Breeding strategies in *Melilotus albus* Desr., a salt-tolerant forage legume

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Abstract

Melilotus albus Desr. is recognized as one of the species with the greatest potential as a forage source for ruminants in saline rangelands. The objectives of the current research were : 1) estimate heritability and genetic correlations in a *M. albus* pre-breeding material sources for traits associated with winter forage production and regrowth capacity, measured in spaced plants in a non-saline environment; 2) evaluate winter forage production in plots of a selected population in sites with contrasting soil salinities; and 3) evaluate the agronomic performance in plots of two selected populations of *M. albus* as blends (mixtures) and as monocultures. Results indicated the presence of genetic variability associated with winter production and regrowth capacity in a pre-breeding population selected only for late flowering. Also, results showed that selection in a non-saline environment did not modify relative salinity tolerance in analyzed populations of *M. albus*. Finally, varietal mixtures of two selected populations showed a slight increase and a more seasonally balanced dry matter (DM) yield than monocultures. Mixtures combined favorable characteristics from two selected populations (i.e., the highest winter forage production, as expressed in the SP1 population, and the highest regrowth capacity, as expressed in the SP2 population). Further results indicated that genotypes of *M. albus* naturalized in Argentina could be used as genetic resources for sweet white clover propagation in saline regions and that alternative breeding approaches could improve forage productivity in saline environments.

Keywords: forage breeding, abiotic stress, blend, mixture, sweet white clover

Introduction

Melilotus albus Desr. (sweet white clover) is a legume with annual and biennial growth habits. It is of Eurasian origin (Turkington, Cavers & Rempel, 1978). In the USA and Canada, it has been used for both forage production and soil improvement, although its use has been in decline since the 1960s as a result of the increased use of nitrogen fertilizers and lucerne/alfalfa (*Medicago sativa* L.) (Smith & Gorz, 1965). *Melilotus* breeding for forage was started in 1930 in the USA, Canada and the European Continent. Breeding continued until 1965 and resulted in several cultivars of *M. albus* and *M. officinalis* (sweet clovers) that have thrived in the Northern Hemisphere (Smith and Gorz 1965). Currently, in the USA and Canada, most of sweet clovers marketed seed coming from common sources (Meyer, 2005). Recently, was released an annual *M. albus* cultivar resistant to rust and adapted to south and central Texas (USA) (Smith et al., 2017). Since 2000, several studies have been carried out to evaluate forage production of species of the genus *Melilotus* in saline environments in Australia (Nichols et al., 2007; Dear, Reed & Craig, 2008; Rogers et al., 2008). Within the genus *Melilotus*, *M. albus* is regarded as a high-priority legume for breeding for salinity tolerance (Rogers et al. 2005), particularly germplasm of *M. albus* collected in Argentina and Uruguay (Evans & Kearney, 2003; Trigg, 2004; Smith et al., 2017). In fact, cultivar "Jota", an annual form of *M. albus* (Trigg 2004), and cultivar "Messina", of the species *M. siculus*, have been commercially released in Australia.

In Argentina, the annual form of *M. albus* has been naturalized (Zuloaga & Morrone, 1999). In addition, it is the only legume forage species sown in saline environments in the north of Argentina due to its good nutritional value and its ability to grow in diverse saline environments, in a wide soil pH range, and under conditions of variable rainfall (Maddaloni, 1986; Ferrari & Maddaloni, 2001). Despite its agronomic importance, there are currently only three cultivars released in Argentina (INASE, 2016). In recent years, the intensification of livestock production in saline areas of Argentina, the need for improved forage productivity in these areas, and farmers' increasing awareness about benefits associated with sown seed developed from identified cultivars, has allowed the inception of a breeding program for *M. albus* (Schrauf et al., 2003; Zabala et al., 2012).

