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Effect of Different Air and Root-zone Temperatures on Nitrogen Fixation and Nodulation of Annual Medics

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Abstract: In the present study three annual medic cultivars (*Medicago polymorpha* cv. Santiago, *Medicago radiata* cv. Radiata and *Medicago rigidula* cv. Rigidula), were evaluated for symbiosis traits under three levels of day/night air temperatures (DNAT, 15/10, 20/15 and 25/20 ±0.2°C) and four root-zone temperatures (RZT, 5, 10, 15 and 20±0.2°C). The experimental design was a 3×3×4 factorial with treatments organized following a randomized complete block design with three replications. The result showed that low root zone temperature (RZT) and day/night air temperature (DNAT) had a severely reduction effect on root length, root dry matter and nodule formation. Plants did not produce any nodule in the root at 5°C RZT and all DNATs. The plants grown at RZT ranging from 10 to 20°C were found to fix some nitrogen. The 10°C RZT seem to be a thermal critical point for *Medicago spp.* nodulation and N2 fixation. DNAT also had the same effect on plant nitrogen fixation. This result confirms the possibility to screen medics for growth and nitrogen fixation at low temperature. *M. rigidula* performs well in nodule formation and nitrogen fixation at low RZT. Therefore *M. rigidula* is probably the most promising for the production of herbage at low temperature. *M. polymorpha* was found to have a significant performance in moderate temperatures but was also found to be the most widespread species and thus may be of high priority in cultivar development. The best temperatures for nitrogen fixation and nodule formation for annual medics were 15°C RZT and 25/20°C DNAT.

Key words: Air temperature, annual medics, nitrogen fixation, root zone temperature, nodulation

INTRODUCTION

The annual medics are Mediterranean pasture legumes developed for the Australian ley-farming system under fairly mild winter temperature prevailing in Southern and Western Australia[1].

Australian "know-how" and experience filtered to North Africa[2] especially in various experience in Tunisia[3], Algeria[4-6], Morocco[7,8] and Libya[9,10]. The same trend occurred in west Asia in Jordan[11], Syria[12] Turkey[13], Iran[14] and Iraq[10].

The introduction of the ley-farming system in North Africa and West Asia was based upon the use of Australian commercial cultivars *Medicago Scutellata* cvs Robinson, Sava, Sair, *M. truncatula* cvs Jemalong, Cyprus Borung and Ghor, *M. littoralis* cv Harbinger, *M. polymorpha* cvs Circle vally, Serena, *M. rugosa* cv Paragosa, etc.[15,16]. The poor acceptance of the ley-farming in North Africa and West Asia based on annual medics pastures had certainly to do with the local socio-economics[17] but also with the appropriateness of cultivars used.

It is clear that medic cultivars available on a commercial basis from Australia have not adapted to most of the agroecological zones encountered in North Africa and West Asia. The Australian cultivars were selected for the mild to cool winter environment prevailing in the Mediterranean austral environment where frost remained exceptional[18]. These commercial cultivars proved to be ecologically well adopted to the low lands of North Africa (Coastal zone of Morocco, Tunisia and Libya) while most of high plateau of North Africa and West Asia have a noticeable occurrence of freezing days and lower winter temperatures. Ecological distribution of annual medics in Middle East[19,20] and agronomical studies confirmed their frost susceptibility[21,22] and hence the need for medics with different biological characteristics from the Australian cultivars[23]. *Medicago rigidula* and *Medicago polymorpha* for West Asia appeared to be strong candidates for the cold Mediterranean countries.

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For Iran and Syria, *M. rigidula* presented the most suitable ideotype[24] . They reported that *M. rigidula* offers the greatest potential for winter survival. Other researchers also showed that *M. rigidula* had the highest tolerance to cold[19,24,25]. Annual mean air temperature for best growth of *M. polymorpha* varies between 10.5 and 27°C[26]. This cultivar is relatively cold tolerance[27]. Krall *et al.*[25] compared commercial Australian annual medic cultivars for winter survival on western high plains. Experimental lines with potential cold tolerance were established in fall 1994 along with commercial cultuvars. On April 1, 1995 survival was as follow: *M. polymorpha* cv. Santiago (0%), *M. polymorpha* line SC03077 (3%), *M. truncatula* cv. Paraggio (3%), *M. truncatula* line SCO7078 (5%), *M. rigidula* line SCO3075 (55 and 80%) for the control *M. sativa* cv. Ladak, a winter hardy alfalfa[28]. Therefore lack of cold tolerance limits the northern adaptation of most species.

