and plant pathologists. *Phytopathology*. News 32:70.
9. Chadha KL (2009). Handbook of Horticulture. IARI Publications, New Delhi.
10. Cooleng T; Bessin, R; Jones, T; Strang, J; Seebold, K. Vegetable production guide for commercial growers, cooperative extension service bulletin ID-36, University of Kentucky, College of agriculture, 2009-2010.Lexington, Kentucky.
11. 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.
12. 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.

13. Dauchet L, Amouyel P, Hercberg S, Dallongeville J (2006): Fruit and vegetable Consumption and risk of coronary heart disease: a meta analysis of cohort studies. *American Society for Nutrition* 136 : 2588-2593.
14. Delp, CJ (1988) Fungicide resistance in North America. APS Press, St. Paul MN, 11-16.
15. Eckert, J W. and Sommer, N F (1967). Control of diseases of fruits and vegetables by postharvest treatment. *Annual Review of Plant Pathology* 5:391-432
16. Ellis CL, Edirisinghe I, Kappagoda T, Burton-Freeman B (2013). Attenuation of meal-induced inflammatory and thrombotic responses in overweight men and women after 6-week daily strawberry *(Fragaria*) intake. a randomized placebo-controlled trial. *Journal of Atherosclerosis & Thrombosis*. 18: 318-27
17. Ezra D, Hess WM and Strobel GA (2004). New endophytic isolates of *Muscodor albus,* a volatile-antibiotic-producing fungus. *Microbiology* 150: 4023-4031.
18. Gabler M F, Mercier J C, Jiménezc J I, Smilanick J L (2010) Integration of continuous biofumigation with *Muscodor albus* with pre-cooling fumigation with ozone or sulfur dioxide to control postharvest gray mold of table grapes . *Postharvest Biology and Technology* 55 :78-84
19. Giampieri F, Alvarez-Suarez J, Maur (2014) .Strawberry and Human Health: Effects beyond Antioxidant Activity. *Journal of agriculture and food chemistry* 62 : 3867-3876
20. Gonza´ lez MC, Anaya AL, Glenn AE, Macı´as-Rubalcava ML, Hernandez-Bautista BE, Hanlin RT (2009). *Muscodor* yucatanensis,a new endophytic ascomycete from Mexican chakah, Bursera simaruba. *Mycotaxon* 110: 363e372.
21. 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
22. Gustavsson J ,Cederberg C, Sonesson U, Otterdijk RV, Meybeck A, (2012). Global food losses and food waste. *Save Food* : 1-29.
23. Janisiewicz W. and S. Jeffers, (1997). Efficacy of commercial formulation of two biofungicides for control of blue mold and gray mold of apples in cold storage. *Crop Protection.*16: 629–633
24. Janisiewicz WJ and Korsten L (2002). Biological Control of post harvest diseases of fruit. *Annual Review of Phytopathology* 40 : 411-441

25. Janisiewicz, WJ and Marchi A (1992). Control of storage rots on various pear cultivars with a saprophytic strain of Pseudomonas syringae. *Plant Disease* 76: 555-560.
26. Kudalkar P, Strobel G, Hassan S, Geary B and Sears J (2012). *Muscodor sutura*, a endophytic fungus with volatile antibiotic activities. *Mycoscience*. 53:319–325
27. Kusari S, Hertweck C, Spiteller M (2012). Chemical Ecology of Endophytic Fungi: Origins of Secondary Metabolites. *Chemistry and biology* DOI:10.1016/j chembiol.2012 .06.004
28. 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.
29. Lacey, LA, Horton, DR, Jones, D C, (2008). The effect of temperature and duration of exposure of potato tuber moth (Lepidoptera:Gelechiidae) in infested tubers to the biofumigant fungus *Muscodor albus*. *Journal of Invertebrate Pathology* 97: 159–164.
30. Lekli I, ,Ray D, Das DK (2012) Longevity nutrients resveratrol, wines and grapes. *Genes and Nutrition* 5 : 55-60
31. Luvisi DA, Shorey HH, Smilanick JL, Thompson JF, Gump BH, and Knuston J (1992). Sulfur dioxide fumigation of table grapes University of California, Division of Agriculture, Department of natural resource, Bulletin No. 1932.
32. 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.
33. 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.
34. 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
35. Mercier J, Leg FS, Smilanickj L (2009). In-package use of *Muscodor albus* volatile-generating sachets and modified atmosphere liners for decay control in organic table grapes under commercial conditions. *Fruits* .65:31-38
36. 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* 25: 173-179.

