PERIODICO di MINERALOGIA Volume 90, No. 5, 2021

Nitrogen fixing activity, plasmid profiles and intrinsic antibiotic resistance of *Rhizobium galegae* strains

BOGIĆ MILIČIĆ*, ĐORĐE KUZMANOVIĆ, NATAŠA RASULIĆ, DRAGANA JOŠIĆ
Institute of Soil Science, Teodora Drajzera 7, 11000 Belgrade, Serbia

**Corresponding author:*
Bogić Miličić, Department for Microbiology, Institute of Soil Science, Teodora Drajzera 7, 11000 Beograd, Serbia

Abstract

In two simultaneous experiments conducted in test tubes on agarized Jensen media, nitrogen fixing activity of three Rhizobium galegae strains specific for G. orientalis Lam. were investigated on G. orientalis plants, and two indigenous strains specific for G. officinalis L. on G. officinalis plants. Inoculation of G. orientalis with the specific strains 801, 802 and 803 grown as single strain inoculum caused a 2.3-2.9-fold increase the yield of dry shoot weight and 6.0-8.6-fold increase in N content, compared to control. The percentage of fixed N in those strains was 83.5-88.4%. The indigenous stains 821 and 822 of G. officinalis prepared as single strain inoculum caused a 1.7-fold increase in the yield of dry shoot weight and 4.6-5.0-fold increase in N content, compared to control. The percentage of fixed N in those strains was 78.4 and 79.8%. An inoculum composed of a mixture of strains 801 and 802 (double strain inoculum) specific for G. orientalis caused the roots of G. officinalis to form inactive nodules (Nod^+ Fix^-) that did not fix N from the atmosphere. In the same way, an inoculum composed of a mixture of strains 821 and 822 (double strain inoculum) specific for G. officinalis caused the formation of likewise inactive nodules (Nod^+ Fix^-) on G. orientalis roots. Plasmid profiles of strains 821 and 822 were identical, as well as plasmid profiles of strains 801 and 803. All strains were found to carry one plasmid with approx. MW of 140-200 MD. IAR tests for eight antibiotics were more discriminative, as they revealed differences between three strains specific for G. orientalis. However, strains 821 and 822 specific for G. officinalis had identical IAR patterns as well as plasmid profiles.

Keywords: *Rhizobium galegae* strains; inoculation; nitrogen fixation; *G. orientalis* Lam.; *G. officinalis* L., plasmid profile, intrinsic antibiotic resistance.

Introduction

Goat's rue, *Galega orientalis* Lam., is a new perennial forage plant with potentially high green mass yield (70-100 $t \cdot ha^{-1}$) and high crude proteins content (22.6-27.6%) at budding and early flowering. Another characteristic is a high dry weight content of 20-22% [9, 10]. Proteins of *G. orientalis* have high nutrient value owing to their high contents of essential amino acids (lysine, leucine, isoleucine, phenylalanine, valine and threonine), which account for 38-47% of their total amino acids. In addition, its proteins have a high content of proline and arginine, while the contents of cystine, methionine and tryptophan is low. Green weight of *G. orientalis* is rich in mineral elements and other important nutrient substances. Phosphorus content is 0.3-0.5%, calcium 0.8-1.15%, potassium 3.6-4.2%, carotene 160-217 $mg \cdot kg^{-1}$; crude fats 2.8% and sugars (mostly glucose and fructose) 5.0-5.5% in dry matter. Of the vitamins, it contains ascorbic acid, 22-80 $mg \cdot kg^{-1}$ [9].

G. orientalis fixes N from the atmosphere owing to its symbiotic relationship with the highly specific rhizobia (*Rhizobium galegae)*. *R. galegae* was earlier believed to belong to the group of fast-growing bacteria *R. leguminosarum*, known today as *R. loti*. Up-to-date genetic and serological research and tests of cross nodulation of host plants have proved that this was in fact a completely new species (*R. galegae*) without a close correlation with any of the known *Rhizobium* species [16, 18, 13, 5]. Those are Gram negative aerobic rods that do not sporulate and have one or two subpolar flagella.

As cultivation of *G. orientalis* in this country is still in an early stage and there is no intrinsic population of *R. galegae* in our soils that is specific for *G. orientalis*, the microbiological laboratory of the Institute of Soil Science in Belgrade has managed to isolate pure cultures of the root nodule bacteria *R. galegae* from "Rhizotorphin" and to produce a new nitrogen microbiological fertilizer called "Gala Azotofiksin", which is essential for *G. orientalis* cultivation in this country [4, 5].

