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Status of Symbiotic Nitrogen Fixation by *Sinorhizobium meliloti* in Alfalfa (*Medicago sativa* L.) under Field Conditions at AL-Kharj, Saudi Arabia

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A survey was carried out for two years in some selected farms at Al-Kharj, Saudi Arabia to assess the status of the nodules under field conditions by *Sinorhizobium meliloti* on alfalfa (*Medicago sativa* L.) plants and monitoring the introduced *S. meliloti* strains activity under Saudi soils conditions. The samples were collected at regular seasonal intervals from the selected areas. The microbiological examination included the determination of total nodules number as well as the effectiveness of N_2-fixation. Results revealed that, soils in the selected areas in Saudi Arabia have sufficient bacteria of the proper kind to nodulate the alfalfa plants. However, these nodules are high in number, small in size, white in color and proven to be ineffective in nitrogen fixation in the most farms. Inoculation of alfalfa seeds with imported *S. meliloti* strains in Saudi Arabia failed to improve the plant's ability to fix its need from the atmospheric nitrogen. The imported strains were always over competing with the native strains in Saudi soils and did not survive the long hot and dry summer season. Seasonal variation of nodulation was observed and it was found that summer season severely affected the nodulation to nearly nil. This reducing effect on nodules number exert a very slow recovery in the second year. Nitrogenase activity was always very low in all the collected samples in both inoculated and non-inoculated farms, which indicates that the ability of fixing nitrogen by *S. meliloti* strains under Saudi soils conditions is very low.

Key words: Alfalfa, *Sinorhizobium meliloti,* nodules count, N_2-ase activity, seasonal variation.

Alfalfa (*Medicago sativa* L.) is a widely cultivated perennial crop which forms nitrogen-fixing symbiosis with *Sinorhizobium* and decreases the utilization of chemical nitrogen fertilizers in agricultural and pasture systems. Symbiotic nitrogen fixation with rhizobia plays a significant role for sustainable nitrogen input into agro-ecosystems. In Saudi Arabia, large areas are newly introduced for growing alfalfa because it has great nutritive value and high digestibility compared to the locally available non-leguminous forage. Alfalfa as a cultivated forage legume accounts for about 30% of the total production of crops in the Kingdom. Indigenous soil populations of *Sinorhizobium meliloti* have received little attention compared to the introduced strains. The effectiveness of *S. meliloti* with Alfalfa was studied in different countries under field conditions and found either effective or non-effective[1-6]. Environmental factors affect the growth of both plants and bacteria. The initiation of infection and the subsequent development of nodules depends on the temperature of the rooting medium[7-10]. From Saudi Arabia, Al-Turki [11] reported the optimum temperature (around 30˚C) suitable for alfalfa rhizobial growth in two different soils. Inoculation at higher temperature (50˚C) decreased rhizobial

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number to reach the lowest after two weeks, as reported by several authors[8,12, 13] . Barakah, *et al.,* [14] reported the positive response of Alfalfa to inoculation with *Sinorhizobium meliloti* strains indigenous to Saudi Arabian soils. The practice of inoculating seeds with strains of *S. meliloti* is unknown among farmers in Saudi Arabia and research on nitrogen fixation as a practical means of improving the yields and the quality of crops is limited. Also, the question often arises as whether or not legumes crop should be inoculated with *Rhizobium* bacteria and what is the possibility to improve the native population in the soil. The objective of this work was to study the symbiotic nitrogen fixation ability of *S. meliloti* strains under Saudi Arabian soils conditions and the effect of seasonal changes on this ability to produce nodulation.

MATERIALS AND METHODS

Location and farms

Most of the farms in the study areas have long-term histories of cultivation of barley, wheat, potato. Recently alfalfa was introduced as a major crop in some fields. The samples were collected from three large alfalfa farms namely : Rafyah , Al-Safi and Sanable at Al-Kharj region, Saudi Arabia. Two pivots in each farm were used as a sampling farm and the field monitoring was performed for two years.