It is generally admitted that temperate legumes nodulate and fix N_2 in a temperature range 10-30°C and the tropical ones in the temperature range 15-35°C. The temperature range for functioning the symbiosis is narrower than that of the plant supplied with combined nitrogen. The influence of high temperature on symbiosis is well documented. There is less information about the influence of cold on nodulation and nitrogen fixation. Nodule initiation is particularly sensitive to low temperature, but nodules formed at a favorable temperature maintain nitrogen fixation when transferred to lower temperature, even 2°C[29].

The nitrogenase enzyme in the nodules is less affected by low temperature than the formation of bacteriods in the internal nodule tissue. For sub-clover (*Trifolium subterraneum*), at 7°C, almost no bacteriod tissue can be observed; the highest amount of bacteriod tissue was formed between 11 and 15°C, but the efficiency of nitrogen fixation per unit of bacteriod tissue and the plant dry weight was the greatest at 19°C[30]. On some legumes, at least, nitrogen fixation is relatively intensive to low root temperature but nodulation is delayed[31].

Annual medic is the most commonly grown forage legume in ley-farming due to its excellent agronomic qualities. However its lack of persistence under severe winter conditions reduced its utilization in the cold regions of Mediterranean countries. Selection for cold tolerance is difficult due to complexity of field evaluation. A method of selection performed under environmentally-controlled conditions has been used for the identification of genotypes having superior cold tolerance. This led us to launch specific studies in developing new medics and their associate rizobium for the farming system of cold Mediterranean countries like Iran. Achieving this objective may allow sheep to graze for longer period during winter after an early germination soon after the early autumn rainfall or end of winter. Therefore, the purpose of this study was to find medics capable of growing early and fixing nitrogen at low temperatures.

MATERIALS AND METHODS

The study was conducted from May until August 2003. Seeds of 3 annual medic cultivars (*Medicago polymorpha* cv. Santiago, *Medicago radiata* cv. Radiata and *Medicago rigidula* cv. Rigidula), which are found in pastures of Iran, were surface sterilized in 95% ethanol with purity and sodium hypochlorite (2% solution containing 20 ml L)[32]. Then seeds were rinsed thoroughly with distilled water and planted in trays containing sterilized sand. Nine-day-old seedlings, at the cotyledon stage, were transplanted into sterilized 11 cm diameter and 14.5 cm deep plastic pots containing the same medium. Then thirty-six randomly selected pots are placed in each of three-controlled environment chambers. Day/night air temperatures (DNAT) in the growth chambers were 15/10, 20/15 and 25/20°C (±0.2°C). The growth chambers light (300 µmol m^2s) was provided by cool white fluorescent tubes. Light intensity across the growth chambers was measured several times during the experiment and was always uniform. The photoperiod was 12:12 h (day/night). This work examined the influenced of four root-zone temperatures (RZT) (5, 10, 15 and 20±0.2°C) and three day/night air temperatures on three cultivars.

In each growth chamber four root-zone temperatures was controlled by circulating cold water around large pots with 12 pots in each tank. Twelve medium sized pots containing plants were put into each of the large pots. A hole was drilled in the bottom of each medium sized pot to allow these pots to drain large pots. After being transplanted into the pots, the plants were acclimatized for 24 h prior to inoculation. The inoculum was produced by culturing *Rizobium meliloti* in liquid medium, in 450 mL sterile flasks. The medium was autoclaved at 121°C for 20 min prior to culture; each inoculated plant received 1 mL of a 4 day old (log phase) culture. The inoculum was cooled to the corresponding RZT and applied by pipette to the root area. Plants were watered with a modified Hoagland's soluttion[44], in which $CaNO_3$ and KNO_3 were replaced with $CaCl_2$, K_2PO_4 and KH_2PO_4, to provide a nitrogen-free solution; prior to each watering the temperature was adjusted to the treatment RZT.

