

THE USE OF METHODS FOR IDENTIFICATION AND STUDYING THE FUNCTIONAL FEATURES OF THE DOMINANT RHIZOSPHERE MICROORGANISMS OF THE BARLEY

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Abstract. Microbial biome of soil biocenosis determine the main functional role in the circulation of substances and energy and represent key components of the transformation of organic residues, both their mineralization and immobilization of nutrients. Therefore, the studying of the biome of soil microorganisms (especially dominant strains) is the scientific basis for the development of measures aimed at enhanced reproduction of chernozem fertility. Two dominant endophytic bacterial isolates were recovered from native *Hordeum vulgare* rhizosphere varieties at central Ukraine area. New isolates were evaluated for their morphological and biochemical characteristics.

So, the aim of the work was to investigate and identify these important dominant strains of bacteria with help of the innovative colorimetric identification system for carbohydrate utilization – KB009 TM HiCarbo Kit. The results of researches based on the study of morphological, biochemical and physiological state of bacteria in regard to sources of carbon and metabolic similarity with standard strain *Azospirillum brasilense*. Methods of research - physiological features were studied by one of the most accurate methodological approaches in determining the physiological diversity of the association of soil microorganisms - the testing system KB009 TM HiCarbo Kit for carbon sources. Thanks to previous investigation, it was learned number of dominant groups of microorganisms, microbiological processes orientation and ecological indexes of biodiversity. And in result of the researches, it was possible to classify allocated dominant strains to the genus *Phyllobacterium* and *Bacillus*.

Key words: carbohydrate utilization; soil microorganisms; KB009 TM HiCarbo Kit; *Phyllobacterium*; *Bacillus*.

INTRODUCTION

The excessive use of inorganic fertilizers and pesticides changed the traditional cultivation practices, especially in second part of 20th century. The situation has become so alarming that now the role of microorganisms in development of sustainable agriculture is being realised [31, 32, 65, 68]. In order to increase the agricultural production, there has been a tendency to adopt microbial agents in the agricultural engineering of biological systems [25, 58, 80].

Cereals are the major food crops in most countries of the world [8, 35]. *Hordeum vulgare* occupies one of the first place in Ukraine by sowing areas [46, 47]. Meeting the needs of the population with high-quality products is of great economic importance. The rhizosphere formation is a vital basis for plant ontogenesis homeostasis [12, 23, 62]. Effective interaction of microorganisms occurs at all levels, starting from molecular [3, 27]. Functionally significant rhizosphere microorganisms determine up to 70% of the optimal development of plants. Biological and functional features of the microbial communities of the rhizosphere are evaluated by their effective interaction in plant-microbial systems [5, 10, 40, 67].

Investigation of biological functional features of plant-microbial interactions, the groups of microorganisms, the orientation of nutrition processes, in particular, the biological transformation of carbon and nitrogen in soil greatly contributes to effective construction of biological systems in agricultural biomes [19, 28, 30, 74]. Furthermore, immobilizing emissions of organic forms in the form of biomass accumulation may play an important role in the

development of sustainable agricultural production methods [15, 60, 71].

The object of research was to study the rhizosphere of *H. vulgare* [2]. The aim of the experiment was to study morphological and functional features of rhizospheric microorganisms, their physiological state in regard to sources of carbon and interaction with plants for their later study as plant growth promotory bacteria (PGPB). The number of dominant groups of microorganisms, microbiological processes orientation and ecological indexes of biodiversity and the dominance of typical black soil bacterial complex in agrocenosis of wheat for different systems of agriculture and cultivation of soil were investigated before [17, 29, 56, 88]. Subsequently, isolated microorganisms were used in laboratory experiments to determine the isolates species using a colorimetric identification system for carbohydrate utilization – KB009 TM HiCarbo Kit [21, 26, 59, 63].

MATERIALS AND METHODS

Study of typical chernozem microbiota was conducted on the basis of stationary field experiment of the Department of Agriculture and Herbiology NULES of Ukraine "Agronomic Research Station". The territory of the studied field is located in the right-bank part of the Forest-Steppe of Ukraine. The terrain is flat. The soil of the plot is typical, low-humus chernozem; by particle size distribution - coarse-grained medium loam.

