Determination of toxicity of various preparative forms of pesticidal fungicides for nodule bacteria inoculants

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Abstract. The aim of our study was to determine the effect of fungicide formulation, brand of fungicide, its concentration in the solution, holding time and temperature regime of the solution on the number of survivors of nodule bacteria of soybean, lupine, peas and lentils in a solution. Bacterial suspensions of soybean nodule bacteria (*Bradyrhizobium japonicum* 634b), lupine (*Bradyrhizobium lupini* 367a), pea (*Rhizobium leguminosarum* 261b), and lentils (*Rhizobium leguminosarum* 712) were studied. Wetting powders Benomil (active substance benomil 500 g/kg, LLC “Soyuzagrohimi”, Russia), Benorad (active substance benomil 500 g/kg, JSC “August”, Russia) and Fundazol (active substance benomil 500 g/l, LLC “Agro-Kemi”, Russia) and concentrates of suspension Maxim KS (active substance fludioxonil, 25 g/l; “Syngenta International AG”, Switzerland), Protect KS (active substance fludioxonil, 25 g/l; LLC “Agro Expert Group”, Russia), Protect Forte VSK (active substance fludioxonil, 40 g/l + flutriafol, 30 g/l; LLC “Agro Expert Group”, Russia) were studied as fungicidal disinfectants. Compatibility was determined after the preparation of tank solutions of biological products and fungicides, followed by an assessment of the percentage of surviving rhizobia depending on the brand of fungicide, its concentration (10 and 20%), the holding time of the solution (2, 4, 8 h) and the temperature regime of the solution (2-5, 16-18, 27 °C).

1 Introduction

Legumes are the main source of plant protein [1]. The average yield of legumes in Russia is much lower than in Europe and the United States [2-3]. This phenomenon is caused, among other things, by the lack of justified regulations for the effective use of microbiological preparations in combination with chemical plant protection products [4-8].

Unfortunately, the study of the compatibility of biological products and fungicides clearly lags behind the emergence of inoculants based on new strains [9-10] and changes in the technology of production of fungicides under the same brand [11-12]. Fungicides (herbicides, fungicides, insecticides, etc.) have long been shown to be effective, their

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application methods have been developed [13-14] and are fixed in domestic agricultural practice. Therefore, if there are doubts about the effectiveness of the combined use of biological and chemical preparations, agricultural producers give preference to the latter [15-16]. In other words, the lack of scientific works on assessing the compatibility of microbiological and chemical methods of processing leguminous seeds [17-18] may cause rejection of biological products, despite their environmental friendliness [19-20], cost-effectiveness [21] and efficiency [22-23].

Analysis of domestic and foreign literature on the toxicity factors of fungicides for bacteria has shown that the active substances of most fungicides are defined by researchers as more or less toxic to rhizosphere microorganisms [26-27], including nodule bacteria [28-29]. There is evidence [30] that the contact of soybean rhizobia on inoculated seeds with such common fungicides as captan and tiram (contact fungicides), as well as benomil, carbendazim, diphenconazole and tebuconazole (systemic fungicides) causes a significant reduction in the number of viable bacteria. According to work [32], fludioxonil has a significant toxic effect on soy rhizobia. The authors of the study claim that the contact of soy rhizobia with fludioxonil on inoculated seeds significantly reduces the number of surviving bacteria compared to the control 24 and 48 hours after inoculation. At the same time, not all active substances of fungicides are unambiguously toxic to all brands and strains of rhizobia - in work [31], the researcher identified pea rhizobia as resistant to benzimidazoles.

There are reports that different brands of fungicides [35] and different temperature regimes during storage of tank solutions have a noticeable effect on the survival rate of bacteria in such solutions. A number of studies have shown the ability of rhizobia to decompose pesticides [36], which, however, is a fairly common ability among rhizosphere microorganisms [37]. According to a number of data [38, 39], slow-growing soybean rhizobia *Bradyrhizobium japonicum* and fast-growing soybean rhizobia *Sinorhizobium fredii* are able to grow on a mineral-vegetable agar medium with the addition of a production concentration of the fungicide Maxim. The growth rate is either not inferior to the growth of bacteria in the control [38], or slightly reduced [39].

