Agr. Nat. Resour. 56 (2022) 899–908

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Volatile organic compounds of *Streptomyces* sp. GMR22 inhibit growth of two plant pathogenic fungi

Eni Dwi Islamiati[a,b,†], Jaka Widada[a,c,†], Tutik Dwi Wahyuningsih[a,d,†], Donny Widianto[a,c,†,*]

[a] *Biotechnology Study Program, Graduate School of Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

[b] *Department of Agroindustrial Technology, Faculty of Technical and Computer Science, Universitas Nahdlatul Ulama, Jakarta 10320, Indonesia*

[c] *Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

[d] *Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

Article Info

Article history:
Received 21 April 2021
Revised 20 July 2022
Accepted 21 July 2022
Available online 12 October 2022

Keywords:
Antifungal activity,
Fusarium oxysporum,
Ganoderma boninense,
Streptomyces sp. GMR22,
VOCs.

Abstract

Importance of the work: Characterization of *Streptomyces* sp. GMR22 volatile organic compounds (VOCs) will improve their application as antifungal compounds against plant pathogenic fungi.

Objectives: To identify the VOCs of *Streptomyces* sp. GMR22 and to assess their bioactive ability against plant pathogenic fungi.

Materials & Methods: The VOCs were analyzed using solid phase microextraction-gas chromatography mass spectrometry (SPME-GCMS). Molecular docking was carried out to predict and select the VOCs as antifungal candidates. The inhibitory effects of selected compounds were tested against *Fusarium oxysporum* and *Ganoderma boninense*. Their hyphal morphology was observed using scanning electron microscopy (SEM).

Results: Based on the SPME-GCMS chromatogram results, *Streptomyces* sp. GMR22 produced 43 VOCs, with one of them (longifolene) having a required binding energy of -7.08 kcal/mol on beta-tubulin, as well as the lowest half maximal inhibitory concentration) of 9.62 ± 0.5 µL/mL and 7.50 ± 0.9 µL/mL for *F. oxysporum* and *G. boninense*, respectively. Furthermore, the compound suppressed the growth of *F. oxysporum* and *G. boninense* on agar surfaces by 62.5% and 55%, respectively. The SEM examination revealed that the hyphae of both fungi appeared wrinkled.

Main finding: Longifolene was considered the most likely VOC of *Streptomyces* sp. GMR22 as an effective antifungal compound. Therefore, VOCs, especially longifolene, may be highly beneficial in agriculture.

† Equal contribution.
* Corresponding author.
E-mail address: donny@ugm.ac.id (D. Widianto)

https://doi.org/10.34044/j.anres.2022.56.5.04

Introduction

Streptomyces sp. GMR22 is an actinobacterium derived from the rhizosphere of eucalypt stands in Wanagama Forest, Gunung Kidul, Yogyakarta, Indonesia (Nurjasmi et al., 2009). Alimuddin et al. (2011) reported that the strain had potent antifungal activity against *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus flavus* and *Fusarium oxysporum*. Furthermore, it has an half minimal inhibitory concentration (IC_{50}) of 62.5 µg/mL against *Candida albicans* (Herdini et al., 2017). *Streptomyces* sp. GMR22 can also produce several secondary metabolites, such as volatile organic compounds (VOCs).

VOCs are characterized by their small molecular weight (< 300 g/mol), low boiling points and high volatility (Vespermann et al., 2007; Effmert et al., 2012) with the latter facilitating their spread through soil pores; consequently, they have a more prolonged interaction distance than metabolites in dissolved form (Westhoff et al., 2017). Chang et al. (2015) showed that they have inhibitory effects on the biosynthesis of DNA, amino acids, and proteins as well as impaired subcellular changes in membrane integrity.

Several studies reported that VOCs produced by *Streptomyces* sp. have antifungal activity (Wang et al., 2013; Wonglom et al., 2019; Lyu et al., 2020; Ayed et al., 2021; Gong et al., 2022). *Sterptomyces alboflavus* TD-1 produced 2-methylisorbeneol, which inhibits the growth of *A. flavus*, *A. ochraceus*, *A. niger* and *Penicillium citrinum* (Wang et al., 2013). *Streptomyces angustmyceticus* NR8-2, *S. yanglinensis* 3-10 and *Streptomyces* sp. strain S97, *S. setonii* WY228 were reported to have antifungal activities against *Colletotrichum* sp. and *Curvularia lunata*, *Aspergillus* sp., *Botrytis cinerea* and *Ceratocystis fimbriata,* respectively (Wonglom et al., 2019; Lyu et al., 2020; Ayed et al., 2021; Gong et al., 2022).

Among the various VOCs produced by *Streptomyces* sp. GMR22, the variants with antifungal activity have not been identified; hence, an alternative approach is needed to determine the compounds having such effects. Molecular docking is an *in silico*-based method that has been used for computational simulation (Ferreira et al., 2015; Ferreira and Schapira, 2017). In addition, it can be used to predict binding between drug compounds/ligands and receptors/proteins (Cob-Calan et al., 2019; Ferreira et al., 2015). The method is widely used in the discovery and development of new drugs with greater, significant activity (Ferreira et al., 2015). The assessment of compounds with antifungal activity should target a particular protein that can serve as a receptor and has an essential function for cell growth. The current study aimed to identify the VOCs produced by *Streptomyces* sp. GMR22 and their ability to inhibit the growth of the phytopathogenic fungi, *F. oxysporum* and *G. boninense*.

