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Endophytic Fungi Isolated from the Mangrove Species *Rhizophora apiculata* and Their Efficacy as Herbicides

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ABSTRACT

Endophytic fungi play an important role on mangrove growth and development, however research on the endophytic fungi of the mangrove *Rhizophora apiculata* is limited. The endophytic fungi produce diverse bioactive compounds involved in the mangrove's adaptation to varied biotic and abiotic stresses and could have applied uses in agriculture or medicine. The purpose of this research was to isolate endophytic fungi of *Rhizophora apiculata* mangrove and to study their activity as herbicides on the weed *Gomphrena globosa*. The fungi were isolated using the surface sterilization method and identified based on morphological characteristics and molecular characteristics using ITS regions of rDNA. Herbicidal activity of the fungal filtrates extracted by ethyl acetate were tested on seed germination and seedling growth of *G. globosa*. Five fungal isolates were obtained, namely *Penicillium citrinum*, *Diaporthe eucalyptorum*, *Diaporthe musigena*, *Colletotrichum queenslandicum*, and *Diaporthe tectonae*. All isolates were able to grow on PDA medium containing 0, 25, 50, 75, and 100 (% v/v) seawater concentrations, but the growth rate varied by species and seawater concentration. In general, all five isolates showed herbicidal activity by delaying seed germination and reducing shoot and root growth. *P. citrinum* showed the highest herbicidal activity compared to the other isolates. Analysis using Gas Chromatography-Mass Spectrometry of the crude extract of *P. citrinum* filtrate identified 7 main compounds: 3-Methoxy-2-methyl-cyclohex-2-enone, Cyclohexane-carbohexaldehyde, 6-methyl-3-(1-methylethyl)-2-oxo, Cyclopropane carboxylic acid, 1-(2-propenyl)-1,1-dimethylethyl ester, 2-Hydroxy-4-isopropyl-7-methoxytropone, Beta-Asarone, Oxane, 4-(2-amino-1-hydroxyethyl)-4-(3,4-dimethoxyphenyl)-, and Diisooctyl-phthalate. These compounds should be studied further to determine which ones are responsible for the herbicidal activity.

1. Introduction

Mangroves are coastal plants able to grow in saline and anoxic soils in climates that have a combination of high temperature and irradiation (Stewart and Popp 1987). Mangroves are able to grow in these extreme environments because they form a mutualistic symbiosis with diverse endophytic fungi. Endophytic fungi spend all or part of their lifecycle inside a healthy host plant without causing disease symptoms (Zhao *et al.* 2010). The endophytic fungi associated with mangroves are reported as the second largest ecological group of marine fungi. Furthermore, endophytic fungi produce diverse bioactive compounds that play a role in mangrove adaptation to various biotic and abiotic stresses (Shearer 2002). The bioactive compounds produced by endophytic fungi can be used in agriculture, medicine, and food industry development (Gunatilaka 2006; Strobel *et al.* 2004;

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Verma *et al.* 2009). The bioactive compounds derived from endophytic fungi can be used as herbicides in agriculture. For example, a bioactive compound from the endophytic fungus *Edeniagomez pompae* isolated from *Callicarpa acuminata* host plants inhibited seed germination, root length, and seed respiration of *Amaranthus hypochondriacus, Solanum lycopersicum*, and *Echinochloa crus-galli* weeds (Marcias-Rubalcava *et al.* 2014).

Weeds can reduce the quantity and quality of agriculture production significantly. Weeds continue to cause yield losses ranging from 10-60% depending on the crop and environment (Yaduraju 2013). World losses due to weeds in rice are estimated at 15% of world rice production (Smith 1983). In Indonesia, the reduction of agriculture production due to weeds is estimated at 10-20% (Solahudin *et al.* 2010). *G. globosa* is an important and cosmopolitan weed from the Amaranthaceae family that can grow on dry and saline soils (Borsch *et al.* 2001; Judd *et al.* 1999). Species of Amaranthaceae are also reported as alien invasive plants in different countries such as Indonesia, Uzbekistan (Kurbonovich 2017) and the United States (Schwartz *et al.* 2016). In Indonesia they are particularly important weeds in sweet corn cultivation (Asih *et al.* 2018; Tjitrosoedirdjo 2005). *G. globosa* is also host of the polyphagous *Liriomyza huidobrensis* (Blanchard). This polyphagous insect is a vector of the Tobacco Mosaic Virus disease (TMV) (Harris 1981), and they infect important agricultural crops such as many bean varieties (Baliadi and Tengkano 2010). Weeds from Amaranthaceae are reported to develop herbicide resistance. They develop inhibitor resistance in photosystem II (PSII) and proto-porphyrinogen oxidase (PPO) in photosynthesis, acetolactate synthase (ALS) in cellular respiration processes, and 5-enolypyruvyl-shikimate-3-phosphate synthase (EPSPS) for the production of aromatic amino acids in plants, which are prime targets for herbicides (Vencill and Grey 2008). Therefore, it is necessary to look for new sources of herbicides to control these weeds.

Mangrove forests are reported to cover 16,530,000 ha globally distributed across Asia (7,441,000 ha), Africa (3,258,000 ha), and the Americas (5,831,000 ha). Mangrove forests in Indonesia represents nearly 50% of the total mangrove forests in Asia, and almost 25% of the world total (Onrizal 2010). In Indonesia, mangroves forests are found in Sumatra, Kalimantan, Papua, Sulawesi, and Java (Pramudji 2000). *Rhizophora apiculata* is one of the dominant mangrove species in Indonesia (Kusmana 2014). It has been reported that *R. apiculata* is host to diverse endophytic fungi. Endophytic fungi *Acremonium* sp., *Cladosporium* sp., *Penicillium* sp., *Pialophora* sp., *Pestalotiopsis* sp., *Phoma* sp., *Chaetomium globosum, Pseudorotium* sp., and *Sporomielia minima* were reported to be isolated from leaves of *R. apiculata* growing in Tamil Nadu, Southern India (Suryanarayanan *et al.* 1998). There are many reports on bioactive compounds from endophytic fungi with anti-microbial activities (Nia *et al.* 2017; Rukachaisirikul 2012). However, the potential use of the fungi as herbicides has not been studied well so far. Therefore, this research was carried out to study the endophytic fungi associated with *R. apiculata* and their potential activity as sources of herbicides.

2. Materials and Methods

2.1. Isolation and Characterization of Endophytic Fungi of *Rhizophora apiculata*

2.1.1. Sampling and Sampling Site

R. apiculata mangrove was obtained from Nirwana Beach, Padang, West Sumatra, Indonesia (01°01'45.4"S and 100°23'03.2"E). The plant organs used for isolation were the leaves, flowers and twigs. The samples were cut using a sterile knife. Each part of the organ was taken separately, stored in a sterile polythene bag on ice during transportation from field to laboratory, and they were immediately used for endophytic fungi isolation after arriving in laboratory (Prihanto *et al.* 2011).

2.1.2. Isolation of Endophytic Fungi

Isolation of endophytic fungi was carried out using the method described by Hallmann *et al.* (2006) with modifications. Leaves, flowers, and twigs from second-order branches of shoots were used for fungal isolation. Each organ was washed in sterile water, then the twigs were cut to 2 cm lengths, the leaves were cut to 2 x 2 cm pieces, and uncut petals were used for fungal isolation from the flowers. Each organ was surface sterilized separately by soaking in 70% ethanol for 1 minute, in 5.3% sodium hypochlorite for 5 minutes, in 70% ethanol for 30 seconds, and then rinsed aseptically using sterile distilled water 3 times. All samples were dried for 6 hours on sterile filter paper in a laminar airflow cabinet. Samples were then further cut into 1 cm long segments for the twigs, and into 1 x 1 cm square pieces for the leaves and

flower petals. Three pieces of each organ were placed on the PDA media that was prepared by using 75% seawater, rose bengal (25 mg/L) as a fungistatic, and chloramphenicol (250 mg/L) as a bactericide in a petri dish (9 cm in diameter). As a negative control, 0.1 ml of sterile distilled water from the final rinse sample was spread over the media. Petri dishes were incubated at 27°C for 7 days. Each treatment was replicated 3 times.

