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Original article

VIABILITY OF NODULE BACTERIA *BRADYRHIZOBIUM JAPONICUM* ON SOYBEAN SEEDS BY TREATMENT WITH FUNGICIDES DURING EXTENDED STORAGE

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Background. With the increase in soybean cultivation areas, inoculants are becoming increasingly sought after. They are not only compatible with the original preparations for seed treatment of soybean but also offer the possibility of applying them for several days or even months before sowing into the soil.
Objective. The viability of new strains of nodule bacteria *Bradyrhizobium japonicum* (strain PC07 and strain B78) was investigated on the surface of soybean seeds treated with fungicides Fever and Maxim XL during the extended storage of inoculated seeds. Additionally, their ability for nitrogen fixation under symbiotic conditions was evaluated.
Methods. Cultivation, serial dilution method, determination of bacterial titer of inoculants, quantification of colony forming units (CFU), gas chromatography.
Results. In laboratory conditions, on the soybean seeds of Almaz and Vasylkivska varieties, the viability of the nodule bacteria *B. japonicum* PC07 and B78, which are more resistant to the active substances of these fungicides in pure culture, decreased. This reduction was dependent on the storage period, the fungicide used, and the strain of inoculant. One day after inoculation on the seeds without the use of fungicide, 68.6–75.4% CFU of the initial number was retained. Seeds treated with the fungicide Fever contained 36.5–38.3% CFU, and those treated with Maxim XL contained 26.2–27.0% CFU. The use of inoculants based on fungicide-resistant strains of *B. japonicum* PC07 and B78 also provided high cell viability – $2.41–2.8\times10^6$ (on the seeds without treatment), $1.40–1.70\times10^6$ (with Fever) and $0.8–1.17\times10^6$ (with Maxim XL) CFU/seed after 5 days of storage. The nitrogen-fixing activity of root nodules in soybean plants of Almaz and Vasylkivska varieties, whose seeds were treated with Fever, decreased on both varieties by 18.4–22.4% and 32.1–39.5%, respectively and for treatment with Maxim XL, the reduction was 24.5–33.7% and 47.7–75.2%, respectively, during the storage of seeds for 5 and 7 days before sowing, compared to control variants (seeds without fungicide treatment).
Conclusions. The utilization of fungicide-resistant strains of *B. japonicum* PC07 and B78 for bacterization of soybean seeds treated with fungicides Fever and Maxim XL provides a high inoculation titer during 5 days of storage. Viability of microbial cells on seeds treated with fungicides significantly diminishes during storage for more than 7–14 days. Insufficient titer of CFU of *B. japonicum* strains PC07 and B78 on the seed surface after 14 days of storage can considerably impede the efficacy of biopreparations. Therefore, it is crucial to seek substances that can enhance the resistance of rhizobia on seeds to the adverse effects of fungicide seed treatment, ensuring a longer period of their viability.

Keywords: *Bradyrhizobium japonicum*; viability of rhizobia; seed treatment fungicide; fungicide Fever; fungicide Maxim XL; soybean seeds.

Introduction

Soybeans are a classic legume, and modern cultivation techniques incorporate various elements, including treatment with chemicals of fungicidal or insecticidal action and inoculation with nodule bacteria *B. japonicum*. The seed treatment conduces to increase the field germination, to obtain even seedlings, to reduce the damage of soybean plants by diseases and pests and, as a result, to obtain a higher yield [1, 2]. Inoculation of seeds with microbial preparations reduces the use of high-value nitrogen fertilizers, prevents negative environmental impacts, increases productivity and improves soybean grain quality [3, 4]. However, seed treatment with pesticides, especially fungicides or trace elements, can rapidly kill the bacteria on the seed, affecting the functioning and productivity of the legume-rhizobial symbiosis.

Currently in the world much attention is paid to the study of the effects fungicides on microorganisms which are bioagents of microbial preparations [5]. Imfelda & Vuilleumierb [6] claims that fungicides can have different effects on microor-

ganisms stimulated plant growth. Deshwal *et al.* [7] and Ahemad & Khan [8] reported the metabolic versatility of the genus *Bradyrhizobium* sp., regarding the utilization of chlorinated aromatics as an energy source. Mishra *et al.* [9] conversely, showed that exposure to compounds such as benomyl, carbendazim, and captan reduces the number of *B. japonicum* populations by 14–45% while the population of phosphate-solubilizing bacteria showed a slight reduction.

Toxicity of fungicides for nodule bacteria depends on the active substances in their composition, and is also related to the components (surfactants, emulsifiers, antiseptics, etc.), which manufacturers add to the preparations to improve the technological properties [10–12].

According to literature sources, not all active substances of fungicides are toxic to various species and strains of diazotrophs. Gaind *et al.* [13] found that on chickpea seed (*Cicer arietinum*) treated with the poison bavistin population *Mesorhizobium ciceri* (SP4) and *Azotobacter chroococcum* (M4) had a more viable compared to rhizobia on seed treated with thiram. A greater amount of *A. chroococcum* (CBD-15) was isolated from seeds treated with thiram. In laboratory conditions *A. chroococcum* (W5) showed better survival on wheat (*Triticum aestivum*) seeds in the presence of the fungicide bavistin compared to thiram and captan. However, all studied strains of diazotrophs showed a decrease in the viability of the population with long-term contact with fungicides.

Contact of soybean rhizobia with fludioxonil on inoculated seeds reduces by more than 25% the number of survivors bacteria compared to control 48 hours after inoculation. Alginate has been shown to promote bacterial protection by reducing the inhibitory effect of the fungicide Carbendazim and Fludioxonil to 24 hours of drying. These results indicate that the use of polymers in bacterial inoculants is a promising alternative to reduce the negative effect of fungicides on cell viability. These results indicate that the use of polymers in bacterial inoculants is a promising alternative to reduce the negative effect of fungicides on cell viability [14].

