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Original Research Paper

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EFFECT OF THE ENVIRONMENTAL ORIGIN OF FOUR CLOVER SPECIES ON GERMINATION IN SALINE CONDITIONS

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ABSTRACT Climate change will significantly undermine crop production, posing a serious threat to food security. Indeed, climatic factors are very favorable to the rise of salts, the concentration of the soil solution and the precipitation of salts in the root zone and the superficial horizon leading to salinization, which limits the rate of germination seeds. Pastoral plants are not escaped this problem. Effective adaptation in this sector will be of crucial importance. The present study deals with the tolerance of four clover plants, in Moroccan areas, subject to an increase in soil salinity during the last years. The effects of 4 levels of salinity, 0, 70, 140 and 220 mM NaCl, on four *Trifolium* species was conducted at germination. We also tested the effects of salts on Na^+ and K^+ accumulation during germination. An interesting result is the ability of *T. isthmocarpum* to germinate on 220 mM of NaCl despite being a glycophyte species. In addition, seeds from *T. isthmocarpum* showed, unlike other species tested, a high ability to recover their germination after NaCl exposure when stress conditions are alleviated. This indicates that *T. isthmocarpum* developed the mechanisms of salt tolerance in seeds to improve adaptation of this species to high salinity.

KEYWORDS : Germination, Environment, Trifolium, Salinity, Tolerance

INTRODUCTION

Salinity is one of the most important problems affecting many soils of the world. Wong et al. (2010) estimated that salinity affects approximately 950 million ha of land globally. Salinity in the soil is the most significant abiotic factor limiting plant productivity, particularly in arid and semi-arid climate (Acosta-Motos *et al.,* 2017). The high salt concentration negatively affects vegetation growth primarily due to the difficulty for plant roots to absorb water which is referred as the osmotic stress, and secondly due to limited availability of essential nutrients which is referred as the ionic stress (Munns and Tester 2008). Beside these biochemical impairments, salinity adversely affects various and physiological processes such as seed germination, seedling growth, flowering and fruiting (Singh *et al.,* 2015). According to Parihar et al. (2004), these damages occur directly due to the negative impact of salinity on photosynthesis, respiration, nutrient assimilation, hormonal imbalance, etc. It's well established that plants respond to salinity stress by adopting several changes in morphology, anatomy, water relations, photosynthesis, the hormonal profile, toxic ions distribution and biochemical adaptation and in part by modulating gene expression, which ultimately leads to the restoration of cellular homeostasis, detoxification of toxins and recovery of growth (Acosta-Motos *et al.,* 2017; Ashraf and Harris, 2004). Seed germination is one of the most fundamental and vital phases in the growth cycle of a plant in arid and semiarid regions (Hadas, 2004). However, this critical stage is negatively influenced by saline conditions (Nasri et al., 2015), which has been reported to reduce or prevent germination but also extend the germination time by delaying the start of germination (Ibrahim, 2016). Salinity affects seed germination through osmotic effects, ion toxicity or a combination of the two (Zhang et al., 2010). *Trifolium* is one of the most important forage legumes among the genera of the Fabaceae family, both in terms of its agricultural value and the number of species (Sabudak and Guler, 2009). Several species of *Trifolium* are cultivated in intensive agricultural systems in association with companion grass species in simple or complex seeds mixtures (Lamont et al., 2001).In addition, *Trifolium* species are a potent natural source used in traditional medicine to treat a variety of disorders (Kolodziejczyk -Czepas, 2016). In Morocco, temperature, rainfall, pH, and phosphorus content were the most determinant factors affecting the distribution of *Trifolium* species (Bennani et al., 2010; 2011; Beale et al., 1993).

The aim of our study was to examine the effects of salinity on seed germination of four *Trifolium* species in different levels of salinity (0, 70, 140 and 220 mM NaCl). The study focused in particular on: 1) the effect of NaCl on seed germination and recovery at different NaCl concentrations; 2) the inter-specific variability in seed germination and in salt stress tolerance; 3) the comparison of salt solution with the osmotic agent Mannitol to differentiate the osmotic from the ion toxicity effects; 4) the effect of salinity on hard and soft seeds ion content.