Sampling procedures

Samples were collected as undisturbed soil cores, 7.5 cm dia. x 50 cm deep by pressing vertically into the soil directly around healthy alfalfa plant in the field. Three alfalfa plants, 0.5-1.0 m apart from each with the rhizosphere soil, were packed in new polyethylene bags to minimize moisture losses during transportation and were kept in ice-boxes in order to avoid any biological changes. Samples were analyzed as quickly as possible for bacteriological (usually done on the next day of collection) and chemical analysis.

Nodulation and N_2-ase activity determination

The soil was gently washed from the roots; nodulation was assessed by estimating the number of nodules per 3 plants, observing nodule location on the roots, as well as the color. N_2-ase activity was assessed according to method after Hardy *et al.,* [15] , using GC (Pay Unicam 4500) equipped with flame-ionization detector. A 1.5 x 4 mm stainless steel column packed with 80-100 mesh porapak R and N_2 was the carrier gas, with operating conditions as follows:

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 analysis

Moisture was determined by weighing soil samples, before and after drying, in an electrical oven at 105 ˚C for 24 h to constant weight. The loss in weight indicates the quantity of moisture present (Table 1). The root rhizosphere temperature was determined by pressing a thermometer vertically into the soil directly around healthy alfalfa plant roots in the field (Table 1) . The mechanical analysis of soil was determined by the hydrometer method [16]. Soil texture was determined on the bases of the clay; silt and sand, and the name of textured class was ascertained from the textured triangle given by Alexander [17] (Table 2).

pH value was determined with a PW 9420 pH meter in saturated soil paste after standing for 2 h. Electrical Conductivity (EC) was determined in soil saturated paste using PW 9506 digital conductivity meter (Table 2).

Statistical analysis

The data was analyzed using SAS ANOVA [18] with least significant difference (LSD) for the mean separation.

RESULTS AND DISCUSSION

Counting of Nodules

Most fields in this region have no previous history of alfalfa plants; wheat and barley are the common cultivated crops. Data presented in Fig. 1 show that *S. meliloti* occur in all alfalfa field sampling farms as shown by nodules formed on the roots of the plant. In the first year the highest number of nodules were found in Rafyah farm (51 nodules per 3 plants) in autumn and winter (rhizosphere root temp. was 20˚C), and start to fall down significantly to reach only 2 nodules per 3 plants in summer (rhizosphere root temp. 46˚C). A slight rise in nodules number was observed in the second year autumn with the same trend to give a very low number of nodules in the summer. Nodules were induced by inoculation of introduced strains of *S. meliloti* in this farm combined with low rate

of urea as nitrogen fertilizer (25 Kg urea ha^{-1} cut^{-1}). Al-Safi farm gave less nodules number than Rafyah farm, even though it was also considered as inoculated farm in this region. On the other hand, indigenous *Sinorhizobial* strains were also observed at Sanable farm (Fig. 1), as shown by nodules on the roots of un-inoculated alfalfa plants. Also the highest number of nodules was observed in the colder time of the year, which recorded 40 nodules per 3 plants and the lowest counts were observed in summer (less than 4 nod./3 plants). Nodulation of alfalfa plants were found to be influenced by the season of the year in this region, nodule numbers were decreased significantly from spring of first year to autumn of the second year. During spring of the first year, the nodulation was less than 24%, 28% and 31% of the observed nodules in winter, at Rafyah , Sanable and Al-Safi farms respectively. The lowest nodulation was found in summer season; during this period of the year nodulation was only 6%, 18% and 12% in the same farm compared to that observed in autumn (Fig. 1). The survey of the second year shows a very low nodule count compared to that observed in the first year. It seems that temperature in the rhizosphere of alfalfa plant roots play an important role in the survival of rhizobia. The introduced strains tend to increase nodulation at the late autumn, winter and the early spring when the climatic conditions were favorable. The native and introduced strains of rhizobia show nearly the same trends in the sensitivity to cold and hot climate. Nodulation in summer season of the second year reduced significantly to nearly nil, with no difference between inoculated and non-inoculated farms. This indicates that the inoculation was with no benefit to the plants and nodules caused by the native strains apparently. The present results are in agreement with Del Papa, *et al.,* [19] and Eardly, *et al.,* [20]. They found that in both North and South America, wherever alfalfa plants have been introduced, ineffective nodules were produced by *Sinorhizobium* strains.

