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M. A. Dorri[1], B. Kamkar[2], M. Aghdasi[3], A. R. Safahani[4]*

[1]Agricultural and Natural Resources Research and Education Center of Golestan, Gorgan, Iran;
[2]Dept. of Agronomy, Gorgan University of Agricultural Sciences and Natural Resources,
Gorgan, Iran; [3]Dept. of Biology, Golestan University, Gorgan, Iran; [4]Agronomy
Department, Payame Noor University , 19395-4697 Tehran, I. R. of IRAN.
*Corresponding Author: safahani_ali@yahoo.com

GERMINATION AND SEEDLING GROWTH OF *SILYBUM MARIANUM* AS A MEDICINAL PLANT UNDER SALINITY STRESS

ABSTRACT

Milk thistle (*Silybum marianum*) is used as a medicinal plant in the treatment of liver diseases. In order to investigate germination and seedling growth in S. *marianum* subjected to NaCl, a three replicated experiment was carried out in a randomized complete block design in the laboratory of Gorgan University of Agricultural Sciences and Natural Resources. Treatments included seven salinity levels (50, 75, 100, 125, 150, 175 and 200 mM) and a control (distilled water). Germination percentage (GP), mean germination time (MGT), seedling vigour index (SVI), shoot, root and seedling weight were measured. The effect of salinity levels was significant on GP, SVI, MGT and seedling weight and length ($p \leq 0.01$). However, the NaCl concentration effect was not significant on shoot : root length ratio and or shoot : root weight ratios. Results showed that germination decreased when salinity increased, while MGT increased. MGT was 1.75 times higher than in the control at the highest salt concentration. MGT difference was not significant between 75 mM NaCl and control, while it dramatically increased by increasing the NaCl concentration from 150 to 200 mM NaCl. Reduction slope and salt tolerance index (STI) were estimated for germination (0.54 and 231.9) and seedling stage (0.24 and 237.4). According to the results, milk thistle could be considered as a valuable medicinal plant in fairly salinized areas.

Key words: germination, medicinal plant, NaCl, *Silybum marianum.*

INTRODUCTION

Chaudhary *et al.* (2010) reported that milk thistle was one of the top ten best-selling medicinal plants in 2007 worldwide. *Silybum marianum*, commonly known as milk thistle, belongs to a family Asteraceae and is one of the oldest and thoroughly researched plants in the treatment of liver diseases (Pradhan and

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Girish, 2006). The dried seeds include four flavonolignans that are collectively known as silymarin. Silymarin is extracted from the dried seeds where it is present in higher concentrations than in other parts of the milk thistle plant (Luper, 1998).

Salinization of soil and water is one of the world's most serious agricultural environmental problems. Salinity of soil or water is one of major stress obstacles for plant growing areas globally; especially in arid and semi-arid regions, these conditions can severely limit plant production (Jamil *et al.,* 2006). Some areas affected by salinity and salt experience a steady increase; however, statistical data show that these regions include almost 50% of the world's fields, which is equal to three times more than under- cultivation areas (Kamkar *et al.*, 2004). These results were due to the effect of salinity stress on quantitative and qualitative parameters. For instance, it was found that increasing salinity stress decreased almost all of growth parameters in *Nigella sativa*, as well as some growth parameters and essential oil amounts in chamomile (Razmjoo *et al.,* 2008).

Germination is one of the most salt-sensitive plant growth stages and can therefore be severely inhibited by increased salinity (Sosa *et al.,* 2005; Ghavami and Ramin, 2007). The negative responses of seed germination under salt stress has been reported by many authors regarding *Ocimum basilicum*, *Eruca sativa*, *Petroselinum hortense*, chamomile, sweet marjoram and *Thymus maroccanus* (Miceli *et al.*, 2003; Belaqziz *et al.,* 2009). Ahmadian *et al.,* (2012) showed that germination decreased from 79.76% to 50.2% as a result of salt concentration increasing. Salinity delays the onset, reduces the rate and increases the dispersion of germination events, leads to reduced plant growth and final crop yield (Ashraf and Foolad, 2005).

