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SYMBIOTIC NITROGEN FIXATION IN ALFALFA (*MEDICAGO SATIVA* L.) BY *SINORHIZOBIUM MELILOTI* AT AL-QASSIM REGIONS, SAUDI ARABIA

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Abstract

The nodulation status in alfalfa (*Medicago sativa* L.) plants by *Sinorhizobium meliloti* under Saudi field condition was assessed in some selected farms in four seasons for two years. In the present study, we also monitored the introduced *S. meliloti* strains' activity under Saudi soil conditions. The samples were collected at regular seasonal intervals from the selected farms. The total number of nodules, morphology of the nodules and the effectiveness of N_2-fixation was assessed. In general, it was revealed that soils in the selected areas in Saudi Arabia have sufficient bacteria of the proper types to nodulate the alfalfa plants. These nodules are high in number, small in size and white in color. The nodules obtained from most of the selected farms are ineffective for nitrogen fixation. Inoculation of alfalfa seeds with imported *S. meliloti* strains failed to fix the atmospheric nitrogen sufficiently and also the growth improvement of alfalfa plants. There was a wide variation in the occurrence of number of nodules among the four seasons in two years. It was also observed that summer season severely affected the nodulation making it nearly zero. This low number of nodules exerts a very slow recovery of nodule formation in the next year. The introduced strains were always over competing with the native strains but they did not survive because of hot and dry summer. Nitrogenase activity of the nodules collected from both the inoculated and non-inoculated farms were always very low in all the collected samples, which indicates that the ability of fixing nitrogen by *S. meliloti* strains in alfalfa under Saudi soils conditions is very low.

Key words: Alfalfa, *Sinorhizobium meliloti,* Nodule numbers and morphology, N_2-ase activity, Seasonal variation.

Introduction

Symbiotic nitrogen fixation with rhizobia is very important throughout the world for nitrogen input into agro-ecosystems. Alfalfa (*Medicago sativa* L.) is a widely cultivated important agricultural perennial crop which forms nitrogen-fixing symbiosis with *Sinorhizobium.* Fixation of nitrogen with *Sinorhizobium* plays a significant role in decreasing use of chemical nitrogen fertilizers in cropping systems. Alfalfa is introduced in large areas of Saudi Arabia to replace the locally available non-leguminous forage, which has less nutritive value and digestibility compared to alfalfa. This crop is accounts for about 30% of the total production of crops as a cultivated forage legume in the Kingdom. In Saudi Arabia, indigenous soil populations of *Sinorhizobium meliloti* have received little attention. The effectiveness of *S. meliloti* with alfalfa was studied in different countries throughout the world. It was recorded either effective or non-effective by different authors (Peterson & Gooding 1941; Nutman, 1969; Hardarson *et al.,* 1981; Mahler & Wollum 1982; Tuzimura & Watanabe, 1982; Alikhani & Saleh- Ranstin, 2002; Al-Barakah & Mridha, 2014). Environmental factors determine the efficacy of growth of both plants and bacteria; especially temperature plays a significant role in this regard. The infection and the further development of nodules depend on the temperature of the growing conditions (Chatel & Parker, 1973; Trabulsi, 1982; Gibson & Jordan, 1983; Bordeleau & Prevost, 1994; Aranjuelo *et. al*., 2001). From Saudi Arabia, Al-Turki (1995) studied in two different soils and reported that the optimum temperature was around 30°C for growth of alfalfa rhizobia. Decrease in rhizobial number due to inoculation at higher temperature (50°C) has been reported by several authors (Trabulsi *et al.,* 1979; Lie, 1981; Bordeleau & Prevost, 1994). Al-Barakah *et al.* (2011) and recently Al-Barakah & Mridha (2014) reported a positive response of alfalfa to inoculation with *S. meliloti* strain indigenous to Saudi Arabian soils. The use of *S. meliloti* for seed inoculation is not known among the farmers in Saudi Arabia and very limited research was done on nitrogen fixation as a practical means of improving the yields and the quality of crops in Saudi Arabia. It was not studied properly whether the inoculation of legume crops with *Sinorhizobium* will provide a positive growth improvement of legume crops in the country. At the same time, it was also not known that whether inoculation of *rhizobia* can improve the native population in the soil. The objectives of this study were to observe the ability of *S. meliloti* to symbiotic nitrogen fixation in alfalfa under Saudi Arabian soil conditions and also to study the seasonal variation for nodulation.

