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ORIGINAL ARTICLE

Trifolium isthmocarpum Brot, a salt-tolerant wild leguminous forage crop in salt-affected soils

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Plant scientists are investigating the potential of previously unexploited legume species where environmental and biological stresses constrain the use of more conventional forage crops or where these species are better suited to the needs of sustainable agriculture. *Trifolium isthmocarpum* Brot., Moroccan clover, occurs as a weed in different habitats in Morocco. It grows in moderately saline areas, where traditional forage legumes cannot be cultivated; however, it has not been widely studied despite its good palatability. The salt tolerance was studied between natural field conditions and glasshouse. The extensive field studies have recorded the species in many different habitats ranging from healthy agricultural lands to abandoned saline areas. The plants maintained high nodulation capacity (ranging between 60% and 97%) and nitrogenase activities (average 2.04 µmol C_2H_4 $plant^{-1}$ h^{-1}) in different habitats. Shoot systems of plants collected from salt-affected soils exhibited higher concentrations of Na^+ and Cl^- than those collected from healthy soils. Greenhouse experiments showed that germination percentage and vigor value of the studied species was not significantly ($P > 0.05$) affected at 160 mM NaCl, and that 25% of the germination ability was maintained when growing on substrats containing 240 mM NaCl. The growth rate of seedlings was not signicantly affected by 160 mM NaCl but was reduced by 38% under 240 mM NaCl. Leaf succulence and indices of leaf water status did not differ among the salt treatments, whereas relative water content was reduced by only 8% and water content at saturation increased by about 12% at high salt concentrations in the growing medium. This study suggest recommending the cultivation of *T. isthmocarpum* in salt-affected soils, which are widespread and pose a problem for the farmers of Morocco and other countries in the world's arid belt.

Key words: NaCl stress, Pasture crop, Salt-affected land, Trifolium isthmocarpum Brot, Wild Legumes

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Key words: NaCl stress, Pasture crop, Salt-affected land, Trifolium isthmocarpum Brot, Wild Legumes

Salinity stress is a major abiotic stresses that limit plant growth, especially in arid and semi-arid regions (Ashraf and Harris, 2004) and this is intractable problems facing farm managers in the world. Approximately 20% of agricultural land and 50% of cropland in the world is salt stressed (FAO,

2008; Flowers and Yeo, 1995). Identifying adapted and productive species for saline areas is a high research priority in many parts of the world, with a major focus of this recent research directed to forage legume species (Rogers *et al*., 2005). Plants of Mediterranean origin are adapted to dry conditions and some display tolerance of soil salinity (Caballero and Cid, 1993). However, an understanding of the range of salinity that various legumes can tolerate is central to their use in active programs for revegetation of saline lands (Behdani *et al*., 2008). Exploration of new wild plant species tolerant to saline conditions has recently become a global issue.

Morocco becomes seriously affected by secondary salinity; 33% of its cultivated land, which comprises 3% of the total land area, is already salinized (Hsissou *et al*., 1999). Salt-affected lands are less productive and protable, particularly if valuable salt-sensitive crops cannot be grown, and soil reclamation is costly. One of the alternative approaches is the use of native species with economic and ecological relevance.

Legumes are a key component of sustainable agriculture and can offer many economic and environmental benefits if grown more widely in crop rotations, because of their ability to fix nitrogen in root nodules in a symbiotic interaction with soil rhizobia. Several environmental conditions limit the growth and activity of nitrogen- fixing legumes. Salinity is the major factor, which threatens legume agriculture in arid and semi-arid climates. However there are plants that grow under saline conditions, and historically these have been opportunistically used as fodder for grazing livestock or as components of mixed rations to replace roughage (Carberry *et al*., 2010; Hameed and Ashraf, 2008). The selection of such economic plants, with appropriate management, could result in the rehabilitation and re-vegetation of salt-affected lands, which cannot be cultivated with traditional crops.

The growth of salt affected plants is often limited by the ability of roots to extract water from the soil and transport it to shoot due to osmotic changes within the plant (Rodríguez *et al*., 1997). Also, the quantity of water moving from the root to the shoot and its speed determine the concentration of substances reaching the shoot (Markhart and Smit, 1990). Salt stress imposes two constraints: a hyperosmotic effect due to lower soil water potential and a hyperionic effect due to direct toxicity of ions over metabolism and nutrition of plants (Hasegawa *et al*., 2000). Important mechanisms that allow plants to cope with salt stress are Na^+ exclusion and/or Na^+ compartmentalisation (Blumwald *et al*., 2000). According to the two-phase growth model, growth reduction during salinity arises by the progressive accumulation of toxic ions in the aerial part of a plant (Munns, 2002). As a consequence, the hyperionic cue is responsible for varietals differences in salt tolerance, which are evident after long-term salinization (Munns, 1993). The present study was focused on salt tolerance of annual legume. While perennial forage species have the advantage of an extended growing period they must tolerate soil salinity concentrations which peak in summer and autumn in a Mediterranean environment and which are avoided by annuals. *Trifolium* is a leguminous genus which includes 237 species, annual and biennial types (Zohary et Heller, 1984), characterized by high seed yields, nitrogen fixation rates superior to other legumes, and value in crop rotations. Generally, *Trifolium* species are recognised as being salt-sensitive (Maas and