Salinity caused a significant reduction in seedling growth of *Thymus maroccanus* (Belaqziz *et al.,* 2009). Salinity affects seedling growth through slower or less mobilization of reserve foods, suspending cell division and enlarging and injuring hypocotyls (Rahman *et al.,* 2008). Reduced seedling growth has also been reported for basil, chamomile and marjoram (Ramin, 2005; Ali *et al.,* 2007; Baatour *et al.,* 2010). Ghaderi-Far *et al.* (2012) reported that milk thistle was sensitive to salinity in the germination stage, but was moderately saline tolerant in the seedling stage. Growth parameters such as plant height, number of leaves per plant and number of capitula per plant on milk thistle were reduced in the instance of a salinity level greater than 9 dS m^{-1} (Ghavami and Ramin, 2008). Comparison of root- to shoot-length ratio for *Calendula officinalis* under salinity levels showed that this ratio decreased in all of the treatments when compared to the control (Torbaghan *et al.,* 2012).

Due to the increasing demand for medicinal plants and traditional medical systems worldwide (including within the pharmaceutical industry), some medicinal plants need to be grown commercially; however, soil salinity and other forms of pollution pose serious threats to crop production (Qureshi *et al.,* 2005).

The increasing attention paid to the health concerns regarding the hazards of synthetic drugs has given rise to an increased interested in plant-based drugs. In order to produce medicinal plants for the pharmaceutical industry, these plants need to be cultivated commercially, especially in marginal and low quality lands. One of the most effective ways to overcome salinity problems is the introduction of salt tolerant crops) Yildirim *et al.,* 2011).

The aim of this study was to evaluate the seed germination and seedling growth response of milk thistle as a medicinal plant exposed to salinity, which its results may be used for decision making about its cultivation where may be lower utilized in agriculture. The findings of this study hopes to contribute to the better utilization of saline habitats.

MATERIAL AND METHODS

The experiment was carried out using a three-replicated randomized complete block design using seven salinity levels (50, 75,100, 125, 150, 175 and 200 mM) and a control in the Agricultural Sciences and Natural Resources seed research laboratory of Gorgan University of Agricultural Sciences and Natural Resources in 2012. Each experimental unit consisted of a petri dish (15 cm diameter) in which 50 S*ilybum marianum* seeds were placed on top of two sheets of filter paper and covered by a single sheet of filter paper. The seeds were disinfected with 1% NaOCl and rinsed with running tap water three times prior to the germination test. Petri dishes were supplied with 10 ml of each treatment solution and distilled water for the control treatment. Finally, Petri dishes were covered with a polyethylene sheet to avoid the loss of moisture through evaporation. The temperature was adjusted to a constant of 20°C.

Germination percentage

The germination period was started 48 hours after the experiment onset and continued until the 14th day. The criterion for germination was radical emergence of two millimetres or more. The germination period was stopped when no seeds germinated within a period of 48 hours, or when all the seeds had germinated. Each time the germinated seeds were counted, the exact time of counting was recorded. Final germination percentage (GP) was calculated using formula of Elouaer and Hannachi, 2012:

$$GP = \sum_{i=0}^{n} \frac{N_i \times 100}{N}$$

where, Ni - number of germinated seeds up to day i and N - the total number of seeds. Mean germination time (*MGT*) for individual replicates was also calculated on the basis of Ellis and Roberts formula, (1981)

$$MGT = \frac{\sum_{i=0}^{n} T_i \times N_i}{F}$$

where Ti, Ni and F were the days on which the germination count was made, the number of seeds germinated on the counting day and the final number of germinated seeds in each replication, respectively.

Germination percentage data were arcsine transformed. Data were subjected to ANOVA procedures and means were separated using the least significant difference (LSD) test at a 5% probability level.

Seedling vigour index

Seedling vigour index (SVI) was calculated following a modified equation (Islam *et al.,* 2009)

$$SVI = \frac{SL \times GP}{100}$$

At the end of the experiment, 10 seedlings were selected from each Petri dish. Then, seedling shoots and root fresh weights, as well as their lengths were recorded, and were oven dried at 75°C for 24 hours to measure seedling shoot and root dry weight.