Materials and Methods

Microorganisms and cultural maintenance

Streptomyces sp. GMR22 was obtained from the Microbiology Laboratory collection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia and was used to produce VOCs. The pathogenic fungi (*F. oxysporum* and *G. boninense*) were collected from the Laboratory of Plant Pests and Diseases, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. *Fusarium oxysporum* and *G. boninense* originated from palm oil and tomato plants, respectively. Then, *Streptomyces* sp. GMR22 was cultured and maintained in the International *Streptomyces* Project 2 (ISP2) (Difco) medium at 28–29°C for 7 d (Wu et al., 2015). The spores were stored in a 20% glycerol solution at -20°C to prepare the stock culture. *Fusarium oxysporum* and *G. boninense* were cultured and maintained in potato dextrose agar (PDA; HiMedia Laboratories Pvt. Ltd.; Mumbai, India) at 28°C.

Effect of volatile organic compounds on F. oxysporum and G. boninense growth

The inhibitory ability assay against the two pathogenic fungi was carried out using the double-dish sets (DDSs) method (Lyu et al., 2020). One part of the Petri dish, containing PDA medium, was inoculated with 10 mm mycelia and then incubated at 28 °C for 1 d. The other part containing ISP2 medium was inoculated with *Streptomyces* sp. GMR22 using the streak-plate method (Cordovez et al., 2015) and incubated at 29 °C for three days. Then, two dishes were arranged face-to-face, sealed with Parafilm and incubated at 28 °C for 5 d. Fungal growth inhibition was examined by measuring the diameter of the fungus growing on the agar surface. For the control treatment, the pathogen was grown alone, and each treatment was carried out in four replications. Student's t-test was performed to determine statistically significant differences compared to controls ($p < 0.05$, $n = 4$).

The inhibitory ability of the actinobacterium against both fungi was calculated using Equation 1:

$$\text{Fungal inhibition} = (DC\text{-}DT)/\ DC \times 100\% \quad (1)$$

where DC is the diameter of the control fungal mycelia and DT is the diameter of the fungal mycelia exposed to the VOCs produced by *Streptomyces* sp. GMR22 (Boukaew et al., 2013).

Ultrastructure analysis of F. oxysporum and G. boninense

The mycelial abnormalities of *F. oxysporum* and *G. boninense* as indications of the effect of the VOCs of *Streptomyces* sp. GMR22 were detected using scanning electron microscopy (SEM). The preparation of the mycelia was done according to the methods described by Boukaew et al (2013). The samples were affixed to SEM stubs, coated with gold and examined using SEM (JSM-6510LA; Jeol Ltd.; Tokyo, Japan). Subsequently, the specimen was dried, coated with pure gold, and then ready to examine using SEM.

Identification of volatile organic compounds using solid-phase microextraction and gas chromatography-mass spectrometry

The analysis of VOCs produced by *Streptomyces* sp. GMR22 was carried out using the solid-phase microextraction and gas chromatography-mass spectrometry (SPME and GCMS) method (Ayed et al., 2021) (GC-Agilent 7890A; MS-Agilent 5975C with triple axis detector, Agilent Technologies, Inc.; CA, USA). In total, 10 µL of spores (1×10^6 spores/mL) were grown on a solid medium in a solid phase microextraction (SPME) vial and the negative control was ISP2 medium without actinobacterial inoculation. Then, the vial was incubated for 7 d at 28 °C. Subsequently, Supelco SPME (Divinylbenzene/Carboxen™/ Polydimethylsiloxane) fiber was used for the extraction of volatile compounds, after which it was inserted into the injector port on the GCMS tool. An HP-5MS column with 30 m length, 250 µm outside diameter and 0.25 µm inside diameter was used in the desorption process at 260 °C for 5 min. Helium gas was used as the mobile phase at a rate of 1 mL/min. The desorption process started with preheating at 40 °C for 5 min, then increasing to 200 °C at a rate of 10 °C/min without holding the temperature. It was increased again to 260 °C at a rate of 25 °C/min and maintained for 5 min. The VOC data were analyzed using the Chemstation E.02.02.1421 software (Agilent, Agilent Technologies, Inc.; CA, USA), while the detected compounds were determined using tentative identification by comparing the mass spectra of each peak with the NIST08 library (MS NIST, 2008).

In silico molecular docking

Molecular docking was used to predict the binding affinity between VOCs and β-tubulin (PDB ID:1JFF: https://www.rcsb.org/3d-view/) as the target protein (Cob-Calan et al., 2019). The proteins and native ligands were separated using the Chimera 1.10.1 software (Pettersen et al., 2004) to provide space for the active sites. The Autodock 4.2 software (Morris et al., 2009) was used for the docking experiment. The method was categorized as valid when the root means square deviation (RMSD) value was < 2Å. The three-dimensional (3D) conformers of the VOCs were obtained from PubChem (Bethesda, MD, USA).

Inhibition concentration assay of volatile organic compounds against F. oxysporum and G. Boninense

Longifolene, β-caryophyllene and dimethyl trisulfide were selected for antifungal activity testing against *F. oxysporum* and *G. boninense*. The compounds were purchased from the Shanghai Macklin Biochemical Co (Shanghai, China). The *in vitro* test was carried out using the method proposed by Lyu et al. (2020) with slight modification. The upper side dish contained the grown fungi, while the tested compounds were put on the bottom dish. The concentrations of compounds used were 2.5 µL/mL, 5.0 µL/mL, 12.5 µL/mL, 25.0 µL/mL, 50.0 µL/mL and 100 µL/mL. Dimethyl sulfoxide was used as the negative control. Subsequently, the Petri dishes were individually sealed with Parafilm and placed in an incubator at 28°C in the dark for 6 d. Inhibition of the VOCs on fungal growth was observed by measuring the diameters of the treatment and control colonies. Each treatment was carried out using four replications. Student's t-test was performed to determine statistically significant differences compared to controls ($p < 0.05$, $n = 4$).