Hoffman, 1977). However, research undertaken on a limited number of species (Rogers *et al*., 1997; Gibberd *et al*., 2001; Nichols *et al*., 2008) suggest that species do vary in their response and that further research may be beneficial in identifying species that are suited to mild or moderate saline. *T. isthmocarpum* is one of these clovers that don't received large researches. *T. isthmocarpum* is grown in moderately saline areas where traditional forage legumes cannot be grown (Dear *et al*., 2003). It is a wild herb, very common in different habitats in Morocco. Several authors mentioned *T. isthmocarpum* in their studies (generally in laboratory) (Chen and Gibson, 1971; Pederson and Windham, 1989; Rogers and West, 1993; Ennabili *et al*., 1996; Badr *et al*., 2002; Dear *et al*., 2003) although not further. Rogers and West (1993) noted the superior tolerance of *Trifolium isthmocarpum* Brot. (Moroccan clover) compared to *T. subterraneum* L. and *T. purpureum* Loisel., but no studies have detailed the mechanisms and physiological response of *T. isthmocarpum* to salinity. It is clear, however, that salt tolerance may differ between laboratory or glasshouse and natural field conditions, owing to the complex interaction of a number of edaphic and climatic factors. The present work investigates the performance of *T. isthmocarpum* under both field and laboratory conditions to evaluate its potential for use as a fodder crop in salt-affected soil in Morocco.

MATERIALS AND METHODS

Fields studies

Plant samples

Three different habitats were chosen. Healthy arable soil (including barley fields in coastal land), salt affected soils and newly reclaimed land. For the vegetation surveys a simplified method describing species presence was performed for the three different habitats during the period December 2010–May 2011. Fifty homogeneous stands (10×10 m^2) were selected, 20 in healthy soils, 20 in salt-affected soil, and 10 in newly reclaimed lands. Specimens were identified using the local standard floras (Fennane *et al*., 1999). Rainfall data were estimated from the National Weather Service.

Nodulation status and nitrogenase activity

The plants collected from each of the three different habitats were analysed to determine nodulation percentage and to enumerate nodule number. The nitrogen- fixing activity (nitrogenase activity) of the legume–Rhizobium symbiosis was determined according to the methods described by Witty and Minchin (1988).

Chemical analysis

Samples of oven-dried plant shoots were ground, 0.1 g was weighed into a 10 ml vial, 10 ml of 0.5 M HNO_3 added, and samples placed on a shaker at 20 °C for 2 days. The extract was used to determine the concentrations of K^+ and Na^+ using a flame photometer (Jenway Ltd, model PFP7; Essex, UK). The concentration of Cl^- was measured following titrametric method (Begum *et al*., 1992). Values were calibrated using a reference plant tissue sample with known concentrations Na^+, K^+ and Cl^-. For each habitat, five soil samples were collected from profiles of 0–25 cm depth. These five were then pooled together to form one composite sample, air dried, and thoroughly mixed. Textures were determined by the hydrometer method, providing quantitative data on the percentage of sand, silt, and clay. The concentrations of soil minerals Na^+, K^+, Fe^{3+}, Ca^{2+}, and Mg^{2+} were determined using a Perkin 403

atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, CT, USA) according to the Analytical Methods for Atomic Absorption Spectrophotometry (1983). Cl^- was quantified following titrametric method (Begum *et al*., 1992). Sulphates were determined spectrophotometrically using a JENWAY 6300 spectrophotometer (Jenway, Dunmow, UK) using barium chloride and hydrochloric acid (5 ml water soil extract + 5ml 1N HCl+0.5 g $BaCl_2$) and the absorbances read at 606 nm; pH and conductivity of the soil samples were determined in saturated soil paste extract by pH and conductivity meters, respectively, and carbonates and bicarbonates by titrating 5 ml of the 1.5 soil/distilled water extract against 0.01 N HCl using phenolphthalein and methyl orange as indicators (Jackson, 1962). Protein content of plants collected was determined following the method of Lowry *et al*. (1951), using bovine serum albumin as a protein standard.

Greenhouse experiments

Seed germination

The seeds of *T. isthmocarpum* were collected from salt-affected land (EC = 6.1 dS/m, determined in saturated soil paste extract by conductivity measurement), at Dar Bouazza N 33° 29' 34.66" W 7° 48' 59.98" (Casablanca province). The seeds were sown in sterilized Petri dishes on a double layer of filter paper moistened with 5 ml of the treatment solution. The treatment solutions for salinity tests were 0 (control), 80, 160, and 240 mM NaCl. Three replicates of 20 seeds were used in each treatment. The dishes were placed in the dark in an incubator set at 25 °C and humidity variation from 78 to 93%. Seeds were considered to be germinated after the radicle reached 1 cm. The germination rate is expressed as the ratio of number of germinated seeds on the total number of seeds (TG = (n / N) x 100) where n: number of germinated seeds, N: total number of seeds placed in germination. Germination speed (vigor value) was calculated using the following formula (Bradbeer, 1988): V = (a/1+b/2+c/3+d/4 +…+x/n) 100/S, where a, b, c,…x, respectively, represent the number of seeds that germinated after 1, 2, 3,…n days of incubation, and S is the total number of germinated seeds. The petri dishes were examined every 24 hours to follow seed germination.

