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Antibacterial activity of endophytic fungi isolated from conifer needles

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Fungi, in particular endophytes are a promising source of new antimicrobial compounds. The aim of this study was to screen the extracts of conifer needle fungal endophytes for antimicrobial activity and taxonomically place fungi producing ones to determined active metabolites. Seventy three strains of endophytic fungi were isolated from plant samples, mainly from needles of conifers, and cultured. Extracts of cultured endophytic strains were tested for antimicrobial properties using a microdilution assay. Their activity was compared to that of the antibiotic ampicillin. Samples that exhibited antimicrobial properties were further examined. Genomic DNA from five active fungal strains was isolated and species-specific DNA regions (ITS regions) were amplified and sequenced allowing us to determine the identity of the samples. Active endophytic fungi were two strains of *Lophodermium pinastri*, two strains of *Lophodermium seditiosum* and one of *Phoma herbarum*. All of these strains are known as parasitic and can be treated as endophytes only according to the lack of symptoms in their host tissue. This work demonstrates an interesting bottom-up approach to the discovery of new antimicrobial compounds.

Key words: Endophyte, antibiotic, parasitic, *Lophodermium*, antimicrobial, *Phoma*.

INTRODUCTION

The problem of drug-resistant pathogens and co-dependent infectious diseases is substantial and still growing. According to the World Health Organization (WHO) Global Burden of Disease report from the year 2013, infectious and parasitic diseases were still the second leading cause of death, causing 18.4% of all deaths worldwide (WHO, 2014). This brings the need for the search of new antibiotic compounds. Naturally, derived products remain the most important source of their discovery. Fungi are a versatile and precious source with an enormous pharmaceutical potential (Schulz et al., 2002; Vaz et al., 2009; Bhagobaty and Joshi, 2012; Wang et al., 2012). Recently, there have been an increasing number of publications regarding investigations of endophytic fungi producing antimicrobial substances (Janeš et al., 2007; Xiaoye et al., 2012). This niche should be meticulously examined and used as a base for sustainable research and development of new antibacterial substances that can both respond to current antimicrobial resistance and anticipate evolving resistance. Plant endophytic fungi have the ability to produce the same or similar compounds to those originating

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from their host plants (Erbert at al., 2012), as well as a great number of diverse bioactive compounds (Devaraju and Satish, 2010), which have been implicated in the protection of its host against pathogens and herbivores (Wicklow et al., 2005). Since then several new such substances were found (Xuanwei at al., 2010; Sumarah et al., 2011; Radić and Štrukelj, 2012). Metabolic interactions of endophytes with host can favor the synthesis of biologically active secondary metabolites (Owen and Hundley, 2004). Isolation of endophytes from their natural habitat can influence their metabolism (Raviraja et al., 2006). Certain factors can cause production of biologically active compounds in culture. Conditions like temperature, composition of culture medium, amount of nutrients and level of aeration influence growth and synthesis of secondary metabolites. Method of fermentation and extraction technique influences the amount and kind of compounds as well (Strobel and Daisy, 2003). In order to identify novel biologically active compounds; their biological activities necesicates testing by a variety of means. In the case of antibacterial activity it can be tested several different ways including disk diffusion methods, E test, and microdilution broth method. The last one, most common and available was used in our case. The most efficient method for determination of fungal species employs amplifying and sequencing of species-specific regions of fungal DNA. Among few such potential regions internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation.

MATERIALS AND METHODS

Bacteria

Escherichia coli, strain ER2738, were obtained from the Department of Pharmaceutical Biology, Faculty of Pharmacy, Ljubljana, Slovenia.

