Comparison of resistance of soybean nodule bacteria strains to pesticide and osmotic stresses

Yu V Laktionov*, Yu V Kosulnikov and A P Kozhemyakov

All-Russian Research Institute of Agricultural Microbiology, Pushkin, St. Petersburg, Russia

* E-mail: Laktionov@list.ru

Abstract. The increasing areas under leguminous crops and the general increase in the intensification of production force agricultural producers to combine seed inoculation and dressing in one step, carrying it out a few days before sowing. In this regard, it is of practical interest to study the resistance of rhizobia strains of inoculants to osmotic and chemical stresses, i.e. the nature of the dynamics of their viability on seeds and in contact with pesticides. The stability of two strains of soybean nodule bacteria (B. japonicum 634 and B. japonicum H9) to osmotic and chemical stresses (fungicidal mordants) was studied. According to the results of the study, pesticidal protectants had different toxicity degrees for the studied strains, which allowed them to be arranged in order of increasing toxicity for rhizobia: Baisad, VSK; Tirada, SK; Oplot, VSK). Soybean rhizobium strain B. japonicum H9 is defined as more osmotically and chemically stable, i.e., more adapted to modern agricultural technologies of soybean cultivation, which ensures the presence of at least 2·10⁴CFU per 1 seed 9 days after inoculation, while the number of viable cells of strain B. japonicum 634b per 1 seed drops to 0 within 3 days after inoculation. Osmotic resistance of the strain allows for effective inoculation of seeds at least 9 days before sowing, and chemical resistance allows for effective combination of an inoculant based on this strain and all the pesticide protectants studied in this work into one working solution.

1. Introduction

Soy is a valuable technical and fodder crop capable of forming a nitrogen-fixing bean-rhizobial symbiosis with nodule bacteria [11,16]. Due to the activity of this symbiosis, legume agriculture can fully meet its need for nitrogen, which is expressed in an increase in plant yield and an increase in protein and oil content in the yield part [1,2].

A necessary condition for the formation of an effective legume-rhizobial symbiosis is the presence of an appropriate type of active and virulent nodule bacteria in the soil. Because soybean in many regions of Russia has been relatively recently included in crop rotations, there are often no corresponding microbial soybean symbionts in sufficient quantities in soils.

To guarantee the formation of active nodules on the roots of plants, such a technique as pre-sowing treatment of plant seeds with preparations of nodule bacteria is used. This technique is called artificial inoculation of plants [3,9,12,14]. Because rhizobia are not able to form protective cysts or spores, they are very sensitive to various physical and chemical stress factors that act on bacteria when they meet pesticidal protectants and when drying on the surface of treated seeds [4].
In several works, the active substances of the protectants (in pure form) have been identified by researchers as more or less toxic to rhizospheric microorganisms [5,19], including nodule bacteria [10]. It has been shown [6] that the contact of rhizobia with such common fungicidal substances as captan and thiram (contact fungicides), as well as benomyl, carbendazim, diphenocouazole, and tebuconazole (systemic fungicides) – negatively affects the viability of nodule bacteria.

It is also known that different strains of rhizobia can differ significantly in their resistance to osmotic stress. It was shown that the genetically determined structure of cellular exopolysaccharides of various strains of \textit{B. japonicum} determines the degree of their resistance to drying and the toxic effects of hydrogen peroxide and novobiocin [12]. There is evidence that the temperature during the drying of the culture of nodule bacteria significantly affects the viability of the latter [17], while the optimal temperatures for different types of rhizobia differ significantly. For example, it has been shown that \textit{S. meliloti} cells best tolerate drying at 37°C, and \textit{B cells. japonicum} is resistant to drying at 30°C [18]. Several researchers claim that drying is harmful not only and not so much in itself, but in combination with osmotic shock, which is associated with an increase in the concentration of salts in the drying solution [8]. It has been shown that different types of nodule bacteria can differ significantly in their resistance to elevated concentrations of salts [13]. The fact that some types of nodule bacteria in response to osmotic stress can secrete long-chain oligosaccharides is of interest, which may contribute to a better transfer of adverse conditions by nodule bacteria.

Accordingly, to increase the probability of the formation of effective nodules on the roots of leguminous crops, inoculant strains must be not only active and virulent, but also resistant to the stresses that bacteria may be exposed to in modern intensive farming systems. In other words, inoculant strains should be not only biologically effective, but also technologically advanced, i.e., adapted to atypical conditions to which bacteria may be exposed during the storage and use of biological products. To identify such promising strains, it is necessary to study collection microorganisms for their resistance to osmotic and pesticide stresses.

The purpose of the work was to compare the degree of resistance of soybean rhizobia of two strains to osmotic and pesticide stress by determining the dynamics of reducing the number of viable cells in contact with pesticide protectants and drying on inoculated seeds.