The following information is known about the effect of other fungicides on the survival of *B. japonicum* bacteria on seeds. Fungicides based on carbendazim, copper oxide, carboxin, thiram, pencycuron had a small negative effect on the survival rate of *B. japonicum* on seeds, as well as soybean yield. Therefore, they are considered compatible with soybean seed inoculation. Revellin *et al.* [15] found that fungicides with the active substances carbendazim + iprodione, metalaxyl, hymexazole reduced the number of *B. japonicum* cells on seeds, and fungicides based on carbendazim with thiram and pencycuron did not have a negative effect on the survival of *B. japonicum* on treated seeds.

Researchers from Brazil noted a decrease in the survival of bacteria on seeds due to co-treatment of seeds with fungicides (based on benomyl, captan, carbendazim, carboxin, difenoconazole, thiabendazole, thiram, tolylfluanid) with inoculation of soybean with active strains *Bradyrhizobium* sp. The number of viable rhizobia cells decreased by 62% in 2 hours and by 95% in a day after seed treatment [16].

Tariq *et al.* resistance of pea rhizobia to benzimidazoles was established [17].

In soybean production technology, seeds are traditionally inoculated in the sowing day or the day before sowing. With the increase of sown areas under soybeans there is a need for pre-treatment with plant protection products (PPP) and treatment with microbial preparations of seed. It is resulting in higher demand for inoculants, which are not only compatible with the original preparations for seed treatment of soybean, but also ensuring the prolonged viability of rhizobium cells on the seed.

Development and improvement of elements of technologies that ensure the survival of bacteria on seeds for a long time after inoculation, especially at the use of chemical synthesis substances is becoming increasingly important [18–20].

Compounds like polymers and alginate can enhance bacterial survival, but their effect in the presence of chemicals is not sufficiently studied [21]. Another (biological) way to prolong the activity of nodule bacteria on the surface of seeds treated with fungicides is possible. This involves obtaining and using strains of inoculants that are insensitive (tolerant) to PPP, including nitrogen-fixing microorganisms, as well as rhizobia with enhanced production of biologically active substances, including exopolysaccharides. Natural polysaccharides serve as a protective biofilm for bacteria and can enhance the resilience of rhizobia to adverse conditions in the external environment. This increases the shelf life of the microbial preparation and, at the same time, serves as an adhesive to the surface of the seed [22, 23]. The use of nodule bacteria with natural resistance to fungicides has the potential to minimize the time between seed fungicide treatment and inoculation. It also ensures a higher quantity of viable rhizobia on soybean seeds. The positive effect allows for time and resource savings during seed preparation.

In last years, Fever and Maxim XL are widely used among soybean fungicides. They are less toxic to nodule bacteria [24, 25]. At the same time to "List of pesticides and agrochemicals approved for use in Ukraine in 2023" includes many of preparations that are used to treat soybean seeds in order to limit the development of phytopathogens and contain the same active substances as Fever and Maxim XL. In addition, some of the registered fungicides are mixed preparations that combine other compounds with prothioconazole, fludioxonil or metalaxyl-M [26].

The tolerance of nodule bacteria to fungicides and their prolonged presence on seeds as inoculants does not rule out possible negative consequences in the formation and functioning of symbiosis.

Therefore, the aim of our work was to investigate the viability of new strains of nodule bacteria *B. japonicum* on the surface of soybean seeds treated with fungicides Fever and Maxim XL, both with and without the use of chemical PPP during the prolonged storage of inoculated seeds. Additionally, we aimed to assess their ability to nitrogen fixation under symbiotic conditions.

Materials and Methods

The objects of the investigate were strains of nodule bacteria *B. japonicum* from the collection of N_2-fixing microorganisms of the Institute of Plant Physiology and Genetics National Academy of Sciences of Ukraine, received by the authors of the article. *B. japonicum* PC07 strain – analytically selected, isolated from nodules of Kyivska 27 variety, which was cultivated for a long time at the agrobiostation of Pavlo Tychyna Uman State Pedagogical University, Ukraine. *B. japonicum* B78 strain obtained as a result of intergeneric conjugation between *Escherichia coli* S17-1 (pSUP5011::Tn5*mob*) and *B. japonicum* 646. Recombinant strain B78 is a derivative of the parental (basic) strain *B. japonicum* 646 (traditional selection). The use of recombinant strains resistant to fungicides in pure culture as model objects is a promising direction in order to identify the possibility of the combined or sequential use of recombinant strains of rhizobia and PPP to increase the productivity of leguminous crops. In pure culture, recombinant strains show a higher level of resistance to a number of fungicides, including Fever and Maxim XL compared to the original parental forms and a number of other investigated strains [24, 25].

Restoration of physiological activity of nodule bacteria *B. japonicum* after storage in the museum collection, determination of bacterial titer of inoculants was carried out by standard microbiological methods [27].

In the laboratory experiment were performed with the soybean (*Glycine max* (L.) Merr.) seeds of Almaz variety (creator – Poltava State Agrarian Academy, Ukraine), included in the Register of plant varieties of Ukraine since 2007 and Vasylkivska variety (at joint selection of The National Scientific Center Institute of Agriculture of NAAS, Institute of Plant Physiology and Genetics of NAS of Ukraine and The Plant Breeding and Genetics Institute – National Center of Seed and Cultivar Investigation).

Soybean seeds were sterilized with 70% ethanol solution and washed with sterile water before starting the experiment. It were treated (50 g portions in glass cups) for 60 minutes with fungicides Maxim XL and Fever, according to the instructions provided by the manufacturers. Solutions of fungicides were prepared based on the concentration of preparations recommended by manufacturers for pre-sowing treatment of soybean seeds.

Maxim XL 035 FS (Syngenta, Switzerland) is a fungicide of contact and penetrating action against a wide range of the most common diseases. Active substances (fludioxonil (25 g/l, phenylpyrrole class), metalaxyl-M (10 g/l, phenylamide class) spread in the soil during germination, are adsorbed by the roots and distributed throughout the plant. The consumption rate of the preparations for the treatment of soybean seeds is 1.0 l/t. The use of the working solution is 10 l/t of seeds.