The number of microorganisms of the main physiological and taxonomic groups was determined by the method of sowing soil suspensions on the appropriate elective nutrients [36, 61]. First isolate

GPA-7 of dominant bacteria we grew up on glucose peptone agar nutrient medium. The composition of glucose-peptone agar (or Zvyagintseva medium) contains: 1000 mL of distill water; 7g of enzymatic peptone; 5.5g of glucose, 4g of NaCl; 0.5g of yeast extract; 0.04g of bromothymol blue. The high-quality composition of the medium is a nutritional base for the growth of bacteria, providing them with all the necessary substances, glucose is included as a carbon source, which allows the accumulation of a wide range of microorganisms. The second isolate Chap-1 was highlighted on Chapek medium, which is more characteristic for growing of fungi, but also some bacteria is possible to grow. Chapek original medium recipe includes: 1000 mL of distill water; 30g of cane sugar; 1g of K₂HPO₄; 0.5g of MgSO₄; 0.5g of KCl; 0.01g of FeSO₄. The qualitative composition of the microbial complex was studied on the basis of the represented morphological and cultural types [38, 75]. The indicator of species saturation was used to determine the degree of dominance, the Shannon (H) and Simpson (C) indices were calculated for the ecological assessment of biodiversity of microorganisms in the soil. A total of 85 morphotypes were described during the flowering phase of barley, from them dominants composed >10% and we selected the two most interesting isolates for farther investigation, in our opinion. All these investigations already have been described and published in previous study [6, 34, 51].

For further identification and classification of the dominant strains selected by us, we conducted morphological and biochemical studies [39, 49].

Morphological features of dominant strains of microorganisms of the rhizosphere we studied with EVOS FL Imaging System, 40x lens. Dyeing by gram was also performed [24, 37, 55].

Traditional methods of using selective nutrient media over the centuries have identified numerous physiological groups of microorganisms and formed an idea of their role in the cycle of substances, soil formation and plant nutrition [13, 52, 73]. However, over the past 20 years, such studies have only fragmentarily proven new knowledge in environmental issues, agronomy etc. In the late 90's for studying the physiological diversity was proposed a method of analyzing the spectrum of consumption of organic substrates by the natural association of microorganisms [20, 43]. This is a method of multisubstrate testing,

which has high performance, good resolution, satisfactory reproducibility and serves as a high-tech and effective tool for assessing physiological diversity [41, 70, 76].

The KB009 TM HiCarbo Kit test system can be used to study the biochemical profile of a wide range of organisms. It includes a combination of 35 tests of carbohydrate power sources of microorganisms. The kit contains part A, part B, each of which contains 12 tests for the utilization of sugars and part C, which contains 11 media with sugars and 1 control (Table 1).

The tests are based on the principle of pH change and substrate utilization. During incubation, metabolic changes occur in organisms, which can be detected by changing the color of the environment.

Ten sets of part A reveal the utilization of such sugars - (KB009A) - Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose.

Ten sets of part B - (KB009B1) - Inulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbitol, Mannitol, Adonitol, Arabitol, Erythritol, alpha-Methyl-D-glucoside.

Ten sets of part C - (KB009C) - Rhamnose, Cellobiose, Melezitose, alpha-Methyl-D-mannoside, Xylitol, ONPG (ortho-nitrophenyl-β-galactopyranose), Esculin, D-Arabinose, Citrate, Malonate, Sorbose and 1 control.

KB009 Hicarbohydrate kit were inoculated with bacterial cultures separately and incubated at 30±2°C for 48 h. according to the instruction for preparing the bacterial suspension for the Kit. After incubation, results were observed and compared according to colour chart of the kit.

The integrated analysis of metabolic datasets covering different levels of molecular organization has become a central task of systems biology [45, 66]. Carbon source utilisation profiling was used for establishing metabolic relationship between our isolates and the standard bacteria by unweighted pair group method with arithmetic mean (UPGMA), sub programme of online software <http://genomes.urv.cat/UPGMA/index.php?entrada=Exemple2> [57, 81], and initial identification of the dominant strains. As standart strain we took *Azospirillum brasilense* [50, 72, 87], the microorganism which widely used as PGPB for wheat crops.

