EFFECTS OF GENETICALLY MODIFIED ALFALFA PLANTS ON THEIR INTERACTION WITH *SINORHIZOBIUM MELILOTI*

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The aim of our study was to evaluate the effect of three types of genetically modified alfalfa (*Medicago sativa* L.) plants grown in soils of different pH on their growth characteristics and interactions with *Sinorhizobium meliloti*. Transgenic alfalfa plants contained the genes *Ov* from Japanese quail (*Coturnix coturnix*) coding for ovalbumine, *AMV*cp-s from Alfalfa Mosaic Virus (*AMV*), coding for the viral coat protein, and combination of genes *uid*A encoding ß-glucuronidase, and *npt*II, encoding neomycinphosphotransferase II, respectively. Seven strains of *Sinorhizobium meliloti* were used to inoculate alfalfa, of which some showed high tolerance to low pH in an *in vitro* assay system. The experiment also included a non-transgenic isogenic alfalfa line SE/22-GT2 as a control. The highest total dry weight (DW) of green matter in neutral pH soil was determined in transgenic line GTAMV1 (containing the *AMV*-cp-s gene). Plants grown in neutral soil produced 28% more green matter DW in comparison with those grown in the acidic soil. Inoculation with N_2-fixing bacteria increased the DW of green matter from 40% (neutral soil) to 87% (acidic soil). The highest agronomic efficiency, defined as the ratio of difference between the total aboveground biomass DW in inoculated and non-inoculated variant to non-inoculated control, was observed in *S. meliloti* strains 7T4 and 4T8. The number of nodules on the roots of plants was significantly affected by the genotype and inoculation with rhizobial strain ($P < 0.05$). The highest number of nodules was observed in transgenic clones containing the *AMV*cp-s gene, i.e. 85% more than in clones with *Ov* gene, and 77% more than in marker genes only containing clones of alfalfa. Alike, transgenic alfalfa lines containing the *AMV*cp-s gene had higher numbers of nodules by 140% in neutral soil and 4-times more in acidic soil in comparison with isogenic non-transgenic lines. The highest N_2-fixation efficiency (11.5%), defined as the ratio of increase in the total N content in aboveground biomass of inoculated variant to total N content in non-inoculated variant, was found in strain 4T5. Our results showed a transgene-dependent (positive) interaction of rhizospheric nitrogenic microorganisms with roots of transgenic alfalfa plants.

Key words: alfalfa, genetic modification, soil parameters, *Sinorhizobium meliloti,* equivalence

INTRODUCTION

Acid soils make up about 40% of world arable land (≈ 10 % in Slovakia), and this area is increasing. As the same is valid for the global area of commercially planted transgenic crops, the importance to study the interaction between soil microbes and transgenic plants in acid soil is also increasing (Blackwood and Buyer 2004; Donegan et al. 1999; Motavalli et al. 2004).

It has been reported that alfalfa (*Medicago sativa* L.) can effectively grow also on soils with low pH if provided by sufficient source of nitrogen (Howieson et al. 2000). One possibility to accomplish this is introduction of strains of *Sinorhizobium meliloti* with improved capacity of colonization in acidic soils (Velázquez et al. 1999). One step to improve alfalfa cultivation under these unfavourable conditions would be the identification and use of acid-tolerant rhizobial strains (Wegener et al. 2001). Rhizobia vary in their ability to grow and compete under acidic soil conditions (Steele et al.

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1999). The aim of the study of Howieson et al. (1988) was to collect nodule material from medic plants found growing on acid soils, to isolate and culture rhizobia on media adjusted over a range of pH values, and to assess their acid tolerance in both the laboratory and field conditions. Strains of *Rhizobium meliloti* isolated onto low pH media were, in general, more acid-tolerant than sister isolates from high pH media, when tested in both the laboratory and field. Ozawa et al. (1999) selected 8 strains from Indonesian soils based on the acid and aluminum tolerance and examined the acetylene reduction activity of the nodules formed by them. Of the eight strains tested, 3 acid- and Al-tolerant isolates, OSP27, BTCC-B71, and BTCC-B75, exhibited a significantly high acetylene reduction activity. If these isolates were superior in competitiveness, they could become candidates for use as inoculum strains in acid soils.

