Identification and characterization of new *Muscodor* endophytes from gramineous plants in Xishuangbanna, China

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**Abstract**
The endophytic fungi *Muscodor* spp. produce volatile organic compounds (VOCs) which can inhibit and even kill pathogenic fungi, bacteria, and nematodes. Nine endophytic fungal strains, isolated from the shoots of gramineous plants including *Arthraxon hispidus*, *Eleusine indica*, *Oplismenus undulatifolius*, and *Oryza granulata*, were identified as *Muscodor* through phylogenetic analysis of the internal transcribed spacer. Through an SPSS K-means cluster analysis, the nine *Muscodor* strains were divided into four groups based on the antifungal activities of the VOCs produced by these fungi determined by a two-section confrontation test. The first group contains the strains Y-L-54, W-S-41, Y-S-35, W-T-27, and Y-L-56, which showed the strongest activity. The second and third groups contain W-S-35 and Y-L-43, which showed stronger and moderate activity, respectively. The fourth group contains W-S-38 and N-L-7, which were the weakest in inhibiting the tested pathogens. Thirty-five compounds and the relative amounts of VOCs were determined by SPME-GC-MS and comparison with the NIST14 mass spectrometry database and Agilent MassHunter qualitative and quantitative analyses. These 35 compounds were classified into two different categories: (a) the product of fatty acid degradation, and (b) the intermediate and final metabolite of the metabolic pathway with the precursor of mevalonic acid. SPSS clustering analysis showed that the chemical components of VOCs might be correlated with their bioactivity rather than their phylogenetic assignment and some of the identified compounds might be responsible for antifungal activity. In conclusion, new *Muscodor* endophytes were recorded in tropical gramineous plants and a number of strains showed remarkable bioactive properties. Therefore, they have important potential applications in the fields of plant disease control.

**KEYWORDS**
antifungal activity, internal transcribed spacer of ribosomal DNA, *Muscodor*, volatile organic compounds
1 | INTRODUCTION

Xylariaceous fungi are the dominant group of endophytic fungi. Strobel et al. found that xylariaceous fungal isolate 620 can produce volatile organic compounds (VOCs) with strong antimicrobial activity, has intertwined rope-like mycelia, and does not produce spores. Therefore, the genus Muscodor was established based on its mycelia type and its production of VOCs (Strobel, Dirks, Sears, & Markworth, 2001; Worapong et al., 2001). Muscodor has a significant inhibitory effect or even lethal effect on a variety of pathogens (fungi, bacteria, and nematodes), therefore Muscodor has important potential applications in the fields of agriculture and environmental protection (Strobel, 2006). Muscodor albus strain QST 20799 and three end products, andante, arubesque, and glissade, were proposed for postharvest fruit, seed, and soil-borne diseases control. This was approved by the United States Environmental Protection Agency in 2005.

Over the course of exploration of endophytic fungal resources, a total of 21 Muscodor species were recorded to date: M. albus (Worapong et al., 2001), M. roseus (Worapong, Strobel, Daisy, & Castillo, 2002), M. vitigenus (Daisy et al., 2002), M. crispsans (Mitchel, Strobel, Hess, Vargas, & Ezra, 2008), M. yucatanensis (Gonzalez et al., 2009), M. fengyagensis (Zhang et al., 2010), M. cinnamomii (Suwannarach, Bussaban, Hyde, & Lumyong, 2010), M. sutura (Kudalkar, Strobel, Riyaz-Ul-Hassan, Geary, & Sears, 2012), M. equiseti, M. musae, M. oryzae, and M. suthepensis (Suwannaracha et al., 2013), M. kashayum (Meshram, Kapoor, & Saxena, 2013), M. darjeelingensis (Saxena, Meshram, & Kapoor, 2014), M. strobelli (Meshram, Saxena, & Kapoor, 2014), M. heveae (Siri-udom, Suwannarach, & Lumyong, 2015), M. indicus and M. ghoomensis (Meshram, Gupta, & Saxena, 2015a), M. tigerii (Meshram, Gupta, & Saxena, 2015b), M. coffeaeum (Hongasan et al., 2015), and M. camporhoe (Meshram, Kapoor, Chopra, & Saxena, 2017). In a previous study of endophytic fungi from Oryza granulata collected from Xishuangbanna, southwest China, which is an area known to contain rich fungal biodiversity, we isolated two Muscodor strains (Yuan et al., 2011). From 2015 to 2016, further exploration of the endophytic fungi from gramineous plants, including Arthraxon hispidus, Eleusis indica, Oplismenus undulatifolius, and Oryza granulata, from Xishuangbanna, we found that nine endophytic fungal strains can produce VOCs with anti-fungal activity, has intertwined rope-like mycelia, and does not produce spores. These phenotypic characteristics suggested that these strains could be belonging to the genus Muscodor. Here, we report the identification, antifungal activity, and analysis of VOCs of these nine strains.

