Journal of Plant Protection Research eISSN 1899-007X

ORIGINAL ARTICLE

Rhizosphere prokaryotic microbiome, resistance to mycoses and productivity of *Glycine max* (l.) Merr. under bacterial inoculation and fungicide application

Galyna Iutynska, Liudmyla Tytova, Svitlana Vozniuk, Nadiia Shevchuk*, Liudmyla Bilyavska

Department of General and Soil Microbiology, Zabolotny Institute of Microbiology and Virology of NASU, Kyiv, Ukraine

DOI: 10.24425/jppr.2025.156887

Received: November 11, 2024
Accepted: February 25, 2025
Online publication: December 02, 2025

*Corresponding address:
nadia.shevchuk.48@ukr.net

Responsible Editor:
Iwona Adamska

Abstract

The use of fungicides to limit the development of soybean mycoses in modern agrocenoses is a common measure. At the same time, the use of symbiotic and phosphate-mobilizing bacteria increases the productivity and quality of the crop. The combination of biological agents and fungicides can have different effects and requires additional research. This study aimed to investigate the rhizosphere microbiome, mycosis resistance and soybean productivity under combined seed treatment with bacterial inoculants and fungicides. Pre-sowing treatment of soybean seeds was carried out with fungicides Maxim Star 025 FS or Kinto Duo, 12 hours after the seeds were inoculated with the complex bioformulation Ecovital based on *Bradyrhizobium japonicum* and *Bacillus megaterium*. The effect of the integrated seed treatment on the rhizosphere microbiome (by high-throughput sequencing), the level of mycosis development and soybean productivity was determined. Under the complex use of biological and chemical preparations, the biodiversity of the rhizosphere microbiome improved, the relative abundance of fungicide-resistant phyla increased, and the number of sensitive taxa decreased. The low abundance of native rhizobia populations indicated the need for pre-sowing inoculation of soybean seeds. The combined use of fungicides and inoculation of seeds with Ecovital contributed to the improvement of the effectiveness of plant protection against ascochitosis and septoria, and to an increase in soybean yield.

Keywords: biodiversity, bioformulation, pesticides, soybean fungal diseases, yield

Introduction

Soybean is the main crop for vegetable protein and oil production in the world. Due to its ability to assimilate atmospheric nitrogen in symbiosis with nodule bacteria, soybean is one of the best predecessors for cereals, vegetables, and other crops (Mashchenko *et al.* 2024).

Among disease agents, phytopathogenic fungi are the most dangerous and widespread plant pathogens. Common fungal diseases of soybean are phytophthora root and stem rot, sclerotinia stem rot (white mold), frogeye leaf spot, anthracnose stem blight, pod and stem blight (Broderick 2020). This forces producers to use chemical fungicides as plant protection products.

One of the effective measures to improve soybean cultivation technology is the use of symbiotic nitrogen-fixing microorganisms that play an important role in the development of legumes, providing them with nitrogen nutrition, protection against pathogens and adaptation to various stresses (Pedrozo *et al.* 2018).

The use of inoculants in combination with chemical plant protection products is an important but poorly understood issue. Pesticides can negatively affect beneficial soil bacteria and can disrupt ecological balance as well as change the composition of the soil microbiome. Biodiversity of the rhizosphere microbiome, formation, functioning and efficiency of soybean-rhizobial symbiosis under conditions of combined fungicide seed treatment and microbial inoculation remain poorly understood.

The aim of this study was to investigate the rhizosphere microbiome, mycosis resistance and soybean productivity with seed treatment of combined fungicides and bacterial inoculants.

Materials and Methods

Vegetative experiments were conducted in a vegetation house on dark gray podzolic soil in ceramic pots with a capacity of 3 kg with four replications. Field studies were conducted in the Kyiv region (Chabany village) on the same soil, replicated four times. In the experiments, soybean of the ultra-early maturing variety Annushka was grown.