Results and Discussion

Effect of volatile organic compounds on F. oxysporum and G. boninense growth

The results of the DDS assays showed that the growth of *F. oxysporum* and *G. boninense* was inhibited (Fig. 1). There was no direct contact in the DDS assay between the two fungi and the *Streptomyces* sp. GMR22, indicating that inhibition was caused by the presence of VOCs. The measurement results for colony diameters showed that 5 d of exposure to the VOCs inhibited the growth of *F. oxysporum* and *G. boninense* by 62.5% and 55%, respectively.

Several studies have reported the inhibition of VOCs on fungal growth. The reported VOCs were produced from microbes (Fernando et al., 2005; Vespermann et al., 2007; Zheng et al., 2013; Wu et al., 2015; Lyu et al., 2020; Pérez-Corral et al., 2020; Gong et al., 2022; Tang et al., 2020; Ayed et al., 2021), as well as synthetically made (Davidse, 1973; Davidse and Flach, 1978; Zhang et al., 2021; Zhao et al., 2021). Two synthetic VOCs, (methyl 2-benzimidazole carbamate (MBC) and -(4'-thiazolyl) benzimidazole were reported to inhibit fungal DNA and RNA synthesis and mitosis, respectively, in fungal hyphae (Davidse, 1973; Davidse and Flach, 1978). 2-(4'-thiazolyl) Benzimidazole was shown to bind to fungal tubulin that caused mitosis inhibition (Davidse and Flach, 1978). Hollomon et al. (1998) revealed that a VOC (phenyl carbamate) could bind to fungal β-tubulin. Dimethyl trisulfide was reported to inhibit the cell wall biosynthesis genes (Tang et al., 2020). Thus, the fungal growth inhibition might have been be caused by the binding of VOC into β-tubulin, DNA-RNA synthesis inhibition or by affecting the genes involved in cell wall biosynthesis.

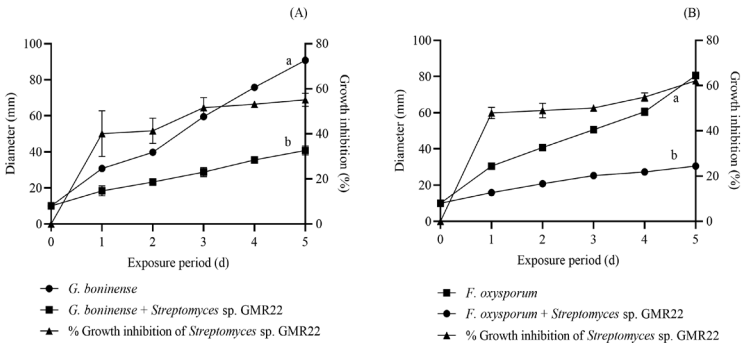

Fig. 1 Inhibition of pathogenic fungal growth by *Streptomyces* sp. GMR22 volatile organic compounds (VOCs): (A) *Ganoderma boninense*; (B) *Fusarium oxysporum*, where growth measured in absence or presence of VOCs, curves with different lowercase letters are significantly ($p < 0.05$) different

Ultrastructure analysis of F. oxysporum and G. boninense

The ultrastructural observations showed that the mycelia of the two pathogenic fungi were substantially damaged. Furthermore, Fig. 2 shows that the damage to the hyphae of *F. oxysporum* and *G. boninense* was caused by exposure to VOCs. The exposed parts were shriveled and incomplete, with their growth morphologically stunted, whereas in the control treatment, the hyphae of the two fungi were intact and normal. In addition, the volatile compounds affected the production of pigments by *F. oxysporum*.

The antifungal effect on the cell wall might have been related to the inhibition of β-glucan synthase and chitin synthase, as reported by Tang et al. (2020) using dimethyl trisulfide as an antifungal VOC. Other studies reported that the blockage of β-glucan synthase caused a decrease in the incorporation of the glucose monomers that linked b-1,3 and b-1,6 glucans, leading to weakening of the cell wall, which then causes fungal cell lysis (Katarzyna et al., 2019; Legentil et al., 2015). Chitin synthase is a protein complex responsible for the elongation of the chitin chain, which is an essential component of the fungal cell wall (Tang et al., 2020). Another plausible explanation is that the VOCs might have inhibited the fungal growth tip through binding to β-tubulin (Davidse and Flach, 1978; Hollomon et al., 1998), which is responsible for microtubule synthesis (Horio and Oakley, 2005; Takeshita et al., 2014).

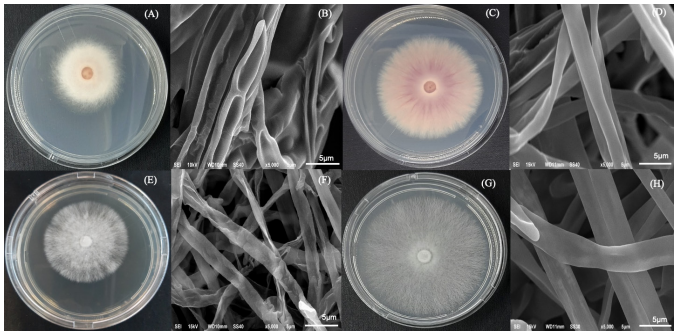

Fig. 2 Colony and hyphal morphology affected by *Streptomyces* sp. GMR22 volatile organic compounds (VOCs): (A) *Fusarium oxysporum* colony after exposure to VOCs; (B) *F. oxysporum* hyphae after exposure to VOCs; (C) *F. oxysporum* colony before exposure to VOCs; (D) *F. oxysporum* hyphae before exposure to VOCs; (E) *Ganoderma boninense* colony after exposure to VOCs; (F) *G. boninense* hyphae after exposure to VOCs; (G) *G. boninense* colony before exposure to VOCs; (H) *G. boninense* hyphae before exposure to VOCs

Identification of volatile organic compounds produced by Streptomyces sp. GMR22

The VOCs produced were diverse and included hydrocarbons, benzenoids, terpenoids, monoterpenoids, sulfur, dihydropyranone and sesquiterpenoids, as shown in Table 1. Furthermore, the dominant compounds were sesquiterpenoid, terpenoid and hydrocarbon in proportions of 58%, 11.6%, and 6.9%, respectively. The results of GCMS analysis indicated that *Streptomyces* sp. GMR22 could produce 43 VOCs.

