Folia Microbiologica (2021) 66:385–397
https://doi.org/10.1007/s12223-021-00854-4

ORIGINAL ARTICLE

Isolation of endophytic fungi with antimicrobial activity from medicinal plant *Zanthoxylum simulans* Hance

Jimmy Kuo[1,2] · Chin-Feng Chang[3] · Wei-Chiung Chi[4]

Received: 1 July 2020 / Accepted: 14 January 2021 / Published online: 5 February 2021

Abstract

Fungal endophytes have been found to exist in many plant species and appear to be important to their plant hosts. However, the diversity and biological activities of these fungi remain largely unknown. *Zanthoxylum simulans* Hance, a popular natural spice and medicinal plant, commonly known as Szechuan pepper or Chinese-pepper, grows on Kinmen Island, Taiwan. In this study, leaf and stem samples of *Z. simulans*, collected in summer and winter, were screened for antimicrobial and anti-inflammatory metabolite-producing endophytic fungi. A total of 113 endophytic strains were isolated and cultured from *Z. simulans*, among which 23 were found to possess antimicrobial activity, belonging to six fungal genera: *Penicillium* (26.09%, 6), *Colletotrichum* (21.74%, 5), *Diaporthe* (21.74%, 5), *Daldinia* (17.39%, 4), *Alternaria* (8.70%, 2), and *Didymella* (4.34%, 1). We also found that the number of species with antimicrobial activity and their compositions differed between summer and winter. Our study demonstrated that *Z. simulans* might contain large and diverse communities of endophytic fungi, and its community composition varies seasonally. In addition, fungal endophytes produce antimicrobial agents, which may protect their hosts against pathogens and could be a potential source of natural antibiotics.

Introduction

Due to continuous evolution of antibiotic resistance in bacterial and fungal pathogens, there is a growing demand for antibiotics, not only for human use, but also for use in livestock (Dos Santos et al. 2015), and the need for the development of new and effective antibiotic chemicals is increasing (Dos Santos et al. 2015; Yang et al. 2015; Liu et al. 2016; Chi et al. 2019). Furthermore, growth in the use of antibiotic chemicals will increase the accumulation of these chemicals in the natural system, potentially causing a threat to human health. Consequently, new treatment methods are needed to solve these problems (Qi et al. 2015). Over the years, researchers have observed that plants harbor eukaryotic fungi of great biological diversity. It is estimated that more than one million plant fungal endophytes are distributed in approximately 300,000 terrestrial plant species on Earth (Jia et al. 2016). These fungal endophytes can produce many interesting secondary metabolites with unique structures and distinct activities, which represent a promising source of antimicrobial and other medicinal therapeutic agents (Deshmukh et al. 2014; Dos Santos et al. 2015).

Microorganisms are a rich source of bioactive compounds used in pharmaceutical, agricultural, and industrial applications (Deshmukh et al. 2014). Accumulated evidence has demonstrated that many of the chemotherapeutic agents produced by medicinal plants are also produced from their endophytes (Stierle et al. 1993). For instance, taxol is one of the most well-known plant-derived anticancer drugs, and is isolated from the bark of the Pacific yew tree *Taxus brevifolia*. The isolation of taxol-producing endophytic fungus *Cladorhinum* sp. from the Pacific yew tree suggests a simpler and more practical way to produce this compound on a large scale (Heinig et al. 2013). Endophytes are microorganisms that include bacteria and fungi living within plant tissues without visibly harming the plant (De Bary 1879). They

Jimmy Kuo and Chin-Feng Chang contributed equally.

✉ Wei-Chiung Chi
joan@email.nqu.edu.tw; joan@nqu.edu.tw

[1] Department of Planning and Research, National Museum of Marine Biology and Aquarium, 94450 Pingtung, Taiwan

[2] Graduate Institute of Marine Biology, National Dong Hwa University, 94450 Pingtung, Taiwan

[3] Department of Biological Science and Technology, China University of Science and Technology, 11581 Taipei, Taiwan

[4] Department of Food Science, National Quemoy University, 89250 Kinmen, Taiwan

have evolved to survive in plant tissues for all or part of their life cycle and may protect the plant hosts from pathogens, insect herbivores, and even abiotic stresses, such as drought, heat and salinity (Liu et al. 2011; Meng and He 2011; Khan et al. 2013). In addition, certain endophytic fungi can produce the same natural products as their plant hosts, as well as a broad variety of other novel bioactive secondary metabolites (Heinig et al. 2013). Soil-born microorganisms have proved to be rich sources of new microbial species and novel bioactive compounds. Since the late 1980s, endophytic fungi from plants have been studied extensively in the search for new drugs. A single drug on the market, emodepside, has resulted from these efforts, and another is now in clinical phase 3 trials, suggesting the potential for identification of new bioactive metabolites from endophytic fungi (Helaly et al. 2018). Many endophytes isolated from medicinal plants produce natural products that are strong fungicides or bactericides (de Siqueira et al. 2011).

The genus *Zanthoxylum* (Magnoliopsida: Sapindales: Rutaceae) includes more than 250 deciduous, evergreen trees, and shrub species, distributed globally in tropical and subtropical regions (Epifano et al. 2011). The genus is economically important owing to alimentary, industrial, and medicinal applications (Wang et al. 2014), the latter including the treatment of abdominal pain, arthritis, asthma, cough, cold, fever, diarrhea, malaria, toothache, and ascarid infections, as well as muscle cramps and spasms (He et al. 2002; Lee and Lim 2008; Wang et al. 2014; Nguyen et al. 2016; Bunalema et al. 2017; Costa et al. 2018; Ekka et al. 2020). In addition, the plants also produce bioactive phytochemical compounds that display anti-inflammatory, antiplasmodial, antiparasitic, antiviral, antibacterial, and antifungal activities (Kumar et al. 2014; Nguyen et al. 2016; Costa et al. 2018; Ekka et al. 2020). *Zanthoxylum simulans* (Chyau et al. 1996) is a prickly shrub native to mainland China (Western Sichuan) and Taiwan (Yang et al. 2002). The stem, branches, and branchlets have spiny thorns, and the bark is aromatic. It is a popular natural spice and has been used in traditional Chinese medicine to treat various diseases, including stomachache, toothache, intestinal worms, eczema, and pruritus, and extensive phytochemical investigations have been carried out on this plant (Wu and Chen 1993; Yang et al. 2002; Wang et al. 2014). Endophytic fungi from this plant have been reported to produce secondary metabolites that inhibit the proliferation of human rheumatoid arthritis synovial fibroblasts (HFLS-RA) (Lyu et al. 2018). However, surprisingly, fungal endophytes of this plant have not been studied in depth. Therefore, the goal of this study was to isolate endophytic fungi from the leaves and stems of *Z. simulans* during summer and winter seasons on Kinmen Island, screen these for antimicrobial activities against indicator pathogens, and finally assay their metabolites for anti-inflammatory activities.

Materials and methods

Collection of plant materials

Leaf and stem samples of *Zanthoxylum simulans* (Fig. 1) were collected from Caidian (Fig. 2) in Kinmen County, Taiwan. A total of ten twigs (each including five leaves) were cut from ten randomly-selected individual *Z. simulans* plants, among which the first five samples were collected in summer (August 2014) and the second five in winter (February 2015). Samples were placed into separated open plastic bags, brought to the laboratory within 30 min, maintained at 4 °C, and processed within 24 h. A voucher sample (NO. Chi 0003) was kept in the Department of Food Science, National Quemoy University, Kinmen, Taiwan.

Isolation of endophytic fungi

Prior to surface sterilization, leaves and stems were thoroughly washed with running tap water to remove surface particles. They were then surface-sterilized following the protocol of Chi et al. (2019), i.e., immersed in 70% ethyl alcohol for 10 s, then soaked in 4% sodium hypochlorite for 30 s, and finally washed twice in sterile distilled water. It was crucial to ensure that the fungal isolates from the plant *Zanthoxylum simulans* were truly endophytes, and were not due to contamination of epiphytes. We confirmed that all the epiphytic microbes were removed, as no microbial growth occurred on control agar plates after 30 days of culture. The surface-sterilized leaf and stem segments were placed on 2% malt extract agar (MEA; BD Biosciences, Franklin Lakes, NJ, USA) containing 0.5 g/L penicillin G (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 g/L streptomycin sulfate (Sigma-Aldrich). The inoculated plates were incubated at 25 °C and observed daily for one month. Fungi with different mycelial morphotypes were isolated and sub-cultured with MEA.

