Endophytic bacteria from activated by exogenic non-pathogenic bacteria

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The results suggest that potato in vitro supports diverse bacterial endophytes. The community composition of the culturable component of the microflora was remarkably different from that revealed by culture-independent method. Introduction of Pseudomonas fluorescens IMBG163 into potato plant tissue resulted in essential rise of endophytic bacterial species number, however, in the further cloning their number was reduced. Endophytic isolates from potato varieties Zagadka and Nigru, induced by the rhizobacterium, exhibited features beneficial for plants.

Keywords*: endophytic bacteria, potato in vitro, biotic stressor, Pseudomonas fluorescens IMBG163.*

Introduction.The plants, including those cultivated in aseptic conditions, are inhabited by endophytic bacteria (endophytes) [1]. Unlike phytopathogens, which also inhabit plant tissues, the endophytes cause no apparent disease to hosting plants. In comparison to free living bacteria, the endophytes form a stronger association with plants and survive in plant tissues during plant vegetation [2].

Numerous types of endophytes are considered to be beneficial for plants i.e. they may participate in protecting the latter from diseases caused by pathogens, insects, and nematodes; they may assist in plants adjusting to adverse environmental conditions [3-5], protect the host plant from harmful effect of heavy metal cations and radionuclides [3, 6], at the same time some of the mentioned bacteria are capable of improving growth and development of plants [7, 8].

The endophytes were proved to be capable of co-existing with almost all plants [9]. The bacteria belonging to a wide range of subgroups (-, -,

-Protobacteria, Flexibacter-Cytophaga-Bacteriodes, gram-positive bacteria with high G+C content, and *Planctomycetales*) have been revealed in tissues in the investigations on endophytic populations in potato plants [10–12]. Alongside with the bacteria studied well by various microbiological and biochemical methods, there are some which can not be secured using traditional methods due to impossibility of their cultivation. Such bacteria are identified using molecular methods by means of investigating their nucleic acids, while not resorting to their cultivation [13]. Non-cultivated bacteria are capable of converting to cultivation under the influence of signals from the environment [14].

The goal of our work was the *in vitro* identification of endophytic bacterial communities of potato plants, Zagadka and Nigru varieties, and the investigation on the effect of inoculation of potato explants by *Pseudomonas fluorescens* IMBG163, beneficial for plants [15] on the communities of endophytes.

Materials and Methods. *Inoculation of potato plants by rhizobacterium.* Potato varieties of domestic selection Zagadka and Nigru were used in our investigation. Rhizobacteria, cultivated in KB medium [16], in the concentration of 10^6 of colony-forming units (CFU) per 1 ml, were used for inoculation. Plant material was inoculated as earlier described [17], then it was sterilized in 70%-ethanol solution and commercial solution BilyznB, followed by three times washing in sterile distilled water. The explants washed were placed on modified agarized hormoneless Murashige and Skoog medium for plant regeneration [16]. The bacteria were cultivated in 16-18-hrs photoperiod, T = 24°C, RH = 80-85%, illuminance 4 000 lx. The regenerant plants were multiplied by the method of clonal multiplication.

Isolation of endophytes. The bacteria were isolated from potato regenerant tissues in aseptic conditions [15]. The powdered material was dissolved and cultivated on 6 times dissolved nutrition agar medium. Bacteria were isolated from potato regenerants (root and stem separately) both bacteria treated and the control samples in two independent experiments.

Cultivation of bacteria. Bacteria were cultivated on glycerol-peptonic agar [16] (*P. fluorescens* IMBG163), mineral agar medium with methanol [19] (pink pigmented bacteria, PPB), and meat infusion agar (MIA) (*Erwinia carotovora* subsp. *atroseptica* and *Pseudomonas syringae*) for 1–5 days at 28 C.

Determining fermentative activity of endophytes. The ability of bacteria to produce ferments of pectin destruction (polygalactorunase and pectiliase), (for pectin whose substrate is polygalacturonic acid) were determined by the ability of colonies to form grooves on the surface of potassium-stabilized polypectate gel as a result of destruction of polygalacturonic acid [20]. Carboxymethylcellulose and natrium polygalacturonate were used as a source of carbon in the concentration of 0.2 % in M9 medium [21]; solution of Congo red (0.1 %) was used to detect cellulase (endoglucanase) activity in the investigated bacteria [22].

