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Effectiveness of complex inoculation of spring wheat with N_2-fixing bacteria *Azospirillum brasilense* and mold-antagonist *Chaetomium cochliodes*

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Aim. To study the specificities of complex inoculation of spring wheat roots with the bacteria of *Azospirillum* genus and *Chaetomium cochliodes* Palliser 3250, and the isolation of bacteria of *Azospirillum* genus, capable of fixing atmospheric nitrogen, from the rhizospheric soil, washed-off roots and histoshere. **Materials and methods.** The phenotypic features of the selected bacteria were identified according to Bergi key. The molecular the polymerase chain reaction and genetic analysis was used for the identification the bacteria. **Results.** It has been demonstrated that during the introduction into the root system of spring wheat the strain of *A. brasilense* 102 actively colonizes rhizospheric soil, root surface and is capable of penetrating into the inner plant tissues. **Conclusions.** The soil ascomucete of *C. cochliodes* 3250 promotes better settling down of *Azospirillum* cells in spring wheat root zone, especially in plant histosphere which induces the increase in the content of chlorophyll *a* and *b* in the leaves and yield of the crop.

Key words: *Azospirillum brasilense*, nitrogen-fixing bacteria, spring wheat, endophytes, chlorophyll, *Chaetomium cochliodes* 3250.

INTRODUCTION

The bacteria of *Azospirillum* genus are some of the most investigated objects among the plant rhizosphere. The manner of their influence on plants is multifaceted, including high N_2-fixing activity, which results in providing nitrogen for the nutrition of plants, the capability to produce biologically active substances which activate chloroplast genesis, stimulate the growth and development of plants, and improve their resistance to unfavorable conditions of environment and malignant agents.

The aim of this work was to study the specificities of *Chaetomium cochliodes* Palliser 3250 promoting the penetration of *Azospirillum* bacteria into spring wheat roots.

MATERIALS AND METHODS

The calculation of *Azospirillum* bacteria was made on Caceras solid agar medium with red congo where they form dark red, little, dry, wrinkled colonies, 0.5−1.5 mm in diameter [1].

The preliminary identification of the selected bacteria was carried out by morphological, cultural, physiological, and biochemical features according to Bergey's [2]. The description of the bacteria of *Azospirillum* genus from the original publications was also used [3].

We used universal oligonucleotide primers which were conformed to conservative positions at 3'- and 5'-ends of 16S rRNA gene: 16S Forward (CGG-CCC-AGA-CTC-CTA-CGG-GAG-GCA-GCA) and 16S Reverse (GCG-TGG-ACT-ACC-AGG-GTA-TCT-AAT-CC). The reaction of amplification was carried out on Applied Biosystems appliance. PCR-products were separated in 1.5 % agarose gel by horizontal electrophoresis. The detection of obtained PCR-products was made on Applied Biosystems ABI Prism 3130 automatic capillary analyzer.

The ability of *C. cochliodes* 3250 to colonize spring wheat root system was investigated in the laboratory experiments with sterile conditions [4]. The staining of mold mycelium in root tissues was made by Kobel method [5].

The content of chlorophylls *a* and *b* in leaves was investigated by the spectrophotometer method [6].

The field experiments with spring wheat (Rassvet breed) were carried on leached chernozem soil characterized by the following agrochemical specificities: humus content – 3.6 %; mobile phosphorus forms (by Kirsanov) – 210–240 mg P_2O_5; metathetical potassium (by Kirsanov) – 160–170 mg K_2O in 1 kg of soil; pH H_20 – 6.5. The area of the plot was 25 sq.m, with four multiple replications.

RESULTS AND DISCUSSION

The rhizospheric soil, washed-off roots and histosphere of spring wheat were used to obtain clean bacteria cultures of *Azospirillum* genus. Eleven most active strains, capable of fixing atmospheric nitrogen, were selected, described by their phenotypic features and investigated with molecular and genetic analysis methods. Currently the GenBank database contains the information about 11 strains of *Azospirillum* genus bacteria. As known from the literature, all the *Azospirillum* strains selected from Ukrainian soils belong to *A. brasilense* and *A. lipoferum*. Therefore, we carried out the phylogenetic analysis of the obtained strains as well as typical strains of *A. brasilense* Sp7 and *A. lipoferum* 59b, obtained from the Bacterial Collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms RAS.