Salt tolerance evaluation

Salt tolerance was assessed as the amount of salinity that caused 50% inhibition of germination. A three-parameter logistic model (Chauhan *et al.,* 2006; Tanveer *et al.*, 2013) was fitted to seed germination percentages obtained at different concentrations of NaCl using Sigma Plot software (v. 11).

$$GP = \frac{a}{\left(1 + {}^{C}\!/_{C_{50}}\right)^{SC_{50}}}$$

where, *GP*: total germination (%) at NaCl concentration levels; a: maximum germination percentage seen at control; C: the electrical conductivity in dSm^{-1}; C_{50}: defined *C* at *Gr* = 0.5, which was 50% of total germination in a potential condition; *S*: represented the response curve steepness.

Salt tolerance index (STI) was also used to evaluate salt tolerance (Steppuhn *et al.*, 2005)

$$STI = C_{50} + SC_{50}$$

C50 and *S* parameters were estimated using the *NLIN* and REG procedures (SAS 9.1, Institute Inc., 1988).

Salinity response threshold for germination and seedling weight was also obtained based on the linear response equation proposed by Maas - Hoffman (1977):

$$Y_r = 100 - b \times (E_c - a)$$

where *a* is salinity tolerance threshold corresponding to the salinity value of E_C, beyond which a reduction in yield starts appearing with respect to non-saline conditions and *b* is the rate of decrease (slope) in *Y* by unit increase of E_C.

RESULTS

Salinity effects were significant in terms of seed germination percentage, seedling vigour and MGT ($p \leq 0.01$, but not significant for both S:R length ratios and S:R weight ratios (Table 1).

The results revealed that the seed germination (%) of milk thistle was affected by an increase in NaCI concentration (Table 1).

Table 1

Mean squares of measured traits in germination and seedling stage: Germination percentage (GP), Mean germination time (MGT), Seedling vigour index (SVI)

S.O.V	DF	GP	MGT	Seedling		S:R ratio		SVI
				Length	Weight	Length	Weight	
Rep	2	8.76	0.019	0.86	0.021	1.43	0.022	0.79
NaCl Con.	7	232.97**	1.55**	5.72**	7.25**	1.06^{ns}	0.15^{ns}	10.35**
Error	14	6.78	0.197	0.249	0.403	0.501	0.097	0.248
CV.%		4.34	12.11	6.13	5.42	14.73	7.09	8.04

**: significant at 0.01 level and ns: no significant

The reduction of germination percentage was observed mainly at the higher levels of NaCI concentration compared to the control, as the lowest germination percentage was related to the highest salinity level (200 mM), where only 42.7% of the seeds were germinated (Table 2).

Table 2

Mean comparisons of germination percentage (GP), Mean Germination Time (MGT) and seedling vigor index (SVI) for milk thistle seeds under different salinity levels

NaCl Conc. [mM]	GP [%]	Sedling		MGT [h]	SVI
		Length [cm]	Weight [mg]		
0	81.33a	9.66a	13.87a	67.68d	7.84ab
50	83.3a	10.08a	13.00a	70.32d	8.39a
75	79.4a	9.48a	13.27a	74.88cd	7.52b
100	82,0a	7.11bc	11.67b	84.96bc	5.84cd
125	83.3a	7.90bc	11.20bc	89.28bc	6.58c
150	77a	7.35bc	10.73bc	96.24b	5.70d
175	64b	6.81c	10.50c	102.96ab	4.36e
200	42.7c	6.74c	9.37d	117.84a	2.88f

Means with at least one the same letter are not statistically different

Seedling weight of milk thistle was more pronounced compared to seedling length as a result of increasing NaCl concentration (determination coefficients of 0.84 and 0.68, respectively) (Table 3).

Table 3

Linear regression between NaCl concentration (X) against shoot, root and seedling length (Y)

Length (Y)	Intercept (a)	B (X)	R^2	CV [%]
Shoot	3.11	-0.0093	0.52**	28
Root	7.02	-0.0088	0.48**	9.8
Seedling	10.13	-0.0182	0.68**	9.8
Seedling weight	14.17	-0.022	0.84**	5.3

**: significant at 0.01 of probability level

Linear regressions are shown for MGT and SVI vs. NaCl in Fig. 4a-b; root, shoot and seedling weight in Fig. 3a-b and for root, shoot and seedling length in Table 3.

