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Review Article

Alfalfa (*Medicago sativa* L.) and *Sinorhizobium meliloti*: prospects of using rhizobial inoculants in Serbia

Dušica Delić[1*], Olivera Stajković-Srbinović[1] and Jelena Knežević-Vukčević[2]

1 Institute of Soil Science, Teodora Drajzera 7, 1100 Belgrade, Serbia

2 Chair of Microbiology, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade

ABSTRACT: It is considered that the symbiotic association between alfalfa (*Medicago sativa* L.) and *Sinorhizobium meliloti* is one of the most efficient interactions between N_2-fixing bacteria and legumes. But even greater efficiency of symbiotic nitrogen fixation can be achieved by selecting the best combinations of host genotypes and nodule bacteria. The Serbian K-28 cultivar has the potential capacity to be a good parental genotype in breeding programs for improved N_2 fixation capability through inoculation with particular compatible strains. On the other hand, *S. meliloti* strain L5 is a broadly effective inoculant with most alfalfa cultivars, with a mean value of fixed N_2 of 35% over a two-year period. Serbian soils are well supplied with autochthonous rhizobial strains which specifically nodulate alfalfa, and this is why the use of rhizobial inoculants in alfalfa production in Serbia is not a common practice. However, the number and effectiveness of such strains depend on soil type, the land use system (alfalfa stand, arable land and lea) and the alfalfa host plant. For this reason, use of rhizobial inoculants with highly effective strains is needed in alfalfa production. Our two-year field experiment showed that shoot dry weight increased by 42-77% in inoculated alfalfa, while the amount of fixed nitrogen increased by 35%. Alfalfa inoculation with effective strains is an alternative approach to improving the long-term productivity of alfalfa. In our five-year trial, the inoculated K-28 alfalfa cultivar showed maximum nitrogen fixation (293 kg N ha^{-1}) in the fourth year of utilisation. During this period, symbiotic nitrogen fixation of alfalfa, determined from fixed N_2 in the second cutting, varied between 32 and 44%. The most effective strain promoted an increase of shoot dry weight by 34-60% in relation to the control in the third and fourth years of utilisation. In addition, *Sinorhizobium* spp. have the potential to be used as plant growth-promoting rhizobacteria (PGPR). Inoculation of Italian ryegrass seeds with a particular rhizobial strain the year before alfalfa growing ensured abundant nodulation and better growth of the alfalfa. In Serbia there are rhizobial inoculants of alfalfa with consistently high quality which permit reduction of mineral N fertiliser use in production of this crop, with consequent economic and ecological benefits.

Keywords: alfalfa, *Sinorhizobium meliloti*, symbiotic nitrogen fixation, rhizobial inoculants

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INTRODUCTION

With respect to total sowing area, leguminous plants (fam. Leguminoseae) take second place in the world just after the family Poaceae. Alfalfa (*Medicago sativa* L.) is one of the most important perennial legumes in agriculture, especially in animal husbandry, due to its excellent nutritive value. The agronomical value of alfalfa is based on its high herbage yield and protein content (20%), high digestibility and capacity for symbiotic nitrogen fixation (SNF), which reduces the need for mineral fertilisers (Zeng *et al.* 2007). Moreover, owing to SNF, alfalfa is of great significance in crop rotation, as it enriches soil with nitrogen (N), in addition

*correspondence: vukmirdusica@yahoo.com

to which it exerts positive influence on multiplication of soil microorganisms needed for mineralisation of soil organic matter (Delić-Vukmir *et al.* 1994; Nešić *et al.* 2008). Alfalfa has the ability to tolerate heavy metals and thus can play an important role in the restoration of nitrogen-depleted soils and be used as a priority pioneer species for the rehabilitation of degraded and contaminated soils (Gardea-Torresdey *et al.* 1999).