Seed treatment

Before sowing, the seeds were treated with one of the systemic and contact fungicides: Maxim Star 025 FS (fludioxonil 18.75 g · l^{-1} + ciproconazole 6.25 g · l^{-1}, manufactured by Syngenta, Switzerland) and Kinto Duo (prochloraz 60 g · l^{-1} + triticonazole 20 g · l^{-1}, manufactured by BASF, Switzerland). The doses of fungicides for seed treatment were used in accordance with the manufacturer's recommendations: Maxim Star 025 FS and Kinto Duo – 1 liter per 1 ton of seeds. Seeds were treated with fungicides by spraying with subsequent mixing.

Twelve hours after the fungicide treatment, the seeds were inoculated with the complex bioformulation Ecovital, that contains three strains of highly effective nodule bacteria *Bradyrhizobium japonicum* UCM B-6018, B-6023 and B-6035, which differ in the spectra of synthesized phytohormones and complement each other. This bioformulation also contains the phosphate-mobilizing *Bacillus megaterium* UCM B-5724, which improves phosphorous nutrition of plants and increases plant resistance to diseases (Iutynska *et al.* 2022; Borzykh *et al.* 2022). Ecovital treatment was carried out by spraying the seeds at the consumption rate of 1.0 liter per 1 ton of seeds.

Experimental design

The vegetative experiment was conducted according to the following seed treatment scheme: 1) control without inoculation and fungicide treatment; 2) seed inoculation with Ecovital; 3) treatment with Maxim Star 025 FS; 4) treatment with Maxim Star 025 FS + Ecovital; 5) treatment with Kinto duo; and 6) treatment with Kinto duo + Ecovital. Pot lighting and thermal conditions were natural. Throughout the experiment, soil moisture was maintained at 60% of the full moisture capacity. The field experiment was conducted according to the following seed treatment scheme: 1) control without inoculation and fungicide treatment; 2) inoculation with Ecovital; 3) treatment with Maxim Star 025 FS + Ecovital; and 4) treatment with Kinto duo + Ecovital. Samples of rhizosphere soil for analysis were taken during the period of budding – beginning of flowering.

Pyrosequencing

To analyze the prokaryotic metagenome of soybean rhizosphere soil, total microbial DNA was isolated using a commercial Power Soil DNA Isolation Kit (MoBio, USA). Sample preparation, creation of 16S rRNA amplicon libraries, and high-throughput sequencing were performed on a GS Junior device (Roche, USA) in accordance with the manufacturer's guidelines (*em*PCR Amplification Method Manual 2012; Sequencing Method Manual for GS Junior Titanium Series 2012).

Taxonomic analysis of nucleotide sequences of amplicons was performed using the computer program module QIIME (version 1.7.0) (Kuczynski *et al.* 2012). Taxonomic identification of operational taxonomic units (OTUs) was performed using the RDP database (http://rdp.cme.msu.edu/). Taxa with a share of 10% or more in the microbiome were considered dominant, and taxa with a representation of 1% to 10% were considered subdominant. The biodiversity of the microbiome was assessed using the main ecological indices: Shannon's (the proportion of a particular species in a community), Menhinik's (species richness index), Simpson's and Berger-Parker's (dominance indexes).

Definition of plant damage by mycoses

Soybean plants affected by phytopathogenic fungi were observed during the periods of budding – beginning of flowering and bean filling. The incidence of soybean plants with ascochitosis and septoria was determined and recorded on a 6-point scale (Trybel 2001). According to the obtained indicators of disease development, the technical efficiency of the protective effect of the studied Ecovital and fungicides was calculated using the formula:

$$E\ (\%) = (D_c - D_e)/\ D_c \cdot 100,$$

where: E (%) represents the technical efficiency of the protective effect, D_c and D_e represent the indicators of disease development in the control (D_c) and experiment (D_e), respectively.

Definition of plant productivity

The productivity of soybean plants in the field experiment was determined by the mass of the seeds obtained (weighing method) with conversion to $c \cdot ha^{-1}$.

Statistical data analysis

All vegetative and field experiments with four repetitions were analyzed by using the MSTATC software. For statistical analysis, the analysis of variance (ANOVA) method was used.

