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PRE-SOWING PROTECTION OF INOCULATED SOYBEAN *Glycine max* (L.) Merr. SEEDS BY WATER-SOLUBLE POLYMER COMPOSITIONS AND THEIR SOLID-PHASE MODIFICATION

Yu.V. LAKTIONOV[1], Yu.V. KOSULNIKOV[1], D.V. DUDNIKOVA[1], V.V. YAHNO[2], A.P. KOJEMYAKOV[1]

[1]*All-Russian Research Institute for Agricultural Microbiology,* 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail laktionov@list.ru, kullavayn@gmail.com (✉ corresponding author), vyachno@yandex.ru, kojemyakov@rambler.ru;
[2]*Ekos Branch, All-Russian Research Institute for Agricultural Microbiology,* 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail vyachno@yandex.ru
ORCID:
Laktionov Yu.V. orcid.org/0000-0001-6241-0273
Kosulnikov Yu.V. orcid.org/0000-0003-1134-3503
Dudnikova D.V. orcid.org/0000-0002-4319-1957
Yahno V.V. orcid.org/0000-0001-7953-3405
Kojemyakov A.P. orcid.org/0000-0002-9657-2454
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Abstract

The effectiveness of some water-soluble polymers as film-forming agents that provide better adhesion of bacteria to seeds (like multicomponent formulations in modern chemical dressings) remains practically relevant. The likely candidate adhesives are low and high molecular weight sodium alginate (FMC polymer), hydroxypropyl methylcellulose (HPMC) (Colorcon®, Colorcon, Inc., USA), polyethylene glycol (PEG), carbomer-carbopol 940 (Necardis SA), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (povidone, PVP) (K15). Film-forming polymers can also improve the shelflife of biologicals, their compatibility with chemical protective agents and resistance to UV radiation, temperature extremes and drying, thus increasing survival of bacteria on the surface of inoculated seeds. These allow practitioners to carry out seed pre-sowing inoculation beforehand. Developed polymer compositions should be more effective than single-component, provided that they remain cost-effective and convenient for practical use. This paper is the first to report the effects of various polymer combinations on inoculated seeds and the improvement of protective properties of the water-soluble polymers by activated charcoal, a solid-phase component. Among the polymers tested, polyvinylpyrrolidone is revealed to be the most effective for rhizobial survival due to longer allowable interval between seed inoculation and sowing. Our objective was to compare survival rate of *Bradyrhizobium japonicum* 634b inoculum for soybean cv. Belgorodskaya 7 seeds as influenced by water-soluble polymers polyvinylpyrrolidone, polyvinyl alcohol, sodium alginate and carboxymethylcellulose as additives. Our findings indicate that 10 % polyvinylpyrrolidone solution is the most effective among the studied polymers. Its use increases more than 10-fold the survival of nodule bacteria on seeds 10 days after inoculation of seed material. Variants with different concentrations of carboxymethyl cellulose and sodium alginate do not ensure bacterial survival on seeds for more than 3 days. It is possible to create an effective polymer-carbon composition with a lower concentration of polyvinylpyrrolidone (7.5 % polyvinylpyrrolidone and 5.0 % activated charcoal). This composition is more effective than polyvinylpyrrolidone without coal, and provides a 20-30 % reduction in bacterial death on inoculated seeds after the first 5-7 days of seed storage.

Keywords: symbiotic nitrogen, *Bradyrhizobium japonicum*, inoculation, soybean, polyvinylpyrrolidone, polyvinyl alcohol, sodium alginate, carboxymethylcellulose

Soybean is a valuable leguminous crop and is of vast food, fodder and agricultural importance [1-3]. Soybean seeds are rich in easily digestible protein (up to 39-42%) and valuable oil (up to 18-23%), and its herbage harvested not later than the bean plumpness phase is a nutritious (22-23 fodder units per 100 g herbage) and vitamin-rich (50-60 mg of carotene per 1 kg herbage) fodder [4, 5]. Due to intensive nitrogen fixation and high crop management practices, soy-

bean plays a positive environment-forming role in crop rotation and considerably increases the companion crop yield [6, 7]. Soybean is considered a good predecessor for cereals, tilled and fodder crops. From the agrotechnological point of view, soybean is very plastic, and, depending on agricultural, soil and climatic conditions, can be cultivated both as cereal and as tilled crop by varying within the extensive range the fertilization and protector application rates [8, 9]. A wide diversity of varieties with different earliness and demands on growth factors allows the soybean plant to easily adapt to growing conditions. All of the above allows us to view soya as a multipurpose crop [10].

