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ISOLATION, IDENTIFICATION AND SCREENING OF PATCHOULI OIL TOLERANT MICROORGANISMS FROM PATCHOULI RHIZOSPHERE AND PHYLLOPLANE

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ABSTRACT

In this study we isolated, screened and identified patchouli oil tolerant microorganism from rhizosphere and phylloplane. Rhizosphere soil samples from patchouli growing plot and patchouli leaves were collected at University of Agricultural Sciences Bangalore. Five organisms out of 21 isolated were found to be tolerant in the initial screening step. These five organisms were identified as *Botryosphaeria* spp, *Aspergillus terreus*, *Phoma* spp., *Fusarium solani complex* and *Penicillium citrinum*. The organisms were tested for patchouli oil tolerance (0.1 to 0.4%). Varying level of tolerance was recorded, highest tolerance index was observed in *Botryosphaeria* spp (0.81 - 0.56) and lowest in *Fusarium solani complex* (0.45 to 0.19).

INTRODUCTION

Rhizosphere is the soil adjacent to the root system of the plant and is influenced by root exudates, host plant and soil environment.[1] Microbial communities in this region are known to influence plant growth including nitrogen fixation, abiotic and biotic stress tolerance, plant growth and secondary metabolite production.[2] Presence of specific rhizobacteria has been reported to enhance secondary metabolite production like essential oil accumulation.[3] Phylloplane hosts a range of microorganism that are unique to the plant and are influenced by plant development and season. Plants emit volatile compounds and hormones that play a role in signalling specific microbial communities. These microbes are known to metabolize compounds released by the plant and have a beneficial association.[4]

Patchouli (*Pogostemon patchouli.* Pellet) is a perennial aromatic shrub, belonging to Lamiaceae family. This plant is a native of Philippines islands[5] and is widely cultivated in Indonesia, Malaysia and Sino Japanese regions.[6] *P. patchouli* is a shrub that grows up to 90-100cm in height with large stem, swollen nodes and leaves are oblong – ovate in shape.[7] The plant is propagated through cuttings and the crop is harvested two to three times year. The crop is cultivated in deep, well drained, fertile, slightly acidic, deep loamy to loamy soils with pH in the range of 5.5-7.5 for good growth.[7] The volatile oil is distilled from dried leaves of patchouli and are extensively used in perfumery due to its oriental notes and strong fixative property. The essential oil is known for its antiseptic, sedative and hypotensive properties and extensively used in soaps, perfumes and detergents.[8]

Aromatic plants like patchouli are known to produce volatile oil that can promote or inhibit the growth of microbial communities. The objective of this study was to isolate, identify and screen organisms for their tolerance to patchouli oil from the rhizosphere and phylloplane of patchouli plants. Tolerance to patchouli oil is the screening method to select microorganism that associate may with the plant readily.

1. MATERIALS AND METHODS

2.1 Isolation of microorganisms from soil

Rhizosphere soil was collected from patchouli plot at Sanjeevani Vatika, University of Agricultural Sciences, GKVK, Bangalore. The soil sample was collected from the upper layer of soil rich in organic matter and high microbial load around patchouli plants. 5g of the soil samples were collected in clean, dry and sterile polythene bag. Serial dilution agar plating[9] and Waksman Direct inoculation methods were employed for the isolation of soil microbes; suspension cultures was diluted up to 10^{-3} . The aliquots were cultured for fungus on Potato Dextrose Agar (Peeled potato 200g, Dextrose 20g) media with 0.1 % patchouli oil. Three plates from each soil samples were incubated for 96 hours at 25 ± 2°C. Each morphologically unique microbial colonies were sub-cultured and purified using standard techniques. Patchouli oil (0.1%) was added to screen for tolerant microorganisms.