ITS rDNA sequencing and phylogenetic analysis

Endophytic fungal isolates that exhibited antimicrobial activity were identified based on sequencing of a fragment spanning 18S to 28S, including internal transcribed spacer 1 (ITS1), 5.8S rDNA, and ITS2. The universal primers pairs ITS1: 5′-TCCGTAGGTGAACCT GCGG-3′ (or ITS5: 5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4: 5′-TCCTCCGCT TATTGATATGC-3′ were used to amplify the ITS rDNA sequence (White et al. 1990). Fungal genomic DNA extraction and PCR amplification

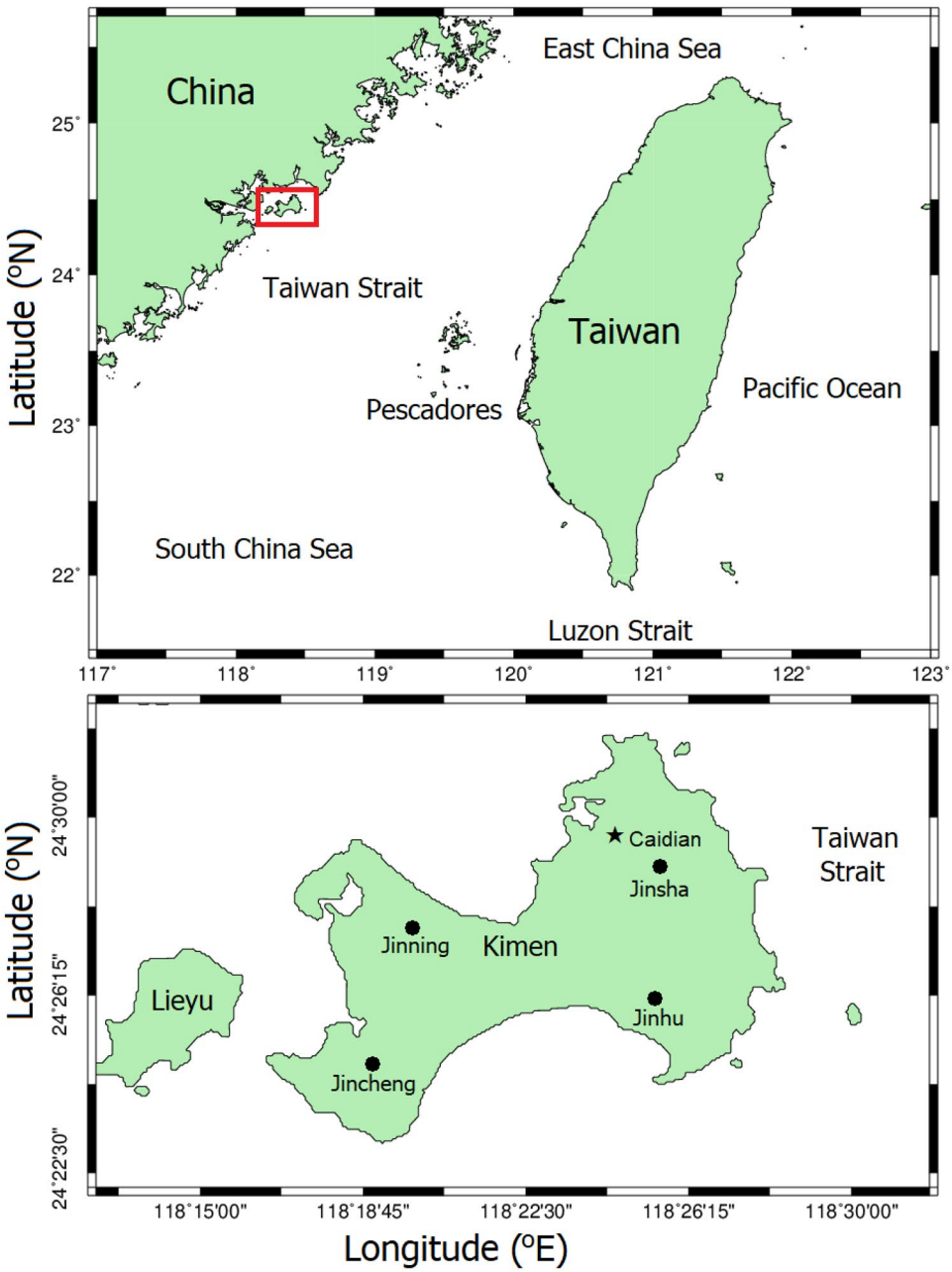


Fig. 1 Distribution of *Zanthoxylum simulans* in Caidian, Kinmen County, Taiwan, from where the samples were collected

were performed following protocols described previously (Chi et al. 2019). All the obtained ITS rDNA sequences were compared against the UNITE Database (User-friendly Nordic ITS Ectomycorrhiza Database) (Nilsson et al. 2019) using the nucleotide BLAST program (Altschul et al. 1990).

Neighbor-joining analysis with Kimura's two-parameter model was performed using MEGA software ver. 7.0.26 (Tamura et al. 2011). The robustness of the phylogenetic tree was evaluated by bootstrap analysis of 1000 randomly-resampled data sets. *Leucoagaricus gongylophorus* SES090115-02 (Basidiomycota) was used as the outgroup.

Preparation of crude extract

The endophytic fungi were cultured by inoculating two agar plugs (8 mm in diameter) of fungi into 250-mL flasks containing 100 ml GYP broth (1 g/L dextrose, 0.2 g/L peptone, and 0.1 g/L yeast extract). Each flask was incubated at 25 °C for 14 days on a shaking incubator at 220 rpm. The culture broth and the mycelia were separated by filtration after incubation; then, the filtered broth was extracted twice with an equal volume of ethyl acetate (EtOAc) and concentrated in a vacuum to dryness (Chi et al. 2019). The crude extracts obtained were kept at − 20 °C and used for

Fig. 2 The natural habitat (**A**), leaves (**B**), flowers (**C**), and fruit (**D**) of *Zanthoxylum simulans*

in vitro antimicrobial, anti-inflammatory, and cell viability assays. Test solutions of endophytic fungi were obtained by dissolving crude extracts in methanol to a final concentration of 0.5 mg/mL.

Indicator microorganisms

The indicator microorganisms used for antimicrobial activity assays throughout this study were as follows: five Gram-negative bacteria, including *Escherichia coli*, *Edwardsiella tarda*, *Pseudomonas anguilliseptica*, *Vibrio alginolyticus*, and *Vibrio parahaemolyticus*; four Gram-positive bacteria, including *Lactococcus garvieae*, *Staphylococcus agalactiae*, *Streptococcus aureus*, and *Streptococcus iniae*; and two fungi, *Candida albicans* (Hazen 1995) and *Cryptococcus neoformans* (Kwon-Chung et al. 2017). Fungal strains *C. albicans* and *C. neoformans* were kindly provided by the Health Bureau of Kinmen County; the nine bacterial strains were supplied by the Institute of Fisheries Science, National Taiwan University.

Indicator strains were cultured as follows: *E. coli* was grown in Luria broth (LB) at 37 °C for 18 h and maintained on LB agar (LA). *E. tarda*, *L. garvieae*, *P. anguilliseptica*, *S. agalactiae*, *S. aureus*, and *S. iniae* were grown in tryptone soy broth (TSB) for 18 h at 37 °C and maintained on tryptone soy agar (TSA). *V. alginolyticus* and *V. parahaemolyticus* were grown in marine broth (MB) for 18 h at 37 °C and maintained on marine broth agar (MA). *C. albicans* and *C. neoformans* were grown in yeast malt broth (YM) for 48 h at 30 °C and maintained on YM agar (YA) (Chi et al. 2019).