The inoculation of alfalfa seeds with efficient and competitive *S. meliloti* has long been practised to increase plant production and to preserve the nitrogen fertility of soils. The acid tolerance of *S. meliloti* in culture medium and in soils is considered as useful criteria to select for strains with improved survival in agricultural acidic soils (Del Papa et al. 2003).

The main objective of our study was to study the impact of different transgenes incorporated and expressed in alfalfa plants on symbiotic ability of plants and compare the differences between transgenic and non-transgenic plants in their interaction with beneficial soil bacteria *S. meliloti* in neutral and acid soil.

MATERIALS AND METHODS

Evaluation of the tolerance of S. meliloti strains to acidity in an in vitro testing system

In the experiment aimed at evaluation of acid tolerance of rhizobial bacteria we used 7 native isolates (7T4, 4T32, 4T8, 5T2, 5T31, 4T5, 7T11) of *S. meliloti* obtained from nodules of alfalfa grown near Trebišov (Eastern Slovakia) in soil of pH = 5.5 and 1 commercial strain (D113, provided by The Genebank of Rhizobia, Praha-Ruzyně, Czech Republic).

The isolates and the commercial strain were cultured in liquid yeast extract-manitol medium (YEM) (Mallik 2000) in 25 ml Erlenmeyer flasks on an orbital shaker for 24 hours. Then, the medium was diluted 1 : 1 000 with sterile redistilled water and 0.5 ml of the suspension was added to modified YEM medium in test tubes (16/160 mm). The pH of YEM media in test tubes were adjusted to 4.3–5.0 in intervals of 0.1 pH units (Ozawa et al. 1999). Four replications for each strain per pH variant were performed. The optical density (OD) of bacterial cells was measured spectrophotometrically using SPEKOL 11 spectrophotometer at a wavelength of 600 nm. The change in cell density was calculated from the difference of OD_0 (at the initiation of culture) and OD_1 (after 24 hours of culture).

Evaluation of the nodulation ability and effect of inoculation by nitrogen-fixing bacteria on transgenic plants of alfalfa grown in acidic conditions

In the inoculation test we used three types of transgenic alfalfa plants (TGPs): a) TGPs containing the *Ov* gene from Japanese quail coding for ovalbumin (Mucha et al. 1991): GTL1/105-3, GTL1/111-1 (herein named GTOV1, GTOV2), b) TGPs containing the *AMV*cp-s gene coding for Alfalfa Mosaic Virus coat protein (Kúdela and Gallo 1995): GTL4/402-2, GTL4/404-1 (GTAMV1, GTAMV2), c) TGPs with introduced marker genes *npt*II, coding for neomycin-phosphotransferase II (selection marker, kanamycin resistance), and *gus* coding for *β*-glucuronidase (visual marker): GTL5/401-1, GTL5/409-1 (GTGUS1, GTGUS2), and an isogenic non-transgenic line: SE/22-GT2. All transgenic lines of alfalfa were derived from the highly regenerable genotype Rg9/I-14-22 selected from cv. Lucia (Faragó et al. 1997). Plants were multiplied *in vitro* micropropagation of selected clonal lines of alfalfa using induction of axillary shoots from nodal explants on MS (Murashige and Skoog 1962) medium + 0.25 $mg.l^{-1}$ IBA (MS0.25). After the acclimatization period, the plants were transfered into plastic pots (75 × 75 mm) filled with soil substrates: natural acidic soil from the area of Pribylina (Central Slovakia, pH = 4.0) supplemented with P, K and Mg according to Burdon et al. (1999), and neutral soil from the fields of RIPP Piešťany (Western Slovakia, pH = 7.0). The plants were inoculated with different inoculation suspensions of nitrogen-fixing bacteria *S. meliloti*. The bacterial strains used were 4 native isolates (showing high tolerance to low pH in liquid culture medium in the previous experiment): 7T4, 4T8, 4T5, 7T11, and 1 standard commercial strain D113. Before use, the inoculation