The temperature of the rooting medium affects both the initiation of infection and the subsequent development of nodules[7, 8]. The failure of *R. trifolii* inoculants to persist through more than one growing season in Western Australian soils has been attributed to the high soil temperatures in summer [9]. Trabulsi [10] found that soil temperature of 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. Higher temperature (≥40°C) showed a marked harmful effect on the survival of rhizobia in the soil and on the

Table 1. Moisture content(M) and seasonal root zone temperature (T) of soils under investigation

Region	Farmsite	Autumn		Winter		Spring		Summer	
		M (%) First year	T(C)	M (%)	T(C)	M (%)	T(C)	M (%)	T(C)
Al-Kharj	Rafyah	10.3	36	8.3	13	6.3	28	10.5	46
	Sanable	11.6	34	10.5	13	22.1	28	9.1	46
	Safi	19.2	36	5.2	14	18.4	29	21.1	46

Table 2. Chemical and physical analysis of soil 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-Kharj	Rafyah	Sandy loam	7.7	4.2	6.9	252	611	45.4	20.1	11.1	0.55	8.1	6.9
	Sanable	Sandy loam	7.7	3.3	11.4	553	514	41.5	22.2	10.2	0.50	7.5	5.2
	Safi	Clay loam	7.8	4.1	30.0	462	470	36.2	21.4	17.5	0.30	19.6	8.4

nodulation and N_2–fixation of alfalfa plants. Al-Turki [11] found that the optimum temperature suitable for alfalfa rhizobial growth in two soils from Saudi Arabia was around 30˚c. Inoculation at higher temperature (50˚C) decreased rhizobial number to reach the lowest after two weeks [8, 12, 13].

Nitrogenase activity

Nitrogenase activity was determined in the root samples with nodules using the acetylene assay as an indirect estimate of N_2-fixation. Maximum N_2-ase activity was obtained after one hour incubation before assay. There was great difference in the activity among samples with no clear trends with nodule numbers, ranging from 0 to 0.61 u mole $g^{-1}h^{-1}$ (Fig. 2). Al-Safi inoculated farm gave the lowest N_2-ase activity (0.04 u mole $g^{-1}h^{-1}$) in winter season of second year. One interesting result was observed in spring and summer, most of the samples gave a result not more than 0.10 u mol $g^{-1}h^{-1}$, with a very low number of nodules (Fig. 1). This result indicates that the ability of the native rhizobia in nitrogen fixation is very low. The N_2-ase activity value for the inoculated alfalfa plants at Rafyah farm, was higher than that found in the non-inoculated farm (i.e. Sanable). The activity of the indigenous rhizobia at Sanable farm, was higher most of the time than found in Al-Safi. Since nodulation of alfalfa tended to change by the seasons as described (Fig. 2).

N_2-ase activity was also effected, and remarkably reduced to less than 20% from the value found in the colder period of the year. Moreover, in autumn season of the second year N_2-ase activity recovered about 50% from its value in the first year (Fig 3). However, this ability to recover was less later on, 20% in winter. The value in the second year was decreased significantly and always less than that observed in the first year,

