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Germination and emergence variability of alfalfa (*Medicago sativa* L.) landraces collected in Southern Tunisia oases

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Abstract

Variability in germination under controlled conditions with and without salt, seeds characteristics and field emergence were evaluated for 20 landraces of *Medicago sativa* ssp *sativa*, a forage legume collected from three sites in Tunisia. Highly significant differences were recorded among the landraces ($p < 0.001$) for all the characters studied except tegument colour. Number of seeds produced per plant ranged between 378 and 460 seeds g^{-1}. Emergence responses of seeds were different among landraces and sites and their values ranged between 12 to 45%. Salt stress (150 mM) caused substantial reduction in final and rate of germination of all the landraces with an average decline of 62.4 and 69.7%, respectively. Final germination values ranged from 26.5 to 75.75% when supplied distilled water. However, final germination values ranged from 10.75 to 32.5% when the seeds were exposed to saline water (150 mM). Rate of germination ranged from 12.75 to 63.25% and from 6.25 to 21.25% at non-saline and salt stress, respectively. Cluster analysis separated the 20 landraces into four clusters. Three landraces in the first cluster had the lowest germination and the greatest field emergence. One landrace composed the second cluster and showed both high germination and field emergence. Two landraces in the third cluster presented greater germination and low emergence. The other landraces constituted the fourth cluster and had intermediates emergence and germination.

Additional key words: arid land; diversity; salt stress; seeds.

Resumen

Variabilidad en la germinación y en la emergencia de variedades autóctonas de *Medicago sativa* en diferentes oasis de Túnez

Se evaluó la germinación en laboratorio con y sin estrés salino, así como las características de las semillas y la emergencia de 20 poblaciones de la leguminosa forrajera *Medicago sativa* ssp *sativa*, procedentes de tres localidades de Túnez. Se observaron diferencias altamente significativas entre las poblaciones ($p < 0,001$) en todos los caracteres estudiados excepto en el color del tegumento. El número de semillas por gramo varió entre 378 y 460. La emergencia de las semillas fue diferente entre las poblaciones y las localidades (variaron entre el 12 y el 45%). El estrés salino (150 mM) causó una fuerte reducción en todas las poblaciones, tanto en la germinación final como en el porcentaje de germinación, con una reducción media del 62,4 y 69,7%, respectivamente. Los valores de germinación finales variaron entre 26,5 y 75,75% y entre 10,75 y 32,5% con agua destilada y estrés salino, respectivamente. El porcentaje de germinación varió desde el 12,75 al 63,25% y del 6,25 al 21,25% en condición no salina y estrés salino respectivamente. El análisis cluster separó las poblaciones en cuatro grupos.

Palabras clave adicionales: diversidad; estrés salino; regiones áridas; semillas.

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important forage crops in the world. It has been originated from the Caucasus region (Michaud *et al*., 1988). The species presents purple flowers, a tap root, an erect growth habit, coiled pods and no winter dormancy. This plant improves the yield and quality of the following crops by atmospheric nitrogen fixation (Bruulsema and Christie, 1987). Furthermore, it reduces diseases

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Abbreviations used: FG (final germination percentage), GR (rate of germination).

and weeds, increases soil organic matter contents and improves water infiltration (Campbell *et al.*, 1990). In Tunisia, this crop is the most cultivated forage legume and has been grown in oases decades ago. Currently, alfalfa is grown on more than 9,720 ha across Southern Tunisia (ODS, 2005).