MATERIALS AND METHODS

1. Plant material

Four *Trifolium* species were studied based on the relative salt sensitivities: *T. campestre, T. glomeratum, T. isthmocarpum, T. tomentosum*. They were collected in Morocco from four different environments and origins subject to soil salinity at Sidi Hajjaj, Sebt Brikiyine two plain located in the South-West of Rabat, the country's capital city, Had-Harrara is a coastal plain located in some 276 km South-West of Rabat and Sidi-Allal Bahraoui is a coastal plain situated slightly to the north of Rabat (Table 1).

Table 1: Geographical coordinates, geology, and climate characteristics of *Trifolium* ecotypes used in the experiment.

Region	Altitude (m)	Latitude	Longitude	T °C Min-Max	Soil pH	Annual Rainfall (mm)	Topography	Sol Type	Grazing Intensity	Bioclimatic stage
Sidi Hajjaj	628	32,92	7,29	6,3 - 39,8	8,8	242	Plain	Sandy loam	Important	Semi-arid with warm winter
Sebt Brikiyine	447	32,29	8,05	4,3 - 40,4	7,9	200	Plain	Sandy	Nul	Aridwith warm winter
Had-Harrara (Oualidia)	108	32,48	9,11	7,9 - 39,2	8,3	294	Coastal plain	Sandy	Moderate	Semi-arid

Sidi-Allal Bahraoui	146	34,04	6,69	4,8 - 29,9	6	399	Coastal plain	Very sandy	Important	Semi-arid with winter temperate

2. Germination

The germination experiments were conducted using three replicates of 80 seeds by each species (20 seeds by ecotype by dish) for each treatment. Seeds were placed in a sterile Petri dishes with double filter paper in distilled water (control) or salt stress conditions (70, 140 and 220 mM NaCl) with three replicates. Twenty seeds of each ecotype were scarified and then disinfected by successive baths. Firstly, the seeds were put in a solution of sodium hypochlorite at 15% for 20 minutes, followed by washing with sterile distilled water abundantly, rinsed with Alcohol 90° for one minute then transferred into sterile Petri dishes with double filter paper in distilled water. The Petri dishes were incubated at 27 °C and with humidity from 78% to 93% in darkness and examined every 24 hours for 7 days to monitor seed germination. The germination was determinate by the emergence of the radicle from the seed coat (Panuccio et al., 2014). The effect of NaCl on germination performance was estimated by measuring different parameters.

a) Final Germination Percent (FGP):

It's the percentage of seeds sown that germinated calculated by: **FGP = Ng / Nt × 100**

Where:

Ng: number of germinated seeds,

Nt: total number of sown seeds.

Final germination percentages were calculated as the mean of three replicates (±ESD) from four ecotypes.

b) Mean daily germination (MDG):

This is an index of daily germination speed and calculated by:

MDG= FGP/d

Where:

FGP: Final Germination Percent,

d: test period

c) Recovery of germination:

Recovery of germination upon transfer of seeds from NaCl solution to pure water attributes the initial repression of germination and its subsequent recovery to osmotic factors. After 7 days, ungerminated seeds from the high concentrations of salt treatments (140 mM and 220 mM) were rinsed three times in distilled water and transferred in Petri dishes with two discs of filter paper saturated with distilled water to study the recovery of germination for 20 days. The recovery percentages (RP) was determined by: **RP = [(ab}(cb)]100**

Where:

a is the total number of seeds germinated after being transferred to distilled water,

b is the total number of seeds germinated in saline solution,

c is the total number of seeds.