Determination of antimicrobial activity

The antimicrobial activities of metabolites of the endophytic fungi against the indicator bacterial and fungal strains were measured using the agar well diffusion method (Rios et al. 1988). Culture broths of bacterial indicators were diluted using beef extract peptone (BEP) medium (5 g/L beef extract, 5 g/L NaCl, 10 g/L peptone, 15 g/L agar) to achieve a final concentration of 1×10^6 CFU/mL and poured into BEP plates. Culture broths of fungal indicators were spread evenly on YA and cultured at 25 °C for 3–5 days. The fungi were diluted using molten Sabouraud agar (SA) to achieve a final concentration of 1×10^5 spores/mL, and 10 mL of this diluted medium was poured into a plate containing 8 mL of solidified SA medium. A sterilized cork borer with an external diameter of 7.8 mm was used to make circular wells in the BEP and SA agar, and 40 µL of the fungal test solutions was added into the wells. The diameters of inhibition zones were measured after incubation of bacterial indicators for 24 h at 37 °C and fungal indicators for 48 h at 25 °C. No activity was observed for any of the indicator microbes in blank tests.

Determination of anti-inflammatory activity and cell viability

Inhibition of inducible nitric oxide synthase (iNOS) activity by treatment with crude extract was determined through decreasing NO release in the supernatants of lipopolysaccharide (LSP)-stimulated cells using the Griess reagent method (Wang et al. 2007) following

previously-established procedures (Chi et al. 2019). Two positive controls, nitroarginine (a non-selective iNOS inhibitor) and aminoguanidine (a selective iNOS inhibitor) plus vehicle (0.1%, DMSO), were added to RAW 264.7 cells (a mouse leukemic macrophage cell line) in the presence of 200 ng/mL LSP. NO formation was measured using the Griess reagent (Wang et al. 2007).

The cell viability of RAW 264.7 cells treated with crude extract was studied using the redox indicator Alamar Blue according to the method described by Chi et al. (2019). The mouse macrophage cell line RAW 264.7 was obtained from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan).

Nucleotide sequence accession numbers

The nucleotide sequences obtained in the present work are available in the GenBank database (MN220648–MN220655 and MN368169–MN368183).

Results

Endophytic fungi isolation and antimicrobial activity analysis

Using the culture-dependent method, a total of 113 endophytic fungi were isolated from all the samples, among which 51 (45.13%) were obtained from the summer samples and 62 (54.87%) from the winter samples. Most of the endophytic fungi (110 isolates, 97.34%) were isolated from leaves, with only 3 isolates (2.65%) isolated from stems.

All crude extracts of the isolated fungi were then tested for antimicrobial activity against 11 indicator microorganisms. Using the agar well diffusion method, 23 isolates (20.35%) were found to possess antimicrobial activity against at least one indicator microorganism (Table 1). The percentages of fungal isolates possessing antibiotic activities obtained from the summer and winter samples were 23.53% (12) and 17.74% (11) (Table 1),

Table 1 Antimicrobial activities of 23 antibiotic-producing endophytic fungi isolated from *Zanthoxylum simulans* Hance against indicator microorganisms using the agar well diffusion method

Culture no.	Sampling season	Antimicrobial activity										
		E. coli	*E. tard*	*P. angu*	*V. algi*	*V. para*	*S. agal*	*S. aure*	*S. inia*	*L. garv*	*C. albi*	*C. neof*
NOU275	SUM	-	-	-	-	-	-	-	-	-	+	-
NOU279	SUM	+	-	-	-	-	-	+	-	-	+	-
NOU283	SUM	-	-	-	-	-	-	+	-	-	-	-
NOU286	SUM	-	-	-	-	-	-	-	-	-	+	-
NOU287	SUM	-	-	-	-	-	-	-	-	-	-	+
NOU291	SUM	-	-	-	-	-	+	-	-	-	+	-
NOU297	SUM	-	-	-	-	-	-	-	-	-	+	-
NOU299	SUM	-	-	-	-	-	-	+	-	-	-	-
NOU304	SUM	-	-	-	-	-	-	-	-	-	+	-
NOU307	SUM	-	-	-	-	-	-	-	-	-	-	+
NOU312	SUM	-	-	-	-	-	-	+	-	-	-	-
NOU315	SUM	-	-	-	-	-	-	+	-	-	-	-
NOU715	WNT	-	-	-	-	-	-	+	-	-	-	-
NOU718	WNT	-	-	-	-	-	+	-	-	-	+	-
NOU721	WNT	+	-	-	-	-	-	-	-	-	-	-
NOU728	WNT	+	-	-	-	-	-	-	-	-	-	-
NOU735	WNT	-	-	-	-	-	-	-	-	-	+	-
NOU741	WNT	-	-	-	-	-	+	-	-	-	+	-
NOU743	WNT	-	-	-	-	-	-	+	-	-	-	-
NOU759	WNT	+	-	-	-	-	+	+	-	-	-	-
NOU760	WNT	-	-	-	-	-	+	-	-	-	-	-
NOU768	WNT	-	-	-	-	-	-	+	-	-	-	-
NOU772	WNT	-	-	+	-	-	-	-	-	-	-	-

SUM summer, *WNT* winter, + with activity, - without activity, *E. coli Escherichia coli*, *E. tard Edwardsiella tarda*, *P. angu Pseudomonas anguilliseptica*, *V. algi Vibrio alginolyticus*, *V. para Vibrio parahaemolyticus*, *S. agal Staphylococcus agalactiae*, *S. aure Staphylococcus aureus*, *S. inia Streptococcus iniae*, *L. garv Lactococcus garvieae*, *C. albi Candida albicans*, *C. neof Cryptococcus neoformans*

respectively. Among the 23 antibiotic-producing fungal isolates, 56.52% (13) exerted activity against Gram-positive bacteria, 21.74% (5) showed activity against Gram-negative bacteria, 8.70% (2) showed activity against both Gram-positive and Gram-negative bacteria, 47.83% (11) showed activity against fungi, and 17.39% (4) showed activity against both bacteria and fungi. Most of the isolates (18 isolates, 78.26%) were able to inhibit one indicator microorganism, and only two isolates (8.70%) inhibited more than three indicator organisms (Table 1).

The percentages of susceptibility of *E. coli*, *P. anguilliseptica*, *S. agalactiae*, *S. aureus*, *C. albicans*, and *C. neoformans* to the 23 antibiotic-producing isolates were 17.39, 4.35, 21.74, 39.13, 39.13, and 8.70% (Table 1), respectively. None of the isolates exhibited activity against indicator microbes *E. tarda*, *V. alginolyticus*, *V. parahaemolyticus*, *L. garvieae*, and *S. iniae*.

Fig. 3 Phylogenetic analysis of fungal endophytes with antimicrobial▶ abilities isolated from medicinal plant *Zanthoxylum simulans*. The phylogenetic tree was constructed based on the ITS-rDNA sequences by the neighbor-joining method, using Kimura's two-parameter model. Bootstrap percentages greater than 70% are shown at branch points. The ITS-rDNA sequence of *Leucoagaricus gongylophorus*, a Basidiomycota species, was used as the outgroup. The scale bar represents 0.05 substitutions per nucleotide position