The experimental design was a 3×3×4 factorial with treatments organized following a randomized complete block design with 3 replications and a total of 3 DNAT, 4 RZT treatments and 3 cultivars. Plants were harvested at 51 days after inoculation and the following data were collected: root length, root dry matter, nodule dry weight, nodule number, nodule number in each clone, nodule set number, nodule set length and diameter, nodule length and diameter, nodule dispersion (root length that has nodule) and plant nitrogen content (Kjeltec system, which includes digestion system 20 and a 1002 distilling unit, Tecator AB, Hoganas, Sweden).

Results were statistically analyzed for variance using the SAS system[33]. When analysis of variance showed significant treatment effects, Duncan's multiple range test was applied to compare the means at P=0.05[34].

RESULTS AND DISCUSSION

All traits were influenced by RZT, DNAT, cultivar (except for nodule number and nodule set diameter) and interactions among those treatments.

M. polymorpha had the highest root length (36.10 cm) and root dry matter (119.70 mg) at 25/20°C DNAT and 15°C RZT (Table 1). *M. radiata* (32.4 cm) and *M. rigidula* (33.3 cm) produced similar root length at 20/15°C DNAT with 15 and 20°C RZT and 25/20°C DNAT with 15°C RZT, but less than *M. polymorpha* at 25/20°C DNAT and 15°C RZT. *M. radiata* had the lowest root length (3 cm) at 20/15°C DNAT and 5°C RZT regime. Low RZT and DNAT had a severely reduction effect on root length and root dry matter (Table 1 and 2). Root dry matter was not difference between *M. polymorpha* (119.7 mg) and *M. rigidula* (116.7 mg) at 25/20°C DNAT and 15°C RZT. *M. radiata* had the lowest root dry matter (7 mg) at 15/20°C DNAT and 15°C RZT. Root dry matter was the same in *M. radiata* at 15/20°C DNAT with 5, 10 and 15°C RZT.

The effect of low temperature on annual medic nodulation and N fixation and assimilation may be mediated via effects on plant physiology and growth. For soybean, decreased aerial temperature resulted in decreased relative growth rate, stomatal conductance, net CO_2 exchange rate, leaf assimilation export rates and root dry matter[35-37]. Many adverse effects of low root zone temperature on chilling-sensitive plants like reduction root length and root dry matter can be attribute to low temperature-induced membrane phase transitions which decrease the activity of membrane-bound enzymes[38,39]. The effect of low temperature on N_2 fixation and NO_3-N assimilation may also be mediated via effects on photosynthesis or translocation as has been demonstrated in the case of limitation to nitrogen activity. Decreased shoot and root respiration and increased carbon level (partly as starch) in mature leaves and stems at low temperatures[40] reduce transportation of energy to root and nodules and decreased nodule formation and function and as a result reduced root growth and development (reduction in root length and root dry matter).

M. rigidula had the highest nodule dry matter (28.7 mg) nodule number (46.6), nodule number in each clone (2.8) and nodule set number (10.2) at 25/20°C DNAT and 15°C RZT. At the same temperatures nodule number in each clone was not statistically different between *M. polymorpha* (2.7), *M. radiata* (2.7) and *M. rigidula* (2.8). The maximum and minimum nodule dispersion were for *M. rigidula* at 20/15°C DNAT and 15°C RZT (8.4) and all varieties at any DNAT and 5°C RZT, respectively (Table 2).

M. rigidula had the highest nitrogen content (11.7 mg) at 15/10°C DNAT and 20°C RZT. Nitrogen content was the same in *M. rigidula* at 25/20°C DNAT with 15°C RZT and 15/10°C DNAT with 20°C RZT (Table 2).