Previous studies conducted by our research group have indicated that selection for late flowering and a greater number of basal branches result in a population with more leaves and higher relative leaf yield, which is positively associated with plant digestibility (Zabala et al., 2012). However, agronomic evaluations suggest that another key factor needed to improve forage

production is increased winter growth. This approach could help to fill the winter feed gap that is common in temperate regions (Humphreys, 1997; Nunes & Smith, 2003). Indeed, the slow initial growth in *M. albus* delays the first grazing or cut towards late spring (seeding occurs in autumn). Therefore, our research group embarked on a breeding program in 2009, with the aim of increasing winter forage production.

In breeding programs for abiotic stress tolerance, the phase/s (i.e selection phase and/or testing phase) at which stress is imposed is still an unsolved issue, and evidence for either argument is inconclusive (Richards 1983, 1992; Ceccarelli et al. 1998). Another important issue is whether the best genotypes of a species selected in a non-saline environment are the best in saline soil too and if it is possible that negative genetic correlations between stress tolerance and forage characteristic appear.

Finally, a wide range of genetic variability is necessary to start any plant breeding program. The efficient use of wild plant genetic resources requires extensive collection, characterization and agronomic evaluation of the available plant material (Schultze-Kraft, 1979; McFerson, 1998). Systematic and detailed procedures should be established to collect representative germplasm (Bennet et al., 1970; Mohammadi & Prasanna, 2003). Most forage breeding programs begin with variable populations, and subsequent selection schemes are developed to increase favorable alleles for agronomic characteristics in the selected genotypes. Thus, determination of genetic parameters such as heritability and genetic correlation is a priority that dictates the best selection scheme (Nyquist & Baker, 1991), with the implied conception of "superior genotype/s". Based on these approaches, the breeding of forage species has achieved substantial genetic gains in forage quantity and quality, but these gains are smaller (in relative terms) than those achieved for crop species (Casler et al., 2000; Casler & Brummer, 2008). For this reason, it is necessary to find alternative breeding approaches to increase forage production (Brummer, 1999; Woodfield & Brummer, 2001). In this sense, evidence exists about mixtures (blends) of cultivars of different species with better agronomic performance than monocultures. Better performance is based on the complementary use of resources (radiation or soil nutrients) in space and time by the mixture components (Willey, 1979; Turkington, 1996). However, little is known about forage performance of blends of the same plant species (Evans et al. 1995). Only two cultivars of *Bromus catharticus* developed with complementary utilization of resources as the selection criterion have been registered in Argentina (INASE, 2003a, b).

The objectives of the present study were to: 1) estimate heritability and genetic correlations in a *M. albus* pre-breeding material sourced from naturalized genotypes for traits associated with winter forage production and regrowth capacity, as measured in spaced plants in a non-saline environment; 2) evaluate winter forage production in plots of a selected population in sites with contrasting soil salinities; and 3) evaluate the agronomic performance in plots of two selected populations of *M. albus* as blends (mixtures) and as monocultures.

Materials and methods

Estimation of genetic parameters

In previous research, Schrauf et al. (2003) collected annual-growth in saline areas in the north central region of "Chaco Ecoregion", Argentina (between the latitudes of 27.47° and 35.39° S, and between the longitudes of 60.59° and 64.22° W). Thereafter, Schrauf et al. (2003) selected plants from between and within progenies over a 2-year period (1998-1999). The aims of the breeding program were to increase total dry matter (DM) yield and leaf DM yield as well as the change in DM yield distribution across the year. Selection for late flowering resulted in selected population ET2 (according to Zabala et al. (2012)). A random sample of 2000 seeds of ET2 was sown in a greenhouse in September 2009 for seed multiplication. Once the seeds grew, a total of 24 healthy plants were individually allocated to 10 L pots filled with a mix of soil and peat (in a 1:1 percentage) using a fully randomized design. Plants were irrigated to pot capacity (i.e., to the field capacity of natural soils) throughout the entire experiment. A fertilizer (nitrogen:phosphorous:potassium [NPK] blended in a 15:15:15 percentage) was applied on two occasions: 1) when potted plants reached 30 cm height, they were fertilized (1 g/pot of fertilizer), and 1 month later, they were fertilized again (2 g/pot of fertilizer). No cuttings were taken during this time. During the flowering period, we changed the pot position every 5 days to allow random mating among plants.

