International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (4), pp. 001-006, April, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Isolating endophytic fungi from evergreen plants and determining their antifungal activities

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Accepted 17 January, 2018

A total of 262 strains of endophytic fungi were isolated from 23 evergreen plant species collected from Zijin Mountain in Nanjing, China. Two-hundred and three of the fungi isolates were classified into 23 taxa in 19 genera based on colony morphology and microscopic observation of mycelia and asexual/sexual spores. The highest richness was obtained from *Cedrus deodara* (28), while the highest diversity of identified species (6) was isolated from *Sabina procumbens*. Some fungi appeared to be host-specific, such as *Botrytis ricini* lt300, *Geotrichum candidum* lt274 and *Lacellina graminicola* lt256, while other strains (e.g. *Alternaria alternata* lt222, *Anthina* sp. Lt147, *Colletotrichum gloeosporioids* lt305 and *Fusarium solani* lt293) were commonly isolated from a range of plants. The richness of the endophytic fungi recovered from plant branches was significantly higher than those from leaves. Moreover, about 70% of the obtained endophytic fungi could produce antifungal metabolites against at least one plant pathogenic fungi. The EtOAc extracts of seven species of *Anthina* sp. lt147, *C. gloeosporioids* lt305, *Ectostroma* sp. Lt144, *Fusarium decemcellulare* lt183, *Fusarium oxysporum* lt173, *Paraconiothyrium brasiliense* lt161 and *C. montemartinii* lt198 showed broad inhibition against the growth of all the six phytopathogens with the inhibition rates from 20 to 80%. These results indicated that endophytic fungi may play an important role in protection of the evergreen plants from disease as well as an excellent resource for searching for natural antifungal compounds.

Key words: Endophytic fungi, evergreen plants, antifungal activity.

INTRODUCTION

Endophytic fungi are microorganisms which grow symptomless within plants that show no obvious sign of infection or disease (Hallmann et al., 1997). They often occur sparsely as hypha in the intercellular fluids and wall spaces of their plant hosts (Bacon and White, 2000). Endophytic fungi have been found in healthy tissues of all the terrestrial plant taxa and even lichens with abundant species (Arnold, 2007).

The interactions between the endophytic fungi and their hosts are complex including mutualism, commensalism, and latent and virulent pathogenicity (Hallmann et al., 1997 and references therein). Endophytic fungi colonizing the plant tissues usually get nutrition and protection from the host plant. In return, they enhance tolerance of the host plants by producing certain functional metabolites (Redman et al., 2002). Certain entophytic fungi might promote growth and improve the ecological adaptability of their hosts by enhancing plant tolerance to environmental stresses and resistance to phytopathogens and/or herbivores (Waller et al., 2005). For example, *Microdochium bolleyi,* an endophytic fungus of *Fagonia cretica*, displayed antifungal activities against plant pathogen *Microbotryum violaceum* (Zhang et al., 2008). Kim et al. (2007) reported that *Fusarium oxysporum* strain EF119, which was isolated from roots of red pepper, showed potent disease control efficacy against tomato late blight, with control value over 90%. In addition, endophytic microorganisms are recognized as an outstanding source of bioactive natural products for

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exploitation in medicine, agriculture, and industry, because there are so many of them occupying literally millions of unique higher plants growing in so many unusual environments (Guo et al., 2008). Particularly, since *Taxomyces andreanae,* an endophytic fungus that produces anti-cancer drug taxol in *Taxus brevifolia* Nutt*,* were discovered (Stierle et al., 1993), many researchers began to study endophytic fungi associated with medicinal plants, including their diversity, ecological distribution and active metabolites (Hu et al., 2008 and references therein; Sun et al., 2008). However, there are rare reports about endophytic fungi in evergreen plants based on literature survey. At present study, we presented the diversity and distribution of endophytic fungi in evergreen plants at the scenic resort of Zijin Mountain, Nanjing, and their antifungal activities against several plant diseases causing phytopathogens.