Table 1. Interpretation of results of the KB009 TM HiCarbo Kit test system

Test	Principle	Original colour of the medium	Positive reaction	Negative reaction
Carbohydrate utilization	Detects carbohydrate utilization	Pinkish Red/Red	Yellow	Red/Pink
ONPG	Detects β-calactosidase activity	Colourless	Yellow	Colourless
Esculin hydrolysis	Detects esculin hydrolysis	Cream	Black	Cream
Citrate utilization	Detects capability of organism to utilize citrate as a sole carbon source	Green	Blue	Green
Malonate utilization	Detects capability of organism to utilize sodium malonate as a sole carbon source	Light Green	Blue	Light Green

RESULTS

Finally, two the most interesting and promising dominant strains were selected for further morphological studies and assigned the respective codes (Table 2).

Carbohydrate test: the color of the medium changes from red to yellow due to the formation of acid, if the test is positive. If the test is negative, the color of the medium remains red (Fig. 1).

ONPG test: the medium changes from colorless to yellow if the test is positive. The medium remains colorless if the test is negative.

Hydrolysis of esculin: the color of the medium changes from cream to black if the test is positive. The middle remains a cream color if the test is negative.

Using of citrate: the color of the medium changes from yellow to green if the test is positive. The medium remains yellowish-green if the test is negative.

Use of malonate: the color of the medium changes from light green to blue if the test is positive. The average level remains light green if the test is negative.

Differentiation of samples on carbon utilization profiling was a useful preliminary test of the assay and it's shown in Table 3.

It is established that such sugars as glucose, sucrose, lactose are the most easily available. Behind them are polyalcohol (glycerin, mannitol, etc.). Polysaccharides, cellulose, hemicellulose, starch can serve as sources of carbon, and after their transformation to pass into digestible by microorganisms' mono- and low molecular weight oligosaccharides. To transform these substances, microorganisms must produce a specific set of

enzymes that perform hydrolytic functions (for example, members of the genera *Aspergillus*, *Bacillus*, *Penicillium*, *Phyllobacterium*, etc.) [53, 77].

According to the results of tests for carbohydrate absorption, we see that isolate GPA-1 utilize sugars such as: Xylose, Dextrose, Galactose, Melibiose, L-Arabinose, Mannose, Rhamnose, Esculin, D-Arabinose, Citrate, Malonate.

The isolate Chap-1 in turn feeds on such sugars: Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Inulin, Glycerol, ONPG, Esculin.

After investigation of dominant strains and standart rhizospheric bacteria strains we measured the stability and assessed the proportion of alters between bacteria metabolic processes over time [69, 83, 91]. This way we can tell how close the given bacteria are in feeding process. We carried out the calculations using the Jaccard index - is a statistic used for gauging the similarity and diversity of sample sets - by using online service (<https://planetcalc.com/1664/>). The Jaccard coefficient can be a value between 0 and 1, with 0 indicating no overlap and 1 complete overlap between the sets.

Carbon source utilisation profiling showed a significant relatedness among the isolates (Table 4). Two dominant strains GPA-1 and Chap-1 were compared with the standard *Azospirillum brasilense* strain [79].

UPGMA hierarchical clustering showed approximately 72 % similarity between Chap-1 and the standard strain, and 44% between GPA-1 and standart strain. Hence it can be inferred that metabolic similarity between the root isolates is very assertive.

