Medicago arborea Project at University of Wisconsin, Madison

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Background. *Medicago arborea* grew on us. The more we worked with it, the more interesting it became, and now it dominates our thinking. In the mid 1980s, the following Plant Introductions (PIs) were obtained from the U.S. Plant Introduction Program. The PIs behave as tetraploids, 2n=4x=32, and a small sample examined had mainly 16 bivalents at metaphase I in pollen mother cells.

PI 199254: US Embassy, Athens, Greece
PI 504540: Greece, 1958
PI 249937: Israel, originally from Mediterranean, 1968
PI 330677: Aegean Islands, Greece, 1983

The objectives in the 1980s were to observe the lack of contractile growth, the woody stems, and the flowering characteristics. (Also, we would like to see some of the black wood that develops in the center of old material, as reported by Bolton in his book entitled 'Alfalfa', 1962.) Only a few seeds were used in the 1980s and the remnant kept in cool, dry storage until the next planting in 1997. .

The objectives of our present research are gene transfer, and genomic relationships in the cross-pollinated, perennial *Medicagos*. While working on these objectives, it is possible to survey levels of heterosis and inbreeding depression *to* provide a base for comparison of these parameters in the self-pollinated annuals. Heterosis and inbreeding depression have been observed in *M. lupulina* and *M. truncatula* (see Vol. 2 and 3 of this website) and our goal is to develop a unified concept for both annuals and perennials.

M. arborea plants are started from seed planted in the greenhouse in late winter, transplanted to the field in late spring, and returned to the greenhouse in the fall. Nodules can be seen in the transplanting operations. Nodulation is natural, because we have not used commercial inoculation for decades. Growing conditions are the same as we use for alfalfa. Seed is produced by hand pollination in the greenhouse, in winter and spring. *M. arborea* under our conditions usually does not flower profusely, and plants are not always in flower at the same time. Crosses are made whenever two or more plants are in flower. Typically two years are required to get seed of a given plant. At this time the plants are getting large and require a lot of management. Hence, they usually are discarded, and we go on to the next seed generation.

Three seed generations of *M. arborea per se* have been produced since 1998; Synthetic-0, Syn-1, and Syn-2. About 15 Syn-0 plants were displayed in Bingham's garden at Poynette, WI, at the Alfalfa Genome Conference in early

August 1999. These plants were transplanted to the greenhouse that autumn, one or two plants at a time, as the temperature at night began to fall below freezing. The plants survived light frosts, but were killed by temperatures that continued to progress below freezing. The same is true for the above ground shoots of *M. sativa* and *M. falcata*. It is the crown that has the potential for winter survival. *M. arborea* lacks a crown, thus leaving all of the growing points basically above ground and killed by freezing temperatures. In our nurseries, some accessions of *M. falcata* are more cold tolerant than *M. arborea* and *M. sativa,* and can remain green several degrees below freezing, but they too are eventually killed. The point is that there is not much difference in the above ground frost tolerance of the species.

In 2004, additional *M. arborea* accessions were obtained from Italy, North Africa, Spain, and Greece. Also, we now have access to the *M. arborea* that has been growing wild in some areas of coastal California after escaping cultivation. Hence, we have a relatively large collection, which we will try to keep in separate gene pools from now on.

Whenever *M. arborea* is intercrossed in the greenhouse, we typically use the pollen accumulated on our pollinating tool to pollinate a raceme of flowers on an alfalfa male sterile plant, 2n=4x=32. The tool is most often a pocketknife blade. This has never been a big operation, mainly because of limited flowering of *M. arborea*. On the average, only about 10 racemes get pollinated on each male sterile each year. On the basis of about 10 ovules per flower, and 10-20 flowers per raceme, between 1000 and 2000 ovules per male sterile are challenged to hybridize each year.

Male sterile clone 6-4ms has been used each year, but has not produced seed, except for one seed in 2004, that grew into a haploid of 6-4ms. Clone 6-4ms was obtained about 35 years ago among Saranac X Saranac crosses, and has been maintained by cuttings over the years. Clone 6-4ms obviously does not produce seed in the cross, but we have kept it in the program as an embedded check for stray pollen. Also, clone 6-4ms essentially kept us going because it formed pods after pollination, and sometimes carried them to dry pods containing small dark aborted seeds.

