A very non-dormant alfalfa (*Medicago sativa* L.) with high multifoliolate expression.*

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Besides forage yield and persistence, forage quality is also very important in alfalfa production. Alfalfa quality depends not only on the environment but also on cultivar and herbage leaf proportion (Lacefield, 2004). Alfalfa leaves have normally three leaflets, and leaves are more digestible and have higher nutritional value than stems. Therefore, one way of improving alfalfa quality can be to increase leaf/stem ratio (LSR) by selecting for a higher frequency of multifoliolate (MF) plants in the population, i.e., plants showing leaves with more than three leaflets (Etzel *et al.* 1988; Volenec & Cherney (1990).

Main objectives of INTA´s alfalfa breeding program at Manfredi Exp. Station (Córdoba, Argentina) are forage yield, plant persistence, multiple pest resistance and forage quality. Regarding the latter, a phenotipic recurrent selection (PRS) program for increasing the number of MF genotypes in an extremely non-dormant [Fall Dormancy (FD) 10] population was carried out from 2008 to 2010. Initial breeding population was composed by 83 trifoliolate (TF) genotypes selected under field conditions for vigor, pest resistance and regrowth rate from FD 10 cultivars Ruano, Mireya and CW1010. These selected plants were transplanted to a pollination cage and intercrossed using honeybees in order to produce seed of the initial (C-0) population. C-0 seed was then planted to initiate PRS for increasing MF expression. Selection was performed at two stages of plant development: a) early vegetative stage, by choosing plants with at least one MF leaf; and b) early flower stage on those previously selected plants, by choosing the ones having a MF score of 4 (6-7 MF leaves stem^{-1}) and 5 (≥ 8 MF leaves stem^{-1}). Scores were assigned according to Sheaffer *et al.* (1995). Each selection cycle started with 1,000 plants. After four cycles of PRS, MF expression (% MF) increased from 6.7% in C-0 to 77.7% in C-4 population (Figure 1)

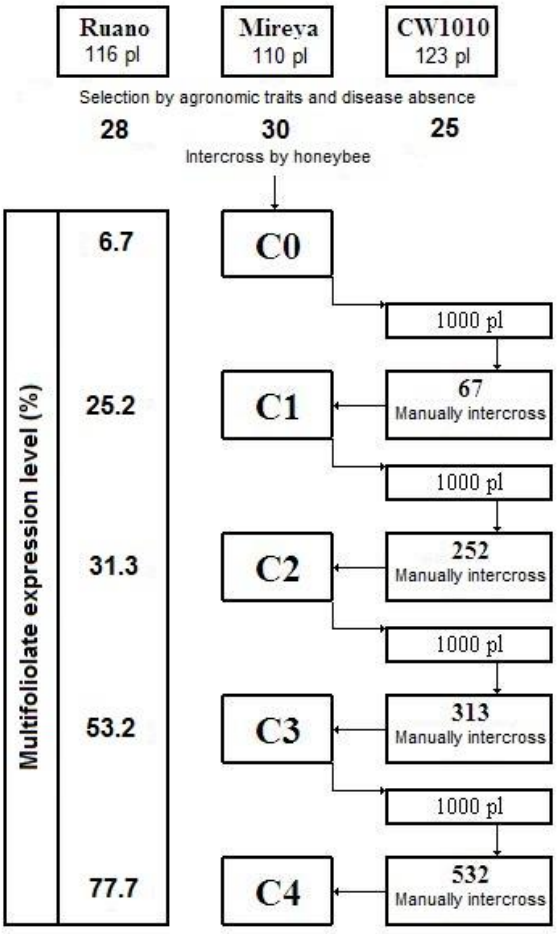

Figure 1 Scheme of phenotypic recurrent selection performed to generate multifoliolate alfalfa populations (C1 to C4). *Note: 67, 252, 313 and 532: number of selected plants in each cycle*

The effect of high MF expression on yield components and quality along selection cycles (C-1 to C-4) was assessed under field conditions using individual plants and dense stand arrangements. Evaluations on individual plants (25 plants plot^{-1}) were performed under two soil moisture conditions (rainfed and irrigated) and two growing seasons (2010-2011 and 2011-2012) using a RCB design with three replications, in which C-1 to C-3 were the treatment populations and C-0 was the check population. Evaluated agronomic traits were forage yield (FY, cumulative kg DM plot^{-1} at 10% blooming), number of stems (S), plant height (H), number of nodes

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per stem (N), number of leaves per stem (LN), number of leaflets per leaf (F), multifoliolate expression (% MF) and LSR. Forage quality was evaluated through estimation of crude protein (CP) and neutral detergent fiber (NDF) content and *in vitro* true DM digestibility (IVTDMD) at 48 hours. For dense stand evaluations, 5-m^2 (1 x 5 m) plots were sown at a seeding rate of 10 kg ha^{-1} in the fall 2012. Using a RCB design with three replications, populations C-3 and C-4 were compared to C-0 and three TF commercial cultivars. Measured traits (plot means) were cumulative forage yield (metric tons DM ha^{-1}), plant height (H), number of nodes per stem (N), leaf/stem ratio (LSR), crude protein (CP), neutral detergent fiber (NDF) and IVTDMD. Results are summarized in Table 1 (individual plants) and Table 2 (dense stand).

