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Survival of rhizobia on seeds, nodulation and growth of soybean as influenced by synthetic and natural seed-applied fungicides

Stefan Martyniuk, Monika Kozieł, Anna Gałązka

Department of Agricultural Microbiology, Institute of Soil Science and Plant Cultivation – State Research Institute
ul. Czartoryskich 8, 24-100 Puławy, Poland

Abstract. Soybean seeds (cv. Aldana) were treated with a synthetic seed dressing composed of carboxin (20%) and thiuram (20%) as the active ingredients or with a natural fungicidal preparation containing 23.8% of natural oil extracted from tea tree, and next day untreated (control) and treated with the above mentioned preparations soybean seeds were pelleted with inoculant containing *Bradyrhizobium japonicum* – the symbiotic bacterium of this plant. Shortly (within 1–2 hours) after seed inoculation and after 24 hours of storing the inoculated seeds at room temperature seed samples were collected to count rhizobial numbers on seeds. At the same sampling times seeds were also sown into pots filled with perlite moistened with water solution of plant nutrients (without N). After 4 weeks of plant growth in a growth chamber numbers of root nodules were counted and fresh and dry mass of shoots were determined. The studied chemical seed dressings had no significant effects on numbers of soybean rhizobia on seeds, and on nodulation and growth of soybean plants when seeds were analyzed or planted shortly after their inoculation with *B. japonicum*. However, the studied preparations significantly reduced numbers of soybean rhizobia on seeds stored for 24 h at room temperature, but only the synthetic one affected adversely nodulation and some growth parameters of soybean plants that developed from the stored seeds.

Key words: soybean, carboxin, thiuram, tea tree oil, seed dressings, *Bradyrhizobium japonicum,* nodulation

INTRODUCTION

Soybean, similarly to other plants belonging to the family *Fabaceae* (*Leguminosae*), can develop symbiotic associations with root-nodule bacteria (rhizobia) that fix atmospheric nitrogen and supply their legume host-plants with this important nutrient. Rhizobia are usually ubiquitous soil microorganisms but in regions or countries, like Poland, where soybean is not an indigenous plant and where this crop is not grown frequently soils are usually void or deficient in rhizobia nodulating soybean (Sadowsky, Graham, 1998; Prěvost, Bromfield, 2003, Martyniuk et al., 2005; Cheminingwa, Vessey, 2006). Under such conditions soil inoculation or pre-sowing pelleting of seeds with inoculants containing root-nodule bacteria specific for soybean results in a significant increase of nodulation, symbiotic nitrogen fixation and seed yield of this crop (Thies at al., 1991; Singleton et al., 1992; Kozieł et al., 2013).

Seeds of leguminous plants, similarly to any other agricultural crops, need to be treated with fungicides or/and insecticides before planting. These chemicals applied as seed dressings protect germinating seeds and young seedlings against various fungal pathogens and pests. However, when rhizobial inoculants are introduced onto chemically treated seeds of a legume crop survival of the bacteria on such seeds can be markedly reduced due to possible toxic effects of pesticides (Curley, Burton, 1975; Rennie, Dubetz, 1984; Strzelec, Martyniuk, 1994; Martyniuk, 2012). Previous studies have shown that interactions between chemical seed dressings and rhizobia on legume seeds depend on the method of inoculant application and on chemical composition of seed protectants, and that it is possible to select pesticides containing active substances which are compatible with rhizobia (Curley, Burton, 1975; Ramadoss, Sivaprakasam, 1989; Martyniuk et al., 2002).

The aim of the study was to compare the influence of a synthetic and a natural fungicidal seed dressing on survival of *Bradyrhizobium japonicum* on seeds, and on nodulation and growth of soybean in a pot experiment.

Corresponding author:
Stefan Martyniuk
e-mail: sm@iung.pulawy.pl
phone +48 81 4786 962

MATERIAL AND METHODS

Materials. *Bradyrhizobium japonicum*, strain PR, used in this study was taken from the Collection of N_2-fixing Bacteria belonging to the Department of Agricultural Microbiology of the Institute of Soil Science and Plant Cultivation in Puławy. Stock cultures of this bacterium were maintained at 4°C on slants of yeast extract-mannitol agar (YEMA) supplemented with 3 g $CaCO_3$ l^{-1} (Vincent, 1970). Yeast extract-mannitol broth (YEMB) was used to proliferate *B. japonicum* in liquid cultures. To prepare a solid-state inoculant containing living cells of *B. japonicum* finely milled brown coal was used as the bacterial carrier. The carrier material was obtained from a commercial producer of rhizobial inoculants in Poland. Portions (3 × 10 g) of the carrier moistened with water (10% w/w) were placed in glass bottles and sterilized by autoclaving in 121 °C for 60 min. Bottles with sterile carrier were than inoculated with 3 ml of YEMB cultures of *B. japonicum* and incubated for 7 days at 28 °C. These inoculants, containing about 5×10^{10} cfu (colony forming units) of viable cells of *B. japonicum* in 1 g, were used to pellet soybean seeds. Numbers (cfu) of *B. japonicum* on inoculated soybean seeds and in the inoculant were counted by standard dilution plate procedures on YEMA containing 25 mg l^{-1} of Congo red (Vincent, 1970, Kozieł et al., 2013).