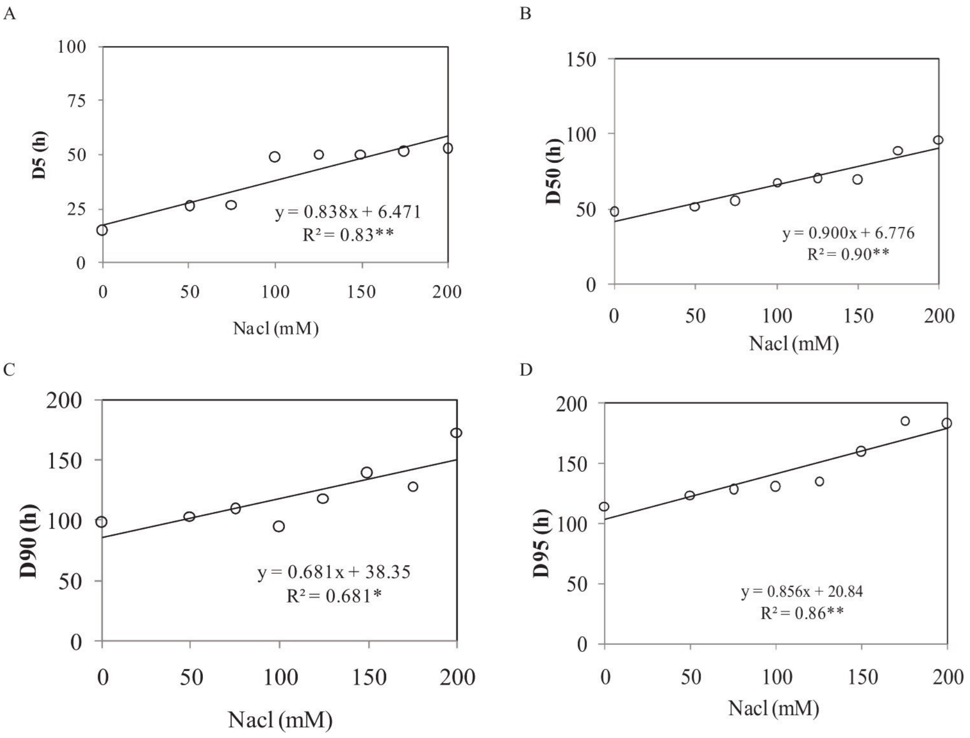

Fig. 1. A linear model fitted for D_5 (A), D_{50} (B), D_{90} (C) and D_{95} (D) *vs.* NaCl concentration (mM)

Germination percentage difference was not significant in the control up to 150 mM concentration (Table 2), but a reduction threshold was seen at 101.2 mM using the linear response equation from this threshold onward, germination percentage declined (Table 4).

Table 4

Threshold, slope, C50 and STI for seed germination percentage and seedling weight of *S. marianum* vs. NaCl

Equation	Threshold [mM]	Slope	C_{50} [mM]	SC_{50}	STI	Significant
$G_p = {}^{G_{max}}\big/_{1+(ec/C_{50})^{SC_{50}}}$	-	-	231.4	0.52	231.92	**
$G_r = 100 - b \times (ec - a)$	101.2	0.54	-	-	-	**
$Yield = {}^{Y_m}\big/_{1+(ec/C_{50})^{SC_{50}}}$	-	-	234.8	2.58	237.38	**
$Y_r = 100 - b \times (ec - a)$	43.64	0.247	-	-	-	**

To clarify germination onset times, D5, 50, 90 and 95% of maximum germination percentage, a linear model was fitted against NaCI concentrations (Figs. 1a-d). This model showed that D5 (time to 5% of maximum germination), D50 (time to 50% of maximum germination), D90 (time to 90% of maximum germination) and D95 (time to 95% of maximum germination) were strongly related to salinity levels (Figs 1a-d).