2 | MATERIALS AND METHODS

2.1 | Endophytic fungi isolation and storage

The gramineous plants were sampled during 2015 and 2016 from Xishuangbanna in Yunnan province of China (E 100°32′–100°44′, N 22°04′–22°17′). The samples were placed in zip-lock plastic bags, stored in a box with ice and transported to laboratory within 48 hr after sampling. Healthy plant tissues were rinsed with tap water, then immersed in 75% ethanol for 3–5 min and then in 1% sodium hypochlorite for 8–10 min, and finally rinsed thrice with sterile distilled water. The surface disinfected tissues were cut into segments of about 5 mm length and the segments were placed on MEA plate (2% malt extract agar with 50 mg/L chloramphenicol). The plates were then incubated at 25°C in darkness. Fungal hyphae emerging from the segments were transferred to new potato dextrose agar (PDA) plates for purification. The purified cultures were grown on PDA slant, then covered with sterile liquid paraffin, and deposited in Fungal Biology Laboratory of Zhejiang University.

2.2 | Phylogenetic analysis based on the internal transcribed spacer of ribosomal DNA sequence

The internal transcribed spacer (ITS) was PCR-amplified using the universal primers, ITS-1 (5′-TCTCTCCGCTATGGATATGC-3′) and ITS-4 (5′-TCTTCTCGCTATGGATC-3′) (White, Bruns, Taylor, & 1990). The PCR amplification products were purified and sequenced bidirectionally on an ABI 3730 sequencer (Applied Bio-Systems) according to Zhang et al. (2010). The ITS sequences of the type strains of the known Muscodor species were retrieved from the National Center for Biotechnology Information’s (NCBI) GenBank. ITS sequences obtained in this study were aligned with sequences of known Muscodor species using clustal X 2.1 (Larkin et al., 2007) and manually corrected using GeneDoc (Nicholas & Nicholas, 1997). Sequences obtained in this study were deposited in NCBI GenBank with Accession Number MG309792–MG309800.

Phylogenetic analyses were carried out using maximum parsimony (MP) approach with PAUP* v. 4.0b10 (Swoford, 2002) and Bayesian analyses (BI) approach with MrBayes v3.2.6 (Ronquist et al., 2012). jModelTest was used to compare the likelihood of different nested models of DNA substitution and select the best-fit model for the dataset. Likelihood settings from the best-fit model (TrN + G) were selected by AIC using jModeltest. The branches were indicated with MP bootstrap proportion (MPBP) and BI posterior probability (BIPP).

2.3 | Determination of the colony characteristics and optimal growth temperature

The tested Muscodor strains (Table 1) were incubated on PDA medium at 25°C for activation. The mycelial disks (5-mm diameter) were excised from the edge of the Muscodor colony, one disk was inoculated on PDA in the center of the Petri dish (9-cm diameter). The inoculated Petri dishes were kept in darkness, with three kept at each of the following temperatures: 15, 20, 23, 25, 28, 30, 35, and 40°C, respectively. After incubation for 15 days, the colony characteristics were observed and the diameters were recorded.
2.4 | Assessment of the antifungal activity of VOCs

To measure antifungal activity, five plant pathogenic fungi, Botrytis cinerea strain ZJUP10, Fusarium oxysporum strain ZJUP28, Penicillium digitatum strain Pd01, Pytium ultimum strain ZJUP22, and Pestalotia diospyr strain ZJUP21 were used. The tested pathogens were collected and preserved in the State Key Laboratory for Rice Biology at Zhejiang University, China.