Fungi

Conifer needles were collected from several plant species (*Abies* sp*., Cedrus* sp*., Juniperus* sp*., Larix* sp*., Metasequoia* sp., *Picea* sp*., Pinus* sp*., Taxus* sp.), samples of leaves, stalks, roots and remaining plant parts of other plant species (*Sambucus* sp*., Calluna* sp*., Centaurea* sp.) were also collected from unpolluted habitats around Slovenia. Samples were kept in sterile test tubes or plastic bags at 4°C and transported to the laboratory as soon as possible. Once in the laboratory, samples were surface sterilized with 70% ethanol for 2 min and 1% sodium hypochlorite for 3 min in a sterile chamber with laminar flow of air to kill epiphytes (microorganisms on surface of samples) and cut in half longitudinally with a sterile razor. Internal surface was then exposed to a solid culture medium comprised of potato dextrose agar (PDA). Explicitly, the PDA medium was composed of potato extract, 0.4% (w/V); glucose, 2.0% (w/v); and agar, 1.5% (w/V) in water. After 2 to 5 weeks incubation it was determined when the fungi from the plant samples formed colonies, and every fungal colony was aseptically transferred onto 3 new PDA plates. Every Petri dish was wrapped with Parafilm and left in a chamber for cultivation of fungi at room temperature (22 to 25°C). Fungal cultures were re-inoculated to a new medium repeatedly every week for 1 to 3 times (Figure 1). Replicates were removed and only strains which differed in morphology (shape, patterns and color are distinguishing among different microorganisms) were kept and submitted to a subsequent antimicrobial test (Méndez et al., 2008). Solid PDA culture media overgrown with fungus (15 ml in 90 mm diameter Petri dish), were homogenized with an electric homogenizer to obtain a particle size of approximately 5 mm. The homogeneous mixture was transferred to a conical flask to which 70 ml of a solvent were added and closed with a glass stopper. The extraction solvents utilized were methanol, ethyl acetate or dichloromethane. The culture of every fungal strain was extracted in each of the three solvents at room temperature for 15 min in an ultrasonic bath, 24 h without ultrasound, and after that 15 min in the ultrasonic bath again. The mixture was filtered through a filter paper and washed three times with 10 ml of the chosen solvent. Samples were completely dried by rotary evaporation under reduced pressure to determine the weight of dry extracts. The extracts were then dissolved in methanol (50%) to a concentration of 1 mg/ml. The samples were stored in closed tubes at -20°C until further analysis.

Broth microdilution method

Broth microdilution test was performed according to the standard procedure (National Committee for Clinical Laboratory Standards, 2003). Bacteria from a freshly grown colony were transferred with a sterile sling and suspended in 10 mL of a sterile 0.9% solution of sodium chloride. The suspension was diluted with 0.9% of NaCl to obtain the absorbance of the final suspension between 0.08 and 0.1 at λ=625 nm. This corresponded to 10^8 CFU/mL of bacteria. 800 µL of that suspension were diluted with 25 ml of 0.02% sterile solution of Tween® 80 to obtain the bacterial suspension that was used as an inoculum in a microdilution test. Broth microdilution method was carried out in a 96-well microplate with round bottom wells and the volume of each well of 200 µL. Each well consisted of 90 µL of Mueller-Hinton broth, 10 µL of inoculum and 10 µL of tested extract (or control). All tests were carried out in duplicates. Controls and extracts were added in consecutive 2-fold dilutions. Sodium salt of ampicillin (Sigma-Aldrich, Germany) with a starting concentration 1 mg/mL was used as an efficiency comparison and an extraction solvent as a positive control. Starting concentration of fungal extracts added were 1 mg/mL. The microplate was then incubated for 24 h at 37°C before the results were evaluated.

Determination of endophyte species

Fungal samples were collected from permanent cultures (approximately 100 mg of fungal tissue) and homogenized using ultratorax homogenizer. QiagenDneasy plant mini kit was used to isolate fungal DNA. ITS regions were amplified with GeneAmp2700 Thermal cycler, using two sets of primers ITS F 5'-AGAAAGTCGTAACAAGGTTTCCGTAG-3', ITS R 5'-TTTTCCTCCGCTCATTGATATGCTT-3' and ITS-g F 5'-TCCGTAGGTGAACCTGCGG-3' (White et al., 1990), ITS-g R 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990). The 30-cycle amplification program contained 30 s of denaturation (95ºC), 30 s of annealing (55ºC) and 1 min of elongation (72ºC). PCR products were analyzed with agarose gel electrophoresis, stained with Cybr gold stain and detected under UV light. Samples were extracted from gel and purified using Qiaex II Gel Extraction Kit. Purity of isolated DNA fragments was checked with spectrophotometer NanoDrop 1000. Purified samples were sequenced by GATC labs. Obtained sequences were analysed using NCBI BLAST software. Strains 2 and 35 demonstrating the highest antimicrobial activity