Germination was started in the control at the shortest time, while this time in 50 mM and 75 mM NaCl was 1.7 times longer than the control and in other concentrations increased more than three times compared to the control which obviously occurred at concentration 200 mM (Fig. 2).

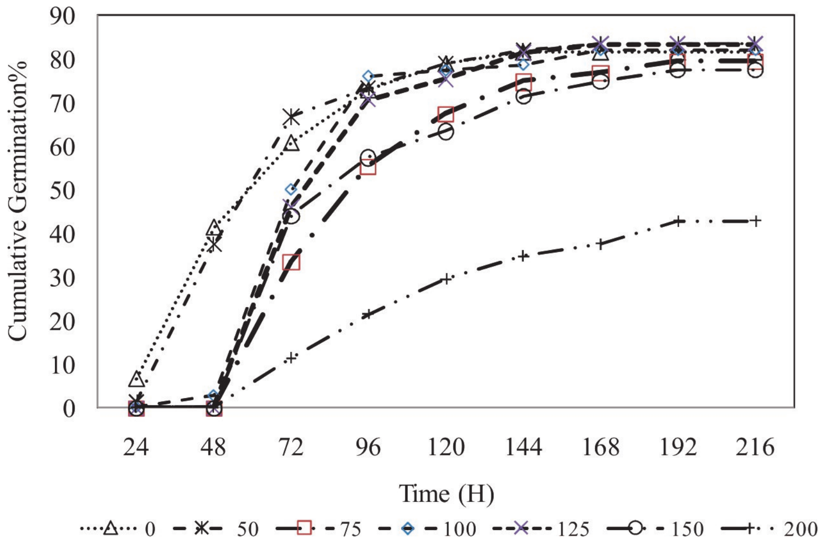

Fig.2. Cumulative germination percentage against time after experiment onset, in different NaCl concentrations (mM)

Similarly, D50, D90 and D95 were shorter in the control (Figs. 1b-d). As NaCl concentration increased, D50, D90 and D95 also increased. D50 in 100 and 125 mM NaCl was delayed 1.4 times longer than the control while it was longer in 175 and 200 mM NaCl so that it was recorded 1.86 and 2.02 times longer than the control, respectively (Fig. 1b).

MGT difference was not significant between 75 mM NaCl and control, while above 75 mM NaCl was significant differences so that MGT dramatically increased by increasing the NaCl concentration from 150 to 200 mM NaCl (Table 2).

SVI in salinity levels of 50 and 75 mM were not significantly different from the control, while it decreased at 200 mM to 63.26% and 65.67% compared to the control, and 50 mM salinity levels, respectively (Table 2).

Mean comparisons showed that there were significant differences between seedlings' length and weight means at a higher than 75 mM salinity level compared to the control (Table 2). Our results showed that NaCl concentration caused more inhibition in shoot growth than in root growth (Fig. 3). Therefore, shoot/root dry weight ratio decreased by increasing NaCl levels.

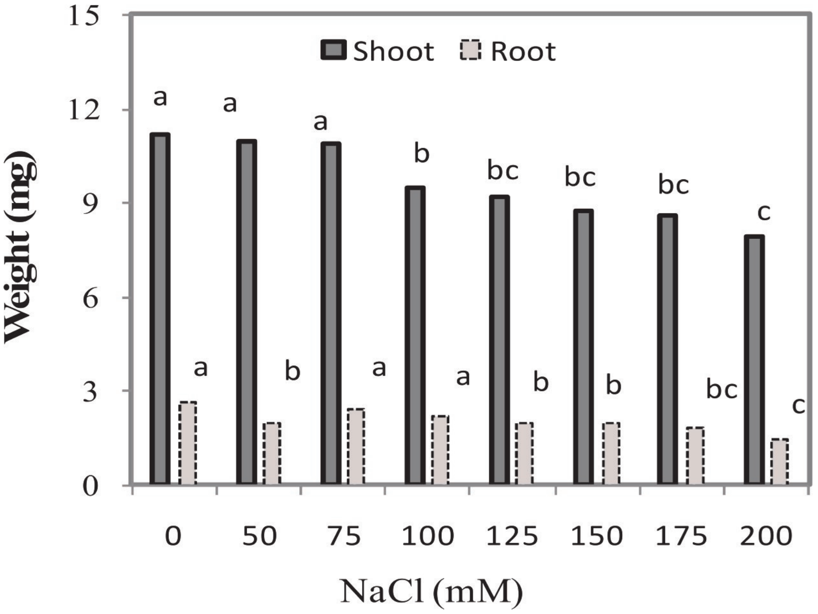

Fig.2. Cumulative germination percentage against time after experiment onset, in different NaCl concentrations (mM)