It is worth noting that the absence of an obvious toxic effect of the fungicide on rhizobia in a joint tank solution does not guarantee the absence of negative consequences for nodule formation in the field [40-42]. For example, a number of studies have described the inhibitory effect of the fungicide Maxim on the intensity of nodule formation in inoculated soybean plants [24], despite the fact that seed treatment and inoculation were separated in time. On the other hand, some authors indicate [39] that the treatment of soybean seeds with the Maxim fungicide with their simultaneous inoculation provides a more intensive nodule formation, an increase in aboveground mass, and a significant increase in yield compared to the variant where the seed treatment was carried out with a solution of only one inoculant.

This study is the first to present data confirming that the compatibility of inoculants and fungicides based on the same active substance is significantly affected by the method of production of the fungicide, i.e. the qualitative and quantitative composition of additional components, which, from the point of view of the manufacturer, increases the manufacturability of the drug itself - the so-called "formulation" of the fungicide (film-forming polymers, adjuvants, surfactants, etc.). Also in this study, the influence of the temperature regime of the tank solution on the survival of rhizobia inoculants was determined and the dynamics of bacterial death on seeds inoculated simultaneously with their treatment was determined. These data supplement the limited information that concerns the compatibility of preparations of nodule bacteria and chemical means of protecting legumes.

2 Materials and methods

Our aim was to determine the effect of formulation of the fungicide, brand of fungicide, its concentration in solution, the holding time of the solution and temperature regime on the dynamics of the death of nodule bacteria of soybean, lupine, peas and lentils in both the tank solution with pesticides, and treated by them seeds.

Strains of soybean nodule bacteria (*Bradyrhizobium japonicum* 634b), lupine (*Bradyrhizobium lupini* 367a), pea (*Rhizobium leguminosarum* 261b) and lentils (*Rhizobium leguminosarum* 712) were obtained from the departmental collection of useful agricultural microorganisms of the All-Russian Research Institute of Agricultural Microbiology (ARRIAM, Saint Petersburg). The choice of microorganism strains was determined by the greatest practical significance of crops (soy, lupine, pea, lentils) in modern Russia and the CIS countries. In Russia on the basis of these strains produce biological products for legumes under the commercial name Risotorphine® (manufacturer is ARRIAM). Bacterial cultures were prepared on a semi-synthetic medium (0.5 g/l K₂HPO₄, 0.2 g/l MgSO₄·7H₂O, 0.1 g/l NaCl, 1.0 g/l yeast extract, 10.0 g/l mannitol), followed by cultivation on a BioSan ES-20/60 shaker incubator, 170 rpm, Latvia. As a seed material for processing with varieties used soybean seeds of the Belgorodskaya 7 variety.

For the preparation of tank solutions with inoculants, the following chemical fungicides were used: wetting powders Benomil (active substance benomil 500 g/kg, LLC “Soyuzagrohimi”, Russia), Benorad (active substance benomil 500 g/kg, JSC “August”, Russia) and Fundazol (active substance benomil 500 g/l, LLC “Agro-Kemi”, Russia) and concentrates of suspension Maxim KS (active substance fludioxonil, 25 g/l; “Syngenta International AG”, Switzerland), Protect KS (active substance fludioxonil, 25 g/l; LLC “Agro Expert Group”, Russia), Protect Forte VSK (active substance fludioxonil, 40 g/l + flutriafol, 30 g/l; LLC “Agro Expert Group”, Russia). The choice of pesticide brands for the study was due to their widespread use in modern Russia and the CIS countries as fungicidal seed disinfectants for legumes.

Variants of working solutions of fungicides and nodule bacteria were mixed (20 % solution of bacterial suspension with 10 % and 20 % solutions of the fungicide of each test brand) and kept in a refrigerator (2-5 °C), at room temperature (16-18 °C) and in a thermostat (27.5 °C). At certain time intervals (0, 1, 2, 4, and 8 hours), a sample of each variant was taken, a series of dilutions was prepared, and sown on Petri dishes with agar nutrient medium (20 g/l agar-agar, 0.5 g/l K₂HPO₄, 0.2 g/l MgSO₄·7H₂O, 0.1 g/l NaCl, 1.0 g/l yeast extract, 10.0 g/l mannitol). After 10 days of thermostating, the number of colony-forming unit (CFU) of rhizobia was calculated and, thus, the titer of bacteria in each variant of the tank solutions was determined.

