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Influence of carmoisine on the viability of *Bradyrhizobium japonicum* in vitro and physiological indices of soybean under symbiosis conditions

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ABSTRACT

The effect of carmoisine (azorubin) on the viability and reproduction of *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan, 1982 *in vitro* and on the physiological parameters of soybean plants under conditions of symbiosis with the bacteria was studied. Sensitivity to carmoisine of selected strains of *B. japonicum* – 634b, 646, 614, M8, PC07, PC09, PC10 and Tn5-mutants of *B. japonicum* 646 – B20, B78, B144 was studied by the "hole method". It was determined that *B. japonicum* strains are not sensitive to carmoisine in concentrations of 0.25-1.0%, because when growing rhizobia on the surface of mannitol-yeast agar, the zones of inhibition of culture growth around the "holes" with solutions of synthetic colorant were absent. In pot experiments, the physiological response of *Glycine max* (L.) Merr. plants of the Almaz variety to inoculation was studied by biological preparations based on *B. japonicum* B78 and PC07 with carmoisine in their composition. It was shown that the studied biological products had no negative impact on plant growth and development compared to the control, and on the contrary, it contributed to the activization of biosynthetic processes, e.g., the soybean stems linear growth, the growth of aboveground mass and root system, and the chlorophylls and carotenoids accumulation during growing season.

Keywords: rhizobia, soybean, *Bradyrhizobium japonicum*, bacterial preparations, colorant carmoisine, chlorophyll

INTRODUCTION

Fixation of atmospheric nitrogen by microorganisms is important for the overall nitrogen balance in the soil. A special role in this process belongs to nodulating bacteria that assimilate molecular nitrogen from the atmosphere in symbiosis with plants. Symbiotic relationships are established between legume plants and nodulating nitrogen fixing bacteria that have colonized their root systems. They are based on a mutual exchange of carbon assimilates and nitrogen compounds (Kots et al., 2011; Mahmud et al., 2020). Comprehensive research and optimization of biological nitrogen fixation processes in agriculture is more and more urgent to help meeting the food demand for a growing world population (Soumare et al., 2020).

Interest in the practical use of agronomically valuable microorganisms arose in the 19th century with the discovery of nodulating bacteria and establishing their role in nitrogen nutrition of legumes. Over a period of more than a century, the idea has evolved from the use of soil from fields where legumes were grown for pre-sowing bacterization, to the selection of active strains of microorganisms and the creation of modern microbial preparations based on them (Patyka, 2003).

One of the most important elements of the process of soybean growth, which affects its yield, is the pre-sowing treatment of seeds with bacterial preparations based on nitrogen-fixing bacteria, that is used in modern intensive technologies and in technologies aiming to obtain environmentally friendly crop products.

Bacterization of soybean seeds stimulates metabolic processes, changes the rate of initial growth reactions, provides intensive development of the root system and promotes the formation of high yields of soybean seeds, even in presence of a background of autochthonous soil population of nodulating bacteria (Patyka, 2003).

Today, there is an increased interest worldwide in bioinoculants for agriculture based on nitrogen-fixing bacteria (Werner and Newton, 2005). In developed countries, the focus is on the introduction of environmentally friendly technologies in agricultural production. In the USA, Japan, Germany, Great Britain, France, and Switzerland, whole branches of industrial production of microbiological preparations for agriculture have been established and are developing successfully for the last 30 years. Scientific and technical advances in the production of microbial preparations are also characterized by the expansion of their range (Santos et al., 2019).

In general, there is a wide range of microbial preparations on the Ukrainian market, especially for soybeans, both of domestic and foreign production. They are available in solid and liquid forms. Most often, substrates based on peat or vermiculite are used for solid bacterial fertilizers, to which adhesives are added to improve the retention of them on seeds surface. The liquid form of the inoculant usually has two components: a strain of nodule bacteria in a liquid nutrient medium and a mixture of physiologically active substances with micro- and macronutrients, or an adhesive. Each preparation form has its advantages and disadvantages, which can be effectively used in specific production conditions (Kots et al., 2016).

The main characteristic of bacterial preparations is the titer of nodule bacteria (the number of viable rhizobia cells per g or ml of the preparation). According to European and American standards, there should be from 10^7 to 10^9 viable cells of nodule bacteria per unit of product to guarantee shelf life, and the number of foreign microflora should not exceed 1% of the number of rhizobia (Volkogon et al., 2006).

The effectiveness of the preparation depends on the virulence, complementarity, competitiveness of the bioinoculum strain, and tolerance to biotic and abiotic environmental factors (Patyka et al., 2004). In fact, nitrogen-fixing bacteria have a wide range of survival compared to other microorganisms. However, most chemicals affect their viability and functional activity in varying degrees (Krutylo, 2008). Selection of nitrogen fixing bacteria for the formulation of bioinoculum includes tolerance to several chemical active substances used for seed treatment as plant protection products against pathogens and phytophagous at the initial stages of organogenesis (Vorobey et al., 2020).

One of the important elements of bacterial preparations technology, regardless of their physical form (liquid or solid), is the uniformity of their application to seeds. This determines the effectiveness of their entry into the soil with seeds and ensures the formation of an active symbiosis between plants and nodulating bacteria on the entire area of legume cultivation. Visual assessment of the preparation uniformity application to the seeds can be provided by using different colorants, adding them to the inoculant. It is important to choose a colorant that will visualize the preparation distribution on the seeds surface and will not lose its coloring properties over time (at least 6 months). Preferably, synthetic dyes are used to control the uniformity of seed treatment by preparations with fungicidal or insecticidal effect (Gorina, 2008). In the case of adding dyes to the preparations of nodule bacteria, it is necessary to select a concentration that will not have a toxic effect on the viability of nitrogen-fixing microorganisms, the host plant, and on legume-rhizobium symbiosis in general.

The colorants are divided into three groups by origin: mineral (inorganic), natural, and synthetic. Synthetic colorants have significant technological advantages over most natural colorants as they give bright, easily reproducible colors, are resistant to light, oxidants, and reducing agents as well as to changes in pH, etc. Synthetic food colorants are classified by chemical structure (azo colorants, triarylmethane, quinophthalone, indigo, and xanthene colorants) and by color of aqueous solution

(Smirnov, 2009).

Azo colorants are usually used in the form of sodium or potassium salts as water-soluble compounds. They are most stable in pH range from 3 to 7. Azo colorants include representatives of different colors and shades, they are easy to manufacture and use, and relatively cheap (Smirnov, 2009).

