PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF NITROGEN-FIXING BACTERIA *Azospirillum brasilense* 137 CAPABLE TO ROOT COLONIZATION OF SPRING WHEAT

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Abstract. The new PGPR strain of genus *Azospirillum*, capable of fixing atmospheric nitrogen, has been isolated from the roots of spring wheat. Phenotypic features were studied and molecular genetic analysis of the new *Azospirillum brasilense* strain was carried out. It has been shown that inoculation of seeds of spring wheat by strain *A. brasilense* 137 promotes active colonization the root surface by this bacteria and increased nitrogen activity in the root zone of this culture.

Keywords: *Azospirillum brasilense*; physiological and biochemical properties; molecular genetic studies; spring wheat.

INTRODUCTION

Azospirillum bacteria are of interest to scientists from different countries due to their ability to stimulate the growth of a wide range of plants (this is plant growth promoting rhizobacteria or PGPR) [7, 18, 20, 26, 27]. They produce plant-growth-regulating substances and intensify the process of biological binding of nitrogen in close interaction with the roots of plants, increasing the yield of agricultural crops and the quality of the resulting products [8, 13, 23]. However, underestimation of the ability of nitrogen-fixing microorganisms to colonize root spheres often leads to a lack of positive effects from the use of microbiological fertilizer [22].

The most promising in agricultural production is the use of diazotrophs for plant inoculation, capable to active colonization of the roots. Such an association provides a more cohesive, and therefore, more effective interaction of bacteria with plants and greater resistance to adverse environmental conditions. It is known that bacteria of the genus *Azospirillum* are capable of developing in the risoplane and penetrate into the zone of the histospheric, breed there and fix atmospheric nitrogen [3, 6]. It should be noted that according to Bashan and his colleagues, the ability of *Azospirillum* to colonize the roots is closely related to their ability to fix the nitrogen of the atmosphere [2]. Thus, the authors have shown that factors that inhibit nitrogen fixation (nitrates) inhibit the spread of bacteria, and the factor that stimulates nitrogen fixation (micro aerophilic conditions) increases the ability of *Azospirillum* to colonize the roots.

The analytical selection of *Azospirillum* strains with high ability to fix atmospheric nitrogen, and to colonize the root zone of plants is a prerequisite for the creation of effective associations: a plant - diazotrophs.

MATERIALS AND METHODS

Study of physiological and biochemical properties of bacteria

The isolates were obtained from rhizospheric soil and roots of spring wheat at the study site of Institute of Agricultural Microbiology and Agro-industrial Production of the NAAS. From these samples, successive dilutions were made in sterile physiological solution (NaCl 0.9 %) and aliquots of 100 µL were seeded in the semisolid culture medium NFb (malic acid – 5.0 g/dm^3; K_2HPO_4 – 0.5 g/dm^3; $MgSO_4 \times 7H_2O$ – 0.2 g/dm^3; NaCl – 0.1 g/dm^3; $CaCl_2 \times 2H_2O$ – 0.02 g/dm^3; KOH – 4.5 g/dm^3; agar – 1.75 g/dm^3, tap water up to 1.0 dm^3, pH 6.8 adjusted with KOH. The cultures that showed bacterial growth in the form of a sub-surface white film, typical of *Azospirillum*, were replic in solid NFb culture medium added with yeast extract (5 g/dm^3) and Congo Red (15 mL/dm^3 an aqueous solution 1 : 400, for the identification of small, dry colonies, scarlet red color [5]. The evaluation of the cultures purity and their viability was carried out by conventional methods of microbiological research. Cultural, morphological, and physiological properties of the nitrogen-fixing bacterial strains were studied according to standard techniques [11]. Identification of microorganism strains in primary species was performed using techniques that are described in the original work of the authors [5].

Determination of antibiotic resistance of bacterial strains was carried out by diffusion method in agar using standard antibiotics discs [15]. Antibiotic-resistant mutants of *Azospirillum* were obtained using the Szybalski method [4].

The nitrogenase activity of the associative nitrogen-fixing bacteria and the potential nitrogen activity in the root zone of wheat were determined with acetylene gas chromatograph Chrom-4 [14].

Electrophoresis of proteins

To prepare protein samples, aliquots of bacterial cultures were resuspended in 100 µL of 8M urea solution, maintained for 30 minutes at room

temperature, 100 μL of Sample Buffer Laemmli 2x Concentrate (Sigma, USA) were added and the mixture was boiled for 10 min.

