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A comparison of procedures for evaluating and selecting alfalfa landrace germplasm for tolerance to salinity

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Abstract

In arid and semiarid regions, salinity may affect alfalfa (*Medicago sativa*) productivity and survival due to either cultivation on salt-affected soils or the use of salinized irrigation water. Exploiting germplasm evolved under salt-stress conditions offers opportunities for crop tolerance improvement. In the first phase of the current study, four reportedly salt-tolerant landraces originated from stress-prone areas of West Asia or North Africa and two reference commercial cultivars underwent three evaluation trials according to different methods, namely, seed germination in saline water, in vitro testing of young plants, and greenhouse evaluation of adult plants in Cone-tainers. Experimental populations obtained by intercrossing landrace genotypes selected according to each evaluation method were subsequently evaluated under stressful field conditions in two regions featuring different salt stress type, namely, southern Tunisia (irrigation with saline water) and northwestern Argentina (rainfed cropping in saline soil). Landrace ranking for salt tolerance differed somewhat depending on the evaluation method, and the proportion of selected plants per landrace depended accordingly on the method. In each field experiment, there were two evaluation phases, and the second phase corresponded in both cases to harsher conditions. The in vitro evaluation and selection resulted in potentially more useful selected germplasm than the other evaluation methods. The field experiments highlighted the large specific adaptation effects that affected the response of salt-tolerant germplasm across the two regions.

Abbreviations: ANOVA, analysis of variance; CREA, Council for Agricultural Research and Economics; DMY, dry matter yield; EC_a, apparent soil electrical conductivity; EC_e, electrical conductivity of the soil saturation extract; EC_{es}, estimated soil electrical conductivity; EC_s, electrical conductivity of the solution; IC(50), salt concentration inhibiting 50% seed germination; INTA, Instituto Nacional de Tecnologia Agropecuaria; NAAIC, North America Alfalfa Improvement Conference; PPM, plant preservative mixture; RCBD, randomized complete block design; SAR, sodium adsorption ratio; Syn 1 (seed), first-generation synthetic (seed); Syn 2 (seed), second-generation synthetic (seed).

1 | INTRODUCTION

Salinity represents a key abiotic stress over large areas in arid and semiarid regions, where the scarcity of water and the hot, dry climates frequently cause salt concentrations that hinder crop growth and production (Abo El-Enein, 1991; Bhattarai et al., 2020). The salinity of soil and water is caused by the presence of soluble salts originating from dissolving rocks and concentrated by evaporation and plant transpiration.

Alfalfa (alias lucerne, *Medicago sativa* L.) is the main perennial forage legume grown in the Mediterranean basin and other temperate-climate drought-prone regions (Annicchiarico et al., 2015). A progressive reduction of irrigation and an expansion of rainfed cropping are expected for alfalfa in different dry regions to cope with the growing demand for nonagricultural water uses and the predicted decrease of rainfall amount due to climate change (Annicchiarico et al., 2011). Nonetheless, irrigated alfalfa is frequent in dry areas to maintain stand density and ensure satisfactory yield. Due to reduced availability, raising costs, and competing demand of good-quality water, unsuitable water such as saline groundwater is increasingly used for alfalfa irrigation in several regions. Poor groundwater quality for alfalfa irrigation is a problem in vast areas of Iran (Qadir et al., 2008), Algeria (Belkhiri & Mouni, 2012; Bradaï et al., 2012), southern Tunisia (Loumerem et al., 2007), and Morocco (Farissi et al., 2011), while the salinization of surface irrigation water is affecting alfalfa production areas in Argentina and the southwestern United States (Cornacchione & Suarez, 2017). Alfalfa germplasm with a long history of growth under salt-affected conditions may have developed some kind of tolerance in various areas, representing a potentially useful donor for breeding (Farissi et al., 2011; Loumerem et al., 2008; Torabi et al., 2011). Alfalfa is rated in fact as moderately sensitive to salinity (Grieve et al., 2012) with a significant reduction of forage yield starting from a soil salt concentration corresponding to an electrical conductivity of the saturation extract (EC_e) of about 6–8 dS m^{-1} (Cornacchione & Suarez, 2015; Scasta et al., 2012).

Genetic variation for salt tolerance exists in alfalfa, of which the impact was confirmed by multi-environment trials in which the top-yielding cultivars differed across saline and nonsaline agricultural sites (Annicchiarico et al., 2011; Fan et al., 2023). This tolerance may rely on various physiological mechanisms, such as sodium and chloride exclusion or better retention of potassium in plant tissues (Benabderrahim et al., 2020; Cornacchione & Suarez, 2015, 2017; Noble et al., 1984; Smethurst et al., 2008). While indirect selection criteria based on some of these mechanisms could be envisaged, the breeding strategy for salt tolerance has essentially relied on direct selection under stress conditions. In general, the selection for crop salt tolerance based on yield performance under field conditions is difficult because of the marked spatial heterogeneity of the salinity level in fields (Pecetti et al., 1995; Richards, 1983). A great deal of the breeding work for salt tolerance in alfalfa has focused on high seed germination under stress, resulting in a range of cultivars that were selected for tolerance at the germination stage (Bhattarai et al., 2020). A seed germination test in saline solutions recommended by the North American Alfalfa Improvement Conference (NAAIC) has long been adopted as a fast screening criterion for germplasm evaluation (Rumbaugh, 1991). However, alfalfa plants may be affected by salinity at three stages, namely, seed germination, seedling growth, and mature plant growth (Smith, 1993). Inconsistencies were reported between the ability of alfalfa cultivars/populations to germinate at high NaCl concentrations and their post-germination performance under salt stress (Al-Niemi et al., 1992; Johnson et al., 1992; Steppuhn et al., 2012). Johnson et al. (1992) suggested that plants selected at salinity levels applied in germination tests may have different mechanisms of tolerance than those selected at different salinity levels during subsequent growth periods, and therefore selection methods including each critical growth stage may be required to develop alfalfa cultivars with increased forage yield in saline environments. Scasta et al. (2012) reaffirmed the difficulty in correlating different screening procedures for salt tolerance in alfalfa, with only slight consistency of cultivar ranking among tested parameters. Because of these difficulties, Peel et al. (2004) proposed a greenhouse protocol for the phenotypic screening and selection of large plant numbers at the adult stage (in the order of 4–5 months of growth). In vitro selection procedures have also been proposed. Some were based on alfalfa cell lines (Safarnejad et al., 1996; Winicov, 1991), but their application was hindered by poor in vitro regeneration. More recent ones envisaged in vitro mass selection performed on seedlings

Core Ideas

- Cultivation on salt-affected soils or the use of salinized irrigation water affects alfalfa productivity and survival.
- Landraces evolved in stress-affected areas are valuable genetic resources for salt tolerance improvement.
- Selections for salt tolerance from different methods in controlled conditions differ for field-based tolerance.
- In vitro evaluation and selection provided potentially more useful selected germplasm than other methods.
- Specific adaptation affected the field response of salt-tolerant germplasm across different regions.

(Campanelli et al., 2013; Ruta et al., 2022), providing encouraging results under in vitro testing conditions that were not verified under field conditions. In vitro screening of alfalfa seedlings has been proposed also for tolerance to other abiotic stresses, such as soil aluminum (Khu et al., 2012) or drought (Tiryaki et al., 2022).

