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Survival and Symbiotic Effectivity of Lyophilized Root Nodule Bacteria

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Abstract: The study has been carried out under the greenhouse and laboratory conditions, using the lyophilization technique to determine the preserving possibilities of 3 native strain isolated from bean root nodules that been widely cultivated in Turkey and was defined as *Rhizobium* bacteria. The strains been developed separately in the Petri dishes with YMA (Yeast Extract Mannitol Agar) sown into lyophilization tubes containing skim milk, frozen at -85°C and dehydrated at -55°C with vacuum. According to the cultural counting results continued for 35 months after lyophilization, while the living cells in R11 strains and R12 strains found as 41×10^9 and 35.5×10^9 cell mL⁻¹, respectively, the best result was determined in the R13 strain as 51.5×10^9 cell mL⁻¹. In the greenhouse trials, the dry weight of plant's green parts, the number of nodules and total nitrogen amounts were evaluated. The tested strains were inoculated with bean varieties peculiar to themselves and the lyophilized cultures gave similar results with the main cultures kept in tubes with YMA.

Key words: Lyophilization, *Rhizobium* bacteria, native strain, symbiotic effectivity of strains

INTRODUCTION

Rhizobium bacteria are preserved under various conditions in order not to lose their nitrogen fixation and genetic change (Antheuniss, 1973; Miyamoto-Shinogara *et al.*, 2006). Since, the cultures used continuously in the production are preserved best in the leaning tubes of agar, it facilitates its reproduction (Antheuniss *et al.*, 1981). However, when preserving cultures in the agar medium, it takes a lot of efforts to transfer them repeatedly into a new medium every other month (Abadias *et al.*, 2001; Miyamoto-Shinogara *et al.*, 2006). In the recent studies, performing the control of cultures' activeness and effectively nodulation has been reported to be essential (Safronova and Novikona, 1996; Willems *et al.*, 2001). When compared to the other preservation methods, the lyophilization method (frozen and dehydrated) has been determined to be used successfully with a widely range (Abadias *et al.*, 2001; Miyamoto-Shinogara *et al.*, 2000).

Although, there are different views on the preservation temperatures of frozen and dehydrated cultures, preservation at low temperatures is recommended by Miyamoto-Shinogara *et al.* (2006), Malik (1990) and Revellin *et al.* (2000). Willems *et al.* (2001) has suggested that *Rhizobium* cultures are preserved at either 15°C in a dry place or at 4°C in the fridge. The vigor tests carried out after lyophilization are usually done in the cultural counting technique and the

results are presented as the number of living cells per milligram (Peter and Reichart, 2001; Ekdawi-Sever *et al.*, 2003). In the preparation of culture collections of *Rhizobium*, lyophilized cultures are reported to be preferred in the greenhouse and field trials (Miyamoto-Shinogara *et al.*, 2006).

This study has been carried out to constitute the long time preservation of native *Rhizobium* strains, whose activeness was determined in the earlier study (Küçük, 2004), isolated from the root nodules of bean varieties that are cultivated widely in Turkey, providing the supply of the essential materials of Turkey's developing microbial inoculant production and the formation of *Rhizobium* cultural collections.

MATERIALS AND METHODS

The strains: In this study, *Rhizobium* R11, R12 and R13 strains, whose activeness was determined from earlier study (Küçük, 2004), were used.

The preparation of lyophilization process: The cultures been purified in Petri dishes with Yeast Extract Mannitol Agar (YMA) then transferred into medium containing 20% skim milk in lyophilization tubes (2 mL) (Malik, 1990). After the tubes having been frozen at -85°C, they were dehydrated at -55°C under vacuum (Jenning, 1999). The samples were accumulated at +4°C.

The vigor tests: During the preservation, a vigor test was performed for the cultures every other month. The cultures were dissolved in the yeast extract mannitol broth at the time of counting (Ekdawi-Sever *et al.*, 2003). At the end of three days development, the number of living cells after lyophilization was obtained by cultural counting technique (Schoung *et al.*, 2006).

