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Selection for late flowering and greater number of basal branches increases the leaf dry matter yield in *Melilotus albus* Desr.

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Abstract. *Melilotus albus* is recognised as an important source of forage for ruminant animals in rangelands, particularly some of the germplasm of *Melilotus* collected in Argentina. This study was designed to evaluate the effects of 2 years of selection in *M. albus* for late flowering and branching on forage yield in a 2-year field plot experiment and to evaluate the effects of selection for late flowering on photoperiodic requirements in a 1-year pot experiment under natural and artificial lighting conditions. Three populations were evaluated, namely original population (T), a population selected for late flowering and greater number of basal branches (ET1), and a population selected only for late-flowering plants (ET2).

The field plot experiment showed that total DM yield per year was higher for ET1 and T than for ET2 in Year 1 and higher for ET1 than T and ET2 in Year 2. Relative leaf yield was higher for ET1 and ET2 than for T in both years. Leaf number was greater for ET1 than for ET2 and T in both years. The number of new basal and total branches was greater in ET1 than in ET2 and T for both years.

The pot experiment showed that days to flowering, calculated as the average of natural and artificial lighting treatments, were higher in ET2 and ET1 (83.4 days $\pm$ 15 and 72.8 days $\pm$ 19, respectively), than in T (61.2 days $\pm$ 21). Supplementary lighting reduced days to flowering compared with natural lighting conditions for all populations (58.7 days $\pm$ 13 *v.* 86.1 days $\pm$ 12).

Results showed that 2 years of selection proved to be efficient in breeding for late flowering and greater number of basal branches in *M. albus*. The longer vegetative stage observed in the improved populations can be explained by the selection of plants which require a longer photoperiod to flower. Selection for late flowering and greater number of basal branches resulted in a population with more leaves and higher relative leaf yield.

Additional keywords: forage legumes, phenology, plant breeding.

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Introduction

Legumes, native or introduced, have been used to increase the nutritional value of ruminant diets, as well as the amount of N in the soil, mainly in tropical and subtropical environments (Clem and Hall 1994; Shelton *et al.* 2005; Nichols *et al.* 2007).

Melilotus albus Desr. is a legume with annual and biennial growth types. It is of Eurasian origin (Turkington *et al.* 1978) and has been naturalised in Argentina (Zuloaga and Morrone 1999). In the USA and Canada, it has been used for both forage production and soil improvement, however, in these countries its use has been in decline since the 1960s, as a result of the increased use of N fertiliser and lucerne (*Medicago sativa* L.) (Smith and Gorz 1965). Studies are being carried out to evaluate the forage production of species of the genus *Melilotus* in Argentina and Australia (Nichols *et al.* 2007; Dear *et al.* 2008; Rogers *et al.* 2008). Within the genus *Melilotus*, *M. albus* is recognised as one of the species with the greatest potential as a source of forage for ruminant animals in rangelands, particularly some of the germplasm of *Melilotus* collected in Argentina (Evans and Kearney 2003; Trigg 2004). This is due to its ability to grow in a wide range of soils (pH from 4.8 to 8.2) and rainfall (from 500 mm/year) (Panigatti 1974; Bruno *et al.* 1982; Maddaloni 1986; Ferrari and Maddaloni 2001) and its relatively good nutritional value.

Genetic selection can be used to increase dry matter (DM) yield by both delaying flowering and increasing the number of

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branches per plant. Variability in flowering date affects the amount of DM produced within a region and creates opportunities to use different plant species or cultivars in regions with different growing season length (Nichols *et al.* 2009). Thus, in areas with increased rainfall in spring, such as in the Central-North region of Argentina, late-flowering cultivars would have greater accumulation of digestible DM before flowering (Nichols *et al*. 2009), compared with early-flowering cultivars. The number of branches per plant is positively correlated with regrowth capacity and plant persistence (Grof *et al.* 1970; Piano and Pecetti 2010), total DM yield (Julier *et al.* 2007), leaf DM yield and grain yield (Bisht *et al.* 1998). In addition, the relative leaf yield, i.e. ratio leaf DM yield to total DM yield, is positively correlated with plant digestibility (Malaviya *et al*. 2004).

