EFFECTS OF STORAGE TEMPERATURE, DURATION AND INOCULATION METHODS ON *BRADYRHIZOBIUM JAPONICUM* GROWTH WITH SOYBEAN

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Abstract

The positive effect of root-nodulating strains of *Bradyrhizobium* spp in soybean in terms of biological nitrogen fixation and plant growth and development is still a main focus in the present. According to the literature, a concentration of 10^5-10^6 bacteria on the seed, will guarantees that the root can be infected during the germination process, and in this way will achieve early nodulation. In this research the effects of storage temperature, duration and inoculation methods on the survival of *Bradyrhizobium japonicum* growth on soybean seeds and the performance of soybean bacterial inoculant on seeds under field conditions were determinate. The results showed that the storage of the inoculated seeds at low temperature (5-10°C) can assure a very good root infection in order to obtain a higher nodule number and after 90 days, whether the inoculation was done in station or factory. The registered results showed values which ranged from $0.03*10^5$ to $5.06*10^5$ CFU/seed. In case of the performance of soybean bradyrhizobia inoculants on seeds under field conditions we determined that from the soybean tap roots, the highest number of nodules was 138, while the lowest number was 42 nodules. Regarding the total weight of the nodules prevailed from the tap roots, the highest registered values was 4748.0 mg. In case of secondary roots, the highest number of nodules was 353, while the lowest number was 103.

Key words: *Bradyrhizobium japonicum*, inoculation, storage temperature and duration, soybean nodules

Root-nodule bacteria included in the group of α-*Proteobacteria*, are fixing atmospheric nitrogen in the symbiosis with leguminous plants inducing the formation of nodules on the roots and are members of the following genera *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium, Azorhizobium* and *Allorhizobium*

Soybean [*Glycine max* L. (Merr.)] is gaining importance as a diversifying crop in cereal dominating cropping system. In Romania, has occupied an area of 165,140 hectares with an annual production of 416.4 million tons and with an average yield of 2.5 tons/ha (FAO, 2017). Inoculation of soybean with *Bradyrhizobium japonicum* (*Bacteria*, α-*Proteobacteria*, *Alphaproteobacteria*, *Rhizobiales*, *Bradyrhizobiaceae*; Rivas *et al*, 2009) is a common agricultural practice in order to allow an efficient nodulation of the plant and to increase nodule number and/or nodule weight. The success of inoculation will improve grain yield and let in soil a higher amount of nitrogen. Besides, the bacteria will protect the roots from the attack of pathogens due to production of diverse microbial metabolites (siderophore, rhizobitoxin) and will increase the uptake of phosphorus and other minerals (Deshwal *et al*, 2003)

The characteristics of *Bradyrhizobium* spp. are rod-shaped, nonspore-forming cells, motile with one polar or subpolar flagelum, aerobic, Gram-negative, cell-sized of 0.5-0.9 μm and 1.2-3.0 μm, the optimum growth temperature is 25-30°C at pH 6-7 (Holt *et al*, 1994).

The seed ready to sow is a technology that offers numerous advantages in the pre-sowing process because it considerably simplifies the producer's tasks and reduces production costs. In this way, the quantity of product used is reduced and also guarantees the application of adequate doses of fungicides, insecticides, inoculants and bio-inductors of high complementarity. Today's technologies are designed to reduce the impact of pesticides on bacteria, increasing the compatibility between the inoculant and the seed treatment chemicals. Seed inoculants are mainly applied by adhering the product before sale or at sowing. Bacterial survival on the seed is mainly affected by three factors: desiccation, the toxic nature of seed

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coat exudates and storage temperatures. Early studies demonstrated that bacteria survival on seeds improves if the peat-based or vermiculite-based inoculants were applied 4 or 8 weeks after inoculant production, respectively (Albareda M. *et al*, 2008).

Despite the long history of inoculation of legumes and clear laboratory demonstration of the ability of a wide range of other beneficial microorganisms to improve crop performance, there are still very few commercially available microbial seed inoculants (O'Callaghan, M., 2016)

The objective of this study was to determine: (1) effects of storage temperature, duration and inoculation methods on the survival of *Bradyrhizobium japonicum* growth with soybean and (2) the performance of soybean bradyrhizobia inoculants on seeds under field conditions.