All reagents used were produced by Sigma Aldridge, USA. Protease activity was detected by the formation of protein coagulates of fat free milk. Ehrlich's reagent was used to determine the auxin content in the bacterial culture medium [23]. Antagonist activity of the isolators and their influence on wheat vegetation was determined as earlier described in [24].

Total DNA was isolated from roots and stems (cingula) of 3-4-week-old test-tube potato plants, the surface of which was sterilized, in accordance with the commonly accepted method [25]. Bacteria DNA isolation was performed using UltraClean™ reagent kit (MoBio Lab., USA).

Analysis of terminal-marked restriction DNA fragment length polymorphism (TRFLP) of potato endophytic populations *in vitro* was performed by the method earlier described [26]. The primers 507F (5 -TGCCAGCAGCCGCGGTA, Cy5 marked on 5 -end) and 1384r (5 -GGTTACCTTGTTACGACTT) were used for amplification of *rrs*-gene fragment. PCR was performed in Tercik thermocycler. (DNA Technology, Russia). PCR product was purified by the set of reagents UltraClean™ PCR Clean-up DNA (MoBio Lab) and was treated by *HhaI* enzyme (Fermentas, Lithuania). Previous *in silico* analysis revealed that the enzymes of apparent endonucleases of restriction which recognize 4 b.p. (*AluI, HaeII, TaqI, HhaI*) form the majority of the marked fragments. TRF aliquots (0.5 ml) were mixed with 1 ml of buffer (to apply on gel)

and 0.3 мl of DNA standards (Amersham, UK) The reaction mixture was denaturated (92 C min) and ice-cooled. The samples l) were applied on 6% denaturing polyacrylamide gel in ALF automatic sequenator (Pharmacia, Sweden). The sizes of fluorescent-marked fragments were calculated using ALF Fragment Manager Software (Amersham). TRF were considered to be positive having not more than 50 units. TRF sizes in the range of 50-600 b.p. were used for analysis.

To determine the nucleotide sequence of rrs gene fragment (16 S rRNA) the Sanger's method was used. Corresponding PLR product was obtained on the DNA-matrix of M1 bacteria-isolator using F507-Cy5 and R1384 primers. The nucleotide sequence was analyzed using BLASTn (NBCI) and Vector NTI 8.0 (Infomax Inc., USA).

Statistic analysis of the data obtained was performed using SigmaPlot 8.0 software.

Results and Discussion. *Rhizobacterial treatment of Nigru potato plants in vitro.* After inoculation the number of *P. fluorescens* IMBG163 bacteria in Nigru potato germ explants equaled $1.34C10^5$ KUO/g of raw plant tissue. The bacteria were observed to yield to agar in the rhizoplane area as a specific halo in a week after cultivation. The plants having such phenomenon did not differ morphologically from the control ones. Both IMBG163 and pink bacteria (M1 isolate) were observed upon determining their presence in plants using microbiological method, i.e. cultivation of leave and root segments on the selective medium of 1-5 vegetative generations. However, the mentioned bacteria were not observed in the control plants. It is possible to suppose that P. fluorescens bacteria are capable of provoking the yield of other types of bacteria out of inner explant plant tissue, coexisting with potato plant of the given variety.

Microbiological testing of Nigru potato plants revealed that the number of *P. fluorescens* IMBG163 positive plants decreased each passage, while the number of plants, of which PPB M1 was isolated, increased. M1 isolate was isolated from all parts of the plant (i.e. leaves, stem, root), it was also detected to oecize the plant from the top to the roots. At the same time, the regenerant plants grew and developed well, which testifies to the tolerance of such bacteria to the potato plant.

Comparative analysis of the defined 400 b.p. long part of the gene, which codes RNA of minor subunit of M1 bacteria ribosome with known sequences of GenBank, (NCBI) allowed making a conclusion on M1 isolate belonging to methylotrophic bacteria and revealed the homology to *Methylobacterium radiotolerans, M. organophilum, M. fujisavense, M. rhodium, M. jeotgli, M. mesophilicum.* The detailed analysis of DNA sequence revealed that end nucleotides on this fragment are located between 791–1234 b.p. The mentioned fragment contained the conservative part of gene, specific for its constant nucleotide number, and variable region (50 nucleotides), identical to *M. radiotolerans.* M1 was noted to be different from other types of methyltrophic bacteria by 3-10 positions in the borders of the mentioned sequence.