The yield and quality of plant mass and grain of *G. orientalis* greatly depend on the efficacy of seed inoculation with specific rhizobia. One of the main factors regarding the yield and quality of plant mass and grain is the N fixing activity of *R. galegae* strains. However, successful inoculation would equally depend on a series of other factors, such as inoculum density and method of application, soil type and general conditions existing for the development of host plants, the ability of inoculum strains to adapt to such conditions and the affinity between the symbionts.

This research aimed to test the nitrogen fixing activity of 3 strains of *R. galegae* from the Institute`s collection that are specific for *G. orientalis* and 2 autochthonous strains of *R. galegae* specific for *G. officinalis*, as well as their effects on the yield and quality of plant mass of *G. orientalis* and *G. officinalis*.

Previous investigations [1] showed that plasmid profile analysis, although not as discriminative as insertion sequence typing or DNA fingerprinting, can be used for rough typing of isolates, and is indeed a method of choice when large number of isolates has to be typed, and the time and or the means are limited.

On the other hand, it has already been demonstrated [17, 7] that different strains of rhizobia vary widely in their IAR to specific antibiotics – which can be used for strain characterization and identification. In combination with plasmid profile, this trait can be used for strain characterization, so their behavior in soil can be monitored if they are used as seed inoculants.

Materials and methods

Two simultaneous trials were carried out on *G. orientalis* and *G. officinalis* plants. Shoots of *G. orientalis* and *G. officinalis* were grown under aseptic conditions in 250 x 20 mm tubes, each containing a single plant on 30 ml agarized mineral Jensen medium. The following treatments were applied to *G. orientalis*: ∅, ∅N, 801, 802, 803 (single strain inoculum) and a strain mixture of 821+822 (double strain inoculum) specific for *G. officinalis*. Each treatment had 8 replications. Strain 801 was isolated from "Rhizotorphin"; strain 802 was procured from Japan (Institute for Fermentation, Osaka, Japan), while strain 803, resistant to high Fe concentrations (>200 $\mu g \cdot ml^{-1}$), was obtained from the parent strain 801 by adaptation to high concentrations of Fe salts.

G. officinalis had the following treatments: ∅, ∅N, 821, 822 and a mixture of 801+802 (double strain inoculum) specific for *G. orientalis*. Strains 821 and 822 were indigenous stains isolated from the root nodules of *G. officinalis* from two sites in Serbia (Beljin near Debrec and Takovo near Ub). Seeds of *G. orientalis* and *G. officinalis* were

inoculated with 0.5 ml liquid culture of *R. galegae* developed previously in Erlenmeyer flasks on a medium containing mannitol and yeast extract (YMB) with aeration on a shaker under 28 °C temperature [12]. Inoculum density was 2.5-3.0·10^9 ml^{-1}.

In nitrogen treatments, N was added in the form of KNO_3 at 0.5% rate (50 mg per 100 ml medium). Plants were grown for 45 days, and then their yield was measured and total N content in shoot dry weight was achieved by means of the Kjeldahl procedure. Percentage of fixed N was calculated using the total difference method. N content in plant shoot dry weight (mg·$plant^{-1}$) was determined according to formula:

$$N = \frac{\text{Plant shoot dry weight}}{100} \text{ x \% N (in plant weight determined by Kjeldahl method)}$$

The amount of fixed N expressed (mg·$plant^{-1}$) was determined according to formula:

$$N_2 fixed = Nyield\ (fix.\ crop) - Nyield\ (control\ crop).$$

Plasmid profile of the strains was obtained by AGE [19]. *Agrobacterium rhizogenes* strain A4M70 that harbors one plasmid of known size of 254 kb (168 MD) was included on each gel as a referent strain for evaluating plasmid size of the strains.

IAR for 8 antibiotics was determined on solid YMA medium to which appropriate concentration of antibiotics was added prior to inoculation. Plates were then incubated for 48h at 28ºC and visually scored for presence or absence of growth of particular strains. IAR of the strains was determined for Ampicillin (1, 5, 10 μg·ml^{-1}); Tetracycline (0.5, 1, 5 μg·ml^{-1}); Penicillin (25, 50, 100 IJ·ml^{-1}); Neomycin (25, 50, 100 μg·ml^{-1}); Streptomycin (1, 5, 10 μg·ml^{-1}); Erythromycin (1, 5, 10 μg·ml^{-1}); Rifampycin (0.1, 0.5, 1 μg·ml^{-1}) and Chloramphenycol (10, 25, 50 μg·ml^{-1}).