Results

In the microbiome of dark gray podzolic soil of the soybean rhizosphere, 23 phyla (including two belonging to *Archaea*), 65 classes, 111 orders, and 271 bacterial species were identified; the proportion of unclassified sequences was 6-26%. In the total number of identified sequences, the absolute dominants were representatives of the *Bacteria* domain (87.5–88.4%), *Archaea* made up 0.5–4.6%, and unclassified sequences made up 7.1–7.9%.

In both the vegetative experiment, and under field conditions, among the identified bacterial phyla, three were absolute dominants with representation: *Proteobacteria* –23–38%, *Actinobacteria* – 18–27% and *Acidobacteria* – 9–13%. Among the identified phyla, three were dominant in both the vegetation and field experiments – Acidobacteria (9.5–13.3%), Actinobacteria (18.2–27.4%) and Proteobacteria (23.3–37.6%) (Table 1). The highest representation of the Proteobacteria phylum was achieved in the field experiment with Ecovital application. This parameter increased by 11.4%, compared to the control. In the vegetation experiment, the representation of Actinobacteria phylum in the variant with Ecovital treatment, was 1.4% higher than the control. The representation of Acidobacteria phylum increased in all experiments with preparations treatment, most of all in the field experiment with Ecovital + Kinto duo application, which was 2.3% higher than the control value.

In the field experiment, an increase the representation of the Proteobacteria phylum occurred in the rhizosphere soil of plants from 26,2% in controle to 35.8–37.6% after seeds treatment with Ecovital both alone and in combination with fungicides.

Comparing soybean seeds treated with Ecovital and fungicides to the untreated control, there were quantitative changes in the rhizosphere microbiome: the representation of subdominant phyla *Firmicutes*, *Gemmatimonadetes* and *Bacteroidetes* increased, and the phylum *Crenarchaeota* decreased, which was probably due to the different sensitivity of these phyla to fungicides.

Attention was paid to the orders *Rhizobiales* and *Bacillales*, since the complex microbial inoculant Ecovital includes representatives of these orders – nodule bacteria *Bradyrhizobium japonicum* and *Bacillus megaterium*. Compared to the control variant, the representation of *Rhizobiales* and *Bacillales* increased in the rhizosphere microbiome with the combined seed treatment.

The genus *Bacillus* was the most represented among the sequences of the order *Bacillales*. When seeds were inoculated with Ecovital, which contains phosphate-mobilizing bacilli, the representation of the genus *Bacillus* in the rhizosphere microbiome increased to 3% compared to 2.5% in the untreated control. This may have been due to the active development of bacteria introduced into the root zone, which is important for the formation of the microbial-plant system (Fig. 1). In the order Rhizobiales, a greater diversity (9–10 species) was found in the rhizosphere soil of plants where complex seed treatment was applied, while in the absence of treatment only 7 species were found (Fig. 2). The most represented was the genus Methylobacterium – 0.5–0.9% in the seed treatment and 0.4% in the control variant. The relative number of representatives of the genera Devosia and Hyphomicrobium for the application of complex treatment was 0.2–0.4%, while in the control it was 0.1%.

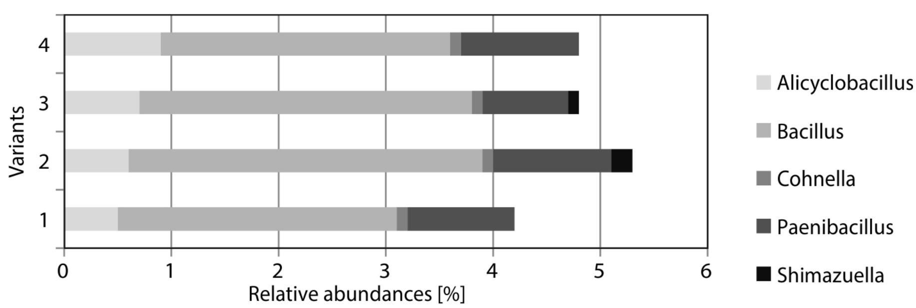

Fig. 1. Relative abundance of *Bacillus* genera in soybean rhizosphere under different seed treatment options: 1 – control; 2 – Ecovital; 3 – Kinto duo + Ecovital; 4 – Maxim Star + Ecovital