Polish cultivar "Aldana" of soybean [*Glycine max* (L) Merrill] was grown in a pot experiment.

Two commercial fungicidal preparations were used to treat soybean seeds. The synthetic one, composed of carboxin (20%) and thiuram (20%), is registered as a seeds dressing for leguminous crops (pea, lupine). The second one contains 23.8% of natural oil extracted from tea tree [*Melaleuca alternifolia* (Maiden & Betche) Cheel] and is registered as an antifungal product, also approved for the use in organic (ecological) agriculture.

Experimental procedure. Surface disinfected soybean seeds (100 g portions in glass beakers) were treated with the above mentioned chemical preparations according to the instructions given by the producers. The synthetic product was applied at the rate of 0.4 ml per 100 g of seeds and it was diluted with water (1:1 v/v) before the use. The natural product (2% solution in water) was applied at the rate of 1 ml/100 g of seeds. Next day the chemically treated seeds were pelleted with *B. japonicum* inoculant, prepared as described above, at the rate of 2 g/100 g of soybean seeds and thoroughly mixed. Numbers of *B. japonicum* (cfu) were counted using 10 g of seed samples (in 3 replications) taken directly (within 1–2 hours) after seed inoculation and after 24 hours of seeds storage at room temperature. At the same time seeds were also sown into plastic pots (1000 ml) containing perlite moistened with 300 ml of N-free liquid medium to support plant growth. Five treated soybean seeds were planted into each pot, in six replications for each treatment, and after emergence four uniform seedlings were left in each pot. The pots were placed in a growth chamber (Heresus HPS 1500–2000) running at 16 h/8 h day/night regime and temperature 22 °C/15 °C, respectively. After four weeks the experiment was terminated to count numbers of nodules on root systems of each seedling and to weight the plants. Fresh and dry mass of shoots and dry mass of roots were described in the paper. All data were subjected to the analysis of variance using Anova test.

RESULTS AND DISCUSSION

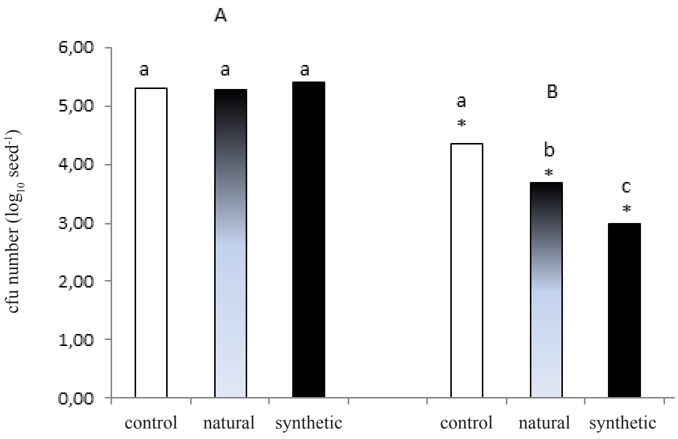
When soybean seeds were analyzed shortly (within 1–2 hours) after their inoculation with *B. japonicum* no significant differences were detected in the numbers of *B. japonicum* colony forming units occurring on seeds treated with the seed dressings and on untreated (control) seeds (Fig. 1). Both chemical seed dressings (synthetic and natural) had also no detrimental effects on nodulation and growth of soybean plants developed from seeds that had been planted shortly following their inoculation with *B. japonicum* (Fig. 2, Table 1). However, seeds examined after their storage for 24 hours at room temperature were colonized with significantly lower numbers of *B. japonicum* than the seeds analyzed shortly after their inoculation, irrespective of the treatments (Fig. 1).

Moreover, at this sampling (after 24 h) significantly less *B. japonicum* cells survived on soybean seeds treated with the studied chemical dressings than on untreated seeds, and the synthetic product containing carboxin and thiuram was more toxic to soybean rhizobia than the natural preparation (Fig. 1). With respect to nodulation of the plants that developed from inoculated seeds stored for 24 h,

Table 1. Effects of seed dressings and seed storage time on fresh and dry weight of soybean plants grown in a pot experiment.

Treatment	1–2 hours			24 hours		
	shoots fresh weight (g plant^{-1})	shoots dry weight (g plant^{-1})	roots dry weight (g plant^{-1})	shoots fresh weight (g plant^{-1})	shoots dry weight (g plant^{-1})	roots dry weight (g plant^{-1})
control	1.11 a	0.22 a	0.08 a	0.95 ab	0.18 a	0.07 a
Natural	1.15 a	0.20 a	0.06 a	1.0 a	0.20 a	0.07 a
Synthetic	1.05 a	0.21 a	0.07 a	0.88 b	0.17 a	0.06 a

Values in columns with the same letter are not significantly different.