Carmoisine (azorubin) is one of the most common azo colorants. It is often used in the food, pharmaceutical, cosmetic, and textile industries (Humeniuk et al., 2013; Grumezescu and Holban, 2017). It is biologically inactive, not carcinogenic, does not interact nor change the bioavailability of active substances, has no unpleasant taste or smell, is soluble or evenly distributed in the dispersion medium, withstands sterilization up to 120 °C, and has high light endurance and coloring ability (Kolmakova, 2008; Leulescu et al., 2021).

As a result of previous laboratory studies, we found no negative effect of carmoisine in concentrations of 0.5 and 1.0% on the germination energy and laboratory germination of soybean seeds Almaz (creator – Poltava State Agrarian Academy, Ukraine) and Vasylkivska (originator – Plant Breeding and Genetics Institute – National Center of Seed and Cultivar Investigation of the National Academy of Agricultural Sciences of Ukraine) varieties (Kukol et al., 2020). However, a slight inhibition of growth processes was observed in the variants with treatment of soybean seeds of Vasylkivska variety with used colorant solutions. Thus, the length of roots in 5 and 8-day-old seedlings was 3.4–10.2% shorter compared to the control. The data obtained indicate the absence or low level of phytotoxicity of synthetic colorant carmoisine on germination of soybean varieties belonging to different maturity groups (Kukol et al., 2020).

The aim of this study was to investigate the effect of carmoisine (azorubin), which was used as a coloring agent for bacterial loose preparation based on vermiculite, on the viability of fungicide-resistant nodulating bacterium *B. japonicum* (Kirchner, 1896), Jordan, 1982 *in vitro* and on the vegetative growth of soybean plants under symbiotic conditions.

MATERIALS AND METHODS

In the laboratory experiment, nodulating bacteria *B. japonicum* strains 634b, 646, 614, M8, PC07, PC09, PC10 (analytical selection) and Tn5-mutants B20, B78, B144 (obtained by transposon mutagenesis (pSUP5011::Tn5*mob*) of *B. japonicum* 646 strain) were studied as test objects. The bacteria were from the Institute of Plant Physiology and Genetics NAS of Ukraine collection of N_2-fixing microorganisms. To restore its physiological activity, *B. japonicum* was grown in biological tubes on a nutrient medium yeast-mannitol agar (YMA) (Netrusov, 2005) for 8 days at +28 °C.

The synthetic colorant carmoisine (azorubin) was used as a substance identifier to control application of the bacterial preparation on the surface of soybean seeds (Sarafanova, 2012). Azorubin is a derivative of coal tar and the chemical formula of the colorant E122 is $C_{20}H_{12}N_2Na_2O_7S_2$ (Smirnov, 2009).

Aqueous solutions of carmoisine were used in the studies – a liquid suspension of bright red (deep crimson) color. The solutions were prepared with different concentrations, in accordance with the range of application rates recommended by the manufacturer ("Ukrasa", Ukraine) for food coloring (not more than 0.5–2.5 g per 1 kg), in particular – 0.25, 0.5 and 1.0 g per 100 ml (in the laboratory experiment). The colorant was pre-sterilized in an autoclave at P = 0.5 atm (t = 110–112 °C) for 30 min.

The sensitivity of soybean nodule bacteria to carmoisine was studied by the "hole method" in a Petri dish (Chervinets et al., 2004). Holes with a diameter of 10 mm were cut in YMA plates by a sterile metal cylinder, 0.2 ml of an aqueous suspension of nodule bacteria was introduced. Sowing was carried out by "continuous lawn". Drygalski spatula was used for uniform distribution of cells on the YMA surface. The carmoisine solution (80 µl) was introduced in the lateral (experimental) 4 holes. Holes in the center of the agar plate filled with sterile H_2O (80 µl) were used as control. Each treatment was repeated 5 times. Petri dishes were incubated in a thermostat for 8 days at a temperature of +28 °C.

The growth rate of *B. japonicum* around the holes was assessed qualitatively using a 4-level scale: intensive growth («+ + +»), weak inhibition («+ + –») more intensive inhibition («+ – –») and complete lack of growth («– – –»). The absence of growth retardation indicated the resistance of microorganisms to carmoisine in the applied concentrations. Colonies were counted after 8–10 days of incubation in a thermostat.

The experiments 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 recommended for cultivation in the forest-steppe zone of Ukraine.

Soybeans were grown on a sandy substrate (10 kg, 8 plants in each pot on) with the introduction of Helrigel nutrient mixture with 0.25 of nitrogen norm (1 norm was 708 mg $Ca(NO_3)_2 \cdot 4H_2O$ per kg of sand) (Grodzinsky and Grodzinsky, 1964), at 60% substrate humidity and natural light. The repeatability of pots in the experimental variants was 7 times. Soybean seeds were externally sterilized for 15 min with 70% ethanol and washed with running water. Pre-sowing inoculation of seeds with a bacterial preparation with the addition of carmoisine was carried out for 1 hour. The bacterial basis of the preparation were active fungicide-resistant strains of nodule bacteria *B. japonicum* B78 and PC07.

Production of the preparation on a loose carrier (vermiculite)

Highly dispersed moisture-bearing mineral carrier vermiculite was sterilized in a steam autoclave (P = 1.2 atm, 1.5 hours) and, after cooling under sterile conditions, enriched with additives (in the same proportion 1:1:1:1 – corn extract, molasses, glucose, sterile tap water (up to 70–75% humidity) and carmoisine in various concentrations (0,25–0,5 g of carmoisine per 130 g of *vermiculite*). Additives were prepared by special technology. The corn extract was diluted with water (1:5), kept at 24 °C for 24 h. Solution was adjusted by 20% NaOH to pH 5.0–6.0 and sterilized at 1.5 atm for 1 hour. Glucose solution (20%) was sterilized at 0.7 atm for 20–30 min, and 80% molasses at 1.5 atm for 1 hour 40 minutes.

Fungicide-resistant strains of nodule bacteria *B. japonicum* B78 and PC07 (Vorobey et al., 2020) were grown in biological tubes on YMA at a temperature of 28 °C for 7 days. Pure rhizobia cultures were washed off from agar by sterile yeast-mannitol medium, and the collected suspension was transferred in flasks with YM medium (10 ml of suspension/350 ml of YM) and cultured during 7 days at a temperature of 28 °C and constant aeration.

The suspensions of nodule bacteria *B. japonicum* were introduced in packages filled with vermiculite, carmoisine and nutritional supplements, which were closed and subjected to light mechanical shaking to mix the components of the preparation. The preparation was kept at room temperature (+18 to +23 °C) for 7 days. The bacterial titer of the preparation on a loose carrier was $2.0 \cdot 10^9$ CFU (colony forming units) per g of the preparation. The amount of preparation used to soybean seeds treatment was calculated based on the application of 200 g of preparation per hectare at a normal sowing rate of seeds (120 kg). The bacterial load in the experiments was 400–500 thousand CFU per seed. Seeds inoculated with a preparation based on strains of *B. japonicum* without carmoisine were used as control.