The antifungal activity of the VOCs produced by the tested Muscodor strains was determined by confrontation culture in a Petri dish with two-sections. A mycelial disk with a diameter of 5 mm was taken from the edge of the Muscodor culture, and then incubated on PDA in one section of the Petri dish at 25°C for 4 days in darkness, and a pathogenic fungal mycelia disk of same size was placed in the other section. The Petri dishes were wrapped with two layers of Parafilm and incubated at 25°C for 4 days in darkness. In parallel, the tested pathogens were grown in the absence of Muscodor, and when their colonies reached the edge of the Petri dish, the diameters of the corresponding pathogenic fungal colonies in the confrontation treatment were measured. To evaluate the viability of the tested pathogens, the abovementioned pathogen colonies were transferred onto new PDA plates and incubated at 25°C to test their viability.

| Strain | Species         | Host          | Tissue | ITS GenBank accession number |
|--------|-----------------|---------------|--------|------------------------------|
| Y-L-54 | Muscodor sp.1   | Oryza granulata | Leaf   | MG309797                     |
| W-S-41 | Muscodor sp.1   | Arthraon hispidus | Sheath | MG309800                     |
| W-T-27 | Muscodor sp.2   | Oplismenus undulatifolius | Stalk | MG309795                     |
| Y-S-35 | Muscodor sp.2   | Oryza granulata | Sheath | MG309796                     |
| W-S-38 | Muscodor sp.3   | Oplismenus undulatifolius | Sheath | MG309799                     |
| N-L-7  | Muscodor sp.4   | Eleusine indica | Leaf   | MG309792                     |
| Y-L-43 | Muscodor sp.4   | Oryza granulata | Leaf   | MG309793                     |
| W-S-35 | Muscodor sp.4   | Oplismenus undulatifolius | Sheath | MG309794                     |
| Y-L-56 | Muscodor sp.5   | Oryza granulata | Leaf   | MG309798                     |

Note. ITS: internal transcribed spacer.

FIGURE 1 Phylogenetic tree of Muscodor spp. based on internal transcribed space sequences. The branches are indicated with maximum parsimony bootstrap proportion and Bayesian analyses posterior probability.
2.5 | Determination of the components and the relative amounts of VOCs

*Muscodor* strains were incubated on PDA in Petri dishes with two sections as described above. The PDA plate without *Muscodor* was considered a control. The VOCs emitted by *Muscodor* were extracted with an SPME syringe (SUPELCO) 50/30 mm divinylbenzene/carboxen/polydimethylsiloxane on StableFlex fiber. VOCs were analyzed by GC-MS (Agilent 6890N/5975B) following procedures described in Zhang et al. (2010). The data were processed using the Agilent MassHunter workstation (Agilent).

Chromatographic peaks were recognized by Agilent MassHunter qualitative analysis B.07.00, and the mass spectrogram was obtained. Then, the compounds were identified by a search performed in the NIST14 database. A quantitative analysis of VOCs produced by *Muscodor* strains was performed through Agilent MassHunter quantitative analysis b.07.01.

2.6 | Data statistical analysis

Statistical analyses were conducted using IBM SPSS 22.0. The antifungal activities of nine *Muscodor* strains against five plant
pathogenic fungi were analyzed using an SPSS K-means cluster analysis. The components and the relative amounts of VOCs produced by these Muscodor strains were analyzed through SPSS clustering analysis with the nearest neighbor element and Pearson correlation, with a range from 0 to 1.

3 RESULTS

3.1 Phylogenetic analysis of the endophytic fungal strains

Phylogenetic analysis based on ITS sequences using MP and BI yielded similar tree topology. The tested nine endophytic fungal strains with the known Muscodor species formed a strong supported cluster with 90% MPBP and 1.00 BIPP (Figure 1). Y-L-56 clustered with M. vitigenus, M. sutura, and M. equiseti with 96% MPBP and 1.00 BIPP. N-L-7, Y-L-43, and W-S-35 clustered with M. coffeanum with 93% MPBP and 0.99 BIPP. W-S-38 formed an independent branch. W-T-27, Y-S-35, and M. suthepensis formed a clade with 100% MPBP and 1.00 BIPP. Therefore, the nine tested endophytic fungal strains were identified as five different Muscodor species: Muscodor sp. 1 to Muscodor sp. 5 (Table 1). ITS sequence analysis also showed that Muscodor sp. 3 (W-S-38), Muscodor sp. 4 (N-L-7, Y-L-43, and W-S-35), and Muscodor sp. 5 (Y-L-56) have considerable sequence differences from known Muscodor species.