d) Toxicity effects

In order to differentiate the osmotic from the toxic effects of ionic elements, we used an osmotic agent (Mannitol). If NaCl has only osmotic effects, it should affect the seeds in the same manner and to the same extent as Mannitol. If not, the effect of NaCl on germination can be explained by the toxic effects of ionic elements. The treatments consisted of putting 20 seeds of the ecotype of each species (showing little or no germination on a very saline medium 220 mM NaCl) on a medium supplemented with 10 ml of: (a) 0 mM NaCl; (B) 220 mM NaCl and (c) 380 mM Mannitol (osmotic potential similar to 220 mM NaCl). Each treatment also contains a 5 mM CaSO4 base. The incubation lasted 20 days.

e) Ion analysis

To determine how the seed coat protects the seed from the toxicity of saline ions, seed responses to increasing concentrations of NaCl with respect to their Na^+ and K^+ ion contents were examined for hard (impermeable) and soft (permeable) seeds. For this reason, no prior treatment was carried out on the seeds used in this experiment, in particular, scarification. The objective is to evaluate the Na^+ and K^+ content in hard and non-hard seeds for each species, and thus test the behavior of the seed via the penetration of saline ions. Knowing that the seeds considered hard were those that did not swell after incubation (they do not compress when tapped slightly with the aid of a forceps), unlike the soaked ones that have swollen after germination in the solution. 80 seeds of each species were incubated for 20 days in 0 mM, 140 mM or 220 mM NaCl solutions, which also contains a 5 mM $CaSO_4$ base. The seeds were removed from Petri dishes, washed 3 times for 30 seconds with iso-osmotic solutions of Mannitol and dried. The oven-dried samples were crushed with mortar. Then, the ions were extracted into 10 ml of 0.5 M HNO3 with stirring for 48 h at 20-25°C. Na^+ and K^+ were determined in dilutions of the extracts using a flame photometer (PFP7, Jenway Essex).

3. Statistical analysis

Data presented as a mean and standard error of the mean (SEM)were statistically analyzed using multi -variance and the significant differences were identified by post hoc Tukey's Honestly Significant Difference (HSD) test at $p<0.05$ using Statistical Package for Social Sciences (SPSS).

RESULTS

1. Final Germination Percentage (FGP)

In general, seeds of all tested species germinated well at control conditions (Table 2), but they showed lower FGPs under all NaCl concentrations (70, 140 and 220 mM NaCl) compared to the control ($p<0.0001$), except for *T. isthmocarpum* at 70 mM NaCl ($p=0.39$). The effect of high level of salinity (140mM and 220mM) on seed germination revealed a considerable interspecific variation. In fact, seed germination was significantly inhibited under these highest levels of salinity for all species; however, *T. isthmocarpum* showed a high and significant FGP compared to the three others plants (Table 2). In addition, the maximum percentage of germination was obtained from the second day for *T. glomeratum* and *T. tomentosum* and from the third day for *T. campestre* and *T. isthmocarpum* under controlled conditions.

Table 2: Effect of treatment with different concentrations of (NaCl) on the germination of seeds for the four *Trifolium* species

	Germination in percentage (%)			
NaCl	*T. campestre*	*T. glomeratum*	*T. isthmocarpum*	*T. tomentosum*
0mM	92,33 ± 2,52a	93,25 ± 1,90a	93,33 ± 1,88a	89,08 ± 2,73a
70mM	70,83 ± 4,95a	77,16 ± 2,36ab	84,81 ± 1,75b§	43,69 ± 0,59c
140mM	42,89 ± 2,81ac	50,95 ± 6,27c	69,24 ± 6,45b	28,23 ± 0,72a
220mM	10,39 ± 2,39a	16,90 ± 2,55a	36,33 ± 2,96b	16,70 ± 0,31a

Values are means ± SEMof three replicates with 20 seeds per replicate from 4 different ecotypes.Values followed by the same small letters in a row are not significantly different (P≤0.05 according to ANOVA, Tukey test). Values marked with (§) indicate a non-significant difference compared to the control (NaCl 0 mM level).