Seeds were set via open pollination of potted plants. In nature, *M. albus* is cross-pollinated by several species of bee. The mating design stipulated that the test population be isolated from other flowering individuals by at least 2.0 km, and the free movement of insects was allowed. At the beginning of flowering period, a hive was placed next to the mating population to enhance pollination by honey bees (*Apis mellifera*).

Progenies derived from these potted plants represented half-sib families. On May 6, 2010, progenies were planted as seeds. Twenty plants (replicates) per half-sib (n = 480) were transplanted

two weeks after emergence in a completely randomized design. Plants were placed 70 cm apart between and within rows. The trial was conducted in the experimental field of the Facultad de Ciencias Agrarias, Universidad Nacional del Litoral, Esperanza City, Santa Fe Province, Argentina (FCA-UNL, 31°25' S, 60°56' W), in an Argiudoll soil with 2.8% organic matter, 0.144% total N, 68 mg/kg of P, 9 ppm of S, pH 6.9 and electrical conductivity of 0.9 dS/m.

From September 22–29, 2010, we measured plant height (PH, cm), leaf number (LN), and number and length of basal branches (NBB, LBB, 10 cm above ground level). Herbage samples of plants were collected to a cutting height of 10 cm above ground level using scissors. All clipped herbage from each plot was weighed fresh and then dried to determine DM content. Leaf DM yield per plant (LDM, g), shoot DM yield (SDY, g) per plant and total DM yield (TDY, g) per plant, and leaf weight (LW, mean of 10 leaves per plant measured in grams [g]) were then calculated. Of these characteristics, DM yield per plant defined winter forage production. PH, LN, and LW was indirectly associated with winter forage production. Number of basal branches and LBB were associated with regrowth capacity (Zabala et al., 2012).

An analysis of variances (ANOVA) was performed (Table 1); heritabilities on an individual plant basis (h_1^2) and associated errors were estimated as functions of variance components (Vencovsky & Barriga, 1992; Holland, Nyquist & Cervantes-Martínez, 2003) according to the following linear model first proposed by Nyquist and Baker (1991):

$$P_{jkm} = \mu + R_j + F_k + a_{(jk)} \qquad (1)$$

where P_{jkm} is the phenotypic plant value of the j^{th} plant of the k^{th} half sib family, μ is the general mean, R_j is the effect of the j^{th} replicate, F_k is the effect of the k^{th} family, and $a_{(jk)}$ is the error term.

Genotypic correlations (r_g) between heritable characteristics were estimated as proposed by Cruz and Regazzi (1997).

$$r_g = COV_g(XY)/\sqrt{(\sigma^2 a_x * \sigma^2 a_y)} \qquad (2)$$

where $COV_g(XY)$ represents the genetic covariance between X and Y, $\sigma^2 a_x$ describes the additive variance of trait X, and $\sigma^2 a_y$ represents the additive variance of trait Y.

Data were analysed using the InfoStat statistical package (Di Rienzo et al., 2013).

[Insert Table 1 here]

Two selected populations were obtained from this experiment. Selection criteria were, in fact, defined based on the results of this experiment (see "Results" section, "Estimation of Genetic Parameters"). Therefore, it is necessary to explain population characteristics in order to understand experiments described in the next sections. Population SP1 was obtained by means of open pollination of plants from half-sib families with the highest winter forage production (associated to less branching capacity). Population SP2 was obtained by open pollination of plants selected from six half-sib families with the greatest branching capacity (less winter forage production).

Agronomic evaluation of selected population SP1

A 2-year field-plot experiment (2012–2013) was carried out to compare the productive performance of a population selected from field experiments discussed in the "Estimation of genetic parameters" section for winter forage production (identified as "SP1") and two lots of *M. albus* material used as controls: 1) a commercial seed (supplied by Oscar Peman y Associated Seed Company) without cultivar identification (identified as population "C"); and 2) the original/source population for the current research (originally developed by our research group and published as Schrauf et al., 2003 and Zabala et al., 2012, and known as "ET2").