The current, composite investigation encompassed two distinct phases. In the first one, four reportedly salt-tolerant landraces originated from salt stress-prone areas of West Asia and North Africa, and two reference commercial cultivars underwent three evaluation trials carried out at the Council for Agricultural Research and Economics (CREA), Lodi, Italy, according to different methodologies, namely, seed germination in saline water, in vitro testing of young plants, and greenhouse evaluation of adult plants as devised by Peel et al. (2004). After these screenings, three sets of selected individuals (one set for each evaluation method) were identified, and the individuals of each set were intercrossed to obtain three experimental populations that were subsequently evaluated in the second phase under stressful field conditions in two regions featuring different types of salt stress, namely, southern Tunisia (featuring irrigation with saline water) and northwestern Argentina (featuring rainfed cropping in saline soil). The objectives of our study were (a) to verify the consistency of salt tolerance of the tested germplasm across the three evaluation methods and (b) to compare the value of the three evaluation methods for salt tolerance selection in terms of actual genetic gains obtained in different salinity stress-prone cropping environments.

2 | MATERIALS AND METHODS

2.1 | Initial plant materials

The study focused on four alfalfa landraces originated in countries of West Asia or North Africa that were highlighted for their possible tolerance to salinity. The landrace Bami originated from a dry area in southeastern Iran and was reported as the most tolerant at the germination stage out of 19 Iranian landraces by Torabi et al. (2011). The landrace Tata originated from an oasis in southwestern Morocco and appeared to be the most tolerant Moroccan landrace to salinity out of those evaluated at germination and early seedling stages by Farissi et al. (2011, 2014). Various possible physiological mechanisms were indicated for its salt tolerance, such as maintenance of enzymatic activity, cell membrane stability, and accumulation of organic solutes (proline and soluble sugars) as osmolytes (Farissi et al., 2011, 2014). The landrace Tamentit originated from an oasis in southwestern Algeria and proved to be tolerant to high salinity stress at germination (Pecetti et al., 2013). The landrace Chenini originated from an oasis in southern Tunisia, and anecdotal evidence gathered by one of the authors from local farmers (S. Tlahig, personal communication, 2014) indicated that this landrace tolerates high salinity levels.

In addition to the four landraces, two reference commercial cultivars were added to the evaluation experiments as a tolerant check and an intolerant check, respectively. The former was Ameristand 801S, which was a benchmark for salt tolerance both at germination and under field conditions (Annicchiarico et al., 2011; Pecetti et al., 2013). This cultivar was obtained through several cycles of recurrent selection for germination at high salinity (https://www.naaic.org/vars2003/Ameristand801S.htm), and its tolerance was reportedly related to a sodium exclusion mechanism from leaves (Smethurst et al., 2008). The cultivar Prosementi was used as the intolerant check based on its poor performance under salt stress both at germination and in field trials (Annicchiarico et al., 2011; Pecetti et al., 2013).

2.2 | Seed germination salt tolerance evaluation

This experiment implemented the NAAIC standard test for assessing cultivar salt tolerance by seed germination under saline conditions (Rumbaugh, 1991) and was carried out at CREA, Lodi, Italy, in 2015. The test contemplates seed germination in each of eight concentrations of NaCl, namely, 0.00%, 0.50%, 0.75%, 1.00%, 1.25%, 1.50%, 1.75%, and 2.00% (w/w) in deionized water. The eight concentrations corresponded to tabulated values of 0.0, 85.6, 128.3, 171.1, 213.9, 256.7, 299.4, and 342.0 mM NaCl solutions, and to an electrical conductivity of the solution (EC_s) of 0.0, 8.3, 11.6, 15.5, 17.2, 23.1, 26.7, and 30.0 dS m^{-1}. Thirty scarified seeds per Petri plate were germinated for each landrace and reference cultivar, adding 4.5 mL of the appropriate salt solution to each plate. From now on, the term "entry" will refer to either landraces or commercial cultivars. The plates were sealed by Parafilm M and placed in the dark in a germination cabinet maintained at 25°C, and the germinated seeds were counted after 7 days. Germination was corrected for hard seeds and then adjusted by dividing the germinated proportion in each plate by the germination of the same entry in the same replication in the control (0.0% NaCl) treatment (Rumbaugh, 1991). The adjusted germination data were analyzed by Probit analysis as indicated by Rumbaugh (1991). Entry mean values were estimated for the NaCl concentration (%) required to inhibit germination of 50% of viable seeds [IC(50)]. Prior to Probit analysis, an analysis of variance (ANOVA) tested the main effects of salt concentration and entry and their interaction for germination percentage. Four completely randomized replications of all the combinations of entries and salt concentrations were available for the data analysis. The software SAS version 9.3 (SAS Institute, 2011) was used for these and all following statistical analyses.

2.3 | Adult plant salt tolerance evaluation (Cone-tainers experiment)

This experiment was carried out from June to October (summer to early fall) 2015 under an open rainout shelter at CREA, Lodi, Italy, and was largely inspired by the screening method proposed by Peel et al. (2004) with some modifications, the most relevant one being the imposition of a variable sodium adsorption ratio (SAR) in the saline solutions used for irrigation (as detailed below). Monthly average maximum and minimum temperatures recorded in Lodi during the study period, together with long-term average values, are reported in Table S1. Scarified seeds of the six alfalfa entries were sown 1.5 cm deep in 3.8 cm × 20.9 cm Ray Leach SC10 Cone-tainers (Stuewe and Sons, Inc.) filled with dried silica sand type 503 (Bacchi S.p.A.) with particle size <0.3 mm. Two seeds per cone were sown, and the emerged excess seedlings were later removed leaving one seedling per cone. The bottom opening of the cones was plugged with a 10 cm × 10 cm square of capillary matting. The 38 perimetral cones of each 98-cone flat were sown (with the cultivar Ameristand 801S) but were considered as border and not evaluated. The remaining 60 cones per flat were assigned to the six tested entries, with 10 plants per entry (in two adjacent rows of five cones each), the position of the six entries being randomized within each flat. The experiment included 32 flats representing as many replications (blocks) of the salt-tolerance experiment in a randomized complete block design (RCBD). Four additional 98-cone flats were randomized among the other 32 and were later used as the replications of a control treatment with no application of saline solutions.

Cotyledon emergence was complete in 5 days, and the first trifoliate leaves emerged 20 days after sowing. Seedlings were grown for 6 weeks before applying any experimental treatment, and in this period, a mild mist irrigation was provided daily. On day 17 after sowing, a fungicide (commercial label Previcur, Bayer CropScience [a.i. propamocarb chloridate 66.5%], at 6 mL m^{-2} in 4 L water m^{-2}) treatment was distributed diluted in the irrigation water. The following day, a fertilizer (Dünger® 70, L. Gobbi s.r.l.) was supplied solubilized in the irrigation water at 0.5 g L^{-1}. It was a powder ternary compound completely soluble in water with 10% N, 45% P_2O_5, and 10% K_2O plus microelements (B, Co, Cu, Fe, Mn, Mo, and Zn). The same fertilization was also applied once a week in the 3 following weeks.