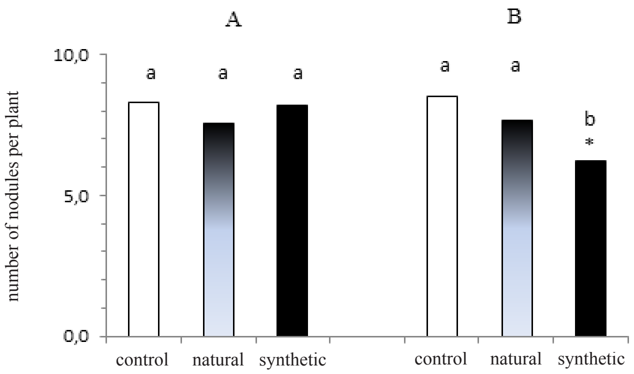


Values with the same letter within groups A and B are not significantly different
The asterisk in B indicates significant difference in relation to A

Fig. 1. Numbers of *Bradyrhizobium japonicum* cfu ($\log_{10}$) on soybean seeds treated with natural or synthetic seed dressing as estimated shortly after inoculation (A) and after 24 hours storage (B).

the natural dressing did not affect markedly nodule numbers on soybean roots as compared to untreated plants, but the synthetic dressing significantly reduced numbers of nodules on roots in comparison to the other treatments (Fig. 2).

Results of the pot experiment have shown that the 24 h storage of inoculated seeds had generally no significant effects on fresh and dry weights of soybean plants which developed both from untreated (control) seeds and from seeds dressed with the chemical preparations, with the exception of the fresh weight of shoots, which had the lowest value in the case of plants treated with the synthetic dressing (Table 1).



Values with the same letter within groups A and B are not significantly different
The asterisk in B indicates significant difference in relation to A

Fig. 2. Numbers of root nodules of soybean plants grown from seeds treated with natural or synthetic seed dressing and planted shortly after inoculation (A) or after 24 hours storage (B).

It was reported in previous studies that some chemical seed dressings containing thiuram, captan or carbosulfan had no substantial effects on the survival of rhizobia, including *Bradyrhizobium japonicum,* on seeds, and on the symbiosis of the bacteria with legumes, particularly when the treated seeds were planted shortly following inoculation (Curley, Burton, 1975; Rennie, Dubetz, 1984; Ramadoss, Sivaprakasam, 1989; Revellin et al., 1993; Strzelec, Martyniuk, 1994). Similar results were published for seed protectants containing a mixture of thiuram and carbendazim (Ramadoss, Sivaprakasam, 1989; Martyniuk et al., 2002). In the present study we have proved that the synthetic dressing composed of thiuram and carboxin was also compatible with *B. japonicum* and had no detrimental effect on nodulation and on soybean plant growth, but only when treated seeds had been planted within a few hours after application of the rhizobial inoculant (Fig. 1 and 2, Table 1). Results presented in Figure 1 show that even on the untreated (control) seeds the number of *B. japonicum* cfu significantly decreased after 24 h storage and this effect was mainly due to seed desiccation (Vincent, 1970; Curley, Burton, 1975; Rennie, Dubetz, 1984; Martyniuk et al., 2002). This figure also shows that the chemical dressings further reduced the survival of the symbiotic bacterium on the stored soybean seeds as compared to untreated seeds, and that the natural fungicide was less toxic to *B. japonicum* than the synthetic one. In spite of this decrease, a good quality of *B. japonicum* inoculant used in this study, ensured that relatively high numbers of the symbiotic bacterium (2–5 × 10^3 cfu/seed) remained on the chemically treated soybean seeds (Fig. 1), which resulted in sufficient nodulation (Fig. 2) and similar performance of soybean plants that developed form chemically treated seeds as compared to the control seed stored for 24 h (Table 1). Results of previous studies have shown that good-quality rhizobial inoculants should provide about 10^3–10^5 of bacterial cells on each seed (Vincent, 1970; Thies at al., 1991; Singleton et al., 1992). Two important, also from practical point of view, results arising from this study deserve underpinning; firstly, chemically treated soybean seeds, and those of other legumes (Strzelec, Martyniuk, 1994; Martyniuk, 2012), that were also inoculated with rhizobial inoculants should be planted as soon as possible after inoculation. This will mitigate eventual toxic effects of chemical seed dressings on rhizobia introduced onto seeds. Secondly, the natural fungicide used in this work seem to be compatible with symbiotic bacteria, but further research is needed, particularly on field effectiveness of this product with respect of its effects on nodulation and protection of ecologically grown legume crops.

CONCLUSIONS

1. The synthetic chemical seed dressing composed of thiuram and carboxin and the natural fungicide containing oil from tea tree had no significant effects on numbers of seed-applied soybean rhizobia, and on nodulation and growth of soybean plants when seeds were analyzed or sown shortly after their inoculation with *B. japonicum*.

2. The studied preparations significantly reduced numbers of soybean rhizobia on seeds stored for 24 h at room temperature, but only the synthetic one affected adversely nodulation and some growth parameters (shoots fresh weight) of soybean plants that developed from the stored seeds.

3. The natural fungicide, the tree oil solution applied onto soybean seeds was more compatible with *B. japonicum* than the synthetic one.

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