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FACTORS WHICH INFLUENCE TOXICITY OF LEGUME SEED DISINFECTANTS TOWARDS BIOLOGICALS BASED ON SYMBIOTIC NITROGEN FIXERS

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Abstract

Symbiotic nitrogen fixers of *Rhizobiaceae* family serve as biologicals for agriculture. This is due to the fact that free-living inoculants which are not crop-specific possess much less nitrogen-fixing ability than the legume—rhizobial symbiosis of a plant and its species-specific symbiont. Despite this, the seedbed inoculation and a wider use of biopreparations of nodule bacteria in legumes are hampered by a number of objective deficiencies of such preparations, for example, the relatively low resistance of rhizobia to adverse environmental factors. These factors include direct contact of bacteria with aggressive substances, i.e. chemical fungicides used for seed treatment. This paper is the first to report that the rhizobia survival rate depends on the temperature of tank solutions and may differ under the effect of disinfectants based on the same active ingredient. That is, methods of disinfectant manufacture significantly affect its toxicity towards nodule bacteria. Our goal was to determine the effect of treaters, its concentration in the solution, the time the solution was kept and the temperature mode on the number of nodule bacteria of soybean, lupine, pea and lentils that survived in the solution. Bacterial suspensions studied were root nodule bacteria of soybean (*Bradyrhizobium japonicum* 634b), lupine (*Bradyrhizobium lupini* 367a), pea (*Rhizobium leguminosarum* 261b), lentil (*Rhizobium leguminosarum* 712), and chemical fungicides were Maxim (fludioxonil, 25 g/l; «Syngenta International AG», Switzerland), Protekt, (fludioksonil, 25 g/l; Agro Expert Group LLC, Russia, Agro Expert Group Kft., Hungary), Protekt Forte (fludioxonil, 40 g/l + flutriafol, 30 g/l; Agro Expert Group LLC, Russia, Agro Expert Group Kft., Hungary). Compatibility was determined by preparing tank solutions of biologicals and disinfectants, followed by determining the percentage of rhizobia that survived, depending on the type of disinfectant, its concentration (10 and 20 %), solution holding time (2, 4, 8 hours) and temperature (2-5, 16-18, 27 °C). Our results show that the resistance of nodule bacteria of various leguminous plants to these pesticides differs and decreases among the nodule bacteria of soybean, lupine, pea, lentils. The pesticide toxicity increases in the order Maxim, Protect, and Protect Forte. The presence of rhizobia in the same solution with disinfectants negatively affects the bacteria survival. The longer the mixture is kept, the less rhizobia remain alive. With increasing temperature of the mixture and the concentration of disinfectants in the solution, their toxicity increases. Low temperatures (2-5 °C) significantly increase the survival rate of rhizobia. The disinfectants Maxim and Protect, prepared on the basis of the same active ingredient with the same concentration, differed sharply in toxicity.

Keywords: symbiotic nitrogen fixers, *Bradyrhizobium*, *Rhizobium,* biologicals, seed dressing agents, treaters, compatibility and toxicity

Leguminous plants are the main source of vegetable protein [1]. The average yield of legumes in Russia is much lower (sometimes by several times) than in Europe and the USA [2, 3]. One of the significant reasons is the low efficiency of the technologies used in the majority of cases. The paradox of the situation is that in the harsh climatic conditions that are characteristic of most of the Russian agricultural land (Ural, Siberia), the need for the most modern farming practices increases by multiple times [4, 5]. These include, in particular, the use of preparations of symbiotic nodule bacteria, which, populating the plant's root system, provide it with the ability to fix atmospheric nitrogen [6-8]. In the Russian market, they are presented but have not yet been widely distributed. Among other reasons, there is a lack of substantiated regulations for the use of microbiological preparations in conjunction with chemical plant protection products. In practice, this inevitably reduces the efficiency and profitability of biopreparations, leads to direct economic losses and unjustifiably discredits the method, which is recognized as an important element of biologization, ecologization and increasing the sustainability of modern agricultural production.

The composition of disinfectants includes the substances that are toxic to microorganisms; therefore, the bacteria, on the basis of which microbiological preparations are made, face unfavorable conditions. Unfortunately, the study of the compatibility of biopreparations and disinfectants clearly lags behind the emergence of new strains that are potentially suitable for practice, forms of biopreparations [9, 10], and changes in the production technology of disinfectants under the same brand [11, 12]. The disinfectants (herbicides, fungicides, insecticides, etc.) have long been proved to be effective, the technologies for their application were tested [13, 14] and entrenched in domestic agriculture. Therefore, if there are doubts regarding the effectiveness of the joint use of biological and chemical preparations, in practice, the latter is preferred [15, 16]. In other words, the lack of scientific works on the assessment of the compatibility of microbiological and chemical methods for the treatment of seeds of leguminous plants [17, 18] may lead to the rejection of biopreparations, despite their environmental friendliness [19, 20], economic efficiency [21] and effect [22, 23] with an increase in the yield of leguminous plants. It should be noted that there are quite a few domestic publications on this issue [24, 25].