Ion toxicity and osmotic effects on germination

The objective of this part was to determine sensitivity to NaCl at germination was attributable to osmotic effects or ion toxicity. Treatments consisted of 10 ml of: (a) 0 mM NaCl; (b) 240 mM NaCl; (c) 480 mM NaCl; (d) 440 mM mannitol (osmotic potential similar to 240 mM NaCl); and (e) 880 mM mannitol (osmotic potential similar to 480 mM NaCl) for 21 days. Three replicates of 20 seeds were used in each treatment.

Growth conditions

Seedlings were transplanted in the greenhouse into pots (20 cm diameter), containing a sterilized substrate of black peat, sand and a clay–loam soil (1:1:1). They watered by the mineral nutrient solution twice a week. The mineral solution contained the following, in mmoles/liter: $CaCl_2$ $2H_2O$, 1.0; $MgSO_4$ $7H_2O$, KNO_3 1.65; K_2SO_4, 0.50, and NaH_2 PO_4 $2H_2O$, 0.65; and micronutrients (in micromoles/liter): $FeSO_4$ $7H_2O$, 27.0; $MnCl_2$ $4H_2O$, 1.13; $CuSO_4$ $5H_2O$, 0.08; $ZnSO_4$ $7H_2O$, 0.19; $NaMoO_4$ $2H_2O$, 0.05, and H_3BO_3, 5.77. The pH of the nutrient solution was adjusted to 7. Pots were arranged in a split-plot design using one control and three saline treatments and three replicates. The environmental conditions during the experiment were 26±5°C

(day) and 14±2°C (night), and the relative humidity ranged between 60% and 70%. The average maximum photosynthetically active radiation (PAR) was 820 µmol m^{-2} s^{-1}. The treatment solutions of NaCl (0, 80, 160, and 240 mM) were applied 2 weeks after transplanting for 4 weeks. We kept only 8 plants per pot to make an average.The NaCl treatments were imposed in increments of 80 mM/day until full treatments had been reached. During the experimental period the average electrical conductivities of the irrigation solutions were 1.05, 6.85 and 11.9 dS m-1 respectively. The pots were flushed thoroughly with distilled water once a week to avoid salt accumulation in the root zone. As the plants grew in size, the volume of liquid was increased and the differences between salt concentrations were kept constant. The irrigation water was supplied in quantities to maintain the electrical conductivity of the drainage water at about ±10% EC of the irrigation water supplied for each treatment.

Growth measurement and nodulation

At the end of experiment, plants were gently washed with deionized water to remove soil from roots. They were divided into shoots (stems and leaves) and roots and fresh weights were measured. The nodulation percentage and the nodule number were determined. The shoots and roots were oven dried at 70 °C for 24 h and the dry weights were determined. The nitrogen- fixing activity (nitrogenase activity) of the legume–Rhizobium symbiosis was determined according to the methods described by Witty and Minchin (1988).

Chemical analysis

Na^+, K^+ and Cl^- were analysed at the end of the experimental period in the shoots of plants. The concentration of Cl^- was measured following titrametric method (Begum *et al*., 1992). The concentrations of K^+ and Na^+ were determined using a flame photometer (Jenway Ltd, model PFP7; Essex, UK). Values were calibrated using a reference plant tissue sample with known concentrations Na^+, K^+ and Cl^-.

Plant water relations measurement

Five mature leaves per treatment for each replicate were sampled at the end of experiment. In these leaves, the following parameters were examined: composite leaf area (LA), using a LI-2000 area meter (LICOR Biosciences, Lincoln, NE), fresh mass (FM), fresh mass at full turgor (TM), measured after immersion of leaf petioles in distilled water for 48 h in the dark, and dry mass (DM), measured after oven drying at 70 °C for 24h to constant weight. Additionally, the specific leaf area (SLA), (the ratio of LA to DM of individual leaves), and leaf tissue density (D = (DM/FM) ×1000 (Inoue *et al*., 1993) were calculated, as were relative water content, (RWC = (FM-DM)/ (TM-DM) ×100), succulence (S = (FM-DM)/LA), water content at saturation (WCS = (TM- DM)/DM), and water saturation deficit (WSD = (TM-FM)/ (TM-DM) × 100).

Final germination rate after seed harvest

Three plants per treatment were followed until fruiting stage. The seeds were harvested from control and each salt treatment. 20 of them were sown in sterilized Petri dishes on a double layer of filter paper moistened with distilled water. The final germination rate is calculated as described above. This parameter is measured to determine the ability of the plant to reproduce after culture under saline conditions.

Statistical analysis

The data were analysed using the Statistical Analyses System (SAS, Inc., Cary, NC) software. Significant differences between treatment means were determined using LSD test at the 0.05 probability level. ANOVA was used to identify overall significant differences.

RESULTS

Fields studies

Habitat characteristics and floristic composition

Table 1 shows variations in soil physicochemical properties of the investigated *T. isthmocarpum* habitats, which ranged from sandy to clay loamy soils, and from healthy (EC = 0.66 dS/m) to salt-affected soils (EC = 6.1 dS/m). Salt-affected soil showed higher concentrations of Na^+, Cl^-, Mg^{2+} et $SO4^{2-}$ than healthy soils by 6, 2, 1.5, and 1.4 fold respectively. Floristic analysis (Table 2) showed the highest incidence of the studied species in healthy soil, followed by salt-affected soil, while the least was recorded at the newly reclaimed land. The species most found on salt-affected soils were *Lolium temulentum* L. et *Melilotus siculus* Turra. The rainfall varied between 560 mm and 390 mm in average.