MATERIALS AND METHODS

Samples collection

Healthy branches and leaves of 23 evergreen ornamentals plant species in Zijin Mountain, Jiangsu province, China, were collected in October 2006 and placed in separate sterile ice-boxes and brought to the laboratory for endophytic fungi isolation.

Isolation and identification of endophytic fungi

Methods used for isolating endophytic fungi were described previously with minor modification (Liu et al., 2001). The plant samples were washed with running water, sterilized successively with 75% ethanol for 3 min and 5% sodium hypochlorite for 5 min, then rinsed in sterile water for 3 times and cut into 1 cm segments for twigs or 1 cm^2 pieces for leaves. Then, the segments were sectioned vertically into two pieces. Twenty plant segments of each plant branch or leaf were incubated at 28±1°C until mycelium is apparent on potato dextrose agar plates (PDA: potato, 200 g; dextrose, 20 g; agar, 15 g; distilled water, 1 L), supplemented with ampicillin (150 μgml^{-1}) and streptomycin (100 μgml^{-1}) to inhibit the bacterial growth, until the mycelium or colony appeared surrounding the segments. Eventually pure mycelia were transferred to PDA slant tubes and stored at 4°C. The isolated endophytic fungi were identified based upon colony morphology and microscopic observation of mycelia and asexual/sexual spores according to the method described in the literature (Barac et al., 2004).

Extraction of endophytic fungal metabolites

The endophytic fungi were cultured on PDA plates for 4 days, then the fresh mycelia were transferred in 1L flask containing 500 ml PD (potato, 200 g; dextrose, 20 g; distilled water, 1 L), medium and cultured at 28°C. After 10 days, the mycelia were discarded by filtration and broths were extracted with ethyl acetate (Liu et al., 2001).

Determination of antifungal activity

The antifungal activity of these strains was evaluated using the *in vitro* plate dilution method (Liu et al., 2001). The extracts mixed with 10 ml of melting PDA medium were poured into Petri dishes (9 cm in diameter) with the final concentration of 2 $mgml^{-1}$. Once the medium had cooled down, each of the 6 test fungal discs (7 mm in diameter), which were taken from the fresh margin of the mycelia, were transferred equally onto the Petri dishes. After culturing at 28°C for 48 h, inhibitory activity of extracts against fungal growth were calculated as the percentage reduction of the hyphal growths in comparison with that of mycelia in the control plates without the extracts. The six target phytopathogenic fungi used in this study were *Fusarium graminearum*, *Alternaria alternata*, *Rhizoctonia solani*, *F. oxysporum*, *Glomerella glycines*, and *Phytophthora capsici*, which were stored in our laboratory.

$$\text{Inhibition (\%)} = (A-B) \times 100 / (A-7)$$

Where A (mm) represents the diameter of the mycelium in the metabolite treated dish; B (mm): represents the diameter of mycelium in water treated dish; 7(mm): the diameter of the discs.

RESULTS

Isolation and identification of endophytic fungi

A total of 262 strains were recovered from 23 species of evergreen plants in Zijin Mountain (Table 1). Moreover, the richness and diversity of the endophytic fungi were different from each plant. The highest richness was obtained from *Cedrus deodara* (28 species) while the lowest from *Magnolia grandiflora*, *Nerium indicum*, *Platycladus orientalis* and *Torreya nucifera* (4 species each). The highest diversity of identified species (6) was isolated from *Sabina procumbens* while the lowest was from *Cyclobalanopsis glauca*, *Llex cornuta*, *Podocaarpus macrophyllus* and *Sabina chinensis* (1 species each).

Except for *Rohdea japonica*, endophytic fungi retrieved from branches were much more than that from leaves. None of the endophytic fungi were obtained from the leaves of *Buxus microphylla, C. deodara, Distylium racemosum* and other twelve plants (Table 1). However, a total of 241 strains of endophytic fungi have been isolated from branches, accounting for 92.0% of the total obtained strains.