As a result of a breeding program of *M. albus* conducted by the Department of Genetics in the Facultad de Ciencias Agrarias (Universidad Nacional del Litoral) and Facultad de Agronomía (Universidad de Buenos Aires) in Argentina, two improved populations were obtained, namely ET1 and ET2. Original collections were subject to selection for late flowering and greater number of basal branches (ET1), and selection only for late flowering (ET2).

The objectives of this study were to evaluate (*i*) the effect of selection for late flowering and branching on forage yield in a field plot experiment, and (*ii*) the effect of selection for late flowering on photoperiodic requirements in a pot experiment under natural and artificial lighting conditions.

Materials and methods

Two experiments were conducted, one in field plots and another in pots, to compare an original population (T) with two improved populations (ET1 and ET2) in terms of total DM yield, leaf DM yield, relative leaf yield, specific leaf area, number of leaves per plant, number of total and basal branches per plant and days to flowering.

Collection and selection within the original population

The breeding program (Schrauf *et al.* 2003), from which the cultivars evaluated in this study were obtained, consisted of collecting annual plants of *M. albus* in Argentina and subsequently multiplying and selecting plants between and within progenies over a 2-year period. The collection of plants was conducted in eight sites of the Centre-North region of Argentina (between latitudes 27.47° and 35.39°S, and longitudes 60.59° and 64.22°W). The multiplication and selection processes were conducted at two locations: Esperanza (latitude 31.25°S and longitude 60.56°W) and Junín (latitude 34.35°S and longitude 60.56°W). The objectives of the breeding improvement program were to increase total DM yield and leaf DM yield and to change DM yield distribution across the year. Four-hundred and eighty plants were collected and then individually planted on two experimental fields on 23 May 2005 (divided equally between both locations: Esperanza and Junín). Average flowering time (i.e. date when the first flower appeared) of collected plants was 65.7 ± 71.8 and 78.5 ± 64.6 days after establishment in Junín and Esperanza, respectively. The number of basal branches between ground level and 10 cm above ground level were 7.6 ± 3.3 and 8.5 ± 4.4 branches per plant across the two sites. An equivalent number of seeds from each of the eight original sites were openly pollinated to obtain population T.

In the first year of selection, all plants flowering before 20 November were eliminated, thus, 65 and 67 plants were selected across the two sites and progenies of open pollination were obtained. In the second year of selection, a sample of each selected progeny was sown on one site (Esperanza), in a 6×5-m plot, with a density of 100 plants/m^2. In the second year, all plants flowering before 20 November were eliminated. On 20 November, plants with three or more branches under 4 cm above ground level were selected ($n = 160$), transplanted to another plot and multiplied under open pollination. Seeds collected from these plants made up population ET1. Remaining plants were multiplied under open pollination and seeds collected from them made up population ET2.

Experiment 1, field plots

Experimental design

A 2-year field plot experiment was carried out to compare the productive performance of three *M. albus* populations (T, ET1 and ET2) in a completely randomised block design with three replicates. The experiment was conducted in the Experimental field of the Facultad de Ciencias Agrarias, Universidad Nacional del Litoral, Argentina (31°25′S, 60°56′W: ~938 mm mean annual precipitation), in an Argiudol soil with 2.8% organic matter, 0.144% total N, 68 mg/kg of P, 9 ppm of S, pH 6,9 and electric conductivity 0.5 dS/m.

Each replicate was a plot of 2.1×3.0 m, sown manually in rows with 15-cm row-to-row spacing (14 rows of 3.0 m). Sowing dates were 24 and 26 May for Year 1 and Year 2, respectively. Sowing density was calculated from seed germination tests, performed according to the International Rules for Seed Analysis (ISTA 2004). Thirty days after sowing, some plants were manually removed from each plot in order to maintain 160–170 plants/m^2 in all treatments.

Two cuttings per year were performed when the average height of 50 randomly selected plants per plot reached 40 cm. This occurred on 15 and 29 August and on 10 and 18 October for Year 1 and Year 2, respectively. Herbage samples were collected to a cutting height of 10 cm above ground level using scissors, from the 10 central rows (2.4 m/row) of each plot. All clipped herbage from each plot was fresh weighed, sub-sampled (250 g) and dried at 60°C until constant weight in a forced air oven to determine DM content. Total DM yield (g/m^2) was then calculated.