Plants grown at the 5°C RZT and all DNAT did not produced any nodule in root. Thus RZT at 5°C strongly inhibited nodulation processes. The grown plants at RZT ranging from 10 to 20°C were found to fix some nitrogen, as indicated by an increase in the total plant N used in this experiment. It can be concluded that *Medicago spp.* nodulation processes and N_2 fixation ability are linearly decreased between RZT of 20°C and 10°C and sharply decreased when RZT drops below 10°C. The 10°C RZT seem to be a thermal critical point for *Medicago spp.* nodulation and N_2 fixation. DNAT also had the same effect on plant nitrogen fixation. Nodule number per plant increased gradually with increasing DNAT from 15 to 25°C. At day/night air temperatures ranging from 15 to 25°C, all the cultivars were found to fix some nitrogen, as indicated by an increase in the total plant N during the experiment. Lie[41] reported that all stages of nodule formation and functioning are affected by sub optimal RZT. Gibson[42] in a review of the data on environmental effects on the legume-*Rhizobium* symbiosis, suggested that low temperatures retard root hair infection more than nodule initiation, nodule development, or N assimilation. With temperate legumes such as *Trifolium parviflorum* and *T. glomeratum*, Kumarasinghe and Nutman[43] found that the onset of infection and rate of infection thread development in root hairs varied greatly with soil temperature. They found that at optimal temperatures (from 18 to 30°C) infections were initiated earlier and in larger numbers than at low (6 to 12°C) or moderately high (36°C) temperatures. We obtained similar results in *Medicago spp.* over the temperatures ranges of 15/10 to

Table 1: Mean comparison of root length, root dry matter, nodule dry matter, nodule number, nodule number in each clone, nodule set number, nodule dispersion and plant nitrogen content above ground of three annual medics under different DNAT and RZT

		RZT (°C) fot nodule number in each clone (per pot)				RZT (°C) for nodule set number (per pot)			
Varieties	DNAT (°C)	5	10	15	20	5	10	15	20
M. polymorpha	15/10	5.9s	14.0k-m	15.40j-l	19.5gh	9.7r-u	12.3p-t	11.3q-u	23.0mn
M. radiata	15/10	7.5rs	9.8p-r	11.00n-p	15.7i-k	8.3tu	9.3s-u	7.0n	17.7op
M. rigidula	15/10	7.9q-s	12.8m-o	15.20j-l	21.0g	13.0p-t	14.7p-s	15.0p-r	36.3jk
M. polymorpha	20/15	8.0q-s	17.8hi	30.30c-e	29.9de	55.3d-f	52.7ef	60.3c	52.3ef
M. radiata	20/15	3.0t	30.3c-e	31.50b-e	33.0b	16.3o-q	56.3c-e	69.3b	44.3hi
M. rigidula	20/15	13.0l-n	29.3e	32.10b-d	33.3b	54.0ef	53.3ef	55.0d-f	60.0cd
M. polymorpha	25/20	7.6rs	25.9f	36.10a	16.5ij	20.7no	28.7l	119.70a	26.0lm
M. radiata	25/20	10.6op	10.2pq	32.40bc	14.1j-m	28.7l	14.7p-s	50.7fg	35.3k
M. rigidula	25/20	14.5j-m	19.3gh	33.30b	11.2n-p	40.3ij	46.3gh	116.7a	25.3l-n
		Nodule dry weight (mg per pot)				Nodule number (per pot)			
M. polymorpha	15/10	0.0n	0.83mn	0.4mn	1.3m	0.0l	2.6k	2.0k	4.3ij
M. radiata	15/10	0.0n	1.3m	0.5mn	3.7l	0.0l	2.3k	2.2k	9.8h
M. rigidula	15/10	0.0n	0.8mn	1.3m	3.3l	0.0l	2.6k	55.0I	14.1f
M. polymorpha	20/15	0.0n	9.3fg	7.7hi	7.3hi	0.0l	23.2c	19.7d	14.8ef
M. radiata	20/15	0.0n	12.3e	8.2h	8.4gh	0.0l	19.3d	14.1f	12.3g
M. rigidula	20/15	0.0n	6.0jk	9.8f	5.2k	0.0l	12.4g	15.9e	10.5h
M. polymorpha	25/20	0.0n	6.7ij	24.3b	2.5l	0.0l	14.8ef	22.8c	4.9i
M. radiata	25/20	0.0n	3.0l	18.7c	5.3k	0.0l	5.3I	21.9c	5.3i
M. rigidula	25/20	0.0n	15.7d	28.7a	1.3m	0.0l	38.0b	46.6a	3.2jk

Means with similar letters in each trait, are not significantly different at 5% probability level according to Duncan's Multiple Range Test.