Based on the cultural, morphological, physiological and biochemical features the selected strains were considered to belong to *Azospirillum* genus, *A. brasilense* strain. The PCR-analysis was used to obtain and sequence the amplificants of 16S rRNA gene.

The comparative analysis of the sequencing results revealed 100 % identity of 16S rRNA of the investigated bacteria with the similar sequences of *A. brasilense* from the GenBank database. Thus the identification of the selected strains using the sequence analysis of 16S rRNA gene is in good agreement with the results obtained during the investigation of phenotypic features.

The selected *Azospirillum* strains were tested in a series of vegetative experiments with spring wheat, where their ability to form associations with plants increasing N_2-fixing activity in the root zone, activating the synthesis of photosynthetic pigments, promoting the growth and development of plants have been studied. As a result we found out the most effective strain to be *A. brasilense* 102.

One of the important conditions of the effective interaction of the introduced associative bacteria strains with plants is the capability of microorganisms to colonize the plant roots actively. To investigate the ability of *A. brasilense* 102 to settle down in spring wheat root zone we obtained a streptomycin stable mutant which did not differ from the primer strain by its cultural, morphological and physiological properties.

We investigated the dynamics of the quantity of mutant cells introduced into the root zone of spring wheat in rhizospheric soil, on the washed-off roots and histosphere of plants in the vegetative experiment. The obtained results testify that *Azospirillum* cells are able to settle down not only in rhizosphere soil and root surface but they also penetrate into the inner tissues of spring wheat plants.

The endophytic property is very useful for micro- and macrosymbionts. The plants, infected with endophytes, get their growth processes activated, their immune status improved and the resistance formed to the stress factors of their environment. The localization of endophytes inside the plant tissues gives the latter some advantages in comparison with rhizosphere microorganisms in terms of the access to nutritious substances and the absence of competition with aboriginal microflora. Also the endophytes, penetrating into the inner tissues, find themselves in more comfortable conditions because they are protected from the negative influence of environment. N_2-fixing endophytes, particularly *Azospirillum*, are of special interest due to their ecological role and possible practical application. The localization of *Azospirillum* bacteria in plant tissues is favorable for N_2-fixing because the microzones of their localization have low partial oxygen pressure which is necessary for active functioning of bacterial nitrogenase and contains available material for this process.

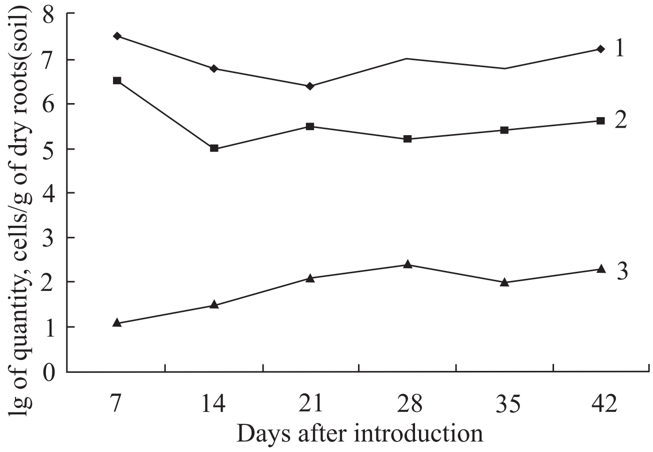
In addition, there is close interaction between micro- and macropartners which contributes to the exchange of signal molecules and metabolites. Particularly, N_2 and phytohormones come into plants without any loss.

However, the issues of bacteria penetration into plant tissues are yet to be investigated in fine detail. Some authors believe that *Azospirillum* bacteria do not have enough pectolytic and proteolytic activity for the abovementioned [7]. At the same time it was demonstrated in thoroughly checked experimental conditions that under the influence of a host-plant the pectolytic activity increased 7–8-fold and the proteolytic activity increased 10–11-fold. According to the available information some mycorrhizal fungi can favor *Azospirillum* bacteria to penetrate into plants [8].

At the Institute of Agricultural Microbiology NAASU the strain of soil ascomycete *Chaetomium cochliodes* Palliser 3250, which manifests high antagonistic activity against the rot agents of culture plant roots, was singled out. We revealed that the soil fungus of *Chaetomium cochliodes* Palliser 3250 actively colonizes the root system of spring wheat forming carposomes on the root surface, penetrating into the root hairs and rhizoderma cells. At the same time the level of micro- and macroelements absorption gets higher, the content of photosynthetic pigments increases, providing for the growth and development of plants. Thus, *C. cochliodes* 3250 is capable of forming endophytic association with spring wheat roots.