3.2 Characteristics of the tested Muscodor colonies and the optimal growth temperature

Colonies of the tested Muscodor strains cultivated at 25°C on PDA medium are shown in Figure 2. All colonies were white, but the colony appearances of different strains were slightly different. Colonies of strains Y-L-54, W-S-41, W-T-27, and Y-S-35 were smooth with sparse aerial mycelia and radialized vegetative mycelia, while aerial mycelia of strains W-S-38, Y-L-56, and W-S-35 were floccus. Strains N-L-7 and Y-L-43 were wooly. None of the strains produced spores on PDA.

The growth temperature of the tested Muscodor strains cultivated on PDA medium is shown in Table 2. There was little difference among the optimal growth temperatures of the tested Muscodor strains on PDA. None of the tested Muscodor strains can grow at temperatures above 30°C. Seven of the Muscodor strains can still grow at 30°C, while the other two strains, W-S-38 and W-S-35, stopped growing. The optimal growth temperature range for the tested Muscodor strains is 20–28°C, except for W-S-38, which shows optimal growth at 20–25°C.

3.3 Antifungal activity of the tested Muscodor strains

The antifungal activity of the tested Muscodor strains was shown in Table 3. The VOCs of Muscodor strain W-S-41 showed the strongest
### TABLE 4
The volatile organic compounds produced by *Muscodor* strains through SPME/GC/MS analysis

| No.  | RT (min) | Possible compounda                        | CAS no. | NIST no. | Lib. match score | Mr   | Peak areab |
|------|----------|-------------------------------------------|---------|----------|------------------|------|------------|
|      |          |                                           |         |          |                  |      | W-S-41    | Y-L-54 | Y-S-35 | W-T-27 | Y-L-56 | W-S-35 | Y-L-43 | W-S-38 | N-L-7 |
| com1 | 3.448    | Propanoic acid, 2-methyl-, methyl ester   | 547-63-7| 34178    | 920               | 102  | 612879    | 446802 | 925157 | 472660 | 144209 | 290250 | /       | /      | /      |
| com2 | 4.799    | 1-Butanol, 3-methyl-                       | 123-51-3| 19490    | 843               | 88   | /         | /      | /      | /      | /      | /      | /      | 809102 | /      |
| com3 | 8.148    | Propanoic acid, 2-methyl-                 | 79-31-2 | 19501    | 917               | 88   | 708184    | 4003722 | 8386293 | 4672700 | 1930881 | 2476244 | /      | /      |
| com4 | 9.521    | 1-Butanol, 3-methyl-, acetate             | 123-92-2| 291294   | 935               | 130  | 113689    | 66375  | 48349   | /      | /      | /      | /      | 81334  | /      |
| com5 | 12.983   | 1-Octen-3-ol                              | 3391-86-4| 19422    | 890               | 128  | /         | /      | /      | /      | /      | 306613 | /      | /      |
| com6 | 13.207   | 3-Octanone                                 | 106-68-3| 163610   | 912               | 128  | /         | /      | /      | /      | /      | 170551 | /      | /      |
| com7 | 16.001   | 4-Nonanone                                 | 4485-09-0| 114360  | 926               | 142  | /         | 21555  | 16037  | 46809  | /      | /      | 58463  | /      |
| com8 | 16.612   | 2-Nonanone                                 | 821-55-6| 114362   | 830               | 142  | /         | 18828  | 21940  | /      | /      | /      | /      |
| com9 | 16.690   | Propanoic acid, 2-methyl-, pentyl ester   | 2445-72-9| 280486  | 780               | 158  | /         | 17140  | 17707  | /      | /      | /      | /      |
| com10| 17.257   | Octen-1-ol, acetate                       | 32717-31-0| 6634    | 929               | 170  | /         | /      | /      | /      | /      | 157307 | /      | /      |
| com11| 18.755   | Octanoic acid, 2-methyl-, methyl ester    | 2177-86-8| 62444   | 821               | 172  | /         | /      | /      | /      | /      | /      | 22396  | /      |
| com12| 21.148   | Bicyclo[2.2.1]heptane, 2-methylene-3-{1-methylethenyl} | 77764-41-1| 150031  | 764               | 148  | /         | /      | /      | /      | /      | 17343  | /      |
| com13| 21.498   | Acetic acid, 2-phenylethyl ester          | 103-45-7| 107577  | 984               | 164  | 36042    | 49418  | 89273  | 22125  | 24542  | 32815  | /      |
| com14| 21.765   | Tridecane, 7-methylene-                   | 19780-80-4| 113992  | 795               | 196  | /         | /      | /      | /      | /      | 21168  | /      |
| com15| 23.252   | 1H-Cyclopropa[naphthalene, 1a,2,3,3a,4,5,6,7b-octahydro-1,1,3a,7-tetramethyl-[1aR-(1aà,3aà,7bà)]-] | 489-29-2| 9244    | 847               | 204  | /         | /      | /      | /      | /      | /      | /      | 10757 |
| com16| 24.136   | Caryophyllene-([II])                      | N/A     | 158185   | 824               | 204  | /         | /      | /      | /      | /      | /      | /      | 12852  | 31956 |
| com17| 24.456   | Biphenylene, 1.2.3.6.7.8.8a, 8b-octahydro-4,5-dimethyl- | 106988-87-9 | 142222  | 819               | 188  | /         | /      | /      | /      | /      | 13314  | /      |
| com18| 24.899   | 1H-Cyclopropa[naphthalene, 1a,2,3,5,6,7a,7b-octahydro-1,1,7a-tetramethyl-[1aR-(1aà,7aà,7aà,7bà)]-] | 17334-55-3 | 249534  | 845               | 204  | /         | /      | /      | /      | /      | 14535  | 33139  | 70109  |