37. Mercier, J., and Jiménez, J.I. 2007. Potential of the volatile producingfungus, *Muscodor albus*, for control of building molds. *Canadian Journal of Microbiology* 53:404-410.
38. Meshram V., Kapoor N., & Saxena S. 2012. *Muscodor strobelli*, new species from South India. *Mycoscience* (in press)
39. MeshramV., KapoorN., and Saxena S (2014). *Muscodor* kashayum *sp. nov. – a new volatile anti-microbial producing endophytic fungus* 4:196-204
40. 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.
41. Mitchell AM, Strobel GA, Moore E, Robinson R, Sears J (2010). Volatile antimicrobials from *Muscodor crispans* a novel endophytic fungus. *Microbiology*. 156: 270–277.
42. N adas, A, Olmo, M and Garcia, J M (2003). Growth of *Botrytis cinerea* and strawberry quality in ozone-enriched atmospheres. *Journal of food science*. 68: 1798-1802.
43. Oyebode O, Gordon-Dseagu V, Walker A(2014). Fruit and vegetable consumption and all-cause, cancer and CVD mortality: analysis of Health Survey for England data. *Journal of Epidemology Community Health*. DOI:10.1136
44. Pusey, PL 1989. Use of *Bacillus subtilis* and related organisms as biofungicides. *Pesticide Science*. 27:133-140.
45. 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*. 4: 307-310.
46. Ramin, A.A., Braun, P.G., Prange, R.K., DeLong, J.M., (2005). In vitroeffects of *Muscodor albus* and three volatile components on growth of selected postharvest microorganisms. *Horticultural Science* 40, 2109–2114
47. Roberts R G (1994). Integrating biological control into post harvest disease management strategies. *Horticultural Science,* 29 : 758-762.
48. Roberts, RG and ST Reymand (1994). Chlorine dioxide for reduction of postharvest pathogen inoculum during handling of tree fruits. *Applied Environment. Microbiology* 60: 2864-2868.

49. Saxena S., Meshram V. and Kapoor N. (2014) *Muscodor tigerii* sp. nov.-Volatile antibiotic producing endophyticfungus from the Northeastern Himalayas *Annals of Microbiology* DOI 10.1007/s13213-014-0834-y
50. Saxena S., Meshram V. and Kapoor N. (2014) *Muscodor darjeelingensis*, a new endophytic fungus of *Cinnamomum camphora* collected from northeastern Himalayas.*Sydowia* 66 (1): 55–67
51. 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.
52. SinghV, Hedayetullah M, Zaman P , Meher J (2014) .Postharvest Technology of Fruits and Vegetables: an overview. *Journal of Postharvest Technology.* 2: 124-135
53. Sommer NF, Fortlage RJ and Edwards DC (2002). Post harvest diseases of selected commodities. In: Kader, A.A. (Ed.), *Postharvest technology of horticultural crops.* 197-249.
54. Spotts, R.A (1984). Environmental modification for control of postharvest decay. *Postharvest Pathology of Fruits and Vegetables* 87: 67-72.
55. Spotts, RA, LA. Cervantes, TJ.Facteau and T Chand-Goyal (1998). Control of brown rot and blue mold of sweet cherry with preharvest iprodione, postharvest Cryptococcus infirmo-miniatus, and modified packaging. *Plant Disease*. 82:1158-1160.
56. Stevens C, Khan VA, JY Lu,Wilson CL,.Pusey PL, Kabwe MK, E. Igwegbe ECK, Chalutz E and Droby S (1998). The germicidal and hormetic effects of UV-C light on reducing brown rot disease and yeast microflora of peaches. *Crop Protection* 17:75-84
57. 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.
58. Strobel (2006). Harnessing endophytes for industrial microbiology. *Current opinion in microbiology.* 9: 240-244
59. Strobel G and Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular biology Reviews.* 491-502
60. Strobel GA, Dirksie E, Sears J and Marksworth C (2001). Volatile antimicrobials from a novel endophytic fungus. *Microbiology.* 147 : 2943-2950

61. Strobel, G.A., Dirkse, E., Sears, J., Markworth, C., (2001). Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. *Microbiol.Reading* 147, 2943–2950.
62. Suwannarach N, Bussaban B, Hyde KD and Lumyong S (2010). *Muscodor cinnamoni*, a new endophytic species from *Cinnamomum bejolghota*. *Mycotaxon* 114: 15-23.
63. 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. *Annuals of microbiology*. 1590-4261.
64. Tan RX and Zou WX (2001). Endophytes: a rich source of functional metabolites. *Natural Products Report* 18: 448-59.
65. Vermeulen SJ, Campbell BM and Ingram J SI (2012).Climate change and food systems . *Annual Review of Enviornmental Resources* 37 :195-222
66. Vinas IJ, Usall N and V Sanchis (1998). Biological control of major postharvest pathogens in apple with *Candida sake*. *International Journal of Food Microbiology*. 40:9-16
67. Williamson B, Tudzynski P and Kan JAL (2007). *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology* 8: 561-580
68. Wilson and P.L. Pusey (1985). Potential for biological control of postharvest plant diseases. *Plant Disease* 69: 375-378.
69. Wisniewski, M E and CL Wilson (1992). Biological control of postharvest diseases of fruits and vegetables: recent advances. *Horticultural Science* 27: 94-98.
70. 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.
71. Worapong, J., Strobel, G., Daisy, B., Castillo, U.F., Baird, G.,and Hess, W.M. (2002). *Muscodor roseus* anam. nov. anendophyte from *Grevillea pteridifolia*. *Mycotaxon* 81:463-475.
72. 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.
73. Zhou K and Raffoul J (2012). Potential anticancer properties of grape antioxidants *Journal of Oncology* DOI: 10.1155/2012/803294