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Fungal Endophytes in Roots of *Aralia* Species and Their Antifungal Activity

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Endophytic fungi were isolated from surface sterilized root tissues of *Aralia elata* and *Aralia continentalis*, collected from farmer's field in Chungnam province, Republic of Korea, in 2005. Based on ITS sequence analysis, 24 fungal genera were characterized from 359 isolates, belonging to 22 Ascomycota, 1 Glomeromycota and 1 Oomycota. *Strumella*, *Rhizopycnis* and *Entrophospora* in *A. elata* and *Rhizopycnis* and *Leptosphaeria* in *A. continentalis* were the most abundant taxa. Out of 24 genera, *Entrophospora*, *Leptodontidium*, *Neoplaconema*, *Paraconiothyrium*, *Rhizopycnis*, *Strumella* and *Tumularia* were new to Korea. A total of 110 isolates were tested for antifungal activities against six plant pathogenic fungi. Out of these, 39 isolates showed antifungal activity against at least one plant pathogenic fungi. Four isolates of *Pyrenochaeta*, 1 isolate of *Entrophospora* and 1 unidentified fungus strongly inhibited the growth of six plant pathogenic fungi.

Keywords : antifungal activity, *Aralia continentalis*, *Aralia elata*, endophytic fungi, ITS sequence.

Endophytes are microorganisms that form symptomless infections within healthy plant tissues (Carroll, 1986; Carroll, 1988). Endophytic microorganism have been found in nearly all plant families (Sieber et al., 1988), which represent many species in different climate regions of the world (Bussaben et al., 2001; Fisher et al., 1992; Larran et al., 2000, 2001, 2002; Luginbuhl and Muller, 1980; McInroy and Kloepper, 1991; Pereira et al., 1999; Petrini and Carroll, 1981; Spurr and Welty, 1975). Dryfuss and Chapela (1994) estimated that there may be at least 1 million species of endophytic fungi alone. It seems obvious that endophytes are a rich and reliable source of genetic diversity and novel, undescribed species. Novel microbes usually have associated with their novel natural products. This fact alone helps to eliminate the problems of dereliction in compound discovery (Strobel and Daisy, 2003).

The presence of endophytes within plant tissues may confer certain advantages to the host plant (Carroll, 1991). Endophytic fungi are of biotechnological interest due to their potential use as genetic vectors (Murray et al., 1992), as a source of secondary metabolites (Fisher et al., 1986; Stierle et al., 1993; Strobel et al., 1996) and a biological control agents (Bacon, 1990; Clay, 1989; Dorworth and Callan, 1996; Schardl et al., 1991). Carroll (1988) has demonstrated the enhancing of the host survival against fungal pathogens in plant-endophyte associations.

Aralia is a genus of the plant family Araliaceae consisting of 68 accepted species. *A. elata* and *A. continentalis* are native to Asia, whose roots are used in traditional medicine and young leaves are used as vegetables in oriental countries. Studies of endophytic fungi in medicinal plants have gradually been accumulated (Huang et al., 2001 and Li et al., 2005). In Korea, however, endophytic fungi in medicinal plants have not been well-studied.

The present study was conducted to isolate and identify fungal endophytes from roots of two aralia medicinal plants (*Aralia elata* and *Aralia continentalis*), to study their diversity and to evaluate their antifungal activity against plant pathogenic fungi.

Materials and Methods

Plant species and sampling. Two plant species, viz., *Aralia elata* and *A. continentalis* were selected for the study. Samples were collected from farmer's field in Gongju, Chungnam province, Republic of Korea in August, 2005. Four plants of each species were selected and five root samples (about 15-20 cm length and 0.5-1 cm diameter) from each plant were randomly excised and brought to the laboratory in separate sterile polyethylene bags.

Isolation of endophytic fungi. Root samples were cleaned under running tap water to remove debris and then air dried and processed within 5 hrs of collection. From each root sample, 10 segments of 1 cm length and 0.5-1 cm thickness were separated and treated as replicates. The root segments were surface sterilized by immersing in 95% ethanol for 1 min, sodium hypochlorite (4% available chlorine) for 4 min and 95% ethanol for 30s and then the surface sterilized samples were washed in sterile water three times to remove

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the surface sterilization agents. Samples were allowed to dry on paper towel in a laminar air flow chamber. Ten segments per plant were placed horizontally on separate Petri dishes containing potato dextrose agar (PDA) supplemented with the antibiotic streptomycin sulfate 0.4 mg/mL and rose bengal chloramphenicol agar (DRBC). After incubation at 25°C for 5, 10 and 25 days, individual hyphal tips of the developing fungal colonies were collected and placed onto PDA media and incubated for 8-10 days and checked for culture purity. Eventually pure cultures were transferred to PDA slant tubes and 20% glycerol stock solution.