A slight increase (0.01 hour per 1 ds × m^{-1}, R^2= 0.74) was observed in MGT by increasing NaCl concentration (Fig. 4a), so that MGT was 1.38 and 1.7 times more than the control at 150 mM and at 200 mM, respectively (Table 2). SVI also had a negative slope of -0.025 and R^2= 0.82 when regressed against NaCl concentrations and a drastic reduction appeared at 200mM compared to the control (Fig. 4b).

A three-parameter logistic model was fitted seed germination percentage against seedling weight of milk thistle data and salinity levels (Fig. 5a-b). According to the model, the salinity response threshold of seed germination and seedling stage in milk thistle were estimated as 101.2 mM and 43.6 mM, respectively (Table 4, Fig. 5a-b).

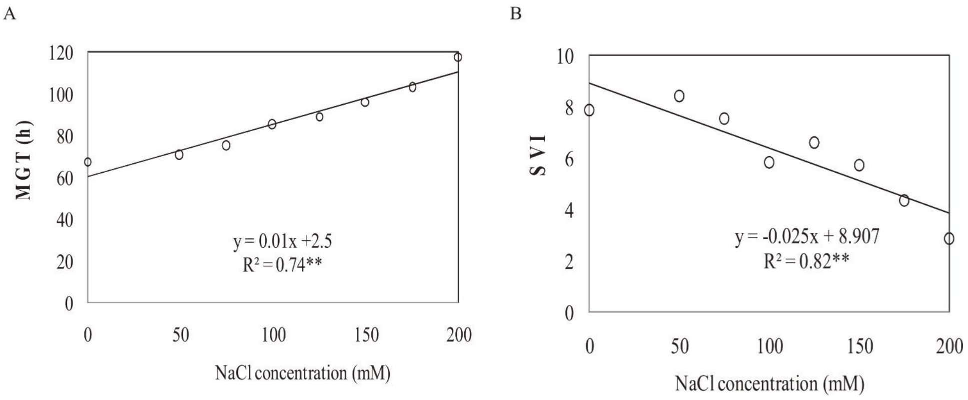

Fig.2. Cumulative germination percentage against time after experiment onset, in different NaCl concentrations (mM)

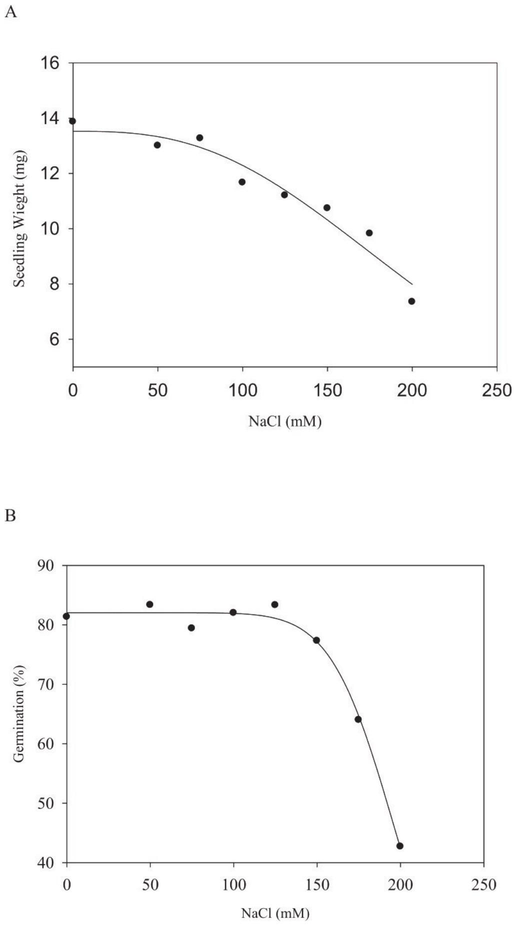

Fig.2. Cumulative germination percentage against time after experiment onset, in different NaCl concentrations (mM)

Curve slope and STI values for the seedling weight were obtained as 0.24 and 237.38 respectively and 0.54 and 231.9 for the seed germination percentage respectively (Table 4).

