World Applied Sciences Journal 30 (11): 1599-1604, 2014
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2014.30.11.14220

Isolation of Endophytic Actinomycetes from Medicinal Plants of the Moscow Region, Russia

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Abstract: Endophytic bacteria are microorganisms that colonize the internal tissues of the plant without causing any external sign of infection or negative effect on their host. In the present study, endophytic actinomycetes were isolated from leaves of medicinal plants collected in Moscow and Moscow region. To isolate actinomycetes we used a method developed by us on the basis of known techniques. The main modification consisted in the pretreatment of leaves with solutions of biologically active substances of plant origin - heteroauxin and zircon. Using the modification method of this study helps to increase numbers of rare actinomycetes isolated from the medicinal plants, which are potentially unique source of novel bioactive metabolites for medicinal and biotechnological practice.

Key words: Endophytic actinomycetes · Actinobacteria · Zircon · Heteroauxin · Medicinal plants

INTRODUCTION

Endophytic bacteria are microorganisms that colonize the internal tissues of the plant without causing any external sign of infection or negative effect on their host [1, 2]. Fungi, bacteria or actinomycetes have been found in endophytic association with plants. Numerous reports have shown that endophytic microorganisms can have capacity to control plant pathogens, insects and nematodes [3]. In some cases, they can also accelerate seedling emergence, promote plant establishment under adverse conditions and enhance plant growth [4]. Endophytic bacteria can produce novel antibiotic compounds and other secondary metabolites. Investigation of the biodiversity of endophytic strains for novel metabolites may identify new drugs for effective treatment of diseases in humans, plants and animals [5].

Medicinal plants are gaining global attention owing to the fact that the herbal drugs are most effective, easily available and with negligible side effects. The world population uses the medicinal plants in health care with homeopathy. It is important to note that some of the endophytic microorganisms can produce the same secondary metabolites as that of the plant thus making them a promising source of novel compounds [6].

Endophytic microorganisms can be derived from any part of the plant like bark, leaves, flowers, fruits, roots, seeds etc. [7]. In the present study, efforts have been made to isolate endophytes inhabiting leaves of medicinal plants growing in the Moscow region.

MATERIALS AND METHODS

Sample Collection: The leaves of the 20 healthy medicinal plants were collected in the forests and fields of Moscow and the Moscow region (Table 1) in spring and summer time. The selection of each plant for isolation of actinomycetes was based on its medicinal properties.

Each sample of healthy leaf of the medicinal plant was placed in a sterile bag, taken to the laboratory and subjected to selective isolation procedures within the next 2-3 days.

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Table 1: Plants used for isolation of endophytic actinomycetes

Plant names	Place of selection
Achillea millefolium	Moscow, natural-historical Park «Kuzminki»
Aloe arborescens	Moscow, home-plant
Anthoxantum odoratum	Moscow region
Arctium lappa	Moscow region
Convallaria majalis	Moscow, natural-historical Park «Kuzminki»
Fragaria vesca	Moscow region
Geranium pretense	Moscow region
Hippophae rhamnoides	Moscow region
Lysimachia nummularia	Moscow region
Matricaria matricarioides	Moscow, natural-historical Park «Kuzminki»
Melilotus officinalis	Moscow region
Mentha arvensis	Moscow region
Plantago major	Moscow region
Rosa cinnamomea	Moscow region
Rubus idaeus	Moscow region
Tanacetum vulgare	Moscow, natural
Taraxacum officinale	Moscow, natural
Trifolium pretense	Moscow region
Urtica dioica	Moscow region
Viola odorata	Moscow, natural-historical Park «Kuzminki»

Selective Media: Endophytic actinomycetes were isolated on organic medium 2 Gause (tryptone – 3.0 g, peptone – 5.0 g, glucose – 10.0 g, NaCl – 5.0 g, agar – 20.0 g) and oatmeal media ISP3 (oat meal – 20.0 g, agar – 20.0 g, trace salt solution - 1 ml) [8].