Two hundred and three strains out of the total 262 isolated endophytic fungi from the 23 evergreen plant species were grouped into 18 genera based on colony color and shape as well as the mycelia and asexual/sexual spores. While 59 strains without asexual spores, that could not be identified by microscopic observation were classified as unidentified strains. Among the 203 identified strains, the most common species were *Fusarium* spp. (22%) and *Anthina* sp*.* (20%). Among the 23 plants, the highest richness of the identified species was obtained from *S. procumbens* (6), followed by *Elaeagnus pungens* (5)*, B. microphylla* (4)*, C. deodara* (4)*, Eriobotrya japonica* (4) and *Ophiopogon japonicus* (4)*.*

The preponderant taxa in evergreen plants of Zijin Mountain were found to be the species in *Alternaria, Anthina, Colletotrichum, Ectostroma* and *Fusarium*, which

Table 1. Endophytic fungi isolated from 23 species of evergreen plants.

Plant species	Fungal taxon																							
	Aa	As		AfBrCa		Cg	CmCs		EsFd		Fo	Fp	Fs			GcLgMsMm			PbPa	Ps	Rs	Ss	Others	Total
B. microphylla		16						1		1						1							1	20
C. deodara												23	1				1	1					2	28
Cyclobalanopsis glauca															1								2(2)	3(2)
D. racemosum													2	1									3	6
E. pungens	2	1				9(1)	(1)				5(1)												4(2)	21(5)
Eriobotrya japonica							(2)		1				1								1		5	8(2)
Ligustrum lucidum											1		7										(1)	8(1)
Llex cornuta		8																					3(2)	11(2)
Llex latifolia	1(1)												(1)										3	4(2)
Magnolia grandiflora		1		1																			2	4
Mahonia bealei					1	2																	3	6
Nerium indicum					1								2	1										4
O. japonicus											2(1)	1(1)					1		1				15	20(2)
O. fragrans		10							4															14
Pinus parviflora	7	10																						17
P. orientalis		2	1																			1		4
P. macrophyllus									10															10
R. japonica						2(1)	(3)																1(1)	3(5)
S. chinensis																3							2	5
S. procumbens					1	1			3								1			4		1	1	12
T. nucifera	2												1										1	4
V. awabuki		4							16															20
Zephyranthes candida											5		1										3	9

A. alternaria lt222 (Aa), *Anthina sp.* Lt147 (As), *A. flavus* lt152 (Af), *B. ricini* lt300 (Br), *C. acremonium* lt301 (Ca), *C. gloeosporioides* lt305 (Cg), *C. montemartinii* lt198 (Cm), *Coprinellus sp.* Lt201 *(Cs)*, *Ectostroma sp.* Lt144 (Es), *F. decemcellulare* lt183 (Fd), *F. oxysporium* lt173 (Fo), *Fusarium poae* lt174 (Fp), *F. solani* lt293 (Fs), *G. candidum* lt274 (Gc), *L. graminicola* lt256 (Lg), *Marssonia sp.* Lt181 (Ms), *Monochaetia monochaeta* lt210 *(*Mm), *P. brasiliense* lt161 (Pb), *Penicillium albicans* lt170 (Pa), *Phacodium sp.* lt202 (Ps), *Rhizoctonia sp.* lt214 (Rs), *Scterotium sp.* lt203 (Ss).

totally occupied 65% of all the isolated endophytic fungi. Moreover, *Aspergillus flavus* lt152*, Botrytis ricini* lt300*, Geotrichum candidum* lt274*, Lacellina graminicola* lt256*, P. brasiliense* lt161*, Phacodium* sp. Lt202 and *Rhizoctonia* sp. Lt214 showed great preference for their host plants but others could exist in many hosts. For example, *Anthina* sp. lt147 was isolated from *B. microphylla, E. pungens, Ilex cornuta, M. grandiflora, Osmanthus fragrans, Pinus parviflora, P. orientalis* and *Viburnum awabuki*; and so as species of *Fusarium*, recovered from eleven species of plants, including *B. microphylla, E. pungens, D. racemosum, E. pungens,* etc.