Table 1 Detected compounds of volatile organic compounds (VOCs) produced by *Streptomyces* sp. GMR22

Retention time (min)	Compound	Class of VOC	Relative area (%)
2.1508	Methylhexanamine	Monoterpenes	0.56
3.3875	2-Methyl-1-butanol	Hydrocarbon	0.78
9.5595	2-Methylenebornane	Terpenoids	0.17
10.1040	Dimethyl trisulfide	Organic trisulfides	0.14
10.4276	2,4 Di-tert-butylphenol	Benzenoids	8.23
10.6119	Eucarvone	monoterpenoids	4.01
11.9022	2-Methyl-2-bornene	Terpenoids	47.91
15.5292	Phenylethyl alcohol	Benzenoids	0.28
15.6897	Benzyl hydrazine	Benzenoids	0.26
16.0643	6-Camphenone	Terpenoids	0.34
18.1038	2-Methylisoborneol	Terpenoids	22.55
18.1620	2H-Pyran	Dihydropyranones	0.35
18.9303	α-Campholenal	Monoterpenoids	0.24
19.8638	1H-indene	Benzenoids	0.91
20.0243	α-Farnesene	Sesquiterpenoids	0.14
20.3335	1-Ethylidene	Hydrocarbon	0.13
20.4822	Santolina triene	Hydrocarbon	0.29
22.3552	β-Chamigrene	Sesquiterpenoids	0.12
23.9690	Longifolene	Sesquiterpenoids	1.01
23.8952	α-Cubebene	Sesquiterpenoids	0.16
24.5968	Ylangene	Sesquiterpenoids	0.13
24.7455	α-Copaene	Sesquiterpenoids	0.24
25.2984	β-Elemene	Sesquiterpenoids	0.54
25.5660	Geosmin	Terpenoids	2.15
26.1190	β-Copaene	Sesquiterpenoids	0.20
26.4400	β-Cubebene	Sesquiterpenoids	0.15
26.7671	β-Caryophyllene	Sesquiterpenoids	0.22
27.3141	Eremophilene	Sesquiterpenoids	1.17
27.4509	γ-Gurjunene	Sesquiterpenoids	0.34
27.5638	Ginsenol	Sesquiterpenoids	0.29
27.8671	Cadina-1(6),4-diene	Sesquiterpenoids	0.59
27.9563	γ-Muurolene	Sesquiterpenoids	0.85
28.0752	Germacrene D	Sesquiterpenoids	1.16
28.2238	α-Selinene	Sesquiterpenoids	0.13
28.5033	Cyclosativene	Sesquiterpenoids	0.68
28.6282	β-Maaliene	Sesquiterpenoids	0.66
28.6520	α-Muurolene	Sesquiterpenoids	0.37
29.0920	γ-Cadinene	Sesquiterpenoids	0.83
29.3893	β-Cadinene	Sesquiterpenoids	1.83
29.6390	Cubebene	Sesquiterpenoids	0.12
29.7936	α-Cadinene	Sesquiterpenoids	0.32
29.9660	α-Calacorene	Sesquiterpenoids	0.21
32.8320	Guai-1(10)-en-11-ol	Sesquiterpenoids	0.39

The most abundant compounds identified in this study were 47.91% 2-methyl-2-bornene, 22.55% 2-methylisoborneol (2-MIB), 8.23% 2,4-di-tert-butylphenol, 4.01% eucarvone and 2.15% geosmin. Among these, 2-MIB, 2-tert-butylphenol, and geosmin have been reported to have antifungal activity against *F. moniliforme* Sheldon, *F. oxysporum* and *Mucor circinelloides*, respectively (Wang et al., 2013; Dharni et al., 2014; Du et al., 2015). However, no published report was identified of 2-methyl-2-bornene as an antifungal compound.

Some other VOCs identified in this study (longifolene, β-caryophyllene, dimethyl trisulfide and α-farnesene) have been reported as antifungals even though their percentages were much lower than those of the abundant compounds. Longifolene inhibited the growth of *Trametes versicolor, Lenzites betulinus, Gloeophyllum trabeum, Trichoderma virens* and *Rhizopus oryzae* (Mukai et al., 2018). The β-caryophyllene of *S. yanglinensis* could inhibit the growth of *A. flavus* and *A. parasiticus* (Passone and Etcheverry, 2014). The antifungal activity of dimethyl trisulfide against *C. gloeosporioides* was demonstrated by Tang et al. (2020). Siddiqui et al. (2017) reported that α-farnesene has great potential antifungal activity because it can inhibit the mycelial growth of various phytopathogenic fungi, such as *Rhizoctonia solani*, *Pythium graminicola*, *Trichoderma harzianum* and *Fusarium oxysporum.*

Analysis of molecular docking on β-tubulin target protein

The taxol binding site on β-tubulin was validated by re-docking using taxol as the native ligand with the co-crystallized (PDB ID: 1JFF). An RMSD value less than 2 Å was used to determine the best docking position between β -tubulin and taxol. The value of RMSD obtained was 1.71 Å (Fig. 3A) which is smaller than 2 Å, indicating that re-docking was qualified. Two types of bonds were detected in the *β-tubulin* -taxol docking complex (Fig. 3B). These bonds were Van der Waals interaction, conventional hydrogen bonds, π-sulfur bonds, π-sigma bonds, π-lone pair bonds, π-stacked and π-alkyl bonds. Van der Waals interactions were observed at 10 amino acid positions (Pro-274, Pro-360, Ser-236, Ser-277, Phe-272, Leu-275, Leu-371, Gln-281, Gln-282 and Asp-226), as shown in Fig. 3. Hydrogen bonds were observed at Thr-276 and Gly-370.