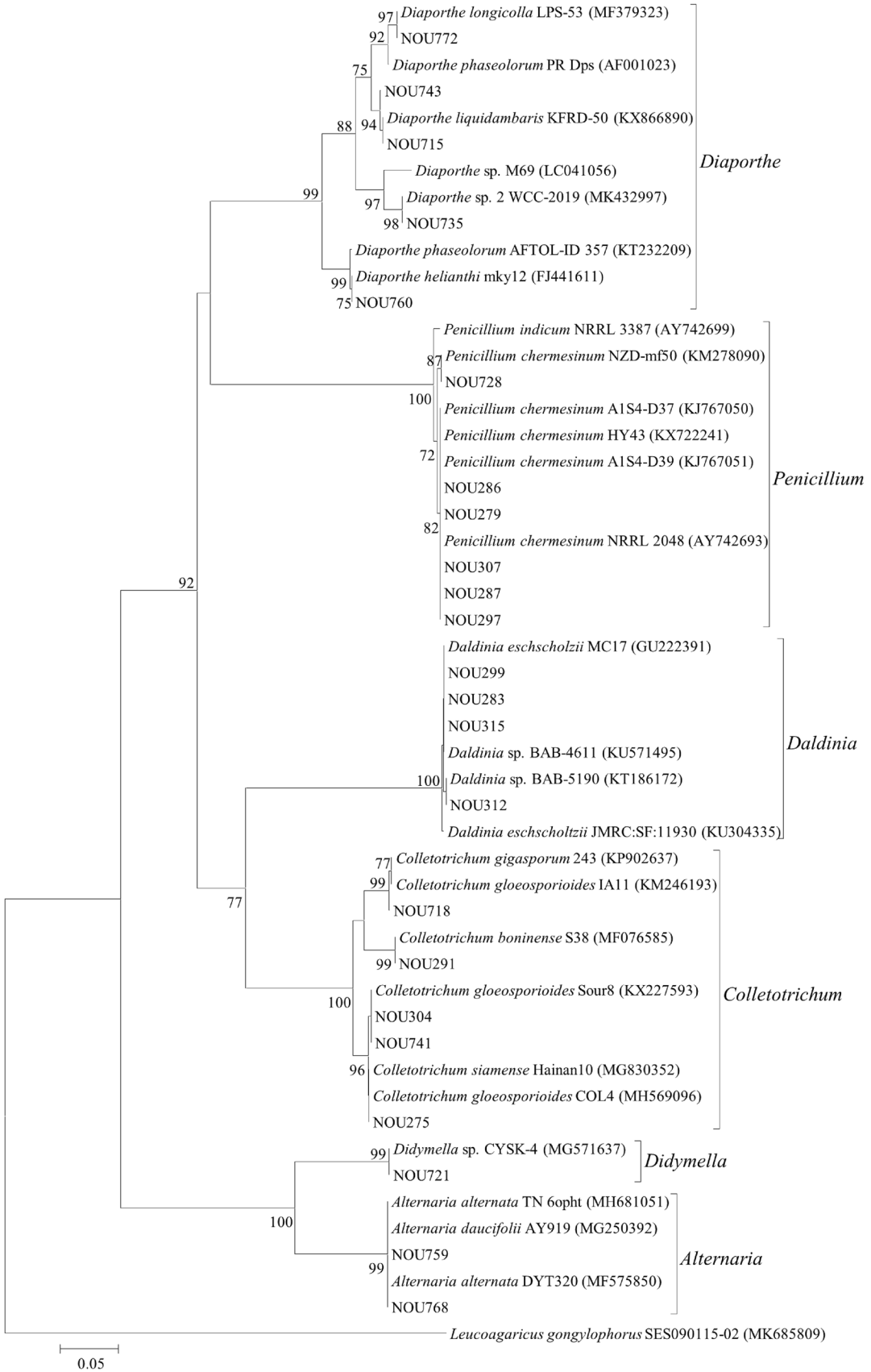
Identification of endophytic fungi producing antibiotics

The ITS rDNA sequences of the 23 antimicrobial substance-producing fungi were sequenced and compared with the UNITE database, a curated database for fungal species identification. All 23 isolates belonged to the phylum Ascomycota (Table 2). According to the morphology and molecular identification from the ITS rDNA sequence (Table 2), *Penicillium* (26.09%, 6 isolates) was the most

Table 2 Blast analysis of ITS-rDNA sequences of 23 antibiotic-producing endophytic fungi isolated from *Zanthoxylum simulans* Hance

Culture no.	Accession no.	Highest match in UNITE database	Accession no.	%Identity	Taxonomy
NOU275	MN220648	*Colletotrichum* sp.	MH569096	100.00%	As; Sordariomycetes; Glomerellales; Glomerellaceae
NOU279	MN220649	*Penicillium chermesinum*	KJ767051	100.00%	As; Eurotiomycetes; Eurotiales; Aspergillaceae
NOU283	MN368169	*Daldinia* sp.	GQ999505	100.00%	As; Sordariomycetes; Xylariales; Hypoxylaceae
NOU286	MN368170	*Penicillium chermesinum*	KM405640	100.00%	As; Eurotiomycetes; Eurotiales; Aspergillaceae
NOU287	MN368171	*Penicillium chermesinum*	KX722241	100.00%	As; Eurotiomycetes; Eurotiales; Aspergillaceae
NOU291	MN368172	*Colletotrichum* sp.	SH1543707	100.00%	As; Sordariomycetes; Glomerellales; Glomerellaceae
NOU297	MN368173	*Penicillium chermesinum*	KM278060	100.00%	As; Eurotiomycetes; Eurotiales; Aspergillaceae
NOU299	MN368174	*Daldinia* sp.	SH1507868	100.00%	As; Sordariomycetes; Xylariales; Hypoxylaceae
NOU304	MN220650	*Colletotrichum* sp.	MG830364	100.00%	As; Sordariomycetes; Glomerellales; Glomerellaceae
NOU307	MN368175	*Penicillium chermesinum*	KX722241	100.00%	As; Eurotiomycetes; Eurotiales; Aspergillaceae
NOU312	MN368176	*Daldinia* sp.	KU571495	100.00%	As; Sordariomycetes; Xylariales; Hypoxylaceae
NOU315	MN368177	*Daldinia* sp.	KU571495	100.00%	As; Sordariomycetes; Xylariales; Hypoxylaceae
NOU715	MN220651	*Diaporthe liquidambaris*	FJ478124	100.00%	As; Sordariomycetes; Diaporthales; Diaporthaceae
NOU718	MN220652	*Colletotrichum* sp.	KP902637	99.63%	As; Sordariomycetes; Glomerellales; Glomerellaceae
NOU721	MN368178	*Didymella* sp.	KY828938	100.00%	As; Dothideomycetes; Pleosporales; Didymellaceae
NOU728	MN368179	*Penicillium chermesinum*	KM278090	100.00%	As; Eurotiomycetes; Eurotiales; Aspergillaceae
NOU735	MN368180	*Diaporthe* sp.	AB245060	96.05%	As; Sordariomycetes; Diaporthales; Diaporthaceae
NOU741	MN368181	*Colletotrichum* sp.	MH517366	100.00%	As; Sordariomycetes; Glomerellales; Glomerellaceae
NOU743	MN368182	*Diaporthe* sp.	KU324784	98.84%	As; Sordariomycetes; Diaporthales; Diaporthaceae
NOU759	MN220653	*Alternaria* sp.	MG250392	100.00%	As; Dothideomycetes; Pleosporales; Pleosporaceae
NOU760	MN220654	*Diaporthe helianthi*	FJ441611	100.00%	As; Sordariomycetes; Diaporthales; Diaporthaceae
NOU768	MN368183	*Alternaria* sp.	U05195	100.00%	As; Dothideomycetes; Pleosporales; Pleosporaceae
NOU772	MN220655	*Diaporthe longicolla*	MF379323	99.32%	As; Sordariomycetes; Diaporthales; Diaporthaceae

As ascomycota


Diaporthe longicolla LPS-53 (MF379323)
NOU772
Diaporthe phaseolorum PR Dps (AF001023)
NOU743
Diaporthe liquidambaris KFRD-50 (KX866890)
NOU715
Diaporthe sp. M69 (LC041056)
Diaporthe sp. 2 WCC-2019 (MK432997)
NOU735
Diaporthe phaseolorum AFTOL-ID 357 (KT232209)
Diaporthe helianthi mky12 (FJ441611)
NOU760
Diaporthe
Penicillium indicum NRRL 3387 (AY742699)
Penicillium chermesinum NZD-mf50 (KM278090)
NOU728
Penicillium chermesinum A1S4-D37 (KJ767050)
Penicillium chermesinum HY43 (KX722241)
Penicillium chermesinum A1S4-D39 (KJ767051)
NOU286
NOU279
Penicillium chermesinum NRRL 2048 (AY742693)
NOU307
NOU287
NOU297
Penicillium
Daldinia eschscholzii MC17 (GU222391)
NOU299
NOU283
NOU315
Daldinia sp. BAB-4611 (KU571495)
Daldinia sp. BAB-5190 (KT186172)
NOU312
Daldinia eschscholtzii JMRC:SF:11930 (KU304335)
Daldinia
Colletotrichum gigasporum 243 (KP902637)
Colletotrichum gloeosporioides IA11 (KM246193)
NOU718
Colletotrichum boninense S38 (MF076585)
NOU291
Colletotrichum gloeosporioides Sour8 (KX227593)
NOU304
NOU741
Colletotrichum siamense Hainan10 (MG830352)
Colletotrichum gloeosporioides COL4 (MH569096)
NOU275
Colletotrichum
Didymella sp. CYSK-4 (MG571637)
NOU721
Didymella
Alternaria alternata TN 6opht (MH681051)
Alternaria daucifolii AY919 (MG250392)
NOU759
Alternaria alternata DYT320 (MF575850)
NOU768
Alternaria
Leucoagaricus gongylophorus SES090115-02 (MK685809)
0.05