On day 42 from sowing, the salinity application started on the 32 flats destined for the stress treatment, which lasted 10 weeks. From that moment on, the flats were submersed one after the other into the saline solution on the days of irrigation. Every week, there were two submersions, on Tuesday and Friday, using the same salt concentration for both weekly irrigations. The saline solution on Tuesday also contained the solubilized Dünger 70 fertilizer as previously described. The irrigation system was built by using two high-density polyethylene pallet bins (Pack Services). The larger bin (120 cm × 80 cm × 60 cm height) was used for the preparation of the salt (and nutrient, every Tuesday) solutions. In every irrigation day, a fresh solution was prepared by filling the bin with 300 L of tap water, into which the computed salt amount was solubilized (see below). During the irrigation phase, this bin was lifted at the higher level (1.70 m from the ground) than the second bin (80 cm × 60 cm × 51 cm height), which was kept at the ground level and acted as the dipping tank for the flats. A pipe connected the upper bin with the lower one, where the solution poured by gravity and a float valve maintained the solution level to a constant height of 21 cm, sufficient to submerge the flats up to the sand top in the cones. Every flat was submersed for 1 min. During the whole irrigation process, the solution in the dipping tank was aerated by an air pump Rambo EP-9000 (Aqua1). The four flats destined for the control treatment were irrigated with the same submersion system and the same biweekly frequency, using a tap water plus fertilizer solution on Tuesdays, and just tap water on Fridays. In both treatments, the sand remained moist between irrigation applications, and there was no sign of drought stress throughout the experiment despite the above-average summer temperatures (Table S1).

In the first week of salt stress, sodium chloride NaCl (Merck Life Science s.r.l.) and calcium chloride $CaCl_2{\cdot}2H_2O$ (VWR International PBI s.r.l.) were solubilized in water. The added amount of the two salts was computed to obtain an EC_s of 6 dS m^{-1}. For the 9 subsequent weeks, we targeted a weekly increment of 3 dS m^{-1} for the EC_s until an EC_s of 33 dS m^{-1} in the final week of the trial, corresponding to about 2.2% or 380 mM NaCl solutions. Every week, a solution sample was taken from the dipping tank contextually to the irrigation and immediately brought to a laboratory (ARAL, Crema, Italy) for the determination of the actual EC_s according to the "Rapporti ISTISAN 2007_31 ISS.BDA.022.REV00 method" (Ottaviani & Bonadonna, 2007) by an immersion conductivity meter (Mettler-Toledo) at 25°C reference temperature. The weekly average values of EC_s from the laboratory analysis (means of two weekly irrigations) are reported in Table 1 for the 10 weeks of salt application, together with the targeted EC_s value for each week and the weekly applied SAR value. Unlike Peel et al. (2004), we did not target a constant SAR value of 3.5 throughout the experiment but, instead, applied an increasing SAR along with increasing EC_s (Table 1). By this choice, we meant to mimic more closely the natural conditions occurring, for instance, in North Africa and West Asia, where SAR values between 10 and 20 are frequent (Belkhiri & Mouni, 2012; Bradaï et al., 2012; Qadir et al., 2010), and a close correlation between electrical conductivity and SAR values is observed (Aliat & Kaabeche, 2013; Saidi et al., 2004; Seilsepour & Rashidi, 2008). Based on the tabulated values reported by Seilsepour and Rashidi (2008), we built a linear

TABLE 1 Targeted and measured (average of two measurements per week) electrical conductivity of the solution (EC_s), and applied sodium adsorption ratio (SAR), of salinized irrigation solutions used to evaluate six alfalfa entries for salt tolerance during a 10-week application of salt stress at Lodi, Italy, in 2015.

Week	Targeted EC_s (dS m^{-1})	Average measured EC_s (dS m^{-1})	Applied SAR
1	6	6.94	6
2	9	9.46	8
3	12	12.69	10
4	15	15.40	12
5	18	18.23	14
6	21	19.70	16
7	24	24.15	18
8	27	25.90	20
9	30	29.05	22
10	33	31.55	24

relationship between EC_s and SAR (SAR = 0.656 [EC_s] + 2.012; $R^2 = 0.858$) that was empirically used to calculate the applied SAR values reported in Table 1. A spreadsheet kindly provided by Dr. Peel (M. D. Peel, personal communication, 2014) enabled us to estimate the amount of sodium chloride and calcium chloride needed to obtain the targeted EC_s once the desired SAR was inputted.

The last saline irrigation was provided on October 3. Three days later, the proportion of apparently surviving plants was recorded for each entry in each replication (flat) of both salt-stress and control treatments. At the same time, the aerial biomass of all plants of each entry in each flat was hand harvested, separating the green tissues from any dry (senesced) tissues. Both green and senesced portions were immediately weighed for fresh weight determination and then placed in an oven at 60°C for 4 days for the dry weight determination. The total plot dry shoot biomass was computed. Afterward, the flats were mist-irrigated once a week with tap water, and the proportion of surviving plants (based on the shoot regrowth) was recorded again for each entry in each replication 3 weeks after the final saline irrigation.

The recorded characters were subject to a combined ANOVA according to the RCBD, testing the main factors entry and salt treatment (stress and control) and their interaction. A second ANOVA compared entry mean values within the salt-stress treatment using the Newman–Keuls test. In this analysis, the two records of plant survival were analyzed as repeated measurements in time (SAS Institute, 2011), introducing the factor time and testing its interaction with entry for survival. A correlation was also computed between the average entry survival values in the two dates. In all analyses, an angular transformation of the proportion values was applied, but the original values were tabulated for ease of understanding.

The final survival proportion of the four tested landraces was the selection criterion for the subsequent breeding work that made use of the surviving plants.

2.4 | In vitro salt tolerance evaluation

The six entries were used for an in vitro evaluation and selection trial using NaCl as the selective agent. Scarified seeds (1200 per entry) underwent three successive preparatory phases: (i) immersion for 3 min in 5 mL of a 98% (v/v) sulfuric acid solution and rinsing in distilled water, (ii) sterilization for 5 min in 50 mL of a 40% (v/v) sodium hypochlorite solution and rinsing three consecutive times in sterile distilled water, and (iii) immersion for 15 min in a 60% (v/v) Plant Preservative Mixture (PPM) solution (Micropoli) followed by transfer onto sterile filter paper for drying. Afterward, the seeds were placed in Petri plates (60 seeds per plate) containing a culture medium (25 mL per plate) formed by distilled water, PPM (0.2% v/v), and microagar (7 g L^{-1}) (Duchefa Biochemie). The plates were kept at 4°C in the dark for 3 days and then transferred for 1 day under growth chamber conditions at a temperature of 24/22°C with a photoperiod of 16/8 h. After removing the seed coat, the germinated seedlings were transferred into sterile round container vessels (Micropoli) containing Murashige and Skoog (1962) medium supplemented with 30 g L^{-1} sucrose (Duchefa Biochemie), 7 g L^{-1} microagar, and one of three different concentrations of sodium chloride (0 [control], 300, and 400 mM NaCl; Duchefa Biochemie), and grown in vitro under growth chamber conditions at a temperature of 24/22°C with a photoperiod of 16/8 h as described by Confalonieri et al. (2014). Ten seedlings were placed per vessel, which represented an experimental unit. The two saline concentrations of 300 and 400 mM NaCl corresponded to tabulated values of 26.7 and 34.2 dS m^{-1} EC and 1.75% and 2.34% NaCl concentration, respectively. Two independent experiments were carried out in sequence using the same protocol. Each experiment was laid out as a six-block RCBD with two replications (vessels) per entry randomized within each block, averaging the entry values prior to statistical analyses. After 4 weeks of exposure to NaCl treatments, all the plants of each vessel in the three treatments (i.e., the control and the two NaCl concentrations) were harvested, and dry weights of shoots and roots were recorded. Dry weights were measured (as mg $plant^{-1}$) after drying separately shoots and roots in an oven at 60°C for 4 days. The plants of the four landraces (Bami, Chenini, Tamentit, and Tata) placed in each vessel of the two salt stress treatments were thoroughly observed prior to harvesting, selecting the visually most vigorous one (which was removed, rinsed, and transplanted for the subsequent selection phase, as detailed below). Shoot and root

measurements for the four landraces in the 300 and 400 mM NaCl vessels were therefore made on all but one living plants after 4 weeks of growth.