The field-plot assay was conducted in two locations, the experimental field of FCA-UNL in Esperanza and in a Natracualf soil of Villa Minetti City (Santa Fe Province, Argentina), with electric conductivity of 4.4 dS/m, pH 8.2 and 34% exchangeable sodium. The experiment was a completely randomized block design with three replicates. Each replicate was a plot of 3 × 5 m, sown manually in rows with 15 cm row-to-row spacing (14 rows of 3.0 m length). Sowing frequency was once per annum for 2 years, and sowing occurred on April 20, 2012 and again on April 25, 2013. Sowing density was 1.2 g/m^2. Two cuttings per year were performed over 2 years (2012–2013), one in late winter and one in late spring of each year. The late-winter cuttings were taken on August 20, 2012 of year 1, and on August 29, 2013 of year 2 in Esperanza. Late-winter cuttings in Villa Minetti were taken slightly later in the season, on September 15, 2012 in year 1 and on September 20, 2013 in year 2. Late-spring cuttings were taken at the Esperanza location on October 20, 2012 in year 1 and on October 28, 2013 in year 2. In Villa Minetti, late-spring cuttings were taken slightly later in the season, on November 12, 2012 in year 1 and on November 18, 2013 in year 2.

Herbage samples were collected up to a cutting height of 10 cm above ground level from the 10 central rows (herbage was taken from only the central 2.4 m of each 3 m row) of each plot. All clipped herbage from each plot was weighed fresh, sub-sampled (250 g used from the total sample), and dried to determine DM content. Subsequently, LDY (g/m^2), SDY (g/m^2), and TDY (g/m^2) were calculated for each cutting sample. Data for each of the 2 years were analysed by using a standard ANOVA test to determine the significance effect of selected population, evaluation site, and the interaction between the two factors. Tukey's test ($p < 0.05$) was used to evaluate the significance of differences between mean values. Statistical analyses were performed with the InfoStat statistical package (Di Rienzo et al., 2013).

Agronomic evaluation of two selected populations (SP1 and SP2) in mixture

Forage yield and seasonal distribution were analysed in different mixtures of two selected populations, SP1 and SP2, according to the additive model proposed by Snaydon (1991). The experiment was conducted in 2014 in an experimental field of FCA-UNL. The experiment was a completely randomized design with three replicates per mixture. Each replicate was a plot of 3 × 5 m, sown manually in rows with 15 cm row-to-row spacing (14 rows of 3.0 m length). Sowing density was calculated from seed weight and a germination test in order to obtain 180 plants/m^2. The following five mixtures were analysed: 1) SP1 (100%), hereafter referred to as "100SP1"; 2) SP1 (75%):SP2 (25%), hereafter referred to as "75SP1:25SP2"; 3) SP1 (50%):SP2 (50%), hereafter referred to as "50SP1:50SP2"; 4) SP1 (25%:SP2 (75%), hereafter referred to as "25SP1:75SP2"; and 5) SP2 (100%), hereafter referred to as "100SP2".

Two cuttings were taken, one in winter (August 14, 2014) and one in late spring (November 1, 2014). Herbage samples were collected as described in the "Agronomic evaluation of selected population SP1" section. Subsequently, LDY, SDY, and TDY (all measured as g/m^2) were calculated. In order to evaluate forage production and plant density accurately, a random sample covering 1 m^2 was taken from each of the five population combinations in two cutting strata, at 10–20 cm above ground level (lower stratum) and at > 20 cm above ground level (upper stratum). Note, plants of the SP1 population were easily distinguished from plants of the SP2 population by their marked hollow on the principal stem, larger leaves and fewer basal branches (see Figure 3 in results).

The LDY, SDY, and TDY values obtained for each of the 2 cuttings were subjected to ANOVA to determine the significance of the effects of population combinations. Differences between mean values were tested using Tukey's test ($p < 0.05$).

