Deliverable 4.2

Drought tolerance, agronomic value, farmers' appreciation and breeding progress of novel genomic and phenotypic selections of lucerne

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Executive summary

This deliverable provides preliminary results on the yielding ability and other agronomic characteristics of novel lucerne (*Medicago sativa* L.) country-specific selections for Algeria, Italy, Morocco and Argentina that were obtained by phenotypic or genomic selection. The selections derived from a common genetic base represented by a reference Mediterranean population obtained by repeated intercrossing of three drought-tolerant landraces or varieties from Italy, Morocco and Australia. The reported first-year results for testing experiments in Italy and Algeria provided clear evidence of the fact that the project CAMA was able to develop candidate varieties with greater yielding ability in drought-prone environments than commercial varieties widely grown in each country. In particular, the genomic selections exhibited a yield advantage over a regionally-relevant commercial variety of 20% for the selection for Italy under managed drought stress in Lodi, and 33% for the selection for coastal Algeria under rainfed cropping in Alger. The forage quality of these selections was comparable to that of the reference cultivars or, limitedly to protein content of the Algerian genomic selection, slightly superior. The country-specific phenotypic selections were less valuable than the genomic selections. The genomic selections for Italy and Algeria showed high interest for variety registration in the respective countries based on these preliminary findings, which will require at least another test year (already planned in each country) to be conclusive. Additional results regarding the value of selections produced by the project are awaited from ongoing evaluation work in Argentina and Morocco.

Table of contents

1. Development of novel genomic and phenotypic selections of lucerne

A major aim of WP 4 of the project CAMA was to select novel drought-tolerant varieties of lucerne, which is the most grown forage legume in the Mediterranean basin, to respond to the need for greater crop diversification that is required for the successful set up of conservation agriculture. Lucerne varieties that could be grown in drought-prone Mediterranean areas under rainfed or limited irrigation conditions are also needed to alleviate the acute regional insufficiency for high-protein feedstuff.

The selection activities were rooted into work carried out in Italy, Algeria and Morocco within the FP6 project PERMED, which allowed to identify three drought-tolerant, stable-yielding cultivars (the Italian landrace Mamuntanas; the Moroccan landrace Erfoud; the Australian variety Sardi 10; see Annicchiarico et al., 2011, Field Crops Res. 120:283-291), and following work carried out within the ArimNet project REFORMA in the same countries and in Argentina at INTA (Instituto Nacional de Tecnologia Agropecuaria, which acted as an external institution in that project and the current one). REFORMA allowed to (i) create an elite Mediterranean reference population by repeated intercrossing of the three aforementioned cultivars, (ii) genotype a random sample of about 150 genotypes (via genotyping-by-sequencing) and phenotype their half-sib progenies in order to develop genomic selection models for drought-stress Italian environments, the coastal area of Algeria, the central part of Morocco and the North-Western part of Argentina, and (iii) set up experiments whose surviving plants were used for country-specific phenotypic selections within CAMA.

Within CAMA we initially performed a final analysis of the data issued by REFORMA, which revealed an outstanding additive genetic variance × environment interaction and low genetic correlation for biomass yield of the half-sib progenies across different drought-prone regions and indicated the need to develop country-specific genomic selection models and the lack of interest of genomic selection for wide adaptation to stressful regions. The predictive ability of genomic selection (as correlation between observed and genome-based modelled data) was fairly low, as it exceeded 0.20 for moderate managed drought (representing Italian stress-prone sites) and the sites of Algeria (Alger) and Argentina (Santiago del Estero) while being even lower for the Moroccan site (close to Marrakech). However, these values were still possibly useful, because phenotypic selection is hindered by very low narrow-sense heritability in this species (see Annicchiarico et al., 2022, The Plant Genome 16:e20264).

The phenotypic selections in Italy, Algeria and Morocco were devised according to a consistent protocol that envisaged selection both among families (i.e., among parents originating them) and within families. The selection targeted the top 10% half-sib families out of the nearly 150 that were evaluated at each site. The selected families had to satisfy both of the following criteria: (i) total dry matter yield over test years > experiment mean + 1 standard deviation (σ).; (ii) final persistence > experiment mean + 1 σ. If the number of families meeting this double criterion was lower than 15, the latter selection criterion was relaxed until reaching 15 selected families, by setting final persistence > experiment mean + 0.5 σ. For each selected family, one plant was visually selected from each of the 4 field replications of the experiment. The 15 × 4 plants were transplanted in an isolated polycross where the Syn 1 seed was produced for the evaluation work. The phenotypic selection performed in Argentina was more complex, as it contemplated an initial selection of 122 surviving plants in the test site (Santiago del Estero) that were used to create a Syn 1 whose plants underwent a second selection stage under a rain-out shelter in Cordoba, with 54 finally selected plants that produced the Syn 1 used for the joint evaluation work.