2.1.3. Identification of Endophytic Fungi

Fungal identification was carried out using combined morphological and molecular characteristics. Morphological identification was conducted based on identification keys of Barnett and Hunter (1998) using cultures grown on PDA prepared without seawater. Colony features such as color, growth pattern, and colony texture; and microscopic characteristics such as hyphae, conidiogenous cells, spores and spore ornamentation were recorded.

Molecular identification was carried out using ITS 1-5.8S-ITS 2 region DNA sequences of ribosomal DNA. Primer pairs used for DNA amplification were ITS 1 (5′-TCC-GTA-GGT-GAA-CCT-GCG-G-3′) as forward primer and ITS 4 (5′-TCC-TCC-GCT-TAT-TGA-TAT-GC-3′) as reverse primer (White *et al.* 1990). Fungal DNA was extracted from 3-day old fungal mycelia grown on sterile cellophane placed on the surface of PDA media without seawater at 27°C. The mycelia were transferred into a 1.5 ml tube and crushed using a sterile pestle. About 500 µl CTAB was added to the tube, then the sample was further crushed until it did not clot. The tube was incubated for 30 minutes at 65°C, then incubated at -20°C for 5 minutes. A total of 500 µl of a mixture of chloroform: isoamyl alcohol (24:1) was added to the tube. The tube was inverted a few times and centrifuged at 25,000 x g at 4°C for 15 minutes. The supernatant was transferred into a new tube and 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The tube was then inverted a few times and centrifuged at 25,000 x g at 4°C for 5 minutes. The supernatant was transferred into a new tube and 50 µl of NaOAc 2 M and 1.5 ml absolute ethanol was added, followed by incubation overnight at -20°C. The fungal DNA pellet was obtained by centrifugation at 25,000 x g at 4°C for 30 minutes. The fungal DNA pellet was washed with 500 µl 70% ethanol, then centrifuged at 10,000 x g at 4°C for 5 minutes. The fungal DNA pellet was dried at 30°C for 30 minutes by vacuum, then dissolved with 50 µl of sterile Tris-EDTA buffer and 10 µl of RNAse prior to incubation at 37°C for 10 minutes. RNAse in the mixture was then deactivated at 70°C for 10 minutes and the DNA stored in a refrigerator at -20°C.

DNA amplification was carried out in 0.2 ml PCR tube by adding 10.5 µl nuclease-free water, 15 µl 2x PCR master mix (Promega), 0.75 µl 10 pmol each of ITS1 and ITS4 primers, and 3 µl DNA templates. The amplification reaction was carried out in 35 cycles with a pre-denaturation reaction at 95°C for 15 minutes, denaturation at 95°C for 30 minutes, annealing at 55°C for 30 seconds, elongation at 72°C for 1.5 minutes, post-elongation at 72°C for 5 minutes and stored at 25°C for 10 minutes. The PCR product was electrophoresed and visualized on 1% agarose gel stained with ethidium bromide (EtBr) under UV light. Successfully amplified PCR products were sent to 1st BASE Laboratories (Seri Kembangan, Malaysia) for sequencing using the same primers.

The sequences obtained were assembled using Chromas Pro 1.41 software (Technelysium, South Brisbane, Australia). Homolog sequences were retrieved from NCBI GenBank database using BLAST (Basic Local Alignment Search Tool) (http://www.blast.ncbi.nlm.nih.gov/blast.cgi). Phylogeny analysis was carried out using the Maximum Likelihood (ML) method with the MEGA 6 software. The strength of internal branch of the phylogenetic tree was tested with bootstrap analysis using 1,000 replications. Bootstrap values of 50% or higher are shown in the phylogenetic tree. The sequence dataset used in phylogenetic analyses are shown in Table 1.

2.1.4. Analysis of Fungal Growth on Various Concentrations of Seawater

The ability of fungi to grow in seawater was studied using PDA containing various seawater concentrations. The aim of this treatment was to select the best fungal growth for biomass production to produce fungal extract and to study fungal growth response on seawater. Five seawater concentrations were used, they were 0, 25, 50, 75, and 100 % (v/v). Each treatment was repeated four times. The plates were incubated at 27°C for seven days. Fungal growth was measured every day and colony diameters were measured on seventh day after inoculation.

Table 1. List of GenBank accession members of sequences used in the phylogenetic analysis

Species name	Species reference	Origin	Accession no.
Diaporthe			
D. citrichinensis	ZJUD34	*Citrus unshiu*	JQ954648
D. citrichinensis	ZJUD85	*Fortunella margarita*	KJ490620
D. bicincta	CBS 121004	*Juglans* sp.	KC343134
D. biguttusis	CGMCC 3.17081	*Lithocarpus glabra*	KF576282
D. ellipicola	CGMCC 3.17084	*Lithocarpus glabra*	KF576270
D. phragmitis	CBS 138897	*Phragmites australis*	KP004445
D. asheicola	CBS 136967	*Vaccinium ashei*	KJ160562
D. eres	CBS 138598	*Ulmus* sp.	KJ210521
D. vaccinii	CBS 160.32	*Vaccinium macrocarpon*	AF317578
D. vaccinii	CBS 122116	*Vaccinium corymbosum*	KC343227
D. longicicola	CGMCC 3.17089	*Lithocarpus glabra*	KF576267
D. longicicola	CGMCC 3.17090	*Lithocarpus glabra*	KF576268
D. apiculatum	LC3187	*Camellia sinensis*	KP267866
D. apiculatum	CGMCC 3.17533	*Camellia sinensis*	KP267896
D. musigena	CPC 17025	*Musa* sp.	JF951138
D. arecae	CBS 535.75	*Citrus* sp.	KC343033
D. persiae	CBS 151.73	*Persea gratissima*	KC343173
D. subclavata	CGMCC 3.17253	*Citrus grandis*	KJ490618
D. mahothocarpus	CGMCC 3.15181	*Lithocarpus glabra*	KC153096
D. betulae	CFCC 50469	*Betula platyphylla*	KT732950
D. betulae	CFCC 50470	*Betula platyphylla*	KT732951
D. ovoicicola	CGMCC 3.17092	*Citrus* sp.	KF576264
D. ovoicicola	CGMCC 3.17093	*Citrus* sp.	KF576265
D. saccarata	CBS 116311	*Protea repens*	KC343190
D. eucalyptorum	CBS 132525	*Eucalyptus* sp.	NR120157
D. brasiliensis	CBS 133183	*Aspidosperma tomentosum*	KC343042
D. caatingaensis	CBS 141542	*Tacinga inamoena*	KY085927
D. longispora	CBS 194.36	*Ribes* sp.	KC343135
D. sclerotioides	CBS 296.67	*Cucumis sativus*	KC343193
D. tectonae	MFLUCC12–0777	*Tectona grandis*	KU712430
D. tectonae	MFLUCC 12–0777	*Tectona grandis*	NR147590
Diaporthella corylina (as *Diaporthe* outgroup)	CBS 121124	*Corylus* sp.	KC343004
Penicillium			
P. steckii	CBS 260.55	Cotton fabric	MH857476
P. sizovae	CBS 413.69	Soil	MH859338
P. steckii	CBS 260.55	Cotton fabric	GU944597
P. steckii	CBS 325.59	Soil	GU944594
P. tropicoides	CBS 122410	Soil of rainforest	GU944584
P. tropicum	CBS 112584	Soil	MH862897
P. hetheringtonii	CBS 122392	Soil	GU944558
P. hetheringtonii	CBS 122392	Soil	NR 111482
P. citrinum	CBS 139.45	-	MH856132
P. citrinum	CBS 252.55	Herbarium specimen	MH857469
P. gorlenkoanum	CBS 408.69	Soil	GU944581
P. miczynskii	CBS 220.28	-	MH854990
P. manginii	CBS 253.31	-	MH855205
P. roseopurpureum	CBS 266.29	-	MH855066
P. shearii	CBS 290.48	Soil	MH856346
P. anatolicum	CBS 478.66	-	MH858863
P. paxilli	CBS 360.48	-	GU944577
P. corylophilum	CBS 330.79	-	GU944557
P. malacaense	NRRL 35754		NR 121344
Talaromyces assiutensis (as *Penicillium* outgroup)	CBS 118440	Soil	JN899320