Results

Estimation of genetic parameters

Differences between half-sib families were statistically significant for plant height (PH, $p < 0.0001$), leaf number (LN, $p = 0.006$), number of basal branches (NBB, $p = 0.04$), LDY per plant ($p = 0.01$), SDY per plant ($p = 0.003$), and TDY per plant ($p = 0.0071$). No differences among half-sib families were found for length of basal branches (LBB, $p = 0.31$) and LW ($p = 0.46$). Heritability values were moderate for all traits except for LBB. Leaf weight heritability was considered to be 0, because its genetic variance component was negative. Heritability values are shown in Table 2. Genetic correlations were high (positive or negative) between almost all traits (Table 3). Genetic correlations were high and negative between traits related to winter DM yield (LDY, SDY, and TDY) and basal branching (NBB). Genetic correlations were high and positive between traits related to winter DM yield, LN, and PH.

[Insert Table 2 here]

[Insert Table 3 here]

Based on initial genetic analysis on an individual plant basis, two populations with different characteristics were obtained. Single plants from half-sib families with the highest winter forage production were selected. Population SP1 was obtained by means of open pollination of 74 plants from the eight half-sib families with the highest winter forage production. Population SP2 was obtained by open pollination of 57 plants selected from six half-sib families with the greatest branching capacity. Plants with three or more branches < 10 cm above ground level were selected, transplanted to another field (5 km from the experimental field of FCA-UNL), and were multiplied there. Almost a half of the total plants were selected in both populations and seeds were harvested from each plant. Seed pool from these plants was used in the subsequent field experiment.

Agronomic evaluation of selected population SP1

For year 1 (2012) in Esperanza, TDY was significantly higher for population ET2 (846.3 g/m^2) than for SP1 and C populations (723.3 g/m^2 and 696.4 g/m^2, respectively). In SP1 population, winter DM yield was 67% of the TDY, much greater than for ET2 (34%) and C (36%) populations (Figure 1). The LDY/TDY percentage for winter cuttings at Esperanza in year 1 ranged from 55%–60% in all plant materials evaluated, while for the spring cutting, the LDY/TDY percentage was 50% in both ET2 and SP1 (vegetative stage) populations, and the LDY/TDY percentage was 38% in population C (flowering stage). Climatic data were obtained from the National Institute of Agricultural Technology (SIGA, 2017) Total precipitation in Esperanza between January and November was 870.9 mm (606.7 mm between January and August). The daily mean temperature was 14.4 °C between April and August and 20.4°C between September and November.

In year 1 in Villa Minetti, TDY was significantly higher for population C (574.3 g/m^2) than for ET2 and SP1 populations (438.9 g/m^2 and 484.8 g/m^2, respectively). In SP1 population, the winter DM yield was 60% of the total, much greater than for ET2 (40%) and C (30%) populations (Figure 1). The LDY/TDY percentage for the winter cuttings ranged from 65%–68% in all plant materials evaluated, while for the spring cuttings, the LDY/TDY percentage was 50% in both ET2 and SP1 (vegetative stage) populations and was 29% in population C (flowering stage) (Figure 1). Total precipitation in Villa Minetti between January and December was 603 mm (330 mm between January and September). The daily mean temperature was 15.8 °C between April and September and 24.8°C between October and December.

[Insert Figure 1 here]

In the second year (2013), Esperanza, the total DM yield was significantly different between three populations: ET2 (731.2 g/m^2), SP1 (512.8 g/m^2), and C (335.5 g/m^2) populations. Winter DM yields for SP1 population were 60% of TDY in Esperanza, significantly different from ET2 (29%) and C (41%) populations (Figure 2). The LDY/TDY percentage for winter cuttings was 52%–60% in all plant materials evaluated, while for the spring cuttings, it was 55%–60% in both ET2 and SP1 (vegetative stage) populations, and was 40% in C population (flowering stage) (Figure 2). Total precipitation in Esperanza between January and November was 775,4 mm (485,7 mm between January and August). The daily mean temperature was 14.6 °C between April and August and 19,6°C between September and November.