Materials and Methods

Location and farms: The sample areas were selected from the farms where barley, wheat, potato were cultivated for a long time and alfalfa was introduced recently as a major crop in some of the fields. The multiple samples were collected from three alfalfa farms namely: Al-Qassim Co.; Al-Gibreen and Hadco Co. in Al-Qassim region, Saudi Arabia. The field observation was also made in four seasons for two years.

Sampling procedures: Samples were collected with soil cores (7.5 cm dia. x 50 cm deep) from around the alfalfa plants by pressing the cores vertically into the soil. Rhizosphere soils from three alfalfa plants (0.5-1.0 m apart) were collected and packed in polyethylene bags. To minimize moisture loss and avoid any biological changes, the bags were left in ice-boxes. The samples were analyzed immediately after collection for microbial study and chemical analysis was performed by following the methods as mentioned by Al-Barakah & Mridha (2014).

Nodulation and N_2-ase activity determination: The number of nodules was counted per 3 plants. The location of the nodules was observed on the roots and the shape, size and color were also noted. By using GC (Pay Unicam 4500) equipped with a flame-ionization detector, the N_2-ase activity was assessed (Hardy *et al.,* 1986). A stainless steel column packed (A 1.5 x 4 mm) with 80-100 mesh porapak R and N_2 was the carrier gas. Temperature: injector 80°C; column 60° C and detector 120°C.

Gas flow rate: N_2 30; H_2 33 and air 330 ml min^{-1}.

Soil chemical and physical analyses: Moisture content was determined (at constant dry weight) by weighing soil samples, before and after drying at 105°C for 24 h. The mechanical analysis of soil was determined by following Page *et al*. (1982). On the basis of the clay, silt and sand, soil texture was determined and the name of textured class was determined following Alexander (1961). The temperature in the root rhizosphere was measured by pressing a thermometer vertically around alfalfa plant. In saturated soil paste (after standing for 2 h) the pH was determined with a PW 9420 pH meter. Electrical conductivity (EC) was determined in soil saturated paste using the PW 9506 digital conductivity meter.

Statistical analysis: The data were analyzed using SAS (1982) ANOVA 18 with least significant difference (LSD) for the mean separation (Anon., 1982).

Results and Discussion

The results of the soil analysis indicated that the soils in the selected three farms were sandy loam. The range of pH (7.6-7.9); EC (dS m^{-1}) (1.8-3.8); $CaCO_3$ (%) (10.3-12.6); total N (ppm) (525-805); total P (ppm) (395-553); Ca^{2+} (ppm) (12.6-25.6); Mg^{2+} (ppm) (4.4-11.4); Na^+ (ppm) (5.9-21.3); K^+ (ppm) (0.30-0.50); Cl^- (ppm) (3.6-10.2); HCO_3^-(%) (5.4-7.1) varied widely and independently in the selected three farms (Table 1). The moisture content and soil temperatures also varied in different seasons of the year (Table 1).

Table 1. Chemical and physical analysis of soils under investigation.

Region	Farm site	Texture	pH	E.C. (dSm^{-1})	$CaCO_3$ (%)	Total N (ppm)	Total P (ppm)	Ca^{++} (ppm)	Mg^{++} (ppm)	Na^+ (ppm)	K^+ (ppm)	Cl^- (ppm)	HCO_3^- (%)
Al-Qassim	Al-Qassim Co.	Sandy loam	7.7	1.8	11.0	805	553	17.6	7.2	5.9	0.50	3.6	7.1
	Al-Gibreen	Sandy loam	7.9	1.9	10.3	539	395	12.6	4.4	10.5	0.30	8.3	6.5
	Hadco	Sandy loam	7.6	3.8	12.6	525	492	25.6	11.4	21.3	0.40	10.2	5.4