However, increasing salt concentration decreases the germination percentage and increases germination time for all species (Figure 1).

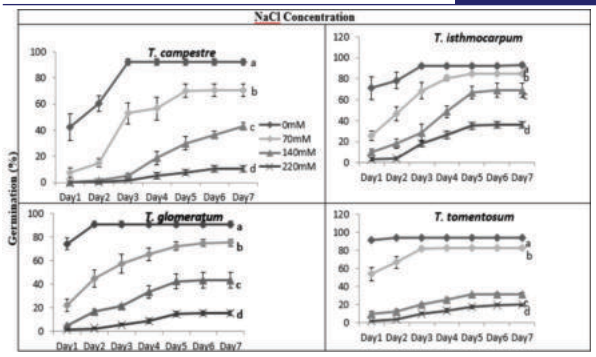

Figure 1: Effect of different NaCl concentrations on seed germination percentage (Mean ± ESM) during 7 days. Values with the same letter at day 7 are not significantly different at P>0.05

2. Mean daily germination (MDG):

For all seed types, significant differences were observed among NaCl treated groups and the control, in terms of mean daily germination (MDG) ($p<0.0001$) (Table 3). The results showed that by increasing the salinity level, (MDG) decreased compared to the control. By contrast, in high salinity (140mM and 220mM), *T. isthmocarpum* had significantly the highest MDG compared to the three others species.

Table 3: Effect of treatment with different concentrations of(NaCl) on Mean daily germination (%)of four *Trifolium* species

	Mean daily germination (MDG)			
NaCl	*T. campestre*	*T. glomeratum*	*T. isthmocarpum*	*T. tomentosum*
0mM	34,91 ± 2,76a	54,19 ± 8,02ac	69,57 ± 8,44bc	78,75 ± 5,67c
70mM	15,84 ± 1,15a	19,95 ± 2,53ac	24,17 ± 1,66bc	23,58 ± 2,17c
140mM	7,37 ± 0,52a	10,76 ± 1,27b	15,46 ± 0,88c	6,84 ± 0,42a
220mM	1,67 ± 0,36a	2,69 ± 0,41ac	7,97 ± 0,53b	3,77 ± 0,48c

Values followed by the same small letters in a row are not significantly different (P≤ 0.05 according to ANOVA, Tukey test). Values are means ± SEM of three replicates.

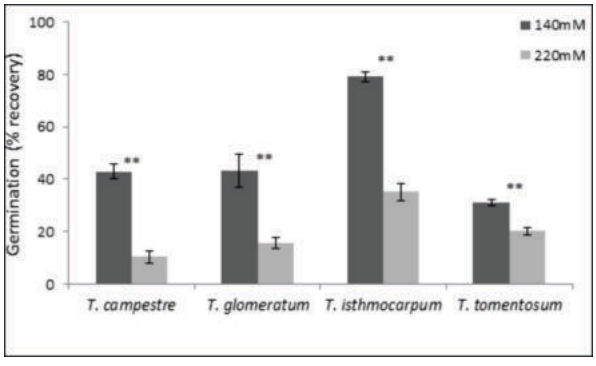

Figure 2: Percentage of recovery germination of *T. campestre, T. glomeratum, T. isthmocarpum, and T. tomentosum* seeds, when osmotic stress due to different concentrations of NaCl (140 mM and 220mM) is alleviated (**P < 0.001).

3. Recovery of germination:

Figure 2 indicated an increase in the germination of seeds of all species during recovery at 140mM NaCl, followed by a significant decrease when the seeds of all the species were treated with 220mM NaCl ($p<0.001$).The germination recovery of seeds of *T. isthmocarpum* treated with 140mM and 220mM NaCl was significantly more important than the germination recovery of *T. campestre*, *T. glomeratum*, and *T. tomentosum* seeds according to ANOVA, Tukey test ($p<0.001$). In fact, seeds of those species did not show a good ability to recover their germination after salt exposure at 140mM and 220mM, and only a maximum of 43.4% and 20.10% respectively germinated after washing with distilled water compared to 79.21% and 35.08% for *T. isthmocarpum*.