2. Materials and Methods

To obtain a liquid culture of nodule bacteria \textit{Bradyrhizobium japonicum} st. 634b and H9 (Departmental collection of useful microorganisms for agricultural purposes FSBSI All-Russian Research Institute of Agricultural Meteorology) cells were transplanted using a loop from a microbial colony into a flask with 250 ml of semi-synthetic nutrient medium based on monosaccharides, yeast extract, and salts (Table 1), followed by its thermostating for 7 days at 24°C on a shaking machine at 180 rpm.

| Medium component | Component concentration (g/l) |
|------------------|-------------------------------|
| Mannitol         | 10.0                          |
| Yeast extract    | 1.0                           |
| \(\text{K}_2\text{HPO}_4\) | 0.5                          |
| MgSO4·7H2O       | 0.2                           |
| NaCl             | 0.1                           |
| CaCO3            | Traces                        |

To study the toxic effects of pesticides on nodule bacteria of inoculants, various variants of work solutions (Table 2) were prepared at the rate of 10 liters of solution per 1 ton of seeds. The solutions contained a bacterial culture at a concentration of 2 l/t and a chemical protectant at two concentrations (manufacturer's recommended full application rate of the pesticide and half that).
The components of tank solutions were mixed in the following sequence: water, bacterial culture, protectant. After mixing, the solution samples were shaken on a vortex for 30 seconds. All operations were performed in a microbiological laminar flow.

The mixed samples were stored at room temperature in the dark. After certain time intervals (1, 4, 8 hours), several 10-fold dilutions of solution variants were sown on Petri dishes with agarized nutrient medium.

To determine the dynamics of the reduction in the number of viable cells, not only in a solution with a pesticide, but also on inoculated seeds, the seed material was treated with aqueous solutions of inoculants, followed by periodic preparation of washes from seeds and their sowing on Petri dishes.

Table 2. Variants of tank solutions in which the dynamics of reducing the number of viable rhizobia over time was studied

| Variant | AR | Norms, l/t | Holding time of tank solution, hour |
|---------|----|-----------|------------------------------------|
| Inoculant + Baisad (full consumption rate) | Bradyrhizobium japonicum; fludioxonyl, 30 g/l + protioconazole, 40 g/l + azoxystrobin, 15 g/l | 2.0 + 1.5 | 1.4.8 |
| Inoculant + Baisad (half consumption rate) | Bradyrhizobium japonicum; fludioxonyl, 30 g/l + protioconazole, 40 g/l + azoxystrobin, 15 g/l | 2.0 + 0.75 | 1.4.8 |
| Inoculant + Tirada (full consumption rate) | Bradyrhizobium japonicum; Thiram, 400 g/l + Diphenoconazole, 30 g/l | 2.0 + 2.0 | 1.4.8 |
| Inoculant + Tirada (half consumption rate) | Bradyrhizobium japonicum; Thiram, 400 g/l + Diphenoconazole, 30 g/l | 2.0 + 1.0 | 1.4.8 |
| Inoculant + Oplot (full consumption rate) | Bradyrhizobium japonicum; Diphenoconazole 90 g/l + tebuconazole, 45 g/l | 2.0 + 0.6 | 1.4.8 |
| Inoculant + Oplot (half consumption rate) | Bradyrhizobium japonicum; Diphenoconazole 90 g/l + tebuconazole, 45 g/l | 2.0 + 0.3 | 1.4.8 |

Seed treatment with different variants of tank solutions was carried out as follows:

1. seed attachments were prepared in Petri dish in the amount of 25 g;
2. seeds were treated with an aqueous solution of the bacterial suspension of the studied strain in a volume of 250 ml (control variants), or in a tank solution that included a protectant and an appropriate protective additive (experimental variants)

The experiment repetition is three-fold.

Statistical data processing was carried out using the Microsoft Excel 10 program. The figures show means (M) and standard errors of means (±SEM). The differences were evaluated by the Student's t-test and considered statistically significant at p < 0.05.

3. Results and Discussion
The studied strains are characterized by different nutrient requirements, which is expressed in a different titer of cultures grown on a standard mannitol-yeast medium. At the end of cultivation, the
titer of strain 634b, other things being equal, was almost 2 times greater ($4.23\cdot10^9$CFU per 1 ml) than the titer of the H9 strain ($2.15\cdot10^9$CFU per 1 ml) (Fig. 1). Accordingly, when preparing a working solution based on these cultures at the rate of 2 l/t, the titer of rhizobia strain 634b was about $8.46\cdot10^8$CFU per 1 ml, and the titer of the H9 strain was about $4.31\cdot10^8$CFU in 1 ml.