abundant genus obtained from *Z. simulans*, followed by *Colletotrichum* (21.74%, 5), *Diaporthe* (21.74%, 5), *Daldinia* (17.39%, 4), *Alternaria* (8.70%, 2), and *Didymella* (4.34%, 1) (Table 2). In addition, a neighbor-joining tree showing the affiliation of the ITS rDNA sequences from these antibiotics-producing fungi to selected reference sequences is presented in Fig. 3. The topology of the phylogenetic tree (Fig. 3) showed that 20 strains in the genera *Diaporthe*, *Penicillium*, *Daldinia*, and *Colletotrichum* formed a major clade I, under a bootstrap confidence value of 92%, while 3 strains in the genera *Didymella* and *Alternaria*, phylogenetically related to order Pleosporales, clustered to form another clade II (bootstrap value, 100%).

Figure 4 compares the taxonomic compositions of fungal endophytes isolated in different seasons at the genus level. We observed that endophytic fungi of genus *Diaporthe* were only present in winter specimens in high abundance (45.45%), whereas endophytic fungi of genus *Daldinia* were only present in summer specimens in high abundance (33.33%). *Alternaria* and *Didymella* were not found in the winter plant samples. Moreover, we found that the patterns of susceptibility of pathogens to summer and winter fungal isolates of the same genus differed; for example, *S. aureus*, *C. albicans* and *C. neoformans* were only susceptible to *Penicillium* isolates collected in summer (Tables 1 and 2). All the above results suggested that endophytic fungi communities might vary in a season-dependent fashion.

Anti-inflammatory analysis

The crude extracts of the 23 endophytic fungal isolates with antimicrobial activity were further evaluated for anti-inflammatory activity using the Griess assay. The cytotoxicities of the fungal crude extracts were determined using an Alamar Blue assay. Fourteen isolates (60.87%) showed low nitric oxide production (< 10%) in the Griess assay, among which 9 isolates (64.29%) did not produce any NO Fig. 5a. The level (% of vehicle) of the Alamar Blue assay ranged from 0 (*Penicillium chermesinum* NQU287; (Biourge 1923)) to 115.49 (*Colletotrichum* sp. NQU741) Fig. 5b; 13 (56.52%) of the 23 test extracts showed a high cell viability (> 80%) Fig. 5b.

In preliminary screening, endophytic fungi with the potential for medicinal use were required to match the criteria of low cytotoxicity (higher values in the Alamar Blue assay) and strong inhibition of iNOS (lower values in the Griess assay) Fig. 5c. Thus, we set the threshold value at 50 for both assay methods in order to identify potentially useful endophytic fungi. A total of 15 endophytic fungi (65.22%) matched the screening conditions Fig. 5c, among

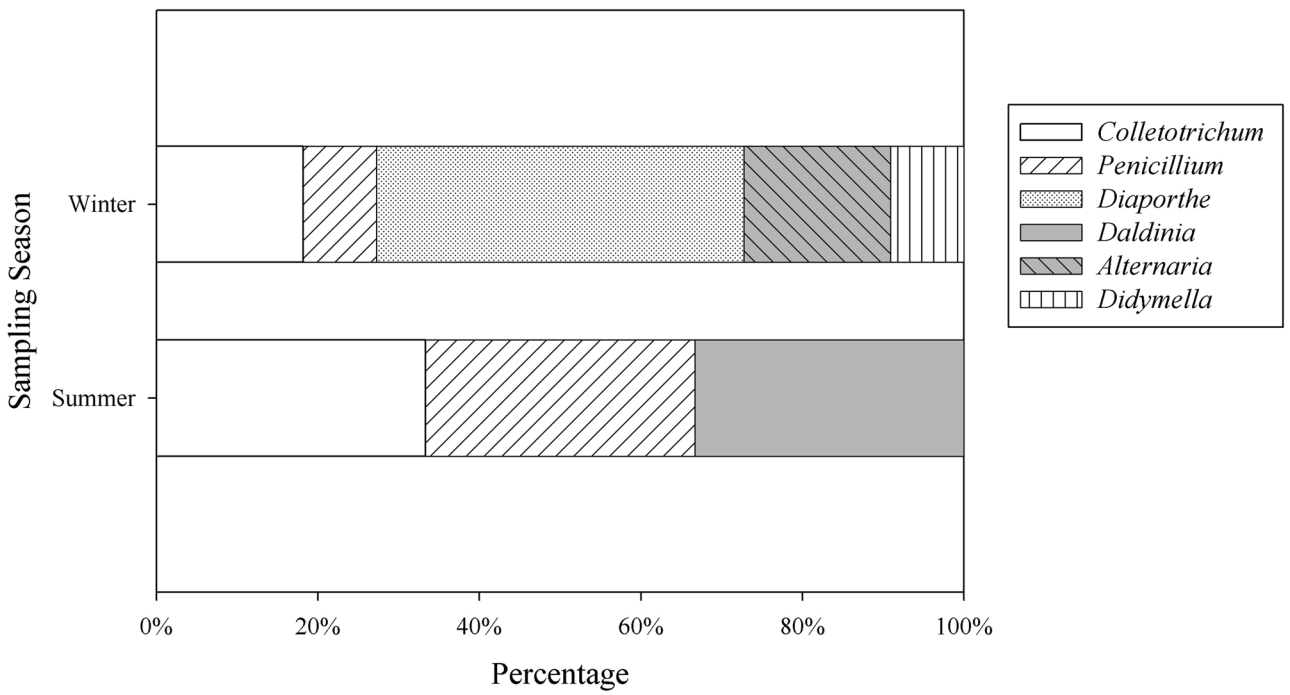


Fig. 4 Taxonomic distribution of endophytic fungi with antimicrobial activities isolated from medicinal plant *Zanthoxylum simulans* in different seasons at the genus level