A combined ANOVA of shoot and root dry weight was performed including the random factor experiment, the fixed factors salinity level and entry, their respective two- and three-way interactions, and the random factor block within experiment. Regardless of the results of this ANOVA, for an ease of data interpretation and given the high correlation between experiments for mean values of each trait ($r \geq 0.97$), salinity level mean values of shoot and root dry weight were compared after averaging across experiments, using the Newman–Keuls test of mean comparison. In a subsequent ANOVA (with the Newman–Keuls test), entry mean values of the stress/control ratio (testing each of the two NaCl stress levels) were compared for both shoot and root dry weight. As the two experiments did not differ ($p > 0.05$) for ratio mean values in a specific ANOVA, entry ratio values (for each salt level) were averaged across experiments and used to express the salt susceptibility of the entries. This ratio represented the inverse of the following sensitivity index applied by Campanelli et al. (2013) for in vitro cultivar testing:

$$\text{Sensitivity index} = [(\text{Trait value under stress} - \text{Trait value in non-stress})/\text{Trait value in non-stress}] \times 100.$$

Scasta et al. (2012) also used a salt control ratio (thus defined) comparable to the current one to evaluate forage production in a greenhouse experiment.

Correlation coefficients were computed for each ratio between the two salinity levels.

2.5 | Selections within evaluation methods

The ultimate goal of each evaluation trial was the subsequent development of salt-tolerant alfalfa germplasm according to each evaluation procedure, using germplasm of the four landraces as a genetic base. For each procedure, we generally aimed at identifying 100 tolerant individuals (as adult plants, young plants, or germinated seeds, according to the trial) for further growth and use as parents of an experimental population. The individuals were chosen in proportion to tolerance (i.e., germination, plant survival, or growth) exhibited by each landrace in the relevant evaluation procedure.

Healthy young seedlings from seeds that germinated in the plates containing either 1.75% or 2.00% NaCl solution represented the selection target for the experimental population selected from the seed germination test. Because of the severe stress for germinating seeds represented by the applied salt concentrations, only 56 seedlings (whose proportion was consistent with the landrace germination ability under stress) could be selected across the four landraces for growth in a growth chamber in polystyrene plugtrays filled with a growing substrate (Semina BR Extra). At the beginning of the spring season, the selected plants were transplanted in plastic pots of 18 cm upper diameter and 18 cm height, filled with the growing substrate, kept in a greenhouse (shaded during summer) with regular mist irrigation and fertilization until the plants were field transplanted in late September. In the following spring, the plants were hand clipped at the first flowering (mid-May) and let regrow under an insect-proof mesh cage in a field isolator. At the onset of the second flowering (mid-June), a bumblebee (*Bombus terrestris* L.) nest (Natupol, Koppert Italia) was introduced in the isolator for pollination, and the first-generation synthetic (Syn 1) seed set was hand harvested subsequently and threshed from each plant at maturity. An equal number of seeds from each surviving and seed-producing plant were taken, scarified, and sown in late August in polystyrene plugtrays filled with the growing substrate. Six-week-old seedlings were field transplanted and, in the next season, the plants were clipped, isolated, and pollinated by bumblebees to obtain the second-generation synthetic (Syn 2) seed, following the same protocol described for the previous generation. The Syn 2 seed lot was coded as "MSI036" in the CREA-ZA germplasm collection and then made available for the following evaluation phase.

Plant selection from the Cone-tainer trial included 100 visually vigorous plants that were alive 3 weeks after the final saline irrigation (and after the aerial clipping and subsequent regrowth). The subsequent phases of plant growth in the chamber and greenhouse, and transplantation in the field were the same as those previously described for the materials selected from the germination trial, so were the Syn 1 and Syn 2 seed generation multiplications under field isolators using bumblebees for the pollination. The Syn 2 seed lot representative of this selection method was coded as "MSI038."

Plant selection from the in vitro trial included 98 plants that were visually selected after 4 weeks of growth in the round vessels with either 300 mM or 400 mM NaCl and showed acceptable growth after the subsequent transplanting in polystyrene plugtrays filled with the growing substrate kept for 4 weeks in a growth chamber with same light/dark and temperature patterns as previously described. The subsequent phases of plant growth and transplantation in the field and the production of Syn 1 and Syn 2 seed generations was the same as described for selection from the germination test. The Syn 2 seed lot thus obtained was coded as "MSI037."

2.6 | Field evaluation of selections

A common set of entries, including the aforementioned experimental populations issued from the preceding phase of the investigation (MSI036, MSI037, and MSI038), the parental

landrace Chenini, the benchmark salt-tolerant cultivar Ameristand 801S, the salt-tolerant Argentinian cultivar Kumen PV INTA, and the Australian cultivar Sardi 10 (not specifically salt tolerant: Annicchiarico et al., 2011) were evaluated in two multi-year field trials performed in one location in Argentina and one in Tunisia, as detailed below. Additional germplasm was independently evaluated in each location. The two trials had the same plot size and plant density, while other experimental and trial management items were the standard for each station and environment (Table S2).

2.7 | Argentina

The experiment was conducted at the experimental station of the National Institute of Agricultural Technology (INTA: Instituto Nacional de Tecnología Agropecuaria) in Santiago del Estero, Argentina (28°01′ S, 64°13′ W). The soil is classified as a Torriorthentic Haplustoll with silty loam texture (Table S2) with pH 7.1. The site has mesothermal and semiarid climate characterized by a long, warm, and rainy spring-summer season and severe drought in winter; the long-term average annual precipitation is 599 mm (Table S3). The total rainfall recorded during the experiment was 1169 mm, with lower annual values than the long-term average and long dry spells from May to September/October (from late autumn to spring) (Figure S1; Table S3). The average maximum and minimum monthly temperatures during the evaluation period (May 2019 to October 2021) were 28.8°C (ranging between 21.1°C in June and 34.7°C in January) and 12.5°C (ranging between 3.3°C in July and 19.7°C in January), respectively.

The experiment was established in May 2019 according to a Latinized row–column design (4 × 3) with three replications. Limited supplemental irrigation with nonsaline water (EC < 1 dS m^{-1}) was applied to ensure the plant establishment during the dry period from sowing to October 2019 and again in the dry period of 2020 (Table S2). Other trial management details are reported in Table S2.

In addition to the common set of entries between the two field evaluations, the trial in Argentina included the salt-tolerant cultivars Salado (from the United States), Salina, and Salinera INTA (from Argentina), and two further cultivars from Argentina, namely, Monarca and ProINTA Super-Monarca.