Electrophoresis of proteins was perfomed by the Laemmli method in 12% SDS-PAGE [16]. After separation, protein bands in the gel were stainied according to the protocol for PageBlue Protein StainingSolution ("Thermo Scientific", Lithuania).

DNA extraction and PCR-amplification of nifD and dnifA genes

The occurrence of diazotrophic bacteria was assessed by PCR-amplification of a 710 bp and 323 bp fragments of nifD gene (nitrogenase enzyme) using specific primers: nifD-up (5'-ATCATCGGTGACTACAAC-3') and nifD-do (5'-ATCCATGTCGCGGCGAA-3') described by Potrich et al. [23] and dnifA-R 5'-CTCCTCGGCCACGGTGC-3' and dnifA-F 5'-CGCGGCGCAGATCGAAT-3' (first published in this article). The latter oligonucleotides were developed with the use of the Vector NTI® software (Thermo Fisher Scientific,Waltham, MA, USA) and primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The reaction volume was 25 μL. Other components were: 1x DreamTaq PCR buffer, 0.1 mM dNTP, 3 mM $MgCl_2$, 0.6 mg/mL bovine serum albumin, 4 % glycerol, DreamTaq polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and distilled water to 25 μL. PCR program was: pre-denaturation, 95 °C for 4 min; amplification for 35 cycles of: 95 °C for 30 sec, annealing 52 °C (nifD-up\nifD-do) and 62 °C (dnifA-F\dnifA-R) - for 30 sec, elongation 72 °C for 30 sec. The final elongation was 72 °C for 7 min. Typically, 5 μL (10-15 ng DNA on reaction) of sample DNA were added to PCR reaction volume.

The DNA samples for PCR were obtained using commercial kits "GeneJET Genomic DNA Purification Kit" (Thermo Fisher Scientific, Waltham, MA, USA) and "DNA-sorb" (SSCIBSM, Ukraine), according to the manufacturer's instructions. Concentration and purity of DNA preparations were determined spectrophotometrically ("Nano Drop 1000C"): in the first case was 23.1 - 34.0 ng / μL (260/280 = 1.9) and in the second - 383.6 - 527.6 ng / μL (260/280 = 2.4). DNA sample of *A. brasilense* Sp7 (ATCC 29145) was used as positive control.

PCR was performed using an "T-personal Combi" thermocycler (Biometra, Germany). PCR amplification products were analyzed by agarose gel (1.5%, w/v) electrophoresis at 5.0 V/cm and revealed by ethidium bromide staining [9]. Digital images were obtained using the gel documenting systems "GelDoc XR Plus" (BioRad Laboratories, USA).

Field experiments

Field experiments were carried out in accordance with the general rules on leached black soil of the experimental field of the Institute of Agricultural Microbiology and Agro-Industrial Production of the National Academy of Agrarian Sciences (Chernihiv, Ukraine). The area of registration site is 10 m^2, the repetition of the experiment is fourfold.

Scheme of experiment: 1. Control (without inoculation); 2. Inoculation by strain *Azospirillum* sp. 137; 3. Inoculation by antibiotic resistant strain *Azospirillum* sp. 137 to kanamycin (KmR); 4. Inoculation by antibiotic resistant strain *Azospirillum* sp. 137 to streptomycin (SmR).

The seeds of spring wheat of the Thera grade were inoculated by a bacterial suspension with a titre of $3x10^5$ cells per seed.

The root sampling was carried out with a periodicity of 15 days. Determination of the number of cells *Azospirillum* sp. 137 was performed by the method based on the suspension-dilution of root samples and on inoculation of selective medium of Caceres, with the addition of streptomycin 500 μg / mL and kanamycin 25 μg / mL.

Statistical analysis

The processing of experimental data was carried out using a table editor Microsoft Excel. Differences were considered significant when the value of the significance level was $p \leq 0.05$.

RESULTS

Bacterial characterization

Studying of potential nitrogen activity (PNA) in the root zone of spring wheat 12 varieties in the phase of earing and flowering for three years showed that the highest values of PNA on the washed roots (risoplane)were characterized by plant varieties: Etude and Varyag (969-1014 nmol C_2H_4 / g of roots / hour).