The genomic selections were performed on 564 plants randomly sorted out of the Mediterranean reference population (all independent from those used for construction of the genomic selection models and the phenotypic selections), which were genotyped by genotyping-by-sequencing. Out of

this initial number, 13 were discarded because of poor quality of the molecular data, while several others were excluded from selection because of very poor vigour. The country-specific genomic selections for Italy, Algeria, Morocco and Argentina targeted 15 parent cultivars per country (consistently with the selection of 15 parent genotypes in the half-sib family-based phenotypic selection) , using for each country the most-predictive of the tested genomic selection models (see details in Annicchiarico et al., 2022, The Plant Genome 16:e20264). The selected parent genotypes were rarely coincident across countries: only nine parents were common to two countries, and no parent was selected for three or four countries. Syn 1 seed was produced in isolation in Lodi for all selections (Figure 1) and then distributed to partners for the evaluation work. The activity of seed multiplication was delayed at first by the Covid19 crisis (whose impact in the area of Lodi was particularly severe), and then by a wave of extraordinarily high summer temperatures during the planned multiplication year that led to production of little seed with poor germination (due to high proportion of abnormal dark seeds). A second year of seed multiplication was required, to produce enough seed for the multi-environment trials.

Figure 1 – Parent plants of country-specific genomic selections in Lodi before seed multiplication (performed by pollination of Bombus bees inside separate insect-proof cages)

2. Evaluation and agronomic value of novel selections of lucerne

Because of the delay in the seed multiplication of the genomic selections, the setup of lucerne trials suffered of a one-year delay and took place in autumn 2022 in Italy (in Lodi, in managed environments), early winter 2023 in Algeria (Alger), and summer 2023 in Argentina (Santiago del Estero). In Morocco (region of Marrakech), no trial set up was possible in late 2022 or early 2023 because of lack of water for seedling establishment (or any other agricultural use) caused by the local dramatic drought, but the trial was established in late 2023. This report focuses on first-year evaluation results in Italy and Algeria, since no evaluation data are available yet from Argentina and Morocco.

2.1 Evaluation of all selections in Lodi

The objective of this activity was to evaluate all the phenotypic and genomic selections along with the genetic base from which the selections originated (the Mediterranean reference population) and a control variety with wide adaptation to Italian environments (Prosementi). Because of limited produced Syn 1 seed, the genomic selection for Morocco was excluded from this study, to ensure sufficient seed available for the planned study in Morocco. The evaluation was performed in pure stand in two managed environments that represented a severely drought-prone and a moisture-favourable growing

environment. Each environment was a large (24.0 m × 1.6 m × 0.8 m deep) bottomless container in concrete filled with local soil, covered by a rainout shelter and equipped with a double-rail irrigation boom (Figure 2). The experiment included four replicates per environment. Each plot comprised 64 plants. The stressful environment provided 315 mm of water from February to October 2023 that mimicked a typical Mediterranean-climate rainfall distribution, with 3 harvests for dry matter yield assessment. The favourable environment provided 700 mm of water over the same period, and allowed for six forage harvests during the evaluation year. The material was also evaluated for final plant survival in both environments, and leaf size, mean number of days from mowing to flowering, autumn dormancy (as plant height in autumn about two weeks after the last harvest), and five forage quality traits, under favourable conditions. The quality traits, recorded as extra-project research work, encompassed protein content, neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and Relative Feed Value (a synthetic measure of the nutritive value of the forage) and were recorded on the first and the last forage harvest.

Figure 2 – Stress-prone (left) and moisture-favourable (right) managed environments for evaluation of lucerne selections in Lodi

The main evaluation results are summarized in Table 1. Total biomass yield over this first test year tended towards significant genotype x environment interaction ($P < 0.10$) mainly due to the fact that significant population differences emerged under severe drought stress (where Prosementi and the phenotypic selection for Italy where bottom-performing) but not under favourable conditions. Because of the temperature pattern, the stressful managed environment was especially indicative of population performance in drought-prone environments of Italy (such as those of Southern Italy). In this environment, the genomic selection for Italy was higher yielding ($P < 0.05$) and tended to show greater final plant survival than the phenotypic selection for Italy and the reference cultivar Prosementi. A trend towards better yielding ability under stress of the country-specific genomic selection relative to the phenotypic one emerged also for Algeria but not for Argentina (in which the phenotypic selection effort had been relatively greater). No yield improvement of the selections over the genetic base used for selection emerged yet over the limited time span of this evaluation. Highest autumn plant height (indicative of lowest autumn dormancy) emerged for the Moroccan selection (consistently with the high autumn and winter temperatures of the region of Marrakesh in which it was selected), whereas lowest autumn plant height was displayed by the semi-dormant variety Prosementi and the genomic selection for Algeria. No significant shift of summer flowering date was observed for any selection relative to its genetic base. Finally, significant population variation for forage quality traits was found only for protein content, for which some selections displayed somewhat higher values than their genetic base although this trait was not explicitly selected for.