DNA extraction. The isolates were grown in liquid shake culture (130 rpm) in V8 broth media for 7 days at 25°C. Mycelia were collected from the cultures by filtration and transferred to sterile plastic tubes. These samples were frozen at −70°C for minimum 1 hour and then freeze dried in a freeze dryer (Eyela FDU-1000, Japan) and stored in hygrometer (Sanpla corporation). Mycelia were ground in 1.5 eppendorf tubes. Extraction buffer 200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 30 mM EDTA, 0.5% SDS and proteinase K were added to each tube and incubated at 37°C for 1 hr. Samples were extracted with 2×CTAB solution (2% CTAB(w/v), 100 mM Tris-HCL (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% PVP (Polyvinyl-pyrrolidone) and chloroform:isoamyl alcohol (24:1), followed by centrifuge for 10 min. Genomic DNA was washed with 70% ethanol, dried and re-suspended in 50 µl sterile distilled water. RNase enzyme added to each sample to remove RNA, followed by incubation at 37°C for 2 hrs. The amount of DNA was examined by electrophoresis through 0.7% agarose gel in TBE buffer stained with ethidium bromide and visualized with UV light. DNA was stored at 4°C until used.

Polymerase chain reaction (PCR). DNA of all fungal samples were amplified by polymerase chain reaction (PCR) using two different ITS primers- ITS1 & ITS4. Amplification reactions were performed in a total volume of 50 µl containing 10x buffer 5 µl, 10x BSA buffer 5 µl, dNTP 5 µl, 1 µl of each primer, 0.3 µl of Taq polymerase, 1 µl of genomic DNA and 31.7 µl of distilled water. PCR amplification was carried out in i-cycler (BIO-RAD, USA) for 30 cycles of 94°C for 1 min denaturing, 55°C for 1 min annealing and 72°C for 1.30 min extension. Initial denaturing at 94°C was extended to 5 min and the final extension was for 10 min at 72°C.

Electrophoresis. Amplification products were separated by electrophoresis in gels containing 1.0% agarose. The electrophoresis was run in 0.5×TBE-buffer and the amplification products were visualized by ethidium bromide staining under UV light. The lengths of the amplification products were estimated by comparing to 100-bp DNA ladder. The PCR products were purified using the wizard PCR prep. DNA.

DNA sequencing and data analysis. DNA sequence was done by using ITS1 and ITS4 primers. Analyses of sequences were performed with the basic sequence alignment BLAST program run against the database (National Center for Biotechnology Information website, http://www.ncbi.nlm.nih.gov). Then phylogenetic trees were constructed and neighbour-joining algorithms were done, using PHYDIT program version 3.0.

Test organisms for antifungal activity. Six phytopathogenic fungi were examined to evaluate antifungal activity of endophytic fungi isolated from aralia plant. They were *Alternaria panax*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Phytophthora capsici*, and *Pythium ultimum*. The phytopathogenic fungi were obtained from the culture stock of Plant Pathology Laboratory, Chungnam National University, Daejeon, Republic of Korea.

Screening for antifungal activity. Dual culture technique was adopted for antifungal activity test against test pathogens on PDA plates. Five-day-old disks (5 mm diameter) of endophytes were placed on 3 points of Petri plates containing PDA medium. Test pathogens were inoculated at the center of PDA plates. Plates were incubated at 25°C for 5-8 days. Antifungal activity was indicative as mycelial growth of test fungus prohibited in the direction of active endophytic fungus. The level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist colony from the fungal growth radius. The width of inhibition zones between the pathogen and the endophytes was evaluated as >10 mm (+++, strong inhibition), 2-10 mm (++, moderate inhibition) and <2 mm (+, weak inhibition).

Results

Identification of fungal taxa. A summary of endophytic fungal taxa isolated from roots of araliaceae plants is presented in Table 1. Four hundred segments were evaluated from roots of *A. elata* and *A. continentalis*. A total of 359 fungal isolates were recovered from the root samples, where 231(64%) isolates were from *A. elata* and 128(46%) isolates were from *A. continentalis* (Table 1).