4. Toxicity

In the Figure 3, the comparison of salt solution (220 mM NaCl) with the osmotic agent (Mannitol) to differentiate the osmotic from the ion toxicity effects, show that responses of germination to treatments with 220 mM NaCl and Mannitol were not similar, showing higher germination percentages for Mannitol statistically significant ($p<0.001$). In addition, by comparison to control treatment, statistically significant differences for 220mM NaCl and Mannitol for all species have been reported.

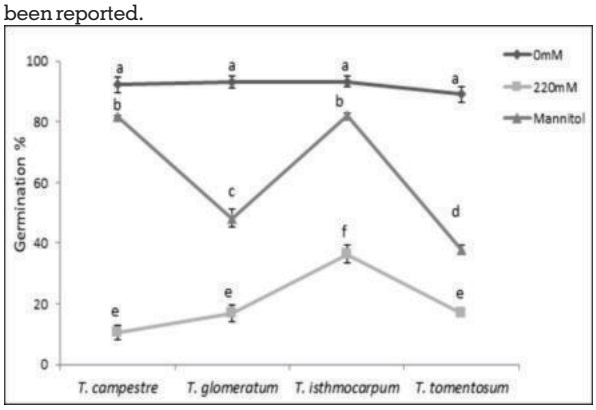

Figure 3: Percentage germination (Mean ± SEM) for the four species seeds exposed to 220Mm NaCl and Mannitol compared to control 0mM. Means not sharing common letters (a, b, c, d, e, or f) are significantly different (p≤0.05) as assessed by ANOVA, Tukey test.

5. Ion content

In this section, the Na^+ and K^+ ion content is evaluated for soft (permeable) and hard (impermeable) seeds.

a) Potassium K^+ (hard seeds)

Potassium content was found to decrease significantly with salinity for *T. tomentosum* and *T. glomeratum*. By contrast, K^+ content was observed to increase significantly as salinity increase in *T. isthmocarpum*. Interestingly, 0, 140 and 120 mM NaCl determined no significant difference in average seed K^+ content for *T. campestre* (Table 4).

Table 4: Effect of different NaCl salt levels on ion concentrations K^+ and Na^+ of hard seeds dry matter(µmol/g d m) 20 days after the initiation of the treatments.

Ion	*NaCl*	*T. campestre*	*T. glomeratum*	*T. isthmocarpum*	*T. tomentosum*
K+	0mM	241,75 ± 13,42a	266,25 ± 3,89a	239,75 ± 1,30a	263,75 ± 5,98a
	140mM	239,75 ± 4,49a	212,50 ± 7,33b	253,50 ± 2,35b	202,25 ± 8,67b
	220mM	211 ± 7,67a	183,50 ± 5,63c	257,75 ± 2,21b	199 ± 14,51b
Na+	0mM	74,25 ± 8,98a	59 ± 4,72b	19,50 ± 0,81d	52,25 ± 4,79f
	140mM	51 ± 8,47a	67,25 ± 4,80bc	20,50 ± 2,47de	48,75 ± 9,15f
	220mM	55 ± 7,83a	77,75 ± 5,87c	35,50 ± 7,15e	51 ± 10,1f

For each ion, means ± ESM followed by the same letter in each column are not significantly different according to ANOVA, Tukey test.

b) Sodium Na^+ (hard seeds)

For *T. glomeratum* and *T. isthmocarpum*, sodium content was observed to increase slightly as salinity increase. Sodium levels were significantly higher at the highest salt concentration 220mM than at 140 mM NaCl by comparaison to the control (0mM NaCl). However, no significant changes were observed for *T. campestre* and *T. tomentosum* (Table 4).

c) Potassium K^+ (soft seeds)

Table 5 indicates that K^+ content was reduced significantly from 0 through 140 to 220mM NaCl for *T. campestre, T. glomeratum* and *T. tomentosum*. However, despite increasing NaCl levels, *T. isthmocarpum* shows the absence of significant differences between mean K^+ concentrations.

d) Sodium Na^+ (soft seeds)

Na^+ content was observed to increase significantly with NaCl concentration in/for all the studied species (Table 5). Na^+ concentration in soft seeds increased markedly with increasing NaCl concentration in each species. By contrast, Na^+ concentrations of hard seeds in saline solution were much lower than in soft seeds.