The analysis of docking between the ligand and β-tubulin showed a high-affinity value, when the negative dock value was increased (Du et al., 2016). The docking results showed that longifolene has the lowest binding energy for β-tubulin (-7.08 kcal/mol), as shown in Table 2, which was lower than that of taxol. Three types of strong bonds were detected in the *β-tubulin*-longifolene docking complex (Fig. 4A). These bonds were Van der Waals interactions, alkyl bonds and π-alkyl bonds. Van der Waals interactions were observed at five amino acid positions (Ser-236, His-229, Asp-26, Glu-27 and Arg-320), as shown in Fig. 4. Longifolene had similar binding site interaction bonds with taxol at Ser-236 and Asp-226. β-Caryophyllene and dimethyl trisulfide have higher binding energies than longifolene and so were chosen for β-tubulin ligand docking analyses to compare their binding affinity. The results showed that β-caryophyllene–β-tubulin had similar binding sites and interaction bonds with taxol at Leu-227 (Fig. 4B) and with dimethyl trisulfide at Ser-236 (Fig. 4C).

The interaction of the ligand with a protein through hydrogen bond interactions or Van der Waal interactions contributed to stable ligand-protein complexes (Ferreira and Schapira, 2017), which might lead to stronger antimicrobial activity. Consequently longifolene, β-caryophyllen and dimethyl trisulfide may have antifungal activities.

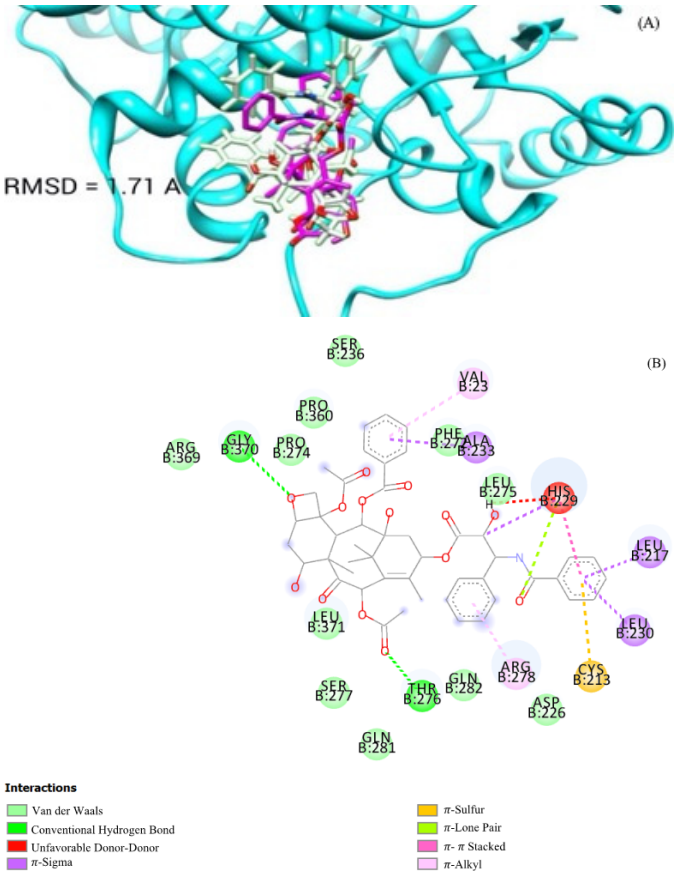

Fig. 3 Active binding sites of taxol to β-tubulin protein: (A) co-crystalized native taxol (pink) and taxol as posed by the AutoDockTools 1.5.6 program (white); (B) 2-dimensional visualization of taxol and β-tubulin interaction, RMSD = root mean square error

Table 2 Binding energy between β-tubulin and taxol and volatile organic compounds as test of ligands

Compound	Molecular weight (g/mol)	No. of H bonds	Binding energy (kcal/mol)
Taxol	853.92	1	-6.44
Longifolene	204.35	-	-7.08
β-Caryophyllene	204.35	-	-6.79
Cubebene	204.35	-	-6.48
β-Cubebene	204.35	-	-6.48
Germacrene D	204.35	-	-6.43
γ-Muurolene	204.35	-	-6.32
β-Cadinene	204.35	-	-6.21
α-Cubebene	204.35	-	-6.11
Eremophilene	204.35	-	-6.06
β-Copaene	204.35	-	-6.00
Ginsenol	222.37	-	-5.97
α-Copaene	204.35	-	-5.95
Cyclosativene	204.35	-	-5.95
Ylangene	204.35	-	-5.93
α-Muurolene	204.35	-	-5.92
Cadina-1(6),4-diene	204.35	-	-5.91
γ-Cadinene	204.35	-	-5.91
1-Ethylidene	142.20	-	-5.90
γ-Gurjunene	204.35	-	-5.90
β-Maaliene	204.35	-	-5.88
α-Selinene	204.35	-	-5.83
β-Chamigrene	204.35	-	-5.72
α-Calacorene	200.32	-	-5.71
β-Elemene	204.35	-	-5.69
α-Cadinene	204.35	-	-5.63
1-Ethylideneoctahydro	164.29	-	-5.50
α-Farnesene	204.36	-	-5.49
Geosmin	182.30	2	-5.05
6-Camphenone	150.21	-	-4.92
Eucarvone	150.22	-	-4.84
2-Methylisoborneol	168.28	1	-4.58
α-Campholenal	152.23	-	-4.54
2.4-Di-tert-butylphenol	150.22	2	-4.49
2-Methyl-2-bornene	150.26	-	-4.35
2-Methylenebornane	150.26	-	-4.31
Santolina triene	136.23	-	-4.00
Phenylethyl Alcohol	122.16	1	-3.67
Methylhexaneamine	115.22	-	-3.58
2-Methyl-1-butanol	88.15	-	-2.99
2H-Pyran	82.10	-	-2.56
Benzylhydrazine	122.17	-	-2.56
Guai-1(10)-en-11-ol	122.17	-	-2.55
Dimethyl trisulfide	126.26	-	-2.50