Table 1. Continued

Species name	Species reference	Origin	Accession no.
Colletotrichum			
C. queenslandicum	ICMP 1778	*Carica papaya*	NR 144796
C. Aotearoa	1CMP 18537	*Coprosma* sp.	NR 120136
C. ti	ICMP4832	*Cordyline sp*	NR 120143
C. clidemiae	ICMP 18658	*Clidemia hirta*	NR 120142
C. alienum	ICMP 12071	*Malus domestica*	NR 120141
C. aenigma	ICMP 18608	*Persea americana*	NR 120140
C. tropicale	ICMP 18653	*T. cacao*	NR 119815
C. maximae	AGMy0254	-	KX9435821
C. tropicale	CBS 124949	*T. cacao*	MH863435
C. aeschynomenes	ICMP 17673	*Aeschynomene virginica*	NR 120133
C. gloeosporioides	ICMP 17821	*Citrus sinensis*	JX010152
C. fruticola	CBS 238.49	-	JX010181
C. musae	ICMP 19119	*Musa* sp.	NR 120132
Monilochaetes infuscans (as *Colletotrichum* outgroup)	CBS 869.96	-	NR 155365

2.2. Analysis of Fungal Secondary Metabolite

2.2.1. Production of Fungal Crude Extracts

All five fungal isolates were grown separately on PDA without seawater at 27°C for seven days. Each fungal isolate was then cut using a cork borer (3 mm diameter) on the part of the active growing culture. The piece of fungal culture from each isolate was transferred into a 250 ml Erlenmeyer flask containing 100 ml of PDB. The flask was incubated in a 121-rpm shaking incubator for 21 days at 27°C (Prihanto *et al.* 2011; Uzma *et al.* 2016). At harvest, the culture was filtered using 0.45 µm filter paper to separate the filtrate from mycelia. The filtrate was put into a separator tube, and ethyl acetate was added at a 1:1 ratio. The tube was shaken for 15 minutes, then left to stand until a separation of the ethyl acetate and the filtrate phases were observed. The ethyl acetate phase was taken and evaporated using a rotary evaporator at 40°C to obtain the crude extract of the fungus (Padhi *et al.* 2015).

2.2.2. Herbicidal Activity Test of Fungal Crude Extract

Each crude extract of fungal isolates was dissolved with DMSO to a concentration of 500,000 ppm. The fungal crude extract was dilute further into concentrations tested for herbicidal activity for each isolate were 0, 100, 250, 500, 750, 1000, and 2,500 ppm. These concentrations were dissolved in 15 ml of 1% agar medium and poured into 9 cm sterile petri dishes at 45°C. Control treatment used 1% agar media containing DMSO. The media was allowed to solidify at room temperature (Marcias-Rubalcava *et al.* 2014).

G. globosa weed seeds were surface sterilized by soaking them in 70% ethanol for seven minutes then rinsing twice with sterile distilled water (Yemets *et al.* 2003). The seeds were planted into agar medium containing various concentrations of crude fungal extract. Ten seeds were planted for each petri dish and each treatment was replicated four times. Seeds were germinated in light intensity of 114 lx for seven days at 27°C. Number of germinated seeds, and length of shoots and roots were recorded every day for seven days.

The level of inhibition activity on shoot and root growth was obtained from the following equation:

$$\text{Percentage of growth reduction} = \frac{A - B}{A} \times 100\%$$

Where:
A = length of shoot or root of control
B = length of shoot or root of treatment

2.2.3. Analysis of Bioactive Compounds using GC-MS (Gas Chromatography-Mass Spectrometry)

The fungal extract with the greatest inhibitory effect on the growth of the germinated seeds was analyzed further to identify the bioactive compounds using a Shimadzu GCMS-QP 2010 Ultra gas chromatograph mass spectrometer with a Rtx-5MS stationary phase (5% diphenyl 95% dimethyl polysiloxane) 30 m x 0.25 mm column. The gas carrier used as the driving phase was helium of ultra-high purity with a pressure of 75.4 kPa. Sample injection volume was 1.5 µl with injector temperature of 230°C, ion source temperature of 200°C, interface

temperature of 270°C, and split 20°C. The program column temperature was 50°C for 3 minutes and increased at a rate of 5°C/minute to 150°C, the final temperature of the column was 270°C for 2 minutes and increased at a rate of 3°C/minute. The MS spectrum of each peak in the chromatogram was compared with the NIST 11 database.

2.3. Data Analysis

Data of fungal growth on PDA media containing different seawater concentrations and fungal crude extract activity on growth of *G. globosa* were analyzed using analysis of variance (ANOVA) with SAS 9.4. The real difference between treatments was followed by the Duncan Multiple Range Test (DMRT) at the level of 5% ($p<0.05$).

3. Results

3.1. Endophytic Fungi Associated with *Rhizophora apiculata* Mangroves

A total of five endophytic fungi were isolated from the leaves, flowers, and twigs of *R. apiculata* mangroves. Three isolates, D-U33, D-U21, and D-U31A, were obtained from leaves. While the Bu-U42B isolate was obtained from flowers and the R-U2B isolate was from twigs (Figure 1). Based on morphological characteristics, all five isolates had septate hyphae, and two isolates were able to produce spores while three isolates were sterile mycelia (Table 2). There was no fungal growth observed in the negative control treatment.

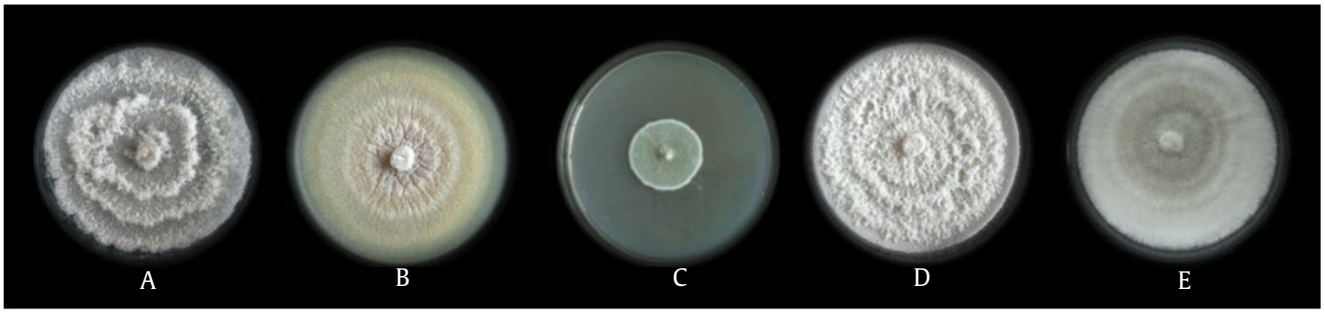

Figure 1. Overview of endophytic fungi on PDA. (A) D-U33, (B) D-U21, (C) D-U31A, (D) Bu-U42B, (E) R-U2B

Table 2. Morphological characteristics of endophytic fungi isolated from *R. apiculata* mangrove

Isolate code	Colony color	The basic color of colony	Surface texture of the colony	Anamorphic structure
D-U31A	Dull green	Bright yellow	Granulated	Hyphal system septate and hyaline. Two branched conidiophores, conidiophores 13.55 µm, hyaline, swollen ends (2.30 µm), widened at the base (3.44 µm) and smooth surface. The cylindrical phialides are rather oval, with 2-6 branch phialides, and 7.32 µm long. Conidia are rounded to slightly rounded (3.3 x 2.72 µm), hyaline, thin-walled, and not insulated
D-U33	White	Yellowish white	Velvety	Hyphal system septate, hyaline, hyphae diameter of 3.27 µm
D-U21	Light gray yellow	Light gray yellow, middle of the thick colony ash	Wrinkles with small spots	Hyphal system septate, hyaline, hyphae diameter of 3.46 µm
Bu-U42B	White	Yellowish white	Velvety	Hyphal system septate, hyaline, hyphae diameter of 2.44 µm
R-U2B	White, middle of the gray colony	White, middle of the colony ash	Breathe	Hyphal system septate, hyaline, hyphae diameter of 2.2 µm, at the end of hyphae there is modification of hyphae with oval shape to irregular shape, brown color, size 7 x 12 µm, between hyphae and hyphae modifications there are clear boundaries. Conidia are oval (2.4 x 6.1 µm), hyaline and thin walled

The letter symbol in the isolate code shows the origin of the endophytic fungus isolation; Leaves (D), Flowers (Bu), Twigs (R)

Based on molecular identification using DNA sequences of ITS regions, the isolates were identified into 5 different species. BLAST analysis showed the five species had between 97 and 100% similarity with sequences available in GenBank (Table 3). The isolates obtained from the leaves were identified as *Diaporthe tectonae* for D-U33, *D. musigena* for D-U21, and *Penicillium citrinum* for D-U31A. The isolates from the flowers and twigs were identified as *D. eucalyptorum* for Bu-U42B, and *Colletotrichum queenslandicum* for R-U2B.