The estimation of the germination potentialities constitutes a crucial preliminary step in potential value evaluation of the species. Germination starts with the quiescent dry seed imbibition; it continuous by the emergence and development of embryo essential structures which are indicative of the ability to produce a normal plant under favorable conditions (Bewley, 1997). Several environmental factors such as temperature, salinity, light, and soil moisture simultaneously influence germination (Ungar, 1995; Huang *et al.*, 2003; Zia and Khan, 2004; El-Keblawy and Al-Rawai, 2005, 2006). Salinity constraint affects seed germination either through osmotic effects, by preventing or delaying germination (Welbaum *et al.*, 1990). This abiotic factor can reduce germination through ion toxicity, which can render the seeds unviable (Huang and Reddman, 1995). Although high salinity may inhibit germination, this effect may itself depend upon the prevailing temperature at the germination stage (Rivers and Weber, 1971; Badger and Ungar, 1989). Since that, tolerance to salinity during germination is crucial for the establishment of plants growing in saline soil of arid regions (Ungar, 1995; Khan and Gulzar, 2003). Meyer and Allen (1999), affirm that seed germination and emergence depend on factors associated with genotype, maturation environment, post-maturation history and germination environment.

It is well known that germination decreases with salt stress in alfalfa, but the effects of genetic variation on germination state are not or poorly documented. The aim of this study was to assess variability in a set of alfalfa landraces collected from Tunisian oases for (i) seed characteristics, (ii) laboratory germination in presence and absence of salt (NaCl) and (iii) field emergence.

Material and methods

Seed collection

Seeds of *M. sativa* ssp *sativa* were collected in June 2004 from 20 southern Tunisian oases, which were situated in three different localities (Gabes, Kebili and Tozeur) (Fig. 1). These areas are included in arid to semi-arid with a typical Mediterranean climate, characterized by irregular rainfall and a harsh dry summer period. Landraces's names, origins and main characteristics are summarized in Table 1.

Two traits were used to characterize seed colour and weight. Four lots of 100 seed from each landrace were separated to estimate tegument colour by visual observations. The following scale was adopted: 1,

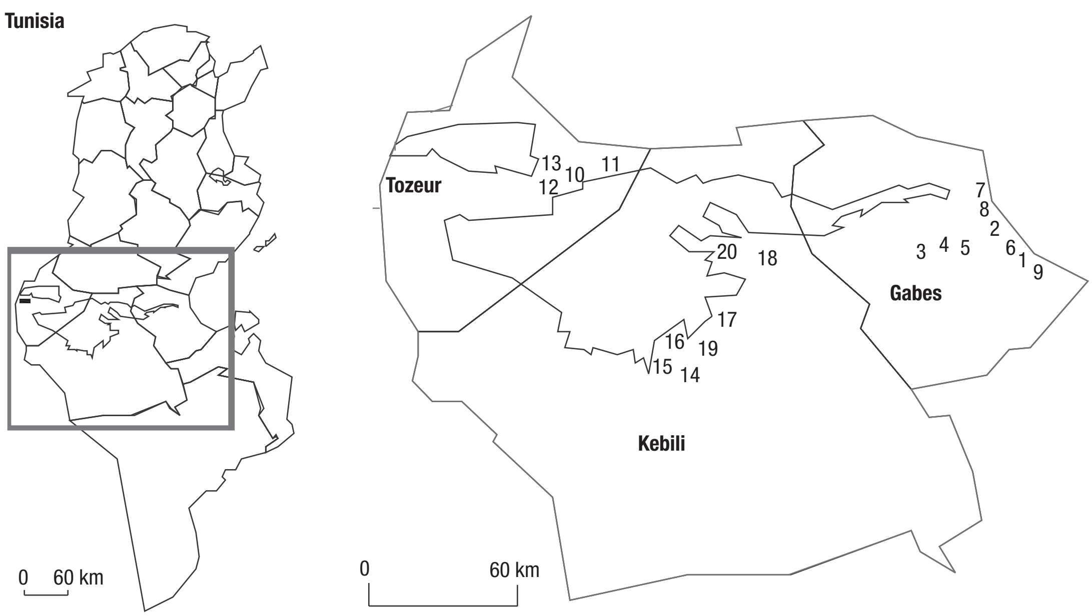

Figure 1. Origin of the 20 landraces of alfalfa. Numbers indicated landrace codes.