2.2 Isolation of microorganisms from phylloplane

Mature leaf samples of patchouli were collected from field and placed in sterile plastic bags and immediately brought to the laboratory. From the basal part of the leaf, 1cm fragment leaf blade was cut. Using direct impression method, the leaves (abaxial and adaxial surface) were placed on petri plates containing potato dextrose agar medium. Plates were kept undisturbed

in dust free chamber at room temperature for a period of 3-5 days. The isolates were again pure cultured in PDA with 0.1% Patchouli oil.[10]

2.3 Identification of fungi

The classical way of identifying fungi is light microscopy. The isolates were sent to Microbial Type Collection Centre (MTCC), Chandigarh, India for identification of the organisms. Phenotypic characteristics like colour, size, texture, morphology etc., were identified.

2.4 Tolerance of the isolated microorganisms to patchouli oil

Spore suspension of the isolates were prepared using sterile distilled water. They were spot inoculated on PDA amended with different concentrations (0.1%-0.5%) of patchouli oil in triplicates. Growth was measured regularly up to 6 days by taking the diametric measurements at right angles to each other. The Tolerance Index (TI) was calculated from the fungal growth in the presence of patchouli oil, divided by the fungal growth in the control (without oil) plate in the same period.[11]

2. RESULTS

3.1 Isolation of microorganisms from rhizosphere

Isolation of microorganisms was carried out using serial dilution and pour plate method. The sample was diluted up to 10^{-3}. By using pour plate method, diluted samples were transferred into Potato Dextrose agar medium. The observation was recorded after 3 days of inoculation was recorded. The isolates displayed variability in colour, texture and size. They consisted of fungi, bacteria and yeast. The colony characteristics of the isolates from rhizosphere are described in table 1 and fig 1. Five fungal, 3 bacterial and 3 types of yeast colonies were isolated on potato dextrose agar.

3.2 Isolation of Microorganisms from Phylloplane

In table 2 and fig 2, the colony characteristics of the isolates from the phylloplane is described. More number of fungal colonies were observed. Totally eight fungal, two bacterial and yeast colonies were isolated using direct leaf impression method. Most of the fungal isolates from rhizosphere and phylloplane were similar. Phylloplane showed more number of colonies compared to rhizosphere. The isolated organisms were pure cultured with media amended with 0.1% patchouli oil. Only five fungal species survived, the bacterial and yeast strains did not show any growth.

3.3 Identification of fungi

Based on the phenotypic characteristics like colour, size, texture, morphology etc. The fungal colonies were identified by MTCC, Chandigarh (Table 3). The fungal isolates identified were *Botryosphaeria* spp, *Aspergillus terreus, Phoma* spp., *Fusarium solani complex* and *Penicillium citrinum. Botryosphaeria* spp., was characterised by aerial- fast-growing mycelium that covered 90-mm-diameter petriplate in 48 h at room temperature and the mycelium turned black, after 10 days of incubation at room temperature. *Aspergillus terreus,* was velvety, brown, cinnamon buff and had radial folds; the reverse was whitish brown. *Phoma s*pp had sticky white radial colony and *Fusarium solani complex* was thick white mycelium with pink radial folds on the reverse side. *Penicillium citrinum* was dark green in colour, numerous small buffy colonies were observed on the plate.

3.4 Tolerance of the isolated fungi to patchouli oil

Out of twenty one organisms (fungi, bacteria and yeast) only five fungal strains survived when sub cultured with 0.1% patchouli oil. These organisms were selected for further tolerance studies. The organisms were treated with 0.1-0.5% patchouli oil. The growth of colonies were recorded at regular intervals (2, 4 and 6 days) by taking the diametric measurements at right angles to each other. The growth rate of each fungal species in different oil concentration was calculated for each replicate. The tolerance of fungal isolates are represented in terms of Tolerance Index in table 4 and growth pattern in fig 4. Organism M1 (*Botryosphaeria*. spp) on treatment with patchouli oil at 0.1, 0.2, 0.3 and 0.4% displayed a tolerance index of 0.81 -0.56. With increasing concentration of patchouli oil, the tolerance levels decreased. M2 (*Aspergillus terreus*) displayed tolerance 0.49-0.45, M3 (*Phoma.* spp) 0.71 -0.43, M4 (*Fusarium solani complex*) 0.45 to 0.19 and M5 (*Penicillium citrinum*) 0.57-0.45.