The phylogenetic analysis shows the best model used in phylogenetic tree construction using Kimura 2-parameter for *Diaporthe* and *Colletotrichum* and Tamura 2-parameter for *Penicillium*. The results shown in the phylogenetic tree supported the result shown in BLAST analysis with D-U33 being closely related to *D. tectonae*, D-U21 closely related to *D. musigena*, and Bu-U42B closely related to *Phomopsis lithocarpus*, which is reported to be a synonym of *D. eucalyptorum* (Figure 2). While D-U31A was shown to be closely related to *P. citrinum* (Figure 3) and R-U2B was closely related to *C. queenslandicum, C. aoteroa, C. clidemiae, C. alienum, C. aenigma, C. tropicale,* and *C. citri maximae* (Figure 4).

3.2. Fungal Growth at Different Seawater Concentrations

All five endophytic fungi isolated from *R. apiculata* were able to grow at different seawater concentrations. The growth, however, varied depending on the isolates and seawater concentrations used (Table 4). In general, all five endophytic fungi showed no abnormal growth colony features in growth medium without seawater treatment. *D. musigena* tended to grow better in media containing seawater. While *D. eucalyptorum* tended to grow better in media without seawater, and growth decreased significantly in media containing 100% of seawater. In contrast, the growth of *D. tectonae, P. citrinum,* and *C. queenslandicum* were in general not affected by the concentration of seawater.

3.3. Herbicidal Activity Test of Endophytic Fungi Crude Extract on *Gomphrena globosa* Seed Germination and Seedling Growth

DMSO, the solution that was used to prepare fungal crude extract concentration and used in the control treatment, did not have any effect on *G. globosa* seed germination or seedling growth. The *G. globosa* seeds in the control treatment germinated one day after planting. In general, extracts from all of the endophytic fungi delayed seed germination of *G. globosa* by one to two days. The delay caused by crude extract of *D. eucalyptorum* in the germination occurred at concentrations of 1,000 and 2,500 ppm, whereas the delay in germination caused by crude extracts of *D. musigena, C. queenslandicum,* and *D. tectonae* was observed only at a concentration of 2,500 ppm. *P. citrinum* crude extract showed strong effect on *G. globosa* seed germination. Seed germination was delayed by 1 day on the lower *P. citrinum* extract concentrations of 100-750 ppm, and was delayed by 2 and 3 days at extract concentrations of 1,000 and 2,500 ppm, respectively (data not shown).

Further effects of crude fungal extract on root and shoot growth were tested on *G. globosa* seedlings. The effects were stronger on roots than on shoots for all the fungi and all treatment concentrations. For shoot length, based on statistical tests at $p<0.05$, *P. citrinum* extract at 100 ppm reduced growth by 8.9%, while *D. eucalyptorum, D. musigena,* and *D. tectonae* extract at 500 ppm reduced growth by 9.7%, 14.5% and 8.5%, respectively. *C. queenslandicum* extract at 750 ppm reduced growth by 22.17%. Increased fungal extract concentrations showed greater reductions in shoot growth (Table 5).

The inhibitory effects on root length appeared at a concentration of 100 ppm in extracts of *P. citrinum, D. eucalyptorum, D. musigena,* and *C. queenslandicum* with inhibitory values of 44.3%, 19.25%, 18%, and 15.27%, respectively. In *D. tectonae* extract, the inhibitory effect appeared at a concentration of 750 ppm, with an inhibitory value of 11.48%. Increased

Table 3. BLAST analysis of endophytic fungi isolated from *R. apiculata* based on ITS1-5.8S-ITS2 region of rDNA

Isolate code	BLAST analysis on the NCBI website						
	Species	Maximum score	Total score	Query cover (%)	Similarity (%)	E value	Accession number
D-U33	*Diaporthe tectonae*	929	929	100	99	0.0	NR_147590.1
D-U21	*Diaporthe musigena*	1,009	1,009	100	98	0.0	JF951138.1
D-U31A	*Penicillium citrinum*	974	974	99	100	0.0	MH856132.1
Bu-U42B	*Diaporthe eucalyptorum*	944	944	99	97	0.0	NR_120157.1
R-U2B	*Colletotrichum queenslandicum*	935	935	99	99	0.0	NR_144796.1

The letter symbol on the isolate code shows the origin of the endophytic fungus isolation; Leaves (D), Flowers (Bu), Branches (R)