To investigate the ability of *C. cochliodes* 3250 to penetrate further into the histosphere of spring wheat of *Azospirillum* bacteria in the vegetative experiment, we examined the quantity dynamics of mutants introduced into the spring wheat root system together with *C. cochliodes* 3250 (Figure).

The obtained results testify to the observed insignificant diminishing and further stabilization of the introduced mutant quantity at the level of (1.6–4.0) · 10^5 bacterial cells in 1 g of rhizospheric soil. *C. cochliodes* 3250 facilitated the increase in the quantity of *Azospirillum* cells up to (3.6–6.4) · 10^6. This fact may be explained by high antagonistic activity of *C. cochliodes* 3250 regarding the phytopathogenic fungi which can provoke root rot, and as we noted earlier, during the introduction in chernozem soil there is considerable limitation of phytopathogenic fungi from *Fusarium* genus, by which *C. cochliodes* 3250 promotes the settling down of *Azospirillum* bacteria in rhizospheric soil. It is necessary to take into account the fact that the most favorable conditions for the development of *Azospirillum* cells are the microaerobic conditions which can be provided by *C. cochliodes* 3250 during the decrease of O_2 in the N_2-fixing zone.



The colonization of spring wheat root sphere with *Azospirillum sp.*^{str}: 1 – root surface; 2 – rhizosphere; 3 – histosphere

The results demonstrated that *Azospirillum* bacteria settled down more actively on the surface of spring wheat roots and their quantity was one order higher than in rhizospheric soil. The influence of *C. cochliodes* 3250 in this case was less considerable.

The quantity of *Azospirillum* bacteria in the inner tissues of spring wheat was much less and amounted to (10–2.5) · 10^2 bacterial cells in 1 g of roots. Under the influence of *C. cochliodes* 3250 the ability of *Azospiril-*

Table 1. The colonization of different spring wheat root spheres with *A. brasilense* 102^{str} (vegetative experiment)

The variants of experiment	The number of bacterial cells (piece/g) in absolutely dry roots (soil)		
	Days after introduction		
	7	14	21
Rhizospheric soil			
Inoculation with *A. brasilense* 102^{str}	3.1 · 106	1.0 · 105	3.2 · 105
Inoculation with *A. brasilense* 102 + *C. cochliodes* 3250	5.6 · 106	1.3 · 106	5.6 · 106
Washed off roots			
Inoculation with *A. brasilense* 102^{str}	3.1 · 107	6.3 · 106	2.5 · 106
Inoculation with *A. brasilense* 102 + *C. cochliodes* 3250	2.0 · 107	7.1 · 106	4.0 · 106
Histosphere			
Inoculation with *A. brasilense* 102^{str}	0.12 · 102	0.32 · 102	1.26 · 102
Inoculation with *A. brasilense* 102 + *C. cochliodes* 3250	4.5 · 105	4.0 · 105	2.5 · 106

Table 2. The influence of spring wheat inoculation with microorganisms on the content of chlorophyll *a* and *b* in the leaves of plants (vegetative experiment)

The variants of experiment	Chlorophyll concentration, mg/100 g of leaves		
	a	*b*	*A* + *b*
Without inoculation (control)	42.56	13.86	56.42
Inoculation with *A. brasilense* 102^{str}	52.87	17.95	70.85
Inoculation of seeds with *A. brasilense* 102^{str} + + *C. cochliodes* 3250	68.85	23.84	92.69
Less essential difference $_{05}$	0.80	1.23	

Table 3. The influence of *A. brasilense* 102 and *C. cochliodes* 3250 on the yield of spring wheat (the average for three years of experiments)

The variants of experiment	The yield, t/ha	The yield increase	
		t/ha	%
Without inoculation (control)	3.71	–	–
Inoculation with *A. brasilense* 102	4.35	0.64	17.2
Inoculation with *C. cochliodes* 3250	4.54	0.83	22.4
Inoculation of seeds with *A. brasilense* 102 + + *C. cochliodes* 3250	4.65	0.94	25.3
Less essential difference $_{05}$	0.17		

lum bacteria to penetrate into the roots increased (by 3–4 order) and in the histosphere of spring wheat there were $4.0 \cdot 10^5$–$2.5 \cdot 10^6$ bacterial cells in 1 g of roots (Table 1).