Table 5: Effect of different NaCl salt levels on ion concentrations K^+ and Na^+ of soft seedsdry matter(µmol/g d m) 20 days after the initiation of the treatments.

Ion	*NaCl*	*T. campestre*	*T. glomeratum*	*T. isthmocarpum*	*T. tomentosum*
K+	0mM	146,50 ± 2,97a	151,25 ± 3,64a	127,25 ± 2,39a	146,75 ± 6,60a
	140mM	117,50 ± 1,79b	97,50 ± 7,28b	136,75 ± 3,25a	98,00 ± 11,67b
	220mM	83,50 ± 1,25c	68,00 ± 5,49c	133,50 ± 6,57a	91,50 ± 16,05b
Na+	0mM	45,75 ± 2,64a	54,75 ± 4,06a	30,50 ± 1,66a	52,75 ± 3,45a
	140mM	535,75 ± 19,55b	525,50 ± 21,87b	448 ± 15,73b	498 ± 13,68b
	220mM	790 ± 19,20c	965,16 ± 62,82c	724,75 ± 18,44c	829,25 ± 37,96c

For each ion, means ± ESM followed by the same letter in each column are not significantly different according to ANOVA, Tukey test.

DISCUSSION

Soil salinity is known to reduce growth and development through osmotic stress, ion toxicity, mineral deficiencies and induced physiological and biochemical disorders in metabolic processes (Hasegawa et al., 2000). A high germination rate is required to avoid the adverse effect of salt stress at early growth stages. In this study, we evaluated salt tolerance of four *Trifolium* species (*T. campestre, T. glomeratum, T. tomentosum* and *T. isthmocarpum*) in the presence of different salt concentrations at seed germination. The effect of salinity was highly significant on seed germination of three tested species *T. campestre, T. glomeratum, T. tomentosum*; the strongest reduction of germination (more than 83%) was observed in the presence of 220 mM NaCl compared to the control. It has been suggested that the decrease in the germination rate with the increase in the salt concentration corresponds to an increase in the external osmotic pressure, which affects the absorption of the water by the seeds and / or an accumulation of the Na^+ and Cl^- ions in the embryo. This toxic effect can altered the metabolic processes of germination and in extreme cases to the death of the embryo by excess of ions (Panuccio et al., 2014). However, *T. isthmocarpum* conserved the highest germination rate compared to the others three species, which suggest an important salinity tolerance. The decrease in MDG of all the species with the NaCl concentration are explained by the time necessary for the seed to set up mechanisms allowing it to adjust Its internal osmotic pressure. Indeed, the highest MDG occurred at high salinity for *T. isthmocarpum* compared to the others three species. The seed transfer experiments on 0 mM of NaCl after pretreatment with 140 mM and 220 mM of NaCl are made to specify the mode of action of the salt on germination. For all the species studied, after transfer of the seeds that failed to germinate under high concentrations of salt to distilled water, they recovered relatively their aptitude of germination. Most importantly, seeds of *T. isthmocarpum* show a good ability to recover their germination after salt exposure compared to the other three species. The results suggested that the effects are initially osmotic, due to the recovery of germination once the salt stress has been removed. This may have ecological significance within highly saline environments for seedling establishment, reflecting a physiological adaptation of *T. isthmocarpum* in particular, if there is insufficient rain to leach salts from the soil. Nevertheless, toxicity due to the accumulation of Na^+ ions have also been demonstrated, as evidenced by the decrease in germinative capacity of the studied species, compared to their controls (0 mM NaCl), even after the return to the environment without salt. The recovery of germination after transfer to fresh water after a period exposure of high salinity was reported more for seeds of halophyte plants than for annual legumes (glycophyte plants) (Khan and Ungar, 2001; Hanslin And Eggen, 2005; Flowers and Colmer, 2008), hence the significance of our results. Our data clearly demonstrated that most probably ion toxicity is more detrimental to seeds compared with the osmotic component of salt stress, as evidenced by different effects of NaCl (220 mM) and Mannitol (380 mM) treatments. In addition, the increase in Na+ in soft seeds with increasing salinity indicates that none of the four species are able to prevent Na^+ from entering seeds. By contrast, the significant increase in Na^+ concentrations with salinity for *T. glomeratum* and *T. isthmocarpum* hard seeds indicated that the two-species may have little control over the uptake and translocation of the salt ion, due to the osmotic adjustment to NaCl salinity, largely and especially conferred by Na+ (Panuccio et al., 2014). The observations that the NaCl *T. isthmocarpum* seeds were able to germinate to higher percentages than the other species seeds, and that they take both salt and water up rapidly and to levels determined by the external salt concentration, are consistent with our hypothesis that sodium was absorbed by seeds, facilitating water uptake and allowing germination under osmotic conditions which would otherwise prevent germination. Using salt as an osmoticum in saline environments appears to allow seeds to germinate more rapidly, and at lower osmotic potentials than they might otherwise be able to tolerate. This may have functional ecological effects, increasing ability to germinate (zhang, 2010) Interestingly, K^+ content was observed to increase significantly as salinity increase in *T. isthmocarpum* hard seeds this result can be explained in the light of previous studies suggesting that maintenance of high K+ concentrations in salt-tolerant species may be one of the mechanisms underlying their superior salt tolerance (Tester and Davenport, 2003). However, the marked decline in K+ with increasing NaCl concentrations for *T. campestre, T. glomeratum* and *T. tomentosum* hard and soft seeds could also indicate some damage to cell membranes and leakage of solutes (Nichols et al., 2009).