Biometric indices – the mass of the aboveground part of plants, roots and the plants height – were measured on the 15th and 20th day after germination, as well as in the following stages: 3 true leaves, budding-beginning of flowering, and full flowering of soybeans. The measurements were repeated 10 times.

The content of photosynthetic pigments in plant leaves was determined by the method of Wellburn (1994). Leaf samples from the middle tiers of five randomized plants per treatment were taken for determination. The measurements were performed three times per analysis for each variant. The pigments were extracted with dimethyl sulfoxide (DMSO) (10 ml of DMSO per 100 mg of plant material). The optical density of the solution was determined on a Shimadzu UV-1900 (Japan) spectrophotometer at 649 (chlorophyll *a* content), 665 (chlorophyll *b* content) and 480 nm (carotenoids) in a

1 cm thick cuvette. Numerical values of wavelength correspond to the maximum absorption of pigments in DMSO. The concentrations of pigments in the extract were calculated and their content in the test material was determined (mg/g leaf fresh weight (FW)). The calculation of the pigment concentration was performed according to the corresponding equations (Chl_a – chlorophyll *a*, Chl_b – chlorophyll *b* and C_{car} – carotenoids):

$$Chl_a = 12.19 \times A_{665} - 3.45 \times A_{649}$$

$$Chl_b = 21.99 \times A_{649} - 5.32 \times A_{665}$$

$$C_{car} = (1000 \times A_{480} - 2.14 \times Chl_a - 70.16 \times Chl_b) \times 220^{-1}$$

The data obtained from all analyses were processed statistically. The significance of the difference between controls and treatments were evaluated using ANOVA. Means were separated by using LSD test at 5% of probability. Differences were considered to be significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Studies have shown that carmoisine diffused relatively quickly into the YMA medium. As a result, the plane of its influence extended to the rhizobia, which were in direct contact with the holes filled by colorant, as well as to cells distant from the holes. The absence of a negative effect of carmoisine on growth activity of nodule bacteria on the entire surface of YMA was noted (Table 1).

Under the conditions of culturing of nodule bacteria with a continuous "lawn" (culturing from the 1st dilution), the growth of *B. japonicum* culture around the holes with carmoisine and near the control hole with H_2O was similar in intensity. As an example, in Figure 1 the growth of pure cultures of *B. japonicum* PC07, M8 and 634b on YMA with colorant content is shown, which confirms the lack of inhibitory effect of carmoisine on the reproduction and growth of nodule bacteria.

Under the conditions of culturing of *B. japonicum* suspensions with a lower concentration of cells (culturing from the 3rd dilution) there was also no inhibitory effect of carmoisine on the reproduction of rhizobial cells, as evidenced by the growth activity of *B. japonicum* colonies on the surface of YMA (Fig. 2).

Table 1. Resistance of pure cultures of *Bradyrhizobium japonicum* to the influence of the synthetic colorant carmoisine (laboratory experiment)

Strain of *Bradyrhizobium japonicum*	Carmoisine concentration, g per 100 ml					
	0.25		0.5		1.0	
	a	b	a	b	a	b
PC07	0	+ + +	0	+ + +	0	+ + +
PC09	0	+ + +	0	+ + +	0	+ + +
PC10	0	+ + +	0	+ + +	0	+ + +
M8	0	+ + +	0	+ + +	0	+ + +
614	0	+ + +	0	+ + +	0	+ + +
634b	0	+ + +	0	+ + +	0	+ + +
646	0	+ + +	0	+ + +	0	+ + +
B20	0	+ + +	0	+ + +	0	+ + +
B78	0	+ + +	0	+ + +	0	+ + +
B144	0	+ + +	0	+ + +	0	+ + +

Note: a – the size of the zones of bacterial growth inhibition, mm; b – bacterial growth rate «+ + +» – intensive growth

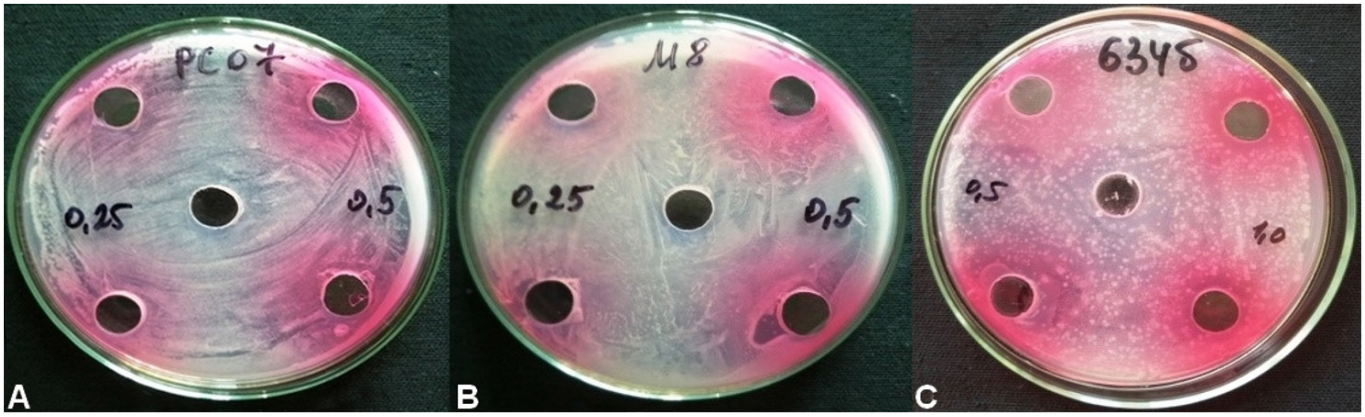

Figure 1. Growth of pure cultures of nodule bacteria *Bradyrhizobium japonicum* strains of analytical selection on the YMA: A – PC07, B – M8, C – 634b. Culturing a continuous "lawn". The concentration of carmoisine in the holes – 0.25, 0.5 and 1.0 g per 100 ml