Table 1. Name, origin and main characteristics of studied alfalfa landraces

Province	Label	Name of oasis	Type of oasis	Collecting date in 2004	No. of seeds g^{-1}	Colour[a]	Longitude	Latitude	Alt (m)
Gabes (n = 9)	P1	Kattana	Littoral	June	460 ± 4.08	2	33° 45’	10° 12’	17
	P2	Chenchou	Littoral	November	413 ± 2.45	2	33° 52’	09° 53’	69
	P3	Chenini-1	Littoral	February	414 ± 1.63	2	33° 53’	10° 04’	21
	P4	Chenini-2	Littoral	February	395 ± 2.45	2	33° 53’	10° 05’	16
	P5	Chenini-3	Littoral	February	415 ± 3.74	1	33° 53’	10° 05’	13
	P6	Tboulbou	Littoral	March	429 ± 4.55	1	33° 49’	10° 07’	16
	P7	Metouia	Littoral	April	452 ± 3.59	1	33° 57’	09° 59’	25
	P8	Ghannouch	Littoral	April	413 ± 1.71	2	33° 55’	10° 03’	23
	P9	Zerkine	Littoral	June	400 ± 5.00	2	33° 41’	10° 15’	32
Tozeur (n = 4)	P10	Essdada	Continental	December	446 ± 2.94	2	33° 59’	08° 13’	60
	P11	Bouhlel	Continental	December	406 ± 4.55	2	33° 59’	08° 14’	47
	P12	Dgach	Continental	December	448 ± 9.14	2	33° 58’	08° 13’	59
	P13	Hammajerid	Continental	December	397 ± 7.18	1	33° 59’	08° 09’	66
Kébili (n = 7)	P14	Zaafrane	Continental	November	423 ± 4.69	2	33°27’	08° 55’	44
	P15	Nouael	Continental	December	399 ± 4.79	2	33° 29’	08° 51’	30
	P16	Jersine	Continental	December	382 ± 6.03	1	33° 31’	08° 49’	20
	P17	Elgolaa	Continental	November	423 ± 6.13	1	33° 28’	09° 00’	56
	P18	Limaguess	Continental	April	378 ± 6.78	2	33° 45’	09°05’	82
	P19	Douz	Continental	March	423 ± 9.54	2	33° 27’	09° 00’	51
	P20	Stiftimia	Continental	April	432 ± 6.61	2	33° 48’	09° 00’	32

[a] Colour of tegument: 1, bicolour (yellow and chestnut); 2, uncoloured (chestnut).

bicolour (yellow and chestnut) and 2, uncoloured (chestnut). The number of seeds per gram was calculated in four samples of 10 g for both landraces.

Field emergence

The seeds of the 20 landraces were established *ex situ* in the experimental field of Arid Lands Institute of Medenine Tunisia (33.50° N; 10.06° E). The edaphoclimatic characteristics of experimental site are similar to oasis’s conditions. The soil is sandy, the salinity of irrigation water is 3 g L^{-1}, the annual rainfall is 180 mm, the minimum mean temperature at winter is 12°C and the maximum mean temperature at summer is 28°C. The experimental site was laid out using randomized complete block design with 4 replicates. The seeds were mechanically scarified to obtain homogeneous germination (Crochemore *et al*., 1998), and then washed with sterile distilled water.

The experiment was started on September 2005 in pots of 2 m^2, 2 m in length and 1 m width. Seeds were sown using a drill at a rate of 100 seeds m^{-1} (200 seeds pot^{-1}). Fertilizer (32 kg ha^{-1} of nitrogen as ammonia and 70 kg ha^{-1} of P_2O_5 as biphosphate) was applied 2 weeks before seeding. Final emergence counts were determined after emergence had stopped (two weeks after seedling). The seeds were considered as germinated when the first two leaves arise from the ground.