Selective Isolation of Endophytic Actinomycetes: The leaves samples were washed in running tap water to remove soil particles. The washed leaves (1 g) were sterilized by treating with 75% ethanol for 5 minutes then with 1% s odium hypochlorite (NaClO) for 20 minutes. The samples were washed in sterile distilled water three times to remove the surface sterilization agents and soaked in 10% sodium hydrocarbonate ($NaHCO_3$) for 5-10 minutes to disrupt the plant tissues and inhibit fungal growth [9, 10, 11, 12, 13, 14].

The treated leaves samples were soaked in solutions of biologically active substances of plant origin - heteroauxin (20 μg/ml) and zircon (1 μg/ml). In the solution of these substances the leaves were rinsed for 20 minutes. The samples of leaves were cut into small pieces, placed in tubes with sterile distilled water (10 ml) and incubated for 1 hour at 28°C, stirring occasionally. The resulting suspensions were serially diluted with sterile distilled water at a ratio of 1:100 and 1:1000, then plated on agar selective media by traditional method and incubated at 28°C for 3 weeks.

Biologically Active Substances of Plant Origin: Heteroauxin is a substance of auxin group, phytohormone, stimulator of a plant growth. Under the influence of heteroauxin cell division is controled in plants. In small concentrations heteroauxin stimulates, in large - inhibits the plant growth [15]. The active substance of heteroauxin is an indolyl-3-acetic acid. In the work with soil actinomycetes we found a stimulating effect of heteroauxin on the germination of spores [16]. So we decided to use heteroauxin for allocation of endophytic actinomycetes from the leaves of medicinal plants.

Zircon - biologically active substance of plant origin, an immunomodulator, which induces root formation and flowering of plants. It has a strong antifungal and antistressful action. Drug Zircon in the alcohol solution (0.1 mg/ml) has been obtained from plants *Echinacea purpourna*. In this work, we used the drug company NEST-M (The Russian Federation) active ingredient of zircon, which is a mixture of hydroxycinnamic acids.

Effectiveness of Surface Sterilization: The surface sterilized samples of leaves were washed by sterile distilled water 3 times. An aliquot of 0,2 ml suspension of the third water portion were spread to organic medium 2 Gause agar plates and incubated at 28°C for 2 weeks, then the plates were observed for microbial growth [9].

Preliminary Identification of Endophytic Actinomycetes: Colonies were isolated to pure culture onto tubes with splay organic medium 2 Gause and ISP3. Every dereplicated colony was registered for determination of number of actinomycetes of different genera. Preliminary identification of isolates were grouped by observing their morphological and cultural characteristics, including the characteristics of colonies on plates, the color of aerial and substrate mycelia and diffusible pigment and spore chain morphology. Morphology of isolates was observed with light microscope OLYMPUS BX–41.

The cell wall type was also determined on the basis of the occurrence of isomers of diaminopimelic acid in order to distinguish the streptomycetes from other spore-forming actinomycetes. The diagnostic sugars of the representatives of each group were also detected [17]. Based on the preliminary grouping, 8 isolates were selected for further research.

16S rRNA Gene Sequence Analysis: The 8 isolates were subjected to 16S rRNA gene sequence analysis for precise genus and species identification. The identities of the organisms were determined based on partial or nearly full length 16S rRNA gene sequence analysis. The genomic DNA of each isolate was extracted by using a

Table 2: Primers used in the study

Primer name	5' – 3' sequence	Target gene	Length of target gene fragment (bp)	Reference
27f	5'-AGAGTTTGATCCTGGCTCAG-3'	16S rRNA	1400-1500	[16]
341f –gcd	5'CCTACGGGAGGCAGCAG			
785f	5'GGMTTAGATACCTGGTAGTCC			
907r	5'CCGTCAATTCCTTTGAGTTT			
1100r	5'GGGTTGCGCTCGTTG			
1492r	5'-TACGGYTACCTTGTTACGACTT-3'			