Nodulation, nitrogenase activity, and inorganic and organic shoot content

The studied plants showed high nodulation percentages (ranging between 60% and 97%) and nitrogenase activities (average 2.04 µmol C_2H_4 $plant^{-1}$ h^{-1}) at three different habitats (Table 3). The highest values of protein content was recorded in plants collected from salt-affected soils and the lowest in plants collected from newly reclaimed land. Shoot systems of plants collected from salt-affected soils exhibited higher concentrations of Na^+ and Cl^- than those collected from healthy soils by more than twofold, and showed a reduction in K^+ content of about 30% (Table 3).

Germination, growth and nodulation

Germination percentage and vigor value of the studied species was not significantly (P > 0.05) affected at 80 or 160 mM NaCl, whereas, 27% germination ability was maintained at 240 mM NaCl (Fig. 1). Shoot dry weight was not significantly affected at 80 and 160 mM NaCl, but a 38% reduction at 240 mM NaCl was recorded (Fig. 2). Root dry weight showed a slight increase at 80 mM NaCl compared to control treatment, however it decreased significantly (P<0.001) by 7% at 160 and 10% at 240 mM NaCl (Fig.2). At different NaCl concentrations, nodulation percentages ranged between 70% and 98%; and the average of nitrogenase activities was 2.55 µmol C_2H_4 $plant^{-1}$ h^{-1} (Table 4).

Ion toxicity and osmotic effects on germination

The calculated osmotic potentials for each solution were: 0 mM NaCl (0 MPa), 240 mM NaCl (−1.3 MPa), 480 mM NaCl (−2.4 MPa), 440 mM mannitol (−1.34 MPa) and 880 mM mannitol (−2.88 MPa). Germination percentage of *T. isthmocarpum* in 440 mM mannitol was higher than in 240 mM NaCl (Fig. 3), indicating possible toxic ion effects of NaCl at this concentration. However, there was almost no germination in 480 mM NaCl or in 880 mM mannitol after 21 days of incubation.

Shoot inorganic contents

Under salt stress, the Na^+ and Cl^- concentrations increased significantly (P <0.05) with increasing salinity levels compared with the control treatments (Table 4). Unlike Na^+ and Cl^-, the K^+ concentration was decreased with increasing NaCl concentration relative to the control treatments,

significantly (P <0.05) at 160 and 240 mM by 22%, and 32% respectively.

Plant water relations and final germination rate after seed harvest

All salt treatments induced a significant decrease in LA, which declined by 35%, 50%, and 58% at 80, 160, and 240 mM NaCl, respectively (Table 5). SLA showed an increase (around 15%) under salinity stress. Leaf density (D) showed a significant (P<0.05) reduction at 240 mM NaCl by 10%. No significant differences were observed among salt treatments in respect of leaf water status, and RWC exhibited 8% reduction at the higher salt concentration. WCS presented significant increase than control (12%) at high salt concentrations. Germination capacity (in distilled water) of grain from control plants was about 90%. It fell to 30% for grains formed on 240 mM NaCl.

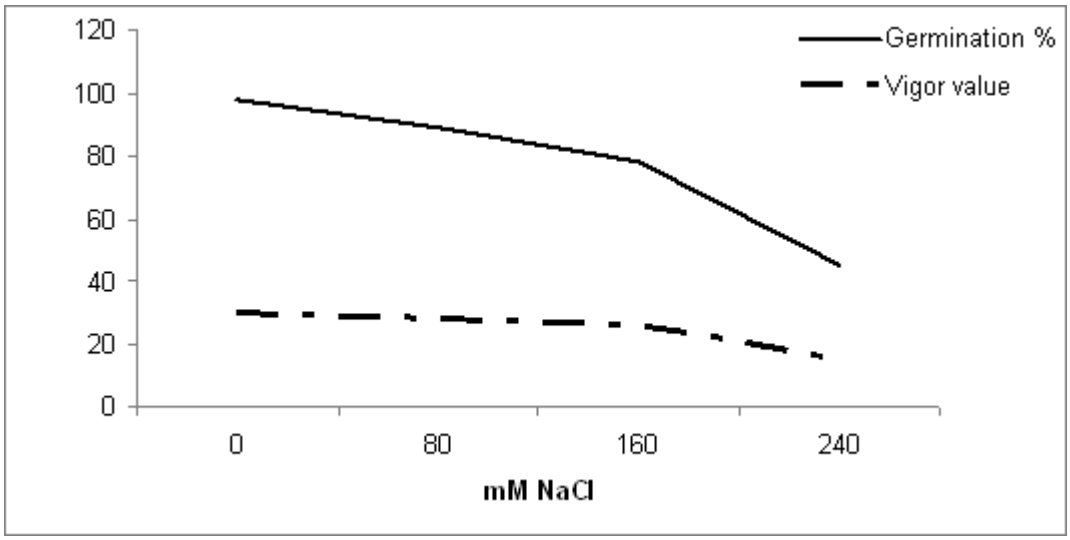

Figure 1 Effect of salinity on germination and vigor value (germination speed) of *T. isthmocarpum*