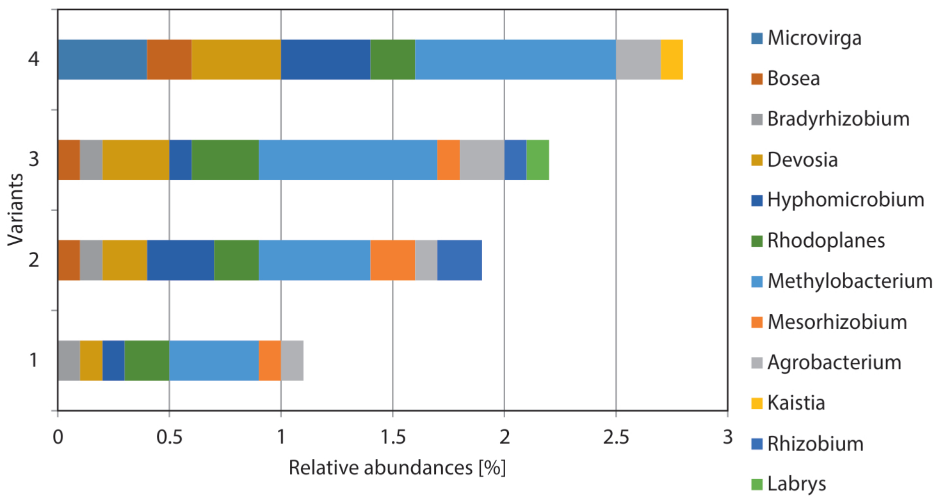

Fig. 2. Relative amounts of *Rhizobiales* genera in soybean rhizosphere with different seed treatment options: 1 – control; 2 – Ecovital; 3 – Kinto duo + Ecovital; 4 – Maxim Star + Ecovital

Table 1. Representation of dominant phyla in the rhizosphere of soybean variety Annushka

Phyla	Representation in the microbiome [%]			
	Control	Ecovital	Kinto duo + Ecovital	Maxim Star + Ecovital
	Vegetative experiment			
Actidobacteria	9.5	9.6	9.9	10.7
Actinobacteria	21.0	22.4	18.2	18.3
Proteobacteria	24.3	23.4	23.3	23.9
	Field experiment			
Acidobacteria	11.0	12.8	13.3	10.4
Actinobacteria	27.4	25.8	21.4	27.2
Proteobacteria	26.2	37.6	36.8	35.8

Note: sensitivity threshold of the device (0.05%), $p < 0.01$

The representation of sequences belonging to the genera *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* was very low – only 0.1% (Fig. 2). The results indicate the need for inoculation of legume seeds with selected, highly active strains of symbiotic bacteria.

In the order *Rhizobiales*, representatives of the genera *Microvirga* and *Bosea* were found, which had not been previously described in the soybean rhizosphere. In the variant with seeds treated with the fungicide Maxim Star and Ecovital, representatives of the genus *Kaistia* were found. They are known to be able to form biofilms (Usui *et al.* 2020), which is important for the existence of a microbial community on plant roots.

The main ecological indices (Table 2) showed an increase in biodiversity in the variants with the use of Ecovital and fungicides. Shannon's and

Table 2. Ecological indices of prokaryotic biodiversity of the soil rhizosphere microbiome

Indexes	Control	Ecovital	Kinto duo + Ecovital	Maxim Star + Ecovital
Menhinick's index	136	140	155	153
Shannon's index	3.4	3.8	3.9	4.0
Simpson's index	0.1	0.05	0.05	0.04
Berger-Parker's index	0.26	0.12	0.16	0.15

Note: LSD – least significant difference

Menhinick's species richness indices increased from 3.4 and 136 in the control variant to 3.8-4.0 and 140-155 in the experimental variants, respectively, while Simpson's and Berger-Parker's dominance indices decreased. This indicated favorable conditions for the formation of a wide genetic polymorphism of prokaryotes in dark gray podzolic soil of soybean rhizosphere under complex seed treatment.