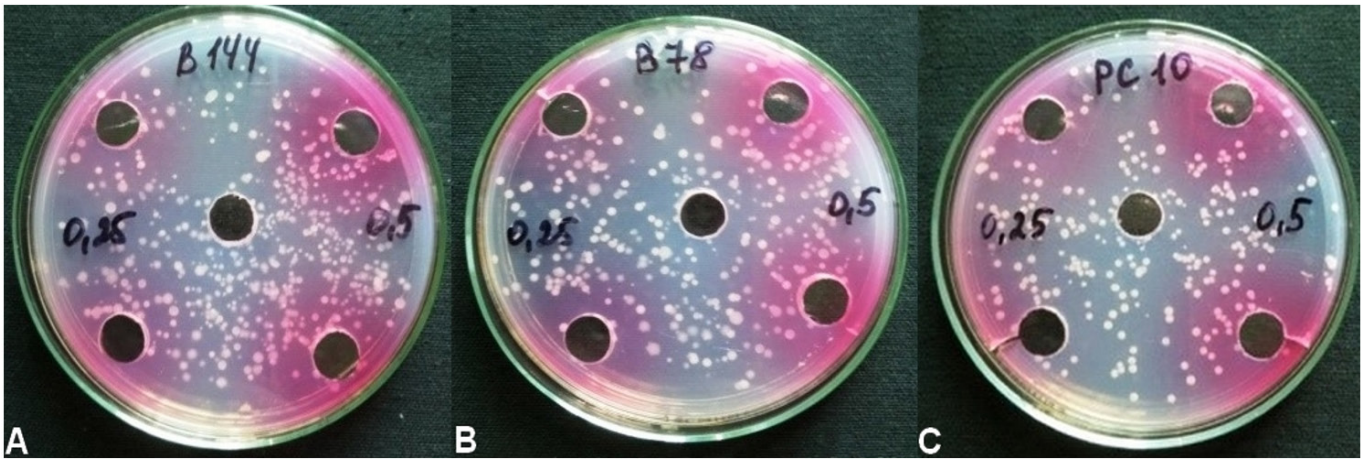

Figure 2. Growth of nodule bacteria *Bradyrhizobium japonicum* strains on the YMA: A – B144, B – B78 (was obtained by transposon mutagenesis) and C – PC10 (analytical selection). Culturing is performed from the 3rd dilution (CFU $\times 10^3$). The concentration of carmoisine in the holes – 0.25 and 0.5 g per 100 ml

The study revealed the absence of a negative effect of the carmoisine within the concentrations recommended by the manufacturer "Ukrasa" (Ukraine) on the growth (viability and reproduction) of nodule bacteria *B. japonicum* strains, which were obtained by different methods of selection.

During the growing season, a positive effect of preparations of nodule bacteria PC07 and B78 with carmoisine content on the growth of soybean plants was noted. For example, in variants with the preparation PC07 + carmoisine at a dose equivalent to 0.25 g per ha of the preparation, the linear growth of the stem was higher by 2.96, 0.5, 21.2, 6.2 and 21.35% and at an equivalent to 0.5 g per ha by 8.77, 2.29, 10.2, 22.5 and 24,7% compared to the control, respectively, depending on development stages of plants (Table 2).

The root system was well developed in plants of all variants of the experiment. In general, pre-sowing inoculation of soybean seeds with bacterial preparations with carmoisine had a positive effect on plants root growth. In the variants with the use of preparations with a higher concentration of carmoisine (0.5 g / 1 ha portion of the preparation) greater mass of roots in comparison with the roots of plants of the control variant of the experiment and the variant with a lower concentration of dye (0.25 g / 1 ha portion of the preparation) was revealed (Table 3). More intense growth of the root system in the early growing season of soybean plants (on the 15th and 20th day after germination) and growth retardation in the 3 true leaf stage were also observed. With the application of preparations with carmoisine, the increase in root weight relative to control plants was by 6.4–13.5% and

Table 2. Dynamics of stem elongation (height in cm) of soybean plants inoculated before sowing with a bacterial preparation with the addition of different concentrations (0.25 and 0.5 g per 1 ha portion of the preparation) of carmoisine (n=15)

Treatment	The stage of plant development				
	15 days after germination	20 days after germination	3 true leaves	budding–beginning of flowering	full flowering
B78 (control)	7.05a	14.21ab	22.44b	27.30a	36.30a
B78 + 0.25 g of carmoisine	7.33ab	12.61a	21.88a	28.15b	38.70b
B78 + 0.5 g of carmoisine	7.63b	14.39b	24.12c	28.70b	41.40c
$LSD_{0.05}$	0.47	1.03	1.90	2.24	2.82
PC07 (control)	7.75ab	16.14a	28.33a	33.65a	42.10a
PC07 + 0.25 g of carmoisine	7.98a	16.23a	34.33c	35.75a	51.09b
PC07 + 0.5 g of carmoisine	8.43c	16.51a	31.22b	41.22b	52.50b
$LSD_{0.05}$	0.51	0.88	2.10	2.66	3.39

Note. Here and in the Table 3, 4 different letters indicate values that differ significantly from each other in the same columns of the table by according to Fisher's test at P≤0.05

Table 3. Dynamics of plant root mass growth (g per plant) of soybean under inoculation with solid-phase bacterial preparations with different concentrations of carmoisine (n=15)

Treatment	The stage of plant development				
	15 days after germination	20 days after germination	3 true leaves	budding–beginning of flowering	full flowering
B78 (control)	0.31a	0.33a	1.82a	2.80a	3.80a
B78 + 0.25 g of carmoisine	0.33a	0.39b	1.84a	2.90a	3.95a
B78 + 0.5 g of carmoisine	0.35a	0.41b	2.02b	3.02a	4.08a
$LSD_{0.05}$	0.05	0.04	0.16	0.40	0.39
PC07 (control)	0.37a	0.40a	2.06a	2.45a	3.49a
PC07 + 0.25 g of carmoisine	0.40ab	0.46b	2.15a	2.28a	3.40a
PC07 + 0.5 g of carmoisine	0.42b	0.48c	2.18a	2.47a	3.58a
$LSD_{0.05}$	0.05	0.04	0.33	0.28	0.34

Note. Significant level at P≤0.05

15.0–24.2%, respectively, on the 15th and 20th day of plant vegetation (after the germination) (Table 3).

A similar trend was observed for the growth of aboveground mass in plants inoculated with preparations B78 and PC07 with carmoisine at the early growing stages and growth slowing at the stages of budding-beginning of flowering and full flowering of soybean (Table 4). For example, under the use of preparations B78 + carmoisine increase in aboveground mass was 4.38–5.26% (on the 15th day after germination), 12.10–13.15% (on the 20th day), 9.02–12.4% (at stage of 3 true leaves), 3.11–3.70% (at the stage of budding-beginning of flowering), and 1.0–1.5% (at full flowering stage) relative to the weight of control plants.

When the preparation PC07 + carmoisine was used, the increase in aboveground mass by stages of development amounted to 6.08–8.7% (on the 15th day after germination), 7.96–12.43% (on the 20th day), 20.0–

22.5% (at the stage of 3 true leaves), 4.94–7.30% (at the stage of budding-beginning of flowering), and to 0.6% (at the full flowering stage) compared to the preparation without the addition of carmoisine (Table 4).