Table 2. Characteristic features of the different isolates

	GPA-1	Chap-1	<i>Azospirillum brasilense</i>
Colony morphology			
Configuration	round	round	round
Margins	smooth	smooth	smooth
Elevation	convex	flat	convex
Shape	circular	circular	circular
Pigmentation	almost transparent	white	milk colour
Cell morphology			
Gram's reaction	-	+	
Shape	Long rods	Short rods	Long rods
Arrangement	Single	Paired	Single

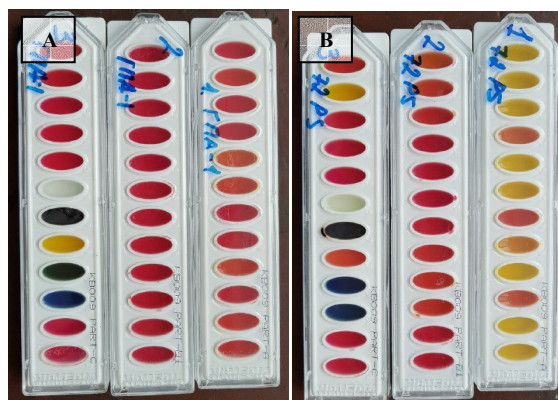


Figure 1. Visual results of the KB009 TM HiCarbo Kit, where: **A.** GPA-1; **B.** Chap-1

Table 3. Results of multi-substrate testing of dominant bacteria and standard strain

№	Test	GPA-1	Chap-1	<i>Azospirillum brasilense</i>
1	Lactose	-	-	-
2	Xylose	+	+	+
3	Maltose	-	+	+
4	Fructose	-	+	-
5	Dextrose	+	+	+
6	Galactose	+	+	-
7	Raffinose	-	+	+
8	Trehalose	-	+	-
9	Melibiose	+	-	+
10	Sucrose	-	+	-
11	L-Arabinose	+	-	-
12	Mannose	+	-	-
13	Inulin	-	+	-
14	Sodium gluconate	-	-	-
15	Glycerol	-	+	-
16	Salicin	-	-	-
17	Dulcitol	-	-	-
18	Inositol	-	-	-
19	Sorbitol	-	-	-
20	Mannitol	-	+	-
21	Adonitol	-	-	-
22	Arabitol	-	-	-
23	Erythritol	-	-	-
24	L-Methyl D-Glucoside	-	-	-
25	Rhamnose	-	-	+
26	Cellobiose	-	-	+
27	Melezitose	-	-	-
28	L-Methyl D-Mannoside	-	-	-
29	Xylitol	-	-	-
30	ONPG	-	+	-
31	Esculin	+	+	-
32	D-Arabinose	+	-	+
33	Citrate	+	+	+
34	Malonate	+	-	-
35	Sorbose	-	-	-

Table 4. Similarity matrix for the dominant bacterial isolates and their Jaccard coefficient values

Similarity matrix computed with Jaccard coefficient	<i>Azospirillum brasilense</i>	GPA-1	Chap-1
<i>Azospirillum brasilense</i>	1	0.44	0.72
GPA-1		1	0.44
Chap-1			1

Based on our investigation two suspected bacterial endophytes from soil samples were recovered, purified and assigned the respective codes. These were observed for their shape, size, pigmentation and margin along with the colony/cell morphology of individual isolates.

Based on our investigation of carbon power sources, Gram staining, microscopy and previous studies, as well as classification according to the determinant of Bergi [85, 89], we concluded that, isolates GPA-1 belong to the genus *Phyllobacterium*, and isolates Chap-1 belong to the genus *Bacillus*. Thus, with the help of our research, we combined the identification method together with the functional component and got a comprehensive approach to the work methodology. In this case, biochemical analysis helps in the identification of bacteria and allows us to assume what other functions the isolates perform. So, we learned the functional features of these bacteria, in addition to biochemical analysis.

DISCUSSION

Despite the fact that modern molecular biological research methods have become relevant now, they still will not show us a complete picture of the life cycle and especially the metabolism of important microorganisms. And given the metabolic similarity between our strains and standard strain we can concluded, the microorganisms identified by system of carbohydrate utilization – KB009 TM HiCarbo Kit are interesting for further studying and researching. The previous studies shown, that genus *Phyllobacterium* and *Bacillus* are classified as plant growth promoting bacteria (PGPB) [86, 90]. They show direct and indirect mechanisms in suppression of plant pathogens and plant growth promoting activities [95].