suspensions were adjusted to the optical density equivalent of 10^8 CFU ml^{-2}. Plants inoculated with a sterile physiological solution were included as negative controls. Plants were grown in a growth chamber at rela-tive air humidity about 90%, photoperiod of 16 h light/8 h dark and a temperature of 23˚C. Pots (4 plants in each) were arranged in a completely randomized block design and each treatment consisted of three replicates. At harvest (12 weeks after the end of acclimatization period), the following parameters were evaluated: the absolute dry weight of aboveground biomass (mg), fresh weight of roots (mg), root system-size (scale 1–9, 1 = very low), root lenght (mm), number of nodules, percentage of active nodules (according to Rice et al. 1977), morphology of nodules (1 – round, 2 – prolonged, 3 – nodules in aggregates), and the total amount of N in the aboveground biomass. The agronomic efficiency (%), characterized by the rate of difference bettwen absolute dry weight of inoculated and non-inoculated variants to non-inoculated control, and nitrogen fixation efficiency (%), calculated as the ratio of increase of total N in aboveground biomass of inoculated variant to total N in noninoculated control, were calculated according to Mengel and Kirkby (2001). The results were subjected to analysis of variance (ANOVA) using the Statgraphics Ver. 5.0 software.

RESULTS AND DISCUSSION

The first *in vitro* experiment aimed to evaluate the tolerance of rhizobial strains to acid conditions. Seven native (7T4, 4T32, 4T8, 5T2, 5T31, 4T5 and 7T11) and one standard commercial strain (D113) of *S. meliloti* have been used. The density of bacteria in liquid medium after 24 hours of cultivation was significantly ($P < 0.001$) affected by the bacterial strain and the pH of medium. Different *Rhizobium* species are known to differ in their tolerance to soil acidity (Cheng et al. 2002) and the hydrogen ion concentration has been shown to be the major factor restricting the survival and growth of rhizobia in soil. Differences have been observed between *R. meliloti* strains in their ability to grow and to nodulate alfalfa under acid conditions (Boisson-Dernier et al. 1994; Rice et al. 1977). The overall density of bacterial cells decreased, in general, with decreasing medium pH down to 60 %. The most profound drop of bacterial cell density caused by the decrease of pH from 5.0 to 4.3 occured with strain 4T5 (by 26% more than the overall average) and the lowest decrease using the strain 4T32 (by 60% less than the overall average). The highest optical density of bacterial cells after 24 hours of cultivation in liquid medium with pH values adjusted to 4.3–5.0 in intervals of 0.1 pH units was observed in strain 4T5 with densities exceeding the mean values by as much as 103% (Fig. 1). Based on differences between the initial numbers of cells and counts at the end of cultivation in culture media with pH values of 4.3 to 5.0 we selected the strains 4T5, 7T11, 7T4 and 4T8 for testing the acidity tolerance directly in acidic soil.

Many studies reported positive correlation between the acid tolerance of rhizobia in nutriet media and competive nodulation in acid soil (Ozawa et al. 1999; Dilworth et al. 2001). Rice (1982) observed the relationship between soil pH, nodulation and yield of alfalfa inoculated with low-pH sensitive strains. The data indicated that the low-pH tolerant selections of *R. meliloti* possessed better N_2-fixing efficiency at low pH as well as greater nodulation potential than the low-pH sensitive strains. Identification of rhizobial strains possessing the combination of high effectiveness and tolerance to mineral stresses aid in the development of inoculum suited for infertile, acid soils (Thornton and Davey 1983).