Since the positive effect of preparations with carmoisine was higher at the early stages of plant development, it is possible that this substance can affect the plants growth rate at the initial stages.

The genotoxicity of 0.25, 0.5, 0.75 and 1.0% solutions of azo dyes at 24 and 48 hours of exposure to plant cells of the test object *Allium cepa* was already researched and published. Carmoisine and metanil yellow were found to cause a decrease in the mitotic index and induced the appearance of different chromosomal aberrations depending on the concentration of the dye and the duration of its exposure (Khan et al., 2020). Investigating the phytotoxic effect of carmoisine on wheat germination, the researchers noted inhibition of growth processes and changes in antioxidant activity in the cells with an increase (up to 0.25%) in the concentration of the dye in solution and the duration of the synthetic compound to the seedlings (Leulescu et al., 2021). Thus, it can be assumed that the concentration of the working solution and the duration of action on biological objects are the determining factors of the intensity and direction of the effect of artificial dyes on plants.

Looking at plant tolerance to carmoisine in the applied concentrations, the colorant can influence the formation and functioning of legume-rhizobial symbiosis and the biosynthetic processes in the host plant. Earlier, we found that by inoculating the soybean variety Almaz with microbial preparations made on the basis of *B. japonicum* PC07 and B78 with the addition of carmoisine (0.25 and 0.5 g per 200 g of the preparation), the number and weight of nodules formed on the roots during vegetation were at the level of the control plants or exceeded them. As a result of the analysis of nitrogen-fixing activity of the formed symbiotic systems, the absence of negative influence of synthetic dye on its level was noted (Kukol et al., 2021).

It should be noted that the use of carmoisine (azorubin) as a coloring agent for biological products has not yet been studied. At this stage, the results of our research open the prospect of further study of this effect of carmoisine not only as a dye, but also as a substance with a growth-promoting effect.

The content of chlorophylls in leaves was used as a test indicator of the effect of pre-sowing inoculation

Table 4. Dynamics of soybean plants aboveground mass growth (g per plant) under inoculation with bacterial preparations with different concentrations of carmoisine (n=15)

Treatment	The stage of plant development				
	15 days after germination	20 days after germination	3 true leaves	budding–beginning of flowering	full flowering
B78 (control)	0.31a	0.33a	1.82a	2.80a	3.80a
B78 + 0.25 g of carmoisine	0.33a	0.39b	1.84a	2.90a	3.95a
B78 + 0.5 g of carmoisine	0.35a	0.41b	2.02b	3.02a	4.08a
$LSD_{0.05}$	0.05	0.04	0.16	0.40	0.39
PC07 (control)	0.37a	0.40a	2.06a	2.45a	3.49a
PC07 + 0.25 g of carmoisine	0.40ab	0.46b	2.15a	2.28a	3.40a
PC07 + 0.5 g of carmoisine	0.42b	0.48c	2.18a	2.47a	3.58a
$LSD_{0.05}$	0.05	0.04	0.33	0.28	0.34

Note. Significant level at P≤0.05

of seeds with bacterial preparations of *B. japonicum* on physiological processes in soybean plants.

Content of chlorophyll *a* and *b* in soybean leaves varied depending on the stage of plant development and preparations used for inoculation of seeds. The highest content of chlorophyll *a* in the leaves was observed at the budding-beginning of flowering stage, and chlorophyll *b* – at the stage of 3 true leaves. At the stage of full flowering of soybean plants, the content of chlorophylls and carotenoids decreased in all variants of the experiment, however, in plants inoculated with preparations based on strain B78, it decreased to a greater extent compared with variants using the PC07 strain (Fig. 3). This could be due to the weakening of the nitrogen-fixing activity of the symbiotic systems *Glycine max* L.– *B. japonicum* during this period. In this case, the supply of plants with nitrogen in the stage of full flowering was mainly due to biological fixation with a growing demand for assimilates for the formation of generative organs (Kiriziy and Vorobei, 2007).

The researchers noted a gradual decrease in the quantity of assimilates per one bean due to an increase in the number of reproductive units and their partial loss (by means of abscission). In general, it was found, that the scale of accumulation of organic matter by soybeans largely corresponds to the development of the microsymbiont. Within this pattern, the distribution of plastic substances between plant organs is also determined by varietal specificity, which in turn consists of quite complex mass transfer between reproductive and vegetative organs at different stages of development and can change under the influence of genetic and phenotypic factors (Tilba and Tishkov, 2016).

It is known that the effective functioning of the symbiotic apparatus in inoculated plants stimulates the accumulation of photosynthetic pigments and increases the photosynthetic rate (Tsvetkova et al., 2020). The increase in the content of pigments in the leaves of soybean plants inoculated with preparations with carmoisine may indicate an intensification of photosynthetic processes in them (Fig. 3). The direct effect of carmoisine on nodule bacteria and activation of their properties (including nitrogen-fixing activity) is not excluded, which may also stimulate to some extent the chlorophyll synthesis, considering that leaves of soybean plants inoculated with preparations without carmoisine, were slightly poorer in the content of green pigments (Fig. 3).

According to the literature, the amount of chlorophyll (*a* + *b*) in leaves ranges from 0.3 to 5 mg/g of fresh weight (Shlyk, 1971). In most variants, the content of chlorophyll *a* + *b* was normal and varied between 2.62–3.54, 2.64–3.50, 1.65–3.15 mg/g, respectively, at the stage of third true leaf forming, budding–beginning of flowering, and full flowering, depending on the preparation used for inoculation.

In general, the content of total chlorophyll in the leaves decreased with advanced maturity. These data indirectly indicate a slight decrease in the number of reaction centers of photosystems, probably due to disruption of the synthesis of structural and enzymatic proteins in case of insufficient nitrogen supply (Kononov and Shkotova, 2012; Tyutereva et al., 2017).