Table 1: Colony characteristics of microorganisms isolated from patchouli soil rhizosphere.

Organism	Colony characteristic : color and texture				Average Number of colonies
	Surface	Reverse	Periphery	Texture	
Fungal Isolates					
MI1	Dark green	Brown	Whitish	Filamentous	1(large)
MI2	Whitish	Pink	White	Cottony	3
MI3	White	Pink	White	Filamentous	1
MI4	Green	Brown	Green	Powdery	4
MI5	Brown	Creamish	White	Powdery	5
Bacterial Isolates					

MI6	White	White	White	Creamy	1
MI7	Yellow	Off-white	Yellow	Creamy	2
MI8	off white	Off-white	off white	Creamy	4
Yeast Isolates					
MI9	White	White	White	Crustaceous Serrated	2
MI10	White	White	White	Crustaceous smooth	1

Table 2: Colony characteristics of microorganisms obtained from patchouli phylloplane.

Organism	**Colony character: colour and texture**				**Average number of colonies**
	Surface	**Reverse**	**Periphery**	**Texture**	
Fungal isolate					
MI1	Pink	Brown	Whitish	Cottony	2
MI2	**Black**	**Black**	**Whitish**	**Filamentous**	**2**
MI3	White	Pink	White	Filamentous	3
MI4	**Fiesta green**	**Creamish**	**Green**	**Powdery**	**1**
MI5	**Brown**	**Creamish**	**White**	**Powdery**	**2**
MI6	Sulphur Yellow	Creamish	Whitish	Crustaceous	1
MI7	Whitish	Cream	Whitish	Filamentous	1
MI8	Greenish black	Black	Green	Filamentous	1
Bacterial isolates					
MI9	Off-white	Off-white	Off-white	Creamy	2
MI10	Pink	Pink	Pink	Creamy	6
Yeast isolates					
MI11	White	White	White	Crustaceous smooth	4

Table 3: Fungi identified (Phenotype based)

Sl.No	**Fungi Identified**	**MTCC NUMBER**
1	*Botryosphaeria spp*	MTCC10850
2	*Aspergillus terreus*	MTCC11026
3	*Phoma spp.*	MTCC 11027
4	*Fusarium solani complex*	MTCC11028
5	*Penicillium citrinum*	MTCC 10849

Table 4: Tolerance of fungal isolates to patchouli oil (0.1-0.4%).

Treatment	**Tolerance index**
T1M1	
T2M1	0.81
T3M1	0.54
T4M1	0.53
T5M1	0.56
T1M2	
T2M2	0.59
T3M2	0.58
T4M2	0.45

T5M2	0.45
T1M3	
T2M3	0.73
T3M3	0.59
T4M3	0.58
T5M3	0.64
T1M4	
T2M4	0.45
T3M4	0.24
T4M4	0.28
T5M4	0.19
T1M5	
T2M5	0.57
T3M5	0.55
T4M5	0.45
T5M5	0.45

Tolerance index rating values indicate: 0.00–0.39 – very low tolerance.
0.40–0.59 – low tolerance.
0.60–0.79 – moderate l tolerance.
0.80–0.99 – high tolerance.
1.00–>1.00 – very high tolerance
[ref: 11]

Key: TreatmentsT1: control, T2:0.1%, T3:0.2%, T4:0.3%, T5:0.4% patchouli oil.

Organism: M1: *Botryosphaeria. spp; M2: Aspergillus terreus; M3: Phoma spp.; M4: Fusarium solani complex; M5: Penicillium citrinum.*