Three types of transgenic plants of alfalfa containing the genes *Ov* for ovalbumine production, *AMV*-cp-s for Alfalfa Mosaic Virus coat protein expression, *gus* for visual marker expression and non-transgenic

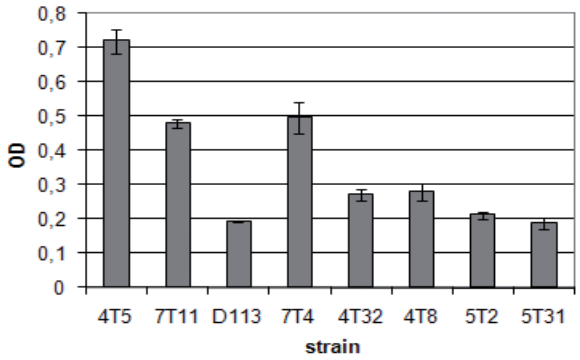

Fig. 1. Comparison of optical density of cells (OD) of nitrogene strains of bacteria *Sinorhizobium meliloti* after 24-hour cultivation in liquid medium of pH values in an intervale 4.3 to 5.0

parental isogenic line SE/22-GT2 have been used to evaluate the nodulation efficiency of transgenic alfalfa plants. Clonal plants of the selected genotypes were inoculated with four selected strains of *S. meliloti* (4T5, 7T4, 7T11, and 4T8), which showed high tolerance to low pH in an *in vitro* assay, and one standard strain D113. Plants inoculated with a sterille physiological solution served as controls.

The variation in green matter dry weight was statistically significantly ($P < 0.001$) affected by the genotype, inoculation and soil pH. Of the tested genotypes, the highest green matter DW was found in transgenic line GTOV1 where the DW exceeded the average DW of all genotypes by 23%. In general, however, for the neutral soil, the highest average values for this trait were found in transgenic lines of alfalfa containing the *AMV*cp-s gene, that was by 26% higher than in lines containing the *Ov* gene, and over 21% higher than in lines containing the marker genes. Regarding comparison of transgenic and non-transgenic alfalfa plants,

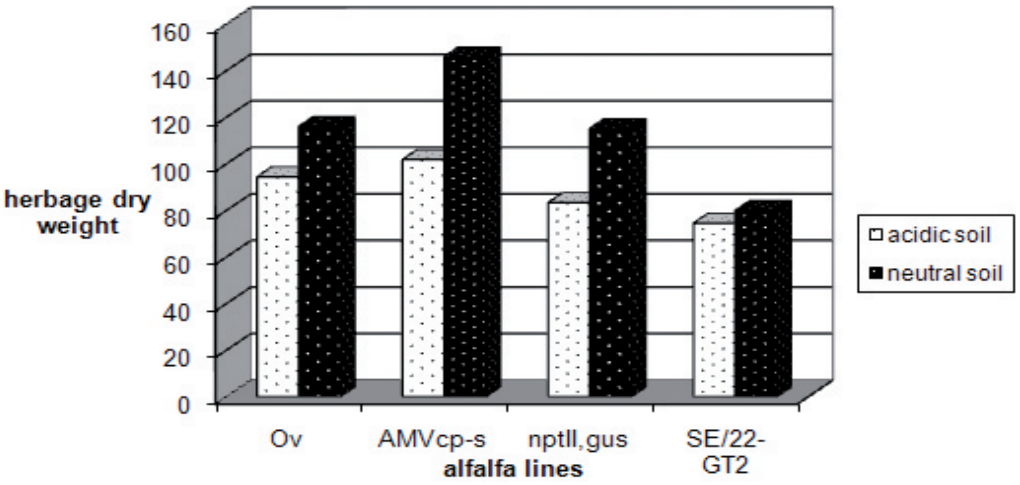

Fig. 2. Comparison of herbage dry weight (mg) of transgenic and non-transgenic lines cultivated in two substrates of soil