The soil salinity of each plot was assessed in March 2019, December 2020, and October 2021, exploiting this information as a covariate for data analysis. At the first observation date, a large saline area was mapped by recording the horizontal (1 m) and vertical (1.5 m depth) apparent soil electrical conductivity (EC_a) through the portable EM-38 DD instrument (Geonics Ltd.). The readings were made according to a grid, which allowed returning later to the same points to locate the plots to be sown and to be further measured for EC_a. At each date, soil electrical conductivity measurements as EC_e were made in the laboratory on selected soil samples taken in the field at 0–30, 30–60, and 60–90 cm depths based on EC_a measurements. The linear regression between EC_a and EC_e was computed for all combinations of EC_a (i.e., horizontal or vertical) and EC_e (i.e., at each of the three sampling depths or averaged across depths) to obtain the estimated electrical conductivity (EC_{es}) for each plot. For the three dates, the best linear regression ($p < 0.05$) was the one between the average EC_e across three depths and the horizontal EC_a.

Before the beginning of the experiment (March 2019), the average EC_{es} in the plots was 9.5 dS m^{-1} (ranging from 6.0 to 13.5 dS m^{-1}). However, due to subsequent salt dynamics, the EC_{es} changed over time. The average values were 27.4 dS m^{-1} (from 22.0 to 33.1 dS m^{-1}) in December 2020, and 25.8 dS m^{-1} (from 23.4 to 29.2 dS m^{-1}) at the end of the trial (October 2021), which represented a nearly threefold increment compared to the initial salinity level.

The forage biomass per plot was evaluated over 16 harvests (Table S2). At each harvest, all plot plants were cut at approximately 5-cm height from the ground, measuring the dry biomass weight after oven drying at 65°C for 48 h. Plot dry matter yield (DMY) data of each harvest were subjected to ANOVA (InfoStat, version 2020; http://www.infostat.com.ar) using a general linear model including alfalfa populations as a fixed factor, column, row, and replications as random factors, and the EC_{es} as a covariate using the values estimated from the records made in December 2020 (i.e., that relative to the intermediate timing of observation). Entry mean values were compared using the least significant difference (LSD) Fisher test ($p < 0.05$). The field experiment in Argentina went through two distinct phases. The first phase corresponded to harvests 1–8, when the provision of some supplemental irrigation and an initially lower soil salinity (as EC_{es}) determined relatively more favorable conditions compared with the second phase (harvests 9–16) when the rainfed trial did not receive any irrigation and the soil salinity (as EC_{es}) sharply increased. Because of the different conditions in the two phases, the DMY cumulated over the harvests 1–8 (phase 1) and the DMY cumulated over the harvests 9–16 (phase 2) were analyzed separately by ANOVA. An additional ANOVA including the fixed factors entry and harvest phase (1 or 2) and the random factor replication was carried out using the SAS version 9.3 (SAS Institute, 2011) to test the occurrence of entry × harvest phase interaction according to Steel and Torrie's (1980) split-plot in time statistical model.

2.8 | Tunisia

The trial was conducted at the experimental station of the Arid Regions Institute (IRA) in Elfjé, Tunisia (33°29′ N, 10°38′ E),

15 m above the sea level and about 6 km from the coast. The soil is loamy sand (Table S2) with pH 7.7 and 0.5% organic matter. The climate is xerothermomediterranean, with a long-term average annual precipitation of 209 mm, falling mainly in autumn and winter (Table S4). The total rainfall recorded from sowing (November 2019) to the last harvest (September 2022) was 275 mm, with remarkable annual variability and severe drought throughout the years 2021 and 2022 (Figure S2; Table S4). The average maximum and minimum monthly temperatures during the evaluation period were 28.7°C (ranging between 18.5°C in January and 39.3°C in August) and 17.3°C (ranging between 8.6°C in January and 26.1°C in August), respectively.

The trial was established in a well-drained soil after a 6-year fallow period according to an RCBD with four replications. Trial management information is reported in Table S2. Each block (nine plots in a 3 × 3 pattern) was managed as a basin of 36 m^2 (6 m × 6 m) bounded with small ridges (20–25 cm high) to allow surface irrigation. Plants were maintained well-watered under an oasis management, irrigating twice a week with saline water (Table S2). The irrigation water had an EC of 9.7 dS m^{-1}, SAR equal to 12, and pH 7.26, while its ion concentrations were $Na^+ = 66$ mEq L^{-1}, $Ca^{2+} = 32$ mEq L^{-1}, $Mg^{2+} = 28$ mEq L^{-1}, $SO_4{}^{2+} = 54.4$ mEq L^{-1}, $K^+ = 0.7$ mEq L^{-1}, $Cl^- = 60$ mEq L^{-1}, and $HCO_3{}^- = 6$ mEq L^{-1}. The EC was determined by a portable conductivity meter SenseLine F430 (ProSense) at 25°C reference temperature.

Together with the common set of entries, the experiment in Tunisia also included the anecdotally salt-tolerant local landrace Mareth and the non-dormant, non-salt-tolerant Australian cultivar Siriver (Annicchiarico et al., 2011).

The restrictions imposed due to the COVID-19 pandemic in 2020 and 2021 affected the trial management, and the plots could only be harvested three times in 2020 and four times in 2021, whereas seven harvests were carried out throughout 2022 (Table S2). At each harvest, plants of the whole plot were manually clipped at ~5 cm height and weighed for the determination of the forage fresh weight. A forage subsample of 300–400 g from each plot was placed in a forced-air oven at 65°C for 3 days to determine the dry matter content and then computing the plot DMY expressed in t ha^{-1}. Because of the loose harvest frequency in the first 2 years of the experiment (harvests 1–7) due to the restrictions, the DMY cumulated over the harvests 1–7 (phase 1) and the DMY cumulated over the harvests 8–14 (phase 2) were analyzed separately by ANOVA. An ANOVA including the fixed factor entry and the random factor block tested the variation among alfalfa population for DMY in each harvest phase. An additional ANOVA including the fixed factors entry and harvest phase and the random factor block aimed to test the occurrence of entry × harvest phase interaction. Finally, a combined ANOVA including the fixed factors entry and location (Santiago del Estero or Elfjé) and the random factor replication (i.e., block) within location was carried out for DMY of harvest phases 1 and 2 of the subset of seven entries that were tested in both locations to assess the occurrence of entry × location interaction. All the analyses of data from Tunisia, as well as the combined ANOVA across locations, were performed using the SAS version 9.3 (SAS Institute, 2011).

TABLE 2 NaCl concentration required to inhibit germination of 50% of seeds [IC(50)] for six alfalfa entries tested for seed germination under saline conditions at Lodi, Italy, in 2015.

Cultivar	IC(50) (% NaCl)
Ameristand 801S	1.906 a
Bami	1.518 b
Chenini	1.408 bc
Tamentit	1.566 b
Tata	1.508 b
Prosementi	1.217 c

Note: Values are the means of four completely randomized replicates. Cultivar means followed by different letters do not belong to overlapping 95% confidence intervals according to Probit analysis.

3 | RESULTS

3.1 | Seed germination salt tolerance evaluation

The significant entry main effect and entry × treatment interaction from ANOVA (data not reported), together with the results of the Probit analysis, clearly pointed to the different germination responses of the tested entries under salt stress. In particular, the computed IC(50) concentration according to Probit analysis clearly separated the tolerant cultivar Ameristand 801S (of known selection history for seed germination under salt stress) and the salt-susceptible cultivar Prosementi (Table 2). The four landraces showed an intermediate response between the two reference cultivars.

Of the selected seedlings used to produce the experimental population MSI036, 71% derived from germination with 1.75% NaCl, and 29% from germination with 2.00% NaCl solution. The proportion of selected seedlings among landraces was about 29% for Tamentit, 27% for Bami, 25% for Tata, and 19% for Chenini, reflecting the trend toward landrace differences reported in Table 2.