Inoculation of seeds with variants of tank solutions was performed as follows:

Preparation of a sample of seeds in a Petri dish in the amount of 25 g.

Inoculation of seeds in a Petri dish with a 20% solution of bacterial suspension in tap water (control version), or in a tank solution with a fungicide (experimental versions).

The number of viable bacteria per 1 inoculated seed was determined as follows:

Placing 8 inoculated seeds from a Petri dish in a test tube with 8 ml of pre-sterilized water, followed by shaking it on a vortex for 1 min.

Preparation of a series of consecutive dilutions of the received flushing from seeds with their subsequent sowing on Petri dishes with agar nutrient medium.

Calculation of the formed bacterial colonies typical for the studied bacterial culture. Using the resulting number to determine the number of bacteria per 1 inoculated seed.

Statistical data processing was performed using Microsoft Excel 10. To confirm the validity of the differences between the variants, the figures show the average values (M)

and standard errors of the averages ($\pm$ SEM). Differences were evaluated by the Student’s t-test and considered statistically significant at $p < 0.05$. The experience is repeated 3 times.

3 Results

It was determined that several brands of benomil - based wetting powders widely used in conjunction with inoculants were significantly toxic to soy rhizobia (fig. 1).

Fig. 1. Effect of 10% solutions of Fundazol, Benomil and Benorad fungicides on the survival of soybean rhizobia *B. japonicum strain 634b* in tank solution.

The graphs show that all 3 fungicides differ significantly in their toxicity to soy rhizobia. So, if 10% Fundazol 8 hours after the preparation of the tank solution reduces the number of bacteria by 3-3.5 times, then 10% Benomil and Benorad for 8 hours of contact with rhizobia reduce the number of the latter by almost an order of magnitude. In other words, all 3 wetting powders, despite the same active substance and its concentration in the fungicide, it was possible to build up a fairly definite series to increase toxicity for soy rhizobia: Fundazol, Benomil, Benorad.

Repeating a similar experiment with rhizobia lentils *R. Leguminosarum strain 2720*, revealed a greater sensitivity of this type of bacteria to the studied fungicides. Of all the variants of the experiment, the colony bacteria for counting were obtained only in the variants with the Fundazol fungicide and only for 4 hours of holding tank samples (fig. 2).

Fig. 2. Effect of 10% and 20% solutions of Fundazol fungicide on the survival of lentils rhizobia *R. leguminosarum* strain 634b in tank solution.

This provision confirms, firstly, the comparative low toxicity of the Fundazol fungicide for rhizobia (in comparison with other benomil - based fungicides), and secondly, the different resistance of different types of rhizobia to the same fungicides. For benomil – based powdered fungicides, soy rhizobia can be called resistant, and lentils rhizobia can be called sensitive.

Mixing of fungicidal concentrates of suspensions with rhizobia preparations also negatively affected the survival of the latter. The resistance of nodule bacteria of different legumes to this pesticide formulation was different and decreased in sequence: soybean rhizobia, lupine rhizobia, pea rhizobia, lentils rhizobia. The toxicity of pesticides increased in this order: Maxim, Protect, Protect Forte (fig. 3).

Fig. 3. The proportion of surviving rhizobia *B. japonicum* 634b (I), *B. lupini* 367a (II), *R. leguminosarum* 260b (III) and *R. leguminosarum* 712 (IV) in a mixture with 10 % (a) and 20 % (b) solutions of fungicides Maxim (1), Protect (2) and Protect Forte (3) (a mixture of cultures and fungicides was kept for 8 hours at 16-18 °C).

The results of the above graph show that the nature and concentration of the active substance of fungicide do not clearly determine the dynamics of reducing the number of

rhizobia. For example, the Maxim fungicide, which is the least toxic for all the studied rhizobia species, contains the same active substance in the same concentration as the much more toxic Protect fungicide. The toxicity of the Protect fungicide for soybean and lupine rhizobia was comparable to that of Protect Forte (see table 1), despite the fact that the latter concentration of fludioxonyl is almost 2 times higher, and there is also a second active substance — flutriaphol.