An important agrobiological feature of soybean is its capability to form nitrogen fixing legume-rhizobia symbiosis [11, 12]. Such symbiosis completely provides the needs of plant in nitrogen [13], however such an intensive nitrogen fixation is possible only in optimal conditions, particularly if active virulent symbiont bacteria are present in soil in an amount sufficient for effective symbiosis [14, 15]. Normally, it can only be achieved through artificial pre-seeding inoculation of soybean with nodule bacteria [16, 17]. It is important not only to choose the right strain of bacteria, which will be most effective in appropriate soil and climatic conditions, but also to properly inoculate the seeds with such a preparation, which, for inoculants used today, means, among other things, mandatory seeding-down of treated seeds on the treatment day [18].

In practice, it is this requirements that is the most difficult for major farms and often neglected. Typically, it results in considerable reduction of effect of inoculation, which is manifested in poor nodulation and further nitrogen deficiency and hence in considerable underharvesting. Therefore, the researchers lately pay their attention to development of methods enabling the increase in the number of nodule bacteria on inoculated seed at the time of its seeding. One of the most promising ways is to combine polymer solutions acting as adhesives, film-formers and rhizobia protectors on treated seeds with inoculant [19, 20]. Polymer solutions must, first, fix the bacteria in polymer films, thus preserving the largest possible number of bacteria on treated seeds, and second, enhance bacterial resistance to adverse environmental factors, such as desiccation, sunlight, rapid temperature changes and seed exudates toxic for rhizobia. In combination, such properties contribute to increase of period between seed inoculation and seeding [21, 22].

The most widespread polymer adhesives in agricultural practice are polyvinylpyrrolidone (povidone), polyvinyl alcohol, sodium alginate and carboxymethylcellulose [23], however their effectiveness as rhizobia protectors is poorly studied. Of particular urgency is the issue of effectiveness of a polymer and its optimal concentrations, as well as recommendations for its application as bacteria protector for a particular rhizobia strain-variety pair.

In this paper, having analyzed the effects of a number of water soluble polymers, we identified polyvinylpyrrolidone as the most effective rhizobia protector (it extends the allowable interval between seed inoculation and sowing) and for the first time demonstrate that a solid-phase component (activated charcoal) improves the protective properties of polymer composition upon preseeding inoculation.

The purpose of this work was the investigation of temporal dynamics of viable rhizobia counts on inoculated soybean seeds as affected by various polymers in different concentration and by the mixes of different components, and also upon optimization of polymer protectors with activated charcoal.

Techniques. In order to obtain *Bradyrhizobium japonicum* preparation, 634b strain (Departmental Collection of Beneficial Agricultural Microorganisms of All-Russian Research Institute of Agricultural Microbiology) was grown in

liquid semisynthetic medium for 1 week at 28 °C on shaker [24].

As polymeric additives to the inoculate, water-soluble polymers were used, i.e. polyvinylpyrrolidone (Sigma-Aldrich, USA), PVA polyvinyl alcohol grades 4-98, 4-88 (Sigma-Aldrich, USA), sodium alginate (Xiamen Huaxuan Gelatin Co., Ltd, China) and carboxymethylcellulose (ZAO Karbokam, Russia). Activated charcoal powder (grade OU-A, OAO Sorbent, Russia) was used as solid-phase component for polymer protectors. Belgorodskaya 7 cv. soybean seeds were inoculated with the preparation in the following way. *B. japonicum* 634b strain (0.25 ml of 20% suspension) was applied to seed portions (25 g) in Petri dishes. The dishes containing inoculated seeds were stored at room temperature in the dark, with periodical collection of seed portions to prepare swabs (for the first time, 1 hour after inoculation, then after 27, 72, 124, 168 and 240 hours).