method of Power Soil DNA Isolation Kit (MO BIO, USA) of Manucharova N. [18]. The 16S rRNA genes from pure cultures were amplified by using the universal primer pair 27F-1492R (SYNTOL, Russian Federation) (Table 2). PCR was carried out with automatically amplificatory 2720 Thermal Cycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 94°C for 4 minutes; 30 cycles of 94°C for 1 minute, 51°C for 1 minute and 72°C for 2 minutes; and the final elongation for completion of unfinished chains at 72°C for 10 minutes [17]. PCR amplification was carried out by using kit of reagent GenPak qPCR Core (OOO «Laboratory Isogen», Russian Federation) contained 3,2 pmol each primer, 1U Taq DNA polymerase, 200 µM deoxynucleoside triphosphates, 2,5 mM $MgCl_2$ and 175 ng of template DNA. Summary volume reaction mix was 20µl.

The PCR products were separated by 1% agarose gel electrophoresis and sequenced on a 3500 Genetic Analyzer (Applied Biosystems, USA).

The nBLAST algorithm [19] was used to determine the closest matches for the 16S rRNA gene sequence data against the nucleotide sequence dataset.

In an attempt to determine the phylogenetic groupings of endophytes, DNA sequences of representative strains were retrieved from GenBank. The rRNA gene sequences of isolates and representative straines were aligned using CLUSTAL X2. Maximum likelihood trees were constructed using MEGA 6 with 1000 bootstrap reanalyses [20, 21].

Antimicrobial Activity: Each strain was tested for antimicrobial activity against 8 test microorganisms, including grampositive and gramnegative bacteria and yeast: *Staphylococcus aureus* FDA 209 P, *Staphylococcus aureus* mutant strain 209P/UF-2, *Staphylococcus aureus INA 00761 (MRSA), Micrococcus luteus ATCC 9341, Bacillus subtilis ATSS 6633, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Saccharomyces cerevisiae Y1334.*

RESULTS AND DISCUSSION

Selective Isolation of Endophytic Actinomycetes: A total of 179 actinomycetes were isolated from the leaf samples of the 20 medicinal plants. It should be noted that after pretreatment of leaves with heteroauxin and zircon actinomycetes were isolated from all the 20 leaves samples, while in the control actinomycetes were isolated only from 14.

In pure culture 120 strains of actinomycetes were isolated. Most of the isolates were obtained from materials of *Hippophae rhamnoides* (11,7%)*, Urtica dioica* (11,7%), *Viola odorata* (10,8%) and *Plantago major* (10%) (Fig. 1). It is important to note, that the colonies grown after leaves pretreatment with heteroauxin, were characterized by better growth. This fact gave the opportunity to isolate in a pure culture approximately 80% of the colonies whereas in the control samples and after pretreatment with zircon we could isolate only 59% and 54%, respectively. It can be explained by the activation of biochemical processes and increasing of germination intensity of spores compared with the pretreatment with zircon and the control.

The pretreatment samples of the leaves with solutions of heteroauxin and zircon promotes more efficient isolation of actinomycetes from the leaves. Application of heteroauxin (20 mg/ml) stimulates the release of actinomycetes 3 times and zircon (1 mg/ml) – 2,5 times compared to the control (Table 3).

Identification of Endophytic Actinomycetes: According to the preliminary morphological identification, the most abundant genus was *Streptomyces* (65%), a finding consistent with other reports from different references [9, 11, 12, 13, 17]. Since the aim of our study was to isolate rare non-*Streptomyces* actinomycetes genera, 41 strains that did not seem to belong to the genus *Streptomyces* according to the morphological criteria, or which classification was in doubt, were further checked for cultural and micromorphological characteristics and were

Table 3: Results of the pretreatment of the herb leaves samples with *solutions of heteroauxin and zircon*

	Control	Heteroauxin	Zircon	In total
Number of grown colonies of actinomycetes	27	84	68	179
%	15	47	38	100

Table 4: Rare actinomycetes isolated from the leaves of different medicinal plants