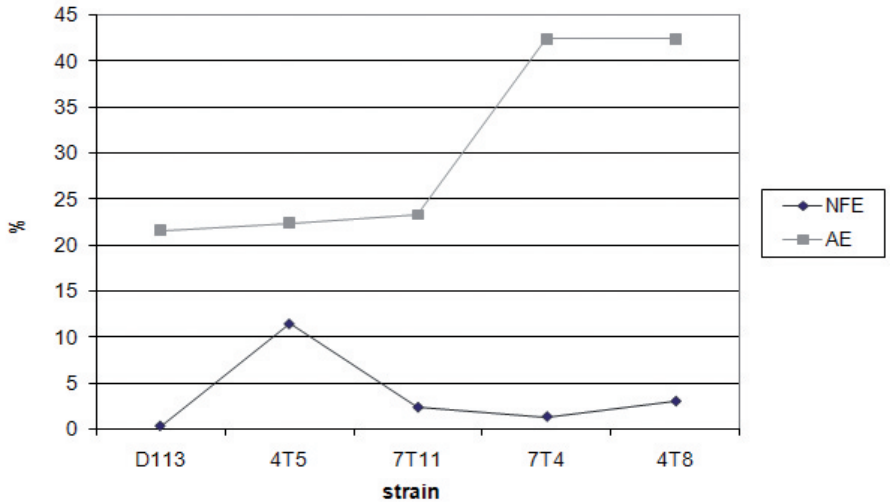

Fig. 3. Comparison of nitrogen fixation efficiency (NFE) and agronomic efficiency (AE) in selected *Sinorhizobium meliloti* strains

all the three types of transformants showed significantly higher herbage dry weight (44-82%) than the non-transgenic kontrol genotype SE/22-GT2. Similarly, in acidic soil, lines containing the *AMV*cp-s gene had by 18% higher green matter DW in comparison with the marker genes containing lines and by 8 % of those containing the *Ov* gene. As in neutral soil, all the types of transgenic alfalfa plants had also in acidic soil higher green matter DW than the non-transgenic control line, however, the increase of herbage DW in this case was not so pronounced (12–37%) as in the neutral soil (Fig. 2). Alike, the highest number of shoots per plant was scored in plants containing the *AMV*cp-s gene when grown in both, neutral and/or acidic soil. The expected effect of genetic modification on plant biomass production may be different. In the study of Donegan et al. (1999) transgenic plants expressing lignin peroxidase had significantly lower shoot weight, higher nitrogen and phosphorus content, than the parental plants or plants with incorporated amylase gene. In our study, the line GTOV1 produced the highest shoot weight in acidic soil. Generally alfalfa plants grown in the neutral soil (pH = 7.0) in our study produced by 28% more green matter DW in comparison with plants grown in acidic soil (pH = 4.0), which reflects the requirements of alfalfa for neutral soils. This confirms the study of Užík and Faragová (2001), who performed selection of alfalfa for higher nitrogenase activity in two consecutive selection cycles and found that in the first generation of plants (Syn 1) the variation in shoot weight of plants was significantlly affected by soil pH, time of harvest and progeny. The shoot weight was higher by 14–45% in neutral soil in comparison to acid soil. Inoculation with selected rhizobial strains increased the shoot weight in neutral soil by 40 % and by 87% in acid soil. According to Hardarson et al. (1981) a significant positive correlation was observed between the percentage of nodules produced by the effective strains and the dry matter production of plants grown at null level of nitrogen in soil. Positive response to inoculation (by yield increase) is determined by a complex of factors including yield potential of a legume, influence of environmental conditions, availability of soil nitrogen and availability of rhizobial populations to provide sufficient N_2 fixation. Rhizobial population size was correlated negatively to pH (Denton et al. 2000). The initial attachment of rhizobia to root surfaces of *Viciaceae* is one of the earliest interactions between symbionts in a complex host-specific infection process (Chovanec et al. 2008). Many authors suggest that host-specificity for the rhizobial partner is already expressed during this adsorption (Caetano-Anollés et al. 1989; Schultze et al. 1994).