For quantitation of viable bacteria on a single soybean seed, 8 inoculated seeds from the Petri dish were placed in a test tube containing 8 ml of sterile water and shaken on a vortex for 1 minute. Tenfold dilutions of swabbed sample were prepared followed by plating onto agar-based nutrient 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 yeast extract, 10.0 g/l mannitol, 16 g/l agar-agar; pH 6.8-7.2) in Petri dishes for incubation at 28 °C and counting of the colonies formed. Based on the bacteria titer in the sample, their count was determined per 1 inoculated seed. The experiment was carried out in 4 biological replicates.

Statistical processing was carried out using Microsoft Excel 10. The figures and tables present mean (M) and standard error of the mean (±SEM). Differences were evaluated by Student's t-test and were considered statistically significant at $p < 0.05$. Analysis of variance was carried out according to Dospekhov [25]. In variants where the polyvinylpyrrolidone was used as protector, the difference from control group exceeded the least significant difference LSD_{05} values and was statistically significant.

Results. Soybean seed surface is an adverse medium for *B. japonicum* (Fig. 1, A). The same figure shows the curve of reduction (RC) of alive rhizobia upon treatment of soybean seeds with bacterial suspension with 100 g/l polyvinylpyrrolidone. Polyvinylpyrrolidone was chosen as a base for protector due to a number of works attributing the polyvinylpyrrolidone such traits as high adhesiveness, water retention, ability to protect bacteria from toxic seed exudates and to enhance overall viability of bacteria on inoculated seeds [22].

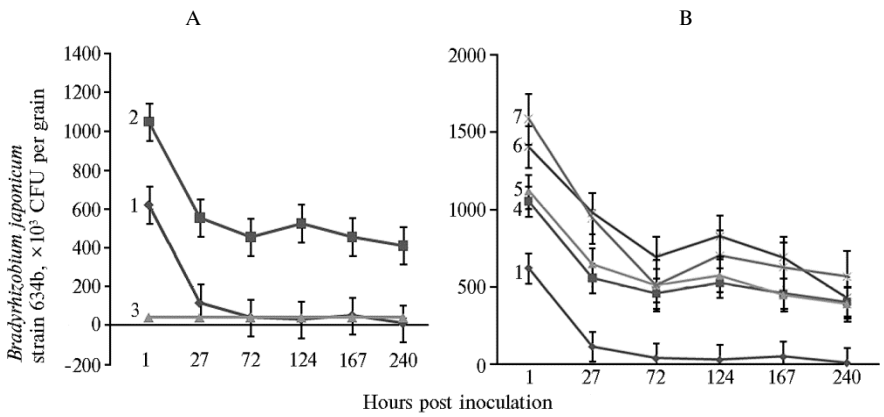

Fig. 1. ***Bradyrhizobium japonicum*** **bacteria strain 634b survivability on inoculated Belgorodskaya 7 soybean seeds in the presence of polyvinylpyrrolidone** (A) **and in its combination with activated char-**

coal within povidone-charcoal compounds (B): 1 — control, 2 — polyvinylpyrrolidone (100 g/l), 3 — number of viable rhizobia per 1 seed required for forming effective symbiosis, 4 — polyvinylpyrrolidone (100 g/l) + activated charcoal (0 g/l), 5 — polyvinylpyrrolidone (100 g/l) + activated charcoal (25 g/l), 6 — polyvinylpyrrolidone (100 g/l) + activated charcoal (50 r g/l), 7 — polyvinylpyrrolidone (100 g/l) + activated charcoal (75 g/l) (lab test).

The testes revealed that adding polyvinylpyrrolidone materially improves the survivability of soybean rhizobia on inoculated seeds. Thus, in control group (CG), the number of viable bacteria on seeds exceeds the threshold required for effective symbiosis (about 40 000 CFU per seed) in 72 hours. With polyvinylpyrrolidone, CG drops to a stable level of 500 000 CFU per seed in 24 hours and does not suffer any considerable drops for at least 10 days.