Molecular methods for fungal identification and classifi-

Table 1. Diversified genera of endophytic fungi isolated from roots of *A. elata* and *A. continentalis* in Korea

Phylum	Genus	No. of isolates from		Total
		A. elata	*A. continentalis*	
Oomycota(1)	*Pythium*	3		3
Glomeromycota(1)	*Entrophospora*	27	3	30
Ascomycota(22)	*Alternaria*	2	1	3
	Ascomycete	3		3
	Aspergillus	1		1
	Cladosporium	2		2
	Cylindrocarpon		5	5
	Drechslera	2		2
	Epicoccum	1		1
	Fusarium	1		1
	Leptodontidium	11	6	17
	Leptosphaeria	1	20	21
	Macrophomina		3	3
	Neoplaconema		5	5
	Paraconiothyrium	7		7
	Penecillium		1	1
	Pyrenochaeta	10		10
	Rhizopycnis	35	37	72
	Scopulariopsis		1	1
	Spirosphaera	4		4
	Strumella	64	5	69
	Tricladium	1		1
	Tumularia		3	3
	Zalerion	9		9
	Unidentified	47	38	85
Total		231(64%)	128(36%)	359

cation were applied. PCR-fragments from the ITS were amplified for DNA sequence analysis in order to discriminate isolates at or close to the species level. BLAST database searches were performed with full-length ITS-fragments queries to reveal relationships to published sequences. Sequenced isolates and BLAST searches are listed in Table 2. Pairwise similarity of scores of fungal endophytes sequences and BLAST searches were mostly >91%. Based on ITS sequence analysis, a wide diversity of fungi was recovered from root samples in this study, comprising three phyla, 24 genera and 28 species. Eighteen genera were isolated from *A. elata* and twelve genera were isolated from *A. continentalis*. Among the 24 genera discovered, 22 were under the phylum Ascomycota, 1 was under Glumeromycota and 1 was under Oomycota (Table 1).

Strumella (n=64) and *Rhizopycnis* (n=35) were the most frequently recovered fungi from *A. elata* and the predominant fungus from *A. continentalis* was *Rhyzopycnis* (n=37) followed by *Leptosphaeria* (n=20). Among the fungi recovered, *Entrophospora, Leptodontidium, Neoplaconema, Paraconiothyrium, Rhyzopycnis, Strumella* and *Tumularia* were new to Korea (Annomymous 2004). Phylogenetic trees were constructed to illustrate the overall molecular diversity of the endophytic fungi associated with aralia and to check sequence data within a phylogenetic context (Fig. 1 & 2). By neighbor-joining algorithm, a total of 85 isolates could not be identified.

Antifungal activity. The antifungal activity of endophytic fungi isolated from aralia plants are shown in Table 3 and Fig. 3. Thirty nine out of 110 isolates showed antifungal activity against at least one plant pathogenic fungi. Four isolates of *Pyrenochaeta*, 1 isolate of *Entrophospora* and 1 unidentified species from *A. elata* were positive against all 6 plant pathogenic fungi tested. Eight isolates were active against five plant pathogenic fungi and four showed activity against four plant pathogenic fungi. Twelve endophytes were less potential and showed antifungal activity against only one pathogenic fungus.

Table 2. Endophytic fungi isolated from roots of two aralia plant species in Korea

Isolates*	Most closely related fungal sequence	Accession no.	Identity (%)
640,652,729,	*Strumella* sp.	AY354268	93
641,642,721,725	*Rhizopycnis vagum*	AF022786	99
643,645,647,671,693,731	*Entrophospora* sp.	AY035666	97
644	*Zalerion varium*	DQ069053	99
646,657,684,728,747,759	*Leptodontidium orchidicola*	AF214578	97
648,664	*Paraconiothyrium brasiliense*	AY642531	94
650,685,	*Spirosphaera cupreorufescens*	AY616233	92
654,655,665,688,699	*Pyrenochaeta lycopersici*	AY649594	95
660	*Drechslera biseptata*	AY004788	92
663,670	*Pythium ultimum*	AY986961	99
666	*Tricladium splendens*	AY204635	96
668,724,726,727,732,748	*Leptosphaeria* sp.	DQ093682	99
673	*Aspergillus tubingensis*	AJ280008	99
675,683,701	*Ascomycete sp.*	AY303611	92
677	*Fusarium oxysporum*	AY928414	99
682,703	*Cladosporium tenuissimum*	AJ300331	99
686,694	*Alternaria tenuissima*	AF494276	100
700	*Epicoccum nigrum*	AF455409	99
723	*Cylindrocarpon macrodidymum*	DQ093687	93
730	*Macrophomina phaseolina*	AF132795	100
733,740	*Neoplaconema gloeosporiodes*	AJ534443	96
734	*Tumularia aquminata*	AY265337	93
745	*Cladosporium cladosporioides*	AY463365	99
751	*Penecillium minioluteum*	L14505	96
752	*Strumella grisula*	AF485078	91
754	*Alternaria alternata*	AY433814	100
756	*Penecillium digitatum*	AF033471	99
757	*Scopulariopsis chartarum*	AY625066	94
649,651,653,656,658-59,661-62, 667, 669,672,674,676,678-81,687,689-92, 695-98,702,704-7,710,722,735-39, 741-44,746, 749-50,753,755,758	Unidentified		