29 pure cultures of nitrogen-fixing bacteria of the genus *Azospirillum* were isolated from the root zone of spring wheat of the studied varieties. The highest nitrogen fixing capacity in pure culture (112 nmol of ethylene / vessel / hour) was characterized by *Azospirillum* sp. 137 (Table 1).

Cultural, morphological characteristics of the new isolate corresponded to the characteristics of the

Table 1. Nitrogenase activity of pure cultures of *Azospirillum* genus, isolated from the washed roots of spring wheat

Strains	Nitrogenase activity, nmol C_2H_4 / g of roots / hour
Azospirillum sp. H271	88.5 ± 15.1
Azospirillum sp. H262	101.5 ± 16.8
Azospirillum sp. H393	86.0 ± 7.8
Azospirillum sp. R931	95.5 ± 18.2
Azospirillum sp. Ch1	111.5 ± 8.4
Azospirillum sp. Sp1	61.0 ± 9.8
Azospirillum sp. S99	70.5 ± 7.9
Azospirillum sp. 137	112.3 ± 8.2

bacteria of the genus *Azospirillum*. Physiological and biochemical properties of this strain were studied in order to determine its species belonging to *A. brasilense* or *A. lipoferum*. It was shown that the strain isolated from rhizoplans of the wheat grade Etude was somewhat different from the type *A. lipoferum* 59 b, *A. brasilense* Sp7 by the ability to absorb carbon sources (Table 2). Thus, the introduction of *Azospirillum* sp. 137 in the medium of Norris led to alkalinization of the nutrient medium, probably due to the use of yeast extract by bacteria.

Under conditions of laboratory experiments on the mineral medium of Kozer examined the ability of *Azospirillum* to use nitrogen sources (Table 3). It is shown that type *A. brasilense* $Sp7^T$ and *Azospirillum* sp. 137 were characterized by abundant growth on medium with peptone and asparagine. *Azospirillum* sp.137 bacteria produced a significant number of polysaccharides that formed a mucous membrane spreading through agar slopes the medium with asparagine and ammonium hydrophosphate. On this basis, the new isolate also differed from the type strains *A. lipoferum* 59 b, *A. brasilense* Sp7.

Sensitivity of bacteria to antibiotics is one of the important systematic features. Isolated *Azospirillum* sp. 137 was resistant to cefalexin, amoxicillin and ciprofloxacin (Table 4).

The greatest bactericidal effect for the new strain and *A. brasilense* strain was shown under the action of levomitsitina (amfenicol). Rifampicin - from the same group of antibiotics that inhibit transcription and protein synthesis, substantially inhibited the growth of all strains examined (growth retardation area was 4.4-5.1 cm).

Table 2. Use of carbon sources by *Azospirillum* strains (growth on the semisynthetic mineral medium with the yeast extract)

Carbon sources	*A. lipoferum* b59	*A. brasilense Sp.7*	*Azospirillum sp*. 137
glucose	ac	ac	n
xyrod	m / ac	ac	al
ramnoza	m / ac	n	al
sucrose	n	n	al
lactose	n	n	al
maltose	n	n	al
mannitol	n	n	al
sorbite	n	n	m / al
dultsit	n	n	al

Note: ac - acidifies of the medium; m / ac - moderately acidifies the medium; al – alkalinizes the medium; m / al - moderately – alkalinizes the medium; n - do not change the pH the medium.

Table 3. Ability of strains of genus *Azospirillum* to *use* a variety of *nitrogen sources* (on the mineral medium of Kozer)

Use of sources of nitrogen		*A. lipoferum* 59b	*A. brasilense Sp.7*	*Azospirillum sp*.137
$(NH_4)_2HPO_4$	Growth	Moderate	Moderate	Plentiful
	EPS	+	++	++++
$NaNO_3$	Growth	Moderate	Moderate	Moderate
	EPS	+	+	++
NH_4NO3	Growth	Moderate	Moderate	Plentiful
	EPS	+	+	++
Urea	Growth	Plentiful	Moderate	Plentiful
	EPS	+	+++	+
Asparagin	Growth	Moderate	Plentiful	Plentiful
	EPS	+	+	++++
Peptone	Growth	Plentiful	Plentiful	Plentiful
	EPS	+	++++	+