MATERIAL AND METHOD

In order to determine the presence and the average number of bacterial colonies of the genus *Bradyrhizobium* per seed (CFU/seed), 13 samples of soybean seeds from Dekabig variety were received. Twelve samples were previously inoculated in laboratory or in station with Rizoliq LLIS + Premax LLI biopreparate (250 ml + 50ml/100 kg seed), while the 13th was the untreated control.

To obtain information about the CFU/seed value for *Bradyrhizobium* spp., YEMA nutritional medium (yeast extract-mannitol-agar) in variants with Congo red with antibiotic (vancomycin) was used.

In order to obtain manageable concentrations of bacteria the serial dilution technique (log dilution) was used. A log dilution is a tenfold dilution, meaning the concentration is decreased by a multiple of ten. To complete a tenfold dilution, the ratio must be 1:10. A sample size of 1 ml was added to 9 ml of sterile distillated water to equal a total of 10 ml. For our experiment we used the dilution 10-3 and 10-4 in five repetitions. Inoculation was done using the spread method with 1 ml of appropriate dilutions and placed on the surface of the nutritive medium. The sample was then evenly coated on the agar surface using a sterile glass spreader (triangle rod). After inoculation the Petri dishes were placed in an incubator at a constant temperature of 28°C for 7 days. After incubation, each bacterial cell from the dilution has formed a single colony which was counted.

The 12 inoculated samples used in the experiment had the following indications:

• V1: seed inoculation carried out at the station 90 day before sowing, storage T= 15-20ºC
• V2: seed inoculation carried out at the station 90 day before sowing, storage T= 15-20ºC
• V3: seed inoculation carried out at the station 60 day before sowing, storage T= 15-20ºC
• V4: seed inoculation carried out at the station 60 day before sowing, storage T= 15-20ºC
• V5: seed inoculation carried out at the station 30 day before sowing, storage T= 15-20ºC
• V6: seed inoculation carried out at the station 30 day before sowing, storage T= 15-20ºC
• V7: seed inoculation carried out at the station 30 day before sowing, storage T= 5-10ºC
• V8: seed inoculation carried out at the station 90 day before sowing, storage T= 5-10ºC
• V9: seed inoculation carried out at the station 60 day before sowing, storage T= 5-10ºC
• V10: seed inoculation carried out in the laboratory 30 day before sowing, storage T= 5-10ºC
• V11: seed inoculation carried out in the laboratory 90 day before sowing, storage T=5-10ºC
• V12: seed inoculation carried out in the laboratory 60 day before sowing, storage T= 5-10ºC

The untreated control had the code NI-T0 (V13).

In order to determine the nodules number on the main and secondary roots, and also their weight, the soybean plants (12 variants; V1-V12; 3 repetitions/variant) from soybean Dekabig variety were received from a private company. After receiving the plants, they were placed in Berzelius glasses with water, in order to easily detach the remaining soil particles adhering to the roots. On the same day, the nodules from the main and secondary roots were counted and weighed.

RESULTS AND DISCUSSIONS

Analysing the effects of storage temperature, duration and inoculation methods on the survival of *Bradyrhizobium japonicum* on soybean seeds the results showed value which ranged from $0.03*10^5$ to $5.06*10^5$ CFU/seed (*table 1*).

Values within the range recommended by the specialized literature (10^5-10^6 CFU/seed) were recorded for the 6 samples kept at a temperature of 5-10ºC after inoculation, regardless of the location where the seed inoculation was made. In case of seeds inoculated under laboratory conditions and stored at 5-10°C, after the determination of CFU/seed it was observed that the highest value was obtained in case of the variant V12 with $2.42*10^5$ CFU/seed (*table 1*). The values obtained for the seeds inoculated in the station ($5.06*10^5$ CFU/seed) were higher compare to the values from seeds inoculated under laboratory conditions, and this being due to the non-uniformity of the seed samples brought to the analysis.

The lowest values, under literature recommendation (10^5-10^6 CFU/seed), were recorded in the case of inoculated seeds stored at 15-20ºC (*table 1*). In this case, the highest CFU/seed values were obtained from the variant

V4 ($0.12*10^5$). In both location, where inoculations were made, the results revealed, that the number of CFU/seed decrease over time.