Table 1 – Total one-year biomass dry matter yield (DMY) and final plant survival in drought-prone or moisture favourable managed environments, and autumn plant height (as a negative indicator of autumn dormancy), days from mowing to flowering and forage protein content, for country-specific genomic (GS) and phenotypic (PS) selections, their genetic base and one widely-adapted Italian cultivar (Prosementi)

Material	DMY, severe drought (t/ha)	DMY, moisture favourable (t/ha)	Plant survival severe drought (%)	Autumn plant height (cm)	Days to flowering	Protein content (%)
GS Algeria	4.50 a	10.52	39.3 a	10.7	23.9 a	28.3 a
GS Argentina	3.97 a	10.88	18.0	12.6 a	20.8	27.7 a
GS Italy	4.58 a	10.21	34.8 a	12.3	20.8	27.4 a
PS Algeria	4.18 a	11.23	29.1 a	12.2	21.8 a	27.6 a
PS Argentina	4.29 a	10.55	19.0	13.2 a	22.3 a	28.3 a
PS Italy	3.89	10.48	16.8	12.7	22.6 a	28.0 a
PS Morocco	4.17 a	10.59	43.2 a	13.8 a	20.6	27.1
Genetic base	4.64 a	11.10	37.9 a	12.5 a	21.9 a	27.1
Prosementi	3.83	10.46	32.2 a	11.7	23.3 a	28.2 a
LSD (P < 0.05)	0.65	NS	20.0	1.4	2.1	0.9

Column means followed by letter ‘a’ do not differ from the top-ranking mean at P < 0.05; NS = non-significant mean differences.

These first-year results are only indicative for a perennial crop such as lucerne, and the second year of evaluation (already planned) will be very important to obtain more relevant results. Importantly, for Italy these preliminary results indicate the development by genomic selection of a candidate variety that has greater drought tolerance than the widely-adapted commercial variety Prosementi (as expressed by a 20% yield advantage under stress: Table 1).

2.2 Evaluation of Algerian selections in Alger

The objective of the research work in Alger was to assess the value of the new selections for Algeria relative to a standard represented by the commercial variety Speed (which probably is the most grown foreign variety in Algeria, in the absence of Algerian commercial varieties). The evaluation trial was performed in two rainfed growing conditions, namely, pure stand, and intercropping of lucerne with the tall fescue variety Flecha (featuring excellent adaptation to drought-prone Mediterranean environments: see Pecetti et al., 2011, J. Agron. Crop Sci. 197:12-20). The experiment was designed as a split-plot with six replicates holding the growing condition on main plot and the lucerne population on sub-plots. Pure stand plots included 81 plants, whereas mixed stand plots included 54 lucerne plants and 108 grass plants. In the growing period from January to July 2023, the rainfall amounted to 427 mm, and there were two forage harvests. Dry matter yield of lucerne and tall fescue, and lucerne number of internodes, internode length, plant height, flowering date and protein content were recorded on both harvests and growing conditions. An overview of the experiment is given in Figure 3. The planned farmer-participatory evaluation of the populations could not be performed.

The evaluation results relative to yield data cumulated over harvests are reported in Table 2. On average, the intercropping condition featured a lucerne content of 45% and did not provide a yield advantage over the pure stand (3.77 vs. 3.96 t/ha). The genomic selection outperformed the phenotypic selection, the genetic base and the reference cultivar Speed for dry matter yield averaged across

conditions ($P < 0.05$). Such a response was substantially consistent across pure stand and mixed stand, given the absence of genotype x environment interaction ($P > 0.10$). In intercropping, the genomic selection tended to produce greater total yield via greater legume yield along with same tall fescue yield. While the genomic selection achieved a distinct genetic progress over the genetic base it originated from (over 36% based on average lucerne yield across growing conditions), the phenotypic selection failed to display a clear yield advantage over its genetic base or the reference cultivar Speed. No significant ($P < 0.05$) difference among populations was observed for any other trait; however, the genomic selection, compared with the phenotypic one, exhibited a trend towards greater internode number (8.7 vs. 8.2) and higher protein content (22.5 vs. 21.8). The latter result agrees with a similar trend observed in Lodi (Table 1).