Successful practice of modification of polymer solutions was described, particularly, in a paper dedicated to influence of ZnO and MgO additives on the effects of carboxymethylcellulose as a compound promoting survivability of soybean nodule bacteria during storage in liquid culture [26]. In our research, adding to the polyvinylpyrrolidone solution of solid-phase filler (activated charcoal) also enabled us to somewhat increase the effectiveness of composition based on polyvinylpyrrolidone (see Fig. 1, B). Optimal charcoal concentration in the final solution was 50 g/l.

Similarly, the optimal concentration of polyvinylpyrrolidone (75 g/l) in combination with activated charcoal (50 g/l) in a povidone-charcoal composition was identified (Fig. 2).

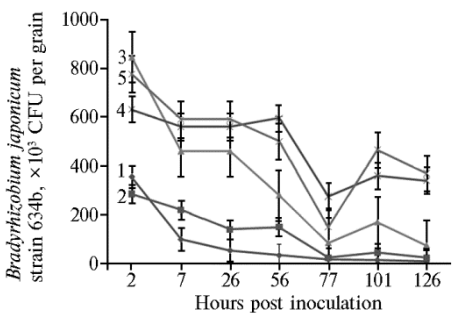

Fig. 2. Survivability of *Bradyrhizobium japonicum* 634b strain on inoculated seeds of soybean Belgorodskaya 7 variety depending on povidone-charcoal composition: 1 — control, 2 — polyvinylpyrrolidone (25 g/l) + activated charcoal (50 g/l), 3 — polyvinylpyrrolidone (50 g/l) + activated charcoal (50 g/l), 4 — polyvinylpyrrolidone (75 g/l) + activated charcoal (50 g/l), 5 — polyvinylpyrrolidone (100 g/l) + activated charcoal (50 g/l) (lab test).

Thus, our experiments showed high effectiveness of polyvinylpyrrolidone as a polymer base for rhizobia protector, though its relatively high cost considerably limits the opportunities for industrial production of polyvinylpyrrolidone-based protectors. Therefore, we have compared a number of analogous inexpensive polymers widespread in agriculture as adhesive that can potentially substitute (in full or in part) expensive polyvinylpyrrolidone in povidone-charcoal mixture.

One of such analogs is polyvinyl alcohol that is described as a seed-encapsulating polymer promoting the resistance of nodule bacteria to stress-factors [27]. Polyvinyl alcohols of both grades were studied according to the methodology similar for studying polyvinylpyrrolidone (Table 1). It turned out that while polyvinyl alcohol improves the survivability of rhizobia on seeds, it is materially inferior to polyvinylpyrrolidone in effectiveness.

We also have tested carboxymethylcellulose and sodium alginate as protectors. These polymers were elected due to the reports of their propitious effect on liquid culture of nodule bacteria during storage [23]. For sodium alginate, a capability of sustaining the viability of growth-promoting bacteria on inoculated glass beads for over 14 years was reported [28]. In our experiments, however, the effect of application of carboxymethylcellulose and sodium alginate as bacteria protectors

was rather insignificant (Table 2).

1. Survivability of *Bradyrhizobium japonicum* 634b strain on inoculated seeds of soybean Belgorodskaya 7 variety when polyvinyl alcohol is applied (10^3 CFU per one seed, $N = 4$, lab test)

Hours after inoculation	Variant					
	control	50 g/l	75 g/l	100 g/l	200 g/l	LSD_{05}
		Polyvinyl alcohol PVA 4-98				
1	255±12.1	300±13.4	470±18.2	580±23.6	845±31.7	35.40
19	25±1.1	25±1.2	30±1.7	55±2.1	105±4.2	5.08
66	0	0	0	5±0.9	20±1.4	2.45
93	0	15±0.7	0	0	0	0
		Polyvinyl alcohol PVA 4-88				
3	85±3.3	180±4.2	255±6.7	305±7.4	425±9.5	8.97
50	0	0	0	0	30	0
76	0	0	0	0	10	0