Results and discussion

Nitrogen fixing activity of the investigated *R. galegae* strains specific for *G. orientalis* and *G. officinalis* are presented in Tables 1 and 2.

Table 1. Nitrogen fixing activity of *R. galegae* specific for *G. orientalis* L. (Test in tubes)

Treatments	Shoot dry weight mg $plant^{-1}$	Index of increase shoot dry weight	N-content in shoot dry weight		N-fixed in shoot dry weight	
			%	mg $plant^{-1}$	%	mg $plant^{-1}$
Ø	15.13 d	1.00	1.22	0.184 d		
ØN	45.52 a	3.01	3.50	1.593 a		
801	39.70 b	2.62	3.53	1.401 b	1.217	86.87
802	44.47 a	2.94	3.58	1.592 a	1.408	88.44
803	34.57 c	2.28	3.22	1.113 c	0.929	83.47
Mixture of strains 821+822	15.42 d	1.02	1.20	0.185 d	0.001	0.54

a-d: Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level ($p \leq 0.05$). Ø-uniniculated plants (control); ØN-plants treated with KNO_3 (control with N)

Data in Table 1 show that all of the three strains tested as single strain inoculum, i.e. 801, 802 and 803 specific for *G. orientalis*, triggered an increase in yield and N content in

plant shoot dry weight. Shoot dry weight of the inoculated plants increased 2.3-2.9-fold, and total nitrogen 6.0-8.6-fold, compared to control. Strain 802 showed higher nitrogen fixing activity than strains 801 and 803. Shoot dry weight and total N content in the plants inoculated with that strain are nearly identical to those in plants that received a full rate of N in the form of KNO_3. The percentage of fixed N in those strains was 83.5-88.4%. The results indicate a high degree of effectiveness of all three strains in symbiosis with *G. orientalis* as the host plant.

A double strain inoculum composed of the indigenous strains 821+822 specific for *G. officinalis* applied to *G. orientalis* did not increase its shoot dry weight and N content, compared to control. The strains form inactive nodules on *G. orientalis* roots (Nod^+Fix^-) that do not fix N from the atmosphere.

This fact suggests that *R. galegae* are root rhizobia highly specific for species of the *Galega* genus. The results of this investigation are consistent with some earlier reports [14, 16, 5].

Unlike the strains specific for *G. orientalis*, the indigenous strains of *G. officinalis* (821 and 822) applied as single strain inoculum provoked a much lower increase in plant shoot dry weight 1.7-fold and N content 4.6- and 5.0-fold than control (Table 2). The percentage of fixed N is also lower 78.4% and 79.8%. The relationship applies to relative values, as well as absolute.

Table 2. Nitrogen fixing activity of *R. galegae* specific for *G. officinalis* L. (Test in tubes)

Treatments	Shoot dry weight mg plant^{-1}	Index of increase shoot dry weight	N-content in shoot dry weight		N-fixed in shoot dry weight	
			%	mg plant^{-1}	%	mg plant^{-1}
Ø	17.22 c	1.00	1.38	0.237		
ØN	36.76 a	2.13	3.96	1.456 a		
821	29.60 b	1.72	3.71	1.096 c	0.861	78.41
822	30.40 b	1.77	3.88	1.179 b	0.942	79.80
Mixture of strains 801+802	18.40 c	1.07	1.31	0.241 d	0.004	1.66

a-d: Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level ($p \leq 0.05$). Ø-uniniculated plants (control); ØN-plants treated with KNO_3 (control with N).

The double strain inoculum of strains 801+802 specific for *G. orientalis* applied to *G. officinalis* was not found to increase in plant shoot dry weight or N content. The strains formed inactive nodules on the roots of *G. officinalis* (Nod^+Fix^-) that did not fix atmospheric N.

G. orientalis, is a weed plant, whose high content of alkaloids (galegine and vasicine) can be toxic in forage [9].

Although our research was conducted in the laboratory, i.e. in test tubes containing agarized Jensen medium, it made evident the role of specific active strains as inoculum in the cultivation of *G. orientalis*. This agrees with our earlier findings in trials conducted in pots on chernozem soil [5]. *G. orientalis* requires fertile soil with neutral pH and is not tolerant to heavily acidic soils due to their deficient potassium and toxicity of aluminum and manganese ions [2, 20, 15]. Acidity is a limiting factor for *G. orientalis* and *R. galegae* to establish a good symbiotic relationship. Many authors have shown that, besides soil factors, inoculation

with active *R. galegae* strains is another important factor in *G. orientalis* production in the field [11, 15, 4, 3].