Note: ++++ - intensive production of exopolysaccharides (EPS); +++ - moderate production of EPS; ++ - insignificant production of EPS; "-" - no growth

Table 4. Sensitivity of *Azospirillum* strains to antibiotics

Antibiotics	Growth retardation area, cm		
	A. lipoferum 59b	*A. brasilense Sp7*	*Azospirillum sp*.137
Amoxicillin	2.0 ± 0.2	0	0
Oksacillin	0	0	0
Ampicillin	0	0	0
Cefalexin	0	2.3 ± 0.2	0
Tseftriakson	5.2 ± 0.2	3.6 ± 0.0	4.0 ± 0.1
Streptomycin	4.4 ± 0.1	3.5 ± 0.0	4.5 ± 0.0
Kanamitsin	4.9 ± 0.1	3.5 ± 0.1	3.4 ± 0.1
Gentamycin	2.1 ± 0.1	0	0.7 ± 0.2
Tetratsiklin	4.4 ± 0.1	1.8 ± 0.1	2.9 ± 0.2
Erythromycin	3.9 ± 0.1	3.4 ± 0.1	3.7 ± 0.1
Levomitsitin	3.7 ± 0.1	4.6 ± 0.0	4.7 ± 0.1
Ciprofloxacin	1.8 ± 0.1	0	0
Nelidiksova acid	2.9 ± 0.3	1.8 ± 0.2	1.9 ± 0.1
Rifampitsilin	5.1 ± 0.2	4.9 ± 0.1	4.4 ± 0.1
Polimiksin	4.5 ± 0.1	2.1 ± 0.1	1.9 ± 0.1

Thus, based on the results of the study of the physiological and biochemical properties of the isolate Azospirillum sp. 137, it can be concluded that this strain belongs to the genus *Azospirillum*, but clarification of the systematic position to the species name required further research.

However, the ability of *Azospirillum* sp. 137 to grow on a nutrient medium NFb without biotin indicates that it belongs to the species *A. brasilense* [11].

Electrophoresis of proteins of *Azospirillum*

Electrophoretic separation of proteins of new isolates was carried out in comparison with type strains of bacteria of the genus *Azospirillum*. The results of this analysis indicate that the protein spectrum of *A. brasilense* 137 and *A. brasilense Sp7*^{T} were similar but differed from the spectrum of the type strain *A. lipoferum* 59 b^T (Fig. 1).

PCR analysis

PCR analysis of target genes of dnifA and nifD of *A. brasilense* 137, standard strains of *A. lipoferum* 59 b^T, *A. brasilense* Sp7^T and strain *A. brasilense* Sp245, confirmed the similarity of the gene fragments of dnifA and nifD of the new strain and strains of species *A. brasilense* (Fig. 2, Fig. 3).

The ability of *Azospirillum* to colonize the root zone of spring wheat

In order to study the ability of *Azospirillum* to colonize the root zone of spring wheat plants antibiotic resistant strains *Azospirillum* sp. 137 to kanamycin (*A. brasilense* 137 (KmR)) and streptomycin (*A. brasilense* 137 (SmR)) have been selected.

The concentration of the antibiotics in the medium in which the abundant growth of the culture occured and the preservation of cultural and morphological characteristics was observed, was 25 μg / mL for kanamycin and 500 μg / mL for streptomycin. So, the colonies of the studied cultures in an agar medium with malate are small, shiny with mother-of-pearl tint. When aging, the culture forms a pink pigment.

In order to investigate the ability of antibiotic resistant mutants to fix the nitrogen of the atmosphere, the potential nitrogen activity of these crops grown on a Dobreiner medium was determined. It is shown that *Azospirillum* sp. 137, *A. brasilense* 137 (KmR) and *Azospirillum* sp. 137 (SmR) contributed to change in color of the medium from green to blue for 3 days cultivating. It should be noted that the potential nitrogen activity of kanamycin and streptomycin resistant strains was slightly lower than wild-type strain of *Azospirillum* sp. 137 (Table 5). But this difference was not significant.