Table 1: Mean variable comparisons for multifoliolate expression among selection cycles (C-1 to C-3) and initial breeding population (C-0) under individual plant conditions. Values are general means of four environmental evaluations (irrigated 2010/11 and 2011/12 and rainfed 2010/11 and 2011/12) carried out in Manfredi, Córdoba, Argentina.

Traits	Alfalfa populations			
	C-0	C-1	C-2	C-3
FY (kg DM $plot^{-1}$ $year^{-1}$)	4.38a	4.11a	3.90a	3.15b
S	75.70a	80.72a	68.00b	60.77b
H (cm)	48.34a	45.64b	43.97b	40.87c
N	9.69a	9.50a	9.67a	9.49a
LN	41.28a	39.64a	41.07a	37.67b
%MF	4.89d	17.08c	39.40b	67.25a
LfN	3.08c	3.28b	3.71b	4.51a
LSR	1.17b	1.30a	1.34a	1.40a
CP (%)	26.60b	28.25a	28.70a	29.25a
NDF (%)	40.47a	39.70b	39.44b	38.91b
IVTDMD (%)	79.29a	80.29a	79.45a	80.72a

Means in the same row followed by the same letter are not significantly different (DGC test, p< 0.05). References: FY: forage yield; S: number of stems; H: plant height; N: number of nodes per stem; LN: number of leaves per stem; %MF: multifoliolate leaf percentage; LfN: number of leaflets per leaf; LSR: leaf/stem ratio; CP: crude protein; NDF: neutral detergent fiber; IVTDMD: *in vitro* true DM digestibility.

Table 2 : Mean variable comparisons among advances selection cycles for multifoliolate expression (C-3 and C-4) and the initial breeding population (C-0) under dense stand conditions. Values are means of two evaluated growing seasons (2012/13 and 2013/14) in Manfredi, Córdoba, Argentina.

Traits	Alfalfa populations		
	C-0	C-3	C-4
FY (ton DM ha^{-1} $year^{-1}$)	12.47a	11.23a	10.70a
H (cm)	45.00a	41.39a	41.05a
N	10.75a	9.57a	9.37a
LSR	1.35b	1.46a	1.49a
CP (%)	23.11b	23.55b	25.00a
NDF (%)	40.93a	39.40a	38.25a
IVTDMD (%)	78.33a	79.89a	80.93a

Means followed by the same letter are not significantly different (DGC test, p< 0,05). References: FY: forage yield; H: plant height; N: number of nodes per stem; LSR: leaf/stem ratio; CP: crude protein; NDF: neutral detergent fiber; IVTDMD: *in vitro* true DM digestibility.

Under individual plant conditions, population C-3 had overall lower (p<0.05) forage yield than population C-0 (Table 1). This negative correlation between %MF and DM yield was also reported by Ferguson and Murphy (1973). However, under dense stand conditions, there were no DM yield differences (p>0.05) between MF

populations and TF cultivars (Table 2), even though populations C-3 and C-4 had the lowest DM yields. For the individual plant trials, over the four environments defined by soil moisture and growing seasons, C-0 and C-1 populations exhibited higher ($p<0.05$) number of stems per plant (S) and higher ($p<0.05$) plant height (H) than C-2 and C-3 populations. On the contrary, under dense stand conditions, there were no differences ($p>0.05$) in H among populations. The relationship between H and MF expression was described in various studies and it is controversial (Ferguson & Murphy, 1973; Bingham & Murphy, 1965; Juan *et al*., 1993a). Regarding the number of nodes per stem (N), there were no differences ($p>0.05$) among MF selection cycles and TF checks, under both individual and dense stands conditions. Number of leaves per stem (NL) was lower ($p<0.05$) in C-3 than in C-0 population and TF checks. Selection for higher MF expression (C-1 to C-3) increased ($p<0.05$) the number of leaflets per leaf (LfN) relative to C-0. However, this may not necessarily correspond to an increase in total plant leaf area because leaflet size might be smaller than in TF plants, as stated by Volenec & Cherney (1990). On the other hand, Etzel *et al.* (1988) concluded that plants with MF expression within the range of 4.1 to 7.3 leaflets leaf^{-1} had larger leaf area, faster leaf expansion after defoliation and lesser stems compared to TF plants. In the present work, total leaf area was not measured. Populations C-3 and C-4 showed higher ($p<0.05$) LSR than C-0 under both individual plant and dense stand conditions. This is consistent with the increase ($p<0.05$) of CP content exhibited by MF populations (C-1 to C-4) relative to C-0 under both individual plant and dense stand evaluations. Similar results were reported by Petkova & Panayotova (2007). On the contrary, when Juan *et al*. (1993b) compared forage quality of cultivars with moderate MF expression to “high quality” (HQ) TF cultivars (selected for lower FDN and FDA), they found no advantages from MF alfalfas. In the present study, the more advanced MF selection cycles showed lower ($p<0.05$) NDF than C-0 under individual plant conditions. Under dense stand conditions, FDN also tended to decrease with selection cycles but no significant differences were detected among C-0 and C-1 to C-3 populations. In a similar way, IVTDMD increased as selection cycles progressed, but differences were not significant. In another study, Yancheva *et al*. (2012) detected higher CP and IVTDMD for three MF experimental populations compared to two TF cultivars.