Plasmid profile of the strains revealed that all investigated strains harbour one high molecular weight plasmid. Plasmids of strains 801 and 803 appear to be of the same size, with relative mobility that corresponds to the MW of 300 kb. Plasmid profiles of the strains 821 and 822 are identical, but different from the strains 801, 802 and 803 in that the plasmid band present in these two strains has the apparent MW of 250 kb, the same as the plasmid band of the control *A. rhizogenes* strain. Strain 802 has the plasmid band with the highest relative mobility, corresponding to the MW of 240 kb.

Growth of unicellular microorganisms, including rhizobia, can be measured using two different parameters: cell mass and cells numbers, which need not coincide. Mass of individual cells may vary at different stages of growth and increases continuously over time, while cell number increase is a process with interruptions, resulting from the process of successive cell partitions at certain time intervals. Generation time for fast-growing rhizobia is 2-4 h, and 6-8 h for slow-growing. In this investigation, the growth of rhizobial strains applied to different antibiotic concentrations was visually determined based on cell mass increase.

Resistance - sensitivity of investigated strains to different concentrations of 8 antibiotics is shown in Table 3.

Table 3. Growth of *R. galegae* strains on the different concentration antibiotics

R.galegae strain	Ampicillin ($\mu g \cdot ml^{-1}$)			Tetracycline ($\mu g \cdot ml^{-1}$)			Penicillin ($IJ \cdot ml^{-1}$)			Neomycin ($\mu g \cdot ml^{-1}$)			Streptomycin ($\mu g \cdot ml^{-1}$)			Erythromycin ($\mu g \cdot ml^{-1}$)			Rifampycin ($\mu g \cdot ml^{-1}$)			Chloramphenycol ($\mu g \cdot ml^{-1}$)		
	1	5	10	0.5	1	5	25	50	100	25	50	100	1	5	10	1	5	10	0.1	0.5	1	10	25	50
801	+	-	-	±	-	-	+	±	-	+	±	-	+	±	-	+	±	-	±	-	-	+	±	-
802	+	-	-	±	-	-	+	+	-	+	±	-	+	±	-	+	±	-	+	±	-	±	-	-
803	±	-	-	-	-	-	+	±	-	±	-	-	±	-	-	+	-	-	+	-	-	+	±	-
821	+	+	±	+	+	±	+	+	±	+	+	±	+	+	+	+	+	±	+	±	-	+	+	±
822	+	+	±	+	+	±	+	+	±	+	+	±	+	+	±	+	+	±	+	±	-	+	+	±

* + good growth; ± weak growth; - no growth

The results of our research given in table 3 show that *R. galegae* strains 821 and 822 specific for *G. officinalis* are generally more resistant to antibiotics than strains 801, 802 and 803 specific for *G. orientalis*. Strain 803 is the most sensitive to Ampicillin, Tetracycline, Neomycin, Streptomycin and Rifampycin, and is generally the most sensitive strain of all strains. Strains 801 and 802 have similar IAR to almost all antibiotics tested, except for Rifampycin and Chloramphenycol; strain 801 is resistant to higher concentrations of Chloramphenycol than strain 802, while the opposite is true for Rifampycin. Strains 801 and 803 differ in IAR for most antibiotics: Ampicillin, Penicillin, Neomycin, Streptomycin, Chloramphenycol while strain 803 being the more sensitive one in all cases. Since strain 803 was derived from strain 801, it appears that adaptation to high levels of Fe^{2+} led to the increased sensitivity to antibiotics, compared to the parent strain.

Strains 821 and 822 have almost identical IAR patterns for the 8 antibiotics tested, and can not be distinguished by IAR.

The attempt to characterize *R. galegae* strains by plasmid profile and IAR to the point of successful identification of individual strains was only partly successful. Due to the high similarity of the strains, both plasmid profiles of the strains and the IAR were not very discriminative. Plasmid profile analysis showed that strains 801 and 803 have the same plasmid profile, which is not unexpected since strain 803 is derived from the strain 801. It seems that adaptation to high levels of Fe^{2+} affected the antibiotic resistance, but not the plasmid content of the parent strain. Although plasmid profile analysis is used for strain characterization and sometimes strain identification in different rhizobia species [16, 8], it

appears from the work of different authors and our previous work that the discriminative value of plasmid profiles for strain identification is more pronounced in rhizobia species that usually harbour several plasmids of lower MW, such as *R. leguminosarum* bv. v*iciae* or *R. leguminosarum* bv. *trifolli* [8, 6]. Rhizobia species that are characterized by the presence of high molecular weight plasmids (mega-large plasmids) and low number of plasmid bands in their plasmid profile are less variable in plasmids profiles [17, 6].