Fever ("Bayer CropScience AG", Germany) is a systemic contact fungicide with the active substance prothioconazole (300 g/l, triazole subclass). The consumption rate of the preparations for the treatment of soybean seeds is 0.2–0.4 l/t. The use of the working solution is 10 l/t of seeds [24].

Two hours after treatment, soybean seeds were inoculated with aqueous monosuspensions of nodule bacteria based on strains of *B. japonicum* PC07 and B78. Bacteria were grown on mannitol-yeast agar (YMA) for 7 days at 28 °C (YMA g/l: K_2HPO_4 – 0.5; $MgSO_4 \times 7H_2O$ – 0.2; NaCl – 0.1; mannitol – 10.0; yeast extract – 0.5; agar – 15.0–17.0; distilled water, sterilized at 1 atm for 30 min, pH 6.8–7.2). The culture from agar was washed off with sterile water, a thick suspension (20 ml) was prepared, which was used to inoculate flasks (volume – 350 ml). Cultivation was carried out for 6 days at a temperature of 28 °C and constant aeration of the growing medium. The finished liquid-phase preparation of nodule bacteria was

diluted to the required concentration and inoculated by them with soybean seeds (per 50 g of seeds – 1 ml of suspension). Inoculated seeds were stored at room temperature (23 ± 2 °C). Bacterial titers of inoculants were CFU 5.1–5.9×10^9/ml.

Samples were analyzed 2 and 4 hours after seed inoculation and 1, 2, 5, 7, 14, 21, 28, and 35 days after inoculation. At the appointed time, 10 inoculated seeds were transferred aseptically into a glass flask containing 100 ml of sterile H_2O. The flasks were shaken in a shaker for 5 minutes. 1 ml of suspension was taken from each flask and a series of successive dilutions in sterile water was prepared. The number of colony-forming units (CFU) on the surface of soybean seeds was determined by serial dilution [27]. Aliquots of 100 μl (0.1 ml) from each dilution of each batch were distributed on yeast mannitol agar plates in Petri dishes and incubated at 28 ± 2 °C. Colonies (CFU) were counted after 7 days. The number of bacterial cells stored on the seeds was determined based on the average number of colonies of the two dilutions closest to the counting range. The studies included an assessment of the survival of bacteria on soybean seeds treated with the fungicides Maxim XL and Fever and without the use of chemical PPP. Variants of the experiment, in which soybean seeds were not treated with fungicides, but only seeds were inoculated with nodule bacteria *B. japonicum* were used the controls.

The dynamics of reducing the viability of microbial cells was calculated as a percentage of the number of microorganisms at each subsequent point of determination to their initial number.

In vegetative experiments, the symbiotic activity of nodule bacteria *B. japonicum* PC07 and B78 was studied after their prolonged presence on the surface of soybean seeds of Almaz and Vasylkivska varieties. Seeds of soybean, inoculated with nodule bacteria *B. japonicum* PC07 and B78 from a laboratory study, were sown in pots filled with substrate (washed river sand). The soybean seeds were stored for 5 and 7 days at a temperature of (23 ± 2 °C) and humidity (60%) before sowing. In each pot, 8 plants were grown under 60% substrate humidity relative to full saturation, and they were exposed to natural light. The inner surface of the pots was sterilized in advance with a 20% H_2O_2 solution. A mixture of Hellriegel [28] with 0.25 norm of nitrogen served as the source of mineral nutrition for the plants. The experiment was repeated 7 times.

The nitrogen-fixing activity (NFA) of root nodules was determined using the acetylene method by Hardy [29] in the stage of three true leaves (on the 35th day after the emergence of uniform plant shoots). Roots with nodules were placed in tightly sealed glass bottles with a capacity of 75 cm^3, where a 10% concentration of acetylene was created. The incubation duration was 1 hour. After incubation, the gas mixture was analyzed using the gas chromatograph "Agilent Technologies 6850" (USA) Network GC System with a flame ionization detector. Gas separation was carried out on a column (Supelco Porapak N) at a thermostat temperature of +55 °C and a detector temperature of +150 °C. Nitrogen was used as the carrier gas (50 ml per 1 min). The volume of the analyzed sample of the gas mixture was 1 cm^3. Pure ethylene obtained from ethyl alcohol and concentrated sulfuric acid heated to 160 °C was used as a standard. The amount of ethylene formed from acetylene under the action of nitrogenase in the incubated sample was expressed in molar units (μmol).

Statistical analysis was performed using MS Excel. The tables show the arithmetic mean values and their standard errors (x ± SE). All differences were considered significant at $P < 0.05$.

Results

The selection of strains for the study was determined by their high symbiotic activity and efficiency under conditions of vegetative and field experiments (2015–2020). We previously conducted studies to determine the resistance of several strains of nodule bacteria *B. japonicum* to plant protection agents using the "agar diffusion method". Both strains PC07 and B78 in pure culture (*in vitro*) were found to be relatively insensitive to the fungicides Fever and Maxim XL [24, 25].

The viability of strains of nodule bacteria *B. japonicum* PC07 and B78 on soybean seeds of Almaz and Vasylkivska varieties was studied depending on the applied fungicide and the storage period of inoculated seeds in the laboratory conditions.

Two hours after inoculation on soybean seeds of the Almaz variety (without fungicide treatment), the bacterial amount decreased by 18.7% (*B. japonicum* PC07) and 14.6% (*B. japonicum* B78) compared to their initial amount on the seeds (100%). This is indicated by the bacterial titer of suspensions (aliquots) obtained by rinsing rhizobia from the seeds. After 2 hours, 81.3% (4.8×10^6 CFU/seed) of *B. japonicum* PC07 cells and 85.4% (4.1×10^6 CFU/seed) of *B. japonicum* B78 cells remained viable. After the next 2 hours, the number of rhizobia on seeds decreased by another 6.8% (*B. japonicum* PC07) and 8.3% (*B. japonicum* B78)

compared to the result obtained in the previous determination point (Tables 1, 2).