Table 1

***Bradyrhizobium* spp. bacteria counting (CFU/seed)**

Variant	Location of inoculation	Days after inoculation	Storage temperature	Bacteria counting (CFU/seed)
V1	Laboratory	90	15-20ºC	$0.03*10^5$
V2	Laboratory	60	15-20ºC	$0.06*10^5$
V3	Laboratory	30	15-20ºC	$0.10*10^5$
V4	Factory	90	15-20ºC	$0.12*10^5$
V5	Factory	60	15-20ºC	$0.06*10^5$
V6	Factory	30	15-20ºC	$0.03*10^5$
V7	Factory	30	5-10ºC	**$5.06*10^5$**
V8	Factory	60	5-10ºC	**$2.56*10^5$**
V9	Factory	90	5-10ºC	**$3.78*10^5$**
V10	Laboratory	90	5-10ºC	**$1.47*10^5$**
V11	Laboratory	60	5-10ºC	**$1.46*10^5$**
V12	Laboratory	30	5-10ºC	**$2.42*10^5$**

In case of the performance of soybean bradyrhizobia inoculants on seeds under field conditions we determined that from the soybean main (sin. primary, tap) roots, the highest number of nodules was recorded for the seed preparation variants V8 (138 nodules), V10 (137 nodules) and V12 (130 nodules), while the lowest number was recorded for V2 (42 nodules) and V1 (47 nodules) (*table 2*).

From the secondary (sin. fibrous, tap) roots, the highest number of nodules was recorded as in the case of the main roots, for V8 (353 nodules), V12 (284 nodules) and V10 (242 nodules) variants, while the lowest number was recorded for V5 (103 nodules), V1 (105 nodules), respectively V2 (133 nodules) (*table 2*).

Regarding the total weight of the nodules prevailed from the main roots, it recorded the highest values for V8 (4748.0 mg), V6 (4252.5 mg) and V10 (4156.4 mg), and the lowest weight was recorded for V5 (1914.1 mg), V1 (1957.9 mg) and V9 (2130.2 mg) variants. Analyzing the total weight of the nodules taken from the secondary roots, the highest registered values of approx. 7630 mg were found for V7 and V8 variants. The lowest values were recorded for V5 (1413.1 mg) and V9 (2284.6 mg) (*table 2*).

It was observed, that the best results regarding the previously analyzed factors, have been recorded in the case of variants V8, V10 and V12. Variant 8 (V8) recorded the best values also at the previous time of sample collection (beginning pod – R3 stage), being from this point of view the recommended inoculation variant.

Table 2

Nodule number and nodule total and average weight (per plant) obtained after seed inoculation

Variant	Main (primary) root		Average weight	Secondary root		Average weight
	Nodules no.	Nodules total weight (mg)	mg	Nodules no.	Total weight (mg)	mg
V1	47	1957.9	56.4	105	3950.0	36.6
V2	42	2788.6	69.8	133	3452.8	26.8
V3	65	3356.6	44.3	172	3357.2	22.7
V4	81	3962.6	45.7	183	4555.7	23.6
V5	66	1914.1	28.5	103	1413.1	13.9
V6	92	4252.5	43.6	187	4615.8	23.9
V7	64	4012.6	78.3	233	7630.3	33.2
V8	138	4748.0	36.0	353	7628.5	20.0
V9	108	2130.2	18.8	151	2284.6	15.2
V10	137	4156.4	29.9	242	3832.2	15.9
V11	104	3076.0	31.1	200	3599.6	20.5
V12	130	3205.4	27.0	284	4489.8	21.9

Comparing the average weight of a nodule collected from the main (primary) root between beginning pod (R3) and full maturity (R8) reproductive (R) stages, was notice that overall the weight of the nodules has increased, and this fact corresponds to a higher

amount of nitrogen that will remain in soil. The highest average weight per node, in the case of the main root, was recorded in the case of variant 7 (V7) for both sampling periods (*figure 1*).