Greenhouse tests: The test subjects include lyophilized R11, R12, R13 strains, inoculated and noninoculated subjects. The *Rhizobium* strains that defined as *Rhizobium* bacteria in the earlier study (Küçük, 2004) were inoculated with bean varieties having high nodulation and nitrogen fixation peculiar to themselves. (R11, R12, R13 strains inoculated with Akman 98, Göynük 98 and Şehirali 90 bean varieties, respectively). The bean seeds (*Phaseolus vulgaris* L.) were provided from Anatolian Agricultural Research Institute in Eskişehir, Turkey. The seeds were soaked into 95% sodium hypochloride solution for 5 min and their surface sterilization were made by washing using sterile distilled water (Daba and Haile, 2002).

Afterwards, the seeds were sown into pots containing sterile peat (3 g). After the lyophilization strains having been developed in yeast extract mannitol broth 10 mL of culture (10^9 cell mL⁻¹) was added to developing roots. At the end of 35 month development period, the roots sections of plants were cut and the numbers of nodules were examined by Mostasso *et al.* (2002) and Daba and Haile (2002).

The green part sections of the plants were dehydrated at 65°C in the oven until they were with stable weight, then weighted and their nitrogen amounts were determined with Kjeldahl method. All parameters were statistically analyzed using an analysis of variance (Yurtsever, 1984).

RESULTS AND DISCUSSION

In the recent study, while lyophilized bacteria cultures were preserved at +4°C, the vigor test was carried out every other month for 35 months. At the end of 35th month, nodulation and nitrogen fixation activity in the tested bean varieties were determined. The living *Rhizobium* numbers obtained from culture counting after the lyophilization process performed every other month are shown in Table 1. The starter values obtained after lyophilization varied between 263.5×10^9 and 35.5×10^9 cell number mL⁻¹ (Table 1). The results in Table 1 show that, the existence of change on the living *Rhizobium* cell numbers performed every other month after the lyophilization process was determined. An analysis of

Table 1: Colony forming units after freeze-drying (10^9 cell mL⁻¹)

Time (Months)	<i>Rhizobium</i> strains		
	R11	R12	R13
1	263.5±1.3	183.5±2.6	211.5±3.6
5	219.5±2.2	180.0±3.0	191.0±2.5
10	185.0±2.5	108.0±2.3	159.5±2.0
15	109.0±2.0	90.0±2.3	111.0±1.2
20	96.0±1.2	86.5±1.0	91.0±0.8
25	76.5±0.9	64.5±0.5	88.5±0.5
30	66.5±3.6	59.0±0.1	69.0±0.5
35	41.0±0.5	35.5±0.5	51.5±0.2
ANOVA	df	Mean squares	
Replication	1	1.0	
Combination	23	8196.0	
Strain (S)	2	4046.4**	
Time (T)	7	24646.1**	
S×T	14	563.7**	
Error	23	9.3	

Significant at the **0.01 levels of probability, respectively

variance of data indicated that strain, time, strain×time (S×T) were significantly (0.01 level) (Table 1). Since the lyophilization is a method preventing the biological originated material from being spoiled, it is preferred in the *Rhizobium* microbiology to preserve the cultures for long time (Antheumiss, 1973; Miyamoto-Shinogara *et al.*, 2000, 2006; Safronova and Novikona, 1996; Willems *et al.*, 2001; Abadias *et al.*, 2001; Jennings, 1999).

Miyamoto-Shinogara *et al.* (2006), Willems *et al.* (2001) and Peter and Reichart (2001) showed that after lyophilization, the starter values being high like 10^9 cell mL⁻¹ originated from their using intensive cell numbers at the start of lyophilization, taking possible bacteria loss number into account during the lyophilization process. In addition, in the performed culture counting after lyophilization every other month continued for 35 months, the decreasing vigor of *Rhizobium* strains when compared to their starter values, as Safronova and Novikona (1996) observed that the deaths occurred in time are thought to be originated from *Rhizobium* bacteria's being quite sensitive to the dry environment.