At the cutting dates, 10 plants per plot were sampled to determine:

– Leaf, shoot and total DM yield (g DM/m^2).
– Relative leaf DM yield (g DM leaf/g DM total).
– Specific leaf area, determined by sampling 10 individual leaves per plant using a Li-Cor LI-3000 leaf area meter (Li-Cor Inc., Lincoln, NE, USA).
– Leaf number per plant.

Fifteen days after cuttings, 10 plants per plot were sampled to determine the number of new branches as follows:

– Number of new basal branches per plant, counted as those between ground level and 4 cm above ground level.
– Number of new total branches per plant, counted as those below cutting height. Branches with at least one fully expanded leaf were considered as new branches.

Statistical analyses

Total, leaf and shoot DM yield, relative leaf yield, specific leaf area and leaf number per plant were analysed using the PROC MIXED of SAS (2003), with the following model:

$$y_{ijklm} = \mu + a_i + \beta(a)_{j(i)} + t_k + c_l + at_{ik} + ct_{li} + ac_{il} + atc_{ikl} + \varepsilon_{ijklm}$$

where y_{ijklm} is the observed value m, in the year i, within block j, for population k and cutting i; μ is the general mean; a_j is the year effect; $\beta(a)_{j(i)}$ is the block effect within the year i; t_k is the population effect; c_l is the cutting effect; at_{ik} is effect of the interaction between year and population; ct_{lk} is the interaction between cutting and population; atc_{ikl} is the interaction between year, population and cutting; and ε_{ijklm} is the residual error term.

The model used to analyse the number of branches did not include the effects of cutting and its interactions because this variable was measured only after the first cutting.

Measurements were means from within each plot, as this was considered the experimental unit. Differences between mean values were tested for significance using a Tukey test ($P < 0.05$).

Experiment 2, pots

Experimental design

During the first year of the experiment, a further 1-year study was conducted in Esperanza, to evaluate days to flowering in populations T, ET1 and ET2 under natural and artificial lighting conditions. Twenty-five plants per population, with each plant in one pot, were evaluated under natural lighting conditions and the same number under artificial lighting conditions. For the artificial lighting condition, six incandescent light bulbs of 100 W were uniformly distributed to provide 4 h of supplementary light per day from 21 July to the end of the experiment.

Seven-day-old seedlings were individually allocated in 10-L pots filled with a mix of soil and peat (1 : 1) in a completely randomised design, on 17 May. Plants were irrigated to pot capacity (i.e. a term analogous to the field capacity of natural soils) throughout the entire experiment. A fertiliser (NPK 15 : 15 : 15) was applied on two occasions: when plants in pots reached 30 cm (1 g/pot), and 1 month later (2 g/pot). No cuttings were performed. Flowering date was recorded for every plant as the date when the first flower appeared. Days to flowering were calculated as the difference in number of days between the flowering date and 21 July. The flowering range was calculated as the difference in number of days between the first and the last dates of flowering for individual plants within each population and under each lighting condition.

Statistical analyses

Days to flowering were analysed using the PROC MIXED of SAS (2003), with the plant as the experimental unit, with the following model:

$$y_{ijk} = \mu + a_i + l_j + al_{ij} + \varepsilon_{ijk}$$

where y_{ijk} is the observed value k, in the population i, and lighting conditions j; μ is the general mean; a_i is the population effect; l_j is the lighting condition effect; al_{ij} is effect of the interaction between population and lighting condition; and ε_{ijk} is the residual error term.

Mean differences as a result of treatment effects were determined using the Tukey test ($P < 0.05$).