Table 1. The proportion of surviving rhizobia in a mixture with 10 and 20% solutions of fungicides (16-18 °C) depending on the time since mixing ($M\pm SEM$).

Time, h	Rhizobia and concentration of fungicide							
	<i>Bradyrhizobium</i>				<i>Rhizobium</i>			
	<i>japonicum</i> 634		<i>lupini</i> 367a		<i>leguminosarum</i> 261b		<i>leguminosarum</i> 712	
	10 %	20 %	10 %	20 %	10 %	20 %	10 %	20 %
Maxim, KS								
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
Protect, KS								
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
Protect Forte, VSK								
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

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

The more toxic the fungicide was for rhizobia, the more clearly the positive effect of low temperatures on the survival of nodule bacteria was manifested. The role of the

temperature factor increased with increasing concentration of the fungicide. Thus, the proportion of surviving soybean nodule bacteria in a mixture with 10% Maxim fungicide solution 8 hours after mixing at 2-5 °C and 16-18 °C was 72.02 and 68.88%, respectively. At the same time, values of 65.73 and 31.12% were obtained for 20% solution of fungicide. The revealed regularity was valid for each studied pair of biological product — fungicide.

The question of how the dynamics of rhizobia death changes in a mixture with a fungicide on treated seeds was studied by treating soybean seeds of the Belgorodskaya variety with joint tank solutions of inoculant *B. japonicum strain 634b* and fungicides Maxim and the Protect with subsequent preparation of flushes from seeds and their sowing on Petri dishes (fig. 5, 6).

Fig. 5. Dynamics of reduction in the number of CFU of *B. Japonicum strain 634b* per 1 soybean seed, when inoculated together with treatment by 10% and 20% solution of the fungicide Maxim.

Fig. 6. Dynamics of reduction in the number of CFU of *B. Japonicum strain 634b* per 1 soybean seed, when inoculated together with treatment by 10% and 20% solution of the fungicide Protect.

The fungicide Protect was expected to be more toxic to rhizobia than the fungicide Maxim. Interestingly, for the fungicide Protect in 10% concentration, there is a slight excess of the number of surviving rhizobia on the treated seeds compared to the control (up to 4 hours). Probably, the formation of the fungicide to some extent contributes to a more uniform application of bacteria to the seeds, as well as their certain fixation in polymer films.

In contrast to the Maxim fungicide, the concentration of the fungicide begins to play a significant role for fungicide Protect in relation to toxicity for rhizobia. The toxicity of fungicide Protect for rhizobia increases almost linearly with an increase in its concentration from 10% to 20% in the composition of the tank solution.

4 Conclusion

Summing up, we can say that among the studied rhizobia, soy nodule bacteria (*Bradyrhizobium japonicum* 634b) were the most resistant to fungicides, and lentils nodule bacteria (*Rhizobium leguminosarum* 712) were the least resistant. Wetting powders based on benomil (Fundazol, Benomil, Benorad) can be defined as incompatible with inoculants. The studied fungicidal suspension concentrates (Maxim, Protect, Protect Forte) showed their relative compatibility with inoculants, since soy rhizobia in a 20% solution of the most toxic Protect Forte perished only by 80% in 8 hours of contact. It is noteworthy that the powders Fundazol, Benomil, and Benorad, as well as the fungicides Maxim and Protect, prepared on the basis of the same active substance with the same concentration, sharply differed in their toxicity for all the studied rhizobia strains.

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 with those additional components (film-forming polymers, surfactants, emulsifiers, antiseptics, etc.) that a particular manufacturer adds to a particular brand of fungicide to improve its technological properties (formulation of the preparative form of the fungicide). It has been shown that the toxicity of pesticides for nodule bacteria increases with an increase in the concentration of fungicides and an increase in the temperature of tank solutions. Accordingly, reducing the time of contact between pesticides and inoculant bacteria and lowering the temperature of tank solutions (2-5 °C) significantly increases the survival rate of rhizobia. It was shown that the dynamics of death of soybean rhizobia in contact with the fungicides Maxim and Protect differs in the conditions of the tank solution and on the surface of the treated seeds. In soybean seeds inoculated together with treatment by 20% Protect Forte fungicide, the number of surviving rhizobia after 8 hours was only 2.5 times less than in the case of seeds treated with only one inoculant.

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