In the studies carried by Safronova and Novikona (1996), in order to preserve *Rhizobium* bacteria, when they compared the methods of lyophilization with the method of keeping in the liquid nitrogen, they determined the lyophilization as the best preserving method for *Rhizobium* bacteria and also stated that the symbiotic characteristics of strains do not change at the end of the study.

The number of counted nodules of tested plants in their flowering period, the dry substance weight dehydrated at 65°C and their total nitrogen extent are shown on Table 2. The highest nodule number which is R12 was obtained from inoculated Göynük 98 bean variety. Nodulation was not detected in the uninoculated

Table 2: Effects of strains on bean varieties

Lyophilize bacteria+ bean varieties	Nodule No. (No. plant ⁻¹)	Shoot dry weight (g plant ⁻¹)	Inc.* (%)	N (%)	Inc.* (%)	Total N (mg plant ⁻¹)	Inc.* (%)
R11+Akman	20.5±0.2	2.04±0.2	68.5	4.10±2.2	45.9	83.43±5.3	146.1
R12+Göynük	25.5±0.7	2.90±0.4	121.3	4.50±2.5	51.5	130.50±6.8	236.3
R13+Şehirali	23.0±0.1	2.40±0.9	87.5	3.86±3.8	43.5	91.72±3.4	166.2
Control (Uninoculation)							
Akman	0	1.21±0.5	-	2.81±3.0	-	33.90±2.5	-
Göynük	0	1.31±0.7	-	2.97±2.2	-	38.80±2.0	-
Şehirali	0	1.28±1.0	-	2.69±0.0	-	34.50±2.2	-
ANOVA							
Source of variation	df	Nodule No. (No. plant ⁻¹)	Shoot dry weight (g plant ⁻¹)	N (%)	Total N (mg plant ⁻¹)		
Replication	1	1587.0	0.011	0.0023	0.0003		
Combination	5	322.4**	0.97**	1.17**	3137.9000*		
Treatment (T)	1	1587.0**	4.11	5.34**	13136.7000*		
Strain (S)	2	6.25	0.24**	0.22**	772.8000*		
T×S	2	6.25	0.151**	0.03	503.6000*		
Error	5	4.34	0.004	0.014	0.1800		

*Inc.: Increase, Significant at the **0.01 levels of probability, respectively

beans. While the highest dry weight 2.90 g pot⁻¹ was given by the inoculated with R12, the least dry weight 1.21 g pot⁻¹ was given by the control (uninoculated) application. An analysis of variance of data indicated that, combination and treatment were significantly on nodule number (Table 2) and combination, strain, strain×time were significantly on shoot dry weight (Table 2), combination, treatment and strain were significantly on N% (Table 2). The results in Table 2 show that, the total nitrogen varied between 83.43 and 130.5 mg plant⁻¹. In the greenhouse trials carried out by Mostasso *et al.* (2002) and Daba and Haile (2002), the variation of the dry weights of bean plants being between 2.75 and 2.68 g pot⁻¹ is harmonious with the our trial's results.

Consequently, according to the cultural counting performed after lyophilization, the starter values of lyophilized cultures varied between 35.5×10⁹ to 263.5×10⁹ cell mL⁻¹. Between three tested strains, the highest vigor value was obtained from R13. The values belong to lyophilized cultures and the main cultures kept in tubes with YMA were found similar to each other. During the preservation of isolates, when taking some factors into consideration such as absence of contamination, having no mutations due to the continuous inoculated that happening continuously, having economically low cost, the ability of being preserved securely for a long time, using the lyophilization technique can be recommended for preservation of Turkish *Rhizobium* strains.

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