Higher green matter DW was observed in alfalfa plants grown in acid soil after inoculation with *S. meliloti* 4T5, 7T11, 7T4, 7T8 and D113. The highest green matter DW and shoot number in acid soil was recorded for lines inoculated with the strain 7T4. Regarding maximal agronomic efficiency, the best results were obtained after inoculation of alfalfa plants with *S. meliloti* strains 7T4 and 4T8 (Fig. 3). Watkin et al. (2000) identified the tolerance to soil acidity in inoculant strains of *R. leguminosarum* bv. *trifolii*. The acid-soil tolerance of six strains was assessed in a three-year cross-row field experiment in an acid sandy soil of pH = 4.2. They found the strains WSM409 and NA3039 that colonised and persisted in acid-soil better than other strains and the strain WSM409 with outstanding characteristics have been proposed for improving clover production on acid soils.

The highest root fresh weight was observed in transgenic alfalfa line GTAMV1 in neutral soil and line GTOV1 in acid soil, respectively. In general, the highest average values of this trait were in transgenic alfalfa plants containing the gene *AMV*cp-s, regardless of the soil pH. Inoculation of plants with *S. meliloti* increased the root weight in average by 25% in comparison with non-inoculated control. The average fresh weight of roots of alfalfa was 23% higher in neutral soil then in acid soil. The transgenic lines of afalfa, in comparison with non-transgenic ones, showed in average by 38% higher root fresh weight in acid soil and by 78% higher in neutral soil. Concerning alfalfa plants grown in neutral soil, the highest average root lenght was recorded in transgenic lines containing the *Ov* gene, while the highest size of root system was observed in lines with the introduced *AMV*cp-s gene. On the other hand in acid soil, both the highest average root length and the strongest root system were observed in transgenic lines containing the *AMV*cp-s gene.

The number of nodules on roots was significantly ($P < 0.05$) affected by both, the genotype and type of inoculation strain. Although the majority of studies addressing potential risks of genetically modified plants cultivation have addressed only aboveground effects (Bruinsma et al. 2003), due to high economic

and ecological importance of symbiotic N_2 fixation, it is also very important to ascertain whether the ability of the infection of plant root hair and formation of nodules is retained in genetically transformed plants of alfalfa and how is the nodulation capacity affected by the expression of transformation event. The highest number of nodules on roots was observed in the transgenic line GTAMV1. Higher values than the average number of nodules were also found in lines GTOV1 and GTAMV2. Inoculation of plants with *S. meliloti* increased the number of nodules from 1.8 (strain D113) to 3.3 fold (strain 7T11). The highest numbers of nodules were again found in transgenic alfalfa lines containing the *AMV*cp-s gene. The nodulation capacity of these plants was by 85% higher than those with incorporated *Ov* gene, and by 77% higher than those with marker genes. Similarly, the highest proportion of active nodules was also found in transgenic plants with introduced gene encoding the *AMV* coat protein in the sense orientation, that contained 70 to 81% of active nodules from the total number of nodules. According to Boisson-Dernier et al. (2001) transformed roots of *M. truncatula* with gene *gusA* and *npt*II can be nodulated successfully by *S. meliloti* and can be colonized by endomycorrhizal fungi. Likewise, transgenic alfalfa containing the gene coding for acidic glucanase and a rice basic chitinase did not seem to negatively affect the *Rhizobium*-alfalfa interaction (Masoud 1996).