Symbiotic nitrogen fixation in legumes is a natural process of supplying the host plant and soil with N by reduction of atmospheric nitrogen (N_2) This process is performed by N_2-fixing nodule bacteria collectively called rhizobia (fam. Rhizobiaceae). Before modern mineral fertilisers, legume SNF represented the primary nitrogen input for agriculture. Symbiotic nitrogen fixation is considered to represent a significant boost to N fertilisation, an additional advantage being that as a natural process, it is not hazardous to the environment (Vance 1997).

Rhizobia nodulate the host plant root in the process called nodulation, in which bacteria are transformed into bacteroids, directly reducing N_2 by the nitrogenase enzyme complex, using considerable energy (Elboutahiri *et al.* 2010). The host plant gets reduced nitrogen from the bacteria (NH_3 followed by amino acids) while at the same time providing them with carbohydrates and all essential nutrients (Rengel 2002; Sytnikov 2013). The exchange of signal metabolites between symbionts has to be compatible in order to result in effective nodulation and nitrogen fixation. Efficiency of nitrogen fixation is a complex trait, involving genes of both symbionts and their interaction (Ferguson *et al.* 2010).

Symbiotic nitrogen fixation in alfalfa. Alfalfa fixes N_2 in symbiosis with the specific nodule bacteria *Sinorhizobium* (*Ensifer) meliloti*, *S. medicae* and *S. tibeticum* (Lajudie *et al.* 1994). It is considered that the alfalfa/*S. meliloti* symbiotic association represents one of the most efficient interactions between N_2-fixing bacteria and leguminous plants. The amount of N_2 usually fixed by alfalfa in the field varies between 140 and 210 kg ha^{-1} $year^{-1}$, with an assessed potential of even 550 kg ha^{-1} $year^{-1}$. Owing to this amount of nitrogen fixed, alfalfa needs smaller amounts of nitrogen fertiliser in comparison with many other legumes and particularly non-legumes in crop rotation. The highest intensity of N_2 fixation is usually achieved at low or moderate levels of nitrogen fertilisation covering no more than 20–30% of plant demands (Provorov & Tikhonovich 2003).

Various alfalfa genotypes and rhizobial strains differ in SNF capacity. Differences of N_2 fixation are reflected in shoot dry weight (SDW) of the host plant, since this phenotypic characteristic is the result of genetic variation in both symbionts and represents an indirect measure of nitrogen fixation intensity, together with the percentage of fixed N_2 (%Ndfa). On the other hand, the activity of bacteroid nitrogenase in plant root nodules is a parameter for direct measuring of existing SNF activity. In our laboratory research, the values of nitrogenase activity in nodules of alfalfa varied from 0.084 mol h^{-1} $plant^{-1}$ to 0.340 mol h^{-1} $plant^{-1}$, whereas the nitrogenase activity of highly effective strains ranged between 0.138 mol h^{-1} $plant^{-1}$ – and 0.213 mol h^{-1} $plant^{-1}$ (Vukmir 1993).

It is known that variability of N_2 fixation has a highly complex genetic basis dictated by interactions among a great number of SNF-enabling genes in both symbionts (Caetano-Anolles & Gressoff 1991; Langer *et al.* 2008). The source of variability in alfalfa lies in its tetraploid genome and cross-pollination, which makes each plant a distinctive genotype (Vitale *et al.* 1996). There is also considerable diversity of the species *S. meliloti* caused by its highly polymorphic genome in natural populations (Langer *et al.* 2008; Elboutahiri *et al.* 2010). The precise extent of influence exerted by the symbionts on SNF variability is not known, although it is important for the selection process. Some reports suggest prevailing influence of the host plant, but other data indicate influence of the bacterial strain as well and their interaction (Rengel 2002). In laboratory experiments of ours, we precisely showed highly significant influence of the *S. meliloti* strain on variations of shoot dry weight, and consequently on SNF variability, in the case of several cloned genotypes of alfalfa cultivars: Serbian Kruševačka K-22 and K-28, and Mediana. Based on two-factor variance analysis (ANOVA) of SDW, we demonstrated highly significant influence of the bacterial strain on variability of SNF (F value around 77), influence much stronger than that of the plant genotype (F value around 15) and interaction of symbionts (F value around 5.9), which suggested a need for a parallel selection of both symbionts (Delić 2003).