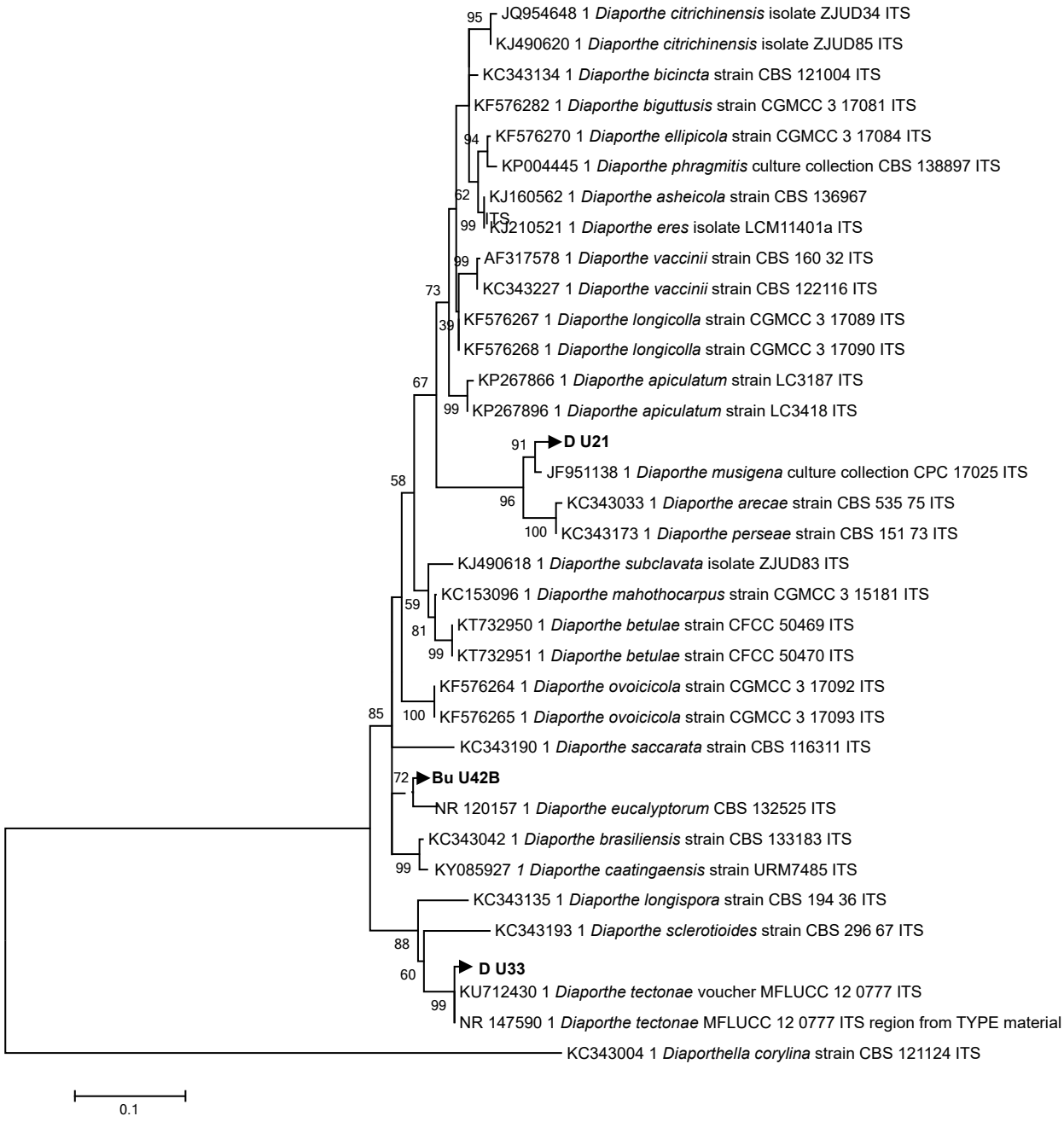

Figure 2. Phylogenetic tree of the genus *Diaporthe* isolated from *R. apiculata* mangrove based on DNA sequences of ITS region of rDNA inferred from Maximum Likelihood method with Kimura 2-parameter + Gamma distribution + Invariable models (K2+G+I). Bootstrap values are indicated on the branches obtained from 1,000 replications. *D. corylina* strain CBS 121124 was used as an outgroup

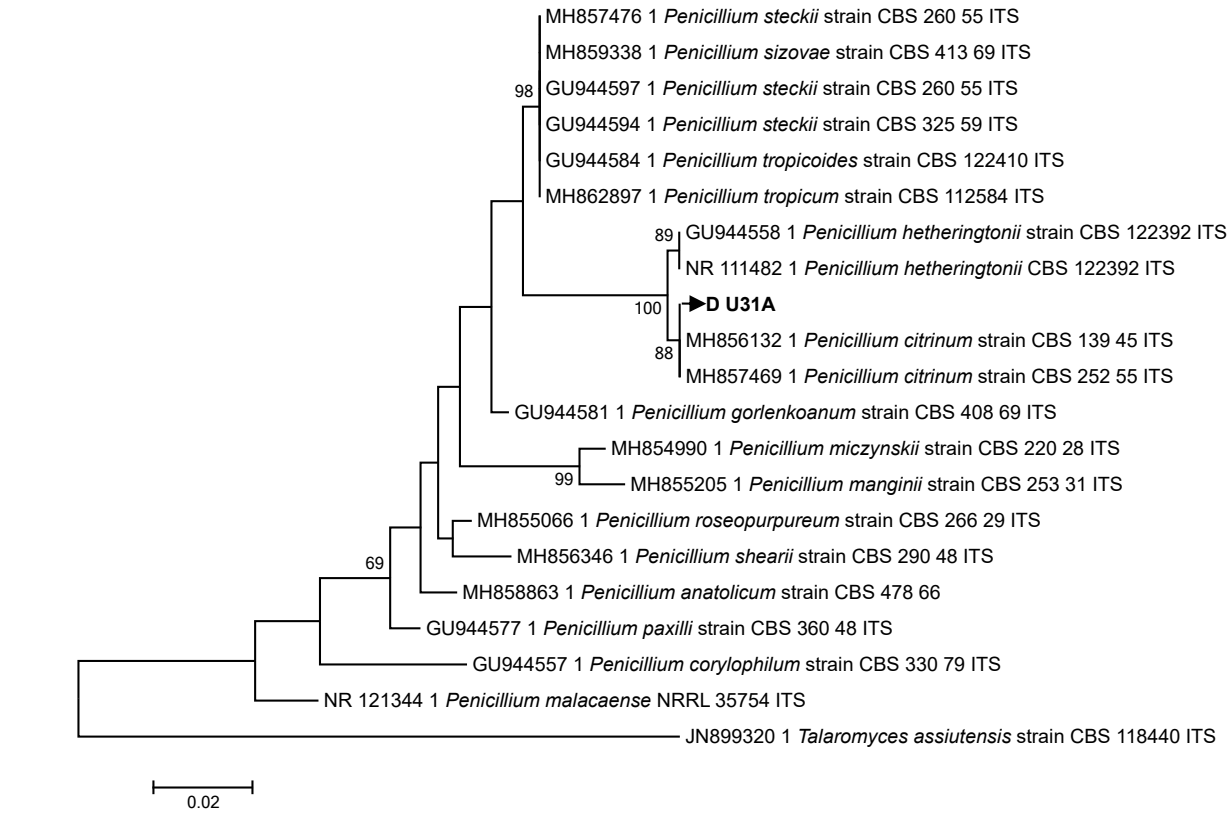

Figure 3. Phylogenetic tree of the endophytic fungi genus *Penicillium* isolated from *R. apiculata* mangrove based on ITS region sequences of ribosomal DNA inferred from Maximum Likelihood method with Tamura 2-parameter + Invariant models (T92 + I). Bootstrap values are indicated on the branches obtained from 1,000 replications. *Talaromyces assiutensis* strain CBS 118440 was used as an outgroup

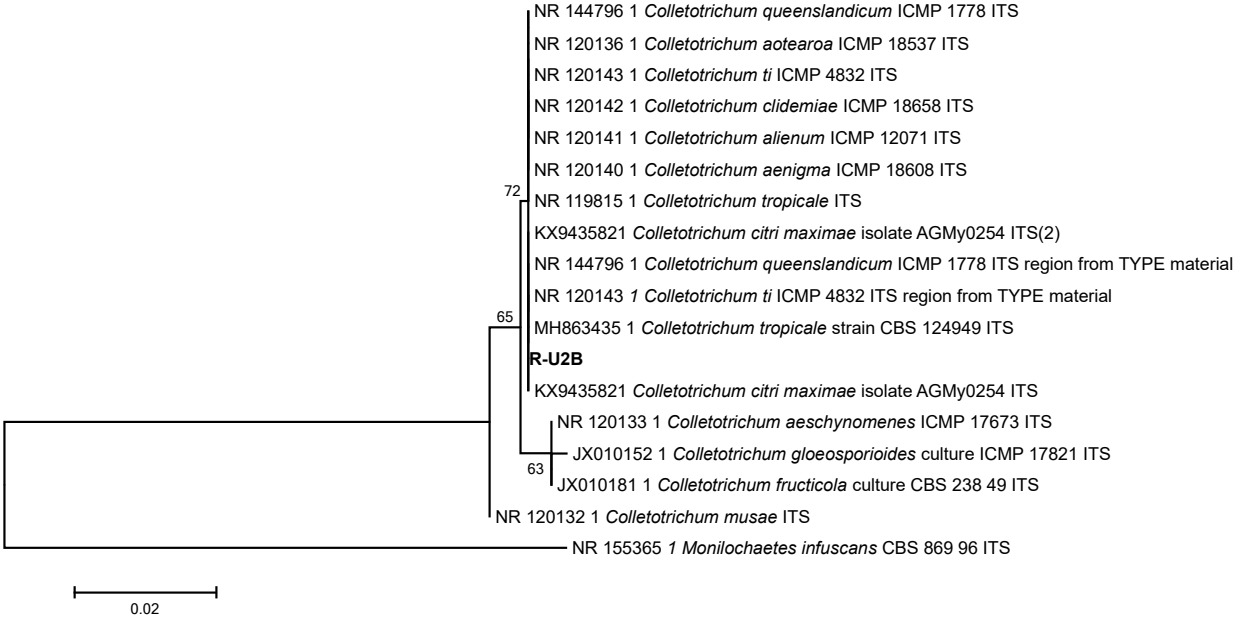

Figure 4. Phylogenetic tree of the endophytic fungi genus *Colletotrichum* isolated from *R. apiculata* mangrove based on ITS region sequences of ribosomal DNA inferred from Maximum Likelihood method with Kimura 2-parameter models (K2). Bootstrap values are indicated on the branches obtained from 1,000 replications. *Monilochaetes infuscans* CBS 869 96 was used as an outgroup

fungal extract concentration resulted in greater reduction in root growth. (Table 6). *P. citrinum* endophytic fungus had strong effects on seed germination, and seedling shoot and root growth of *G. globosa* (Figure 5).