Table 1. Means and standard errors in Experiment 1 of total DM yield (TDM), leaf DM yield (LDM), Shoot DM yield (SDM), relative leaf DM yield (RLDM), specific leaf area (SLA), leaf number (LN), number of basal and total branches (NBB and NTB)

Within rows, means followed by the same letters are not significantly different ($P < 0.05$)

	ET1	ET2	T
Year 1			
Cutting 1			
TDM (g/m^2)	206.4 (49)a	221.0 (49)a	206.4 (59)a
LDM (g/m^2)	103.42 (20)a	105.4 (23)a	95.1 (27)a
SDM (g/m^2)	103.00 (29)a	115.6 (26)a	111.3 (33)a
RLDM	0.50 (0.03)a	0.48 (0.02)ab	0.46 (0.03)b
ELA (mm^2)	59.86 (6)a	55.5 (5)a	57.3 (6)a
LN	52.2 (12)a	54.6 (8)a	47.3 (15)a
Cutting 2			
TDM (g/m^2)	208.8 (47)a	116.0 (29)b	202.5 (29)a
LDM (g/m^2)	110.7 (30)a	63.2 (15)b	70.8 (10)b
SDM (g/m^2)	98.1 (19.6)ab	52.8 (14)b	131.65 (20)a
RLDM	0.53 (0.04)a	0.54 (0.03)a	0.4 (0.02)b
ELA (mm^2)	54.8 (9.7)a	52.5 (4.1)a	55.6 (6.6)a
LN	59.5 (10.3)a	39.6 (10.6)b	36.4 (14.6)b
NBB	3.97 (1.0)a	1.1 (0.9)b	1.7 (1.1)b
NTB	15.60 (3.2)a	4.2 (1.7)b	8.56 (2.0)c
Year 2			
Cutting 1			
TDM (g/m^2)	305.0 (92)a	307.0 (79)a	337.1 (37)a
LDM (g/m^2)	157.8 (52)a	138.8 (38)a	129.6 (21)a
SDM (g/m^2)	147.2 (42)b	168.2 (43)b	207.5 (19)a
RLDM	0.52 (0.03)a	0.45 (0.03)b	0.38 (0.03)c
ELA (mm^2)	62.5 (2.9)a	59.9 (6.8)a	63.2 (3.2)a
LN	74.2 (9.9)a	69.5 (10.2)ab	65.8 (17.5)b
Cutting 2			
TDM (g/m^2)	281.2 (60)a	147.8 (14)b	126.3 (57)b
LDM (g/m^2)	151.1 (36)a	79.5 (11)b	53.7 (23)b
SDM (g/m^2)	130.1 (26)a	68.4 (7)b	72.6 (33)b
RLDM	0.54 (0.03)a	0.54 (0.04)a	0.43 (0.01)b
ELA (mm^2)	59.1 (9.01)a	56.3 (6.02)a	56.1 (5.56)a
LN	65.1 (12.6)a	41.9 (13.5)b	30.5 (12.9)c
NBB	4.47 (1.6)a	1.2 (1.1)b	1.9 (1.6)b
NTB	16.1 (4.7)a	5.1 (2.0)a	5.83 (1.9)a
TDM (g/m^2)	206.4 (49)a	221.0 (49)a	206.4 (59)a
LDM (g/m^2)	103.42 (20)a	105.4 (23)a	95.1 (27)a

Results

Experiment 1

Total, leaf and shoot DM yield, relative leaf yield, and specific leaf area and leaf number

No block effect was observed ($P = 0.12$). There was an interaction between population and cutting ($P < 0.01$), year and cutting ($P < 0.05$) and between population, year and cutting ($P < 0.05$) for total, leaf and shoot DM yield, relative leaf yield and leaf number. Because of these interactions, populations were analysed separately for these variables in each year and each cutting. No differences between populations occurred for specific leaf area.

Dry matter yield for the first cutting in both years did not differ between populations. In the second cutting of Year 1, total and leaf DM yield was higher for ET1 than ET2 and T ($P = 0.0032$) (Table 1). Shoot DM yield was higher for T than ET2 ($P < 0.0001$). In the second cutting of the Year 2 total, leaf and shoot DM yield was higher for ET1 than ET2 and T ($P < 0.0001$) (Table 1). Per year, i.e. the sum of yield in both cuttings, total DM yield was higher for ET1 and T than for ET2 in Year 1 (415.3, 408.9 and 336.9 g/m^2, respectively), and higher for ET1 than T and ET2 in Year 2 (586.2, 453.9 and 463.4 g/m^2, respectively).