Laboratory germination

Seeds were surface sterilized with 0.58% sodium hypochlorite solution for 1 min, subsequently washed with distilled water and air-dried before being used in the germination experiments to avoid fungus attack. Seeds were germinated in 10 cm Petri dishes fitted with two layers of Whatman No. 1 filter paper moistened initially with water. Germination experiments were conducted in a culture room at 25°C and 74% relative humidity under a 16 photoperiod (31.68 µmoles quanta m^{-2} s^{-1}) provided by cool white fluorescent tubes. Seeds were germinated in distilled water or in presence of NaCl solutions at concentration of 150 mM. A completely randomized design was used in the germination tests. For each treatment four replicates of 25 seeds (100 seeds per landrace per treatment) were used. During 10 days the germinated seeds (2 mm protrusion of radicles) were counted and removed every 2 days.

The rate of germination (GR) was estimated using a modified Timson index of germination velocity (Khan and Ungar, 1984):

$$GR = \Sigma G/t,$$

where G is the percentage seed germination at one-day intervals and t is the total germination period.

The final germination percentage (FG) was calculated as the cumulative number of germinated seeds with normal radicles (Larsen and Andreasen, 2004):

$$FG = \Sigma n,$$

where n is number of seeds germinated at each counting.

The reduction in germination (R) caused by salinity was estimated using the difference between two germination percentages, under distilled water and under salinity:

$$R\ (\%) = [(FG_0 - FG_{150})/FG_0] \times 100,$$

where FG_0 and FG_{150} are the final germination in % respectively at 0 and 150 mM salt concentrations.

Statistical analysis

Data were analyzed through analysis of variance procedure using SPSS 16.0. The ANOVA was used to test the effects of the landrace, salinity and their interaction on laboratory germination traits. The confidence interval was calculated at the threshold of 95%. Cluster analysis classifications of the 20 landraces on five measured traits in seedling emergences stage was determined by Ward's minimum variance method based on Euclidean distance (Ward, 1963).

Results

Weight and colours of seeds

The ANOVA analysis showed that the number of seeds per gram was significantly different between the studied landraces (Table 1, Table 2). This number ranged from 378 seeds g^{-1} for Limaguess landrace (P18) to 460 seeds g^{-1} for Kattana (P1). However, no significant difference was assessed between landraces for tegument colour. Two teguments types were observed: completely chestnut and bicolour tegument (yellow and chestnut) with frequencies of 35% and 65%, respectively.

Field emergence

Data for field emergence of the landraces were highly significant (Table 2). The emergences of seeds were different among landraces (Fig. 2) and among geographic origin (Fig. 3). The maximal mean of emergence percentage was detected for landraces from Gabes and Kebili oases, with mean values of 27.1 and 32.6%, respectively. However, in region of Tozeur this percentage did not exceed 20.4%. For all landraces, the emergence values ranged between 12 (landrace Zaafrane) and 45% (landrace Stiftimia) (Fig. 2).

Final germination

Table 3 shows the statistical significance concerning the effects of salinity, landraces and their interaction on FG and RG. The FG and RG of the studied landraces under salt stress were significantly lower than those grown under non saline conditions (Table 4, Fig. 3). With distilled water, germination percentages ranged from 26.5 to 75.75%. Landraces Zaafrane, Nouael, Hammajerid and Chenini-1 exhibited the highest FG under normal conditions compared to the other landraces (Table 4). However, in presence of salt at concentration of 150 mM NaCl, FG ranged between 10.75% for Hammajerid and 32.5% for Jersine (Table 4). The

Table 2. Analysis of variance (one-way ANOVA test of landrace effect)

Dependent variable[a]	Sum of squares	df	Mean square	F[b]	Significance[c]
FG at non-saline conditions	15,138.238	19	796.749	16.531	0.000***
GR at non saline-conditions	14,083.137	19	741.218	40.568	0.000***
FG at 150 mM NaCl	3,735.138	19	196.586	68.676	0.000***
GR at 150 mM NaCl	2,080.050	19	109.476	63.465	0.000***
Field emergence	5,573.138	19	293.323	3.492	0.000***
Tegument colour	16.800	19	0.884	ns	ns
Number of seeds g^{-1}	39,312.937	19	2,069.102	72.505	0.000***

[a] FG: final germination percentage GR: Germination rate. [b] ns: non-significant. [c] ***: significant at 0.001.