Therefore, M1 is the most probable to be the representative of *M. radiotolerans.*

Various *Methylobacterium* strains are often isolated from ground, fresh water, as well as from buds, leaves, roots, and tissue cultures in vitro of different plants [5, 27, 28]. *M. radiotolerans* are considered to be the suppressors of pathogenic fungi as well as to be heavy metals tolerant [29].

In two months after inoculation, total DNA obtained from two types of leaves (treated by *P. fluorescens* IMBG163 and the control ones) was studied using the T-RFLP method. Results showed the increase in endophytes number due to the presence of P. fluorescens (Table 1).

Experiment with Zagadka potato plants. Minding the fact that inoculation of Nigru potato plants by *P. fluorescens* IMBG163 could initiate the yield of M1 bacterial isolate, the IMBG163 effect on grouping of endophytes of Zagadka potato plant *in vitro* has been verified. According to our studies, after transplanting the inoculated explants on MC nutrition medium intensive formation of sprouts was observed on the 5^{th} day and on the roots – on the 8^{th} day, like the control plants. However, after three weeks potato regenerant plants inoculated by IMBG163 were observed to grow intensively. Control plants grew slower.

Table 1
Distribution of terminal-marked restriction fragments (TaqI) of rrs-gene of Nigru potato endophytes (b.p.)

Variant	32	38	49	52	51	62	64	77	87	219
Control plants (no treatment)	+	–	–	+	–	+	+	+	+	–
Treated plants (Pseudomonas fluorescens IMBG163)	+	+	+	+	+	+	+	–	+	+

Table 2
Distribution of terminal-marked restriction fragments (HhaI) of rrs-gene of Zagadka potato endophytes (b.p.) of two generations (b.p.)

Variant	39	64	68	262	332	364	432	435	482	539	556
Eyehole	+	–	–	–	+	–	+	–	–	–	+
Stem, R1, control	+	–	–	–	–	–	+	–	–	–	+
Stem, R1, Pseudomonas	+	+	–	–	–	–	+	–	–	+	+
Stem, R2, control	+	–	–	–	–	–	+	–	–	–	+
Stem, R2, Pseudomonas	+	–	–	–	–	–	+	–	–	+	+
Root, R1, control	–	+	–	–	–	–	+	–	–	–	+
Root, R1, Pseudomonas	–	+	+	+	–	+	+	+	+	–	+
Root, R2, control	–	+	–	–	–	–	–	–	–	–	–
Root, R2, Pseudomonas	–	+	+	+	–	–	–	–	–	–	–

Four root morphological types ($1.0C10^4$ KUO/g) and two stem morphological types (20 KUO/g each) were singled out. In the second vegetative generation of plants the number of root morphological types decreased – two root morphological types of bacteria ($2.0C10^3$ KUO/g) were singled out on nutrition medium. According to the microscopic analysis data, the bacteria are presented by gram-positive bacilli, diplococci and gram-negative bacilli. The latter dominated among the isolates.

TRFLP analysis of bacteria DNAs isolated from potato plants revealed that the structure of endophytic association of the stem is different from that of the root (Table 2). The comparison of microbiological and TRFLP methods shows the presence of grouping of endophytes which have not been detected on nutrition medium, i.e. those that are not cultivated at current conditions. At least four bacterial stems pass from eyehole to the root and five pass from eyehole to the stem, and two and one type of bacteria from eyeholes are not preserved in root tissues and potato plant stem after introducing them *in vitro*. Six types of endophytes were discovered only on IMBG163 infected plants, which changes the endophytic association of first generation plants roots comparing to the control. Eight possible types of bacteria were distinguished in the roots of first generation plants, infected by pseudomonades, while only three types were distinguished in the control. The structure of endophytic association of stem bacteria changes slightly (increases by two points) after pseudomonas introduction and is more or less stable during

Table 3
Characterization of potato endophytes in vitro, isolated after inoculation by Pseudomonas fluorescens