Relative leaf yield was higher for ET1 and ET2 than for T in both cuttings of both years ($P < 0.0001$) (Table 1). The leaf number was greater for ET1 than for ET2 and T ($P < 0.0001$) in cutting 2 in Year 1 and Year 2. This variable was greater for ET1 than T ($P < 0.0001$) in cuttings 1 in Year 2 (Table 1).

Figure 1 shows the air temperature and rainfall recorded in the two experimental years. Total DM yield was higher in Year 2 than in Year 1 for all populations, which was explained by the higher air temperatures and higher rainfall in June, July and August in Year 2 compared with those recorded in the same months of Year 1 (Fig. 1). For these 3 months, mean air temperature was 3 degrees higher in Year 2 compared with Year 1, while rainfall was 96 mm in Year 2 and 47 mm in Year 1 (Fig. 1).

The PROC MIXED of SAS (2003) was used to perform a slide effect test for the triple interaction (i.e. population, cutting and year) observed for total DM yield, relative leaf yield and leaf number. The slide effect test analyses the contribution of each factor within the triple interaction. The slide effect test showed that the effect of cutting was significant in population ET2 and T for these variables (Table 2). For relative leaf yield and leaf number, the triple interaction is explained by differences in the performance of the populations across the 2 years and across the two different cutting times, while in total DM yield, across cutting 2 only (Table 2).

Number of new basal and total branches

The number of new basal and total branches was greater in ET1 than in ET2 and T for both years ($P < 0.0001$) (Table 1).

Experiment 2

Differences in days to flowering were statistically significant between lighting treatments ($P < 0.0001$) and populations ($P < 0.0001$). There was no interaction between lighting condition and population ($P = 0.809$). Days to flowering, calculated as the average of natural and artificial lighting treatments, were higher in ET2 and ET1 (83.4 days ± 15 and 72.8 days ± 19, respectively), than in T (61.2 days ± 21). Supplementary lighting reduced days to flowering compared with natural lighting conditions for all populations (58.7 days ± 13 *v.* 86.1 days ± 12). The flowering range under

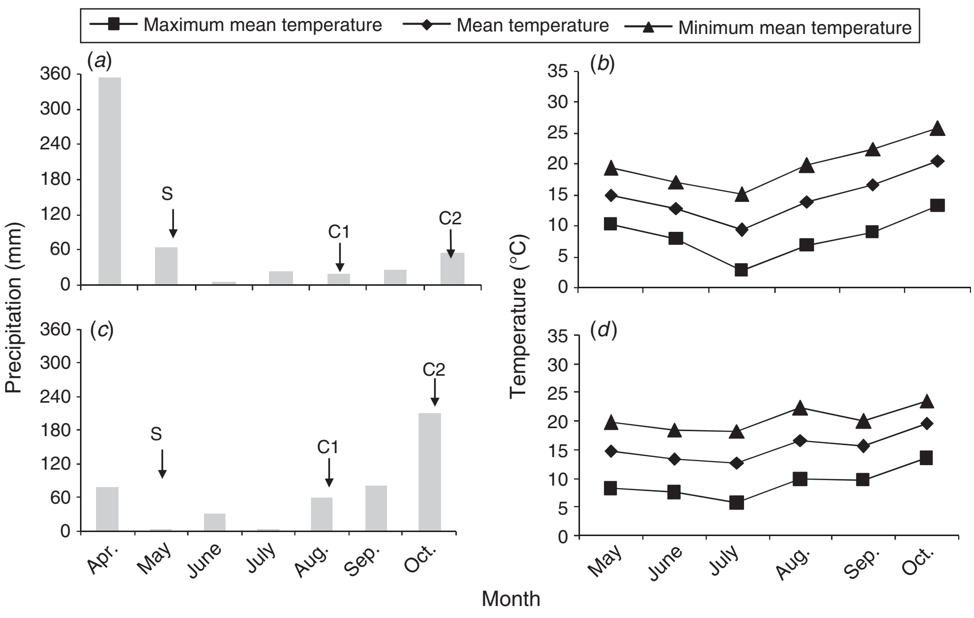

Fig. 1. Precipitation and mean temperatures in Year 1 (Experiments 1 and 2) and Year 2 (Experiment 1): (*a* and *c*) precipitations; (*b* and *d*) —▲— maximum mean temperature; —◆— mean temperature —■— minimum mean temperature. Meteorological data were supplied by EEA Rafaela, INTA, Argentina. S: seeding; C1: cutting 1, and C2: cutting 2.

natural and artificial light was shorter for ET1 and ET2 than for T (Table 3).