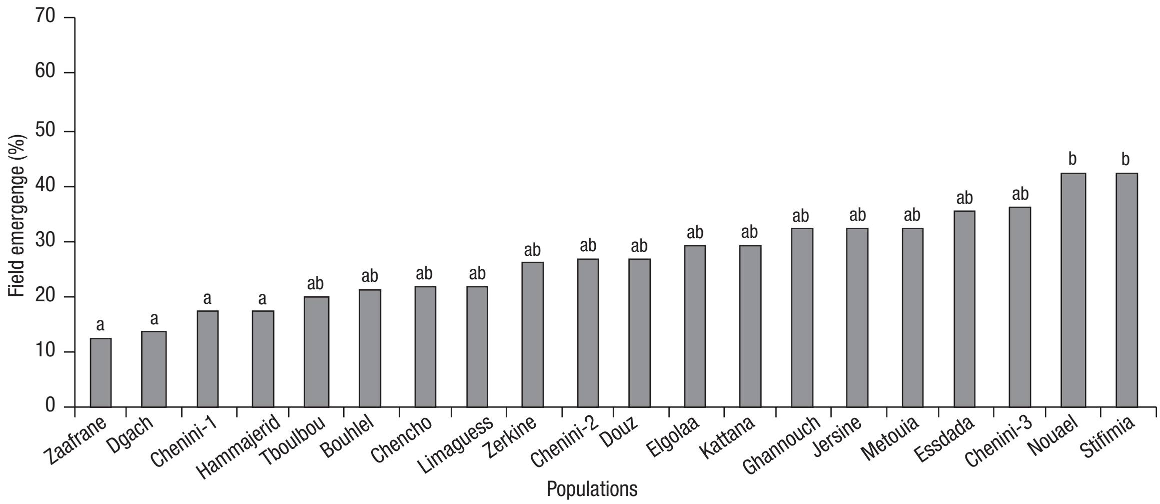

Figure 2. Field emergence percentage of 20 alfalfa landraces at oasis conditions of Tunisia. Different letters indicate significant differences between landracelandraces ($p < 0.05$).

interaction between landrace and salinity effects was also significant (Table 3). In the basis of landraces originality, the alfalfa responses to salt stress were significantly different, especially for final percentage of germination (FG). Salt stress (150 mM) caused drastic reduction in FG of all the landraces with an average decline of 62.4%. The significantly highest average FG under salt condition was obtained with Jesine landrace (32.5%).

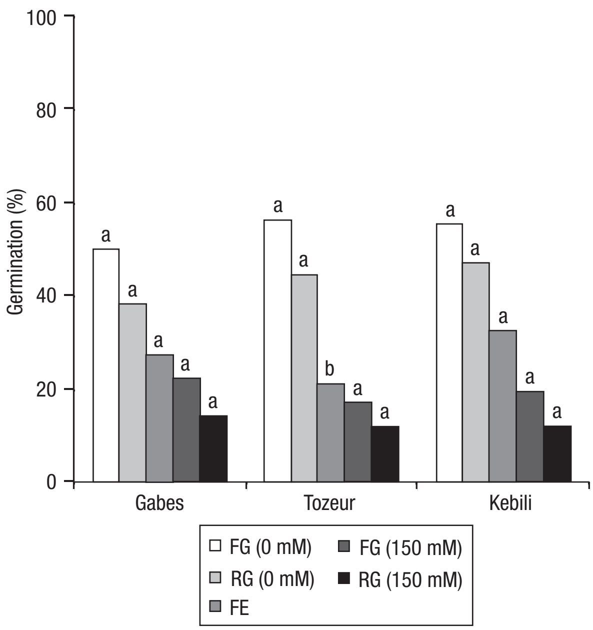

Figure 3. Mean of germination and emergence of alfalfa seeds across sites of collection. FG: final germination. FE: final emergence. RG: rate germination.