*All isolate number started with 050

Discussion

In this experiment, endophytic fungi were isolated from root samples of *A. elata* and *A. continentalis*, because previous investigations support that the extent of endophytic fungi are higher in roots. Stefan et al. (2001) isolated fungi from common reed. The majority (74.5%) of isolates were originated from root tissue, whereas only 20.2% and 5.3% of the isolates were from stem and leaves respectively. Seena and Sridhar (2004) isolated endophytic fungi from cotyledons, seed coats, roots, stems and leaves of 2 sand dune wild legumes from the south east coast of India and endophytic fungi colonized over 90% of root and stem segments.

The number of fungal species recovered is probably a significant underestimate of the species richness actually present, although it has frequently been observed that a relatively small number of species make up a dominant component of endophytic taxa in both temperate and tropical ecosystems (Bills, 1996). Investigations of species composition in woody plants have shown that a large number of endophytic species can be recovered from a single host species; usually one and at the most a few species dominate the community, while the majority of the species are rare (Tejesvi et al., 2005). This was also the case in this investigation. A few fungal genera such as *Strumella, Rhizopycnis*, *Leptosphaeria* and *Entrophospora* were dominant. Many of the taxa isolated infrequently in this study were also isolated from roots of *Picea abies* (Holdenrieder and Sieber, 1992) and *Pinus sylvestris* (Fisher et al., 1991).

Molecular analysis of fungal rDNA at the sequence level

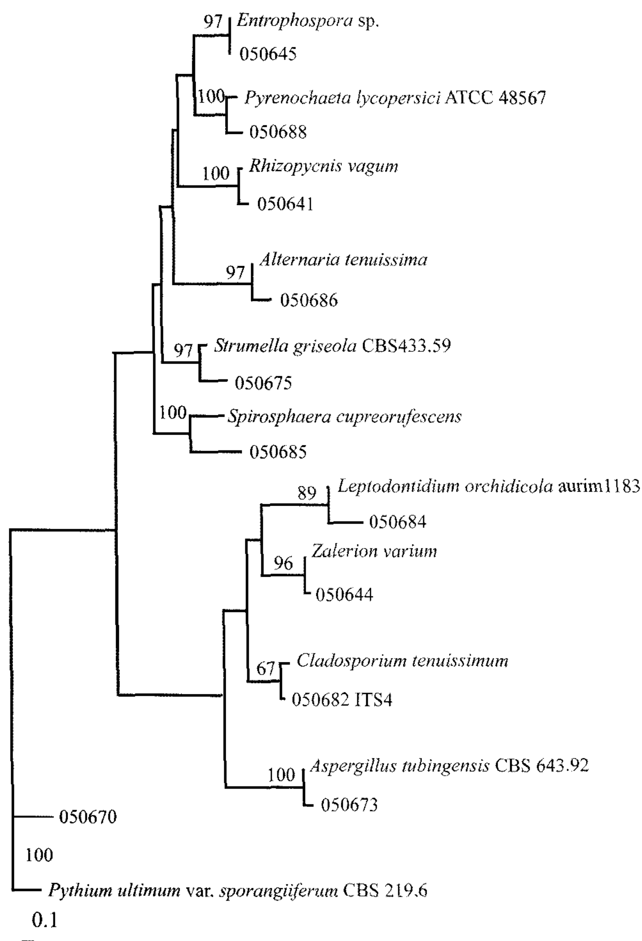

Fig. 1. Phylogenetic tree showing the relationship between *A. elata* endophytic fungi and reference strains. The tree was constructed on the basis of rDNA sequence (ITS1 and ITS4) fragment by using neighbour-joining method. The boostrap analysis was performed with 1000 repetations.