In further laboratory studies, we identified three plant/strain compatibility groups significantly different in N_2 fixing activity. Among 20 investigated symbiotic pairs, the most effective group consisted of three pairs: cultivar K-28 with *S. meliloti* strains L3Si, L5 and 218. We also identified the most effective *Sinorhizobium* strains for other cultivars: strains L5, LR1ks and 218 for the Slovakian Vanda cultivar; strain L3Si for the Slovenian Soča cultivar; and strains L3Si, 218 and L5 for the Bosnian BL-88 and Serbian Kruševačka K-28 cultivars. The intensity of N_2 fixation of one strain was different with different host genotypes due to different compatibility of the symbionts. Our results indicated that the Serbian K-28 cultivar has the potential to be a good parental genotype in breeding programs for improved N_2 fixation capability through inoculation with compatible strains L3Si and L5, as well as with strain 218, which would represent commercial strains for use in microbiological fertilisers for the given cultivar (Delić *et al.* 2010a). These results were confirmed by us in a two-year field trial with the same symbionts (Delić *et al.* 2013). Considerable variation in N_2 fixation effectiveness of *Sinorhizobium* spp. strains, from ineffective to significantly effective, was detected depending on the alfalfa cultivars and the year of utilisation, the first year being the most important due to the crucial role played by successful establishment of the alfalfa stand.

In the first and second year, the SDW of plants inoculated with effective strains was enhanced by 49-68% and 35-86%, respectively, compared to the uninoculated control. The percentage of nitrogen fixed (Ndfa%) in plant mass was 33 and 38% in the first and second years, respectively. We selected the highly effective strain L5, which was a broadly efficient inoculant with most alfalfa cultivars (including BL-88 and K-28) over a two-year period, with the ability to significantly improve the cultivars' yield in field conditions. Cultivar BL-88 had an indiscriminate relationship to all strains and could be a good genotype in breeding programs for improved N_2-fixing capacity, followed by cultivar K-28.

Nitrogen fixation potential of soils in Serbia. The nitrogen fixation potential of the soil (number and activity of *Sinorhizobium* spp. strains) provides information indicating whether there is a need for artificial inoculation of alfalfa as a way to improve nitrogen fixation. In addition, the given potential clarifies the possibility of isolating and selecting highly effective and competitive strains, as well as strains tolerant to adverse environmental conditions which are potential active agents of damage to inoculants specific for alfalfa.

Soils in Serbia are well supplied with autochthonous *S. meliloti* strains, but the number and distribution of these bacteria are variable and depend on the soil type, particular physico-chemical properties of the soil, the land use system (alfalfa stand, arable land and lea) and presence or absence of the alfalfa host plant. Generally, soil types with good physical and chemical characteristics, like chernozem and fluvisol, are the richest in *S. meliloti* (10^5 g^{-1} soil), while poor soils like stagnosol have the lowest number of this rhizobial species, which is particularly sensitive to acidic conditions (Zahran 1999). In all soil types *S. meliloti* numbers varied according to the land use system: the number was the highest in alfalfa stands and decreased parallel with increase in the time elapsed from the presence of host plants during crop rotation (Vojinović *et al.* 1989; Vukmir 1993; Delić-Vukmir *et al.* 1994). Furthermore, in the case of stagnosol, strains of *S. meliloti* were detected only in growing alfalfa fields, which emphasizes influence of the host plant on the presence of *S. meliloti* in acidic soils (Delić-Vukmir *et al.* 1994). A more recent study similarly indicates positive influence of the host plant on autochthonous populations of *S. meliloti* in soil under the influence of ash from a power plant (damaged soil): only samples from an alfalfa field contained *S. meliloti* (Delić *et al.* 2010b).