The period from inoculation until the end of the first day can be noted as "adaptive" for rhizobia on the seed surface, as a gradual (slow) decrease in the amount of microbial cells has been observed. A short-term "stabilization" of the amount of CFU during the first day on the surface of soybean seeds was characteristic only for soy rhizobia that did not come into contact with fungicides. As a result of the research, it was determined that after the first day, the amount of nodule bacteria on the seeds of the control variant decreased on average by 31.4% (PC07) and 24.6% (B78). On the soybean seeds of the Almaz variety, there remained 68.6% (PC07) and 75.4% (B78) of CFU, respectively (Table 2). On the soybean seeds of the Vasylkivska variety, there were 69.2% (PC07) and 74.9% (B78). Therefore, a certain amount of nodule bacteria *B. japonicum* PC07 and B78 during the first day, even under optimal seed storage conditions in the laboratory, lost viability without contact with artificially synthesized compounds (Tables 3, 4).

The reduction in the population of *B. japonicum* on untreated with PPP seeds (control variants) after 24 hours of storage with chemical seed treatments is primarily explained by desiccation, according to the researchers [30].

According to the results of our research, the number of CFU on soybean seeds of the Almaz variety after 5 days was 47.4% (*B. japonicum* PC07) and 53.1% (*B. japonicum* B78). On the seeds of the Vasylkivska variety, it was 48.2% (PC07) and 53% (B78). A rapid decrease in the number of viable nodule bacteria on soybean seeds (without fungicide treatment) was observed on the 7th day of storage. As a result, from the initial number of the rhizobium strain PC07 on soybean seeds of both varieties, 8.5–8.8% remained ($4.2–5.2\times10^5$ CFU/seed), and on seeds inoculated with the strain B78, 15.2–15.6% remained ($7.5–7.6\times10^5$ CFU/seed) (Tables 1–4). The number of bacteria left on the seeds after 7 days was still sufficient for the formation of legume-rhizobial symbiosis. This is due to the fact that the initial number of bacteria that entered the seed was quite significant 4.8×10^6 and 5.9×10^6 CFU/seed.

It is known that high-quality rhizobial inoculants should provide no less than $10^5–10^7$ bacterial cells per seed [31]. The high efficiency of symbiosis was provided by viable rhizobias on granulated seeds in the amount of 2.47×10^7/seed for large-seeded and 1.13×10^5/seed for small-seeded legumes [32].

After 14, 21, and 28 days were recorded 1.16% (69.0×10^3 CFU), 0.02% (1.2×10^3 CFU) and 0.005% (0.03×10^3 CFU) viable rhizobia of the *B. japonicum* PC07 strain on soybean seeds without treated with fungicides, as well as 1.31% (63.0×10^3 CFU), 0.43% (2.1×10^3 CFU) and 0.013% (0.65×10^3 CFU) rhizobia of the *B. japonicum* B78 strain.

At the same time, on the seeds of the Vasylkivska variety without fungicide treatment, the indicators of the number of viable rhizobium cells on the seeds were 1.2% (60.0×10^3 CFU), 0.025% (12.5×10^2 CFU), 0.003% (0.15×10^2 CFU) viable rhizobia of the *B. japonicum* PC07 strain *B. japonicum* PC07, а також 1.2% (60.0×10^3 CFU), 0.39% (19.5×10^2 CFU), 0.015% (7.5×10^2 CFU) rhizobia of the *B. japonicum* B78 strain.

On 28th and 35th days, a critically small number of living rhizobial cells was observed on seeds inoculated with PC07, in fact, the CFU titer dropped to null. However, in the aliquot obtained from the rinsing of seeds of the Almaz and Vasylkivska varieties, inoculated with *B. japonicum* B78 (see Tables 1, 3) on day 28, $6.5–7.5\times10^2$ CFU were detected.

The next stage of the research was to determine the number of viable rhizobia on seeds treated with fungicides after the specified storage period. After 2 and 4 hours on seeds treated with the fungicide Fever, 63.8% and 51.0% CFU of the PC07 strain, as well as 61.5% and 55.7% CFU of the B78 strain, were detected, respectively. On seeds treated with the fungicide Maxim XL, the indicators were 62.5% and 46.2% for the PC07 strain, and 53.3% and 51.1% for the B78 strain, respectively. The number of viable rhizobia on the seeds of both soybean varieties, after contact with fungicides for 4 hours of seed storage, also decreased compared to the control (without the use of chemical PPP).

There was also a decrease in the titer of CFU rhizobia from the first to the fifth day of seed storage (in 5 days). On seeds treated with the fungicide Fever, 30.2% of the PC07 strain rhizobia and 32.3% of the B78 strain rhizobia remained.

Under the conditions of contact of nodule bacteria with the fungicide Maxim XL on Almaz soybean seeds, the CFU values were 20.0% (PC07) and 23.3% (B78) of the initial cell number of these strains (Table 1). On the Vasylkivska soybean seeds treated with the fungicide Maxim XL after 5 days of storage, the CFU values were 21.0% (PC07) and 22.5% (B78) (Table 4). Thus, no significant differences in the number of viable rhizobia on untreated and fungicide-treated soybean seeds were found over 28 days, depending on the soybean variety (see Tables 1–4).