IAR for 8 antibiotics also showed that there are very little differences between investigated strains. Nevertheless, strains 801, 802 and 803 can be distinguished by IAR patterns. IAR patterns of these strains are very similar, but not identical.

Unfortunately, strains 821 and 822, specific for *G. officinalis*, could not be distinguished by neither plasmid profile analysis nor IAR pattern. We assume that the differences between these strains are too low to be revealed by these methods, and probably some of the more discriminative methods such as rDNA typing or DNA fingerprinting must be used for strain characterization in order to be able to monitor these strains after potential inoculation in soil.

Conclusion

Rhizobium galegae are fast-growing rhizobia highly specific for plant species of the *Galega* genus (*G. orientalis* and *G. officinalis*) that are not closely correlated to other fast-growing rhizobia. A mixture of *R. galegae* strains 801 and 802 specific for *G. orientalis*, applied as a double strain inoculum to *G. officinalis*, forms inactive nodules (Nod^+Fix^-) that do not fix N from the atmosphere. A mixture of autochthonous *R. galegae* strains 821 and 822 specific for *G. officinalis* similarly forms inactive root nodules (Nod^+Fix^-) on the roots of *G. orientalis*. Treatments with strains 801, 802 and 803 specific for *G. orientalis* as single strain inoculum increased plant dry weight 2.3-2.9-fold, and N content 6.0-8.6-fold, compared to control. Strain 802 showed higher nitrogen fixing activity than strains 801 and 803. The percentage of fixed N in all strains was 83.5-88.4%). Treatments with the autochthonous strains 821 and 822 specific for *G. officinalis* as single strain inoculum increased plant dry weight 1.7-fold and the content of N 4.6- and 5.0-fold, compared to control. The percentage of N fixed by these strains was 78.4% and 79.8%. Artificial inoculation of *G. orientalis* with its specific rhizobia (*R. galegae*) is essential for achieving good cultivation results in this country. Plasmids of strains 801 and 803 appear to be of the same size, with relative mobility that corresponds to the MW of 300 kb. Plasmid profiles of the strains 821 and 822 are identical, but different from the strains 801, 802 and 803 in that the plasmid band present in these two strains has the apparent MW of 250 kb, the same as the plasmid band of the control *A. rhizogenes* strain. Strain 802 has the plasmid band with the highest relative mobility, corresponding to the MW of 240 kb. *R. galegae* strains 821 and 822 specific for *G. officinalis* are generally more resistant to antibiotics than strains 801, 802 and 803 specific for *G. orientalis*. Strain 803 is the most sensitive to Ampicillin, Tetracycline, Neomycin, Streptomycin and Rifampycin, and is generally the most sensitive strain of all strains. Strains 801 and 802 have similar IAR to almost all antibiotics tested, except for Rifampycin and Chloramphenycol; strain 801 is resistant to higher concentrations of Chloramphenycol than strain 802, while the opposite is true for Rifampycin. Strains 801 and 803 differ in IAR for most antibiotics: Ampicillin, Penicillin, Neomycin, Streptomycin, Chloramphenycol with strain 803 being the more sensitive one in all cases. Since strain 803 was derived from strain 801, it appears that adaptation to high levels of Fe^{2+} led to the increased sensitivity to antibiotics, compared to the parent strain.

Acknowledgement

This research was sustained by the Ministry of Science and Environmental Protection of the Republic of Serbia, Project, No TR-006875B.