Looking through the last studies in our research field scientist discovered [1] that a symbiotic relationship exists between plants and beneficial soil microorganisms wherein the microbes help the plants in nitrogen acquisition, water uptake, and survival during stress [48]. According to estimates, rhizobia contribute to 50% of the biological nitrogen fixation on

earth [93]. Various functions performed by beneficial soil microorganisms include accumulation and cycling of organic compounds, stimulation of nutrient mineralization, and production of plant growth hormones. Species, such as *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and other *Bacillus sp.*, are widely used for the commercial production of PGPR. Various fermentation technologies have been used to formulate potential PGPR isolates using organic and inorganic carriers. Thus we can see importance and useful properties of species *Bacillus* under drought stress.

Another recent investigation tell us about rhizosphere samples under wheat [7], that were collected in the Global Change Experimental Facility in Central Germany, which comprises plots with conventional and organic farming systems under ambient and future climate. Phosphate-solubilizing bacteria were selectively isolated on Pikovskaya medium, phylogenetically classified by 16S rRNA sequencing, and tested for in vitro mineral phosphate solubilization and drought tolerance using plate assays. The culture isolates were dominated by members of the genera *Phyllobacterium*, *Pseudomonas* and *Streptomyces* [4]. Cultivation-derived species richness and abundance of dominant taxa, especially within the genera *Phyllobacterium* and *Pseudomonas*, as well as composition of *Pseudomonas* species were affected by wheat growth stage. Phosphate-solubilizing *Phyllobacterium* species were assigned to *Phyllobacterium ifriqiense* and *Phyllobacterium sophorae*. It is the first time that phosphate solubilization potential is described for these species. Since *Phyllobacterium* species showed the highest drought tolerance along all isolates, they may play an increasingly important role in phosphate solubilization in a future dryer climate [78].

Inoculation of *A. Brasilense* stimulated root carboxylate exudation, which was positively correlated with root length and area [18]. These positive correlations are probably mediated by the effect of carboxylates on the rhizosphere microbial community. This indicates a positive feedback in which *A. Brasilense* inoculation stimulates root carboxylate exudation, influencing the rhizosphere microbial community [33]. It results in positive effects on plant root architecture. The root length of inoculated plants was positively correlated with P supply, indicating that P supply positively affects the microbial community, modulating the interaction between *A. brasilense* and *Z. Mays* [42]. Therefore, we can conclude that the similarity of the dominant bacteria selected by us may have similar properties with *A. brasilense*.

Franco-Duarte and other signed up [22] that there were two factors determine the potential use of microorganisms in biotechnological processes, and also the pathogenicity of other strains are their genetic features and biochemical abilities. In the near future, industrial application as well as treatment of infection, will be possible after characterization, identification,

and following taxonomic classification of the biological material. It is necessary to emphasize that taxonomy and systematics, very often used interchangeably, are in fact two different terms. Although systematics deals with the diversity of organisms, relationships, and possible interactions, taxonomy is a classification of organisms in a hierarchical structure of homogeneous groups that consist of descendants of the nearest common ancestor. Despite a high degree of phenotypic similarity, every assemblage of an individual shows some degree of phenotypic diversity due to genotypic variation. Therefore, the broader the research aimed at the characterization of an individual microorganism, the more precise its identification, and thus the classification and systematics [11]. Accordingly, the “polyphasic” methodology is centered on morphological and biochemical data complemented with molecular techniques data. The combination of the classical approach together with 16S rRNA genes, molecular fingerprinting techniques, and/or other molecular markers is considered an extremely important foundation for the identification and classification of microbes.