Discussion

This study was designed to evaluate the effect of selection in annual *M. albus* for basal branching and late flowering on forage yield and selection for late flowering on photoperiodical requirement.

Table 2. Cutting and population effect ($P < 0.05$) in the slide effect test of PROC MIXED of SAS for the triple interaction in total DM yield, relative leaf yield and leaf number
F, test statistic; *P*, probability

		Total DM yield		Relative leaf DM yield		Leaf number	
		F	*P*	*F*	*P*	*F*	*P*
		Cutting effect					
Population	Year						
ET1	1	0.01	n.s.	1.46	n.s.	1.16	n.s.
ET1	2	0.61	n.s.	1.56	n.s.	2.34	n.s.
ET2	1	11.8	0.0026	16.18	0.0007	36.8	<0.0001
ET2	2	27.0	<0.0001	26.3	<0.0001	23.0	<0.0001
T	1	0.02	n.s.	13.84	0.0014	31.8	<0.0001
T	2	47.6	<0.0001	6.32	0.0206	42.3	<0.0001
		Population effect					
Year	Cutting						
1	1	0.15	n.s.	16.53	0.0008	16.5	0.0008
1	2	5.77	0.0105	5.77	<0.0305	5.77	<0.0305
2	1	0.69	n.s.	36.77	<0.0001	36.7	<0.0001
2	2	15.0	0.0001	29.66	<0.0001	29.6	<0.0001

Table 3. Days to flowering (means and standard errors) and flowering range in different populations and lighting conditions

	Populations		
	T	ET1	ET2
Natural illumination			
Flowering time	74.2 (10.5)	90.25 (9.5)	96.25 (4.6)
Range	50	22	9
Photoperiod of 14 h			
Flowering time	47.83 (13.7)	57.91 (8.9)	68.38 (6.0)
Range	36	28	15

Experiment 1

The number of basal branches is a trait with high heritability in legumes with similar as well as those with different morphology than *M. albus* (Williams 1987; Beveridge *et al.* 2003; Johnson *et al.* 2006; Liu *et al.* 2006; Julier *et al.* 2007). The breeding program implemented over a 2-year period to increase the number of basal branches in ET1 compared with ET2 and T was successful (3.3 more basal branches per plant in ET1 than in T, Table 1). Our results are in agreement with those obtained in Canada with the biennial 'Artic' cultivar of *M. albus*, which was also successfully selected for greater number of basal branches (Kirk 1931 in Smith and Gorz 1965).

A greater number of basal branches were also associated with low vigour for some melilotus mutant plants (Smith and Gorz 1965). However, this was not observed in the population ET1. In Experiment 1, leaf DM yield in cutting 2 was higher for ET1 than ET2 and T. This was due to the greater capacity of basal branching of ET1 which, in turn, increased the number of leaves per plant. All three populations tested in our study showed apical dominance, with slow growth of basal branches before the first cutting and with accelerated rate of branching after the first cutting, particularly in ET1. *Melilotus albus* is, in general, not tolerant to frequent defoliation (Smith and Gorz 1965; Evans *et al.* 2004), and therefore, the improved cultivars of melilotus with greater number of branches have potential to achieve higher tolerance to defoliation, as reported for other species (Harris 1978).

The population ET1, which can be considered of prostrate growth habit, had higher total DM yield than the population T, in only 1 of the 2 years tested. Evans *et al.* (2004) reported that cultivars of melilotus with erect growth habit had higher DM yield than cultivars with prostrate habit. However, these authors reported that as cutting frequency increased, reduction in DM yield was higher in the erect cultivars than in the prostrate cultivar and relative leaf DM was lower in the former than in the latter cultivars. This is consistent with results from our experiment with low cutting frequency (only two cuttings per year).