(Continues)
| No. | RT (min) | Possible compound | CAS no. | NIST no. | Lib. match score | Mr | Peak area<sup>b</sup> | W-S-41 | Y-L-54 | Y-S-35 | W-T-27 | Y-L-56 | W-S-35 | Y-L-43 | W-S-38 | N-L-7 |
|-----|----------|------------------|--------|----------|-----------------|----|-----------------|-------|-------|-------|--------|-------|--------|-------|-------|-------|
| com19 | 25.016  | (-)-Tricyclo[6.2.1.0(4,11)]undec-5-ene, 1,5,9,9-tetramethyl-1(isocaryophyllene-II) | N/A    | 154067   | 847             | 204 | /               | /     | /     | /     | /      | /     | /      | /     | /     | /     | 59052 | /     | 29412 |
| com20 | 25.208  | Cyclohexane, 1-ethyl-1-methyl-2,4-bis(1-methylethenyl)-[3R-(3a,4a,7a)]- | 515-13-9 | 22550 | 928             | 204 | /               | /     | /     | /     | /      | /     | /      | /     | /     | /     | 26202 | /     |       |
| com21 | 25.466  | 4,4-Dimethyl-3-(3-methylbut-3-enyldiene)-2-methylenecyclo[4.1.0]heptane | 79718-83-5 | 195379 | 755             | 202 | /               | /     | /     | /     | /      | /     | /      | /     | /     | /     | 13282 | /     | 21468 |
| com22 | 25.763  | 1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8-tetramethyl-[3R-(3a,3a,7a,8a)]- | 469-61-4 | 22526 | 924             | 204 | 13487           | /     | /     | /     | /      | /     | /      | /     | /     | /     |       | /     |       |
| com23 | 26.307  | Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-[4-methyl-3-pentenyl]- | 17699-05-7 | 141044 | 935             | 204 | 10461           | 26174 | 27232 | 11430 | /       | 19950 | /      | /     | /     | /     |       | /     | 24561 |
| com24 | 26.392  | Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-[1-methylethenyl]-, [1S-(1a,4a,7a)]- | 3691-12-1 | 9225 | 936             | 204 | 16491           | /     | 10527 | /     | /      | /     | /      | /     | /     | /     | 14387 | /     |       |
| com25 | 27.461  | Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-[1R-(1R*,4Z,9S*)]- | 118-65-0 | 249403 | 885             | 204 | 13040           | /     | /     | /     | /      | /     | /      | /     | /     | /     | 76483 | /     |       |
| com26 | 27.551  | 1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-(Z)- | 28973-97-9 | 141110 | 901             | 204 | /               | /     | /     | /     | /      | /     | /      | /     | /     | /     | 36032 | /     | 129446 |
| com27 | 27.677  | 2-Isoisopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8-octahydronaphthalene | N/A    | 193570 | 911             | 204 | 49505           | /     | 10706 | /     | /      | /     | /      | /     | /     | /     | 40370 | /     |       |
| com28 | 27.936  | 1R,3Z,9S-2,6,10,10-Tetramethylbicyclo[7.2.0]undeca-2,6-diene | N/A    | 140074 | 851             | 204 | 24180           | /     | /     | /     | /      | /     | /      | /     | /     | /     | 20364 | /     |       |
| com29 | 27.987  | Calarene epoxide | N/A    | 151460 | 745             | 220 | /               | /     | /     | /     | /      | /     | /      | /     | /     | /     | 16160 | 38668 | 72480 |