Table 1: Viability of *B. japonicum* strains (CFU/seed) on the surface of soybean seeds treated with fungicides and without using chemical plant protection products during extended storage in laboratory (Almaz variety)

Option	Initial number of CFU/seed	Storage time of inoculated seeds								
		2 h	4 h	days after inoculation						
				1	5	7	14	21	28	35
PC07 (without fungicide)	$(5.9 \pm 0.24) \times 10^6$	$(4.8 \pm 0.24) \times 10^6$	$(4.4 \pm 0.25) \times 10^6$	$(4.1 \pm 0.21) \times 10^6$	$(2.8 \pm 0.15) \times 10^6$	$(5.2 \pm 0.23) \times 10^5$	$(69.0 \pm 3.31) \times 10^3$	$(12.0 \pm 0.52) \times 10^2$	$(0.3 \pm 0.01) \times 10^2$	$(0.02 \pm 0.001) \times 10^2$
Fever+PC07	$(4.7 \pm 0.24) \times 10^6$	$(3.0 \pm 0.16) \times 10^6$	$(2.4 \pm 0.12) \times 10^6$	$(1.8 \pm 0.08) \times 10^6$	$(1.4 \pm 0.07) \times 10^6$	$(0.2 \pm 0.01) \times 10^5$	$(10.7 \pm 0.46) \times 10^3$	$(2.0 \pm 0.12) \times 10^2$	$(0.1 \pm 0.004) \times 10^2$	0
Maxim XL+PC07	$(4.0 \pm 0.22) \times 10^6$	$(2.5 \pm 0.13) \times 10^6$	$(1.9 \pm 0.08) \times 10^6$	$(1.1 \pm 0.05) \times 10^6$	$(0.8 \pm 0.04) \times 10^6$	$(0.2 \pm 0.01) \times 10^5$	$(0.9 \pm 0.05) \times 10^3$	0	0	0
B78 (without fungicide)	$(4.8 \pm 0.28) \times 10^6$	$(4.1 \pm 0.19) \times 10^6$	$(3.7 \pm 0.19) \times 10^6$	$(3.6 \pm 0.17) \times 10^6$	$(2.6 \pm 0.12) \times 10^6$	$(7.5 \pm 0.37) \times 10^5$	$(63.0 \pm 2.85) \times 10^3$	$(21.0 \pm 0.91) \times 10^2$	$(6.5 \pm 0.39) \times 10^2$	$(0.5 \pm 0.02) \times 10^2$
Fever+B78	$(5.2 \pm 0.23) \times 10^6$	$(3.2 \pm 0.16) \times 10^6$	$(2.9 \pm 0.12) \times 10^6$	$(1.9 \pm 0.10) \times 10^6$	$(1.7 \pm 0.08) \times 10^6$	$(0.6 \pm 0.03) \times 10^5$	$(24.0 \pm 1.02) \times 10^3$	$(10.5 \pm 0.60) \times 10^2$	$(5.0 \pm 0.23) \times 10^2$	$(0.3 \pm 0.01) \times 10^2$
Maxim XL+B78	$(4.5 \pm 0.22) \times 10^6$	$(2.4 \pm 0.12) \times 10^6$	$(1.9 \pm 0.08) \times 10^6$	$(1.2 \pm 0.06) \times 10^6$	$(1.1 \pm 0.06) \times 10^6$	$(0.2 \pm 0.01) \times 10^5$	$(1.0 \pm 0.04) \times 10^3$	$(0.1 \pm 0.005) \times 10^2$	0	0

Table 2: Dynamics of viability reduction (%) the strains of *B. japonicum* on the surface of soybean seeds treated with fungicides and without using chemical plant protection products during extended storage (laboratory experiments, Almaz variety)

Option	Storage time of inoculated seeds								
	2 h	4 h	days after inoculation						
			1	5	7	14	21	28	35
PC07 (without fungicide)	81.3	74.5	68.6	47.4	8.8	1.16	0.02	0.005	0
Fever+PC07	63.8	51.0	38.3	30.2	0.42	0.22	0.004	0.0002	0
Maxim XL+PC07	62.5	46.2	26.2	20.0	0.04	0.02	0	0	0
B78 (without fungicide)	85.4	77.1	75.4	53.1	15.6	1.31	0.43	0.013	0.0009
Fever+B78	61.5	55.7	36.5	32.3	1.13	0.46	0.02	0.0096	0.0006
Maxim XL+B78	53.3	51.1	26.6	23.3	0.51	0.022	0.0002	0	0

Table 3: Viability of *B. japonicum* strains (CFU/seed) on the surface of soybean seeds treated with fungicides and without using chemical plant protection products during extended storage in laboratory (Vasylkivska variety)

Option	Initial number of CFU/seed	Storage time of inoculated seeds								
		2 h	4 h	days after inoculation						
				1	5	7	14	21	28	35
PC07 (without fungicide)	$(5.0 \pm 0.14) \times 10^6$	$(4.1 \pm 0.22) \times 10^6$	$(3.7 \pm 0.20) \times 10^6$	$(3.46 \pm 0.20) \times 10^6$	$(2.41 \pm 0.10) \times 10^6$	$(4.2 \pm 0.13) \times 10^5$	$(60.0 \pm 2.25) \times 10^3$	$(12.5 \pm 0.22) \times 10^2$	$(0.15 \pm 0.01) \times 10^2$	0
Fever+PC07	$(5.2 \pm 0.25) \times 10^6$	$(3.3 \pm 0.15) \times 10^6$	$(2.7 \pm 0.10) \times 10^6$	$(1.9 \pm 0.09) \times 10^6$	$(1.6 \pm 0.06) \times 10^6$	$(0.2 \pm 0.02) \times 10^5$	$(13.0 \pm 0.12) \times 10^3$	$(1.6 \pm 0.13) \times 10^2$	0	0
Maxim XL+PC07	$(5.0 \pm 0.12) \times 10^6$	$(3.1 \pm 0.12) \times 10^6$	$(2.3 \pm 0.06) \times 10^6$	$(1.35 \pm 0.03) \times 10^6$	$(1,05 \pm 0.02) \times 10^6$	$(0.17 \pm 0.02) \times 10^5$	$(0.09 \pm 0.03) \times 10^3$	0	0	0
B78 (without fungicide)	$(5.0 \pm 0.20) \times 10^6$	$(4.3 \pm 0.20) \times 10^6$	$(3.8 \pm 0.19) \times 10^6$	$(3.7 \pm 0.12) \times 10^6$	$(2.6 \pm 0.18) \times 10^6$	$(7.6 \pm 0.30) \times 10^5$	$(60.0 \pm 3.5) \times 10^3$	$(19.5 \pm 0.10) \times 10^2$	$(7.5 \pm 0.30) \times 10^2$	$(0.4 \pm 0.01) \times 10^2$
Fever+B78	$(4.9 \pm 0.24) \times 10^6$	$(3.3 \pm 0.13) \times 10^6$	$(2.7 \pm 0.10) \times 10^6$	$(1.9 \pm 0.14) \times 10^6$	$(1.6 \pm 0.06) \times 10^6$	$(0.6 \pm 0.02) \times 10^5$	$(22.3 \pm 1.12) \times 10^3$	$(15.6 \pm 0.80) \times 10^2$	$(4.4 \pm 0.20) \times 10^2$	0
Maxim XL+B78	$(5.2 \pm 0.20) \times 10^6$	$(2.8 \pm 0.10) \times 10^6$	$(2.7 \pm 0.05) \times 10^6$	$(1.4 \pm 0.04) \times 10^6$	$(1.17 \pm 0.08) \times 10^6$	$(0.3 \pm 0.01) \times 10^5$	$(1.3 \pm 0.03) \times 10^3$	$(0.1 \pm 0.005) \times 10^2$	0	0