Soils in Serbia contain strains with significant nitrogen fixation activity. For example, 77% out of all *S. meliloti* strains isolated from soils in the Kruševac region were effective in nitrogen fixation (Delić-Vukmir *et al.* 1995). Previous research indicated that soils with very good physico-chemical properties contain highly effective *S. meliloti* strains, and that the host plant exerts positive influence on strain effectiveness (Vojinović *et al.* 1989). However, our investigations showed that there is no rule linking strain effectiveness with soil types or land use: *S. meliloti* strains with moderate effectiveness were isolated from stagnosol, which is not typical for that soil type, while strains from fluvisol varied in effectiveness. Moreover, we isolated highly effective strains from arable land and lea, indicating that land use and the alfalfa host plant did not influence strain activity. *S. meliloti* strains originating from an alfalfa stand established on stagnosol showed moderate activity (Delić-Vukmir *et al.* 1995), while 30% of strains isolated from an alfalfa field under the influence of ash had high and moderate nitrogen fixing efficiency (Delić *et al.* 2010b), which indicates the possibility of selecting effective strains that could improve damaged soils. Our detection of effective nitrogen fixation by alfalfa in acidic soils deserves attention because these soils cover 50% of arable land in Serbia (Delić 2014).

Selection of strains for high-quality rhizobial inoculants. Use of rhizobial inoculants in alfalfa production represents an important way to achieve enhancement of SNF. They contain competitive living rhizobia selected for very high N_2 fixation potential and/or other particular traits (Vojinović 1964; Ben Rebah *et al.* 2007). Artificial rhizobial inoculation reduces the need to use N mineral fertilisers, increases the alfalfa yield, enhances total N content of the plants and seed protein content and supplies non-leguminous crops and soils with N.

Development of high-quality inoculants is one of the main aims in production of microbiological N fertilisers and involves strain selection, selection of a carrier, mass multiplication, formulation of the inoculants and packaging and marketing.

Rhizobial strains show high diversity in terms of morphological, biochemical and genetic characteristics, as well as in regard to symbiotic properties such as competitiveness, virulence and activity. They also react differently to adverse environmental conditions: high temperature, low soil pH, drought and the presence of pesticides, antibiotics and heavy metals (Hefny *et al.* 2001). Strain diversity allows selection of strains with particular characteristics, among which high strain effectiveness is the most important, but the ability to survive on seeds and capacity for nodulation of host roots in the presence of mineral nitrogen are also important properties of rhizobia as a potential agent of microbiological fertilisation (Rengel 2002). Selection of effective strains is performed in plant trials under controlled conditions and in the field. An alternative approach to selection lies in genetic improvement of the selected strains (Xavier *et al.* 2004).

Our long-standing studies of morphological, biochemical and genetic characteristics of rhizobial strains from Serbian soils and their symbiotic properties in particular (Vojinović *et al.* 1989; Delić-Vukmir 1994; Miličić *et al.* 2006a; Stajković-Srbinović *et al.* 2012) have made possible the identification of more than 60 rhizobial strains (including *S. meliloti*, *S. medicae* and *S. tibeticum*), all of which are deposited in the Rhizobial Strain Collection of the Institute of Soil Science in Belgrade. The Collection is included in the IBP World Catalogue of *Rhizobium* Collections and World Directory of Collections of Cultures of Microorganisms. It constitutes a basic fund for scientific research and permanent selection of effective strains of *Rhizobium* spp. to produce microbial N fertilisers for different legumes, including alfalfa.

In order to obtain a high-quality inoculum, we paid great attention to selection of strains with favourable traits: high N_2 fixing capacity, virulence and competitiveness; occupation of a significant proportion of nodules; high activity of nitrogenase and associated enzymes; ability to achieve a high number of cells in inocula; strain mobility; and tolerance to adverse environmental conditions (Miličić *et al.* 2005; Miličić *et al.* 2006a,b; Delić *et al.* 2007; Delić *et al.* 2010b). Considerable efforts of our research team have also been directed towards selecting optimal combinations of rhizobial strains and alfalfa genotypes for high yield and plant shoot quality. As already mentioned, our two-year field experiments showed significant potential ability of particular *Sinorhizobium* strains to improve the yield of alfalfa cultivars in field conditions (Delić *et al.* 2013).