In Villa Minetti, year 2, TDY was significantly higher for both ET2 and SPI populations (396.3 g/m^2 and 357.4 g/m^2, respectively) than for C population (252.8 g/m^2). The winter DM yields from the SP1 population were 59% of the TDY, significantly different from ET2 winter DM yield (37%) and C winter DM yield (34%) (Figure 2). The LDY/TDY percentage for the year 2 winter cutting was 55%–60% in all plant materials evaluated, while for the spring cuttings, ~~it~~ was 50%–55% in ET2 population, ranged from 59%–65% in SP1 population (vegetative stage), and ranged from 29%–35% in C population (flowering stage) (Figure 2). Total precipitation in Villa Minetti between January and December was 520,6 mm (267,8 mm between January and September). The daily mean temperature was 15.1 °C between April and September and 26,3°C between September and December.

[Insert Figure 2 here]

Agronomic evaluation of two selected populations (SP1and SP2) in mixture

Mean values (± standard deviations [SD]) for plant densities in different mixtures were 196 ± 55 plants/m^2 (100SP1), 217 ± 55 plants/m^2 (75SP1:25SP2), 193 ± 72 plants/m^2 (50SP1:50SP2), 167 ± 67 plants/m^2 (25SP1:75SP2), and 169 ± 79 plants/m^2 (100SP2). Plant proportions of each mixture component were 79% SP1:21% SP2 (in the 75SP1:25SP2 mixture); 42% SP1:58% SP2 (in the 50SP1:50SP2 mixture); and 32% SP1:68% SP2 (in the 25SP1:75SP2 mixture). Figure 3 shows the two plant types in selected populations SP1 and SP2. For the first cutting, the SP1 monoculture PH was significantly higher than in all other populations (Figure 4a).

[Insert Figure 3 here]

[Insert Figure 4 here]

Dry matter yield for the first cutting differed between seed mixtures. The SP1 monoculture (100SP1) DM yield was higher that of the 25SP1:75SP2 mixed population and that of the SP2 monoculture (100SP2) (Figure 4c). The LDY/TDY percentage was much lower in the 100SP1 (the SP1 monoculture) (Figure 4e). The contributions of each component of the seed mixtures to TDY are shown in Figure 5a. Regarding the DM yield in different strata (lower and upper), the

50SP1:50SP2 and 25SP2:75SP1 seed mixtures showed the most balanced yield between strata, with vegetative growth occurring nearly equally in both strata (Figure 5b).

[Insert Figure 5 here]

Prior to the second cutting, mean values (± SD) for plant stand for the different seed mixtures were 120 ± 39 plants/m^2 (100SP1), 189 ± 33 plants/m^2 (75SP1:25SP2), 133 ± 42 plants/m^2 (50SP1:50SP2), 123 ± 22 plants/m^2 (25SP1:75SP2), and 150 ± 41 plants/m^2 (100SP2).

The proportions of plants contained in 2-cultivar mixtures at the second cutting were 69% SP1:31% SP2 (in the 75SP1:25SP2 mixture), 40% SP1:60% SP2 (in the 50SP1:50SP2 mixture), and 20% SP1:80% SP2 (in the 25SP1:75SP2 mixture). There were no differences in PH and LDY/TDY percentage (Figures 4b, 4f). Dry matter yield from the 50SP1:50SP2 and 25SP1:75SP2 mixtures, plus the SP2 monoculture (100SP2), was higher than DM yield from the other seed combinations (Figure 4d).

Contributions of selected populations to TDY were not correlated with the proportion of them contained in the different mixtures. In the plots with the highest DM yield, SP2 contributed the most DM to the DM yield in the mixtures (up to 80% of DM yield) (Figure 5c). Regarding the DM yield in different strata, both monocultures and mixtures showed a higher DM yield in the lower stratum (Figure 5d).

Figure 6a shows TDY for both cuttings taken from all five seed combinations (two monocultures, and three mixtures), and Figure 6b shows the contribution of each cultivar to TDY per cutting. The highest DM yield was found in the 50SP1:50SP2 mixture. The most seasonally balanced contribution between strata was achieved in the 50SP1:50SP2 and the 25SP1:75SP2 2-cultivar mixtures (Figure 6b).