Development of ascochitosis and septoria on soybean plants

Reduced development of soybean diseases, ascochitosis and septoria, in all variants with combined seed treatment, was established (Fig. 3). The lowest levels of septoria (5.1-5.2%) and ascochitosis (0.3-0.5%) development from the end of flowering to the beginning of bean formation were seen with the application of Maxim Star or Kinto duo in combination with Ecovital. The effectiveness of combined treatments against septoria ranged from 63.4 to 64.1%, and against ascochitosis – from 91.8 to 95.1%.

The integrated seed treatment significantly suppressed the development of mycoses during the period of bean formation. Under the action of fungicides Maxim Star or Kinto Duo followed by inoculation with Ecovital, the percentage of affected plants was 21.3%, while in the untreated control it was 60.1%.

Productivity of soybean plants

Soybean productivity increased in all treatment variants during the 2 years of research (Table 3). When seeds were inoculated with Ecovital, an increase in

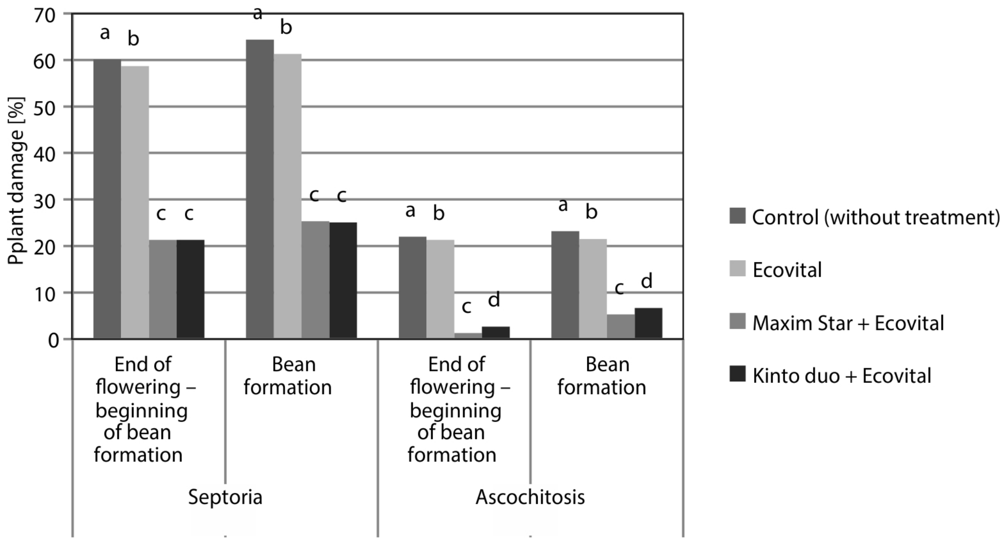

Fig. 3. Efficiency of combined application of fungicides and Ecovital in limiting the development of soybean mycoses of the Annushka variety
Note: Diagrams with the same letters have no significant difference at the 0.05 level of probability (*LSD* test), *LSD* – least significant difference

Table 3. Productivity of soybean variety Annushka under the combined use of fungicides and Ecovital

Variant	Yield					
	1st year		2nd year		average value	
	$c \cdot ha^{-1}$	yield increase, $c \cdot ha^{-1}$	$c \cdot ha^{-1}$	yield increase, $c \cdot ha^{-1}$	$c \cdot ha^{-1}$	yield increase, $c \cdot ha^{-1}$
Control (without treatment)	18.2 a	–	17.6 c	–	17.9 e	–
Ecovital	18.4 b	0.2	19.4 d	1.8	18.9 f	1.0
Maxim Star + Ecovital	21.6 b	3.4	24.6 d	7.0	23.1 f	5.2
Kinto duo + Ecovital	24.3 b	6.1	29.3 d	11.7	26.8 f	8.9
$LSD_{0.05}$	0.07	–	0.06	–	0.07	–

Note: LSD – least significant difference.
a–f – values for the same parameter, different years and different seed treatment variants marked with different letters have statistically significant differences (p 0.05), ANOVA post hoc Tuckey's test

yield was observed by an average of 5.6% compared to the control variant. The yield increase under complex treatment with fungicides and biological product on average ranged from 29.1 to 49.7%. The highest productivity was in the variant with the use of the fungicide Kinto duo with subsequent inoculation and amounted to 24.3 and 29.3 c · ha^{-1} in the years of research.