2. Survivability of *Bradyrhizobium japonicum* 634b strain on inoculated seeds of soybean Belgorodskaya 7 variety when carboxymethylcellulose or sodium alginate are applied (10^3 CFU per one seed, $N = 4$, lab test)

Hours after inoculation	Variant					
	control	carboxymethylcellulose		sodium alginate		LSD_{05}
		25 g/l	50 g/l	25 g/l	50 g/l	
3	105±3.7	155±4.6	175±4.2	215±8.4	478±11.7	10.58
50	25±3.4	98±6.9	150±8.4	200±12.1	153±9.8	20.26
76	0	5±0.9	34±1.7	9±1.2	71±3.1	1.04

In addition, it should be noted that sodium alginate and particularly carboxymethylcellulose make the solutions much more ***viscous*** than polyvinylpyrrolidone does in the same concentrations hence these polymers are not only ineffective as rhizobia protectors but also much less feasible practically. Other authors in their papers also demonstrate that sodium alginate and carboxymethylcellulose that successfully support the bacteria survivability in liquid culture during storage were ineffective protectors on seeds [23]. In the same study, polyvinylpyrrolidone was an extremely effective polymer protector of rhizobia on seeds, but inhibited bacteria stored in liquid culture [23]. At the same time it had been reported that in 4% concentration polyvinylpyrrolidone successfully protects *Azotobacter vinelandii* cells in liquid culture during prolonged storage [29], and salutary effect of polyvinylpyrrolidone on the stored nodule bacteria culture was reported [30], which may indicate the species (strain) specificity of effect of the polymer on bacterial cultures.

Given the high cost of polyvinylpyrrolidone, we have studied the possibility of its at least partial substitution with sodium alginate and carboxymethylcellulose in polymer-charcoal composition (Fig. 3).

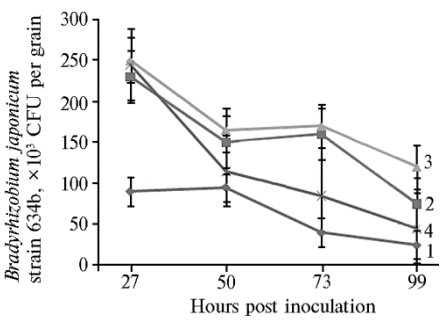

Fig. 3. Survivability of *Bradyrhizobium japonicum* 634b strain on inoculated seeds of soybean Belgorodskaya 7 variety depending on partial substitution of polyvinylpyrrolidone for carboxymethylcellulose or sodium alginate in povidone-charcoal composition: 1 — control, 2 — polyvinylpyrrolidone (25 g/l) + activated charcoal (50 g/l) + , 3 — polyvinylpyrrolidone (25 g/l) + activated charcoal (50 g/l) + carboxymethylcellulose (50 g/l), 4 — polyvinylpyrrolidone (25 g/l) + activated charcoal (50 g/l) + sodium alginate (50 g/l) (lab test).

Adding of sodium alginate to povidone-charcoal composition has reduced the latter's effect to some extent, and carboxymethylcellulose has almost not affected

its effectiveness, which shows that polyvinylpyrrolidone within povidone-charcoal composition cannot be substituted with another polymer even partially.

Thus, soybean seed surface is an adverse medium for *Bradyrhizobium japonicum* strain 634b. The count of these bacteria per inoculated seed drops from 620 000 (1 hour after treatment) to 115 000 (27 hours after treatment). Bacteria destruction can be considerably slowed down through use of polymeric additives to inoculants. The most effective protector of rhizobia among the studied polymers is polyvinylpyrrolidone. When applied in concentration of 100 g/l, 500 000 viable rhizobia remained on a single seed for 10 days. Polymeric basis of this protector may be modified with activated charcoal, which enhances the effects of povidone-charcoal composition 1.5-2.0 times (optimal concentration of povidone in the composition is 75 g/l, concentration of activated charcoal is 50 g/l). Potential full or partial replacements of povidone (polyvinyl alcohol, sodium alginate, carboxymethyl cellulose) in the proposed composition are ineffective.

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