Germination rate

The index of germination velocity calculated by using a modified Timson's index showed that the germination rate decreased under high salinity (Table 4, Fig. 3). A two-way ANOVA made on germination rate indicated significant main effects of landrace, salinity and their interaction (Table 3). Germination rate ranged from 12.75 to 63.25% and from 6.25 to 21.25%, at 0 and 150 mM NaCl, respectively (Table 4). Salt stress (150 mM) inhibited GR for all landraces with a reduction average of 69.7%. The reduction of germination rate induced by salinity constraint showed inter-landrace variation. The highest reduction percentage was observed for Hammjerid landrace (88.3%). However, Essdada landrace presents the lowest reduction percentage (31.4%).

For all landraces, salt stress caused a reduction in the rate and percentage of germination. It was shown that there was no significant difference between landraces' origins on germination traits (Fig. 3). It has also shown that emergence mean were inferior to final germination ones under non-saline condition.

Cluster analysis

Multivariate analysis performed on averages of all variables revealed four main groups (Fig. 4). The first cluster comprised three landraces which were collected from Gabes and Tozeur. The landraces in this cluster had the medium germination percentage (15.91%) under salt stress of 150 mM NaCl and had lower values

Table 3. A two-way ANOVA of the effects of salinity, landrace, and their interaction on germination of *Medicago sativa*

Source	Dependent variable	Sum of squares	df	Mean square	F	Significance[a]
Salinity (S)	Final germination	43,956.900	1	43,956.900	1.722E3	0.000***
	Rate of germination	35,016.806	1	35,016.806	3.502E3	0.000***
Landraces (L)	Final germination	11,248.525	19	592.028	23.190	0.000***
	Rate of germination	10,495.119	19	552.375	55.249	0.000***
(L × S)	Final germination	7,624.850	19	401.308	15.720	0.000***
	Rate of germination	5,668.069	19	298.319	29.838	0.000***

[a] ***: highly significant at 0.001.

for rate of germination (8.3%) as compared with the other landraces. However, the field emergence was greater than the general mean (29.66%).

The second cluster includes a single landrace (Nouael from Kebili province) which showed an average of 71% for final germination and of 61% for germination rate under normal conditions. Under salt stress, it showed medium values of germination percentages.

The third cluster is made up of two landraces, Chenini-1 and Zaafrane. On average, this group is marked by the higher final and rate germinations under normal conditions (FG, 73.75 and 75.75%; RG, 58 and 63%) and salt stress (FG, 29 and 28.75%; RG, 21 and 22.25%). However, it was the lowest in field emergence of all landraces.

Cluster four comprised 14 landraces (the largest group). The landraces in this cluster were collected from the three provinces. On average, these landraces had medium values for all studied traits in both saline and non saline conditions.

Table 4. Mean and standard deviation (SD) of final (FG) and rate (RG) germination of the 20 alfalfa landraces under non-saline and saline condition