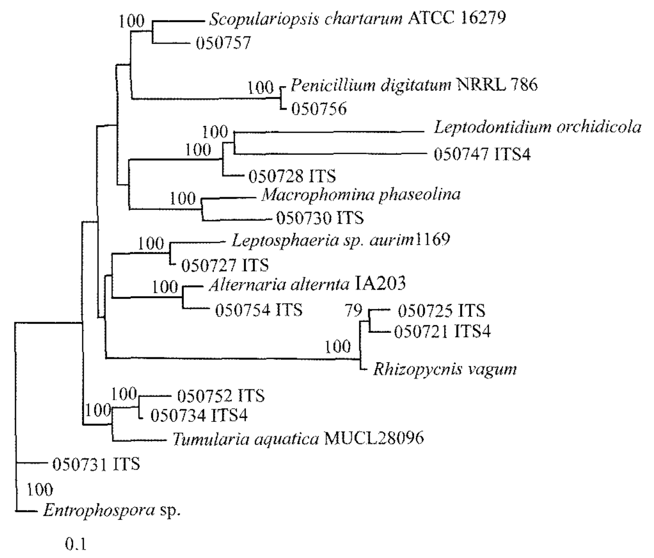

Fig. 2. Phylogenetic tree showing the relationship between *A. continentalis* endophytic fungi and reference strains. The tree was constructed on the basis of rDNA sequence (ITS1 and ITS4) fragment by using neighbour-joining method. The boostrap analysis was performed with 1000 repetations.

provides a powerful technique for assessing fungal diversity at the genus level. The collection of fungi described here includes a wide variety of Ascomycota isolates and a very few fungi belonging to Glomeromycota and Oomycota. Stefan et al. (2001) also identified 26 different fungal ITS sequences from common reed, 23 of them probably from Ascomycetes. Carter et al. (1999) were recovered in a similar proportion of Ascomycetes and Basidiomycetes from the rhizospheres of wheat and oat. Diversity of endophytic fungal community was evaluated by Rubini et al. (2005) from cacao in Brazil and Paul et al. (2006) from white dandelion in Korea. Both Brazil and Korea, the endophytic fungi associated with cacao and white dandelion were mainly belonging to Ascomycetes group.

Endophytic fungi were tested for antfungal activity by dual culture method. *Pyrecochaeta*, *Entrophospora*, *Strumella* and an unidentified fungus showed strong inhibition of mycelial growth of plant pathogenic fungi. Antifungal activities of plant endophytic fungi and actinomycetes have been reported by several groups (Fisher et al., 1984; Huang et al., 2001; Lu et al., 2000; Park et al., 2003; Paul et al., 2006; Naik et al., 2007; Li et al., 2005 and Taechowisan et al., 2003). In the study, it was found that 39 (35.5%) isolates showed antifungal activity against at least one plant pathogenic fungi. Li et al., also (2005) isolated endophytic fungi from Chinese medicinal plants where 30% of fungi showed antifungal activity. It means Korean medicinal plants are also a source of bioactive antifungal agents, other than medicinal value. Tian et al. (2004) studied endophytic actinomycetes from rice plant and tested their antipathogen activities. About 41.2% of the isolates showed antagonism against at least one rice pathogen. Similar results also found by Huang et al. (2001) and Phongpaichit et al. (2006). Phongpaichit et al. (2006) isolated 377 endophytic fungi from *Garcina* species and 70 (18.6%) displayed antimicrobial activity against at least one test microorganism with inhibition zones that ranged from 7 to 19 mm. Aghighi et al. (2004) tested 110 isolates for antifungal activity and identified 14 isolates which showed activity against at least one fungal isolate. Naik et al. (2007) found that endophytic fungi *Chaetomium globosum, Penecillium chrysogenum* and *Streptomyces* sp. have the antagonistic activity against five test pathogens and the antagonism might be due to the production of biologically active compounds.