The chlorophyll *a/b* ratio in a normally developed photosynthetic apparatus is 2.5–3.0, as noted by Shlyk (1971). Other literature data show that in most plants the chlorophyll *b* content as well as the chlorophyll *a/b* ratio varies in a wider range (chlorophyll *a/b* = 2–5). The theoretically possible lower threshold of this index is 1.91 (Syvash et al., 2018). According to the stages of soybean development, the chlorophyll *a/b* ratio in plants of the present study was 3.55–3.69, 3.84–5.23, 3.95–4.83, respectively, and was the lowest at early growing stage (third true leaf forming) and the highest in the budding-beginning of flowering stage (Table 5). Our results show slightly higher values of chlorophyll *a/b* ratio at the stage of formation of third true leaf compared to results of Syvash et al. (2018). At the following stage of soybean plants development, the chlorophylls ratio remained at a high level, as this period is accompanied by an intensification of physiological processes (growth of aboveground mass, formation of photosynthetic apparatus and nitrogen fixing system). The maximum *a/b* ratio fluctuated within

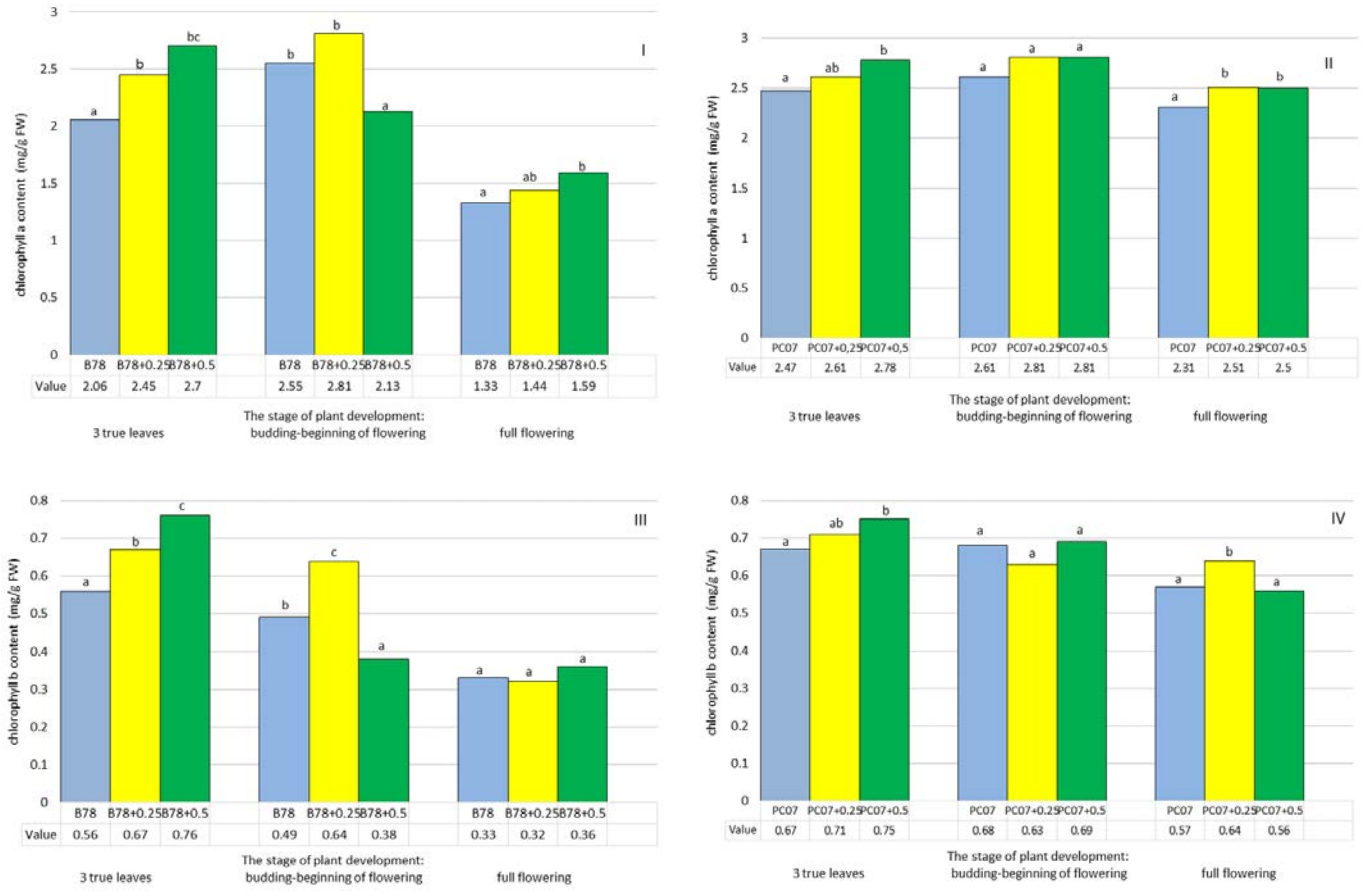

Figure 3. Dynamics of chlorophyll accumulation in the leaves of soybean plants of the Almaz variety: I, II – chlorophyll *a*; III, IV – chlorophyll *b* (control B78; B78+0.25 g carmoisine per 1 ha portion of the preparation; B78+0.5 g carmoisine per 1 ha portion of the preparation; control PC07; PC07+0.25 g carmoisine per 1 ha portion of the preparation; PC07+0.5 g carmoisine per 1 ha portion of the preparation) (n=10)

Note. Here and in the Figure 4 columns of diagram followed by the same letter within variants of each plant development stage are not significantly different according to Fisher's test at P≤0.05

the norm and was 5.23, which indicates the absence of a negative effect of the carmoisine on the formation of the photosynthetic apparatus of soybean leaves (Table 5). Chlorophyll *b*, like carotenoids, plays a protective role (Volodarez, 2012; Tyutereva et al., 2017).

Photosynthetic productivity of plants is largely determined by the level of plastid pigments accumulation in the assimilating organs. Their content, as well as their ratio are characteristics of the physiological state of the leaf and the whole plant. The amount of pigments, i.e. chlorophylls and carotenoids in plants, changes during ontogenesis, during adaptation to environmental conditions, and under the influence of various stressors (Syvash et al., 2018). The main amount of chlorophyll *a* and *b* is part of the light-harvesting complexes and provides absorption and transmission of light energy to the reaction centers, where photosynthetic reactions take place (Scheer, 2004). The greatest efficiency of the photosynthetic apparatus is provided by the ratio of pigments chlorophyll *a* – 50%; chlorophyll *b* – 30%; carotenoids – 20% (Matveeva and Kvasko, 2010).

A necessary companion of chlorophylls are carotenoids, which are not directly involved in photosynthetic reactions, but play the role of collecting antennae of light energy. It is believed that carotenoids have high antioxidant activity and prevent photodestruction of the pigment complexes (Tanaka et al., 2008; Ivanov et al., 2013).