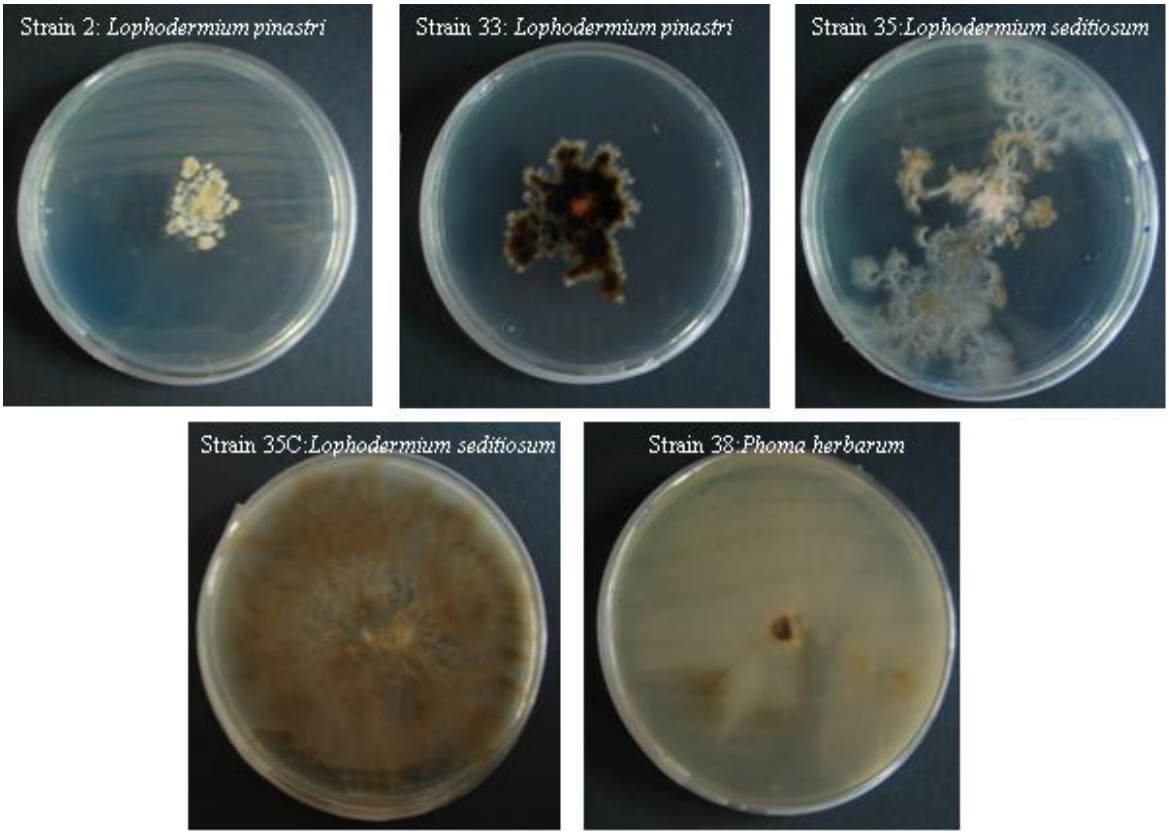

Figure 1. Fungal strains showing antibacterial activity growing on PDA plates.

Table 1. Results of the broth microdilution test with extracts of solid culture medium with mycelium with concentration 1 mg/ml and their dilutions in ratio 1:1.

Extract	1	2	3	4	5	6	7	8	9	10	11	12
Ampicillin Na	-	-	-	-	-	+	+	+	+	+	+	+
Dichloromethane extract 2	-	-	-	+	+	+	+	+	+	+	+	+
Dichloromethane extract 38	-	-	+	+	+	+	+	+	+	+	+	+
Dichloromethane extract 33	-	-	+	+	+	+	+	+	+	+	+	+
Dichloromethane extract 35	-	-	-	+	+	+	+	+	+	+	+	+
Dichloromethane extract 35C	-	+	+	+	+	+	+	+	+	+	+	+
Positive control	+	+	+	+	+	+	+	+	+	+	+	+
Negative control	-	-	-	-	-	-	-	-	-	-	-	-

(+) Opaque culture medium after incubation indicating bacterial growth; (-) clear culture medium after incubation, indicating bacterial growth inhibition. Numbers 1 to 12 represent consecutive 2-fold serial dilutions. Starting concentration of ampicillin/dichlormetane extracts in column 1 is 10 µg/well.

were also sent for morphological identification to CBS-KNAW Fungal Biodiversity Centre, Netherlands.