3.2 | Adult plant salt tolerance evaluation (Cone-tainer experiment)

The salt-stressed plant material differed widely ($p < 0.001$) from the control for shoot dry biomass (1.25 g $plot^{-1}$ vs. 4.56 g $plot^{-1}$), proportions of green over total aerial biomass and plant survival, both recorded 3 days after the final saline irrigation (55.1% vs. 100%, and 78.7% vs. 100%, respectively), and proportion of plant survival 3 weeks after the

TABLE 3 Plant survival proportion 3 days and 3 weeks after the last saline irrigation, and dry matter proportion of green (non-senescent) over total aerial biomass 3 days after the last saline irrigation, for adult plants of six alfalfa entries in a trial with irrigation by submersion in solutions with weekly increase of electrical conductivity during 10 weeks at Lodi, Italy, in 2015.

Cultivar	Plant survival proportion 3 days after last irrigation	Green biomass proportion 3 days after last irrigation	Plant survival proportion 3 weeks after last irrigation[a]
Ameristand 801S	0.78 b	0.59 abc	0.13 c
Bami	0.73 bc	0.46 d	0.10 cd
Chenini	0.93 a	0.65 a	0.28 a
Tamentit	0.92 a	0.61 ab	0.23 b
Tata	0.70 bc	0.48 cd	0.07 cd
Prosementi	0.66 c	0.51 bcd	0.04 d

Note: Values are the means of 32 randomized complete blocks. Column means followed by different letters are different at $p < 0.05$ according to the Newman–Keuls test.
[a]Based on shoot regrowth after aerial biomass clipping.

last saline irrigation (14.1% vs. 100%). Data from the control treatment indicated that there were no differences among entries in shoot dry biomass. The entry × treatment interaction was significant ($p < 0.05$) for all traits and was clearly attributable to the fact that entries lacked variation in the control treatment while being much diversified under stress. As indicators of entry response under salt stress, the values of proportions of green over total aerial biomass and plant survival at both recording dates are reported in Table 3. The ANOVA with repeated measurements of plant survival revealed a significant ($p < 0.001$) effect of the time on survival but no entry × time interaction. Despite the lack of such interaction and the high correlation between the survival 3 days and 3 weeks after the last stress imposition ($r = 0.98$, $p < 0.001$), both records are reported in Table 3 because they likely provide complementary information. The survival observed 3 weeks after the end of the stress imposition decreased remarkably (from 78.7% to 14.1% on average) as a likely effect of ion buildup in the plant cells that caused severe plant mortality (Munns, 2005). On the whole, this experiment highlighted the possible salt tolerance of the landraces Chenini and Tamentit, which had better survival than the reference cultivar Ameristand 801S, as well as modest salt tolerance for the landraces Bami and Tata, especially in terms of green tissue maintenance under stress (Table 3).

The final selection of 100 plants that produced the experimental population MSI038 reflected the performance of the four landraces according to this evaluation method. The proportion of selected surviving plants was 33% for Chenini and Tamentit, and 17% for Bami and Tata.

3.3 | In vitro salt tolerance evaluation

The ANOVA revealed significant variation between experiments ($p < 0.05$), salinity levels ($p < 0.001$), and entries ($p < 0.001$) for both shoot and root dry weight. The experiment × entry interaction was not significant for the two traits, while the experiment × salinity level interaction was significant ($p < 0.05$) for both traits, and the experiment × salinity level × entry interaction was significant ($p < 0.001$) only for shoot weight. Despite the presence of interactions, however, given the high correlation between experiments mean values for each trait ($r \geq 0.97$), salinity levels were compared on average values across experiments for an ease of data interpretation. Growth at either salt-stress concentration reduced remarkably shoot and root development compared with the control treatment, with greater reduction at the higher salinity level and a much more pronounced effect on roots (Table 4). The greater effect of salinity on root growth was highlighted by the root/shoot ratio under stress compared with that in the control treatment (Table 4).

The stress/control ratio values clearly separated the salt susceptible control cultivar Prosementi from the other germplasm for shoot dry weight at 400 mM NaCl concentration and for root dry weight at both salt concentrations (Table 5). Salt susceptibility as indicated by low stress/control ratio emerged for the landrace Tata according to shoot dry weight at 300 mM NaCl concentration. The landrace Chenini exhibited the greatest salt tolerance according to the ratio for root dry weight at 300 mM NaCl, but all landraces performed comparably for shoot or root weight at 400 mM NaCl concentration (Table 5). On the whole, our results indicated that the screening at 300 mM NaCl was not sufficient to discriminate susceptible germplasm (such as the cultivar Prosementi) based on the shoot dry weight ratio and produced ratio values not correlated with those observed at 400 mM NaCl for this trait ($r = -0.26$, NS). However, testing at 300 mM NaCl was sufficient to produce large differences for entry root weight ratio whose values were fully consistent with those obtained for 400 mM NaCl salinity level ($r = 0.91$, $p < 0.05$).

The 98 finally selected survived plants that gave rise to populations MSI037 derived for 56% and 44%, respectively, from material evaluated at 300 and 400 mM NaCl concentration.

TABLE 4 Mean values of shoot and root dry weight, percent of decrease of shoot and root weight under salt stress compared to the control, and root/shoot ratio at each salinity level, recorded across two experiments on 4-week-old plants of six alfalfa entries grown in vitro at three salinity levels at Lodi, Italy, in 2015.

Salinity level	Shoot dry weight (mg plant^{-1})	Decrease (%)	Root dry weight (mg plant^{-1})	Decrease (%)	Root/shoot ratio
Control	12.29 a		3.15 a		0.256
300 mM NaCl	10.10 b	17.8	0.99 b	68.6	0.098
400 mM NaCl	8.35 c	32.0	0.62 c	80.3	0.074

Note: Values are the means of two experiments, six randomized complete blocks, and six cultivars replicated twice in each block. Column means followed by different letters are different at $p < 0.05$ according to the Newman–Keuls test.

TABLE 5 Stress/control ratio of shoot dry weight and root dry weight of 4-week-old plants of six alfalfa entries evaluated at 300 mM NaCl and 400 mM NaCl salt stress concentration in an in vitro test at Lodi, Italy, in 2015.

	Shoot dry weight ratio		Root dry weight ratio	
Cultivar	300 mM/control	400 mM/control	300 mM/control	400 mM/control
Ameristand 801S	0.87 a	0.70 a	0.30 c	0.21 a
Bami	0.86 a	0.68 a	0.34 b	0.23 a
Chenini	0.85 a	0.69 a	0.39 a	0.23 a
Tamentit	0.80 a	0.70 a	0.29 c	0.18 a
Tata	0.72 b	0.75 a	0.34 b	0.20 a
Prosementi	0.81 a	0.55 b	0.22 d	0.14 b

Note: Values are the means of two experiments with three salinity levels [0 (Control), 300, and 400 mM NaCl], six randomized complete blocks, and two replicates of each cultivar within each block. Column means followed by different letters are different at $p < 0.05$ according to the Newman–Keuls test.

They originated for about 14% from Tata and for 28% or 29% from the other three landraces.