This study presents the first results confirming that the compatibility of inoculants and disinfectants based on the same active substance is significantly influenced by the method of manufacturing the disinfectant, that is, the qualitative and quantitative composition (formulation) of additional components (film-forming polymers, adjuvants, surfactants, etc., which, in the opinion of the manufacturers, improve the manufacturability of a disinfectant), as well as the temperature regime of the tank solution. These data supplement the limited amount of information concerning the compatibility of preparations of nodule bacteria and chemical products of protection of legumes.

The authors' goal was to determine the effect of treaters, their concentration in the solution, the time the solution was kept and the temperature mode on the number of nodule bacteria of soybean, lupine, pea, and lentils that survived in the solution.

Techniques. Strains of root nodule bacteria of soybean (*Bradyrhizobium japonicum* 634b), lupine (*Bradyrhizobium lupini* 367a), pea (*Rhizobium leguminosarum* 261b), and lentil (*Rhizobium leguminosarum* 712) were obtained from the departmental collection of useful microorganisms of agricultural purpose of the All-Russia Research Institute for Agricultural Microbiology (ARRIAM, St. Petersburg). The preparations were made on a semi-synthetic medium (0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l NaCl, 1.0 g/l of yeast extract, 10.0 g/l of mannitol) with subsequent cultivation (28 °C, 170 rpm, orbital shaker incubator ES-20/60, "BioSan", Latvia).

The following chemical fungicides were used: Maxim, SC (fludioxonil, 25 g/l; Syngenta International AG, Switzerland), Protekt, SC (fludioxonil, 25 g/l; Agro Expert Group LLC, Russia, Agro Expert Group Kft., Hungary), Protekt Forte, WSC (fludioxonil, 40 g/l + flutriafol, 30 g/l; Agro Expert Group LLC, Russia, Agro Expert Group Kft., Hungary).

Fungicides and nodule bacteria were mixed (20% solution of a bacterial suspension with 10% and 20% solutions of the disinfectant of each examined brand). After certain intervals (0, 1, 2, 4, and 8 hours), the titers of bacteria were determined by seeding on Petri dishes with semi-synthetic medium (the composition is shown above) with the addition of 20 g/l of agar-agar. The mixtures of cultures and fungicides were kept in a refrigerating chamber (2-5 °C), at room temperature under the laboratory conditions (16-18 °C) and in a thermostat (27.5 °C). After 10 days (time of growth of nodule bacteria on Petri dishes), the formed colonies (CFU) were counted.

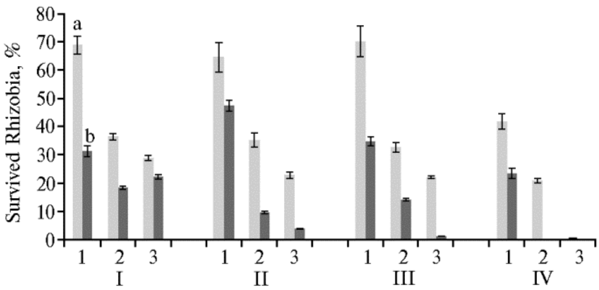

Fig. 1. The proportion of survived rhizobia *Bradyrhizobium japonicum* 634b (I), ***Bradyrhizobium lupini* 367a** (II), ***Rhizobium leguminosarum* 261b** (III) **and *Rhizobium leguminosarum* 712** (IV) **mixed with 10%** (a) **and 20%** (b) **solutions of fungicides Maxim** (1), **Protekt** (2) **and Protekt Forte** (3) (the mixture of cultures and fungicides was kept for 8 h at 16-18 °C).

Data were processed using the Microsoft Excel 10 software. To confirm the reliability of differences between the variants in the figures and in the table, mean values (M) and standard errors of the mean (±SEM) are presented. The differences were assessed by Student's *t*-test and considered statistically significant at $p < 0.05$. The repetition of the experiment is threefold.

Results. The selection of the strains of microorganisms was determined by the greatest practical importance of crops (soybean, lupine, pea, and lentils) in modern Russia and the CIS countries. In Russia, based on these strains, biopreparations are produced for legumes under the commercial name Rizotorfin® (manufactured by ARRIAM).

Mixing chemical disinfectants with rhizobia preparations negatively influenced the survival rate of the latter. The resistance of nodule bacteria of various leguminous plants to pesticides was different and decreased in the following order: nodule bacteria of soybean, lupine, pea, lentils. The toxicity of the pesticides increased in the following order: Maxim, Protekt, Protekt Forte (Fig. 1).