References

1. A. HARTMANN, N. AMARGER: *Genotypic diversity of an indigenous Rhizobium meliloti field population assessed by plasmid profiles, DNA fingerprinting, and insertion sequence typing. Can. J. Microbiol.*, **37**: 600-608 (1991).
2. A.J. HOLDING, J.F. LOWE: *Some effects of acidity and heavy metals on Rhizobium leguminosarum plant association. Plant and Soil.* Spec. Vol. 153-166 (1971).
3. B. MILICIC, DJ. KUZMANOVIC, D. RADIN, D. JOSIC: *Nitrogen fixing activity of Rhizobium galegae strains specific for Galega orientalis Lam. and Galega officinalis L. Monograph, papers of the ninth Congers of Yugoslav society of Soil Science*. Novi Sad, Jun 23th-27th, p 457-463 (1997).
4. B. RADENOVIĆ: *Yield and quality fodder crop (Galega orientalis Lam.) in agricultural condition Bačke in our country. Proceeding papers of eighty Yugoslav Symposiums of fodder crops.* Novi Sad, **26**, 273-279 (1996).
5. B. MILICIC, DJ. KUZMANOVIC, D. RADIN, B. RADENOVIC: *Gala Azotofiksin- a new microbial fertilizer in the production of Galega orietalis Lam. in our country. Proceeding papers of eighty Yugoslav Symposiums of fodder crops.* Novi Sad, **26**, 449-455 (1996).
6. D. JOŠIĆ: *Characterization of indigenous strains and mutants resistant on antibiotics rhizobia Rhizobium meliloti and investigation nitrogen fixation in simbiosis with alfafa (Medicago sativa L.). Master thesis.* Biology faculty, Belgrade (1999).
7. E. TAS, P. LEINONEN, A. SAANO, L.A. RASANEN, S. KAIJALAINEN, S. PIIPPOLA, S. HAKOLA, K. LINDSTROM: *Assessment of competitiveness of Rhizobia infecting Galega orientalis on the basis of plant yield, nodulation and strain identification by antibiotic resistance and PCR. Appl. Environ. Microbiol.*, **62**: 529-535 (1996).
8. G. LAGUERRE, P. MAVINGUI, M.R. ALLARD, M.P. CHARNAY, P. LOUVRIER, S.I. MAZURIER, L. RIGOTTIER-GOIS, N. AMARGER: *Typing of Rhizobia by PCR DNA Fingerprinting and PCR-Restriction Fragment Length Plymorphism Analysis of Chromosomal and Symbiotic Gene Regions: Application to Rhizobium leguminosarum and Its Different Biovars. Appl. Env. Microbiol.* **62**(6):2029-2036 (1996).
9. H. NOMMSALU: *The nutritive value of fooder Galega (Galega orientalis Lam.). Fodder Galega (Galega orientalis Lam.) research in Estonia,* Saku, 25-34 (1994).
10. H. RAIG: *Advances in the research of the new fodder crop Galega orientalis Lam. Fodder Galega (Galega orientalis Lam.) research in Estonia,* Saku, 25-34 (1994).
11. H. RAIG: *Experience with a new introduced fodder plant G. orientalis in the Estonia,* SSSR, Talinn, 15 pp. (1982).
12. J.M. VINCENT: *A manuel for the practical study of root nodule bacteria. IBP Hand book No 15, Blackwell Sci. Pub. Oxford,* England (1970).
13. J.P.W. YOUNG: *Phylogeny and taxonomy of rhizoba. Plant and Soil,* **186**, 45-52 (1996).
14. K. LINDSTROM, B.D.W. JARVIS, P.E. LINDSTROM, J.J. PATEL: *DNA homology, phage-typing and cress-nodulation studies of rhizobia infecting Galegae species. Can. J. Microbiol.*, **29**, 781-789 (1983).

15. K. LINDSTROM, M. SARSA, J. POKUNEN, P. KANSANEN: *Symbiotic nitrogen fixation of Rhizobium (Galega) in acid soils and its survival in soil under acid and cold stress. Plant and Soil,* **87**, 293-303 (1985).
16. K. LINDSTROM: *Rhizobium galegae, a new species of legume root bacteria. Inter. J. of System. Bacteriology,* 39, 3, 365-367 (1989).
17. M. SHISHIDO, I.L. PEPPER: *Identification of dominant indigenous Rhizobium meliloti by plasmid profiles and intrinsic antibiotic resistance. Soil Biol. Biochem.,* **22**(1): 11-16 (1990).
18. P. SOMASEGARAN, H.J. HOBEN: *Handbook for Rhizobia, Metods in Legume-Rhizobium Technology.* Springer-Verlag New York, Berlin, Heildeberg, London, Paris, Tokyo, Hong Kong, Barcelona, Budapest. (1994).
19. R. WHEATCROFT, C. WILLIAMS: *Extraction of whole plasmid DNA for agarose gel electrophoresis. J. Gen. Microbiol.,* **124**: 433-437 (1981).
20. T.A. LIE: *The effect of pH on different phases of nodule formation in pea plants. Plant and Soil,* **31**, 391-406 (1969).