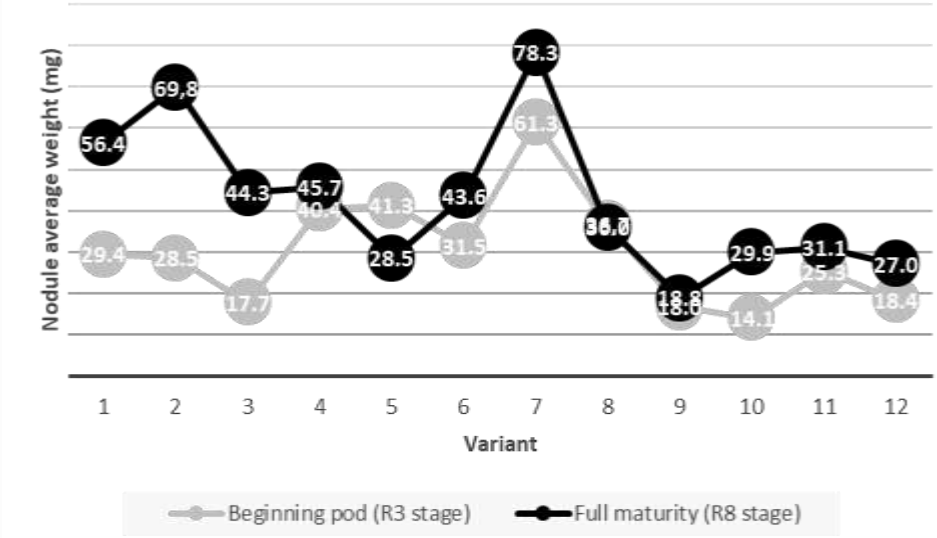

Figura 1. **Comparison of the nodule average weight collected from the main root between R3 and R8 reproductive (R) stages**

In the case of nodules collected from the secondary roots between the same reproductive (R) stages we notice that the average weight of a nodules was fluctuating, and the highest average weight per nodule was registered in the case of variant 1 (V1), followed by V7 (*figure 2*).

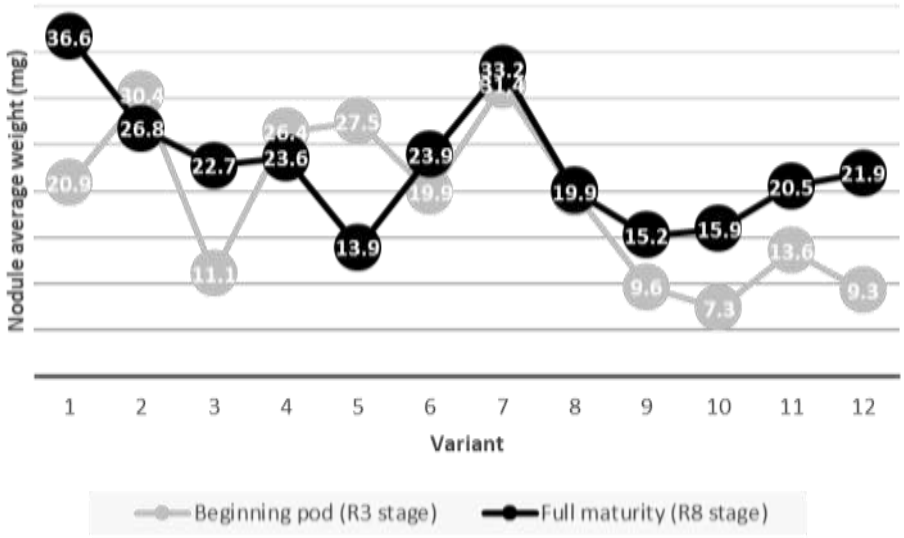

Figura 2. **Comparison of the nodule average weight collected from the secondary roots between R3 and R8 reproductive (R) stages**

CONCLUSIONS

Analyzing the effects of storage temperature, duration and inoculation methods on the survival of *Bradyrhizobium japonicum* on soybean seeds the results revealed, that the number of CFU/seed decrease over time, but if the inoculated seeds are stored at low temperature (5-10°C), the number of bacteria can assure a good root infection even after 90 days. If the inoculated seeds are stored 30 days at 15-20°C, will not assure the minimal number of bacteria necessary for a good nodulation.

In case of the performance of soybean inoculated with *Bradyrhizobium* spp., stored at 5-10°C at least 30 days and then sowed on fields, the results obtained at level of main and secondary roots showed much better results as nodules number and average weight in comparacy with the seed material stored at higher temperature (15-20°C).

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