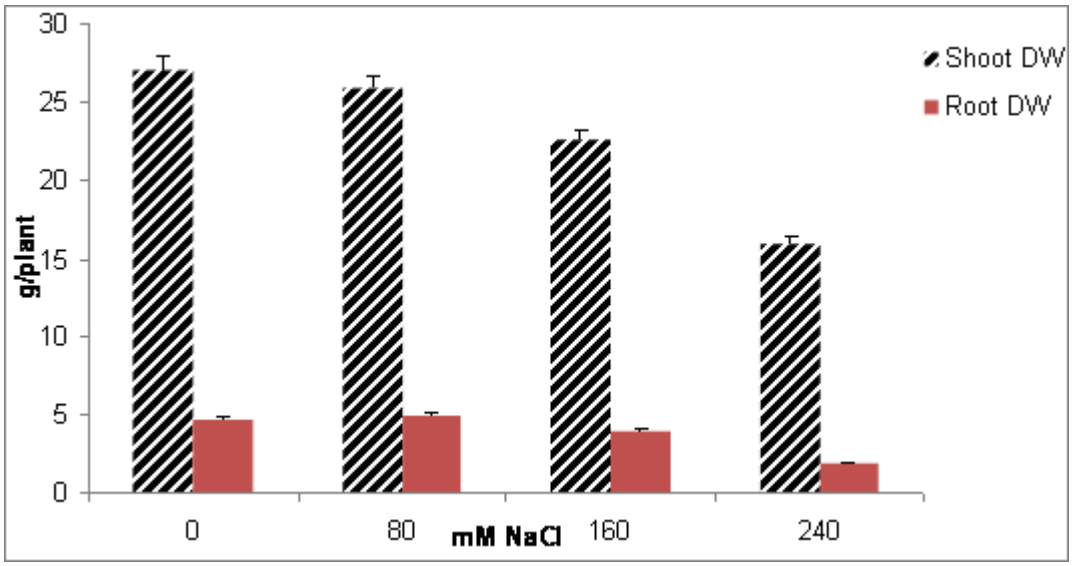

Figure 2 Effect of salinity on root and shoot dry weights of *T. isthmocarpum*

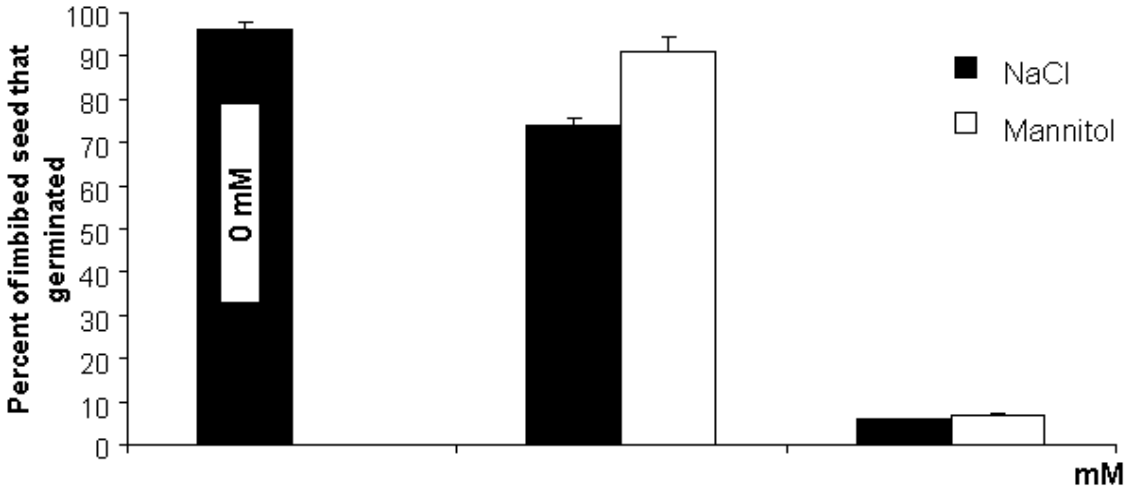

Figure 3 Germination percentage of *T. isthmocarpum* in NaCl and Mannitol concentrations after 21 days (values are the means of three replicates)

Table 1. Soil physicochemical characters of three habitats where *T. isthmocarpum* was recorded

	healthy soil	salt affected soil	Newly reclaimed land
K (mg/kg)	921±26.8	998.5±12	1.85±0.07
Na (mg/kg)	411±31,2	2476±78	9.34±0.3
Mg (mg/kg)	671±17.5	1022±24	6.9±0.5
Ca (mg/kg)	181.2±11.3	198±18	16.8±0.5
Fe (mg/kg)	26.5±2.2	57.9±1.9	40.6±1.1
SO4 (mg/kg)	2026±11.1	2999±12	179.2±17
Cl (mg/kg)	2441±22.3	4867±15.9	26.5±0.9
HCO3 (mg/kg)	262±3.7	377±5.8	28.8±3.4
EC (dS/m)	0.66±0.02	6.1±0.7	0.57±0.02
pH	7.08±0.2	7.5±0.11	7.78±0.9
Soil texture	Clay loamy	Clay loamy	Sandy
Rainfall average	560 mm	500 mm	390mm

Table 2. Percent presence of plant species (having > 10% in at least one stand) associated with *T. isthmocarpum* at the three habitats.