Counting of nodules: The occurrence and counting of nodule data are presented in Fig. 1. Data presented in Fig. 1 shows that *S. meliloti* occurred in all alfalfa plants collected from all the selected locations and it was also observed that *S. meliloti* is very persistent in all the sampling. The average number of nodules obtained from different farms ranged from 2 to more than 50 nodules /3 plants. Out of the four seasons, the total number of nodules was highest during the autumn season (up to 55 nodules per 3 plants). The number of nodule decreased significantly in summer (3 nodules per 3 plants), probably because of high soil temperature (44 °C) in root rhizosphere (Table 1). In the second year, the nodulation decreased significantly and the recovery was not very satisfactory. The decrease of nodulation may be due to the meager number of *S. meliloti* that survived during long hot summer conditions or little ability of the introduced strains to form active nodules. In the second year, because of low recovery (less than 13%) of active nodules the N_2-fixation was not enough. The lowest number of nodules was observed in Hadco and Al-Gibreen sites in comparison to Al-Qassim Co. sites (Fig. 1). The soil samples of these farms were not inoculated with *S. meliloti* strains. In Al-Gibreen sites, the number of nodules observed in the first year was recovered in the second year also and the recovery percentage was less at Hadco sites. The number of nodules recorded in this region decreased significantly during the summer season of the first year and very few nodules were found. Again, high temperature may have been the reason of poor nodulation in this region. More or less the same trends were observed at Al-Kharj region sites in the second year, as the number of nodules was very low in most of the time in the second year (Al-Barakah & Mridha, 2014). This indicates that the inoculation of alfalfa plants with introduced strain did not benefit the plants. The present results are in agreement with Del Papa *et al.* (1999) and Eardly *et al.* (1992). They found that in both North and South America, wherever alfalfa plants have been introduced, ineffective nodules were produced by *Sinorhizobium* strain and Al-Barakah & Mridha (2014) have also reported the same trends from Saudi Arabia.

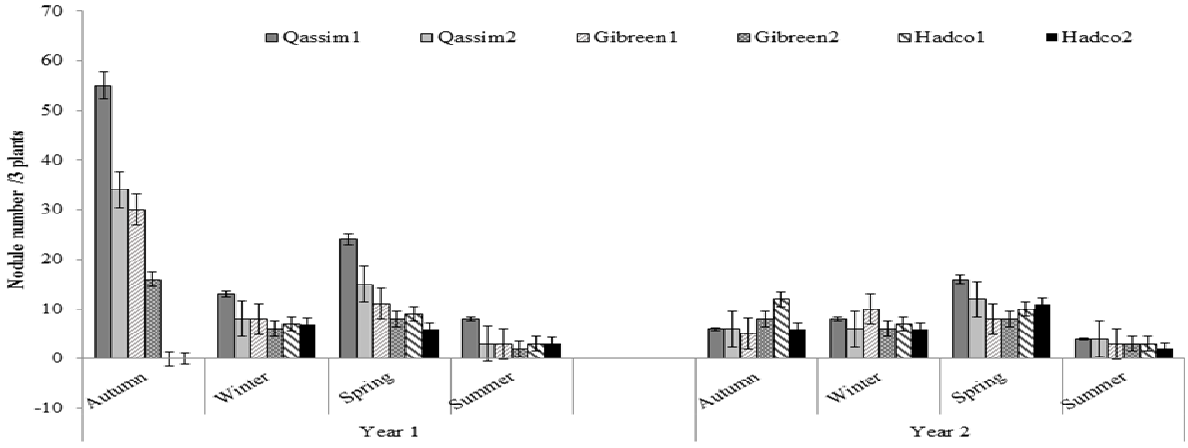

Fig. 1. Nodule number in alfalfa at Qassim regions.

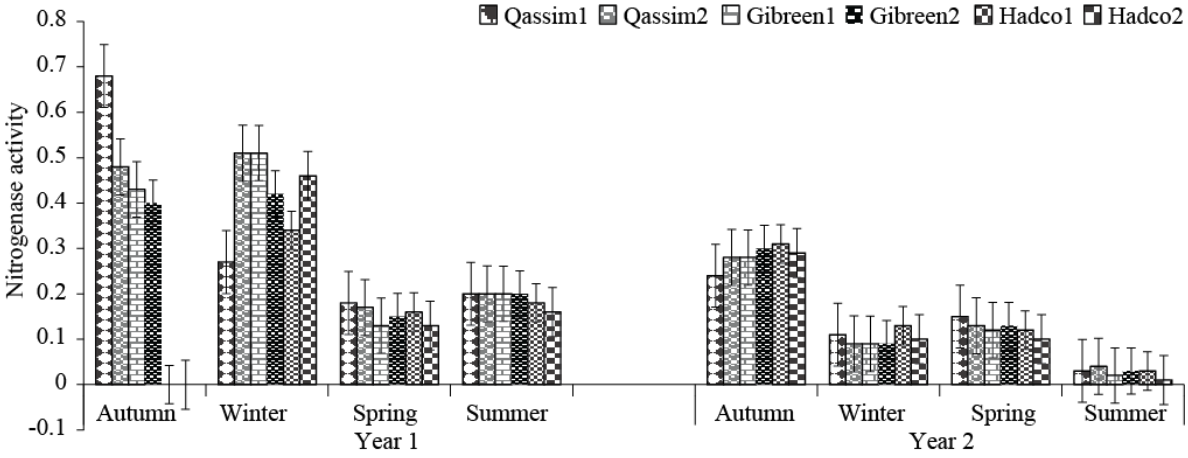

Fig. 2. Nitrogenase activity in alfalfa nodule at Qassim regions.