DISCUSSION

Germination percentage was significantly decreased by salt stress in milk thistle. Seed germination remained relatively unaffected by salinity up to 125 mM NaCl. Castroluna *et al.* (2014) evaluated some *Medicago* varieties' responses to salinity and reported that the germination percentage of Vedor variety between the control (70%) and 50 mM of NaCl or in moderately salinity (77%) showed no significant differences. This result supported our observations and was confirmed in our results (Table 2).

In a research was reported that the lowest concentration of NaCl did not significantly affected radish seed germination (Kaymak *et al.,* 2009). Furthermore, Ghavami and Ramin (2007) stated that seed germination was adversely affected under saline conditions. High concentrations of NaCl may have increased osmotic potential, meaning the seeds would have been unable to imbibe the water required for germination.

NaCl concentration effect on germination percentage up to 150 mM was not significantly different from the control, while, germination time was delayed with increasing NaCl concentrations, which obviously occurred at higher concentrations than 50 mM (Fig. 2). Although the germination percentage of seeds exposed to 50, 100 and 125 mM were close to seed germination in the control (81.33%), germination in the control reached a maximum value after a MGT of 2.82 d, while MGT for 50, 100 and 125 mM was delayed by 0.11, 0.72 and 0.9 d, respectively, compared to the control (Table 2). These observations suggest that 50, 75, 100 and 125 mM NaCl did not have a severe impact on the maximum seed germination percentage of milk thistle, while salt stress severity delayed germination time (MGT) (Fig. 4a). Fricke *et al.* (2006) reported that salt stress caused reduced cell turgor and depressed rates of root and leaf elongation, suggesting that NaCl concentrations acted primarily on water uptake. Furthermore, salt and osmotic stresses are responsible for both inhi-bition or delayed seed germination and seedling establish-ment (Almansouri *et al.*, 2001). Therefore, seed germination time is affected by the severity of salinity stress.

Rajakumar (2013) showed that salt stress effect on the seed germination percentage of *Oryza sativa* up to 50 mM NaCl was the same to that of germination in the control, while a reduction in shoot and root length was observed at this salinity level. Kaya *et al.* (2003) reported that root growth in *Carthamus tinctorius* L. was more sensitive and adversely affected compared to shoot growth under saline conditions, while our results in the case of *S. marianum* showed that shoot growth was more sensitive than root growth. This was the case particularly for those levels above75 mM NaCl (Fig. 3a). According to Greenway and Munns (1980), after some time in 200 mM NaCl, a salt-tolerant species such as sugar beet might have a reduction of 20% in dry weight, whereas a moderately tolerant species such as cotton might have a reduction of 60% in dry weight.

Our results showed that dry weight had a reduction of 53% in 200 mM NaCl in relation to the control (Table 2); thus, this plant could be suggested as a species moderately tolerant to saline conditions.

Afzali *et al.* (2009) reported no significant difference in the root and shoot dry weights between the control and 80 mM NaCl treatments in chamomile; however, the adverse effect of NaCl was more pronounced for root biomass compared to shoot biomass. Rapid root extension enabled *Trigonella* seedlings to exploit moisture in dry habitats (Ramoliya *et al.,* 2004), which is a mechanism for coping with salt stress. Thus, shoots were found to be more sensitive to salinity than roots. Similar to these observations, it has previously been reported that soil salinity suppresses shoot growth more than root growth (Ramoliya *et al.,* 2004).

The highest significant differences were related to control and 150 to 200 mM concentrations (Table 2). It is possible that salt solution provided entry of the ions to the seeds, which may have been toxic to the embryo or the developing seedlings (Almodares *et al.,* 2007). The inhibitory effect of NaCl on growth parameters could be attributed to the osmotic effect of NaCl (Salter *et al.*, 2007).