Table 4: Dynamics of viability reduction (%) the strains of *B. japonicum* on the surface of soybean seeds treated with fungicides and without using chemical plant protection products during extended storage (laboratory experiments, Vasylkivska variety)

Option	Storage time of inoculated seeds								
	2 h	4 h	days after inoculation						
			1	5	7	14	21	28	35
PC07 (without fungicide)	82.0	74.2	69.2	48.2	8.5	1.2	0.025	0.003	0
Fever+PC07	64.0	51.5	37.8	31.0	0,04	0.25	0.03	0	0
Maxim XL+PC07	61.8	45.9	27.0	21.0	0.035	0.019	0	0	0
B78 (without fungicide)	86.0	76.5	74.9	53.0	15.2	1.2	0.39	0.015	0.0008
Fever+B78	63.4	56.0	37.0	32.5	1.2	0.44	0.018	0.008	0
Maxim XL+B78	54.0	52.2	27.0	22.5	0.6	0.02	0.0002	0	0

In our opinion, the "effectiveness" of their contact with fungicides on seeds after 5 days of complex pre-sowing treatment (fungicide + bio-preparation) can be characterized as a "prolonged survival period" due to the physiological features of these microsymbionts.

After 7 days of storage there was a sharp decline in the survival of rhizobia. The number of CFU PC07 strain on seeds treated with Fever decreased 26 times, and B78 strain in 12.7 times compared to the number of cells on seeds without the use of fungicides. At the same time, on the seeds of the treated Fever were kept 20 000 (PC07) and 59 000 microbial cells/seed (B78).

On seeds treated with Maxim XL, the number of rhizobia PC07 strain after 7 days was non-competitive and insufficient for the effective formation of legume-rhizobial symbiosis. Under similar conditions, nodule bacteria *B. japonicum* B78 proved to be more viable (the number of CFU was 23 000/seed).

A similar trend of decreasing viable rhizobia cell number is observed in the soybean seeds of the Vasylkivska variety. The soybean seeds of the Almaz and Vasylkivska varieties, treated with the fungicide Maxim XL and inoculated with the rhizobia strains PC07 and B78, are not advisable for use as planting material after 14 days of storage due to insufficient CFU for the formation of an effective legume-rhizobial symbiosis (see Tables 1–4).

In the conditions of a vegetation study, the symbiotic activity of the nodule bacteria was determined after 5 and 7 days of storage of soybean seeds (Almaz and Vasylkivska varieties), treated with fungicides and inoculated with the strains *B. japonicum* PC07 and *B. japonicum* B78.

It has been established that fungicide-resistant nodule bacteria PC07 and B78, which remained on soybean seeds and retained viability, preserved their functional activity. Symbiotic organs (nodules) formed on the roots of soybean plants of both varieties with their participation. The intensity of nitrogen-fixing activity of soybeans is presented in Table 5.

The nitrogen-fixing activity of root nodules in soybean plants of the Almaz and Vasylkivska varieties, whose seeds were treated with Fever + *B. japonicum* PC07, decreased on both varieties by 21.7–22.4% and 36.0–39.5% for seed storage, respectively, 5 and 7 days before sowing, compared to the control variant (seeds without fungicide treatment). Under similar conditions, the use of Fever + *B. japonicum* B78 on both soybean varieties resulted in a smaller reduction in nitrogen fixation indicators in soybean root nodules, specifically by 18.4–20.0% and 32.1–34.6%, depending on the seed storage period.

Table 5: The nitrogen-fixing activity (NFA) of soybean root nodules (μmol C_2H_4/(plant·hour)) formed with the participation of *B. japonicum* strains after seed storage in laboratory conditions (at a temperature of 23 ± 2 °C) (the stage of three true leaves, vegetative experiment)

Option	Almaz variety						Vasylkivska variety					
	Storage time of inoculated seeds											
	days after inoculation											
	5			7			5			7		
	NFA	%	A*, %	NFA	%	A*, %	NFA	%	A*, %	NFA	%	A*, %
PC07 (without fungicide)	2.40 ± 0.12	100		1.23 ± 0.05	100		2.19 ± 0.08	100		1.09 ± 0.06	100	
Fever + PC07	1.88 ± 0.07	78,3	21,7	0.80 ± 0.04	65,0	36,0	1.70 ± 0.07	77,6	22,4	0.66 ±0.03	60,5	39,5
Maxim XL + PC07	1.59 ± 0.06	66,3	33,7	0.31 ± 0.02	25,2	74,8	1.47 ± 0.07	67,1	32,9	0.27 ± 0.01	24,8	75,2
B78 (without fungicide)	2.29 ± 0.10	100		1.34 ± 0.06	100		2.05 ± 0.14	100		1.07 ±0.06	100	
Fever + B78	1.87 ± 0.09	81,6	18,4	0.91 ± 0.03	67,9	32,1	1.64 ± 0.08	80,0	20,0	0.70 ± 0.02	65,4	34,6
Maxim XL + B78	1.73 ± 0.06	75,5	24,5	0,78 ± 0.02	58,2	41,8	1.50 ± 0.05	73,2	26,8	0.56 ± 0.01	52.3	47.7

Note. A* – decrease in NFA (%) relative to the control.