Landraces	Final germination (%)				Rate germination (%)				R (%)[a]	
	0 mM NaCl		150 mM NaCl		0 mM NaCl		150 mM		FG	RG
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD		
Kattana	55.50	1.29	19.25	1.71	45.50	2.38	12.50	1.29	65.3	72.5
Chenchou	50.50	27.78	24.50	1.29	46.50	4.20	15.25	0.96	51.5	67.2
Chenini-1	73.75	1.89	29.00	1.83	58.00	4.55	21.00	1.83	60.7	63.8
Chenini-2	55.00	3.65	25.25	1.71	46.00	1.41	20.00	1.41	54.1	56.5
Chenini-3	26.50	3.11	21.00	0.82	12.75	2.75	6.50	1.29	20.8	49.0
Tboulbou	61.75	2.06	28.00	2.94	46.25	3.78	16.00	1.83	54.7	65.4
Metouia	46.00	2.58	11.00	1.41	32.00	4.32	8.00	0.82	76.1	75.0
Ghannouch	28.50	2.08	12.50	1.29	22.50	1.29	6.50	1.29	56.1	71.1
Zerkine	51.50	4.51	28.75	2.75	33.25	2.22	21.25	2.06	44.2	36.1
Essdada	30.75	5.12	14.25	0.96	17.50	10.66	12.00	0.82	53.7	31.4
Bouhlel	44.50	3.70	11.50	1.29	36.50	5.26	7.25	0.96	74.2	80.1
Dgach	61.25	2.06	18.75	1.71	51.75	2.06	11.00	0.82	69.4	78.7
Hammajerid	69.00	4.08	10.75	0.96	53.25	1.71	6.25	0.50	84.4	88.3
Zaafrane	75.75	4.65	28.75	2.22	63.25	4.72	22.25	1.26	62.0	64.8
Nouael	71.00	2.58	16.50	2.52	61.75	8.54	13.75	1.26	76.8	77.7
Jersine	41.25	1.71	32.50	1.29	41.50	1.29	15.25	1.71	21.2	63.3
Elgolaa	55.25	3.30	21.75	1.71	39.25	2.50	11.50	1.29	60.6	70.7
Limaguess	54.50	3.11	15.25	0.50	48.75	2.22	12.25	1.71	72.0	74.9
Douz	58.25	2.22	12.50	1.29	48.25	3.59	7.25	0.96	78.5	85.0
Stiftimia	51.75	3.30	17.50	1.29	43.75	1.71	10.75	0.96	66.2	75.4
Mean	53.11	15.11	19.96	7.03	42.41	13.86	12.83	5.26	62.4	69.7

[a] R (%) indicates the percentage of reduction in saline condition.

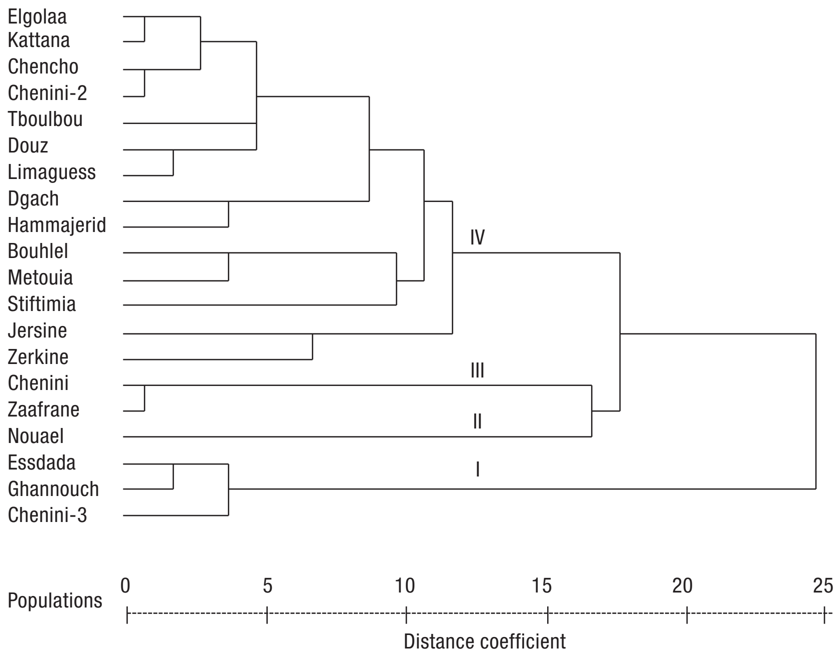

Figure 4. Cluster analysis classifications of 20 landraces native to Tunisian oases determined by Euclidian distance on five measured traits (FG, RG under 0 and 150 mM NaCl, and field emergence) in seedling emergences stage.