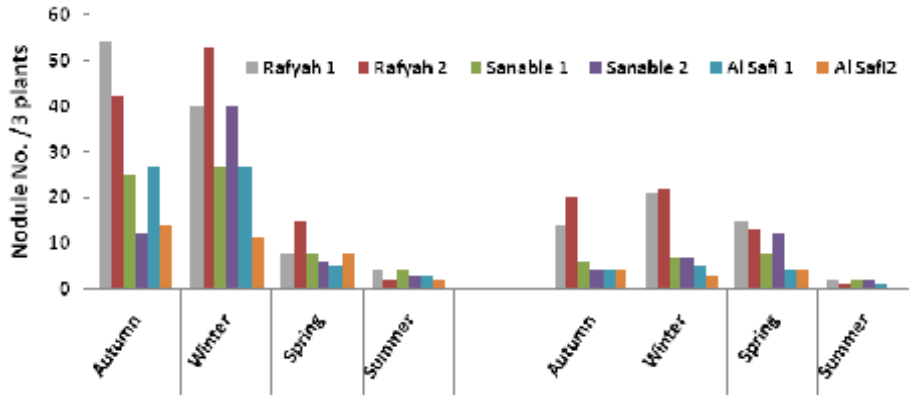

Fig. 1. Nodule numbers in Alfalfa at Al-Kharj region

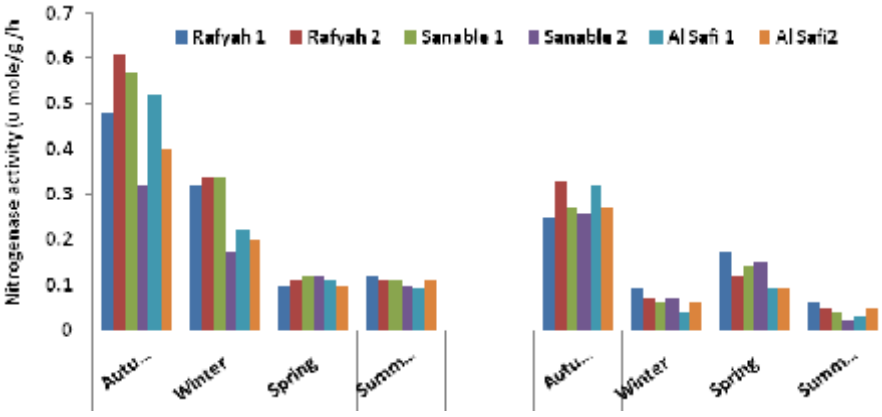

Fig. 2. Nitrogenase activities of nodules of Alfalfa at Al-Kharj region

and dropped to 0 in the summer of the second year. This is not surprising since nodulation was also very low at the same time. It is interesting to note that the N_2-ase activity obtained in this study in both years in this region was less than 1 u mole $g^{-1}h^{-1}$. This value is very low and not enough to fix nitrogen or to give an indication of the rhizobial activity. It seems that most of the observed nodules are very poor in nitrogen fixation, as they may be performed by native strains despite the use of inoculation.

High roots zone temperature have been reported to cause a significant decrease in the number of nodules formed and N_2-ase activity [21]. Rhizosphere temperatures in all the study farms were more than 40°C especially in summer season. Most of Saudi soils are light in texture, with pivot irrigation, a shallow root system well developed in the upper 10-15 cm of the soil. The high temperature and long sunny day in summer may have sterilized the soil and because of that the survival of the rhizobia decreased. Alexander and Chamblee [22] reported that less effective inoculation resulted in some tests when the seed of legumes were subjected for 3 or more hours to sunlight or if it subjected for 2 or more weeks to 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 the inoculation under such conditions will be a complete failure and with no benefit. The native strains adapted to these conditions well re-covered 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 u mol $g^{-1}h^{-1}$), despite the number of nodules observed on the plant roots. It may be concluded that recently introduced *S. meliloti* strains were unable or not effective in fixing nitrogen. 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.