Isolate	Host plant				
(INA accession no.)	Genus and species	Family	Experiment	Closest cultivated species (GenBank accession no.)	Similarity (%)
INA01099	*Aloe arborescens*	*Aloe*	Control	Nocardiopsis umidischolae strain B410-71 (EU849610.1)	97 %
INA01100	*Aloe arborescens*	*Aloe*	Zircon	Nocardiopsis quinghaiensis strain YIM 28A4 (NR_044303.1)	99%
INA01101	*Aloe arborescens*	*Aloe*	Heteroauxin	Nocardiopsis sp. 20041 (AY336516.1)	99 %
INA01102	*Mentha arvensis*	*Lamiaceae*	Heteroauxin	Nocardiopsis quinghaiensis strain YIM 28A4 (NR_044303.1)	99%
INA01103	*Mentha arvensis*	*Lamiaceae*	Control	Nocardiopsis exhalans strain VTT E-063001 (EU430537.1)	99%
INA01104	*Lysimachia nummularia*	*Primulaceae*	Control	Nocardiopsis tropica strain VKM Ac-1457 (NR_024957.1)	97%
INA01097	*Fragaria vesca*	*Rosaceae*	Heteroauxin	Nocardiopsis dassonvillei subsp. dassonvillei strain y47 (KF306364.1)	98%
INA01105	*Arctium lappa*	*Compositae*	Heteroauxin	Nocardiopsis viridoflava (AY117435.1)	99 %

Table 5: Distribution of antimicrobial activity of the isolated endophytic actinomycetes of different genera

		Number of active strains							
Genera	Number of tested isolates	*Staphylococcus aureus* FDA 209 P	*S. aureus* 209P/UF-2	*S. aureus (MRSA)*	*Micrococcus luteus ATCC 9341*	*Bacillus subtilis ATCC 6633*	*Escherichia coli ATCC 25922*	*Pseudomonas aeruginosa ATCC 27853*	*Saccharomyces cerevisiae Y1334*
Streptomyces	79	33	30	24	26	26	6	1	16
Micromonospora	33	13	21	13	14	6	7	1	14
Nocardiopsis	8	1	4	4	2	3	0	0	5
Total	120	47	55	41	18	35	13	2	35

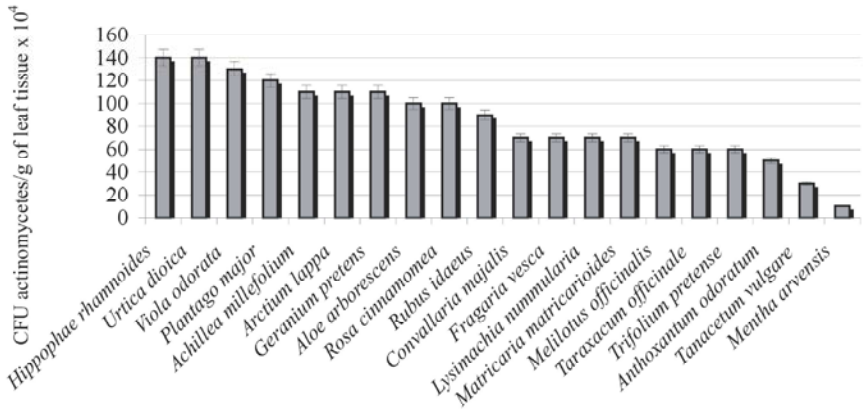

Fig. 1: Distribution of isolated endophytic actinomycetes in twelve medicinal plants

examined for the presence of isomers of diaminopimelic acid. Based on these results *Micromonospora* was the dominant genus (27,5%) and other organisms (7,5%) that could not be accurently indentified to the genus level. Eight strains were subjected to 16S rRNA gene sequence analysis.

Phylogenetic Analysis of Endophyte rRNA Gene Sequences: The nucleotide sequences obtained in this study were deposited in GenBank and have the accession numbers: INA01099 - KJ425228, INA01100 - KJ425229, INA01101 - KJ425230, INA01102 - KJ425231, INA01103 - KJ425232, INA01104 - KJ425233, INA01097 - KJ425227, INA01105 - KJ425234.