Table 5. The pigments content and their ratio in soybean leaves under inoculation with loose bacterial preparations with different content of carmoisine

Treatment	The stage of plant development					
	3 true leaves		budding–beginning of flowering		full flowering	
	Chlorophylls					
	a+b	*a/b*	*a+b*	*a/b*	*a+b*	*a/b*
B78 (control)	2.62	3.67	3.03	5.23	1.65	4.06
B78 + 0.25 g of carmoisine	3.13	3.62	3.45	4.35	1.75	4.46
B78 + 0.5 g of carmoisine	3.46	3.55	2.38	4.17	1.95	5.24
PC07 (control)	3.14	3.66	3.28	3.84	2.88	4.02
PC07 + 0.25 g of carmoisine	3.31	3.67	3.43	4.44	3.15	3.95
PC07 + 0.5 g of carmoisine	3.54	3.69	3.50	4.07	2.93	4.83
	Carotenoids (*c*)					
	c	*(a+b)/c*	*c*	*(a+b)/c*	*c*	*(a+b)/c*
B78 (control)	0.59	4.48	0.69	4.37	0.40	4.13
B78 + 0.25 g of carmoisine	0.67	4.64	0.75	4.59	0.42	4.14
B78 + 0.5 g of carmoisine	0.75	4.61	0.58	4.07	0.47	4.12
PC07 (control)	0.67	4.67	0.73	4.50	0.58	4.99
PC07 + 0.25 g of carmoisine	0.69	4.78	0.76	4.50	0.60	5.17
PC07 + 0.5 g of carmoisine	0.73	4.83	0.72	4.86	0.58	5.24

The carotenoids content in the leaves of the studied plants varied in the range of 0.40–0.76 mg/g. The high carotenoids content in leaves, the highest amount of chlorophylls (*a+b*) and these pigments ratio were under application of the preparations PC07 + carmoisine. These results showed that the dynamics of the carotenoids content was characterized by the same pattern as the content of chlorophyll *a* and *b* (Fig. 4).

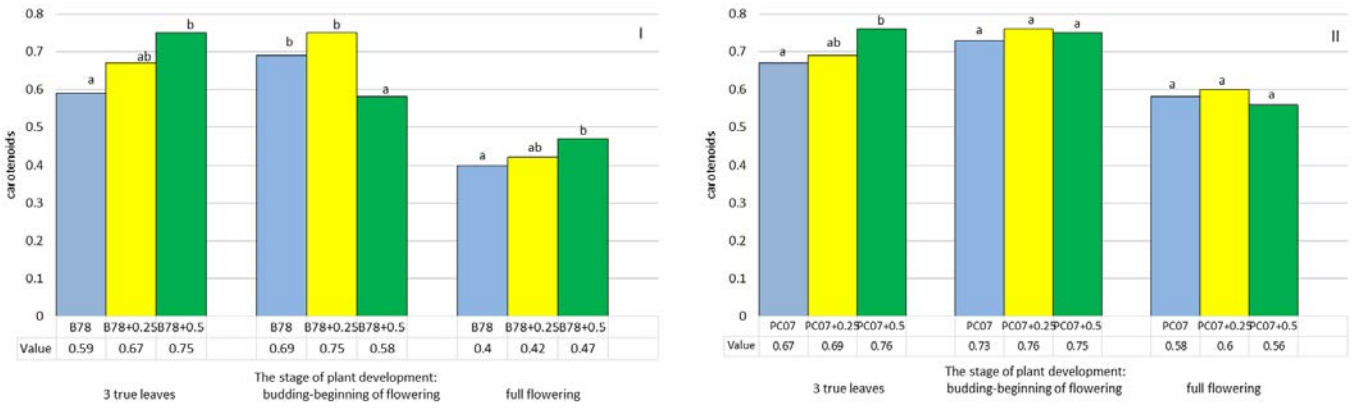

Figure 4. Dynamics of carotenoids accumulation in the leaves of soybean plants of the Almaz variety: I – control B78, B78+0.25 g carmoisine per 1 ha portion of the preparation, B78+0.5 g carmoisine per 1 ha portion of the preparation; II – control PC07, PC07+0.25 g carmoisine per 1 ha portion of the preparation, PC07+0.5 g carmoisine per 1 ha portion of the preparation) (n=10)

The ratio of the sum of chlorophylls (*a+b*) to the sum of carotenoids in higher plants also responds to different factors and varies widely. According to the literature, for most plants this value is from 4.6 to 8.0 (Lichtenthaler and Babani, 2004) and it increases under the influence of negative factors. In our experiments, the ratio of chlorophylls *a+b* to the sum of carotenoids was 4.12–5.24, and lower values were characteristic for plant leaves inoculated with *B. japonicum* B78 with carmoisine and without it.

CONCLUSION

Based on the analysis of all indices obtained, carmoisine, as a substance-identifier for quality control (uniformity) of bacterial loose preparation application on seeds can be recommended for use in the studied concentrations. It was proved that carmoisine had no detrimental effect on the viability and reproduction of fungicide-resistant nodule bacteria *B. japonicum* and physiological parameters of soybean plants under symbiotic conditions.

REFERENCES

Chervinets, Iu.V., Bondarenko, V.M., Shabanova, N.A., Samoukina, A.M., Chervinets, V.M. (2006) Bacteriocinogenic highly antagonistic strains of lactobacilli. Journal of Microbiology, Epidemiology and Immunobiology, 7, 78-82.

Gorina, I.N. (2008) Control over the uniformity of pre-sowing seed treatment. Plant Protection and Quarantine, 2, 8.

Grodzinsky, A.M., Grodzinsky, D.M. (1964) Short reference book on plant physiology, Kyiv: Nauk. Dumka.

Grumezescu, A.M., Holban, A.M., eds. (2017) Natural and artificial flavoring agents and food dyes, 7. Academic Press. DOI: https://doi.org/10.1016/C2016-0-00380-7

Humeniuk, O.L., Shupylo, K.O., Semeniuk, O.Y., Zinchenko, Y.S. (2013) Use of food coloring by the domestic producers of a food's industry. Herald of Chernihiv State Technological University. Series: Technical Sciences, 2, 244-248.

Ivanov, L.A., Ivanova, L.A., Ronzhina, D.A., Yudina, P.K. (2013) Changes in the chlorophyll and carotenoid contents in the leaves of steppe plants along a latitudinal gradient in South Ural. Russian Journal of Plant Physiology, 60 (6), 856-864. DOI: https://doi.org/0.7868/S0015330313050072

Khan, I.S., Ali, M.N., Hamid, R., Ganie, S.A. (2020) Genotoxic effect of two commonly used food dyes metanil yellow and carmoisine using *Allium cepa* L. as indicator. Toxicology Reports, 7, 370-375. DOI: https://doi.org/10.1016/j.toxrep.2020.02.009

Kiriziy, D.A., Vorobei, N.A., Kots, S.Ya. (2007) Relationships between nitrogen fixation and photosynthesis as the main components of the productivity in alfalfa. Plant Physiology, 54 (5), 666-671. DOI: https://doi.org/10.1134/S1021443707050032

Kolmakova, N.S. (2008) Recent studies in the field of safety of synthetic dyes. Market trends. Beer and Beverages, 5, 56-57.