**Figure 1.** Titer of nodule bacteria *Bradyrhizobium japonicum* (strains 634b and H9) during their cultivation on nutrient medium (96 hours).

At the same time, strain H9 showed significantly greater osmotic resistance compared to strain 634b, which resulted in significantly greater cell viability on treated seeds. Viable rhizobia of strain 634b cannot be isolated from washes from seeds within 3 days after treatment, while strain H9 is characterized by the presence of at least $20\cdot10^3$CFU per 1 seed even 9 days after seed treatment (Fig. 2).

**Figure 2.** Titer dynamics of nodule bacteria *Bradyrhizobium japonicum* (strains 634b and H9) after application on soybean seeds (after 1-9 days).
Preparation and exposure of work solutions of cultures of both strains and a number of pesticidal protectants revealed the following patterns:

1. the studied pesticidal protectants differ in their toxicity for the studied strains;
2. as the pesticide concentration increases, its toxic effect against nodule bacteria increases;
3. the studied strains of rhizobia differ in their degree of resistance to chemical (pesticide) stress.

It is shown that 4 hours after the preparation of the work solution of bacterial cultures and the full rate of consumption of the pesticide Baisad, the titer of rhizobia strain 634b is reduced by almost 10 times (from $846 \cdot 10^6$CFU up to $75 \cdot 10^6$CFU in 1 ml), while the titer of the H9 strain drops by less than 4 times ($431 \cdot 10^6$CFU up to $70 \cdot 10^6$CFU in 1 ml) (Fig. 3).

**Figure 3.** Titer dynamics of nodule bacteria *Bradyrhizobium japonicum* (strains 634b and H9) in the same tank solution with different concentrations of the fungicide Baisad.

The pesticide Tirada was identified as a significantly more toxic pesticide for strain 634b, since after 4 hours of contact of rhizobia with the full rate of consumption of the protectant, the number of viable bacteria of this strain decreased by almost 30 times, while the titer of strain H9 decreased by no more than 4 times, which was typical for the variant with the pesticide Baysad (fig. 4).
Figure 4. Titer dynamics of nodule bacteria *Bradyrhizobium japonicum* (strains 634b and H9) in the same tank solution with different concentrations of the fungicide Tirada.

The pesticide Oplot was identified as the most toxic of all the pesticides studied, since at the full rate of protectant consumption after 4 hours of contact of strain 634b bacteria with the pesticide, the number of viable cells was about $10 \cdot 10^6$ CFU in 1 ml, i.e. decreased by more than 80 times. At the same time, under the same conditions, the titer of the H9 strain was about $30 \cdot 10^6$ CFU, i.e. fell no more than 12 times (Fig. 5).

Figure 5. Titer dynamics of nodule bacteria *Bradyrhizobium japonicum* (strains 634b and H9) in the same tank solution with different concentrations of the fungicide Oplot.
4. Discussion
In modern agricultural technologies, the question of the joint use of microbiological preparations and plant protection products is increasingly being raised.

The pesticides studied differ in varying degrees of toxicity for nodule bacteria inoculants. The most toxic of the studied brands of protectants is the Oplot fungicide, the least toxic is the Baisad fungicide, the Tirada fungicide occupies an intermediate position in its toxicity for rhizobia. At the same time, strain $H9$ is more resistant to chemical stress than strain $634b$, which is expressed in the presence of at least $30 \times 10^6$CFU in 1 ml of the work solution with the full norm of the Oplot fungicide, while under the same conditions the titer of strain $634b$ was no more than $10 \times 10^6$CFU per 1 ml. Accordingly, the $H9$ strain is more technologically advanced compared to the $634b$ strain, i.e. more suitable for combining with existing intensive agricultural technologies of soybean cultivation.

5. Conclusion
Thus, it was shown that the studied strains of nodule bacteria $B. japonicum$ st. $634b$ and $H9$ are characterized by different resistance to osmotic and chemical stresses. The $H9$ strain is significantly more osmotically stable, which ensures the presence of at least $20 \times 10^3$CFU per 1 seed 9 days after inoculation, while the number of viable cells of strain $634b$ per 1 seed drops to 0 after 3 days after inoculation. Therefore, the $H9$ strain is preferable for the development of microbiological preparations, which will allow combining the inoculation process with chemical fungicide treatment.

Acknowledgments
The article was made with support of the Ministry of Science and Higher Education of the Russian Federation in accordance with agreement № 075-15-2022-320 date 20.04.2022 on providing a grant in the form of subsidies from the Federal budget of Russian Federation. The grant was provided for state support for the creation and development of a World-class Scientific Center “Agrotechnologies for the Future”.