The microbiota thriving in the rhizosphere, the thin layer of soil surrounding plant roots, plays a critical role in plants adaptation to the environment [82]. By 2050 the world's population is expected to reach 9.5 billion and, to ensure global food security, crop production has to increase by 60% in the same timeframe [9]. A promising strategy proposes to achieve this task by capitalising on the microbiota inhabiting the rhizosphere, the thin layer of soil surrounding plant roots. The rhizosphere microbiota plays a crucial role in plants adaptation to the environment by facilitating, for example, plant mineral uptake and enhancing plants tolerance to both abiotic and biotic stresses [64]. Thus, to fully unlock the potential of rhizosphere microbes for sustainable crop production, it is necessary to study the microbiota thriving at the root-soil interface in the light of the evolutionary trajectories of its host plants. And they investigated how plant genotypes adapted to different eco-geographic niches may recruit a distinct microbiota once exposed to a common environment. In summary, these data indicate that the higher taxonomic ranks of the *H. vulgare* rhizosphere microbiota are conserved across soil types as well as wild and domesticated genotypes such phyla as Acidobacteria, Actinobacteria, Bacteroidetes and Proteobacteria. So we can see that in such fundamental work there wasn't selected genus *Phyllobacterium*, and genus *Bacillus*.

In a study by Coutinho and Bophela [16] the phyllobacterial community in a tropical Brazilian rainforest was dominated by undescribed species and it was estimated that between 2 and 13 million of these species inhabit this habitat. They also showed that between 0% and 5% of the bacterial species in tropical tree canopies were common to all tree species studied. Thus, phyllobacteria on different tree species are

phylogenetically diverse. However, their metaproteomes are functionally convergent concerning traits for survival on the leaf surface, they share a common set of core functional proteins that are required for survival and fitness [94]. Apart from these mechanisms, bacterial inoculation has been shown to prevent a significant drop in water potential, in parallel with a simultaneous increase in root growth, plant biomass, and leaf area. The *Phyllobacterium brassicacearum* strain STM196 inoculated *Arabidopsis thaliana* showed changes in the transpiration rate and there was also reproductive delay which improved the plant's resistance to drought.

All plants in nature harbor a diverse community of rhizosphere bacteria which can affect the plant growth [84]. The samples were isolated from the rhizosphere of wild barley *Hordeum spontaneum* at the Evolution Canyon, Israel. The bacteria which have been living in close relationship with the plant root under the stressful conditions over millennia are likely to have developed strategies to alleviate plant stress. Rhizobacteria can affect plants in various ways [44]. Besides facilitating biotic stress alleviation also abiotic stress tolerance. It is generally accepted that bacteria through various mechanisms can acquire genetic information from the surrounding environment. Moreover, recombination frequencies and mutation rates tend to increase under stressful conditions. Rates of evolutionary change may therefore be enhanced in adverse environments. So from this work we can understand how important is a community of rhizosphere bacteria, especially dominant strains, who have worked out the stress resistance mechanism of a specific plant in specific environmental conditions for many years.

One more scientific work indicates, that stalk rot is one of the most serious and widespread diseases in maize, and effective control measures are currently lacking [14]. Therefore, this study aimed to develop a new biological agent to manage this disease. An antagonistic bacterial strain was isolated from rhizosphere soil and identified as *Bacillus methylotrophicus* based on morphological and biochemical characterization and 16S ribosomal RNA and *gyrB* gene sequence analyses [54]. TA-1 exhibited a strong antifungal effect on the growth of *Fusarium graminearum* mycelium, with 86.3% inhibition at a concentration of 108 CFU per mL. Thus we can see the importance of the genus we selected from rhizosphere of *H. vulgare*.

And the last comparative study [92] is about endophytic bacteria, which were isolated from lotus tissues and tested for antagonistic activities against the pathogenic fungus *F. oxysporum*. Among the putative endophytic *Bacillus* strains identified, suspensions of the strain B-36 showed the highest inhibition rate against *F. oxysporum* growth. Pot assays indicated that B-36 was effective in controlling *F. oxysporum*-inducing lotus rot.

In conclusion based on the investigations of recent studies, it can be argued that in modern agriculture,

focused on organic farming, one way to increase crop yields is the widespread using of fertilizers based on active strains of various microorganisms. And it is the first time that selected by us dominant strains are described for the specie of *H. vulgare* wheat. And we exactly know their metabolic pathways of carbon nutrition. The antagonistic properties of the promising strains selected by us, their phylogenetic analysis and assessment of biological efficiency deserve further research. So our investigation has extreme importance, since earlier microorganisms of these genera were not previously used in our country in the composition of biofertilizers under spring barley wheats.

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