In addition, inoculation with effective strains represents an alternative approach to improving the long-term productivity of alfalfa. In our five-year trial, inoculated alfalfa cultivar K-28 fixed the maximum amount of nitrogen (293 kg N ha^{-1}) in the fourth year of utilisation. Throughout 5 years of utilisation, the SNF of alfalfa determined from fixed N (%Ndfa) in the second cutting varied between 32 and 44%. The most effective strain gave an increase of SDW by 34-60% in relation to the control plants in the third and fourth years of utilisation (Delić *et al.* 2010c).

Formulation of inoculant. Formulation of the inoculant involves introduction of the active agent into a suitable carrier together with additives, the aim of which is to stabilize and protect the microbial cells during storage and transport. Generally, sterile peat has been found to be the preferred carrier in powder-form inoculants due to its ensuring a high sustaining level of rhizobia, higher yields in the field and the chance of extending culture production by diluting the broth without any reduction in quality of the final inoculant (Xavier *et al.* 2004).

In Serbia peat has been used as the carrier in powder formulations for almost 50 years, mainly because of low cost, simple production and good effectiveness in nitrogen fixation. Our investigations of carriers indicated that the most significant stimulating effect on the growth of *S. meliloti* strains was achieved using a peat carrier enriched with lucerne meal and charcoal. In this carrier, the bacterial titre was about 2.5×10^9 rhizobia per gram of inoculant depending on the *S. meliloti* strain applied. A negative effect on strain growth was recorded for peat carriers mixed with Agrostemin and Minazel, as well as for peat with Agrostemi and charcoal (Miličić *et al.* 2006c).

Quality of inoculants. Characteristics of high-quality inoculants are: a sterile carrier medium, a defined bacterial culture, effectiveness, high numbers of live rhizobia and absence of microbial contaminants in the inoculum. Other desired characteristics are long shelf-life and low cost (Xavier *et al.* 2004).

In Serbia quality control of rhizobial inoculants as final products is performed by the manufacturer. Characteristics of the inoculant must comply with legislation in order to be included in the Register of Plant Nutrients. Our inoculant for alfalfa with a particular *S. meliloti* strain as the active agent has about $2.5\text{-}3 \times 10^9$ cells per gram of carrier and has been certified. Field trials of its effectiveness showed that the inoculant increased alfalfa shoot dry yield and total N content by more than 50% in comparison with the unfertilised control (Delić *et al.* 2011; Delić *et al.* 2013). Moreover, our inoculant can be applied in the next season because reduction of the bacterial titre (to about 1.5×10^8 cells per gram) did not influence the inoculant's quality (unpublished results).

Use of rhizobial inoculants in alfalfa production in Serbia. The use of rhizobial inoculants in alfalfa production in Serbia is not a common practice because our soils usually contain autochthonous rhizobial strains which specifically nodulate alfalfa. However, application of mineral N fertiliser per hectare of alfalfa is reduced by participation of fixed N (Delić *et al.* 2010c; Delić *et al.* 2013). This means that inoculant use could decrease size of the financial investment in alfalfa production in view of the costs of rhizobial inoculants versus mineral N fertilisers. Moreover, current research indicates that rhizobia have the potential to be used as plant growth-promoting rhizobacteria (PGPR) with legumes as well as non-legumes, supplying the plants with vitamins and growth substances, in addition to which they can influence phosphorus availability and have a synergistic effect on vesicular-arbuscular mycorrhizal fungi (Antoun & Prevost 2000; Mehboob *et al*. 2009; Delić *et al*. 2012; Stajković *et al*. 2009; Stajković-Srbinović *et al*. 2014). In an investigation of ours, we detected a beneficial effect on yield and N-assimilation in Italian ryegrass caused by inoculation of the plants with particular *S. meliloti* strains. Furthermore, inoculation of Italian ryegrass seeds with a particular rhizobial strain the year before alfalfa growing ensured abundant nodulation and better growth of the alfalfa (Delić *et al*. 2012).