Eight strains subjected to 16S rRNA gene sequence analysis belonged to the genus *Nocardiopsis*. The percentage of 16S rRNA gene sequence similarities (97 to 99 %) of these isolates to the closest type strains of the NCBI database are presented in Table 4.

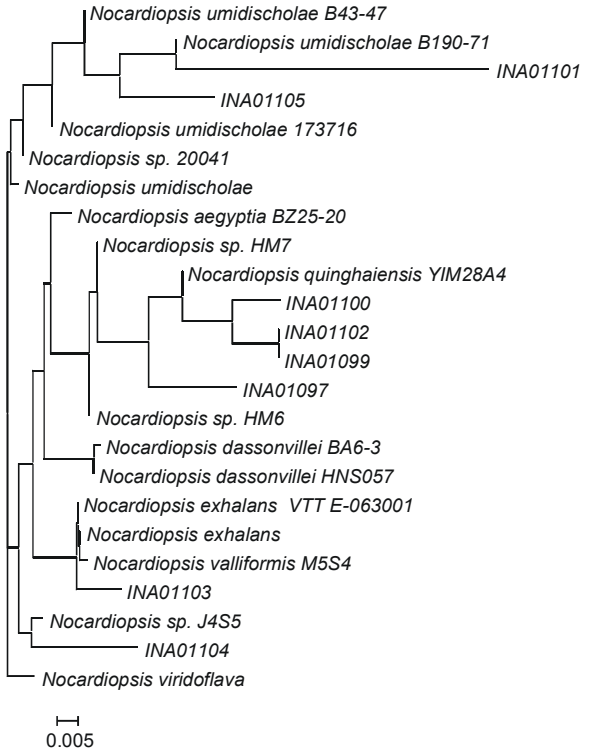

Fig. 2: Neighbor-joining tree of the isolated strains of Nocardiopsis and related species based on 16S rRNA gene sequences. Bar. 0.005 substitutions per nucleotide position. The unrooted tree was constructed as described in Materials and Methods

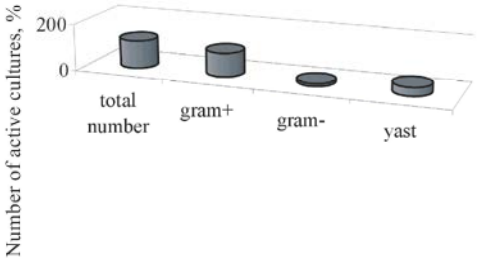

Fig. 3: Antimicrobial activity of the isolated strains of endophytic actinomycetes

The endophyte 16S rRNA gene sequences (~ 1028 bp) were aligned with reference sequences and phylogenetics reconstructed using a neighbor-joining method for a tree of maximum likelihood. The resulting phylogenetic tree (Fig. 2) showed isolates and reference sequences clustered according to established taxonomic orders with high bootstrap support.

Antimicrobial Activity of the Isolated Strains: Eight test microorganisms were used to determine the antibacterial activity of the endophytes. The results of studying of antimicrobial activity of 120 strains on solid media showed that 70 strains (85%) have been active against Gram-positive pathogenic bacteria. Among them, 11 strains (9%) were active against Gram-negative bacteria and 36 strains (30%) - against yeast (Fig. 3, Table 5).

CONCLUSIONS

In summary, a modified method for isolating cultures of actinomycetes from the leaves of medicinal plants, consisting of the pretreatment of the leaves with a solution of heteroauxin or zircon, promotes more effective isolation of actinomycetes compared with control. After pretreatment with heteroauxin and zircon actinomycetes were isolated from all the samples of the plants. Colonies, that have grown after pretreatment of the leaves with heteroauxin, have shown better growth. This fact gave the opportunity to isolate in pure culture nearly 80% of grown colonies. Isolated cultures belonged to the genera *Streptomyces, Micromonospora* and *Nocardiopsis*. Most of the isolated cultures (85%) have antimicrobial activity against the pathogenic bacteria.

Thus, using the modification method of this study helps to increase numbers of rare actinomycetes isolated from the medicinal plants, which are potentially unique source of novel bioactive metabolites for medicinal and biotechnological practice.

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