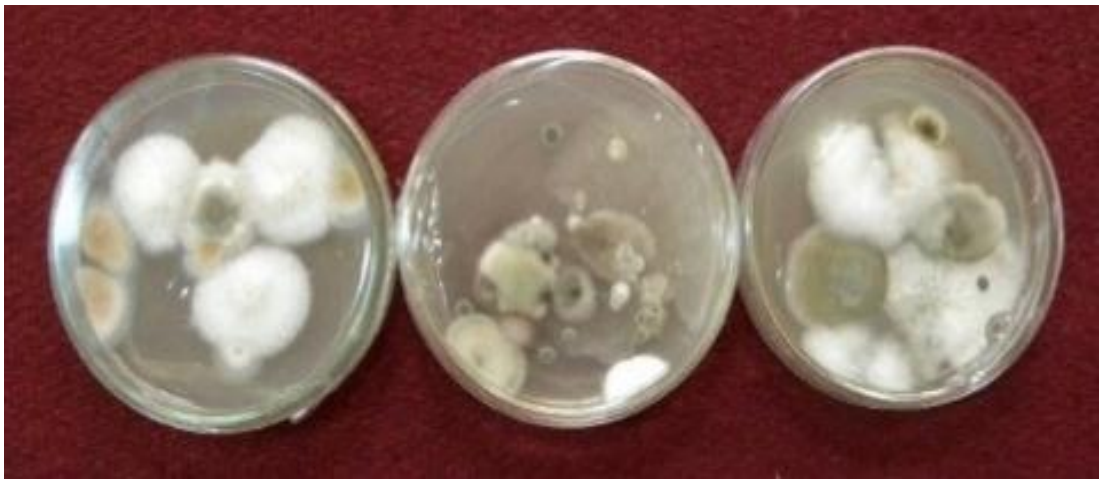


Fig 1: Microbial colonies isolated from patchouli Rhizosphere.

Fig 2: Microbial colonies isolated from