	Healthy soil	Salt affected soil	New reclaimed land
Trifolium isthmocarpum Brot.	35	30	11
Aegilops bicornis Forsk.	8	5	0
Anthemis arvensis L.	5	0	1
Astragalus acutirostris S.Watson	12	4	0
Avena brevis Roth.	7	10	1
Brassica juncea L.	26	16	7
Chamaerops humilis L.	3	17	0
Cytisus aeolicus Guss. ex Lindl.	6	0	2
Echium candicans L.	4	0	1
Festuca arundinacea Schreb.	0	8	8
Lavandula angustifolia Mill.	30	0	0
Lentiscus pistacia L.	16	0	2
Lolium temulentum L.	29	33	7
Lotus maritimus L.	28	26	9
Lotus ornithopodioides L.	32	12	1
Lotus corniculatus L.	41	22	17
Medicago sativa L.	38	25	19
Medicago truncatula Gaertn.	20	14	7
Melilotus siculus Turra.	36	35	12
Olea brachiata L.	8	0	1
Ononis mitissima L.	4	0	2
Ononis natrix L.	4	0	0
Onopordum acanthium (Brot.) Vasc.	7	10	3
Ormenis praecox (Link.) Briq.)	7	0	0
Pistacia lentiscus L.	18	14	7
Plantago ovata Forssk.	8	0	7
Scorpiurus sulcatus L.	7	10	1
Trifolium angustifolium L.	40	0	0
Trifolium arvense L.	19	0	1
Trifolium bocconei Savi.	3	0	0
Trifolium campestre Schreb.	16	0	2
Trifolium fragiferum L.	8	0	7
Trifolium glomeratum L.	36	3	2
Trifolium lappaceum L.	24	5	5
Trifolium repens L.	13	0	0
Trifolium scabrum L.	4	6	2

Table 3. Nodulation, nitrogenase activity, shoot ion content and protein content of *T. isthmocarpum* collected from different habitats.

	Healthy soil	Salt affected soil	New reclaimed land
Nodule plant^{-1}	69±4.2	56±1.8	45±2.6
Nodulation (%)	97±7.2	80±3.6	60±8.5
Nitrogenase activity (µmol C_2H_4 plant$^{-1}h^{-1}$)	3.1 ±0.02	1.00±0.07	2.02±0.08
Protein (g/kg dry wt.)	267±9.8	318±5.5	230±2.1
Na^+ (mmol/g DM)	1.14±0.01	2.48±0.18	0.31±0.04
Cl^-(mmol/g DM)	0.68±0.12	1.7±0.09	0.4±0.02
K^+ (mmol/g DM)	1.87±0.06	1.33±0.05	1.14±0.01

Table 4. Effect of salinity on nodulation and ions shoots content of *T. isthmocarpum*

	Salinity (NaCl) concentration				L.S.D (P< 0.05)
	0 mM	80 mM	160 mM	240 mM	
Nodule plant^{-1}	79±2.1	70±1.1	67±0.7	50±3.4	0.45
Nodulation (%)	98±0.2	91±2.4	82±0.6	70±7.2	1.34
Nitrogenase activity (µmol C_2H_4 plant$^{-1}h^{-1}$)	3.5±0.05	3.2 ±0.05	2.3±0.02	1.2±0.06	0.78
Na^+ (mmol/g DM)	0.24±0.01	1.17±0.01	2.32±0.09	3.8±0.02	2.43
Cl^-(mmol/g DM)	0.5±0.12	0.67±0.11	1.8±0.09	2.6±0.01	2.05
K^+ (mmol/g DM)	1.87±0.02	1.83±0.03	1.57±0.01	1.40±0.01	1.67

Table 5. Effect of NaCl concentration in the nutrient solution, on leaf area (LA), specific leaf area (SLA), density of the leaf tissue (D), relative water content (RWC), succulence (S), water content at saturation (WCS) and water saturation deficit (WSD) of *T. isthmocarpum* ; and final germination rate after seed harvest

	salinity (NaCl) concentration in the nutrient solution				LSD (p<0.05)
	0 mM	80 mM	160mM	240mM	
LA (cm²/leaf)	7.9	5.1	3.9	3.3	0.77
SLA (m²kg^{-1})	1018.6	1200	1170	1159	14.6
D (g kg^{-1})	90.5	84.6	82.5	81.2	12.2
RWC (%)	89.9	82.1	80.4	80.7	2.8
S (mg H_2O cm^{-2})	0.02	0.014	0.011	0.011	0.001
WCS (g H_2O g^{-1} DM)	12.87	13.11	13.96	14.7	1.1
WSD (%)	22.5	16.9	17.7	17.1	1.1
Final germination rate (%) after seed harvest on distilled water	90	66	47	30	

DISCUSSION

Fields studies

Habitat characters and floristic composition

The studied species *T. isthmocarpum* grow in a wide range of edaphic conditions which prove its adaptability and ecological amplitude. *T. isthmocarpum* was mentioned by some authors that is found in a broad range of landscapes in Morocco and is known to be tolerate in coastal areas and moderate clay saline soils (Sauvage,