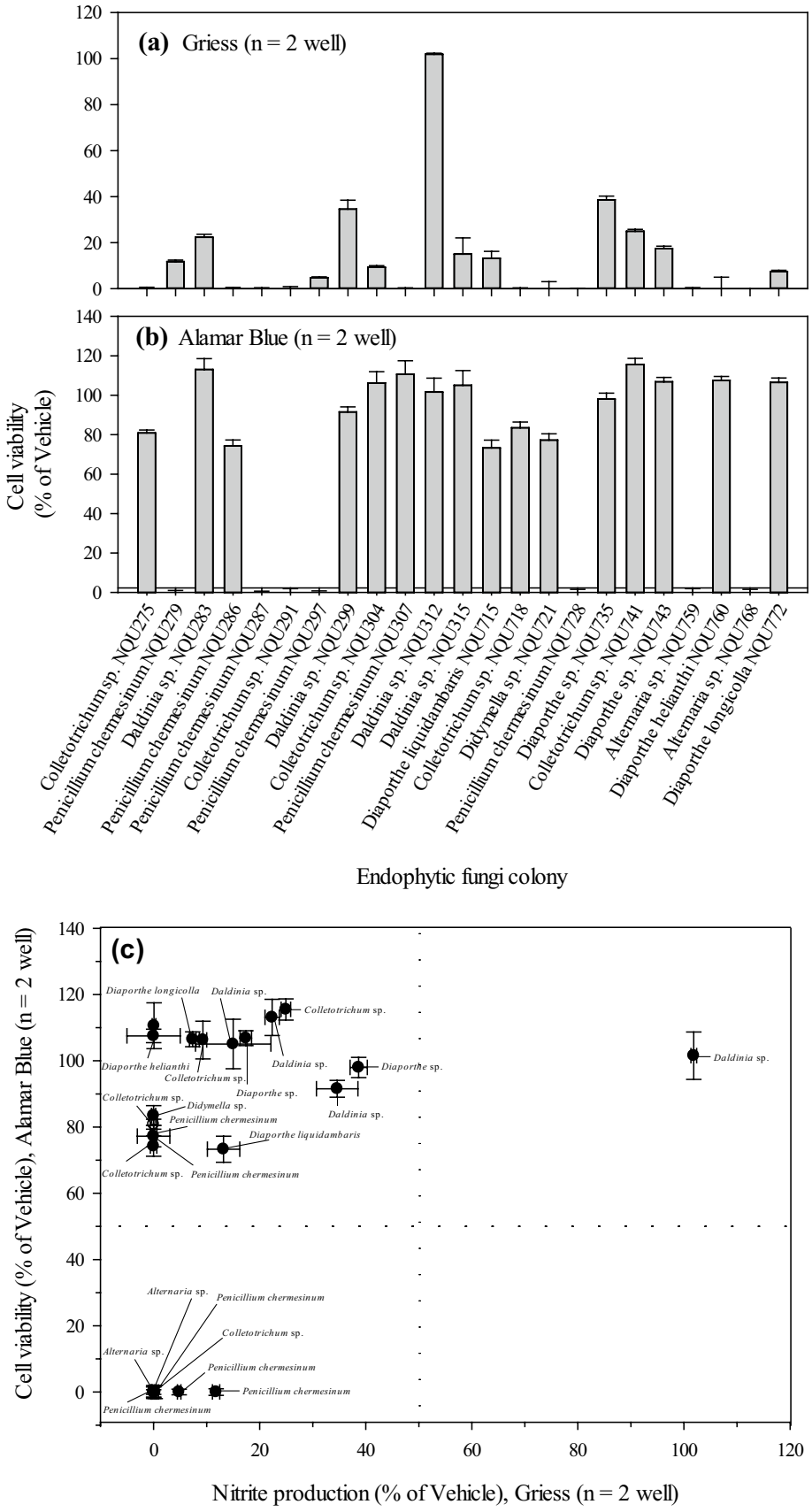


Fig. 5 Nitrite production (**a**), cell viability (**b**), and nitrite production against cell viability with a threshold value of 50 for both assay methods for identification of colonies with anti-inflammatory potential (**c**) of 23 endophytic fungi colonies collected in two sampling seasons based on low NO production and lower cytotoxicity

which 8 and 7 fungi colonies were isolated from plant samples obtained in winter and summer, respectively.

The best anti-inflammatory activity was observed for two isolates, *P. chermesinum* NQU307 and *D. helianthi* NQU760 (Muntanola-Cvetkovic et al. 1981), their extracts having both a high NO inhibition effect (< 15%) and a low cell toxicity (> 100% cell viability) Fig. 5a, b. Our results indicated that crude extracts from the fermentation broth of endophytic fungi isolated from medicinal plant *Z. simulans* are a potential source of natural anti-inflammatory active compounds.

Discussion

Many previous studies have indicated that endophytes of plants, especially medicinal plants, are a promising and untapped source of functional metabolites (Cui et al. 2011; Vieira et al. 2014; Dos Santos et al. 2015; Yang et al. 2015; Liu et al. 2016). However, only very small numbers of plant endophytes and their metabolites have been identified and characterized. In this study, endophytic fungi of *Zanthoxylum simulans* from Kinmen Island were thoroughly screened for metabolites with antimicrobial activity, and a total of 113 endophytic fungi were isolated. The crude ethyl acetate extracts of 23 isolates (20.35%) were observed to exhibit antimicrobial activity towards at least one indicator microorganism. Previous studies have demonstrated antimicrobial activities of fungal endophytes from medicinal plants *Indigofera suffruticosa* Miller (Dos Santos et al. 2015), *Cephalotaxus hainanensis* Li (Liu et al. 2016), and *Melastoma malabathricum* L (Mishra et al. 2016), and reported that 27.69%, 34.74%, and 26.37% of the fungal endophytes possessed antimicrobial activity, respectively; these results were consistent with our findings. In contrast, the percentage of fungal endophytes possessing antimicrobial activity in our study was lower than the percentages for *Aquilaria sinensis* (46.4%) and *Cephalotaxus hainanensis* Li (80·95%), and higher than that of *Baccharis trimera* (12.8%) (Cui et al. 2011; Vieira et al. 2014; Yang et al. 2015). In general, the percentage of endophytic fungi obtained from medical plants that possess antimicrobial activity varies widely from study to study (Luo et al. 2015). However, it is worth noting that the percentage can be affected by the number of indicator microbes used, the culture conditions of the endophytic fungi, and the assay method employed (Kennedy et al. 2009; Chen et al. 2012; Luo et al. 2015).

In this study, more than half (13 isolates, 56.52%) of the isolates with antimicrobial activities were specific to Gram-positive bacteria *S. aureus* and *S. agalactiae*, and only five isolates (21.74%) were found to produce inhibitors against Gram-negative bacteria *E. coli* and *P. anguilliseptica*. These results were in agreement with many previous studies in which the antibacterial activities of endophytic fungi obtained from medicinal plants were analyzed, and exhibited more potent activity against Gram-positive bacteria than Gram-negative bacteria (Phongpaichit et al. 2006; de Siqueira et al. 2011; Kaul et al. 2012). The outer layer of the cell wall of Gram-negative bacteria is composed of lipopolysaccharide, in contrast to the thick layer of peptidoglycan present in Gram-positive bacteria. Owing to differences in the cell-wall composition, antibacterial activity against Gram-positive and Gram-negative bacteria differs (Chi et al. 2019). In addition, ethyl acetate extracts are comparatively less-polar natural products than other solvents such as methanol, and these less-polar natural products might be responsible for the antimicrobial activity of the majority of the extracts against Gram-positive bacteria (Borquaye et al. 2016).

Some fungi, such as *Diaporthe helianthi* NQU760, and *Diaporthe longicolla* NQU772 (Santos et al. 2011), isolated from *Z. simulans* are well-known plant pathogens (Heller and Gierth 2001; Li et al. 2017). Several previous studies have shown that plants harbor diverse communities of endophytic fungi with antimicrobial activity, some of which are plant pathogens (Ashour et al. 2011). For example, *Diaporthe helianthi*, which has been reported to cause severe sunflower disease (Battilani et al. 2003), was isolated from the Brazilian plant *Luehea divaricata*, and found to produce natural products with antimicrobial activity (Specian et al. 2012), while Wagenaar and Clardy (2001) isolated antibiotic-producing *Diaporthe longicolla*, which can cause Phomopsis seed decay in soybean plants (Li et al. 2017). Barley pathogen *Ramularia collo-cygni*, which spends most of its life cycle as an endophyte and turns necrotrophic after months of symptomless growth in the host, was found to produce secondary metabolites with antimicrobial activity (Dussart et al. 2020). These pathogenic isolates were obtained from *Z. simulans* plants that did not exhibit any visible injury; therefore, a symbiotic relationship may be established between pathogenic fungi and host, with the fungi changing themselves into endophytes, which is in agreement with the hypothesis that endophytic fungi develop from pathogenic fungi of plants (Hormazabal and Piontelli 2009).

Zanthoxylum spp. include around 250 species, which have been used in Asia, Africa, and America to treat a number of diseases in humans and animals (Javier Patino et al. 2012). Among the *Zanthoxylum* genus, *Zanthoxylum bungeanum* is the only species in which endophytic fungi have been studied. Li et al. (2016) studied the diversity and antifungal activity of endophytic fungi in *Z. bungeanum* and found that *Alternaria* (30.85%), *Fusarium* (13.72%), and *Phoma* (12.77%) were the most abundant genera. However, this result differed from our findings that the most frequent species with antimicrobial activity found in *Z. simulans*

were *Penicillium* (26.09%), *Colletotrichum* (21.74%), and *Diaporthe* (21.74%) (Table 1). In contrast, Araujo et al. (2018) reported that *Colletotrichum*, *Penicillium*, and *Diaporthe* were the three most frequent endophytic genera cultured from Brazilian Amazon rubber tree *Hevea guianensis*, which was consistent with our results.