phylloplane of patchouli leaves.

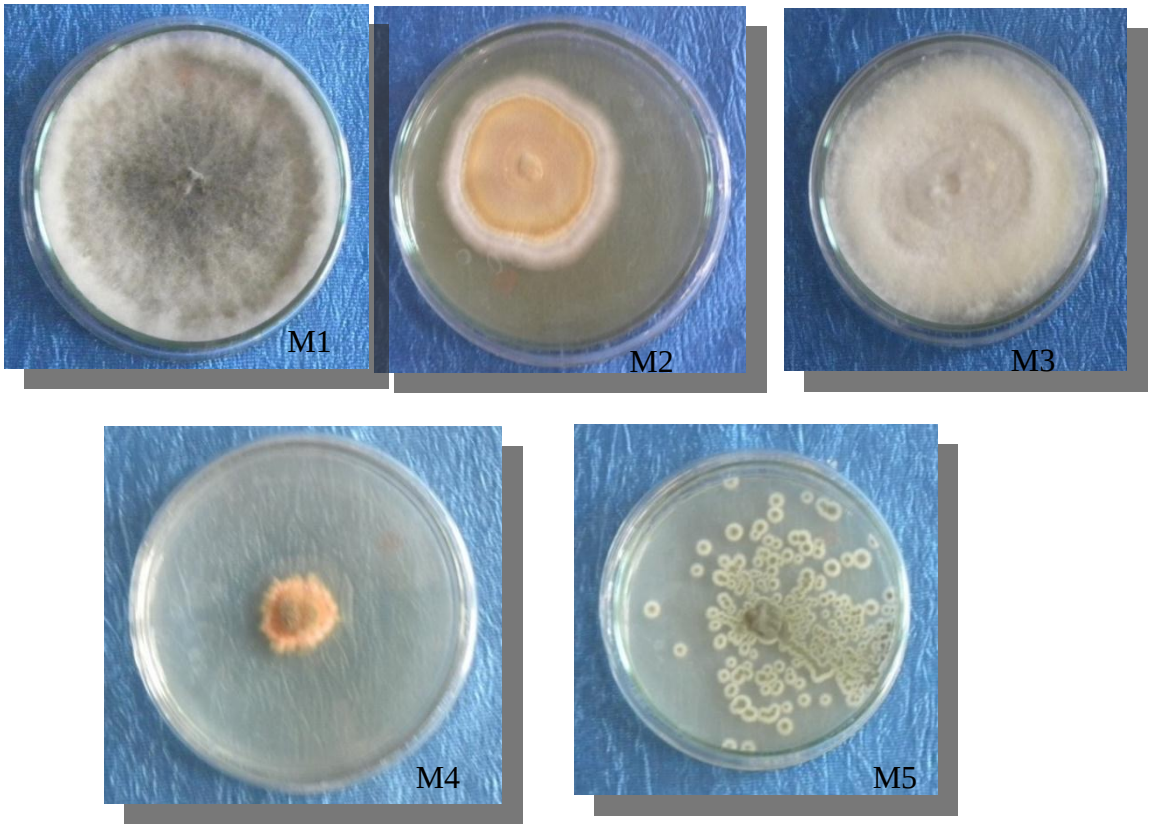
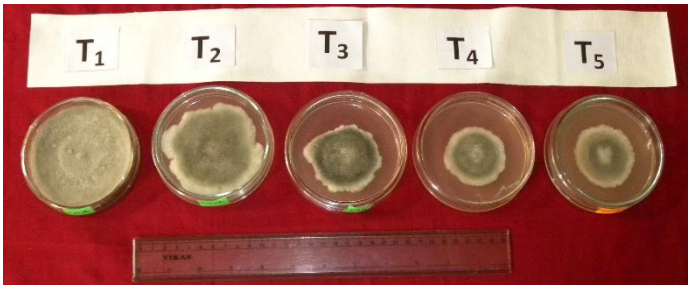
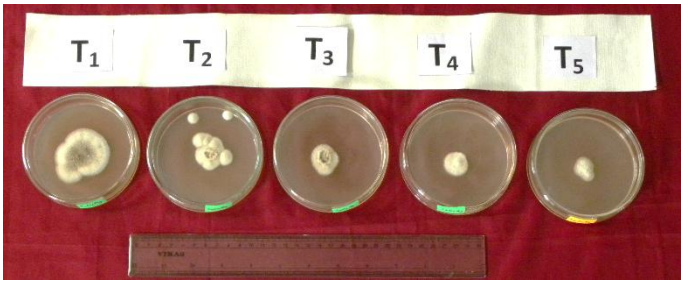