The survival rate of rhizobia in a mixture with disinfectants also significantly depended on the temperature at which the mixture was kept. The more toxic for rhizobia was the disinfectant (Fig. 2), the more clearly the positive effect of low temperatures on the survival of nodule bacteria was manifested. The role of the temperature factor grew with increasing concentration of the disinfectant. Thus, the proportion of surviving nodule bacteria of soybean in a mixture with a 10% Maxim fungicide solution 8 hours after the mixing at 2-5 °C and 16-18 °C was 72.02 and 68.88%, respectively. At the same time, the values of 65.73 and 31.12% were obtained for a 20% solution of the fungicide. The revealed pattern turned out to be valid for each examined biopreparation—disinfectant pair.

In some cases, the active ingredient of the fungicide was not the main factor determining the dynamics of reducing the number of rhizobia. For example, the Maxim disinfectant, which is very low-toxic for all studied rhizobia species, contains the same active ingredient and is in the same concentration as the much more toxic preparation Protekt (Table). At the same time, the toxicity of the Protekt fungicide for soybean and lupine rhizobia was comparable to

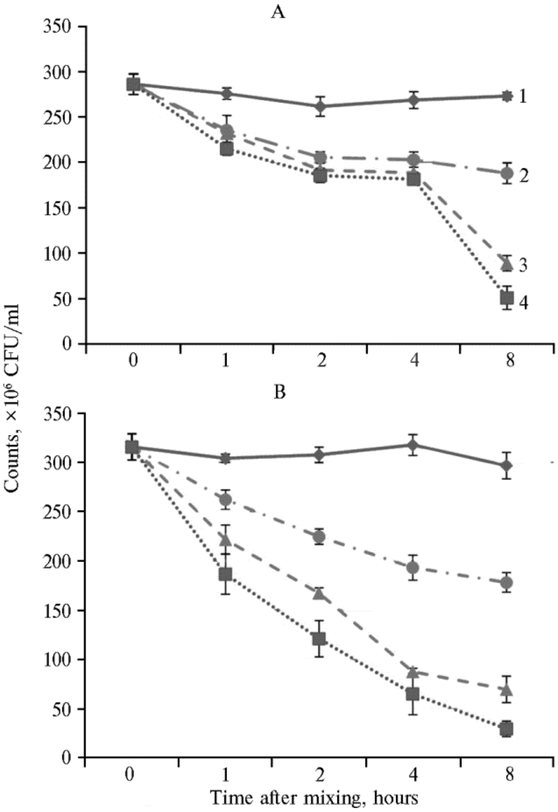

Fig. 2. The number of colonies of *Bradyrhizobium japonicum* 634b in solution with 20% Maxim (A) **and Protekt Forte** (B) **fungicides, depending on the exposure time and temperature of the mixture:** 1 — room temperature (control), 2 — 2-5 °C, 3 — 16-18 °C, 4 — 27.5 °C.

Protekt Forte, despite the fact that the latter has almost 2 times the concentration of fludioxonil and the second active ingredient, flutriafol (see Table). The control was a 20% working solution of bacterial suspensions in tap water; all the differences between the experimental and corresponding control variants are statistically significant at $p < 0.05$.

The analysis of domestic and foreign literature on the factors of toxicity of disinfectants for bacteria showed that the active substances of most disinfectants (in a pure form) were identified by researchers as toxic to rhizosphere microorganisms to some extent [26, 27], including nodule bacteria [28, 29]. It is reported [30] that contact of soybean rhizobia on inoculated seeds with such common fungicidal substances as captan and thiram (contact fungicides), as well as benomyl, carbendazim, difenoconazole and tebuconazole (systemic fungicides), causes a significant reduction in the numbers of viable bacteria.

The proportion of rhizobia surviving in a mixture with 10 and 20% solutions of fungicides, depending on the time from the moment of mixing (the mixture was kept at a temperature of 16-18 °C) (M±SEM)

Hours	Rhizobia and fungicide concentration							
	Bradyrhizobium				*Rhizobium*			
	japonicum 634		*lupini* 367a		*leguminosarum* 2616		*leguminosarum* 712	
	10 %	20 %	10 %	20 %	10 %	20 %	10 %	20 %
Maxim SC								
2	79.02±4.95	67.31±3.56	82.56±5.64	73.76±4.26	83.43±5.23	81.6±5.26	81.67±5.27	63.33±3.21
4	75.52±4.20	65.91±3.24	75.36±4.58	69.92±3.98	74.11±4.13	67.2±3.89	71.67±7.13	60.00±3.14
8	68.88±3.98	31.12±1.72	64.48±3.67	47.36±2.58	70.19±3.98	34.7±1.94	41.67±2.10	23.33±0.79
Protekt SC								
2	75.20±4.45	50.30±3.12	60.69±3.33	33.49±1.49	62.58±3.09	31.11±1.21	81.67±5.28	13.41±0.26
4	58.57±2.89	32.47±1.32	48.43±2.98	25.63±0.71	52.02±2.27	22.14±0.76	71.67±7.16	3.66±0.19
8	36.25±1.97	18.23±0.45	35.06±1.73	9.43±0.14	32.52±1.67	13.98±1.05	41.67±2.13	0.00
Protekt Forte WSC								
2	69.73±3.64	52.93±3.16	55.03±3.57	38.76±2.03	39.00±1.99	17.00±0.54	2.62±0.16	0.00
4	46.12±2.31	27.73±0.86	46.64±2.75	18.12±0.41	36.00±1.75	14.00±0.34	1.07±0.12	0.00
8	28.68±0.95	22.03±0.69	22.65±0.74	3.69±0.10	22.00±0.68	1.00±0.12	0.12±0.10	0.00