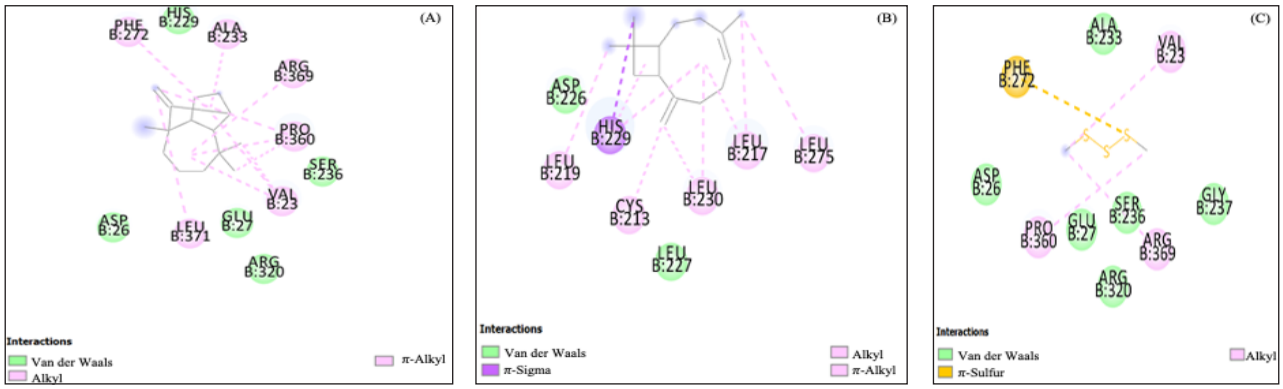

Fig. 4 Two-dimensional visualization of ligands and β-tubulin protein interaction: (A) longifolene; (B) β-caryophyllene; (C) dimethyl trisulfide

Inhibition concentration assay of volatile organic compounds against F. oxysporum and G. boninense

Longifolene, β-caryophyllene and dimethyl trisulfide were selected for antifungal activity testing against *F. oxysporum* and *G. boninense*. Longifolene and β-caryophyllene were used since their binding energies were lower than taxol; therefore, both compounds were predicted to be able to compete with taxol in binding to the β-tubulin. Dimethyl trisulfide was used as a control because it had a higher binding energy than taxol (Table 2). The results showed that longifolene had the strongest ability to inhibit *F. oxysporum and G. boninense,* with mean IC_{50} values of 9.62 ± 0.5 µL/mL and 7.50 ± 0.9 µL/mL, respectively (Table 3). It can be inferred from these results that longifolene might be the most effective VOC of *Streptomyces* sp. GMR22 as an antifungal compound.

Table 3 Maximal half inhibitory concentration (IC_{50}) for two tested fungi

Compound	IC_{50} (µL/mL) on mycelial growth	
	Fusarium oxysporum	*Ganoderma boninense*
Longifolene	9.62±0.5	7.50±0.9
β-Caryophyllene	32.04±0.2	55.20±0.01
Dimethyl trisulfide	80.00±0.02	75.50±4.4
Dimethyl sulfoxide	ni*	ni*

ni = no inhibition at 100 µL/mL
Values are mean ± SD, $n = 4$ in each group

Longifolene is a sesquiterpene as well as the primary component of *Pinus densiflora* and *Pinus thunbergia* heartwood and has antifungal activity (Mukai et al., 2018). The current study confirmed the antifungal activity of longifolene. Although several studies reported that it has anti-bacterial activity (Hassan et al., 2020; Zhang et al., 2021); however, its antifungal activity has not yet been fully explored. In addition, longifolene has been reported to have the termiticidal and anti-tick properties (Hassan et al., 2020; Zhang et al., 2021) and is commercially available as a termiticide. However, commercial fungicides containing longifolene as an active ingredient are not yet available. Since, *Streptomyces* sp. GMR22 can also produce longifolene, this actinobacterium could be considered for use as a fungal biocontrol in agriculture. The ability of *Streptomyces* sp. GMR22 to overproduce longifolene should be investigated so that this strain could be applied as an effective fungal biocontrol agent.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was funded by Program Rekognisi Tugas Akhir Universitas Gadjah Mada (Grant No. 2488/ UN1.P.III/ DIT-LIT/PT/2020). Dr Ahmad Suparmin assisted with editing the manuscript.

References

Alimuddin, A., Widada, J., Asmara, W., Mustofa. 2011. Antifungal production of a strain of *Actinomycetes* spp. isolated from the rhizosphere of Cajuput plant: Selection and detection of exhibiting activity against tested fungi. I. J. Biotech. 16: 1–10.