Fig 3: Fungal isolates obtained from rhizosphere and phylloplane of patchouli.

M1=*Botryosphaeria spp.* (MTCC10850), M2=*Aspergillus terreus* (MTCC11026), M3=*Phoma spp.* (MTCC 11027), M4=*Fusarium solani complex* (MTCC11028), M5=*Penicillium citrinum* (MTCC 10849).



M1



M2

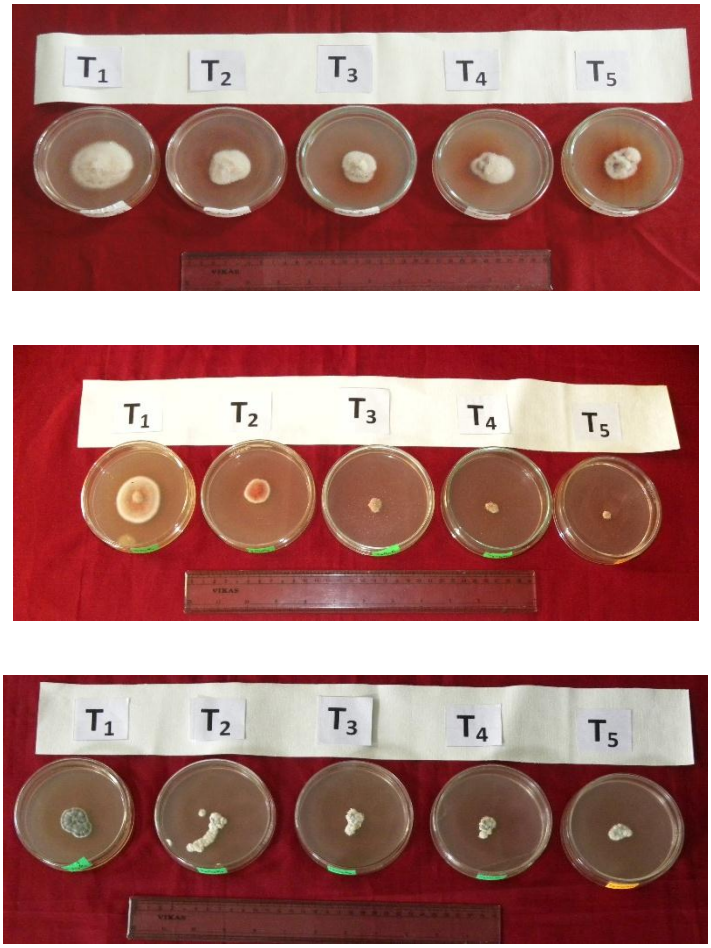


Fig 4: Tolerance of the isolated fungi to Patchouli oil.

Key - T1: control, T2:0.1%, T3:0.2%, T4:0.3%, T5:0.4% patchouli oil)

Organism: M1: *Botryosphaeria*. spp*; M2: Aspergillus terreus; M3: Phoma* spp.; *M4 Fusarium solani complex; M5 Penicillium citrinum.*

3. DISCUSSION

3.1 Isolation of Microorganisms from rhizosphere

Serial dilution plate technique proposed by[9] was used to isolate the microorganism. 0.1% patchouli oil was added as an initial screening step. Presence of more number of fungi than bacteria and yeast were recorded. Adamovic[12], studied the microbial abundance in rhizosphere of medicinal and aromatic plants. Similarly[13] reported distinctive microbiome associated with various medicinal plants which were screened for pharmacological properties. Presence of different types of organisms in rhizosphere and rhizoplane of rice and other plants were reported by[14] and[15] respectively. In table 1, the colony characteristics of five fungal, three bacterial and five yeast isolates described.

4.2 Isolation of microorganism from phylloplane

Using direct leaf impression technique, microorganism from patchouli leaves were isolated. The leaves were imprinted on media containing 0.1% patchouli oil. The colony characteristics of organism isolated suggested they can tolerate atmospheric challenges and accumulation of oil in the leaf glands as the plant grows. Microbial colonization of leaves are mostly saprophytes or endophytes and these organisms are known to play various roles like plant protection[16, 17, 18] production of secondary metabolites[19] and insect control.[20] Impact of phylloplane organism has not been reported till date in patchouli.

4.3 Identification of fungi

Isolation and identification of organisms from the rhizosphere and phylloplane showed the presence of same or similar type of microbes especially in fungi. Based on their phenotypic characteristics, fungal colonies were identified by MTCC as *Botryosphaeria* spp., *Aspergillus terreus, Phoma* spp., *Fusarium solani complex, Penicillium citrinum* (Annexure 1). These are common soil saprophytes and have been reported in research work.[21, 22, 23&24]

4.4 Tolerance of the microorganism to patchouli oil

Among the five fungal species, *Botryosphaeria. s*pp., showed highest tolerance towards patchouli oil at 0.1% followed by *Phoma* spp. *Aspergillus terreus, Fusarium solani complex* and *Penicillium citrinum* was found to have low level of tolerance to patchouli oil. Tolerance of fungal isolates in a hydrophobic environment indicate their adaptability to patchouli oil. Fungi are ubiquitous members of rhizosphere, aerial environments and often are dominant and adapt easily to unique environments. Reduction in growth may be due to increased energy demand to combat the stress and production of proteins that help in osmoregulation in a hydrophobic environment. Extensive research publications are available on tolerance of microbial species to heavy metals, saline.[22, 25] Similarly[26] reported varying tolerance level of different *Aspergillus* spp.to chromium and lead.

The present study is the first report on isolation of patchouli oil tolerant microorganisms from a known ecological niche.

CONCLUSION

The results showed presence of common microorganism in patchouli rhizosphere and phylloplane. This investigation also revealed presence of more number of organisms on the phylloplane which is a stressful environment due to changes that happen within the plant and

climate. Five fungi were found to be tolerant to patchouli oil and their tolerance level varying from high to very low. Further studies need to be done to understand the adaptability of organism to volatile oils and if there can be beneficial association that enhances the oil quality and quantity.

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