3.4. Analysis of Selected Fungal Crude Extract Compounds by Gas Chromatography Mass Spectrometry (GC-MS)

Analysis of compounds by GC-MS was carried out only on *P. citrinum* crude extract because it showed the greatest inhibitory effect. There were 30 peaks of compounds in the chromatogram of the extract, 7 of which had an area of more than 4% and 3 of them were dominant compounds (>10% peak area) (Figure 6). The seven compounds having a percentage of area greater than 4% are suspected to be the compounds mostly likely responsible for the growth inhibitory effect. The identification of these compounds based on comparison with the NIST 11 database are presented in Table 7. The three dominant peaks in the GC-MS chromatogram of the extract of *P. citrinum* based on the spectrum pattern are Beta-Asarone, Oxane, 4-(2-amino-1-hydroxyethyl)-4-(3,4-dimethoxyphenyl), and Diisooctyl phthalate. The other suspected compounds contained in the extract are 3-Methoxy-2-methyl-cyclohex-2-enone, Cyclohexane-carbohexaldehyde, 6-methyl-3-(1-methylethyl)-2-oxo, 2-Hydroxy-4-isopropyl-7-methoxytropone, and Cyclopropane-carboxylic acid, 1-(2-propenyl)-1,1-dimethylethyl ester (Figure 6 and 7).

Table 4. Colony diameter (cm) of endophytic fungi isolated from *R. apiculata* mangrove at various concentrations of seawater

Isolate	Concentration of seawater (%, v/v)				
	0	25	50	75	100
Diaporthe tectonae	7.23^{BCb}	8.88^{Aa}	8.37^{Aab}	8.18^{Aab}	8.01^{Aab}
Diaporthe musigena	8.83^{Aa}	7.1^{Bc}	8.21^{Aab}	7.75^{Abc}	6.07^{Bd}
Penicillium citrinum	8.11^{ABa}	8.14^{ABa}	8.37^{Aa}	8.48^{Aa}	8.41^{Aa}
Diaporthe eucalyptorum	2.85^{Da}	2.88^{Ca}	3.09^{Ba}	2.83^{Ca}	2.91^{Ca}
Colletotrichum queenslandicum	7.15^{BCa}	7.63^{ABa}	7.21^{Aa}	6.60^{Ba}	7.78^{Aa}

The numbers followed by the same capital letters in the same column and lowercase letters in the row show no significant difference based on DMRT at a level of <5%

Table 5. The shoot length (cm) of *G. globosa* treated with various concentrations of crude extract of different endophytic isolates

Isolate	Origin of isolates	Concentration of crude extract (ppm)						
		0	100	250	500	750	1,000	2,500
DMSO (Control)		2.64^{a}	2.6^{ab}	2.58^{abc}	2.59^{ab}	2.57^{abcd}	2.56^{abcd}	2.57^{abcd}
Penicillium citrinum	Leaves	2.58^{abcd}	2.34^{defg}	2.18^{ghi}	2.07^{hij}	1.81^{kl}	1.26^{o}	0.77^{p}
Diaporthe eucalyptorum	Flowers	2.57^{abcd}	2.4^{abcdef}	2.35^{cdefg}	2.32^{efg}	1.98^{ijk}	1.61^{mn}	1.44^{no}
Diaporthe musigena	Leaves	2.61^{ab}	2.5^{abcde}	2.5^{abcde}	2.24^{fgh}	2.05^{hij}	1.58^{mn}	1.31^{o}
Colletotrichum queenslandicum	Branches	2.53^{abcde}	2.5^{abcde}	2.44^{abcdef}	2.37^{bcdefg}	2.00^{ijk}	1.7^{lm}	1.57^{mn}
Diaporthe tectonae	Leaves	2.58^{abcd}	2.53^{abcde}	2.44^{abcdef}	2.35^{cdefg}	2.32^{efg}	2.09^{hij}	1.95^{jk}

The numbers followed by the same capital letters in the same column and lowercase letters in the row show no significant difference based on DMRT at a level of <5%

Table 6. The root length (cm) of *G. globosa* treated by various concentrations of crude extract of different endophytic isolates

Isolate	Origin of isolates	Concentration of crude extract (ppm)						
		0	100	250	500	750	1,000	2,500
DMSO (Control)		5.7^{ab}	5.9^{a}	5.76^{ab}	5.51^{abc}	5.73^{ab}	5.68^{ab}	5.64^{ab}
Penicillium citrinum	Leaves	5.64^{ab}	3.14^{jkl}	2.44^{no}	2.19^{op}	1.59^{qr}	0.5^{s}	0.38^{s}
Diaporthe eucalyptorum	Flowers	5.66^{ab}	4.57^{g}	3.37^{jk}	3.03^{klm}	2.76^{lmn}	2.25^{o}	1.29^{r}
Diaporthe musigena	Leaves	5.61^{ab}	4.6^{fg}	4.14^{i}	3.43^{jk}	2.43^{no}	2.31^{no}	1.8^{pq}
Colletotrichum queenslandicum	Branches	5.63^{ab}	4.77^{efg}	4.52^{gh}	3.45^{jk}	2.65^{mno}	2.47^{no}	0.63^{s}
Diaporthe tectonae	Leaves	5.75^{ab}	5.75^{ab}	5.65^{ab}	5.36^{bcd}	5.09^{cde}	5.03^{def}	3.51^{j}

The numbers followed by the same capital letters in the same column and lowercase letters in the row show no significant difference based on DMRT at a level of <5%