Table 5. Nitrogenase activity of the *Azospirillum* strain 137 and its antibiotic resistant mutants

Strains	Nitrogenase activity, nmol C_2H_4 / g of roots / hour
A. brasilense. 137	16.94 ± 2.21
A. brasilense. 137 (Km^R)	14.10 ± 3.71
A. brasilense. 137 (Sm^R)	15.31 ± 1.27

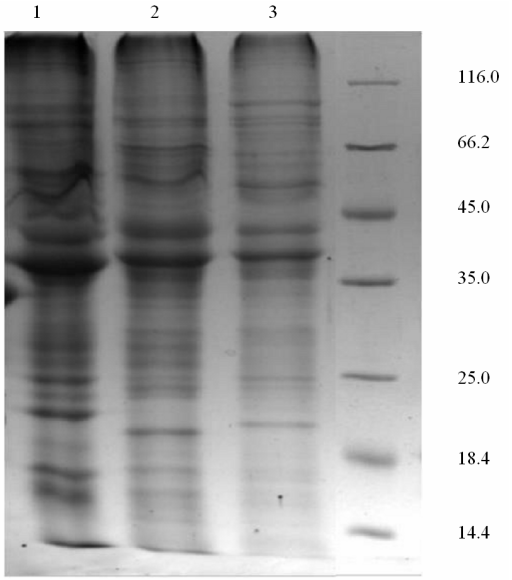

Figure 1. The result of electrophoretic separation of proteins of strains: 1 - *A. lipoferum* 59 b^T; 2 - *A. brasilense* 137; 3 - *A. brasilense* Sp7^T

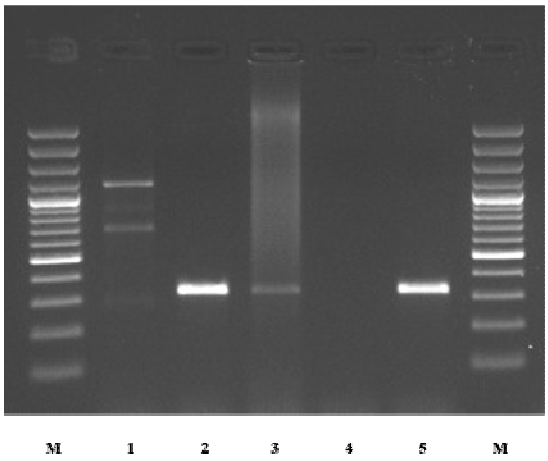

Figure 2. Results of PCR with primers for strain dnifA gene: 1- *A. lipoferum* 59 b^T, 2- *A. brasilense* Sp7^T, 3- *A. brasilense* Sp245, 4- negative control of isolation, 5- *A. brasilense* 137, M-marker "100 bp Plus DNA Ladder"

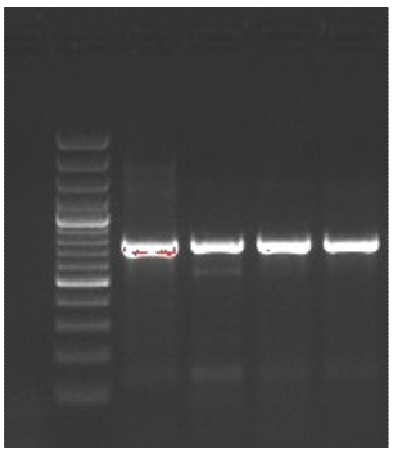

Figure 3. Results of PCR with primers for nifD strains gene: 1- *A. lipoferum* 59 b^T, 2- *A. brasilense* Sp7^T, 3- *A. brasilense* Sp245, 4- *A. brasilense* 137, M- marker "100 bp Plus DNA Ladder"