Thus, the combined use of chemical preparations and complex bacterial inoculation of seeds effectively protected soybean crops from ascochitosis and septoria pathogens. It also contributed to increasing the diversity of the rhizosphere microbiome and increasing plant yields.

Discussion

It is important to study the biodiversity of microbial communities and optimize their functioning in modern agrocenoses, especially in the management of crop diseases. According to the literature, about 100 phyla of prokaryotes have been identified in the soil and rhizosphere, about 10 of which are dominant (Agyekum *et al.* 2023). Among the representatives of 21 bacterial phyla, nine were dominant: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, and *Proteobacteria*. The presence of archaea in the soybean rhizosphere is rarely reported in the known literature, but in these studies there were two phyla of archaea.

Among the genera of the order *Rhizobiales*, representatives of the genera *Microvirga* and *Bosea* were found which belong to a group of new rhizobial symbiotrophs capable of nodule formation and nitrogen fixation (Pulido-Suárez *et al.* 2022; Shi *et al.* 2024).

The issue of an integrated approach to combining plant growth promoting bacteria with fungicides is currently relevant and is considered to be a way to increase the effectiveness of mycosis control and increase the productivity of agricultural plants (Ons *et al.* 2020; Mielniczuk and Skwaryło-Bednarz 2020). The positive effect of using nodule bacteria and PGPB together with fungicides has been described in vegetative and field experiments with chickpea (Dubey *et al.* 2015).

The results confirmed that the combined use of Kinto Duo and Maxim Star fungicides and Ecovital seed inoculation increased the effectiveness of plant protection against mycoses, resulting in better growth and higher yields. Also, better developed plants produced more root exudates, which activated the indigenous soil-born microbiota and contributed to the diversity of the rhizosphere microbiome.

Conclusions

The combined treatment of seeds with microbial inoculant and fungicides in the prokaryotic microbiome of soybean rhizosphere increased the representation of chemical resistant phyla *Bacteroidetes*, *Firmicutes*, and *Gemmatimonadetes*, while sensitive phyla *Armatimonadetes* and *Crenarchaeota* decreased compared to the untreated control. Using complex biological and chemical preparations, the biodiversity of the rhizosphere microbiome increased, which was confirmed by the higher values of Shannon's and Menhinik's indices and a decrease in the Simpson's and Berger-Parker's indices.

Combined use of fungicides Kinto Duo or Maxim Star and inoculation of seeds with Ecovital increased the effectiveness of plant protection against ascochitosis and septoria and increased soybean yield.

References

Agyekum D.V.A., Kobayashi T., Dastogeer K.M.G., Yasuda M., Sarkodee-Addo E., Ratu S.T.N., Xu Q., Miki T., Matsuura E., Okazaki S. 2023. Diversity and function of soybean rhizosphere microbiome under nature farming. Frontiers in Microbiology: 14. DOI: https://doi.org/10.3389/fmicb.2023.1130969

Broderick K. Soybean Disease Update 2020. Crop production clinic proceedings march 19, university of nebraska–lincoln Institute of Agriculture and Natural Resources Cropwatch. DOI: https://cropwatch.unl.edu/2020/soybean-disease-update

Borzykh O.I., Sergiienko V.G., Tytova L.V., Biliavska L.O., Boroday V.V., Tkalenko G.M., Balan G.O. 2022. Potential of some bioagents in fungal diseases controlling and productivity enhancement of tomatoes. Archives of Phytopathology and Plant Protection 55 (15): 1750–1765. DOI: https://doi.org/10.1080/03235408.2022.2116685

Dubey S.C., Singh V., Priyanka K., Upadhyay B.K., Singh B. 2015. Combined application of fungal and bacterial bioagents, together with fungicide and *Mesorhizobium* for integrated management of Fusarium wilt of chickpea. BioControl 60: 413–424. DOI: https://doi.org/10.1007/s10526-015-9653-8

emPCR Amplification Method Manual 2012. Lib-L for GS Junior Titanium Series. Method Manual. 454 Life Sciences Corp. A Roche Company Branford, p. 12.