3.4 | Field evaluation of selections

The combined ANOVA across locations on the common set of seven entries revealed significant ($p < 0.01$) differences between locations and entry × location interaction for DMY of both harvest phases. The level of DMY was remarkably higher in Tunisia than in Argentina, as also made evident by the yield values of all the entries evaluated in the two locations (Table 6). This was a likely consequence of the oasis-type management with continuous irrigation (although with saline water) in the former site compared with the rainfed management with limited supplemental irrigation (applied only in the first evaluation phase) of the Argentinian site (Table S2). Higher temperatures throughout the years (in the presence of adequate water availability) and provision of mineral fertilization (Table S2; Figures S1 and S2) further contributed to make Tunisian conditions suitable to higher forage yield than the Argentinian ones.

The two evaluation phases of the experiment in Argentina differed significantly ($p < 0.01$) in DMY, and there was a significant ($p < 0.05$) entry × phase interaction. In the first phase, which took place under lower salt stress than the second phase according to electrical conductivity observations, the DMY variation among entries was narrow and did not reach statistical significance at $p < 0.05$. During the subsequent phase (harvests 9–16), the very limited irrigation and the estimated soil salinity (as EC_{es}) constantly exceeding 20 dS m^{-1} reduced the overall DMY and emphasized the differences in stress response among entries (Table 6). The DMY of the experimental population MSI037 was noticeable, exceeding that of salt-tolerant cultivars such as Ameristand 801S and Salina PV. Other cultivars did not differ statistically from MSI037, possibly due to an uncontrollably high residual error, but showed a clear trend of yield disadvantage.

Similar to Argentina, the two evaluation phases in Tunisia differed ($p < 0.001$), and the entry × phase interaction was significant ($p < 0.01$). In the Tunisian experiment, the entries differed ($p < 0.001$) in both phases. In the first phase (performed under loose harvest frequency), the experimental population MSI038 derived from the selection in Cone-tainers yielded as much as the two local salt-tolerant landraces Chenini and Mareth (Table 6). However, in the second phase, when the harvest regime was more intensive, the two local landraces strengthened their relative performance, while MSI037, deriving from the in vitro selection, also drew attention for its total DMY (Table 6). The salt-tolerant control cultivar Ameristand 801S was significantly ($p < 0.05$) outperformed by Chenini, Mareth, and MSI038 in the first phase and by Mareth in the second phase. The Argentinian salt-tolerant cultivar Kumen PV INTA was outperformed by

TABLE 6 Cumulated forage dry matter yield, in each of two evaluation phases in two field experiments performed from 2019 through 2022 in salt-stressed environments of Argentina and Tunisia, of three alfalfa experimental populations selected in the preliminary screening trials of the current study (MSI036, MSI037, and MSI038) and evaluated together with other commercial cultivars or landraces.

		Santiago del Estero (Argentina)		Elfjé (Tunisia)	
Population/cultivar	Origin	Phase 1[a] (t ha^{-1})	Phase 2[b] (t ha^{-1})	Phase 1[c] (t ha^{-1})	Phase 2[d] (t ha^{-1})
MSI036	Selection: at germination in saline solution	7.42 a	4.11	27.45	41.39
MSI037	Selection: in vitro in salinized medium	9.33 a	8.12 a	30.48	44.15 a
MSI038	Selection: in Cone-tainers with salinized irrigation	7.22 a	5.08	34.03 a	41.80 a
Chenini	Salt-tolerant Tunisian landrace	6.49 a	3.16	35.52 a	47.45 a
Kumen PV INTA	Salt-tolerant Argentinian cultivar	8.01 a	5.83 a	31.35	29.15
Ameristand 801S	Salt-tolerant US cultivar	7.19 a	4.45	27.75	40.55
Sardi 10	Australian cultivar	9.41 a	6.54 a	30.10	35.15
Mareth	Salt-tolerant Tunisian landrace	–	–	35.42 a	48.49 a
Siriver	Australian cultivar	–	–	31.27	27.23
Monarca	Argentinian cultivar	8.80 a	6.04 a	–	–
ProINTA SuperMonarca	Argentinian cultivar	7.95 a	5.46	–	–
Salado	Salt-tolerant US cultivar	8.45 a	6.75 a	–	–
Salina PV	Salt-tolerant Argentinian cultivar	7.30 a	3.96	–	–
Salinera INTA	Salt-tolerant Argentinian cultivar	9.07 a	6.91 a	–	–
LSD ($p < 0.05$)		2.99	2.62	3.08	7.03

Note: Values from Argentina are the means of three replicates, and those from Tunisia are the means of four replicates. For the harvest schedule and experiment management in each phase, see Table S2. Colum means followed by the letter "a" do not differ from the top-ranking mean at $p < 0.05$ according to LSD.

[a]Cumulated over harvests 1–8.

[b]Cumulated over harvests 9–16.

[c]Cumulated over harvests 1–7.

[d]Cumulated over harvests 8–14.

all selections (MSI036, MSI037, and MSI038) and by the Tunisian landraces in the second phase (Table 6).

The aforementioned entry × location interaction implied an evident cross-over effect between the landrace Chenini, which was remarkably high yielding in its area of origin (south Tunisia) and bottom ranking in Argentina, and the salt-tolerant Argentinian cultivar Kumen PV INTA, with opposite behavior in the two locations (Table 6).

4 | DISCUSSION

Our results confirmed that alfalfa breeding for improved salt tolerance is not precluded by a lack of genetic variation for this trait, in agreement with several earlier studies (Al-Khatib et al., 1994; Cornacchione & Suarez, 2017; Fan et al., 2023; Noble et al., 1984; Smethurst et al., 2008). Developing salt-tolerant alfalfa by exploiting germplasm that evolved under saline conditions is a major avenue for crop salt tolerance improvement given the existing variation in salt tolerance among landraces collected in salt-prone regions (Farissi et al., 2011; Loumerem et al., 2008; Torabi et al., 2011). Our breeding work was based on the assumption of possible variation for salt tolerance among and within landraces that evolved in stress-affected regions. The mean differences among the four evaluated landraces in the three screening trials supported the existence of partly different mechanisms of adaptation to salinity among them, which resounded in the selection pressure applied in each screening trial. The relative importance of different existing salt tolerance mechanisms (Smethurst et al., 2008) may vary depending on the evaluation method and the growth stage of the plant. In fact, establishment, survival, and biomass yield under stress may be considered distinct but interconnected facets of alfalfa tolerance to salinity (Scasta et al., 2012). Because of possible differences in landrace salt tolerance responses across evaluation methods, different proportions of selected plants per landrace depending on the method were advanced to selection. We did not directly assess the variation for salt tolerance within alfalfa landraces but relied on it by picking the best genotypes within landrace in each screening trial. Alfalfa landraces reportedly exhibited sizeable within-population variation for tolerance to another important abiotic stress such as drought (Annicchiarico, 2007).