References
[1] Zavalin A A, Blagoveshchenskaya G G, Kozhemyakov A P 2006 Contribution of legumes to the supply of biological nitrogen and organic matter to the soils of Russia In the collection: Innovation and technological foundations of the development of agriculture (Kursk) 312-315
[2] Kokorina A L, Kozhemyakov A P 2010 Bean-rhizobial symbiosis and the use of microbiological preparations of complex action is an important reserve for increasing the productivity of arable land (St. Petersburg)
[3] Kosulnikov Yu V, Laktionov Yu V 2018 On factors affecting the toxicity of seed protectants for symbiotic nitrogen fixers in the composition of biological products Agricultural Biology 53(5) 1037-1044 doi: 10.15389/agrobiology.2018.5.1037rus
[4] Laktionov Yu B, Kosulnikov Yu V, Dudnikova D V 2018 The effect of water-soluble polymers on the survival of lupin nodule bacteria (RHIZOBIUM LUPINI) Grain farming in Russia 3 17-26. https://doi.org/10.31367/2079-8725-2018-57-3-22-26
[5] Alam S, Kumar A, Kumar A, Prasad S, Tiwari A, Srivastava D, Srivastava S, Tiwari P, Singh J, Mathur B 2018 Isolation and Characterization of Pesticide Tolerant Bacteria from Brinjal Rhizosphere Int.J.Curr.Microbiol.App.Sci. Special Issue 7 4849-4859
[6] Campo R J, Araujo R S, Hungria M 2009 Nitrogen fixation with the soybean crop in Brazil: Compatibility between seed treatment with fungicides and Bradyrhizobial inoculants SYMBIOSIS 48 154–163
[7] Chang W S, Park K M, Koh S C, So J S 2008 Characterization of the Bradyrhizobium japonicum galE gene: Its impact on lipopolysaccharide profile and nodulation of soybean FEMS Microbiol. Lett. 280 242–249
[8] Chen M, Alexander M 1973 Survival of soil bacteria during prolonged desiccation Soil Biol. Biochem. 5 213–221
[9] Daba S, Haile M 2002 Effects of rhizobial inoculant and nitrogen fertilizer on yield and nodulation of common bean under intercropped conditions. *J. Plant Nutr.* 25 1443-1445

[10] Deshmukh V V, Raut B T, Mane S S, Ingle R W, Josh M S 2014 Compatibility of Bradyrhizobium japonicum isolates with agrochemicals. *American International Journal of Research in Formal, Applied & Natural Sciences*, March-May 6(1) 55-62

[11] De Micco V, Buonomo R, Paradiso R, De Pascale S, Aronne G 2012 Soybean cultivar selection for Bioregenerative Life Support Systems (BLSSs): theoretical selection. *Advances in Space Research* 49 1415-1421 (doi: 10.1111/plb.12056)

[12] Głodowska M, Schwinghamer T, Husk B, Smith D 2017 Biochar based inoculants improve soybean growth and nodulation. *Agricultural Sciences* 8(9) 1048-1064 (doi: 10.4236/as.2017.89076)

[13] Miller K J, Wood J M 1996 Osmoadaptation by rhizosphere bacteria. *Annu. Rev. Microbiol.* 50 101-136

[14] Mulas D, Seco V, Casquero P A, Velázquez E, González-Andrés F 2015 Inoculation with indigenous Rhizobium strains increases yields of common bean (Phaseolus vulgaris L.) in northern Spain, although its efficiency is affected by the tillage system. *Symbiosis* 67 113–124

[15] Noh J G, Jeon H E, So J S, Chang W S 2015 Effects of the Bradyrhizobium japonicum waaL (rfaL) Gene on Hydrophobicity, Motility, Stress Tolerance, and Symbiotic Relationship with Soybeans. *Int. J. Mol. Sci.* 16 16778–16791

[16] Regar M K, Meena R H, Jat G, Mundra S L 2017 Effect of different rhizobial strains on growth and yield of soybean [Glycine max (L.) Merrill]. *International Journal of Current Microbiology and Applied Sciences* 6(11) 3653-3659 (doi: 10.20546/ijcmas.2017.611.427)

[17] Sleesman J P, Leben C 1976 Bacteria desiccation: effect of temperature, relative humidity, and culture age on survival. *66* 1334-1338 p.

[18] Yadav J, Verma J P, Rajak V K, Tiwari K N 2011 Selection of effective indigenous *Rhizobium* strain for seed inoculation of chickpea (*Cicer aritenium* L.) production. *Journal of Bacteriology* 1 24-30

[19] Yousaf S, Khan S, Aslam M T 2013 Effect of Pesticides on the Soil Microbial Activity. *Pakistan J. Zool* 45(4) 1063-1067