The diversity of fungi obtained from healthy root tissues of *A. elata* and *A. continentalis* growing in a relatively

Table 3. Antifungal activity of selected endophytic fungi from *A. elata* and *A. continentalis* against six plant pathogenic fungi

Isolate number	Fungi	Plant pathogenic fungi[a]					
		A	B	C	F	P	Py
050640	*Strumella griseola*	++[b]			+		++
050643	*Entrophospora* sp.	++		+++	++		+++
050645	*Entrophospora* sp.	++	+	++	+++	++	
050647	*Entrophospora* sp.	+	+	+		++	+
050648	*Paraconiothyrium brasiliense*	+++					
050649	Unidentified		++	++			
050652	*Strumella griseola*	++		++	+	++	
050654	*Pyrenochaeta lycopersici*	+++	+++	+++	+++	+++	++
050655	*Pyrenochaeta lycopersici*	+++	+++	+++	+++	+++	++
050659	Unidentified	+++	++		++	++	++
050660	*Drechslera biseptata*	+++	++		+	++	+
050662	Unidentified			+++	++	++	
050665	*Pyrenochaeta lycopersici*	++	++	++	++	++	++
050666	*Tricladium splendens*		++	++	+	+++	++
050667	Unidentified						+
050668	*Leptosphaeria* sp.	++	++			+++	
050669	Unidentified	++	+			++	++
050671	*Entrophospora* sp.	+	+	+		++	+
050674	Unidentified				++	++	
050675	*Ascomycete* sp.				+	+	++
050676	Unidentified	++	++		++	++	+
050679	Unidentified	++	++	++	+		++
050682	*Cladosporium tenuissimum*					++	+
050687	Unidentified		+			+	
050688	*Pyrenochaeta lycopersici*	++	++	++	++	++	++
050689	Unidentified					++	
050690	Unidentified	+++	+++	++	+++	+++	+++
050691	Unidentified		+			+	
050693	*Entrophosphora* sp.	++	+++	++	++	++	+++
050697	Unidentified		++				
050698	Unidentified		++				
050701	*Ascomycete* sp.					++	
050724	*Leptosphaeria* sp.	++	++	++		+	+
050731	*Entrophospora* sp.	+			+	++	++
050733	*Neoplaconema gloeosporioides*	++				+	
050736	Unidentified		+	+	++	++	
050748	*Leptosphaeria* sp.	++	+	++	++	++	
050749	Unidentified	++	++	++		+++	++
050752	*Strumella griseola*	+		++			

[a]A; *Alternaria panax* , B; *Botrytis cinerea*, C; *Colletotrichum gloeosporioides*, F; *Fusarium oxysporum*, P; *Phytophthora capsici*, Py; *Pythium ultimum*

[b]Inhibition zone. +, <2 mm; ++, 2-10 mm; +++, >10 mm

narrow range of area suggests that an even broader flora of fungal endophytes might be found across diverse environments. The use of molecular tools aided in rapid identification of our cultured fungi to the genus level. It is clear that fungal endophytes isolated from aralia plant species may benefit the host, and further studies will be carried out to evaluate the potential use of endophytes in biological control of plant diseases.

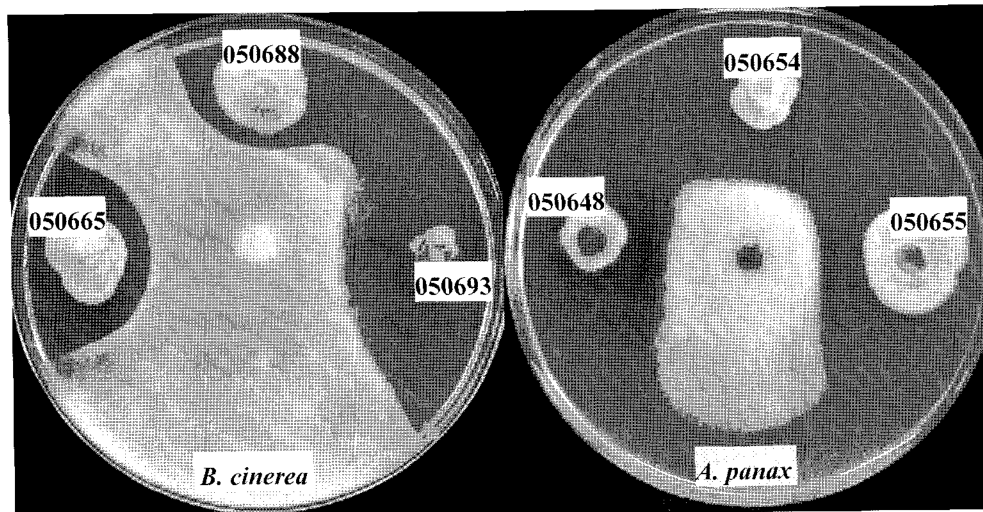

Fig. 3. Isolates 050665, 050688 and 050693 (left) showing antifungal activity against *Botrytis cinerea* and isolates 050648, 050654 and 050655 (right) showing antifungal activity against *Alternaria panax.*

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