Thus, it is possible to make a conclusion that forming the endophyte association with spring wheat roots, *C. cochliodes* 3250 promotes the settling down of *Azospirillum* bacteria in rhizospheric soil and histosphere of spring wheat.

The complex inoculation of spring wheat with *A. brasilense* 102^{str} and *C. cochliodes* 3250 affected the chloroplast genesis of the culture (Table 2). During this period the plants were characterized by high content of chlorophylls *a* and *b* in the leaves.

Thus, during the introduction into the root system of spring wheat the strain of *A. brasilense* 102 actively colonizes rhizospheric soil, root surface and is capable of penetrating into the inner plant tissues. The soil ascomucete of *C. cochliodes* 3250 promotes better settling down of *Azospirillum* cells in spring wheat root zone especially in plant histosphere which induces the increase in the content of chlorophyll *a* and *b* in the leaves of the culture.

The usage of *A. brasilense* 102 and *C. cochliodes* 3250 had high positive effect on the yield of spring wheat plants (Table 3).

Thus, the soil saprophyte fungus *C. cochliodes* 3250 promotes better settling down of *Azospirillum* cells in spring wheat root zone especially in plant histosphere which induces the increase in the content of chlorophyll *a* and *b* in the leaves and yield of the culture.

Ефективність спільної інокуляції пшениці ярої азотфіксувальними бактеріями *Azospirillum brasilense* та грибом-антагоністом *Chaetomium cochliodes*

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Мета. Вивчення особливостей спільної інокуляції коренів пшениці ярої бактеріями *Azospirillum* та грибом *Chaetomium cochliodes* Palliser 3250, а також виділення з ризосферного ґрунту, відмитих коренів та гістосфери пшениці ярої активних штамів бактерій роду *Azospirillum*, здатних фіксувати атмосферний азот. **Методи.** Фенотипові ознаки ідентифікували відповідно до визначника бактерій Берджі. Проведення молекулярно-генетичного аналізу з використанням полімеразно-ланцюгової реакції дозволило віднести їх до *Azospirillum brasilense*. **Результати.** Показано, що штам *A. brasilense*

102 при інтродукції в кореневу систему пшениці ярої активно колонізує ризосферний ґрунт, поверхню коренів і здатний проникати у внутрішні тканини рослин. Висновки. Ґрунтовий сумчатий гриб *C. cochliodes* 3250 сприяє кращій приживаності азоспірил в кореневій зоні пшениці ярої, особливо в гістосфері рослин, що позначається на збільшенні вмісту хлорофілів *a* і *b* в листках і врожайності культури.

Ключові слова: *Azospirillum brasilense*, діазотрофи, пшениця яра, ендофіти, хлорофіл, *Chaetomium cochliodes* 3250.

Эффективность совместной инокуляции яровой пшеницы азотфиксирующими бактериями *Azospirillum brasilense* и грибом-антагонистом *Chaetomium cochliodes*

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Цель. Изучение особенностей совместной инокуляции корней яровой пшеницы бактериями *Azospirillum* и грибом *Chaetomium cochliodes* Palliser 3250, а также выделение из ризосферного грунта, отмытых корней и гистосферы яровой пшеницы активных штаммов бактерий рода *Azospirillum*, способных фиксировать атмосферный азот. **Методы.** Фенотипические признаки идентифицировали соответственно определителю бактерий Берджи. Проведение молекулярно-генетического анализа с использованием полимеразно-цепной реакции. позволило отнести их к *Azospirillum brasilense*. **Результаты.** Показано, что штамм *A. brasilens*e 102 при интродукции в корневую систему яровой пшеницы активно колонизирует ризосферную почву, поверхность корней и способен проникать во внутренние ткани растений. **Выводы.** Почвенный сумчатый гриб *C. cochliodes* 3250 способствует лучшей приживаемости азоспирилл в корневой зоне яровой пшеницы, особенно в гистосфере растений, что отражается на увеличении содержания хлорофиллов *a* и *b* в листьях и урожайности культуры.

Ключевые слова: *Azospirillum brasilense*, диазотрофы, яровая пшеница, эндофиты, хлорофилл, *Chaetomium cochliodes* 3250.

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