Kononov, A.S., Shkotova, O.N. (2012) Influence of nitrogen fertilizer forms on chlorophyll content in single-species and mixed legume-cereal agrocenoses. Bulletin of the Bryansk State University, 4 (1), 103-106.

Kots, S.Ya., Morgun, V.V., Patyka, V.P., Malichenko, S.M., Mamenko, P.M., Kirizii, D.A., Mykhalkiv, L.M., Beregovenko, S.K., Melnikova, N.M. (2011) Biological nitrogen fixation: legume-rhizobial symbiosis. Vol. 2. Kyiv: Logos.

Kots, S.Ya., Vorobey, N.A., Kyrychenko, O.V., Melnykova, N.N., Mykhalkiv, L.M., Pukhtaievych, P.P. (2016) Microbiological Preparations for Agriculture, Kyiv: Logos.

Krutylo, D.V (2008) Nodule bacteria – heterotrophic and symbiotrophic ways of life. Agricultural Microbiology, 7, 147-161.

Kukol, K.P., Vorobey, N.A., Kots, S.Ya. (2020) Influence of carmoisine on growth energy and laboratory germination of soybean seeds. Scientific Reports of NULES of Ukraine, 85 (3). DOI: http://dx.doi.org/10.31548/dopovidi2020.03.003

Kukol, E., Vorobey, N., Pukhtaievych, P., Kots, S. (2021) The effect of biopreparations of nodules bacteria with carmoisine on the formation and functioning of the soybean – *Bradyrhizobium japonicum* symbiotic systems. Plant Varieties Studying and Protection, 17 (2), 123-129. DOI: https://doi.org/10.21498/2518-1017.17.2.2021.236517

Leulescu, M., Rotaru, A., Moanta, A., Iacobescu, G. E. (2021) Azorubine: physical, thermal and bioactive properties of the widely employed food, pharmaceutical and cosmetic red azo dye material. Journal of Thermal Analysis and Calorimetry, 143 (6), 3945-3967. DOI: https://doi.org/10.1007/s10973-021-10618-4

Lichtenthaler, H.K., Babani, F. (2004) Light adaptation and senescence of the photosynthetic apparatus. Changes in pigment composition, chlorophyll fluorescence parameters and photosynthetic activity. In: Papageorgiou, G. C., Govindjee, eds. Chlorophyll a fluorescence. Dordrecht: Springer, pp. 713-736. DOI: https://doi.org/10.1007/978-1-4020-3218-9_28

Mahmud, K., Makaju, S., Ibrahim, R., Missaoui, A. (2020) Current progress in nitrogen fixing plants and microbiome research. Plant, 9 (1), 1011. DOI: https://doi.org/ 10.3390/plants9010097

Matveeva, N.A., Kvasko, O.Yu. (2010) The content of photosynthetic pigments in transgenic chicory plants with gene of the tuberculosis antigen Esat6. Bulletin of Donetsk National University. Series A: Natural Sciences, 2, 249-253.

Netrusov, A.I., ed. (2005) Workshop on Microbiology. Moscow: Akademiya Publ.

Patyka, V.P., ed. (2003) Biological Nitrogen. Kyiv: Svit.

Patyka, V.P., Krutylo, D.V., Kovalevs'ka, T.M. (2004) Effect of aboriginal populations of soy nodule bacteria on symbiotic activity of introduced strain *Bradyrhizobium japonicum* 634b. Microbiological Journal, 66, 14-21.

Santos, M.S., Nogueira, M.A., Hungria, M. (2019) Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. AMB Express, 9 (1), 205. DOI: https://doi.org/10.1186/s13568-019-0932-0

Sarafanova, L.A. (2004) Nutritional supplements: Encyclopedia. 2nd edition, rev. Sankt-Petersburg: GIORD.

Scheer, H. (2004) Chlorophylls and carotenoids. In: Wiley Encyclopedia of Chemical Biology, John Wiley & Sons Inc., pp. 498-505. DOI: https://doi.org/10.1002/9780470048672.wecb083

Shlyk, A.A. (1971) Determination of chlorophyll and carotenoids in extracts from green leaves of higher plants. In: Pavlinova, O. A., ed. Biochemical Methods in Plant Physiology. Moscow: Nauka. pp. 154-170.

Smirnov, E.V. (2009) Food Dyes Handbook. Sankt-Petersburg: Professiya.

Soumare, A., Diedhiou A.G., Thuita, M., Hafidi, M., Ouhdouch, Y., Gopalakrishnan, S., Kouisni, L. (2020) Exploiting biological nitrogen fixation: a route towards a sustainable agriculture. Plant, 9 (8), 1011. DOI: https://doi.org/10.3390/plants9081011

Syvash, O.O., Mykhaylenko, N.F., Zolotareva, E.K. (2018) Variation of chlorophyll a to b ratio at adaptation of plants to external factors. The Bulletin of Kharkiv National Agrarian University. Series Biology, 3 (45), 49-73.

Tanaka, Y., Sasaki, N., Ohmiya, A. (2008) Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. The Plant Journal, 54 (4), 733-749. DOI: https://doi.org/10.1111/j.1365-313X.2008.03447.x

Tilba, V.A., Tishkov, N.M. (2016) Biology of soybean: possibilities to optimize the separate production processes. Oilseeds, 3 (167), 78-87.

Tsvetkova, Y. V., Lyashko, M. U., Strazhnikova, I. I. (2020) Affect of Rhizotorphin on chlorophyll content and productivity of introduced soybean cultivars. RUDN Journal of Agronomy and Animal Industries, 15 (1), 7-18. DOI: https://doi.org/10.22363/2312-797X-2020-15-1-7-18

Tyutereva, E.V., Dmitrieva, V.A., Voitsekhovskaja, O.V. (2017) Chlorophyll bas a source of signals steering plant development. Agricultural Biology, 52, 843-855. DOI: https://doi.org/10.15389/agrobiology.2017.5.843eng

Volkogon, V.V., ed. (2006) Microbial preparations in agriculture: theory and practice. Kyiv: Agrarna nauka.

Volodarez, S.O. (2012) Phytoncide activity in connection with the chlorophyll content in woody plant leaves in the urban environment. Industrial Botany, 12, 167-171.

Vorobey, N.A., Kukol, K.P., Kots, S.Ya. (2020) Fungicides toxicity assessment on *Bradyrhizobium japonicum* nodule bacteria in pure culture. Microbiological Journal, 82 (3), 45–54. DOI: https://doi.org/10.15407/microbiolj82.03.045

Wellburn, A.R. (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolutions. Journal of Plant Physiology, 144 (3), 307-313. DOI: https://doi.org/10.1016/S0176-1617(11)81192-2

Werner, D., Newton, W.E. (2005) Nitrogen fixation in agriculture, forestry and the environment. Dordrecht: Springer Science & Business Media.