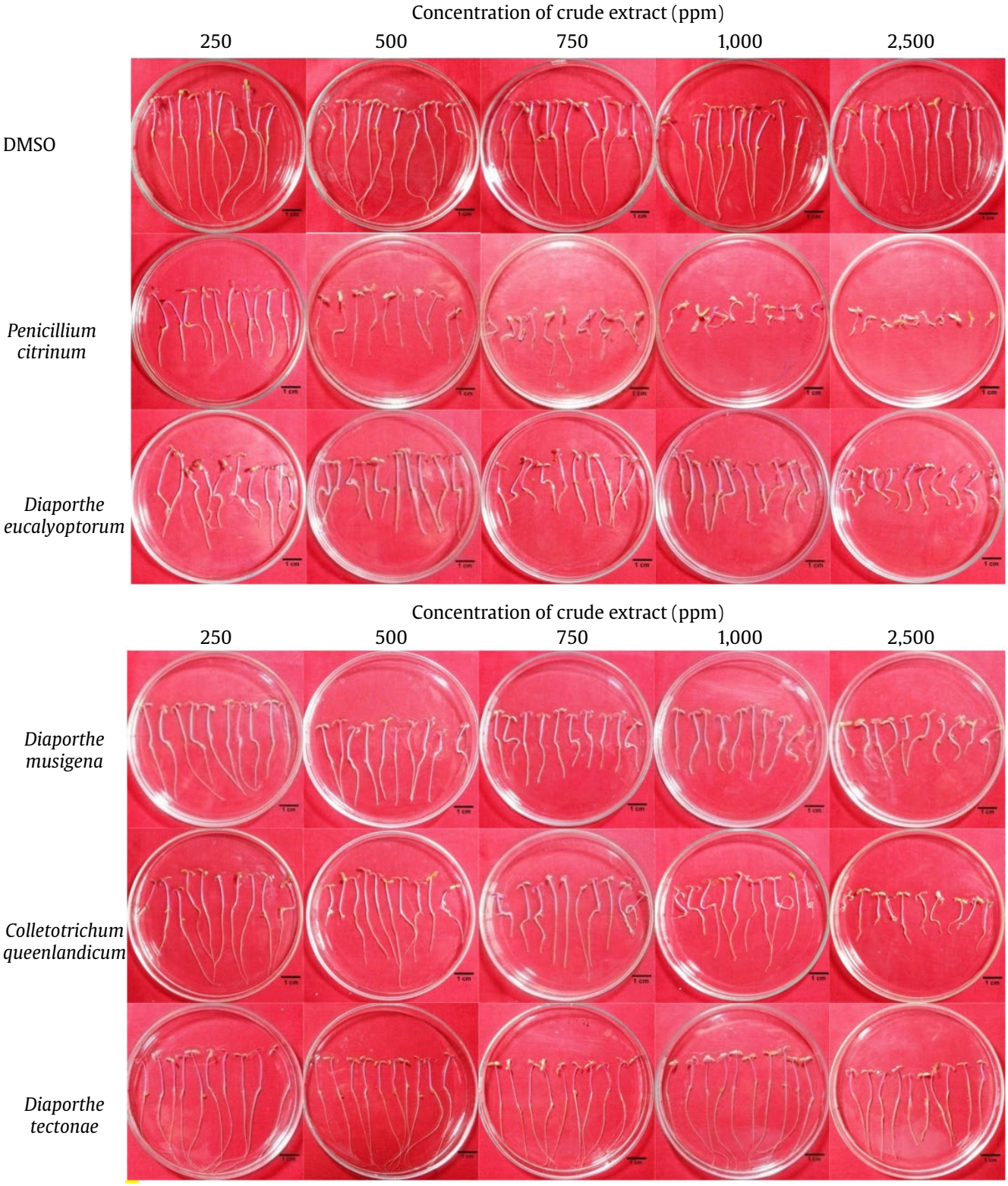

Figure 5. Growth of *G. globosa* affected by various concentrations of crude extract of endophytic fungi

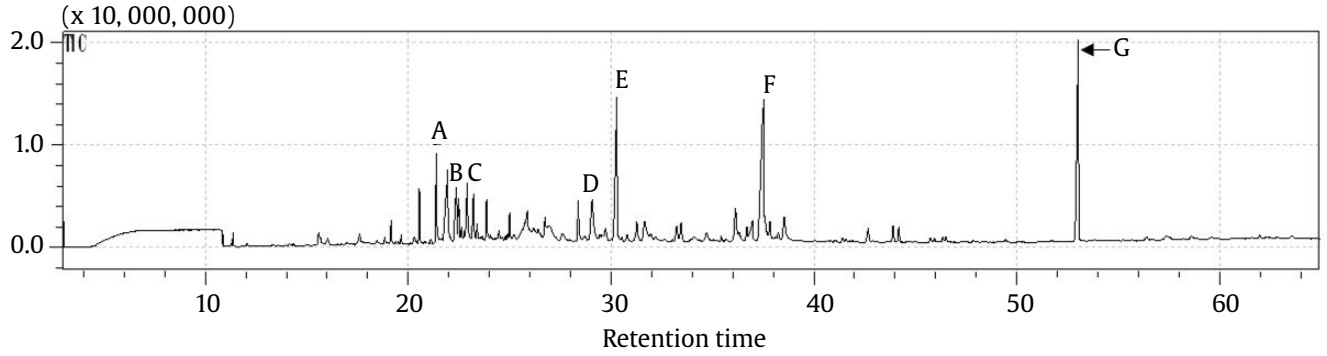

Figure 6. GC-MS chromatogram of crude extract of *P. citrinum* extracted by ethyl acetate. (A) 3-Methoxy-2-methyl-cyclohex-2-enone, (B) Cyclohexane-carbohexaldehyde, 6-methyl-3-(1-methylethyl)-2-oxo, (C) Cyclopropane carboxylic acid, 1-(2-propenyl)-1,1-dimethylethyl ester, (D) 2-Hydroxy-4-isopropyl-7-methoxytropone, (E) Beta-Asarone, (F) Oxane, 4-(2-amino-1-hydroxyethyl)-4-(3,4-dimethoxyphenyl)-, (G) Diisooctyl-phthalate

Table 7. Suspected compounds in crude extract of *P. citrinum* extracted by ethyl acetate

Name of the compound	Area (%)	Retention time	Chemical formula	Molecular weight	Similarity with NIST-MS database (%)	Chemical structure (Figure 7)
3-Methoxy-2-methyl-cyclohex-2-enone	6.09	21.935	$C_8H_{12}O$	140,180	85	A
Cyclohexane-carbohexaldehyde, 6-methyl-3-(1-methylethyl)-2-oxo	4.23	22.380	$C_{11}H_{18}O_2$	182,263	77	B
Cyclopropane carboxylic acid, 1-(2-propenyl)-1,1-dimethylethyl ester	4.14	22.908	$C_8H_{14}O_2$	142,196	86	C
2-Hydroxy-4-isopropyl-7-methoxytropone	4.62	29.072	$C_{11}H_{14}O_3$	194,227	79	D
Beta-Asarone	11.59	30.27	$C_{12}H_{16}O_3$	208,257	75	E
Oxane, 4-(2-amino-1-hydroxyethyl)-4-(3,4-dimethoxyphenyl)-	18.95	37.514	$C_{15}H_{23}NO_4$	281,352	73	F
Diisooctyl-phthalate	15.71	53.039	$C_{24}H_{38}O_4$	390,564	97	G

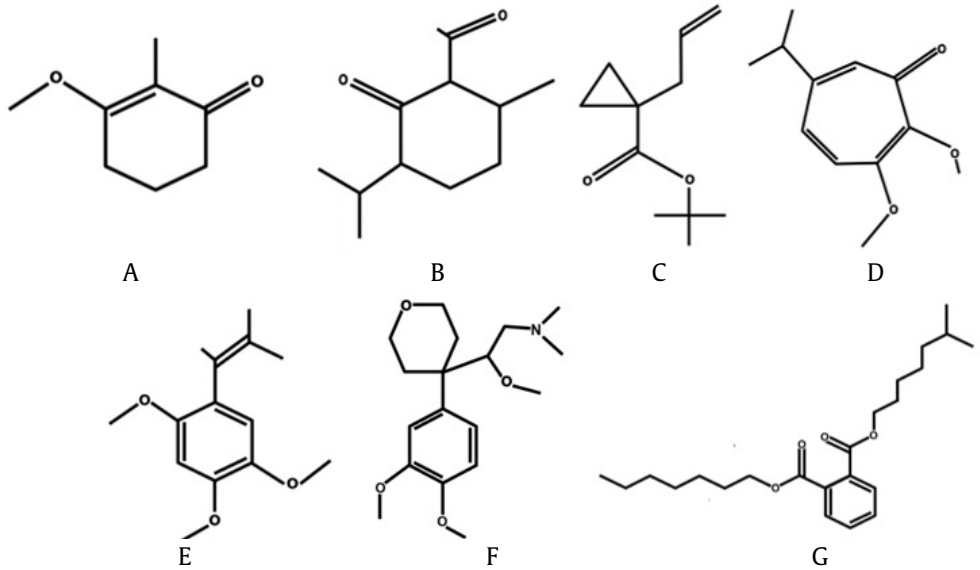

Figure 7. Chemical structures of suspected compounds (A) 3-Methoxy-2-methyl-cyclohex-2-enone, (B) Cyclohexane-carbohexaldehyde, 6-methyl-3-(1-methylethyl)-2-oxo, (C) Cyclopropane carboxylic acid, 1-(2-propenyl)-1,1-dimethylethyl ester, (D) 2-Hydroxy-4-isopropyl-7-methoxytropone, (E) Beta-Asarone, (F) Oxane, 4-(2-amino-1-hydroxyethyl)-4-(3,4-dimethoxyphenyl)-, (G) Diisooctyl-phthalate