1975). Average annual rainfall was important (about 500 mm) during the study on Salt-affected soil, which resulted in a good seed emergence. In fact, the precipitations are diluting the salinity of the soil. Nichols *et al*. (2008) showed that soil salinity levels near the surface in southern Australia are highest when the first germinating rains for the growing season commence. This led Nichols *et al*. (2008) to suggest the extent and timing of these rainfall events, with their effects on leaching of salts from the surface soil, are important for germination on land subject to salinity. Thus, if rainfall is sufficient to leach salts from the soil surface, germination can occur in soil with relatively low salinity and only the most salt-sensitive species will fail to establish. If, however, these rains are sufficient for germination but insufficient to leach salts, high seedling losses will occur for all but the most salt-tolerant species. *Melilotus siculus* has already been mentioned as a species tolerant to high salt concentrations (Rogers *et al*., 2008; Nichols *et al*., 2009). It was highly salt-tolerant and its biomass at 450 mM NaCl was 30% of control, moreover it was still growing after 14 days in stagnant seawater with 550 mM NaCl (Teakle *et al*., 2012). *Melilotus siculus* was the most salt tolerant species, with germination in 300 mM NaCl (89±2.7%) (Nichols *et al*., 2009). The competitive ability of T. *isthmocarpum* in comparison with this plant confirms its salt tolerance.

Nodulation, nitrogenase activity, and shoot content of protein and ions

The nodulation percentage varied among individuals collected from different habitats. Giller (2001) provides a general overview of environmental constraints to nodulation and nitrogen fixation, as indicative of the importance of environmental stresses to rhizobia. This variation can be also explained by the different prevailing environmental conditions. Nodulation status depends on many factors, including the legume stage and edaphic factors, and, especially, moisture content (Graham and Vance, 2000). One of the interesting finding in this study was the important nodulation percentage and nitrogenase activity recorded in the *T. isthmocarpum* plants, which gives the species economic importance as it can be used to enhance soil fertility. The lowest nodulation percent (68%), recorded at the newly reclaimed land, is likely to be due to decreases in population levels of rhizobia in this habitat. Salinity is known to principally affect the infection process, by inhibiting root hair growth and by decreasing the number of nodules per plant, and the amount of nitrogen fixed per unit weight of nodules (De Lorenzo *et al*., 2007). Nitrogen is one of the major limiting nutrients for most crops and other plant species (Newbould, 1989), but, on the other hand, saline environments are generally deficient in nitrogen (Cechin and Fumis, 2004).

Edaphic factors have long been known to influence soil rhizobia and a number of reviews have already documented the importance of different edaphic stresses to rhizobial persistence, subsequent nodulation, and nitrogen fixation. The nitrogen- fixing ability of the studied species, especially in salt-affected areas, predestines it to re-vegetate salt-affected soils (without the need to apply any chemical nitrogen), an important process for the stabilization and reclamation of the plant growth substrate (Severin and Stal, 2008).The increased protein content of plants collected from salt-affected soils suggests a mechanism for salt tolerance. Proteins in plants grown under saline conditions may provide a storage form of nitrogen that is reutilized when stress is over, and may play

a role in osmotic adjustment (Singh *et al*., 1987). A higher content of soluble proteins has been observed in salt-tolerant than in salt-sensitive cultivars of sun flower (Ashraf and Tufail, 1995), barley (Ashraf and Harris, 2004) and finger millet (Uma *et al*., 1995). The problem of salinity on utilization of legumes species is the obligation to select species salt tolerant and the *Rhizobium* compatible and tolerant. In harsh environments (such as saline or acidic soils), rhizobia may experience nutrient deficiency, even in the presence of nutrients. Under severe stress, the requirement for certain essential elements, such as calcium (Reeves *et al.*, 1993) and phosphorus (Zahran, 1999), increases and elevated levels of these elements enhance nodulation and N2 fixation.

Greenhouse experiments

Germination and growth

The selection of species tolerant to salinity needs first to study the behavior of their seeds during germination. The high germination percentage and vigor value (germination speed) of *T. isthmocarpum* recorded under salinity treatment is a very important character from the ecological point of view. Nichols *et al*. (2008) suggested that annual pasture legumes adapted to saline environments must have high salinity tolerance as seedlings or mechanisms to avoid germination at times of high salinity. The ability to germinate and establish seedlings on saline land is particularly important for annual pasture legumes, which must repeat this process each year. Rogers and West (1993) stated that seed populations of *T. isthmocarpum* are sensitive to 60 mM. In contrast, in this study, seeds were collected from plants living in saline soils, which would be expected to exhibit salt tolerance during the germination stage, as a result of natural selection. Many studies have shown that populations collected from saline sites are more salt tolerant than populations collected from non-saline sites (Ashraf *et al*., 1994; Hameed and Ashraf, 2008; Rogers and Evans, 1996). Little inhibition in seedling growth was recorded in treatment containing up to 80 mM NaCl, but 240 mM NaCl was inhibitory to plant growth. Salinity impacts on plants by disrupting cell function, through the toxic effects of specific ions, by osmotic effects, or both (Munns 2002, 2006). Harmful effects of salinity are thought to result from low water potentials, nutrient deficiencies, ion toxicities, or a combination of these factors. Salt stress imposes two constraints: a hyperosmotic effect due to lower soil water potential and a hyperionic effect due to direct toxicity of ions over metabolism and nutrition of plants (Hasegawa *et al*., 2000). Important mechanisms that allow plants to cope with salt stress are Na^+ exclusion and/or Na^+ compartmentalisation (Blumwald *et al*., 2000). According to the two-phase growth model, growth reduction during salinity arises by the progressive accumulation of toxic ions in the aerial part of a plant (Munns, 2002). As a consequence, the hyperionic cue is responsible for varietals differences in salt tolerance, which are evident after long-term salinization (Munns, 1993).