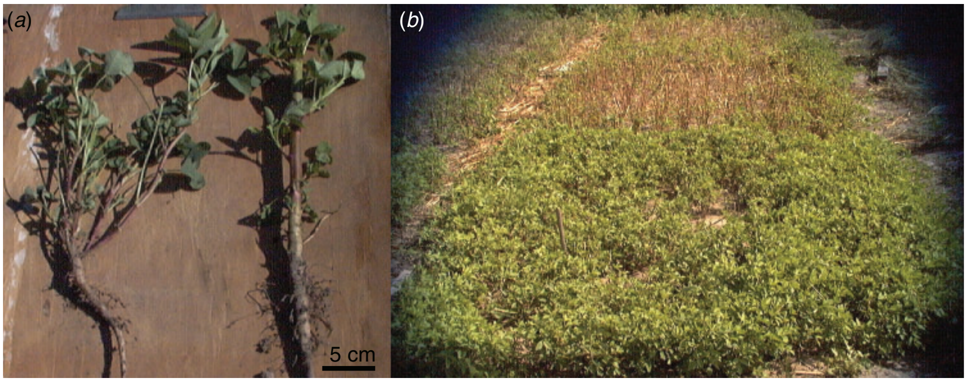

Fig. 2. (*a*) Plants with different branching capacity after cutting 1, ET1 (left) and ET2 (right); (*b*) plots of populations 30 days after cutting 1 in Experiment 1 and Year 1, ET1 (front) and ET2.

As the number of flowering branches increases, plant digestibility of legumes decreases, mainly due to an increase in the stem yield (Ru and Fortune 2000; Rochon *et al.* 2004). The rate of reduction in digestibility is particularly drastic for *M. albus* as flowering of branches progresses (Turkington *et al.* 1978; Maddaloni and Ferrari 2001). Therefore, digestible DM yield can be increased by delaying the flowering date in *M. albus*. In Experiment 1, relative leaf yield, which is positively correlated with digestible DM yield, was higher for the two improved populations than for the original population. The higher relative leaf yield of ET1 and ET2 is in agreement with their later flowering dates, compared with T, observed in Experiment 2.

The triple interactions observed between populations, cutting and year for total DM yield, relative leaf yield and number of leaves in Experiment 1 showed that populations differed in their performance across cuttings and years. The slide test effect allowed that the contribution of each factor to the interaction to be isolated and quantified. Thus, the results show that the differences in performance between cuttings in ET1 compared with ET2 and T was the most important factor contributing to the triple interaction.

Experiment 2

There is scope to change the date of flowering in forage species due to the relatively high heritability of this trait in plants (Casler and van Santen 2010), especially in annual *M. albus*, (Stevenson and Long 1926 in Turkington *et al.* 1978). Results from Experiment 2 support the aforementioned findings. Two years of direct selection for days to flowering proved to be efficient for improved populations ET1 and ET2, compared with the original population. Improved populations (average of ET1 and ET2) flowered 30 days after original population (Table 3). It is worth noting that population ET2 improved only for late flowering. The longer vegetative stage observed in the improved populations ET1 and ET2 would be the result of the selection of plants which require a higher photoperiod to flower, since photoperiod is the main factor affecting flowering date in *M. albus* (Smith 1942; Kasperbauer *et al.* 1962).

This study shows that enough variation exists within the Argentine germplasm of annual types of *M. albus*, to develop improved cultivars (Fig. 2). Selection for late flowering and greater number of basal branches resulted in a population (ET1) with higher leaf and relative leaf yield, which is positively associated with plant digestibility. The population ET1 was registered under the name of 'Faraón UBA-UNL' in the INASE (National Institute of Seeds, Argentina), becoming the second cultivar of *M. albus* to be registered in Argentina (INASE, e. 7/12 No. 78.791 *v.* 7/12/2005, Boletín Oficial No. 30.797). Further studies are required to test the genetic variation of total DM yield in the population ET1, in order to determine the feasibility of selection for this trait.

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