The most significant reduction in nitrogen-fixing activity compared to all other experimental variants was observed due to the combined application of Maxim XL + *B. japonicum* PC07. Nitrogen-fixing activity of soybean root nodules in both varieties lagged behind the indicators of the control variant by 32.9–33.8% and 74.8–75.2% during the storage of treated seeds for 5 and 7 days before sowing, respectively.

Other results were observed in the variant with the treatment of soybean seeds Maxim XL + *B. japonicum* B78 compared to using Maxim XL + PC07. The decrease in nitrogenase activity in symbiosis with both varieties was 24.5–26.8% (5 days of seed storage before sowing) and 41.8–47.7% (7 days of storage of treated seeds) compared to control variants.

Discussion

The study of the viability of new strains of nodule bacteria *B. japonicum* on soybean seeds after treatment with modern fungicides during prolonged storage is extremely relevant.

Currently, there is a wide range of pesticides, including fungicides [26]. Among them, Fever and Maxim XL fungicides are quite common and widely used plant protection agents in modern Ukraine. Many new fungicidal preparations for use in agricultural crop cultivation technologies also utilize the traditional active substance fludioxonil (active substance in Maxim XL) and prothioconazole, a subclass of triazolinthiones (active substance in Fever).

At the same time, there is limited information in the literature regarding the impact of these fungicides on nodule bacteria on seeds when they come into direct contact.

We deemed it worthwhile to study the impact of the fungicides Maxim XL and Fever on the viability of the new strains *B. japonicum* PC07 and B78, which were selected by us for their agronomically valuable symbiotic and cultural properties.

Based on our conducted research, it was determined that the survival rate of the nodule bacteria from both strains on seeds treated with Fever was somewhat higher compared to the application of the Maxim XL preparation. However, it was lower than on seeds in the control variant (without treatment with chemical PPP). It is possible that this is associated with the greater toxicity of the components of Maxim XL. The results of artificial modeling of the effects of Maxim XL and Fever on these rhizobial strains confirm this assumption.

In laboratory conditions, it was demonstrated that growth inhibition zones around the "well" containing 1 production norm of Fever are absent, and strains PC07 and B78 are assessed as insensitive to this fungicide. With the use of 2 production norms of the fungicide, the inhibition zone around the "wells" for strain PC07 was 7.9 mm, and for strain B78, it was 9.0 mm. Therefore, the strains were identified as having low sensitivity to Maxim XL [24, 25].

Araujo *et al.* [35] and Hungria *et al.* [36] assert that the toxicity of pesticides against nitrogen-fixing microorganisms can be more pronounced when seeds are inoculated prematurely, allowing *Bradyrhizobium* cells to be in contact with treated soybean seeds for several days, weeks, or even months. Seeds inoculated with *B. elkanii* SEMIA 587 and treated with Standak Top (containing substances with fungicidal action: pyraclostrobin, thiofanate-methyl, and the insecticide fipronil) showed almost no viable microbial cells after 15 days.

Martyniuk [37] notes that soybean seeds and seeds of other agricultural crops, treated with fungicides and inoculated with rhizobial inoculants, should be planted as soon as possible after bacterization. The author suggests that this would help prevent possible toxic effects of chemical fungicides on the rhizobia.

Researchers did not find significant differences in the number of CFU of *B. japonicum* on soybean seeds treated with fungicides compared to untreated seeds (control) two hours after inoculation [38]. After storing the seeds for 24 hours, the number of microbial cells of *B. japonicum* was significantly lower compared to seeds analyzed later after inoculation. After 24 hours, the fungicide-treated seeds had fewer live cells of *B. japonicum* compared to untreated seeds. The authors suggested that the fungicide containing carboxin (20%) and thiram (20%) is more toxic to soybean rhizobia compared to the biofungicide, which primarily consists of tea tree oil (23.8%) [38].

The dynamics of the decrease in the number of viable cells of nitrogen-fixing microorganisms depend on environmental conditions and vary among species and strains of rhizobia [39]. The crucial parameter capable of influencing the survival of rhizobia on treated legume seeds is moisture. This indicator requires particular attention under conditions of prolonged storage of inoculated seeds [18]. However, in this study, the main factors affecting the series of parameters we investigated were fungicides with different compositions, genotypes of microsymbionts, and the duration of

storage of treated seeds before sowing, while maintaining a constant moisture level. Further exploration involves the possibility of conducting a series of experiments involving the selection and combination of different storage conditions (moisture, temperature) for inoculated seeds, and studying the viability of rhizobia in the presence of fungicides. Sartori *et al.* [40] demonstrated that pre-inoculation of soybean seeds for 30 days can negatively impact the recovery of CFU of *B. elkanii*, biological nitrogen fixation, plant growth, and the mass of thousands of grains. The only pesticide seed treatment that was similar to the untreated control in both greenhouse and field experiments was a combination of pyraclostrobin + thiophanate-methyl + fipronil, while the other two were negative for one or more variables. Survival rates are influenced by the initial condition of cells in the inoculant, their quantity, age, purity, strain, and type of inoculant [41, 42].

Sandini *et al.* [43] indicated that some combinations of fungicides and insecticides may adversely affect the physiological quality of seeds stored for up to 51 days before sowing, but none of them questioned the formation of nodules involving *B. elkanii* and soybean yield in field conditions.

So, the researchers emphasize the crucial measures for successfully addressing the issue of symbiotic binding of atmospheric nitrogen and creating a productive legume-rhizobial symbiosis. It's important to select inoculant-strains with a high degree of tolerance to PPP and adverse environmental factors. The inoculants should have a high bacterial titer (number of CFU/mL of the microbial preparation). The number of viable rhizobial cells on untreated seeds and those treated with artificially synthesized compounds should be sufficient for optimal root infection of plants.