Nutrient deficiencies can occur in plants when high concentrations of Na^+ in the soil reduce the amounts of available K^+, Mg^{2+}, and Ca^{2+} (Al-Abdoulhadi, 2012) or when Na^+ displaces membrane-bound Ca^{2+}. In addition, Na^+ may have a direct toxic effect, such as when it interferes with the function of potassium as a cofactor in various reactions. Many of the harmful effects of Na^+, however, seem to be related to the structural and functional integrity of membranes (Hasegawa *et*

al., 2000). The present study showed that salinity reduced shoot growth more than root growth. These results agree with the generalized assumption that root growth is almost always less affected than shoot growth by salinity. Indeed, root dry weight showed a slight increase at 80 mM NaCl compared to control treatment, as a way of tolerance to salinity to seek a non saline space. Dry matter reduction due to salinity has been reported as more severe in the shoot than in the root for some cultivated legumes such as alfalfa (Esechie *et al*., 2002). However, these generalizations were based on a few case studies of short-term effects (5–14 days), and were more important in grass species. More long-term studies with a wider taxonomic base would be needed to reach general conclusions on the differential allocation of biomass to root and shoot in response to salinity.

Ion and osmotic effects

Toxic ion effects of NaCl, rather than osmotic effects, appear to be the main cause of reduced germination of *T. isthmocarpum* in 240 mM NaCl. In fact, it showed higher germination percentages in an iso-osmotic 440 mM mannitol solution. Rogers *et al*., (2009) report a similar finding for *T. michelianum*, *T. subterraneum* and *Medicago Polymorpha*, which lower germination occurred in a 300 mM NaCl treatment than in iso-osmotic solutions of 550 mM mannitol. The inability to germinate in 880 mM mannitol suggests that osmotic effects alone could inhibit germination at 480 mM NaCl.

Water relations and shoot contents of protein, Na^+, K^+, and Cl^-

The reduction in LA under saline conditions was due to reduced growth. Maintenance of RWC under salinity (decreased by only 3% at high salt concentrations) indicates the good salt tolerance of the studied species. No change in leaf succulence was observed in the present study that indicates the development of a water storage tissue. WCS showed an increase at higher salt concentration because the solute content of cells is higher in saline than non-saline conditions, due largely to the accumulation of Na^+ and Cl^-. The marked increase in Na^+ with increasing salinity indicates that studied species is not able to prevent Na^+ from entering cells. However, concentrations accumulated remains smaller (2.32 mmol/g dry mass on 160 mM) than those recorded on the same species and same salt concentration (3.35 mmol/g dry mass) (Rogers *et al*. 2009). The marked decline in K^+ at high level of NaCl concentrations could also indicate some damage to cell membranes and leakage of solutes (Munns 2002, 2005).

The increased solute content of cells in salt-treated plants causes more water to be taken up than under control conditions (Munns *et al*., 2006). Greenhouse experiments confirm the increase of protein content under salinity stress, which was found also in field-grown plants. Proteins may be synthesized in response to salt stress or may be present constitutively at low concentrations and increase when plants are exposed to salt stress (Pareek *et al*., 1997). A higher content of soluble proteins has been observed in salt-tolerant than in salt-sensitive cultivars of non-legumes such as barley (Hurkman *et al*., 1989) and sun ower (Ashraf and Tufail, 1995). Among the tolerance mechanisms, salt inclusion or salt exclusion has long been recognized in different plants in response to salinity. (Liphschitz and Waisel, 1982; Amarasinghe and Watson, 1989; Munns *et al*., 2006; Errabii *et al*., 2007). The present study supposed that *T. isthmocarpum* uses a salt

exclusion mechanism for maintaining growth under saline conditions, as it accumulated less level of Na^+ and Cl^- than other legumes species such as *T. campestre*, *T. michelianum*, *Medicago sativa* and *Lotus* creticus in the same NaCl concentrations (Rogers *et al*., 2009; Siringam *et al*., 2009).

Toxic ion effects of NaCl, rather than osmotic effects, appear to be the cause of reduced germination of *T. isthmocarpum* in 240 mM NaCl. Evidence for this comes from its higher germination percentage in an iso-osmotic 440 mM mannitol solution. Nichols (2009) reports a similar finding for *T. michelianum*, *T. subterraneum* and *Medicago polymorpha*, in which lower germination occurred in 300 mM NaCl treatment than in iso-osmotic solutions of mannitol. The inability of *T. isthmocarpum* to germinate in 880 mM mannitol suggests that osmotic effects alone could inhibit germination at 480 mM NaCl. Seeds germination capacity is strongly dependent on the salt concentration of the culture medium of mother plants, a total loss of viability occurring in seeds harvested from plants grown in the presence of 240 mM NaCl. Salinity has effects on germination and emergence, preventing the seed access to oxygen, and therefore breathing.

CONCLUSION

The high ability of the studied species to germinate, grow, and fix nitrogen under salt stress in both field and laboratory studies recommends its cultivation as a fodder crop and as a soil melioration plant on salt-affected soils.

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