Antifungal activity

The antifungal activity of the EtOAc extracts of these endophytic fungal broths was summarized in Table 2. Of the 262, 183 extracts (70%) showed antifungal activity against at least one of the plant diseases causing fungi. Seven extracts of the

Table 2. Antifungal activities of endophytic fungi against six plant pathogenic fungi.

Species	Strains	Test phytopathogenic fungi					
		R.s	***F.o***	***F.g***	***A.a***	***P.c***	***G.g***
Alternaria sp.	lt222	++	++	+++	++	++	-
Anthina sp.	lt147	++	++	+	++	+	++
B. ricini	lt300	-	-	+	++	-	-
C. gloeosporioides	lt305	-	++	-	++	-	-
Colletotrichum sp.	lt198	+++	+++	+++	+++	+++	+++
Coprinellus sp.	lt201	-	-	-	++	-	-
Ectostroma sp.	lt144	+++	+++	+++	+++	+++	+++
F. decemcellulare	lt183	+++	+++	+++	+++	+++	+++
F. oxysporium	lt173	+++	+++	+++	++	+++	+++
F. poae	lt174	++	++	+++	++	++	+++
L. graminicola	lt256	-	++	++	++	++	-
P. brasiliense	lt161	+++	+	+	++	+	++
P. albicans	lt170	-	-	-	-	-	+
Phacodium sp.	lt202	+	-	-	-	+	-
Rhizoctonia sp.	lt214	-	-	-	-	-	+++
V. montemartinii	lt195	+++	++	++	++	+++	+++
Unidentified	lt165	-	-	-	+	-	-
	lt187	-	-	-	+	-	-
	lt200	-	-	-	++	+	-
	lt205	-	-	-	-	-	+
	lt217	-	++	++	-	+	-
	lt221	-	-	-	-	-	+++
	lt254	-	++	-	+	-	++
	lt262	-	-	-	-	-	++
	lt297	-	-	-	-	-	+

Note: R.s: *R. solani*, F.o: *F. oxysporum*, F.g: *F. graminearum*, A.a: *A. alternate*, P.c: *P. capsici*, G.g: *G. glycines..* Growth inhibition: -, <20%; +, 20-50%; ++, 50-80%; +++, >80%.

species *Anthina* sp., *Colletotrichum gloeosporioides* lt305, *Ectostroma* sp. lt144, *F. decemcellulare* lt183*, F. oxysporum* lt173*, P. brasiliense* lt161 and *C. montemartinii* lt198*,* inhibited the growth of all the six phytopathogens with the inhibition rates from 20% to 80%. In particular, strain lt305, lt144, and lt183 showed great potential in producing antifungal metabolites against the phytopathogens with growth inhibition rates higher than 80%. Nine species of fungal endophytes which hadn't been identified so far also showed good bioactivities, such as lt221 that could strongly inhibit the growth of *G. glycines,* and lt217 and lt254 to *F. oxysporum* (growth inhibitions higher than 50%).

DISUCUSION

Endophytes commonly thrive in plant tissues, where certain microorganisms reveal tissue-specificity distribution (Davis et al., 2003). This study showed that there are more species of endophytes isolated from branches than from leaves, which is coincident with the result described by Collado et al. (2000) who found that the colonization percentage of endophytes in *Quercus ilex* and *Quercus faginea* leavers were 25% and 26.3%, while in branches 76.3% and 81.9%, respectively. The variance of the colonization and distribution of certain endophytes in branches and leaves could be caused by the difference of structures and nutrients of the host tissues (Rodrigues, 1994).