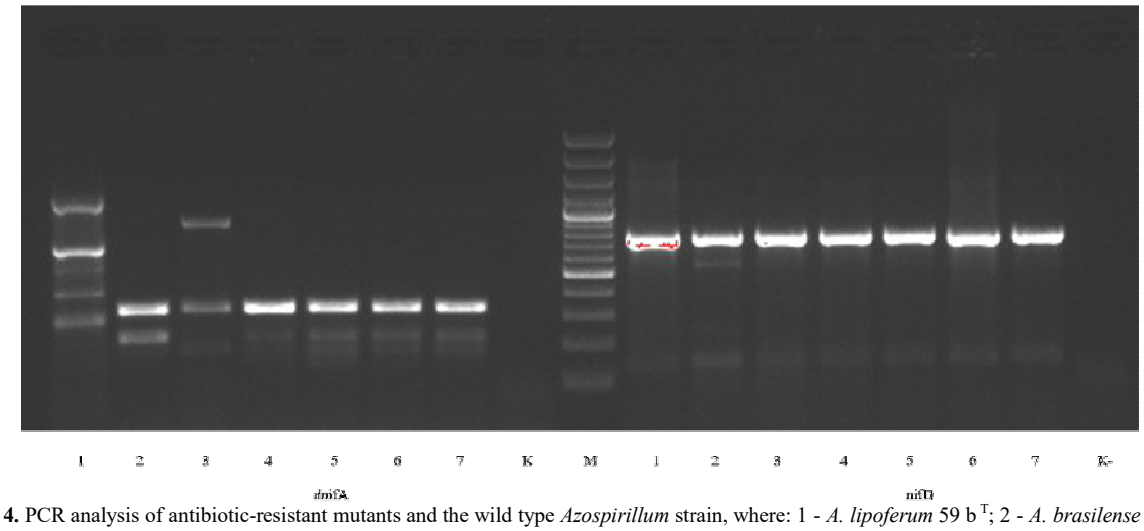

Figure 4. PCR analysis of antibiotic-resistant mutants and the wild type *Azospirillum* strain, where: 1 - *A. lipoferum* 59 b ^{T}; 2 - *A. brasilense* Sp7^{T}; 3 - *A. brasilense* Sp245; 4 - *A. brasilense* 77; 5 - *A. brasilense* 137; 6 - *A. brasilense* 137 (SmR); 7 - *A. brasilense* 137 (KmR)

PCR analysis of antibiotic-resistant mutants using target genes dnifA and nifD showed that the studied gene fragments of strains resistant to kanamycin and streptomycin and the wild type *Azospirillum* strain were identical.

The next step in our work was to detect *Azospirillum* in the root zone of spring wheat during the growing season for inoculation by antibiotic resistant bacteria under field conditions.

The ability of *Azospirillum* to absorb on the surface of wheat roots was investigated.

During the vegetation, root samples were taken every 15 days starting from 28 days after sowing. The number of bacterial cells on the roots was determined and graphs of changes in the number of *Azospirillum* in the root zone of wheat were constructed.

The first single shoots of wheat plants appeared on 10-15 days, the mass appearance was observed on the 25th day after sowing.

At the washed roots (Fig. 5), the number of both kanamycin and streptomycin-resistant mutants, which developed on the surface of the wheat roots, did not differ significantly in dynamics throughout the growing season.

The number of cells was 23x10^{3} for *Azospirillum* sp. 137 (KmR) and 28x10^{3} for *Azospirillum* sp. 137 (SmR) in the root zone at the end of vegetation of plants.

The use of kanamycin resistants and streptomycin resistants mutants for inoculation shows significant increase in the number of *Azospirillum* (1.7 and 2 times, respectively) in the flowering phase, with a general decrease in the amount of bacteria on the roots during the entire period of vegetation.

In view of the correlation between the parameters of the ability of *Azospirillum* to colonize the roots and their ability to fix the nitrogen of atmosphere, we have studied the potential nitrogenase activity (PNA) in the root zone of wheat in the flowering phase (Fig. 6).

Thus, it has been shown that the values of PNA in the rhizosphere soil after use of both antibiotic resistant strains and wild strains (*Azospirillum* sp. 137) was 85.6 - 136.8 nmol ethylene / g of roots / 24 hours. Also, no difference was revealed in comparison with the indexes of nitrogenase activity of the rhizosphere in the control. However, nitrogen activity on the washed roots of inoculated plants significantly (in 2.7 - 4.9 times) exceeded that in the risoplane in the control.

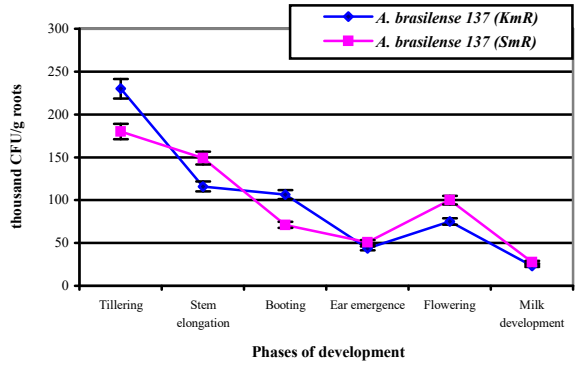

Figure 5. Dynamics of the number of *Azospirillum* on the washed roots of wheat after inoculation of seeds whith *A. brasilense* 137 (KmR) and *A. brasilense* 137 (SmR)