Table 2: Mean comparison of nodule number in each clone, nodule set number, nodule dispersion and plant nitrogen content above ground of three annual medics under different DNAT and RZT

		RZT (°C) for nodule number in each clone (per pot)				RZT (°C) for nodule set number (per pot)			
Varieties	DNAT (°C)	5	10	15	20	5	10	15	20
M. polymorpha	15/10	0.0g	2.0de	1.5f	1.5f	0.0l	1.0jk	1.0jk	1.4f-i
M. radiata	15/10	0.0g	2.0de	1.5f	1.5f	0.0l	1.03i-k	0.7k	2.4e
M. rigidula	15/10	0.0g	2.0de	1.5f	2.3cd	0.0l	1.03i-k	0.9k	2.6e
M. polymorpha	20/15	0.0g	2.0de	2.7ab	2.5bc	0.0l	3.7d	2.7e	2.5e
M. radiata	20/15	0.0g	2.0de	2.0de	2.5bc	0.0l	2.8e	1.8f	1.7fg
M. rigidula	20/15	0.0g	1.7ef	2.7ab	2.0de	0.0l	1.1h-k	1.8f	1.5f-h
M. polymorpha	25/20	0.0g	1.5f	2.7ab	2.0de	0.0l	2.5e	4.4e	1.8f
M. radiata	25/20	0.0g	1.5f	2.7ab	2.0de	0.0l	1.3g-j	5.8b	1.3g-j
M. rigidula	25/20	0.0g	1.5f	2.8a	2.0de	0.0l	3.5d	10.2a	1.4f-i
		Nodule despersion (per pot)				Plant nitrogen content			
M. polymorpha	15/10	0.0l	1.9k	2.3jk	3.1g-i	1.20m	2.8k	3.7j	5.3I
M. radiata	15/10	0.0l	1.9k	1.7k	3.3f-h	0.14n	2.1l	1.0m	3.0k
M. rigidula	15/10	0.0l	2.0k	2.7ij	5.4de	3.90j	5.2I	7.0g	11.7a
M. polymorpha	20/15	0.0l	5.0e	5.0e	4.9e	2.50kl	6.2h	7.9e	9.7c
M. radiata	20/15	0.0l	6.5c	5.4de	6.7c	1.90l	5.0I	7.7ef	7.8e
M. rigidula	20/15	0.0l	5.9d	8.4a	5.1e	4.70I	7.5ef	7.9e	9.5c
M. polymorpha	25/20	0.0l	3.6fg	3.8f	2.8h-j	5.20I	9.2cd	9.6c	8.6d
M. radiata	25/20	0.0l	3.3f-h	5.9d	2.7ij	3.00k	5.1I	6.6gh	7.3ef
M. rigidula	25/20	0.0l	7.8b	8.1ab	1.8k	5.20i	10.9b	11.1ab	10.5b

Means with similar letters in each trait, are not significantly different at 5% probability level according to Duncan's Multiple Range Test.

Table 3: Correlation coefficients for annual medic traits under different DNAT and RZT

Traits	Leaf area	Stem node number	Brunch number	Leaf number	Leaf dry matter	Stem dry matter	Root dry matter	Root length
Stem node number	0.77**							
Brunch number	0.71**	0.51**						
Leaf number	0.92**	0.84**	0.81**					
Leaf dry matter	0.96**	0.71**	0.73**	0.89**				
Stem dry matter	0.85**	0.63**	0.87**	0.71**	0.89**			
Root dry matter	0.75**	0.62**	0.64**	0.69**	0.83**	0.80**		
root length	0.58**	0.69**	0.62**	0.62**	0.60**	0.66**	0.75**	
plant height	0.78**	0.70**	0.79**	0.81**	0.77**	0.87**	0.56**	0.56**

**: significant at 1% probability level

25/20°C DNAT and 5 to 20°C RZT. All cultivar traits reduced in 25/20°C DNAT when RZT increased from 15 to 20°C.

There were positive correlation among all traits at P=0.01 (Table 3). The positive correlation between plant nitrogen content and other traits showed that increasing in each measured traits could increased plant nitrogen rate. Thus as a result rate of plant nitrogen of annual medic varieties depends to developing other traits including nodule number, nodule set number nodule length and diameter, nodule dispersion in root, nodule number in each clone and root length.

This result confirm the possibility to screen medics for growth and nitrogen fixation at low temperature. *M. rigidula* performed well in this conditions and is probably the most promising for the production of herbage at low temperature. The best temperatures for nitrogen fixation and nodule formation in annual medics were 15°C RZT and 25/20°C DNAT.

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