(Continues)
| No. | RT (min) | Possible compound\(^a\)                                      | CAS no. | NIST no. | Lib. match score | Mr  | Peak area\(^b\)  |
|-----|----------|-------------------------------------------------------------|---------|----------|------------------|-----|------------------|
|     |          |                                                             |         |          |                  |     | W-S-41           |
|     |          |                                                             |         |          |                  |     | Y-L-54           |
|     |          |                                                             |         |          |                  |     | Y-S-35           |
|     |          |                                                             |         |          |                  |     | W-T-27           |
|     |          |                                                             |         |          |                  |     | Y-L-56           |
|     |          |                                                             |         |          |                  |     | W-S-35           |
|     |          |                                                             |         |          |                  |     | Y-L-43           |
|     |          |                                                             |         |          |                  |     | W-S-38           |
|     |          |                                                             |         |          |                  |     | N-L-7            |
|     |          |                                                             |         |          |                  |     |                  |
| com30 | 28.083 | Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-[15-(1a,7a,8aa)]- | 3691-11-0 | 70226 | 948              | 204 | 251410           |
| | | | | | | | 30941 |
| | | | | | | | 36361 |
| | | | | | | | 17437 |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | 211194 |
| | | | | | | | 10861 |
| com31 | 28.648 | Spiro[5,5]undeca-1,8-diene, 1,5,5,9-tetramethyl-1-(R)- | 19912-83-5 | 249575 | 814              | 204 | /               |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | 90834 |
| | | | | | | | 118980 |
| | | | | | | | / |
| | | | | | | | 321594 |
| com32 | 29.427 | Patchouli alcohol                                           | 5986-55-0 | 141042 | 796              | 222 | /               |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | 18101 |
| com33 | 31.561 | 1H-Cycloprop[ez]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-[1ar-(1a,4a,4aa,7a,7aa,7ba)]- | 552-02-3 | 141116 | 857              | 222 | 342257           |
| | | | | | | | 175943 |
| | | | | | | | 344106 |
| | | | | | | | 129530 |
| | | | | | | | / |
| | | | | | | | 13024 |
| | | | | | | | 120395 |
| com34 | 34.293 | 2-Propanoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester | 5466-77-3 | 291525 | 921              | 290 | /               |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | 793815 |
| com35 | 37.096 | Hexadecanoic acid, methyl ester                             | 112-39-0 | 158970 | 886              | 270 | /               |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |
| | | | | | | | / |

\(^a\) The chemical nomenclature was adopted according to NIST14 database.

\(^b\) The corresponding compounds existed in controls were deleted. Minor peaks with signal-to-noise ratio < 3 were cutoff.
antifungal activity, with complete inhibition and killing of mycelial growth of Botrytis cinerea, Penicillium digitatum, and Pythium ultimum. In addition, the VOCs of Muscodor strain W-S-41 was able to effectively inhibit two other pathogens: Fusarium oxysporum and Pestalotia diospyr. The VOCs of Muscodor strain N-L-7 showed the weakest antifungal activity, only partially or slightly inhibiting Penicillium digitatum, Botrytis cinerea, and Pestalotia diospyr, and showing no inhibition activity on Fusarium oxysporum and Pythium ultimum.

Among the targeted pathogens, P. ultimum was the most sensitive to VOCs produced by Muscodor. When exposed to the VOCs produced by Muscodor, P. ultimum was completely inhibited and even killed by seven of the VOCs. Penicillium digitatum (sensitive to six VOCs) and B. cinerea (sensitive to five VOCs) also showed sensitivity to the VOCs produced by Muscodor. Fusarium oxysporum and P. diospyr were the least sensitive to the VOCs of Muscodor; they were only partially inhibited by the VOCs.

Through the SPSS K-means cluster analysis based on antifungal activities, the tested Muscodor strains can be divided into four groups: I–IV. The first group contains the strains W-S-41, Y-L-54, Y-S-35, W-T-27, and W-S-41, which showed the strongest antifungal activity. These strains completely inhibited and killed B. cinerea, P. digitatum, and P. ultimum and moderately or partially inhibited F. oxysporum and P. diospyr. Strain W-S-35 belongs to the second group, which showed stronger antifungal activity. It completely inhibited P. digitatum and P. ultimum, moderately inhibited B. cinerea,
and partially inhibited F. oxysporum and P. diospyr. The third group contains strain Y-L-43, which showed weak antifungal activity. It completely inhibited P. ultimum, moderately inhibited B. cinerea, and weakly or did not inhibit F. oxysporum, P. digitatum, and P. diospyr. The fourth group, contains strains W-S-38 and N-L-7, which were the weakest at inhibiting the five tested pathogens.