RESULTS

Seventy three (73) fungal strains were isolated from 73 plant samples. Extracts were prepared from the culture on the solid PDA medium of each of these strains. Microdilution tests (Table 1) showed 5 of them (extracts from strains marked: 2, 33, 35, 35C and 38) holding an antimicrobial activity against *Escherichia coli* ER2738 (Table 2). Only dichloromethane extract, but not methanol or ethyl acetate extracts, showed antibacterial activity. The second strongest antibiotic effect after ampicillin was

Table 2. Data about sampling of plant parts from host plants of endophytic fungi.

Sample number	Host	Herbal origins	Location
Lophodermium pinastri (2)	*Sambucus nigra* L. Black elder	Leaves	N 45° 39,224' E 15° 11,209'
Lophodermium pinastri (33)	*Pinus nigra* Arnold European black pine	Needles	N 45° 32,083' E 14° 46,491'
Lophodermium seditiosum (35)	*Pinus nigra* Arnold European black pine	Needles	N 45° 32,049' E 14° 46,510'
Lophodermium seditiosum (35C)	*Pinus nigra* Arnold European black pine	Needles	N 45° 32,049' E 14° 46,510'
Phoma herbarum (38)	*Pinus sylvestris* L. Scotch pine	Needles	N: 46° 02,383′ E: 14° 30,817′

demonstrated by the extracts of samples 2 and 35, followed by samples 33 and 38. Extract of sample 35C has shown weaker antibiotic effect. Fungal DNA was successfully extracted and amplified producing DNA fragments of anticipated lengths of around 600 BP corresponding to the ITS region. Sequencing of the fragments was performed in 2 parallels to ensure the correct nucleotide order. Sequences of all 5 samples were analysed with BlastN tool. All of our sequences had at least 99% identity to designated species and 92 to 98% identity to next related species. According to this analysis sample 2 and sample 33 are two different strains of *Lophodermium pinastri*, 35 and 35C are two different strains of *Lophodermium seditiosum* and sample 38 is *Phoma herbarum*. Morphological identification of samples 2 and 35 determined fungal species to be *Lophodermium pinastri* and *Lophodermium seditiosum* which matches the results of genetic species determination. All sequences were submitted to the Genebank database under the accession numbers: KC608049, KC608050, KC608051, KC608052 and KC608053.

DISCUSSION

Over 70 isolated endophytic strains revealed a very morphologically diverse coexistence of fungi in limited number of host plant samples. Fungal extracts were prepared with three solvents possessing different polarity in order to enable extraction of a broad spectrum of compounds. Dichloromethane as the least polar of them showed the best results indicating that active antibiotic compounds in the fungi are likely to be lipophilic. All of the five sample extracts that demonstrated antibiotic activity were less potent than ampicillin. However, active compounds are likely to be diluted in these extracts and could possess an even stronger antibiotic effect than ampicillin if purified. Interestingly, four of the five investigated fungal strains belonged to the same genus and only two species. Furthermore, *L. pinastri* and *L. seditiosum* are parasitic fungi rather than symbiotic and can be treated as endophytes only by certain measures (Rodriguez and Redman, 2008; Rodriguez et al., 2009). None of these fungal species has previously been known to produce antimicrobial substances. Small differences between ITS sequences of samples 2 and 33 and sequences of samples 35 and 35C showed that they belong to different strains. Macroscopic observation of the fungi growing on PDA plates also indicated that these strains are not duplicate isolates of the same strain. Further work towards isolation of active molecules from these extracts should be performed in the future. However, this study proves endophytic fungi to be an important source of potential new antimicrobial drugs and encourages the use of ITS region sequencing as a suitable method for endophytic fungi systematization.

Conclusions

According to the growing need for new antimicrobial compounds, endophytic fungi could represent a diverse and rich source of new compounds. This research resulted in discovery of five fungal strains that possess the potential for potent antibiotic compounds. These strains would be interesting candidates for upcoming isolation of active compounds.

Conflict of interests

The authors did not declare any conflict of interest.

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