[Insert Figure 6 here]

Discussion

Heritability values obtained were moderate (between 0.3–0.5) for PH, NBB, LN, and SDY and low for the other traits (< 0.3). Only the heritability of LW was 0, due to negative estimates of variance. Negative variances suggest no variability in data or sampling errors (i.e., insufficient number of leaves taken per cutting) occurred (Searle, 1971). These results indicate the presence of variability

in traits associated with winter production (as measured by PH, LN and DM yield) and regrowth capacity (as measured by NBB) in a pre-breeding population selected only for late flowering. This was confirmed by the effectiveness of phenotypic selection for increasing winter DM yield in SP1 population and regrowth capacity in SP2 population. Mass selection and phenotypic recurrent selection starting from wild germplasm, are the most widely used breeding method in *Melilotus* sp. (Smith and Gorz, 1965; Trigg, 2004; Zabala et al., 2012; Smith et al., 2017). Results of this work and others indicated a high possibility of obtaining good genetic gains for forage traits using wild sweetclover germplasm from Argentine and simple breeding methods.

In the agronomic evaluation during 2012-2013, the principal weather factors that affected the forage production was the precipitation. In 2013 forage dry matter accumulation was lower than 2012 in all evaluated materials, according lower precipitation in 2013. Even though total forage production was lower in SP1 than ET2 population, SP1 showed advantage over ET2 and C in winter forage production. Greater winter forage production in SP1 could be associated with a greater rate of growth at low temperatures as was found in subterranean clover (Evans et al., 1992)and *Medicago polymorpha* (Del Pozo et al., 2002). . Increased growth rates at low temperatures in plants is due to an increase in photosynthetic rate, modulated for proteome changes in chloroplasts and differential accumulation of soluble and structural carbohydrates (Frankow-Lindberg & Von Fircks, 1998; Goulas et al., 2003; Antolín, Hekneby & Sánchez-Díaz, 2005).

Forage parameters such as winter DM yield and regrowth capacity are associated with growth habits, specifically with shoot branching (McSteen & Leyser, 2005; Julier et al., 2007). The degree of shoot branching (NBB) has been shown to be an inheritable trait in populations of *M. albus* (Kirk, 1931 in Smith & Gorz, 1965; Zabala et al., 2012), and also in other populations of legume species (Liu et al., 2006; Julier et al., 2007; Van Minnebruggen et al., 2014). Moreover, shoot branching is a key aspect in terms of yield potential. In the present study, two contrasting populations (in terms of branching capacity, or NBB) were obtained. SP1 population yielded fast initial growth, strong apical dominance with a principal shoot, and many large leaves. SP2 population yielded lower initial growth, several thin stems in the lower stratum, and a greater number of leaves than SP1, but the plant itself was smaller.

With regard to leaf production, SP1 population yielded a lower LDY/YDY percentage than the SP2 population. Negative correlations between shoot length and leaf/stem percentage were found by Julier et al. (2007) in *Medicago truncatula* (an annual “model” legume). However, it is likely that the leaf/stem percentage in the current SP1 population (~50%) did not negatively affect