3.4 | The components and the relative amounts of VOCs produced by the tested Muscodor strains

Thirty-five compounds and the relative amounts of VOCs were determined (Table 4), which were classified into two categories. One is the product of fatty acid degradation, such as methyl isobutyrate, 3-methyl-1-butanol, 2-methylpropanoic acid, 3-methyl-1-butyl acetate, 1-octen-3-ol, 3-octanone, and 2-nonanone. The other category is terpenes, such as monoterpenes (1-ethyl-1-methyl-2,4-bis(1-methylethenyl)-cyclohexane) and sesquiterpenes (caryophyllene), which are the intermediate and final metabolites of the metabolic pathway with the precursor of mevalonic acid.

A chemical dendrogram (Figure 3) was created using SPSS clustering analysis. The dendrogram showed that components of the VOCs from Y-L-54 and Y-S-35 were similar. In addition, the components of the VOCs from Y-L-43 and N-L-7 were also similar.

4 | DISCUSSION

Typical fungal identification mainly depends on morphological characters and phylogenetic analyses. The identification of Muscodor can be challenging, as Muscodor spp. do not produce sporogenous structures. Therefore, phylogenetic analyses are essential tool for identification of Muscodor. Owing to the lack of informative sequences of the reference species except for ITS, the ITS sequence analysis in this study was carried out and was able to identify the tested strains as Muscodor, and support the strains as belonging to five different Muscodor species. Nonetheless, ITS is not diverged enough to informatively discern the phylogenetic relationships among Muscodor species. In a study on xylariaceous fungi, Jaklitsch & Voglmayr interpreted the phylogenetic relationships of five genera from Xylariales, employing ITS, LSU, rp2, and tef1 (Jaklitsch & Voglmayr, 2012). Thus, further multigene phylogenetic analyses are required for interpretation of the phylogenetic relationships among Muscodor species and designation species names for these strains.

We found that the Y-L-54 and Y-S-35 strains, which belong to different Muscodor species, produce the most similar VOC components. On the other hand, N-L-7, Y-L-43, and W-S-35, which are strains of the same Muscodor species, produce different VOC components. This indicates that the VOCs might not be correlated with their phylogenetic assignment, at least among these Muscodor strains. Thus, the five clusters were divided with a dashed line according to antifungal activity (Figure 3). Cluster A (strains Y-L-54, Y-S-35, W-T-27, and W-S-41) and cluster D (strain Y-L-56), which showed the strongest antifungal activity. Cluster C (strain W-S-35), which showed stronger antifungal activity. Cluster B (strain W-S-38) and Cluster E (strains N-L-7 and Y-L-43) showed weak or the weakest antifungal activity. The peak area percentage of VOC components for the five clusters was obtained (Figure 4), and the specific compounds (com1, com3, com5, com6, com8, com 9, com 10, com 12, com22, com25, and com35) in the clusters marked with varying shades of blue might be responsible for antifungal activity, and these compounds are mostly esters, alcohols, and small molecular weight acids, in particular, methyl isobutyrate and 2-methylpropanoic acid, which were produced by all highly active strains (strains W-S-41, Y-L-54, Y-S-35, W-T-27, Y-L-56, and W-S-35). The most active group of compounds identified in this study are consistent with the previous study that the esters, alcohols, and acids produced by Muscodor spp. are remarkable bioactive properties (Mitchel et al., 2008; Strobel et al., 2001).

This study found that Muscodor endophytes are widespread in tropical gramineous plants and a number of strains can inhibit or kill a variety of plant pathogens. Therefore, they have important potential applications in the fields of plant disease control. Moreover, endophytic Muscodor species interacting with host plants, possibly leads to the change in VOCs of the host plants. Different VOCs have different biological effects; for example, methyl isobutyrate and 2-methylpropanoic acid have stronger antifungal activity. On the other hand, caryophyllene has been shown to attract insects (Xiao et al., 2012). Thus, the ecological effects of Muscodor endophytes and plant interactions deserve further study.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interest.

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