When evaluating MF alfalfa populations, it is important to define the way in which MF expression is measured. In the present work, populations C-1, C-2 and C-3/C-4 were respectively classified as “low expression”, “moderate expression” and “high expression”, according to Sheaffer *et al*. (1995). From a commercial viewpoint, an alfalfa cultivar with ≥ 60% of the plants having at least one MF leaf is considered as “high expression”. Thus, differences in how MF expression is estimated may explain to some extent the controversial literature results.

When conducting a selection program, it might be important to estimate if the genetic variability is reduced in the resulting selected populations. In this study, intra and inter genetic variability between C-0 and C-4 populations was assessed using SSR markers. Forty genotypes from each one, keeping the original proportion of MF expression (6.7% and 77.7% for C-0 and C-4, respectively), were used. DNA extraction followed the modified CTAB protocol suggested by Doyle & Doyle (1987). SSR analysis was performed on 25 pairs of primers -mainly originated from *M. truncatula* (Julier *et al*., 2003)- that were successfully amplified and showed clear, strong, single bands for each allele. So, presence or absence of each allele was determined in every genotype. Program ATetra (version 1.2) for autotetraploid species was used to calculate the within- and between-population genetic diversity (Van Puyvelde *et al*., 2010). This program calculates the expected heterozygosity within-population according to Hardy-Weinberg equilibrium (HE) and Nei’s genetic diversity value (Nei 1978). Among-populations, variability was estimated through the population differentiation index or Nei’s GST (Nei 1973). Significance level of HE was calculated by the DGC test (Di Rienzo *et al*., 2002).

For the within-population genetic diversity, 20 markers amplified in a single region and five showed two regions of amplification, which were named ‘a’ and ‘b’ based on molecular weight. These regions were sufficiently distant and did not show any allelic relationship, and so contributed to avoid reading errors when fragments are of similar weight. Between these two regions, only the one that showed greater polymorphism i.e. higher number of alleles (assuming that this would represent a higher discriminative power), was chosen. Overall, the 25 SSR loci gave a total number of 185 PCR fragments (alleles), with molecular weights ranging from 80 to 310 bp. The number of fragments per locus ranged from 3 to 11, with an average of 6.28 alleles locus^{-1}. By determining the number of fragments per locus within each population (C-0 and C-4), the loss of alleles during the selection process was assessed. For the vast majority of SSR markers, the numbers of alleles per locus was the same in both populations. Only for four markers, one or two alleles present C-0 were not detected in C-4. These lost alleles in C-4 had in C-0 a frequency lower than 5%. Given the high multiallelic

degree of SSR in alfalfa, this small loss is considered not significant to differentiate the two populations. This is reinforced by the estimation of HE values for each SSR marker, which ranged from 0.565 to 0.889 in C-0 and from 0.491 to 0.877 in C-4. These values are considered moderate to high, and are consistent with the total number of alleles found for each marker. The overall HE estimation was 0.723 for C-0 and 0.726 for C-4. The DGC test did not detect HE significant differences between the two populations. Therefore, no genetic diversity was lost during the selection process.

It is concluded that four cycles of PRS were effective to significantly increase MF expression without producing inbreeding effects. The more advanced selection cycles (C-3 and C-4) had higher forage quality than the initial population (C-0). SSR markers were highly polymorphic and efficiently revealed the level of genetic diversity in C-0 and C-4 populations. Nei's GST value between-populations ranged from 0.002 to 0.033. Overall GST was 0.013, which means that only 1.3% of the total genetic diversity was between-populations and 98.7% was within-populations.

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