389 the forage quality. Quality analysis in other field experiments indicated that digestibility of forage
390 in SP1 is nearly 60% with a leaf/stem ratio of 50% (data not published). In another study, Espinosa
391 et al. (2012) found that genotypes with longer primary branches and lower branching capacity had
392 higher aerial DM yield. In the present research, we found negative correlations between winter DM
393 yield and regrowth capacity. Taken together, these findings suggest that the use of varietal mixtures
394 could be an alternative approach to achieving better DM yields. In addition, we found that genetic
395 correlations were high and positive between direct traits (LDY, SDY, and TDY) and low between
396 indirect (LN and PH) traits associated with winter forage production.
397 In the current study, we have shown that selection in a non-saline environment does not
398 necessarily modify the relative salinity tolerance in selected populations of *M. albus*. Selected
399 populationSP1 showed good performance in sites with different salinity and sodicity levels. This
400 result is important, because spatial and temporal variation in saline soils is high (Hein, Hein &
401 Quaino, 1989; Cabido et al., 1994; Corwin et al., 2003). In fact, in breeding programs for salt
402 tolerance in forage and crops, it is necessary that the selected genotypes have good agronomic
403 performance in non-saline patches (Munns et al., 2012). Based on this pre-requisite, Richards
404 (1983) supported alternative selection schemes for high yield in non-saline substrates, because high
405 yield in non-saline patches offsets the yield loss in saline patches. In *M. albus*, we propose an
406 alternate breeding scheme, with selection for forage traits in a non-saline environment and
407 subsequent yield screening in a saline environment. Indeed, similar strategies have been proposed in
408 breeding programs aimed at creating forage suitable for growing in water-limited environments
409 (Kirigwi et al., 2004; Rebetzke et al., 2009).
410 In the varietal mixture evaluation, mixture 50SP1:50SP2 showed a slightly increased DM
411 yield. Indeed, the two mixtures 50SP1:50SP2 and 25SP1:75SP2 combine the most favorable
412 characteristics of the two selected populations (i.e., SP1 high winter forage production and SP2 high
413 regrowth capacity). Generally, forage production in annual forms of *M. albus* is greater in spring
414 than winter. This combination of traits produced a more seasonally balanced DM yield proportion
415 between cuttings. Complementarity in time between SP1 and SP2 is common in genotypes with
416 different growth habits or growth rates (Alcock & Morgan, 1966; Turkington, 1996). In fact, Evans
417 et al. (1995) found better agronomic performance in a mixture of white clover varieties combining
418 the fastest initial growth of the Mena variety with the greatest persistence of the S184 variety.
419 Therefore, increased DM yields and a more seasonally balanced DM yield for the 50SP1:50SP2 and
420 25SP1:75SP2 mixtures could translate into greater agronomic performance. On the other hand, it is

possible that genotypes with different growth habits use incident light on the canopy in a better way (Rhodes, 1969), as was demonstrated in the current research by the equal contribution from the two different strata to TDY that was demonstrated by the 50SP1:50SP2 and 25SP1:75SP2 mixtures in the first cutting.

Conclusion

We researched key aspects of plant breeding to improve selection efficiency, not only in *M. albus* but also with a view to improving selection efficiency in other species, for saline environments. First, we estimated the levels of genetic variability in forage characteristics in pre-breeding material originating from naturalized genotypes. Jointly with previous works (Evans & Kearney, 2003; Schrauf et al., 2003; Trigg, 2004; Zabala et al., 2012), we found that naturalized populations of *M. albus* in Argentina have enough genetic variability to be used to release new cultivars adapted to large livestock areas with saline limitations. Second, we used an alternative breeding strategy in the present research. This strategy could be applied to some salt-tolerant native or naturalized species bred in saline environments, specifically salt-tolerant species included in tolerance groups IB and II according to Greenway and Munns (1980). Groups IB and II included salt-tolerant species as *Lotus* sp., *Elymus* sp., *Festuca* sp., *Melilotus* sp., etc., that show optimal growth in the absence of salt stress. Such species collected in diverse saline habitats have physiological mechanisms that provide their salt tolerance. We recommend that breeding programs should begin from these collections, with alternating cycles of selection for forage characteristics in a non-saline environment followed by testing in a saline environment. The design of selection schemes in non-saline conditions could to minimize environmental variability, which is more difficult to achieve in saline soils. In addition, alternate testing in saline environment is necessary to evaluate genetic correlations between forage traits and salinity tolerance. Particularly in sweet clover, simple selection schemes can be followed initially as mass selection. Other breeding methods has been successfully evaluated in *Melilotus* breeding as mass selection in self-pollinated progenies, recurrent selection, backcrossing and synthetics from lines (Smith and Gorz, 1957). Finally, selection of complementary genotypes changes the traditional concept of superior genotype (Turkington, 1996) and could be applied in future breeding schemes of *M. albus*. Mixture components are not generally selected by genotypic complementarity, although there have been some breeding methods developed that support this approach (Hamblin, Rowell & Redden, 1976; Hill, 1996; Annicchiarico, 2003; INASE 2003a, b).

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Disclosure

The authors report no conflicts of interest in this work.

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