The present study suggests that the *T. isthmocarpum* has a good ability to germinate at higher salt concentrations as reported by our previous study (Bennani and Al Faiz, 2014) . Results suggest that seeds take salt up, causing inhibition of germination at subtoxic concentrations; however, when returned to pure water, this extra salt may be able to increase germination rates. This high salinity tolerance of *T. isthmocarpum*, during germination, may be explained by the existence of a significant gradient in the accumulation of potentially toxic (Na^+) and non -toxic essential (K^+) elements in seeds. However, further work is required to determine the

adaptive mechanisms of this trait. In fact, salt tolerance conferred by different genes (Amin et al., 2016 ; Kurotani et al. , 201 5). The genotypes of *T. isthmocarpum* may have been subjected to the effect of genetic selection if we taken into consideration the environmental origin of the seeds collection. A recent review suggested that traits that are hypothesized to contribute to salinity tolerance (e.g. including ion exclusion, osmotic tolerance and tissue tolerance) are more genetically tractable and genes underlying these can be discovered using molecular genetics tools and genomics (Roy et al., 2014). Some studies have shown the existence of gene alleles encoding key effectors of salt tolerance (Ahmad et al., 2017). For example, the vacuolar Na^+ /H^+ antiporter gene AtNHX1 is not as highly inducible in Arabidopsis as its homologous gene is in halophytes, and high level AtNHX1 expression driven by the strong CaMV 35S promoter could significantly improve Arabidopsis salt tolerance (Xiong and Zhu, 2002). This work provided support for further studies to examine both Na^+ and K^+ elements to understand the salt damage and tolerance in *Trifolium* species. Molecular genetics is needed to detect whether the species has developed a gene that allows tolerance after contact for years with saline soil, especially for *T. isthmocarpum* species.

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