Discussion

Many reports have been documented regarding genetic diversity of *Medicago sativa* using different traits; morphological and yield (Julier and Huyghe, 1997; Bolaños-Aguilar *et al*., 2000; Julier *et al*., 2000), digestibility (Crochemore *et al.*, 1996, 1998), nutritive content (Lenssen et *al*., 1991), histological (Guines *et al*., 2003), RAPD markers (Crochemore *et al*., 1998), AFLP markers (Riday *et al.,* 2003), SSR markers (Diwan *et al*., 2000) and allozyme markers (Jenczewski *et al*., 1999). These studies have shown that alfalfa was characterized by a high inter-population variability. However, few studies have reported the diversity on germination stage. The present study of alfalfa landraces concerned germination aspects in field and laboratory. Our results showed important variability on final and germination rate under distilled water and under high level of salinity among the 20 studied landraces. For field emergence, the native alfalfa landraces exhibited high genetic diversity under Tunisian oases conditions (variation coefficient is 43.62%).

Alfalfa is one of the most common Fabaceae found in many regions in arid Tunisia producing large seed numbers. In their natural habitats seeds mature at the end of June. The arid lands in Tunisia are divided into three regions, where well oases were installed (Fig. 1). In the present study, the studied characters were not significantly modified between these three ecological regions, except for field emergence which was lower in Tozeur landraces than Gabes and Kebili ones. This stability of germinal traits between different origins agrees with results of Kiss and Kiss (1998) obtained with *Schizaea pusilla* populations, which showed little differences among geographically populations origins. The variation observed in our study can be attributed more to a genotypic difference than to adaptation in a particular environment. Meyer and Allen (1999) affirm that seed germination and emergence depend on factors associated with genotype, maturation environment, post-maturation history and germination environment. Other authors found that germination was affected by seed dormancy and seed reserves (Forcella *et al*., 2000), and parental environment during seed development (Fenner, 1991).

Alfalfa seeds have the ability to tolerate moderate salinity during germination stage. A two-way ANOVA of germination traits indicated a significant main effect of population, salinity and their interaction. So the variability structure was different between non saline and saline conditions. This can be attributed to different responses among landraces grown under salt stress condition. In our study, germination in laboratory condition showed that the highest germination percentage obtained in the non saline conditions and salt stress substantially affected germination in all landraces. The reduction percentage of germination traits could exceed 80% under salt stress for some landraces. But in total, the average reductions for the final percentage and rate of germination were 62.4 and 69.7% respectively. The detrimental impact of salinity on the germination of alfalfa results in decreasing both the rate and final percentage of germination. These results corroborate several other studies, revealing that halophytes, as glycophytes, are especially sensitive to salt during the germination stage (Ungar, 1982, 1995; Katembe *et al*., 1998; Khan *et al*., 2002). Two different mechanisms of these effects had been reported: (i) an osmotic inhibiting one and (ii) ion toxicity (Khan and Rizvi, 1994; Khan and Ungar, 1998; Song *et al*., 2005).

The alfalfa complex shows a large genetic variability due to both natural and human selection under various climates and locations (Julier *et al*., 2000). Several authors have used multivariate analysis to group populations in genetic diversity studies (García *et al*., 1997; Bayuelo-Jiménez *et al*., 2002; Mohammadi and Prasanna, 2003) and cluster analysis to determine the relative importance of classification (Berdahl *et al*., 1999; Bregard *et al*., 2001). In our previous work, using agro-morphological traits the mainly studied landraces have been grouped into three different clusters (Benabderrahim *et al*., 2009). Herein, depending on germination traits these landraces were grouped into four clusters comprising the lowest final and rate germination but greater field emergence (cluster I), the greater germination and field emergence (cluster II), the greater germination and low emergence (cluster III) and the medium in field emergence and germination percentages (cluster IV).

From the present studies it is evident that the emergences in field and seeds germination in laboratory of alfalfa vary among landraces, but not between localities. Salt stress decreases both rate and percentage of germination, but recovery is possible as long as salinity is low. Also, the germination response to salt conditions is different among the 20 landraces of alfalfa. It can be concluded that study of germination traits is useful; and could have agronomic repercussions that would lead to manage a landrace of alfalfa under variable geographic conditions.

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