Ayed, A., Kalai-Grami, L., Ben Slimene, I., et al. 2021. Antifungal activity of volatile organic compounds from *Streptomyces* sp. strain S97 against *Botrytis cinerea*. Biocontrol. Sci. Technol. 31: 1330–1348. doi.org/10.1080/09583157.2021.1947982

Boukaew, S., Plubrukam, A., Prasertsan, P. 2013. Effect of volatile substances from *Streptomyces philanthi* RM-1-138 on growth of *Rhizoctonia solani* on rice leaf. BioControl 4: 471–482. doi.org/10.1007/s10526-013-9510-6

Chang, P.K., Hua, S.S.T., Sarreal, S.B.L., Li, R.W. 2015. Suppression of aflatoxin biosynthesis in *Aspergillus flavus* by 2-phenylethanol is associated with stimulated growth and decreased degradation of branched-chain amino acids. Toxins 7: 3887–3902. doi.org/10.3390/toxins7103887

Cob-Calan, N.N., Chi-Uluac, L.A., Ortiz-Chi, F., Cerqueda-García, D., Navarrete-Vázquez, G., Ruiz-Sánchez, E., Hernández-Núñez, E. 2019. Molecular docking and dynamics simulation of protein β-tubulin and antifungal cyclic lipopeptides. Molecules 24: 3387. doi.org/10.3390/molecules24183387

Cordovez V, Carrion VJ, Etalo DW et al. Diversity and functions of volatile organic compounds produced by Streptomyces from a disease-suppressive soil. Front. Microbiol. 2015: 6: 1–13. doi.org/10.3389/fmicb.2015.

Davidse, L.C. 1973. Antimitotic activity of methyl benzimidazol-2-yl carbamate (MBC) in *Aspergillus nidulans*. Pestic. Biochem. Phys. 3: 317–325. doi.org/10.1016/0048-3575(73)90030-8

Davidse, L.C., Flach, W. 1978. Interaction of thiabendazole with fungal tubulin. BBA-GEN Subjects 534: 82–90. doi.org/10.1016/0304-4165(78)90456-7

Dharni, S., Sanchita, S., Maurya, A., Samad, A., Srivastava, S.K., Sharma, A., Patra, D.D. 2014. Purification, characterization, and in vitro activity of 2,4-Di- tert -butylphenol from *Pseudomonas monteilii* psf84: Conformational and molecular docking studies. J. Agric. Food. Chem. 62: 6138–6146.

Du, H., Lu, H., Xu, Y. 2015. Influence of geosmin-producing *Streptomyces* on the growth and volatile metabolites of yeasts during Chinese liquor fermentation. J. Agric. Food Chem. 63: 290–296. doi.org/10.1021/jf503351w

Du, X., Li, Y., Xia, Y.L., Ai, S.M., Liang, J., Sang, P., Ji, X.L., Liu, S.Q. 2016. Insights into protein-ligand interactions: mechanisms, models, and methods. Int. J. Mol. Sci. 17: 144. doi.org/10.3390/ijms17020144

Effmert, U., Kalderás, J., Warnke, R., Piechulla, B. 2012. Volatile mediated interactions between bacteria and fungi in the soil. J. Chem. Ecol. 38: 665–703. doi.org/10.1007/s10886-012-0135-5

Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S., Savchuk, S.C. 2005. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil. Biol. Biochem. 37: 955–964. doi.org/10.1016/j.soilbio.2004.10.021

Ferreira, L., dos Santos, R., Oliva, G., Andricopulo, A. 2015. Molecular docking and structure-based drug design strategies. Molecules 20: 13384–13421. doi.org/10.3390/molecules200713384

Ferreira de Freitas, R., Schapira, M. 2017. A systematic analysis of atomic protein-ligand interactions in the PDB. MedChemCommun 8: 1970–1981.

Gong, Y., Liu, J.Q., Xu, M.J., Zhang, C.M., Gao, J., Li, C.G., Xing, K., Qin, S. 2022. Antifungal volatile organic compounds from *Streptomyces setonii* WY228 control black spot disease of sweet potato. Appl. Environ. Microbiol. 88: e023117-21. doi.org/10.1128/aem.02317-21

Hassan, J.u., Kaleem, I., Rasool, A., Xu, K., Tahir, R.A., Lv, B., Li, C. 2020. Engineered *Saccharomyces cerevisiae* for the de novo synthesis of the aroma compound longifolene. Chem. Eng. Sci. 226: 115799. doi.org/10.1016/j.ces.2020.115799

Herdini, C., Mubarika, S., Hariwiyanto, B., Wijayanti, N., Hosoyama, A., Yamazoe, A., Nojiri, H., Widada, J. 2017. Secondary bioactive metabolite gene clusters identification of anticandida-producing *Streptomyces* sp. GMR22 isolated from Wanagama Forest as revealed by genome mining approach. Indonesian J. Pharm. 28: 26–33.

Hollomon, D.W., Butters, J.A., Barker, H., Hall, L. 1998. Fungal β-tubulin, expressed as a fusion protein, binds benzimidazole and phenylcarbamate fungicides. Antimicrob. Agents Chemother. 42: 2171–2173. doi.org/10.1128/AAC.42.9.2171

Horio, T., Oakley, B.R. 2005. The role of microtubules in rapid hyphal tip growth of *Aspergillus nidulans*. Mol. Biol. Cell 16: 918–926. doi.org/10.1091/mbc.e04-09-0798

Katarzyna, Z., Wiater, A., Waśko, A., Pleszczyńska, M., Paduch, R., Jaroszuk-Ściseł, J., Bieganowski, A. 2019. A report on fungal (1→3)-α-D-glucans: Properties, functions and application. Molecules 24: 3927. doi.org/10.3390/molecules24213972

Legentil, L., Paris, F., Ballet, C., Trouvelot, S., Xavier, D., Vetvicka, V., Ferrieres, V. 2015. Molecular interactions of β-(1→3)-glucans with their receptors. Molecules 20: 9745–9766. doi.org/10.3390/molecules20069745