In this study, we found that in summer and winter, the composition of fungal endophytes with antimicrobial activity (Table 1) and the patterns of susceptibility of pathogens to these fungi differed (Fig. 4). It should be noted that we only identified fungal isolates with antimicrobial activity, which therefore did not represent the diversity of endophytic communities. However, a possible explanation of our result could be that the composition and dominance of endophytic species in *Z. simulans* differs in different seasons. This result was in line with several previous studies of the diversity of fungal endophytes (de Souza Sebastianes et al. 2013), which indicated that the diversity and richness of fungal endophytes depend not only on the season but also on the geographical location (González and Tello 2011) and the part of the plant from which the sample was obtained (Gazis and Chaverri 2010).

As shown in Fig. 5a, b, we found that crude extracts of *Penicillium chermesinum* NQU307 and *Diaporthe helianthi* NQU760 possessed strong anti-inflammatory efficacies. A recent study showed that four compounds obtained from *Diaporthe* sp., an endophytic fungus from the Arjun tree *Terminalia arjuna* (Roxb.), showed anti-inflammatory activities (Patil et al. 2017). It has also been reported that plastatin and puteosporin, two naturally occurring PLA_2 (porcine pancreatic phospholipase A_2) inhibitors, from *Penicillium chermesinum*, isolated from a soil sample collected in Nova Scotia, Canada, exhibited anti-inflammatory activities (Singh et al. 1985). These results indicated that crude extracts from fermentation broth of endophytic fungi obtained from medicinal plant *Z. simulans* are a potential source of natural anti-inflammatory active compounds. In addition, it should be noted that these two fungi isolated in this study also exhibited antimicrobial activity. Therefore, they should be good candidates for further isolation and identification of their secondary metabolites, which may provide new drug leads.

Conclusions

As a renowned traditional medicine, the biology, ecology, and metabolite production of endophytic fungi associated with *Zanthoxylum simulans* have not been exploited. The present study confirmed the presence of inhibitors in crude extracts of endophytic fungi obtained from *Z. simulans* for the first time and also provided a foundation for the development and utilization of fungal resources obtained from this medicinal plant for the potential improvement of human welfare. Further studies to characterize the bioactive constituents of the extracts should be conducted in order to gain a greater understanding of the potential and mechanisms of these natural inhibitors.

Acknowledgements The authors thank Guei-Jane Wang, who performed the determination of antioxidant activity, and Hsi-Tsun Chen, who serves in Kinmen County Forestry Bureau and assisted with the identification of plant samples. We are also grateful to Ching-Chang Hung for providing images of the plant.

Author contribution WCC took charge of the experimental design. Collection of leaves and stems of *Zanthoxylum simulans* in Kinmen was carried out by WCC. WCC and CFC isolated and identified endophytic fungi. WCC and KJ interpreted the results, conducted phytochemical screening, and wrote and finalized the manuscript.

Funding Financial support was received from the Kinmen County Fisheries Research Institute (grant number 106G019-1) by W.-C. Chi.

Data availability Specimens of fungi after identification were deposited at the Department of Food Science, National Kinmen University, Kinmen, Taiwan, Republic of China. All data generated during the study are interpreted in the manuscript.

Compliance with ethical standards

Conflicts of interests The authors declare that they have no competing interests.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Araújo KS, Brito VN, Veloso TGR, Leite TS, Pereira OL, Mizubuti ESG, de Queiroz MV (2018) Diversity of culturable endophytic fungi of *Hevea guianensis*: a latex producer native tree from the Brazilian Amazon. Afr J Microbiol Res 12:953–964

Ashour M, Yehia MH, Proksch P (2011) Utilization of agro-industrial by-products for production of bioactive natural products from endophytic fungi. J Nat Prod 4:108–114

Battilani P, Rossi V, Girometta B, Delos M, Rouzet J, André N, Esposito S (2003) Estimating the potential development of *Diaporthe helianthi* epidemics in Italy. Bull OEPP 33:427–431

Biourge P (1923) Les moisissures du groupe *Penicillium*. Cellule 33:7–331

Borquaye LS, Darko G, Oklu N, Anson-Yevu C, Ababio A (2016) Antimicrobial and antioxidant activities of ethyl acetate and methanol extracts of *Littorina littorea* and *Galatea paradoxa*. Cogent Chem 2:1161865

Bunalema L, Fotso GW, Waako P, Tabuti J, Yeboah SO (2017) Potential of *Zanthoxylum leprieurii* as a source of active compounds against drug resistant *Mycobacterium tuberculosis*. BMC Complement Altern Med 17:89

Chen YH, Kuo J, Sung PJ, Chang YC, Lu MC, Wong TY, Liu JK, Weng CF, Twan WH, Kuo FW (2012) Isolation of marine bacteria with antimicrobial activities from cultured and field-collected soft corals. World J Microbiol Biotechnol 28:3269–3279

Chi WC, Pang KL, Chen WL, Wang GJ, Lee TH (2019) Antimicrobial and iNOS inhibitory activities of the endophytic fungi isolated from the mangrove plant *Acanthus ilicifolius* var. xiamenensis. Bot Stud 60:4

Chyau CC, Mau JL, Wu CM (1996) Characteristics of the steam-distilled oil and carbon dioxide extract of *Zanthoxylum simulans* fruits. J Agr Food Chem 44:1096–1099

Costa RS, Filho OPS, DiasJúnior OCS, Silva JJ, Hyari ML, Santos MAV, Velozo ES (2018) In vitro antileishmanial and antitrypanosomal activity of compounds isolated from the roots of *Zanthoxylum tingoassuiba*. Rev Bras Farmacogn 28:551–558

Cui JL, Guo SX, Xiao PG (2011) Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*. J Zhejiang Univ Sci B 12:385–392

De Bary A (1879) Die Erscheinung der Symbiose. In: Trubner KJ (ed) Vortrag auf der Versammlung der Naturforscher und Ärtze zu Cassel. Verlag, Strassburg, pp 1–30

de Siqueira VM, Conti R, de Araújo JM, Souza-Motta CM (2011) Endophytic fungi from the medicinal plant *Lippia sidoides* Cham. and their antimicrobial activity. Symbiosis 53:89–95

de Souza Sebastianes FL, Romao-Dumaresq AS, Lacava PT, Harakava R, Azevedo JL, de Melo IS, Pizzirani-Kleiner AA (2013) Species diversity of culturable endophytic fungi from Brazilian mangrove forests. Curr Genet 59:153–166

Deshmukh SK, Verekar SA, Bhave SV (2014) Endophytic fungi: a reservoir of antibacterials. Front Microbiol 5:715

Dos Santos IP, da Silva LCN, da Silva MV, de Araújo JM, Cavalcanti Marilene S, Lima VL (2015) Antibacterial activity of endophytic fungi from leaves of *Indigofera suffruticosa* Miller (Fabaceae). Front Microbiol 6:350

Dussart F, Creissen HE, Havis ND (2020) *Ramularia collo-cygni* – an enemy in waiting. eLS. John Wiley & Sons, Ltd, pp 1–8

Ekka G, Jadhav SK, Quraishi A (2020) An Overview of genus *Zanthoxylum* with special reference to its herbal significance and application. In: Akram M, Ahmad RS (eds) Herbs and spices. IntechOpen, Rijeka, pp 1–17

Epifano F, Curini M, Marcotullio MC, Genovese S (2011) Searching for novel cancer chemopreventive plants and their products: the genus *Zanthoxylum*. Curr Drug Targets 12:1895–1902

Gazis R, Chaverri P (2010) Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. Fungal Ecol 3:240–254

González V, Tello ML (2011) The endophytic mycota associated with *Vitis vinifera* in central Spain. Fungal Divers 47:29–42

Hazen KC (1995) New and emerging yeast pathogens. Clin Microbiol Rev 8:462–478

He W, Van Puyvelde L, De Kimpe N, Verbruggen L, Anthonissen K, Van der Flaas M, Bosselaers J, Mathenge SG, Mudida FP (2002) Chemical constituents and biological activities of *Zanthoxylum usambarense*. Phytother Res 16:66–70

Heinig U, Scholz S, Jennewein S (2013) Getting to the bottom of Taxol biosynthesis by fungi. Fungal Divers 60:161–170