The possible advantage of salt-tolerance selection performed under controlled conditions to alleviate the difficulty

of screening for salt tolerance in the field (Bhattarai et al., 2020; Flowers, 2004) was another driver of our breeding work. Breeding for salt tolerance in alfalfa has largely relied on high seed germination under stress, as pointed out by Bhattarai et al. (2020), who reported that almost 80% of the registered cultivars with improved tolerance to salinity in the United States were selected for salinity tolerance at the germination stage. Our screening (Table 2) was based on the standardized test described by Rumbaugh (1991). The responses to germination of Ameristand 801S, Tamentit, and Prosementi were consistent with those in an earlier study (Pecetti et al., 2013), confirming the repeatability of this evaluation method. However, the salt tolerance under field conditions of the material selected according to this method was not quite satisfactory. This result may be related to the reported possible inconsistencies between germination behavior and biomass production of adult plants under saline conditions (Al-Niemi et al., 1992; Johnson et al., 1992; Steppuhn et al., 2012). Accordingly, the cultivar Ameristand 801S, which was obtained by recurrent phenotypic selection for germination at high salinity, exhibited outstanding salt tolerance according to the germination test but not according to other evaluation methods or the field experiments. Screening in the Cone-tainers was suggested as a relatively simple method for growing alfalfa plants under saline stress for a period of months (Peel et al., 2004), thus approaching an actual crop condition. A possible limitation of this method is its bypassing the need for the plant to germinate or grow in early stages under high salinity levels similar to other greenhouse methodologies (Scasta et al., 2012) and the adopted in vitro selection method. As pointed out by Shavrukov (2013), plants may face either a salt stress or a salt shock depending on whether NaCl is applied gradually or in a single step, with a differential gene expression in the two cases. The genetic response to salinity can be thus influenced by the method of salt application. In the current study, a salt shock was applied in both the germination test and the in vitro trial, while gradual salt stress was applied in the Cone-tainer trial. We selected final survivors in Cone-tainers (under very high selection intensity) in all landraces, although with differences in overall response among populations (Table 3). The increase in plant mortality here observed after 3 weeks from the end of salt application in the Cone-tainer trial was compatible with a toxicity effect reported to drive a second stress phase due to ion accumulation in leaf tissues (Munns, 2005), after a first phase of the salt stress affecting plant growth mainly through osmotic imbalances. This trend of plant response was consistent with the pattern indicated by Shavrukov (2013) for a salt stress driven by gradual stressor application. Cornacchione and Suarez (2017) found a high correlation between plant salt tolerance and shoot Na^+ concentration, but the correlation provided only a partial explanation of the relative salt tolerance among populations, suggesting that other mechanisms may have been active. Although appealing because of its screening on adult plants, the selection in the Cone-tainers produced material only somewhat more salt tolerant than that based on the germination test (Table 6).

Shoot and leaf growth are generally reported to be more sensitive than root growth to salinity stress (Bhattarai et al., 2020; Cornacchione & Suarez, 2017; Kang et al., 2019). However, an opposite trend was observed in the current in vitro trial, with the root dry weight being more reduced than the shoot weight at both applied salinity levels (Table 4). We cannot exclude that the specific growth environment created by the in vitro medium accounted for this finding. Safarnejad et al. (1996) and Campanelli et al. (2013) reported an increased level of proline in alfalfa material issued by in vitro selection for tolerance, which could promote salt tolerance by better osmotic adjustment. Proline biosynthesis was suggested to be a primary mechanism of tolerance in roots but not in shoots of *Medicago* (Kang et al., 2019). In methodological terms, 300 mM NaCl appeared to be a salinity concentration able to screen in vitro only for root growth. However, our selection made across the two salt concentrations was justified by the strong correlation of landrace root ratios between concentrations and the consistent root response of landraces between concentrations (Table 5).

We could not ascertain whether or not the different proportion of parental landraces in the three experimental populations affected their performance in the field experiments due to any landrace differences in yield potential. The Cone-tainer screening indicated, however, that the four landraces did not differ in adult plant biomass in the control treatment of that trial.

The experimental population derived from the selection in vitro (MSI037) had significantly better yield than the two other experimental populations in the harsher second evaluation phase in Argentina but only a nonsignificant trend of yield advantage in the second phase in Tunisia (Table 6). Moreover, in the second Argentinian evaluation phase, MSI037 yielded at least comparably with salt-tolerant commercial cultivars or cultivars originated from the region where the evaluation occurred and likely provided, therefore, with specific adaptation, which seemed to have played an important role in our field experiments.

The two landraces Chenini and Mareth showed a trend of yield advantage in the harsher evaluation phase in Tunisia (Table 6) as a possible effect of their long-standing adaptation to local climate and stress conditions. This finding reinforced the importance of adapted landrace germplasm for salt tolerance breeding but also emphasized the need of a diversification in germplasm exploitation for a wider usefulness of selected materials under salt stress. As already indicated, a strong cross-over interaction featured the entries Chenini and Kumen PV INTA across Tunisia and Argentina, suggesting specific adaptation to the different type of salt stress and/or region-specific climate characteristics. Strong specific adaptation to the region of landrace origin or

cultivar selection may occur in alfalfa even across much closer regions than the current ones, such as, for instance, across areas of northern Italy (Annicchiarico & Piano, 2005). In the current study, such contrasting adaptive responses may be due to the different type of salinity stresses, the different climates of the two regions, or both of them. In Tunisia, the salinity stress is mostly generated by plentiful irrigation with saline water, with little salt accumulation in the upper soil layers because of the large irrigation water volumes and the light soil texture (both factors contributing to salt leaching towards deeper layers: Gelaye et al., 2019). In Argentina, the soil was already salinized at the beginning of the experiment, and the soil salinity built up further during the trial because the lower precipitations relative to the evaporation demand facilitated the capillary rise of the water with the previously dissolved salts, a phenomenon that is enhanced in soils with smaller pore size such as clay and loamy soils (Li et al., 2013). A major difference between these regions was the extent of drought stress experienced by the crop, which was negligible in Tunisia due to the continuous irrigation and remarkable in Argentina. Such a difference may well produce large genotype × environment interaction for biomass yield, as reported for alfalfa landraces and cultivars across sites of the Western Mediterranean basin (Annicchiarico et al., 2011). In a recent study (Annicchiarico et al., 2022), 127 alfalfa half-sib families sorted out of a broadly based Mediterranean reference population exhibited no correlation for multi-year biomass yield ($r_g = 0.14$, $p > 0.05$), hence, large genotype × environment interaction, across southern Tunisia and an Argentinian site close to the current one but featuring much lower soil salinity. Finally, slight differences in autumn dormancy between Tunisian germplasm (around 10) and Argentinian cultivars (around 8) may have had a bearing on specific adaptation responses to a warm environment featuring plentiful irrigation and high number of harvests (Annicchiarico et al., 2011), as only a completely winter-active material can take the full advantage of the very mild and rainfall-favorable autumn–winter seasons in Tunisia (Figure S2). Pending further verification, the experimental population MSI037 may represent an interesting compromise of a broadly adapted cultivar across diverse salt stress-prone areas. The apparent misadaptation of Chenini to the Argentinian location was somehow offset in MSI037, of which this Tunisian landrace represented over one fourth of the basic germplasm, possibly by other contributing landraces. Bami and Tamentit, in particular, also contributed for over one fourth each to the original genetic base of MSI037. Both of them were characterized by very good tolerance at germination (Pecetti et al., 2013; Torabi et al., 2011), and such a tolerance mechanism might have been useful under the soil salinization conditions at sowing in Argentina.

In conclusion, this study confirmed the challenges associated with alfalfa selection for salt tolerance, particularly with respect to germplasm evaluation and selection procedures, and possible genotype × environment interactions associated with different type of salinity stress and/or climatic conditions. It highlighted, however, the opportunities offered by using elite landrace germplasm evolved in salt-prone regions as a genetic resource. In vitro evaluation and selection are more demanding in terms of needed equipment compared with the assessment in the Cone-tainers but, when set up, is quicker, possibly less expensive, and provides potentially more useful selected germplasm. Selection based on germination in salinized water is quicker and less expensive than the other methods but was not as promising as the in vitro selection according to the current findings.

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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SUPPORTING INFORMATION

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