It's crucial to search for substances that would enhance the resistance of rhizobia on seeds to the negative effects of fungicides and provide a longer period of their viability. This could contribute to more effective and sustained symbiotic relationships with the plants.

Conclusions

Based on our conducted research, it is necessary to highlight the positive results. Inoculation of soybean seeds with fungicide-resistant *B. japonicum* strains PC07 and B78 under stressful conditions (fungicides, prolonged seed storage, laboratory seed storage conditions) proved to be successful. Since, under the defined conditions, rhizobia cells maintained viability on soybean seeds for an extended period. At the same time, no synthetic chemicals were applied to enhance the survival of rhizobia [18–20].

In laboratory conditions at 23 ± 2 °C, the viability of nodule bacteria *B. japonicum* strains PC07 and B78 on soybean seeds gradually decreased. It depended on the seed storage period, the fungicide used, and the inoculant strain. The authors found that the reduction in the number of viable cells of nodule bacteria on soybean seeds does not depend on the soybean variety used. Since the number of CFU in the aliquot wash from the seeds of Almaz and Vasylkivska soybean varieties did not exhibit significant differences.

The high viability of *B. japonicum* strains PC07 and B78 on soybean seeds treated with fungicides is observed for 5 days without the use of auxiliary substances with the function of protecting cells from negative factors. Under the defined conditions of the laboratory study, storing seeds for more than 7–14 days provokes a rapid decrease in the number of viable microbial cells on the seed surface.

By using the initial inoculation bacterial titer (CFU $5.1–5.9\times10^9$/ml) for seed bacterization treated with fungicides, the rhizobial cells that remained viable on the seeds after 5–7 days were functionally active.

Under the conditions of symbiosis with the new strains of rhizobia, nitrogen-fixing activity of the root nodules was determined in the stage of the formation of the three true leaves. In the future, it would be worthwhile to investigate the effectiveness of symbiosis, particularly in terms of soybean seed yield. It is worth analyzing the dependence of the survival of rhizobial cells on seed from external influencing factors (humidity, temperature) during prolonged storage of treated seeds (fungicide + rhizobia).

Research on new fungicide-resistant strains of *B. japonicum* as the bacterial basis for preparations on solid carriers appears promising. This could aim to increase the number of viable rhizobia cells on seeds during prolonged seed storage before planting. To reduce fungicidal stress on rhizobia and prevent rapid drying of the inoculant on the seed surface, one can try adding natural substances to the inoculant.

Interests disclosure

The authors declare no conflict of interests.

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ЖИТТЄЗДАТНІСТЬ БУЛЬБОЧКОВИХ БАКТЕРІЙ *BRADYRHIZOBIUM JAPONICUM* НА НАСІННІ СОЇ, ПРОТРУЄНОМУ ФУНГІЦИДАМИ, ПРИ ДОВГОТРИВАЛОМУ ЗБЕРІГАННІ

Проблематика. Зі збільшенням посівних площ під соєю все більше стають затребуваними інокулянти, не тільки сумісні з оригінальними протруйниками насіння сої, але й з можливістю нанесення біопрепарату за кілька днів або навіть місяців до посіву в ґрунт.
Мета. Дослідити життєздатність нових штамів бульбочкових бактерій *Bradyrhizobium japonicum* (штами РС07 і В78) на поверхні насіння сої, обробленого фунгіцидами Февер і Максим XL, протягом тривалого зберігання інокульованого насіння, а також оцінити їх здатність до фіксації N_2 за умов симбіозу.

Методика реалізації. Культивування, метод серійного розведення, визначення бактеріального титру інокулянтів, кількості колонієутворювальних одиниць (КУО), газова хроматографія.

Результати. У лабораторних умовах на насінні сої сортів Алмаз і Васильківська життєздатність бульбочкових бактерій *B. japonicum* PC07 і B78, які у чистій культурі є більш стійкими до діючих речовин зазначених протруйників, зменшувалася і залежала від терміну зберігання насіння, використаного фунгіциду та штаму-інокулянту. Через 1 добу після інокуляції на насінні без застосування протруйників виявлено 68,6–75,4 % КУО від початкової кількості. На насінні, обробленому фунгіцидом Февер, – 36,5–38,3%, фунгіцидом Максим XL – 26,2–27,0% КУО. Після 5-ти діб зберігання насіння кількість популяцій клітин дорівнювала 2,41–2,8×10^6 (непротруєне насіння), 1,4–1,70×10^6 (протруєне Февером (протіоконазол, 300 г/л)) та 0,8–1,17×10^6 (протруєне Максимом XL (флудіоксоніл, 25 г/л, металаксил-М, 10 г/л)) КУО/насінину. Азотфіксувальна активність кореневих бульбочок рослин сої сортів Алмаз і Васильківська, насіння яких було оброблене Февером, зменшилася на обох сортах на 18,4–22,4 і 32,1−39,5 %, а за обробки Максимом XL – на 24,5–33,7 і 47,7–75,2 % за зберігання насіння відповідно 5 і 7 діб до посіву порівняно з контрольними варіантами (насіння без обробки фунгіцидами).

Висновки. Застосування фунгіцидостійких штамів *B. japonicum* PC07 і B78 для бактеризації насіння сої, обробленого препаратами Февер і Максим XL, забезпечує високий інокуляційний титр протягом 5-ти діб зберігання насіння. Життєздатність бактеріальних клітин на протруєному насінні істотно зменшується при його зберіганні понад 7–14 діб. Недостатній титр КУО штамів *B. japonicum* PC07 і B78 на поверхні насіння після 14-ти діб його зберігання може зменшити ефективність застосування біопрепаратів. Актуальним є пошук речовин, які б сприяли підвищенню стійкості ризобій на насінні до негативного впливу протруйників і забезпечували б триваліший термін їх життєздатності.

Ключові слова: *Bradyrhizobium japonicum*; життєздатність ризобій; протруйник фунгіцидної дії; фунгіцид Февер; фунгіцид Максим XL; насіння сої.