Helaly SE, Thongbai B, Stadler M (2018) Diversity of biologically active secondary metabolites from endophytic and saprotrophic fungi of the ascomycete order Xylariales. Nat Prod Rep 35:992–1014

Heller A, Gierth K (2001) Cytological observations of the infection process by *Phomopsis helianthi* (Munt.-Cvet) in leaves of sunflower. J Phytopathol 149:347–357

Hormazabal E, Piontelli E (2009) Endophytic fungi from Chilean native gymnosperms: antimicrobial activity against human and phytopathogenic fungi. World J Microbiol Biotechnol 25:813–819

Javier Patino LO, Angelica Prieto RJ, Enrique Cuc SL (2012) *Zanthoxylum* genus as potential source of bioactive compounds. In: Rasooli I (ed) Bioactive compounds in phytomedicine. InTech Europe, Rijeka, pp 185–218

Jia M, Chen L, Xin HL, Zheng CJ, Rahman K, Han T, Qin LP (2016) A friendly relationship between endophytic fungi and medicinal plants: a systematic review. Front Microbiol 7:906

Kaul S, Gupta S, Ahmed M, Dhar MK (2012) Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites. Phytochem Rev 11:487–505

Kennedy J, Baker P, Piper C, Cotter PD, Walsh M, Mooij MJ et al (2009) Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish waters. Mar Biotechnol 11:384–396

Khan AL, Kang SM, Dhakal KH, Hussain J, Adnan M, Kim JG, Lee IJ (2013) Flavonoids and amino acid regulation in *Capsicum annuum* L. by endophytic fungi under different heat stress regimes. Sci Hortic 155:1–7

Kumar V, Kumar S, Singh B, Kumar N (2014) Quantitative and structural analysis of amides and lignans in *Zanthoxylum armatum* by UPLC-DAD-ESI-QTOF-MS/MS. J Pharm Biomed Anal 94:23–29

Kwon-Chung KJ, Bennett JE, Wickes BL, Meyer W, Cuomo CA, Wollenburg KR et al (2017) The case for adopting the "species complex" nomenclature for the etiologic agents of *Cryptococcosis*. mSphere 2:e00357–16

Lee S-J, Lim K-T (2008) Glycoprotein of *Zanthoxylum piperitum* DC has a hepatoprotective effect via anti-oxidative character in vivo and in vitro. Toxicol In Vitro 22:376–385

Li P, Wu Z, Liu T, Wang Y (2016) Biodiversity, phylogeny, and antifungal functions of endophytic fungi associated with *Zanthoxylum bungeanum*. Int J Mol Sci 17:1541

Li S, Darwish O, Alkharouf NW, Musungu B, Matthews BF (2017) Analysis of the genome sequence of *Phomopsis longicolla*: a fungal pathogen causing Phomopsis seed decay in soybean. BMC Genomics 18:688

Liu XZ, Song WL, Zhang K, Ye YC, Dai CC (2011) Effects of two kinds of endophytic fungi infection on water stress of seedlings of *Chrysanthemum morifolium*. Acta Hortic Sin 38:335–342

Liu YH, Hu XP, Li W, Cao XY, Yang HR, Lin ST, Xu CB, Liu SX, Li CF (2016) Antimicrobial and antitumor activity and diversity of endophytic fungi from traditional Chinese medicinal plant *Cephalotaxus hainanensis* Li. Genet Mol Res 15

Luo ZP, Lin HY, Ding WB, He HL, Li YZ (2015) Phylogenetic diversity and antifungal activity of endophytic fungi associated with *Tephrosia purpurea*. Mycobiology 43:435–443

Lyu ZZ, Liu JK, Qg Q, Xj Z (2018) Isolation of endophytic fungi from *Zanthoxylum simulans* and screening of its active strain. Zhongguo Zhong Yao Za Zhi 43:1434–1440

Meng JJ, He XL (2011) Effects of AM fungi on growth and nutritional contents of *Salvia miltiorrhiza* Bge. under drought stress. J Agric Univ Hebei 34:51–55

Mishra VK, Singh G, Passari AK, Yadav MK, Gupta VK, Singh BP (2016) Distribution and antimicrobial potential of endophytic fungi associated with ethnomedicinal plant *Melastoma malabathricum* L. J Environ Biol 37:229–237

Muntanola-Cvetkovic M, Mihaljcevic M, Petrov M (1981) On the identity of the causative agent of a serious Phomopsis-Diaporthe disease in sunflower plants. Nova Hedwigia 34:417–435

Nguyen PH, Zhao BT, Kim O, Lee JH, Choi JS, Min BS, Woo MH (2016) Anti-inflammatory terpenylated coumarins from the leaves of *Zanthoxylum schinifolium* with α-glucosidase inhibitory activity. J Nat Med 70:276–281

Nilsson RH, Larsson KH, Taylor AFS, Bengtsson-Palme J, Jeppesen TS, Schigel D et al (2019) The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res 47:D259–D264

Patil M, Patil R, Mohammad S, Maheshwari V (2017) Bioactivities of phenolics-rich fraction from *Diaporthe arengae* TATW2, an endophytic fungus from *Terminalia arjuna* (Roxb.). Biocatal Agr Biotech 10:396–402

Phongpaichit S, Rungjindamai N, Rukachaisirikul V, Sakayaroj J (2006) Antimicrobial activity in cultures of endophytic fungi isolated from *Garcinia species*. FEMS Immunol Med Microbiol 48:367–372

Qi H, Wang WX, Dai JL, Zhu L (2015) In vitro anthelmintic activity of *Zanthoxylum simulans* essential oil against *Haemonchus contortus*. Vet Parasitol 211:223–227

Rios JL, Recio MC, Villar A (1988) Screening methods for natural products with antimicrobial activity: a review of the literature. J Ethnopharmacol 23:127–149

Santos JM, Vrandečić K, Cosić J, Duvnjak T, Phillips AJ (2011) Resolving the *Diaporthe* species occurring on soybean in Croatia. Persoonia 27:9–19

Singh PD, Johnson JH, Aklonis CA, Bush K, Fisher SM, O'Sullivan J (1985) Two new inhibitors of phospholipase A2 produced by *Penicillium chermesinum*. Taxonomy, fermentation, isolation, structure determination and biological properties. J Antibiot 38:706–712

Specian V, Sarragiotto MH, Pamphile J, Clemente E (2012) Chemical characterization of bioactive compounds from the endophytic fungus *Diaporthe helianthi* isolated from *Luehea divaricata*. Braz J Microbiol 43:1174–1182

Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260:214–216

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Vieira ML, Johann S, Hughes FM, Rosa CA, Rosa LH (2014) The diversity and antimicrobial activity of endophytic fungi associated with medicinal plant *Baccharis trimera* (Asteraceae) from the Brazilian savannah. Can J Microbiol 60:847–856

Wagenaar MM, Clardy J (2001) Dicerandrols, new antibiotic and cytotoxic dimers produced by the fungus *Phomopsis longicolla* isolated from an endangered mint. J Nat Prod 64:1006–1009

Wang C, Wan J, Mei Z, Yang X (2014) Acridone alkaloids with cytotoxic and antimalarial activities from *Zanthoxylum simullans* Hance. Pharmacogn Mag 10:73–76

Wang GJ, Chen SM, Chen WC, Chang YM, Lee TH (2007) Selective inducible nitric oxide synthase suppression by new bracteanolides from *Murdannia bracteata*. J Ethnopharmacol 112:221–227

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, New York, pp 315–322

Wu SJ, Chen IS (1993) lkaloids from *Zanthoxylum simulans*. Phytochemistry 34:1659–1661

Yang HR, Hu XP, Jiang CJ, Qi J, Wu YC, Li W, Zeng YJ, Li CF, Liu SX (2015) Diversity and antimicrobial activity of endophytic fungi isolated from *Cephalotaxus hainanensis* Li, a well-known medicinal plant in China. Lett Appl Microbiol 61:484–490

Yang YP, Cheng MJ, Teng CM, Chang YL, Tsai IL, Chen IS (2002) Chemical and anti-platelet constituents from Formosan *Zanthoxylum simulans*. Phytochemistry 61:567–572