The nodules, irrespective of the type of genetic transformation, inoculation suspension used and soil pH, were prevalently those of round-shaped. The activity of nodules was, besides other factors, affected by the density of cells in inoculation suspension and parameters of soil substrate. In acid soils, the proportion of non-effective nodules is reportedly increasing, which might be caused, in part, by decreasing the diversity in the population of effective N-fixing bacteria due to acidic environment, or due to the loss of symbiotic capacity of effective rhizobia (Barber 1980). Inoculation with low-pH-tolerant strains at 10^5 cells per seed or greater prevented ineffective (white) nodulation (Rice 1982).

The variability in the content of N compounds in aboveground plant parts (stems and leaves) was statistically significantly ($P < 0.001$) affected by the genotype, inoculation and soil pH. The highest content of N-compounds was determined in the line GTOV2 and in plants inoculated with *S. meliloti* 4T5. Inoculation with the strain 4T5 increased the total N content in aboveground matter by 12% in neutral soil and up to 23% in acid soil in comparison with non-inoculated control (Fig. 4). According to Kanižai et al. (2007) biennial field experiments of parameter comparison for non-inoculated and inoculated alfalfa in ecological

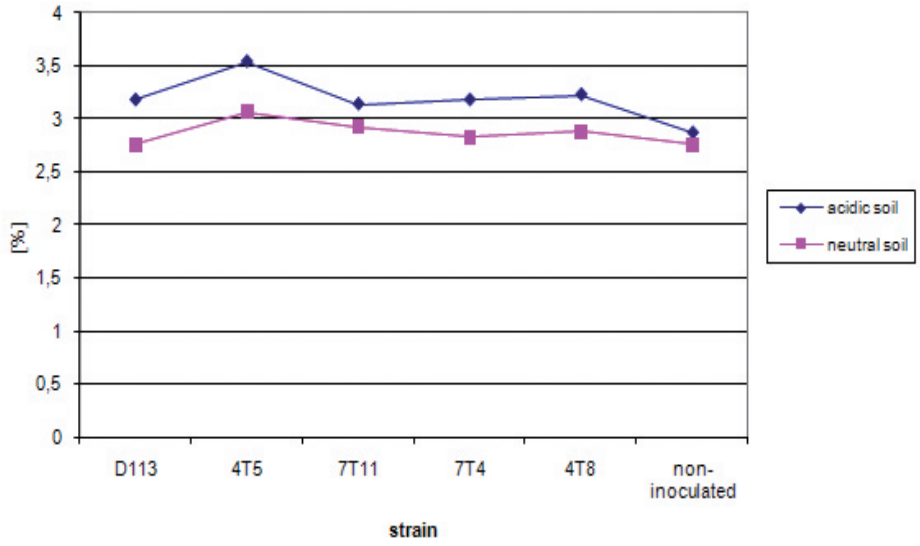

Fig. 4. Comparison of total N content in aboveground matter in alfalfa plants after inoculation with different strains of *Sinorhizobium meliloti*

cultivation led to conclusion that green mass yield was very significantly higher in inoculated alfalfa, and dry matter yield was statistically very significantly higher in both testing years. Nitrogen concentration in aboveground part of plants in both testing years was significantly higher in inoculated alfalfa. Protein yield of inoculated alfalfa was two times higher (very significantly) than in alfalfa not inoculated with highly effective strain of nodule bacteria. The highest average values for total N content were obtained in transgenic lines of alfalfa with introduced *Ov* gene and marker genes in both, neutral and acid soils, respectively. This is in accordance with results of Faragová et al. (2004) who found the highest total N content in dry matter of shoots (stems plus leaves) observed in the transgenic line of alfalfa containing the *Ov* gene. The maximum N_2-fixation efficiency (11.5 %) was recorded for strain 4T5 (Fig. 3).