4. Discussion

4.1. Endophytic Fungi Associated with *Rhizophora apiculata* Mangroves

Based on molecular and morphological analysis, there were five endophytic fungi that were successfully isolated from the *R. apiculata mangrove*. These were from the genera *Penicillium, Colletotrichum*, and *Diaporthe*. Three out of the five fungal species obtained belonged to the genus *Diaporthe*. The existence of mangrove endophytic fungi from the same genera has been reported by Rajamani *et al.* (2018). Endophytic fungi from the genus *Diaporthe, Penicillium,* and *Colletorichum* were reported from the investigation of 20 mangroves from South Andaman Island, India. These fungal genera have been found in all mangroves studied so far. Zhou *et al.* (2018) reported that endophytic *Diaporthe* spp. were isolated from the mangroves *R. stylosa* and *R. mucronata*, with their presence reaching 27.61%. The genus *Colletotrichum* was recorded as the largest group inhabiting the mangrove species *Acanthus ebracteatus* Vahl and *Xylocarpus granatum* J. Koenig (Rajamani *et al.* 2018). Xing and Guo (2011) reported that a total of 295 fungal isolates were obtained from four *Rhizophoraceae* mangrove species, i.e., *Ceriops tagal* (67 isolates), *Rhizophora apiculata* (57 isolates), *R. stylosa* (80 isolates), and *Bruguiera sexangula* var. rhynchopetala (91 isolates), on the south coast of China. These isolates were classified into 38 taxa by morphological characteristics.

The low number of isolated endophytic fungi obtained in this study compared to that of other studies could be caused by the long storage time on ice during transportation from field to laboratory that was located on a different island. Another possibility was also most of the endophytic fungi obtained in this study were mycelia sterilia, mitosporic fungi without known spore stage, with similar colony features. This similarity in colony appearance could have led to underestimating the number of species in the isolation process. Of the five species, only 2 species produced spores (Figure 1 and Table 2).

Endophytic fungi isolated from mangroves are of special interest because they are from hosts that grow in a unique ecosystem. Mangrove forests grow in the dynamic transition zone between terrestrial and marine habitats (Bandaranayake 2002; Gopal and Chauhan 2006; Shearer *et al.* 2007). Therefore, the characteristics of fungal growth in various seawater concentrations are important to be studied. The results of this study showed that all of the five fungi were able to grow on PDA media without seawater as well as with different concentrations of seawater, even though the growth rate varied depending on the isolate and the seawater concentration tested. These findings are in agreement with those reported by Jennings (1983).

4.2. Herbicidal Activity on Crude Extract of Endophytic Fungi

G. globosa is a weed native to Central America and classified as an invasive plant in Cuba, Hawaii and Costa Rica (Ventosa-Febles 2017). Now, the weed has found on waste grounds and disturbed sites in tropical and subtropical countries. Apart from competing with the crop for water, light, nutrients and space, the weeds could also harbor pests which attack the crop thereby reducing the yield and increasing production cost (Suryanarayanan 2019). Therefore, weed control is important strategy in agriculture and forestry. The most common method of weed management is by the application of selective chemical herbicides. The applications of synthetic chemical herbicides have negative effect on environment and cause chemical resistance by the weeds.

Fungal endophytes produce an array of novel metabolites exhibiting various bioactivities including herbicides. There are many studies of the species diversity and bioactive metabolites of endophytes, but only a few studies on fungal endophytes addressing their effect as herbicides (Kowalski *et al.* 2015). Crude extracts of the mangrove endophytic fungi in this study showed herbicidal activity by inhibiting germination and seedling growth of *G. globosa*. The crude extract that showed the best results was derived from *P. citrinum*. This finding was in agreement with Fawole and Yahaya (2017), who reported that crude extract of *P. citrinum* shows herbicides activity by reducing biomass of *Amaranthus hybridus* and *Phyllantus amarus*. Ahmad *et al.* (2020) reported that culture filtrate of endophytic fungi, *Alternaria* sp., *Aspergillus* sp., and *Drechslera* sp. isolated from roots of *Parthenium hysterophorus* aggressive weed (family Asteraceae) showed phytotoxic activity on three species of important weed namely *Chenopodium album, Avena fatua,* and *Convolvulus arvensis*. Culture filtrate of all the three endophytic fungi showed significant reduction in seed germination and growth parameter of the three weed species. An endophytic *Chaetomium globosum* isolated from *Amaranthus viridis* leaves produce azaphilone derivatives chaetomugilin D and chaetomugilin J, which both show phytotoxic activity in lettuce (*Lactuca sativa*) seed germination (Piyasena *et al.* 2015).

Further analysis of crude extract of *P. citrinum* obtained in this study as the best isolate using GCMS indicated that the extract contained diverse compounds. There were 30 peaks in the extract and 7 of them had an area of more than 4% and 3 of them were dominant compounds (>10% peak area) (Figure 6). The seven compounds having a percentage of area greater than 4% were successfully identified in this study. They are Beta-Asarone; Oxane, 4-(2-amino-1-hydroxyethyl)-4-(3,4-dimethoxyphenyl); Diisooctyl phthalate; 3-Methoxy-2-methyl-cyclohex-2-enone; Cyclohexane-carbohexaldehyde, 6-methyl-3-(1-methylethyl)-2-oxo; 2-Hydroxy-4-isopropyl-7-methoxytropone; and Cyclopropane-carboxylic acid, 1-(2-propenyl)-1,1-dimethylethyl ester. We have not found any references reporting any herbicidal activity from any of the compounds identified from *P. citrinum* in this study.

Beta-Asarone is a chemical compound of the phenyl propanoid class. These compounds are reported to be present in *Acorus gramineus* plants and have antifungal and radioprotective activity through their interactions with several molecular targets (Chellian *et al.* 2017, Lee *et al.* 2004). Diisooctyl phthalate is an ester of phthalic acid. This compound has been found in the methanol extract of endophytic *Penicillium* from *Tabebuia argentea* (Murugan *et al.* 2017) and endophytic fungi from *Taxus yunnanensis*. These compounds have antibacterial activity (Chen *et al.* 2009). Five of the seven compounds have no known role. None of the compounds identified from *P. citrinum* in this study have been reported as herbicides. This experiment, however, has shown that *P. citrinum* isolated from *R. apiculata* mangrove species produces herbicidal substances that are active in controlling *G. globosa*. This experiment also succeeded in identifying new compounds derived from endophytic fungi. These compounds may be potential herbicides, and we are now in progress to analyze them further.

The GCMS is an important technique for identifying metabolites produced by various fungi and bacteria, because the technique is easy and effective in identifying mixed compounds (Wani *et al.* 2010). However, the compounds detected in GC-MS are compounds with small molecular weights only (<500 Da). Therefore, the substances responsible for the herbicidal activity in this experiment could be compounds with large molecular weights which could only be detected by LC-MS or by isolation of the active compounds that are then determined from their chemical structure.

In conclusion, a total of five endophytic fungi of *R. apiculata* were isolated from leaves, flowers, and twigs of mangroves. Five fungal isolates were identified, namely *P. citrinum*, *D. eucalyptorum, D. musigena, C. queenslandicum,* and *D. tectonae*. All fungal isolates were able to grow at different seawater concentrations with a varied growth rate depending on the isolate and seawater concentration. All five isolates showed herbicidal activity against *G. globosa* weed by delaying seed germination and reducing the growth of shoots and roots. Among the five isolates, *P. citrinum* showed highest herbicide activity compared to the other isolates. Further analysis of the fungal crude extract of *P. citrinum* culture filtrate showed 7 main compounds identified by GC-MS, namely 3-Methoxy-2-methyl-cyclohex-2-enone; Cyclohexane-carbohexaldehyde, 6-methyl-3-(1-methylethyl)-2-oxo; Cyclopropane carboxylic acid, 1-(2-propenyl)-1,1-dimethylethyl ester; 2-Hydroxy-4-isopropyl-7-methoxytropone; Beta-Asarone; Oxane, 4-(2-amino-1-hydroxyethyl)-4-(3,4-dimethoxyphenyl)-; and Diisooctyl-phthalate. *R. apiculata* is a potential source of endophytec fungi that produce herbicidal compounds.

Conflict of Interest

The authors declare no conflicts of interest. All experiments were undertaken in this study in compliance with the current laws of the country where they were performed.

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