The temperature of the rooting medium plays a significant role in both the initiation of infection and the subsequent development of nodules (Gibson & Jordan, 1983; Bordeleau & Prevost, 1994). Chatel & Parker, (1973) reported that the failure of *R. trifolii* inoculants persisted through more than one growing season in Western Australian soils because of high soil temperatures in summer. Trabulsi (1982) found that soil temperature (20-30 °C) seems to be more suitable, not only for the survival of *S. meliloti* in the soil, but also for nodulation and symbiotic nitrogen fixation on alfalfa. He also mentioned that high temperature (≥40°C) produced negative effect on the survival of rhizobia in the soil and on the formation of nodules as well as N_2–fixation in alfalfa plants. Al-Turki (1995) studied the growth of alfalfa in two soils from Saudi Arabia and found that the optimum temperature for alfalfa rhizobial growth was around 30°C. Inoculation at higher temperature (50°C) decreasing rhizobial number was also reported by several authors (Trabulsi *et al.,* 1979; Lie, 1981; Bordeleau & Prevost, 1994) as was reported during our study.

Nitrogenase activity: Nitrogenase activity of nodules was determined using the acetylene assay. Maximum N_2-ase activity was obtained after one hour incubation before assay. Significant differences in the N_2-ase activity were found among the samples (Fig. 2). The range of N_2-ase activity was 0.09 to 0.68 µmol $g^{-1}h^{-1}$ among the samples. It was also observed that the non-inoculated samples gave the lowest N_2-ase activity in all seasons. In the first year, the samples collected from Hadco and Al-Gibreen sites produced low N_2-ase activity (0.09 µmol $g^{-1}h^{-1}$) in spring, while the inoculated samples of Al-Qassim sites showed higher N_2-ase activity in autumn (Fig. 2). In comparison to the values obtained in the first year the N_2-ase activity was less in the second year. These values were regarded as low and not enough to ensure a nitrogen fixation by alfalfa plants. N_2-ase activity was also affected because of temperature. It was reduced to less than 20% in comparison to colder period. In autumn season of the second year, the N_2-ase activity recovered about 50% in relation to first year. However, this ability to recover was less in winter. The value in the second year decreased significantly and was always less than that observed in the first year. In the second year, it decreased to zero in the summer seasons. It was also indicated that the N_2-ase activity in both the years in this region was less than 1 µmole $g^{-1}h^{-1}$. This value is very low and not enough to fix nitrogen or to give an indication of the rhizobial activity.

Munevar & Wollum (1981) and Aranjuelo *et. al*. (2001) reported that high temperature in the root zone may cause a significant decrease in the production of number of nodules and their N_2-ase activity. In summer season, rhizosphere temperatures were high (40°C) in all the study areas. The high temperature and long hot day in summer may have sterilized the soil and because of that the survival of the rhizobia decreased. Alexander and Chamblee (1965) reported that inoculation is less effective, when the seed of legumes were kept for 3 or more hours under sunlight or for 2 or more weeks under hot sun light. In our results, the summer temperature and the long day length were very harmful on the rhizobial strains and reduced significantly its survival percentage in soil. For that reason, inoculation under such conditions will be a complete failure and with no benefit. The native strains adapted to these conditions recovered well and may have nodulated the plants. However, these strains prove to be very poor in the ability of fixing nitrogen under field conditions as they formed ineffective nodules on alfalfa plant roots. Field data show a very low value in N_2-ase activity in the first year and the second year (less than 1 μmol $g^{-1}h^{-1}$), despite the number of nodules observed on the plant roots. It may be concluded that recently introduced *S. meliloti* strains were not capable of fixing nitrogen as was reported by Al-Barakah & Mridha (2014). According to the data collected in this study, alfalfa plants in Saudi Arabia did not benefit from the symbiotic association. The ability of *S. meliloti* to fix nitrogen by native or introduced strains were non-effective especially during summer season, therefore, the mineral nitrogen fertilization may be the suitable source of nitrogen for the growth of alfalfa plants grown in Saudi Arabian soils (Al-Barakah & Mridha, 2014).

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