CONCLUSION

Symbiotic nitrogen fixation is a renewable source of nitrogen, particularly in alfalfa production, and an environmentally friendly way of supplying the alfalfa plant and soil with nitrogen.

In spite of good results, use of rhizobial inoculants is not usual in Serbian practice because our soils contain autochthonous rhizobial strains which specifically nodulate alfalfa. This eliminates the farm work involved in applying inoculation during sowing. In spite of that, we recommend use of alfalfa rhizobial inoculants because of their considerable positive influence on quantity and quality of the alfalfa yield and for enrichment of the soil with plant nutrients for subsequent crops as a result of their plant growth-promoting abilities.

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Botanica SERBICA

REZIME

Lucerka (*Medicago sativa* L.) i *Sinorhizobium meliloti*: perspektive za rizobijalne inokulante u Srbiji

Dušica Delić, Olivera Stajković-Srbinović i Jelena Knežević-Vukčević

Simbiozna asocijacija između lucerke i *Sinorhizobium meliloti* je jedna od najefikasnijih interakcija između azotofiksirajućih bakterija i leguminoza. Povećana efikasnost simbiozne azotofiksacije može biti postignuta selekcijom najboljih kombinacija geotipova lucerke i rizobijalnih bakterija. Srpska sorta K-28 ima veliki potencijal kao dobar genotip u programu oplemenjivanja lucerke za povećanu sposobnost azotofiksacije inokulacijom sa specifičnim kompatibilnim efikasnim sojevima. S druge strane, soj L5 je visoko efikasan, promiskuitetan inokulant jer je sa većim brojem sorti lucerke ostvario efikasnu azotofiksaciju sa prosečnom vrednošću fiksiranog azota od 35% u dvogodišnjem periodu gajenja lucerke. Zemljišta u Srbiji su dobro snabdevena autohtonim rizobijalnim sojevima koji specifično nodulišu lucerku i to je glavni razlog zbog čega primena rizobijalnih inokulanata u proizvodnji lucerke u Srbiji nije uobičajena praksa. Međutim, njihov broj i efikasnost zavise od tipa zemljišta, načina iskorišćavanja zemljišta (lucerište, njiva i ledina) i biljke domaćina. Zbog toga je u proizvodnji lucerke potrebna primena rizobijalnih inokulanata sa visoko efikasnim sojevima. U dvogodišnjem eksperimentu u kome je lucerka tretirana rizobijalim inokulantom, suva nadzemna masa je povećana za 42 do 77% a količina fiksiranog azota je izosila 35%. To ukazuje da je primena rizobijalnih inokulanata alternativni pristup za unapređenje produktivnosti lucerke. Najveća količina azota od 293 kg N_2 ha^{-1} je zabeležena u našem poljskom petogodišnjem ogledu. U tom periodu, simbiozna azotofiksacija lucerke, određena u drugom otkosu, je varirala između 32 i 44%. Najefikasniji soj *S. meliloti* je omogućio povećanje suve nadzemne mase za 34 do 60 % u odnosu na kontrolu, u trećoj i četvrtoj godini iskorišćavanja. Pored toga, sojevi *Sinorhizobium* spp. imaju potencijal da budu korišćeni kao bakterije koje poboljšavaju rast biljke. Inokulacija italijanskog ljulja rizobijalnim inokulantom specifičnim za lucerku, godinu dana pre gajenja lucerke, uticala je na povećanu nodulaciju i bolji rast lucerke. U Srbiji postoje rizobijalni inokulanti specifični za lucerku sa konstantno visokim kvalitetom a koji su sposobni da smanje primenu mineralnog azotnog đubriva u proizvodnji lucerke što pruža značajnu ekonomsku i ekološku korist.

Ključne reči: lucerka, *Sinorhizobium meliloti*, simbiozna azotofiksacija, rizobijalni inokulanti