The results of the present study revealed that salt stress inhibited seedling growth (root and shoot length, root and shoot dry weight); however, seedling weight was influenced more than seedling length. The same results regarding the reduction of seedling growth were reported by Jeannette *et al.* (2002) and Shannon *et al.* (2000). Mer *et al.* (2000) observed that by increasing salinity, plumule length decreased in wheat, barley, pea and cabbage. The researchers noted that the decrease in growth of young seedlings as a result of increasing salinity occur mostly because of a decrease in water absorption by radicle and by accumulation of salts solution in the cell. Additionally, in our study, we found that roots were less affected by salinity compared to shoots. This could suggest an advantage for milk thistle, in that their roots could create a more tolerant plant against water deficits occurring as a result of salinity stress.

It has been reported that the optimal growth of halophytic plants takes place in low salt concentrations (Esechie *et al.*, 2002), while high salinity levels will cause a distinct decrease in plant growth. At present study seedling dry weight at the highest NaCl concentration (200 mM) decreased 32.44% when compared to the control. Afzali *et al.* (2009) reported that the reduction in shoot dry weight in *Matricaria chamomilla* at the highest NaCl concentration (190 mM) was about 32% when compared to the control.

While seed germination after 100 mM NaCl continued and was even more than the control at 125 mM (83%), SVI showed that it was 1.28 times lower than the control at 125 mM NaCl, and this index reduced at 200 mM NaCl to 2.91 times lower than the control. These results suggested that milk thistle seeds were able to germinate when exposed to fairly high salinity levels, but were not able to produce a healthy and normal seedling.

Linear regression was implemented to establish the relationship between salt stress and some traits in the seedling growth stage. Mavi *et al.* (2010) found a significant correlation between MGT and rate of emergence. They also reported significant correlations between the mean germination time (MGT) of cucurbit seeds in the laboratory and mean emergence time (MET) and final emergence in the field. These results are in agreement with Rahman *et al.* (2008), who reported that ascending salt concentrations not only prevented the germination of seeds, but also extended germination time by delaying the starting of germination. Salt stress weakened the germination percentage and also prolonged the emergence of seeds in four vegetables species*: Beta vulgaris, Brassica oleracea, Amaranthus paniculatus* and *Brassica campestris* (Jamil *et al.,* 2006). Jeannette *et al.* (2002) reported that a faster rate of germination allowed the emerging seedlings to accumulate more biomass relative to the control.

Results suggest that milk thistle has different behaviour in the germination stage relative to the seedling stage when is exposed to NaCl. The results confirmed that milk thistle was more tolerant in the seedling stage due to a lower curve slop and higher STI. Seydi (2003) stated that the salt tolerance index is considered a reliable criterion for salt tolerance. Plant responses to salt and drought are closely related and the mechanisms of their reactions overlap (Zhu 2011). Since salt stress is usually associated with drought stress, an approach for establishing a suitable time selection for integrated irrigation in water limited areas should be considered.

CONCLUSION

By increasing NaCl concentration, milk thistle germination was prolonged and seed germination percentage decreased. Additionally, root and shoot length and weight were reduced by enhancing salt severity. Our results showed that when salinity varied from 50 to 150 mM, germination (%), seedling length and weight reduced, MGT increased (Table 2). The findings suggest that seed germination continued even in the highest salinity concentration levels; however, a drastic decrease in SVI and an increase in MGT will lead to a weak emerging seedling.

The effect of salt concentration on shoot weight was higher than on root weight. In other words, milk thistle roots were able to restore and grow under moderate salinity conditions such as those shown in this experiment, so that these changes may reflect an adaptive advantage for the plant in terms of acquiring limited resources. Overall the results indicated that S*ilybum marianum* as a valuable medicinal plant could be considered as a moderately tolerant to salinity during seedling growth. Recognizing this may help breeders to produce a plant with better adaption abilities.

Understanding the ways in which plants can tolerate salt stress conditions is necessary for growing and developing plants on marginal agricultural lands that are unproductive.

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