Iutynska G.O., Goloborod`ko S.P., Tytova L.V. Dubynska O., D. 2022. Effectiveness of endophytic-rhizobial seed inoculation of *Glycine max* (L.) Merr. cultivated in irrigated soil. Journal of Central European Agriculture 23 (1): 40–53. DOI: https://doi.org/10.5513/JCEA01/23.1.3397

Kuczynski J., Stombaugh J., Walters W.A., Gonzalez A., Caporaso J.G., Knight R. 2012. Using QIIME to analyze 16S rRNA gene sequences from Microbial Communities. Current Protocols. p. 28. In: "Bioinformatics". DOI: https://doi.org/10.1002/0471250953.bi1007s36

Mashchenko Yu.V., Sokolovska I.M., Kovalenko V.O. 2024. biotechnological practices for growing corn for grain under different predecessors in the conditions of the Ukrainian Steppe. Podilian Bulletin: Agriculture, Engineering, Economics 2 (43): 9–15. DOI: https://doi.org/10.37406/2706-9052-2024-2.1

Mielniczuk E, Skwaryło-Bednarz. 2020. B. Fusarium head blight, mycotoxins and strategies for their reduction. Agronomy 10 (4): 509. DOI: https://doi.org/10.3390/agronomy10040509

Ons L., Bylemans D., Thevissen K., Cammue B.P.A. 2020. Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. Microorganisms 8 (12): 1930. DOI: https://doi.org/10.3390/microorganisms 8121930

Pedrozo A., Oliveira N.J.G. Alberton O. 2018. Biological nitrogen fixation and agronomic features of soybean (*Glycine max* (L.) Merr.) crop under different doses of inoculant. Acta Agronómica 67 (2): 297–302. DOI: https://doi.org/10.15446/acag.v67n2.56375

Pulido-Suárez L., Flores-Félix J.D., Socas-Pérez N., Igual J.M., Velázquez E., Péix Á., León-Barrios M. 2022. Endophytic *Bosea spartocytisi* sp. nov. coexists with rhizobia in root nodules of *Spartocytisus supranubius* growing in soils of Teide National Park (Canary Islands). Systematic and Applied Microbiology 45 (6): 126374. DOI: https://doi.org/10.1016/j.syapm.2022.126374

Sequencing Method Manual for GS Junior Titanium Series. 2012. Method Manual. 454 Life Sciences Corp. A Roche Company Branford, p. 26.

Shi N., He T., Qin H., Wang Z., You S., Wang E., Hu G., Wang F., Yu M, Liu X, Liu Z. 2024. *Microvirga sesbaniae* sp. nov. and *Microvirga yunnanensis* sp. nov., Pink-Pigmented Bacteria Isolated from Root Nodules of *Sesbania cannabina* (Retz.) Poir. Microorganisms 12 (8): 1558. DOI: https://doi.org/10.3390/microorganisms12081558

Trybel S.O. 2001. Metody otsinky efektyvnosti [Efficiency assessment methods]. p. 94-96. In: "Metodyky vyprobuvannya i zastosuvannya pestytsydiv" (Trybel, S.O., ed.) [Test methods and application of pesticides]. Kyiv: Svit. P. (in Ukrainian)

Usui Y., Shimizu T., Nakamura A., Ito M. 2020. Metabolites Produced by *Kaistia* sp. 32K Promote Biofilm Formation in Coculture with *Methylobacterium* sp. ME121 Biology 9 (9): 287. DOI: https://doi.org/10.3390/biology9090287