Interestingly, the comparison of transgenic alfalfa plants with the non-transgenic control SE/22-GT2 showed for all three sets of genetically transformed lines generally somewhat higher values for all the evaluated parameters, except for average root length and number of nodules in acid soil. The most of differences were, however, statistically ($P < 0.001$) not significant. From the economic as well as ecological point of view the most important trait of alfalfa is its nitrogen fixing ability. Therefore, to study the capability of infection of plant roots with *S. meliloti* and formation of nodules is of primary importance. As shown in Fig. 5, the highest average number of nodules per plant was observed in transgenic alfalfa plants containing the gene *AMV*cp-s, both in neutral (by 140%) and acid soil (4 times), respectively. The average number of nodules in non-transgenic line exceeded those in transgenic lines containing *Ov* and marker genes by 24−48% in neutral soil, but was from 3 to 4-times lower in these lines in acidic soil. To explain the higher nodule forming ability of transgenic alfalfa lines containing the *AMV*cp-s gene would need further research on genetic and physiological levels.

CONCLUSION

In this paper we report on selection of a set of strains of *S. meliloti* tolerating lower values of pH, detection of compatible combinations of these highly-effective rhizobial strains with three types of genetically transformed alfalfa plants and characterization of their interaction with acid tolerant rhizobia in soils with different pH values. In an *in vitro* assay system four highly effective rhizobial strains (4T5, 7T4, 7T11 and 4T8) of *S. meliloti* tolerating pH values as

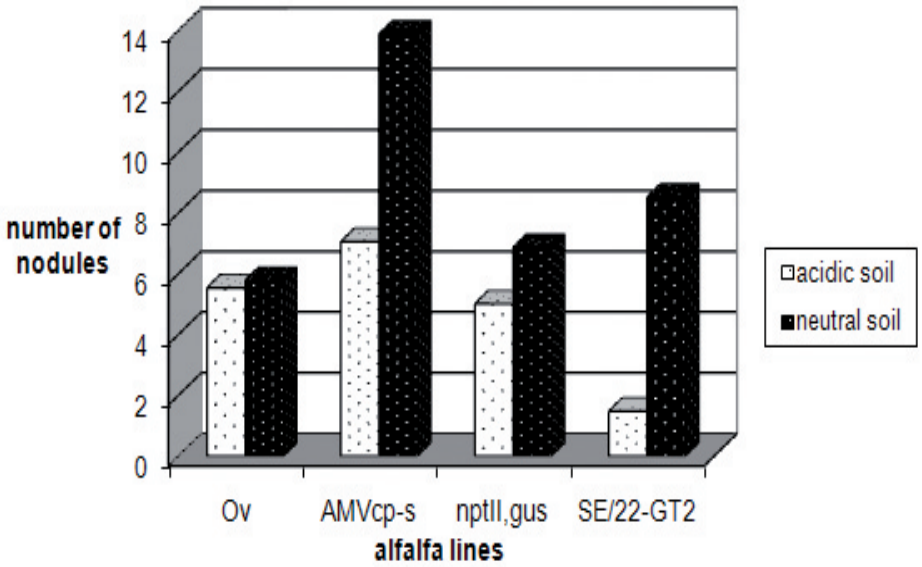

Fig. 5. Comparison of number of nodules of non-transgenic and transgenic lines with gene Ov, AMVsp-s, nptII and gus cultivated in two substrates of soil

low as 4.3 have been selected. An integral part of this work was also the study of the level of equivalence of genetically transformed alfalfa plants with their non-transgenic isogenic counterpart on the level of interaction with rhizosheric microorganisms. Three types of transgenic alfalfa plants containing different transgenes (*Ov*, *AMV*cp-s, and *gus*) were characterized for their growth characteristics and nodulation ability after inoculation with selected low pH tolerant *S. meliloti* strains in neutral (pH = 7.0) and acid (pH = 4.0) soils, respectively. Our results confirm the substantial equivalence between the transgenic alfalfa plants and their non-transgenic counterparts in terms of nodulation ability, even transgenic lines containing the gene *AMV*cp-s had higher numbers of nodules by 140% in neutral soil and 4-times more in acidic soil in comparison with non-transgenic parental lines.

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