On the whole, these preliminary results indicated the successful genomic selection of a candidate variety for rainfed environments of coastal Algeria, where it provided a 33% yield advantage in the ordinary condition of pure stand relative to the widely grown commercial cultivar Speed (based on results in Table 2).

Figure 3 – Lucerne evaluation experiment in Alger during a forage harvest

Table 2 – Total one-year biomass dry matter yield (DMY) of lucerne in pure stand (PS), mixed stand with tall fescue (MS) and averaged across conditions, total DMY of tall fescue and the mixture in MS, and lucerne DMY proportion in MS, for genomic (GS) and phenotypic (PS) selections for coastal Algeria, their genetic base and one cultivar widely grown in Algeria (Speed) evaluated under rainfed conditions in Alger

Material	Average lucerne DMY (t/ha)	Lucerne DMY in PS (t/ha)	Lucerne DMY in MS (t/ha)	Tall fescue DMY in MS (t/ha)	Mixture DMY in MS (t/ha)	Lucerne proportion in MS
Genomic selection	3.56 a	4.86 a	2.26 a	2.09	4.35	0.519 a
Phenotypic selection	2.63 b	3.89 ab	1.36 b	2.12	3.48	0.391 b
Genetic base	2.60 b	3.44 b	1.75 b	2.01	3.76	0.466 ab
Speed	2.54 b	3.66 b	1.42 ab	2.06	3.48	0.409 b
LSD $P < 0.05$	0.58	1.04	0.63	NS	NS	0.099

Column means followed by different letter differ at $P < 0.05$; NS = non-significant mean differences.

2.3 Comparison of the Algerian phenotypic selection vs. the cultivar Speed across different water regimes

This experiment, also set up in early 2023 and representing additional research work relative to the planned project activity, aimed to further verify whether the new material selected for Algeria was specifically adapted to drier conditions than those ordinarily adopted for the crop in the region (which contemplate abundant irrigation during summertime). The newly selected material was represented by the phenotypic selection for Algeria, which was compared to the variety Speed (largely adopted by local farmers) across three water regimes involving high, fair, and no drought stress. High drought stress was represented by rainfed cropping (the perspective crop management devised for CAMA selections). Fair and no drought stress involved irrigations according to the level of the Readily available water (RAW). Fair drought stress, which tended to represent the current crop management, inplied the restoration of the soil water content to field capacity once RFU was depleted to 20% of its maximum amount. No drought stress (envisaged as a control) implied the restoration of the soil water content to field capacity once RFU was depleted to 50% of its maximum amount. Over the period from January to August 2023, all regimes received 430 mm of rainfall, with the addition of 86 mm of irrigation for fair drought stress and 171 mm of irrigation for no stress. The main results of the experiment, which included two forage harvests, are provided in Table 3. The phenotypic selection tended to outyielded Speed under high stress and, quite unexpectedly, outyielded Speed under no stress – thereby showing wide adaptation across water regimes despite its selection under rainfed conditions. This experiment, which will continue in 2024, supported further the value of the selections generated by the project.

Table 3 – Total one-year biomass dry matter yield (DMY) of a novel phenotypic selection and one cultivar widely grown in Algeria (Speed) evaluated under three water regimes in Alger

Material	High stress (rainfed)	Fair stress (moderate irrigation)	No stress (abundant irrigation)
Phenotypic selection	4.50 a	4.28 a	5.36 a
Speed	3.87 a	4.71 a	4.42 b
LSD P < 0.05	1.11	2.98	0.92

3. Conclusions and future prospects

The reported results for Algeria and Italy are just preliminary and require at least a second test year (already planned for all experiments) to be considered fully reliable. So far, they provided definite evidence of the fact that the project CAMA was able to develop candidate varieties with greater yielding ability and adaptation to rainfed or drought-prone environments than current commercial varieties widely grown in these countries. In particular, this conclusion holds for the genomic selections developed for each of these countries, whose yield advantage over the reference commercial cultivars under stress achieved 20% for the selection for Italy and 33% for the selection for coastal Algeria. The forage quality of these selections proved comparable to that of the reference cultivars or, limitedly to protein content of the Algerian genomic selection, slightly superior. These selections have high potential interest for variety registration in the respective countries, upon verification of the results after an additional testing period. In this perspective, additional Syn 1 seed will be produced in Italy for both of these selections, which, although in limited amount, could form the basis for the obtention of Syn 2 seed to be used for further seed multiplications aimed to variety registration and commercial seed production. Additional results regarding the value of selections produced by the project are awaited from the ongoing evaluation trial in Argentina and Morocco.