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REFERENCES

1. Bottomly, P.J., Jenkins,M.B. Some characteristics of *Rhizobium meliloti* from alfalfa fields in Oregon. *Soil Sci. Soc. Am. J.* 1983; **47:**1153-1157.
2. Peterson, H.B., Gooding, T.H. The geographic distribution of *Azotobacter* and *Rhizobium meliloti* in Nebraska soils in relation to certain environmental factors. Univ. Nebraska. *Agric. Exp. Stn. Bull.* 1941; 121: 3-24.
3. Nutman, P.S. Microbiology of Broad balk Soils; Legume nodule bacteria. Rothamasted Report, Part 2. 1969. pp.179-181.
4. Hardarson,G., Heichel, G.H., Vance, P.C., Barness, D.K. Evaluation of alfalfa and *Rhizobium meliloti* for compatibility in nodulation and nodule effectiveness. *Crop Sci.* 1981; **21**: 562-567.
5. Mahler,R.L., Wollum, A.G. Seasonal variation of *Rhizobium meliloti* in alfalfa hay cultivated fields in North Carolina. *Agron. J.* 1982; **74:** 428-431.
6. Tuzimura, K., Watanabe, I. The effect of rhizosphere of various plants on growth of *Rhizobium*. *Soil Sci. and Plant Nutrition(Tokyo).* 1982; **8**:13-17.
7. Gibson, A.H., Jordan, D.C. Ecophysiology of nitrogen fixing systems. pp.301-390 *In: Nitrogen Fixation* (Lange OL, Nobel PS, Osmond CB, Evans, H. eds.) London. 1983.
8. Bordeleau, L.M., Prevost, D. Nodulation and nitrogen fixation in extreme environments. *Plant and Soil*, 1994; **161**:115-125.
9. Chatel, D.L., Parker, C.A. The colonization of host root and soil by rhizobia. I. Species and strain differences in the field. *Soil Biol. Biochem.* 1973; **5:** 425-432.
10. Trabulsi, I.Y. Effect of soil temperature and soil alkalinity on the survival of *Rhizobium* in the soil and the nodulation of alfalfa. *J. Coll. Sci. King Saud Univ.* 1982; **13 (1)**: 5-15.
11. Al-Turki, A. Microbiological studies on symbiotic nitrogen fixation between *Rhizobium meliloti* and alfalfa in some soils of Saudi Arabia. M.Sc.Thesis, Agriculture College, King Saud University, Saudi Arabia. 1995.

12. Trabulsi, I.Y., Abd El-Samea, M.E., Fathi, M.A. Effect of soil temperature on the survival of *R. meliloti* and nodulation of alfalfa . *J. Coll. Agric. King Saud Univ.* 1979; **1**:113-123.
13. Lie, T.A. Environmental physiology of the legume-rhizobium symbiosis. pp.104-134. *In*: *Nitrogen Fixation.1-Ecology* (Broughton W J, ed.) Clarndon Press. Oxford. 1981.
14. Barakah, F.N., Abdel-Aziz, R.A., Radwan, S.M.A. Response of Alfalfa to Inoculation with *Shinorhizobium meliloti* strains Indigenous to Saudi Arabian Soils. *American-Eurasian J. Agric. & Environ. Sci.* 2011; **10(2):**193-199.
15. Hardy, R.W.F., Holsten, R.D., Jackson, E.K., Burns, R.C. The acetylene-ethylene assay for nitrogen fixation : *Laboratory and field evaluation. Plant Phsiol*. 1968;**43:**1185-1207.
16. Page, A.L., *Method of Soil Analysis (Part II). Chemical and Microbiological Properties. Amer. Soc.Agron.* Inc.Medison Wisconson. 1982.
17. Alexander, M. Introduction to Soil Microbiology, John Wiley and Sons Inc., New York. 1961.
18. SAS Institute. SAS user's Guide-Statistical. SAS Institute, Cary, North Carolina. 1982.
19. Del Papa, M. F., Balague, L. J., Sowinski, S. C., Wegener, C., Segundo, E., Abarca, F. M., Toro, N., Niehaus, P. A., Aguilar, O. M., Martinez-Drets, G., Lagares. A. Isolation and characterization of alfalfa-nodulating rhizobia present in acidic soils of central Argentina and Uruguay. *Appl. Environ. Microbiol*. 1999; **65:**1420-1427.
20. Eardly, B. D., Young, J. P., Selander, R. K. Phylogenetic position of Rhizobium sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and nifH genes. *Appl. Environ. Microbiol.* 1992; **58:**1809-1815.
21. Munevar, F., Wollum, A.G. Effect of high root temperature and *Rhizobium* strains on nodulation, nitrogen fixation and growth of soybean. *Soil Sci.Soc.Amer. J.* 1981;**45:**1113-1120.
22. Alexander, C.W., Chamblee , D.S. Effect of sunlight and drying on the inoculation of legumes with *Rhizobium* species. *Agronomy J.* 1965; **57**: 550-553.