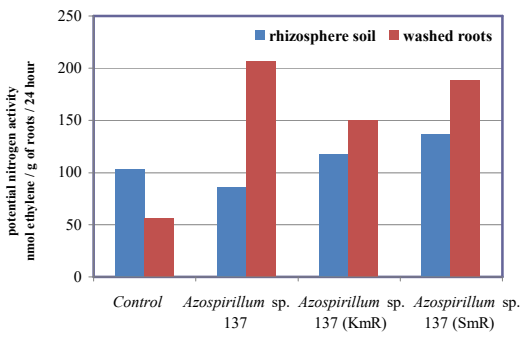

Figure 6. Potential nitrogenase activity in the root zone of wheat, inoculated by antibiotic resistant strains of genus *Azospirillum* and wild strains

The PNA value in wheat rhizoplane inoculated with antibiotic-resistant strains was 2.7 - 3.3 times higher than the nitrogenase activity on the wheat roots of the control variant. This indicates the preservation of the properties of nitrogen fixation by antibiotic resistant strains under the conditions of the present experiment.

DISCUSSION

Phenotypic and molecular methods are used to identify strains of nitrogen-fixing bacteria of the genus Azospirillum, associated with roots and the rhizosphere of different plants [1, 17, 25]. Thereby, we identified a new isolate with high nitrogen activity in a pure culture (112 nmol of ethylene / bottle for 24 hours) derived from the washed roots of wheat of the Etude grade, in which the rhizospalen also showed high PNA (969 nmol C_2H_4 / g of roots / hour).

According to the results of the study of the physiological and biochemical properties of the isolate *Azospirillum* sp. 137, it can be concluded that it belongs to the genus *Azospirillum*. It was shown that this isolate differs from the type strains *A. lipoferum* 59 b and *A. brasilense* Sp7 in the ability to absorb carbon and nitrogen sources and in resistance to antibiotics. However, the ability of *Azospirillum* sp. 137 to grow on a nutrient medium NFb without biotin indicates that it belongs to the species *A. brasilense.* Using molecular genetic methods, the similarity of the dnifA and nifD gene fragments of the new strain and strains of the *A. brasilense* species was shown.

Azospirillum brasilense is a plant growth promoting rhizobacterium (PGPR) that is being increasingly used in agriculture. Recent research has elucidated key properties of *A. brasilense* that contribute to its ability to adapt to the rhizosphere habitat and to promote plant growth. They include synthesis phytohormonal substances, nitric oxide, carotenoids, a range of cell surface components as well as the ability to change physiological properties and undergo phenotypic variation [10]. Plant-bacteria associations have been studied for many decades. However, a complete understanding of the mechanisms used by plant growth-promoting bacteria had remained somewhat elusive, often making it difficult to take full advantage of these complex relationships to improve the growth of plants in an applied setting. Other previous studies shoewed the ability of *Azospirillum* to form associations with plant roots [12, 21, 24]. The results of our work are consistent with the data concerning the number of azospirils in rhizosphere of barley and rye [19]. Thus, it was shown that 15 days after inoculation, the amount of *Azospirillum* l in the rhizosphere soil was 10^5 colony forming units (CFU) / g of soil irrespective of the titer of the inoculum (10^4, 10^5, or 10^8). Unfortunately, the author did not conduct research on the number of cells in the root zone of plants throughout the period of vegetation.

The ability of the bacteria *Azospirillum brasilense* 137 to survive in the root zone of spring wheat, which was grown under conditions of field experiment on the leached black soil in the Polissya region of Ukraine after inoculation of seeds by the culture of kanamycin-, and streptomycin-resistant mutants was studied. It was shown that during the growing season there is a decrease in the number of bacteria of the genus *Azospirillum* from 1.8 - 2.3 x 10^5 to 2.3 - 2.8 x 10^4 on the surface of the washed roots.

It was ascertained that the nitrogen activity on the washed roots of plants, inoculated with antibiotic resistant strains and wild strains, was 2.8 - 7.5 times higher than the given index in the control.

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