Through the investigations of species composition in woody plants, Petrini *et al.* (1992) thought that a few species dominated the community, although a large number of endophytes could be obtained from a single host species, while the majority of the species are rare. Similar result was also observed in this study, such as *F. oxysporum* and *C. gloeosporioides* in *E. pungens* (62%), *Anthina* sp. in *B. microphylla* (80%), and *F. poae* in *C. deodara* (82%). Moreover, seven species such as *A. flavus* lt152*, B. ricini* lt300*, G. candidum* lt274*, L. graminicola* lt256*, P. brasiliense* lt161*, Phacodium* sp. Lt202 and *Rhizoctonia* sp. Lt214. showed strong host specificity, and three strains including *B. ricini* lt300*, G. candidum* lt274 and *L. graminicola* lt256 were isolated as endophytes for the first time. However, some endophytic fungi of *Alternaria*, *Colletotrichum* and *Fusarium* have been reported as endophytes or pathogens of many plants (Liu et al., 2007; Tejesvi et al., 2006; Wang et al., 2007) as well as soil inhabited fungi (Moliszewska and Pisarek, 1996), but the relationship between the fungi in plant and in soil is still unclear.

In general, endophytic fungi do not cause apparent symptoms of infection, but some saprobic or pathogenic fungi can have endophytic stages in part of or even during the whole life, under specific environmental conditions (Wang et al., 2009). Whether endophytes isolated from the evergreen plants became soil fungi after plants dead and decayed or soil fungi invaded into plants and colonized as endophytes? What is the real ecological role of endophytic fungi in the evergreen plants? These questions need to be studied in the future. In this study, 70% of endophytic fungi showed antifungal activity against at least one of the test plant pathogens, which was coincidence with our previous report (Liu et al., 2001). Some species of *Colletotrichum*, *Ectostroma* and *Fusarium* were demonstrated with strong ability to synthesize antifungal metabolites that could be used as biocontrol agents in control of plant diseases. In fact, several studies have been carried on to disclose the antimicrobial activities of fungal *Colletotrichum* and *Fusarium*, but rare on *Ectostroma* sp. For examples, Lu et al. (2000) recovered two *Colletotrichum* spp., which showed strong inhibition against *F. oxysporum* phytopathogenic mycelium growth, while Kim et al. (2007) isolated a strain of *Fusarium* sp. from roots of red pepper, which could control the most potent disease of tomato late blight by inhibiting the growth of *Pythium ultimum*, *Phytophthora infestans* and *P. capsici.* The broad antifungal activity showed by endophytes, especially these with strong bio-activity and wide distribution, such as *Colletotrichum* spp. and *Fusarium* spp., might contribute for stress-resistance by reducing disease symptoms. The present results also indicated that evergreen plants of Zijin Mountain were also a good source of bioactive microorganisms, besides horticultural and medicinal values. As reported, endophytic fungi could produce many novel active natural products (Liu et al., 2001; Strobel, 2000; Zhang et al., 2006), so further investigations will be carried out to isolate and purify the bioactive constituents from these strains.

Conclusion

Endophytic fungi as a member of plant constituents exist in each evergreen plant at the present study. However, the internal relationship between endophytes and plants are complex, some fungi show strong host-specificity, such as *B. ricini* lt300, *G. candidum* lt274 and *L. graminicola* lt256, while other strains (e.g. *A. alternata* lt222, *Anthina* sp. Lt147, *C. gloeosporioids* lt305 and *F. solani* lt293) exhibit in a range of plants. In addition, the richness of the endophytic fungi in plant branches is significantly higher than those in leaves. Moreover, about 70% of the endophytic fungi isolated from the evergreen plants have the potential in producing antifungal metabolites, and the EtOAc extracts of *Anthina* sp. lt147, *C. gloeosporioids* lt305, *Ectostroma* sp. Lt144, *F. decemcellulare* lt183, *F. oxysporum* lt173, *P. brasiliense* lt161 and *Colletotrichum montemartinii* lt198 show broad inhibition against the growth of all the six phytopathogens with the inhibition rates from 20 to 80%. These results suggest that endophytic fungi may play an important role in protection of the evergreen plants from disease as well as an excellent resource for searching for natural antifungal compounds.

ACKNOWLEDGEMENTS

This work was financed by grants from the Jiangsu Provincial Fund for Hi-Tech Research (BG2007330) and a Student Fellowship of Syngenta, UK (0208151088).

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