Note. All differences between the experimental and corresponding control variants are statistically significant at $p < 0.05$.

Not all active ingredients of disinfectants are unequivocally toxic in relation to all species and strains of rhizobia. Thus, in the work by Tariq *et al.* [31], pea rhizobia are defined as benzimidazole-resistant. According to another study [32], fludioxonil has a significant toxic effect on soybean rhizobia. The authors state [32] that the contact of soya rhizobia with fludioxonil on inoculated seeds significantly reduces the number of surviving bacteria as compared with the control 24 and 48 hours after the inoculation. The addition of alginate polymer to the inoculant significantly increased the survival rate of rhizobia in contact with fludioxonil [32]. It allows suggesting that, in the authors' experiment, the best survival rate of rhizobia mixed with the Maxim disinfectant compared to the Protekt disinfectant is not associated with the greater toxicity of the additional components in the composition of the latter, but with the protective effect on the polymer rhizobia in the Maxim preparation. This assumption is supported by the fact that some water-soluble polymers actually increase the overall resistance of rhizobia to adverse environmental conditions; in particular, the addition of sodium alginate and carboxymethylcellulose to the bacterial suspension significantly increases the shelf life of the bacterial preparation [33]. Apparently, not only the composition and concentrations of the active ingredients of the disinfectant are important but also the composition and concentrations of the additional components (film-forming polymers, surfactants, emulsifiers, antiseptics, etc.), that is, the so-called formulation of the preparative form of the disinfectant. A number of studies have confirmed the strongest influence of film-forming polymers, adjuvants, and surfactants on the survival of bacteria in biological preparations [34].

There are the reports that different brands of disinfectants [35] and different temperature regimes during storage of tank solutions significantly affect the survival of bacteria in such solutions. A number of studies have shown the ability of rhizobia to decompose pesticides [36], which, however, is quite common among rhizosphere microorganisms [37]. According to the available data [38, 39], the slow-growing rhizobia of soybean *Bradyrhizobium japonicum* and the fast-growing rhizobia of soybean *Sinorhizobium fredii* can grow on a mineral and plant agar medium with the addition of the production concentration of the fungicide Maxim. At the same time, the intensity of their growth is either not inferior to that in the control [38] or slightly decreases [39].

It should be noted that the absence of a clear toxic effect of a disinfectant with respect to rhizobia in a joint tank solution does not at all guarantee the prevention of negative consequences for nodulation [40, 41]. A number of works describe the inhibitory effect of the Maxim fungicide on the intensity of nodule formation in inoculated soybean plants [24], while seed disinfecting and inoculation were separated in time. At the same time, some authors highlight [39] that inoculation of soybean seeds with the treatment with Maxim fungicide provides more intensive nodulation, increase in the aerial mass and reliable yield increase, compared with inoculation "in its pure form".

Thus, it can be stated that among the studied rhizobia, the nodule soybean bacteria (*Bradyrhizobium japonicum* 634b) were the most resistant to the disinfectants, and the nodule lentil bacteria (*Rhizobium leguminosarum* 712) were the least resistant. In turn, among the used disinfectants, the least toxic for rhizobia was the fungicide Maxim, and the most toxic – Protekt Forte. The disinfectants Maxim and Protekt, prepared on the basis of the same active ingredient with the same concentration, differed sharply in toxicity. Probably, the toxicity of these fungicides for nodule bacteria is associated not only and not so much with the active substances in their composition but also with those additional components (film-forming polymers, surfactants, emulsifiers, antiseptics, etc.) that the manufacturers add to a disinfectant of this or that brand to improve its technological properties (formulation of the preparative form of the disinfectant). The presence of rhizobia in the same solution with disinfectants negatively affects the survival of bacteria: the longer the mixture was kept, the less amount of viable rhizobia remained. With increasing temperature of the mixture and the concentration of disinfectants in the solution, their toxicity increases. Low temperatures (2-5 °C) significantly increase the survival rate of rhizobia.

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