Lyu, A., Yang, L., Wu, M., Zhang, J., Li, G. 2020. High efficacy of the volatile organic compounds of *Streptomyces yanglinensis* 3-10 in suppression of *Aspergillus* contamination on peanut kernels. Front. Microbiol. 11: 142. doi: 10.3389/fmicb.2020.00142

Mukai, A., Takahashi, K., Ashitani, T. 2018. Antifungal activity of longifolene and its autoxidation products. Eur. J. Wood Wood Prod. 76: 1079–1082. doi.org/10.1007/s00107-017-1281-9

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S. and Olson, A. J. (2009) Autodock4 and AutoDockTools4: automated docking with selective receptor flexiblity. J. Computational Chemistry 2009, 16: 2785-91. doi: 10.1002/jcc.21256

NIST MS Search User Guide. 2008. National Institute of Standards and Technology: Gaithersburg.

Nurjasmi, R., Widada, J., Ngadiman, N. 2009. Diversity of actinomycetes at several forest types in Wanagama I Yogyakarta and their potency as a producer of antifungal compound. I. J. Biotechnol. 14: 1196–1205.

Passone, M.A., Etcheverry, M. 2014. Antifungal impact of volatile fractions of *Peumus boldus* and *Lippia turbinata* on *Aspergillus* section *Flavi* and residual levels of these oils in irradiated peanut. Int. J. Food Microbiol. 168–169: 17–23. doi.org/10.1016/j.ijfoodmicro.2013.10.009

Pérez-Corral, D.A., Ornelas-Paz, J.D.J., Olivas-Orozco, G.I., et al. 2020. Antagonistic effect of volatile and non-volatile compounds from *Streptomyces* strains on cultures of several phytopathogenic fungi. Emir. J. Food. Agric. 32: 879–889. doi.org/10.9755/ejfa.2020.v32.i12.2222

Pettersen, E.F, Goddard, T.D, Huang, C.C, Couch, G.S, Greenblatt, D.M, Meng, E.C, Ferrin T.E. 2004. UCSF Chimera–A visualization system for exploratory research and analysis. J. Comput Chem. 25:1605–1612. doi.org/10.1002/jcc.20084

Siddiqui, S.A., Islam, R., Islam, R., Jamal, A.H.M., Parvin, T., Rahman, A. 2017. Chemical composition and antifungal properties of the essential oil and various extracts of *Mikania scandens* (L.) Willd. Arab. J. Chem. 10: S2170–S2174. doi.org/10.1016/j.arabjc.2013.07.050

Takeshita, N., Manck, R., Grün, N., de Vega, S.H., Fischer, R. 2014. Interdependence of the actin and the microtubule cytoskeleton during fungal growth. Curr. Opin. Microbiol. 20: 34–41. doi.org/10.1016/j.mib.2014.04.005

Tang, L., Mo, J., Guo, T., Huang, S., Li, Q., Ning, P., Hsiang, T. 2020. *In vitro* antifungal activity of dimethyl trisulfide against *Colletotrichum gloeosporioides* from mango. World. J. Microbiol. Biotechnol. 36: 1–15. doi.org/10.1007/s11274-019-2781-z

Vespermann, A., Kai, M., Piechulla, B. 2007. Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Appl. Environ. Microbiol. 73: 5639–5641. doi.org/10.1128/AEM.01078-07

Wang, C., Wang, Z., Qiao, X., Li, Z., Li, F., Chen, M., Wang, Y., Huang, Y., Cui, H. 2013. Antifungal activity of volatile organic compounds from *Streptomyces alboflavus* TD-1. FEMS Microbiol. Lett. 341: 45–51. doi.org/10.1111/1574-6968.12088

Westhoff, S., Wezel, G.P.v., Rozen, D.E. 2017. Distance-dependent danger responses in bacteria. Curr. Opin. Microbiol. 36: 95–101. doi.org/10.1016/j.mib.2017.02.002

Wonglom, P., Suwannarach, N., Lumyong, S., Ito, S., Matsui, K., Sunpapao, A. 2019. *Streptomyces angustmyceticus* NR8-2 as a potential microorganism for the biological control of leaf spots of *Brassica rapa* subsp. *pekinensis* caused by *Colletotrichum* sp. and *Curvularia lunata*. Biol. Control 138: 104046. doi.org/10.1016/j.biocontrol.2019.104046

Wu, Y., Yuan, J., Yaoyao, E., Raza, W., Shen, Q., Huang, Q. 2015. Effects of volatile organic compounds from *Streptomyces albulus* NJZJSA2 on growth of two fungal pathogens. J. Basic Microbiol. 55: 1104–1117. doi.org/10.1002/jobm.201400906

Zhang, Q., Lin, G., Duan, W., Zhao, S., He, J., Lei, F. 2021. Synthesis, antifungal activity and 3D-QSAR study of novel (*E*)-longifolene-derived tetralone oxime ethers. ChemistrySelect 6: 4515–4520. doi.org/10.1002/slct.202100898

Zhao, S., Lin, G., Duan, W., Zhang, Q., Huang, Y., Lei, F. 2021. Design, synthesis, and antifungal activity of novel longifolene-derived diacylhydrazine compounds. ACS Omega 6: 9104–9111. doi.org/10.1021/acsomega.1c00217

Zheng, M., Shi, J., Shi, J., Wang, Q., Li, Y. 2013. Antimicrobial effects of volatiles produced by two antagonistic *Bacillus* strains on the anthracnose pathogen in postharvest mangos. Biol. Control. 65: 200–206. doi.org/10.1016/j.biocontrol.2013.02.004