Isolate variant	Auxin isolation	Antagonism to		Enzymatic activity		
		E.carotovora subsp. atroseptica	P. syringae pv. syringae	Pectinase	Cellulase	Protease
47–57, 59, 60, 64S, 72, 76R	–	–	–	–	–	–
58, 67S, 75R	–	–	–	–	+	–
61S	–	–	–	+	+	–
62, 68S	–	–	+	+	+	–
63S	–	+	+	+	+	+
65S	–	–	+	+	+	–
66R	–	–	+	+	+	–
68R	–	–	+	+	+	–
70R	–	+	–	+	–	–
71R	–	+	–	–	–	+
73R	–	+	–	+	+	–
Pseudomonas fluorescens IMBG163	–	+	+	–	–	–
Klebsiella oxytoca IMBG26	+	–	–	+	+	–

N o t e : S – stem, R – root

cultivation. Such difference in structure of endophytic groupings of root and leave endophytes is known [10, 11] and may be explained by a higher metabolic activity of root.

7 of 10 types, discovered in the first generation of plants, including three types of eyehole-specific bacteria, were discovered among endophytes in the second generation of treated plants using the TRFLP method. At the same time, control plants of the second generation revealed only six types of endophytes out of seven, determined in the first generation, as well as three endemic bacteria which proceeded from eyeholes to regenerant plants. Therefore, the structure of root bacteria association is slightly simplified at clonal micromultiplication.

Multiplication of endophytes induced by various factors, e.g. phytopathogens, to the limit when the bacteria may be recorded by sensitive methods, is well-known today [14], it is also well-known that unfavorable growth conditions complicate the structure of bacterial groupings [30]. We were the first to show the increase in varieties of endophytes in potato plants *in vitro* under the influence of non-pathogenic bacteria.

Study on endophytic bacteria in potato plants. A series of isolates of gram-negative bacilli, most commonly isolated from the stem root of test-tube potato plants of different varieties were selected for further investigation of bacteria activated by *P. fluorescence* IMBG 163 introduction. Isolates 47-65 were isolated from stem tissues of regenerant plants of Zagadka, Povin, Asterix, Bilyna, and Chervona Ruta varieties of the first vegetative generation after IMBG163 treatment, and the isolates 66 and 76 were isolated from potato plants of the second and third vegetative generations.

Almost 30% of selected isolates were revealed to have antagonist activity to phytopathogens, such as *E. carotovora* subsp. *atroseptica* and *P. syringae*, or at least to one of them (Table 3).

Unlike IMBG 163, which was used for treatment of potato explants, the isolates were detected to have such enzymatic activities as pectinase, cellulase, and

protease activities, which are significant for interaction with plant cell walls and are possibly associated with biochemical processes involved in modulating the plant stressor-resistance [31]. Some potato endophytic bacteria isolates increased dry mass of the wheat as much as IMBG 163 did [32].

Therefore, having analyzed potato stem and root tissues *in vitro*, the number of bacteria was shown to decrease in vegetative generations during clonal micromultiplication. Inoculation of vitroplants with *P. fluorescens* IMBG163 was shown to modify the structure of endophytic groupings and activate the bacteria which are presumable to increase the population and therefore may be determined using classical methods. At least 30% of pseudomonas-induced endophytic isolates *in vitro* were shown to have antagonist activity to bacterial pathogens. Minding the fact that some isolates stimulate the growth of bacteria, the possibility that these isolates can be pathogenic sparring-partners exists. Taking into account that the number of endophytes in potato decreased during clonal micromultiplication and that *M. radiotolerans* migrated from generation to generation, these two facts clearly point at the necessity to control methylotrophs in potato cutting.

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Эндофитные бактерии картофеля *in vitro*, активированные экзогенными непатогенными бактериями

Резюме

Микробиологическим и независимым от культивирования бактерий методами показано присутствие как культивированных, так и некультивированных форм эндофитных бактерий в растениях—регенерантах картофеля, выращиваемых в условиях in vitro. Ведение ризобактерий Pseudomonas fluorescens ИМБГ163 в растения картофеля параллельно с растительным материалом в культуру in vitro повышало количество видов эндофитных бактерий корней первого вегетативного поколения растений, но при последующем клональном микроразмножении растений количество выявленных эндофитов уменьшалось. Эндофитные изоляты сортов картофеля Загадка и Нигру, активированные ризобактерией, обладали полезными для растений свойствами.

Ключевые слова: эндофитные бактерии, картофель in vitro, биотический стрессор, Pseudomonas fluorescens ИМБГ163.

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УДК 576.5: 582.951:577.2
Надійшла до редакції 18.09.06