The summary of seed production over the years in the *M. sativa X M. arborea* crosses is as follows. From 1998 through 2004: zero seed on 6-4ms, WIms-1, WIms-2, and WIms-3, which are all in 6-4ms cytoplasm. We try new male steriles each year, and in 2003, 12 seeds were obtained on MBms, which is from a cross of a male sterile plant of Magnum III X Blazer XL plant B17. Hence, this male sterile is of commercial cultivar origin, and it should be possible for anyone to obtain similar male steriles by using these two cultivars. We find almost 20% functional male steriles in Magnum III, and about 10% functional male steriles in Blazer XL. It would be interesting to use male steriles selected directly out of the

respective cultivars. It is worth noting that most plants in both cultivars segregate for maintainer genes.

We did not hurry to grow-out the 12 seeds of the MBms X *M.arborea cross*, because we assumed that they probably were selfs. However, the grow-out in 2004 revealed one maternal haploid of MBms, only one probable self, six apparent partial hybrids with variegated flowers, and four apparent partial hybrids with maternal flower color, but expressing some other hybrid characteristics. Sterility and abnormal genetic segregations discussed in the following could be due elimination of some chromosomes during seed development. Root-tip chromosome counts will be made this winter, but we know from surveying pollen mother cells this past summer, that several of the progeny are aneuploids with fewer than 32 chromosomes.

At this point we are treating the findings as a significant lead, and will not draw any firm conclusions until the crossing results are repeated, and/or DNA results are in hand. This work reminds us of the leads we had over decades of tissue culture and regeneration research in alfalfa, and the same rule applies here: The findings are no good unless they are repeatable. In the meantime, however, the novel variation recovered from the cross is interesting, and we are backcrossing some phenotypes into breeding lines.

Progeny of the *M. sativa* X *M. arborea* cross are designated sac-1 through sac-10. No single plant looks like a complete hybrid, but every plant is expressing one or more traits of apparent hybrid origin. It is as if the genome of *M. arborea* is scattered over the ten progeny, although we do not know whether the whole genome is represented. Transfer of pieces of the genome could be an advantage in the long run if some desirable traits are separated from the undesirable. In effect, some backcrossing already may be done. There is much that we do not know at this point, so we will keep crossing and learn as we go. The MBms male sterile is maintained as a clone, and it can be used again. We do not have the original *M. arborea* plants, but we do have the progeny of the plants that were used in the productive cross. For all we know, only one of the *M. arborea* pollen parents may have functioned in the cross with MBms, and we may have to recover the ability by screening progeny. When the crosses are repeated, we will identify individual *M. arborea* genotypes instead of using bulk pollen. This will help in the in DNA marker research.

All six of the variegated sac progeny were test-crossed with pollen from a cream flowered plant (homozygous recessive at the p locus), before the greenhouses were demolished on May 1, 2004, to make way for new greenhouses in about 18 months. This is another problem; we have lost the original greenhouse. The six variegated sac progeny ranged from nearly sterile as seed and pollen parents, to having about 50% fertility compared with normal alfalfa. Importantly however, some seed was obtained on every plant used as a seed parent. The test-cross was to study segregation at the P locus, where the plants with variegated flowers

are theoretically in the duplex condition (PP--). If there is preferential pairing, then there will be no segregation. If there is random pairing and tetrasomic segregation, then there will be 5:1 segregation. This appears simple enough, but the results so far have not fitted either segregation.

Observations and comments

The cool winter greenhouse may be a factor in the wide cross. We have noticed over the years that crosses with *M. arborea* and *M. truncatula* occasionally resulted in pod formation and retention in the winter greenhouse. Moreover, pods sometimes would be carried all the way to maturity and contain tiny aborted dark seeds. This is not surprising because Fridriksson and Bolton in 1963 reported that *M. arborea* and several other species initiated embryo development when crossed with alfalfa. The reason for success with MBms could be due to a special female gametophyte with a permissive endosperm balance situation. Brink and Cooper at Wisconsin began research on the role of the endosperm in seed development in the 1930s. They published a benchmark review of the subject in 1947. Other benchmarks include research on the indeterminate female gametophyte in maize by Kermicle, and the importance of the endosperm balance number (EBN) in the Solanums by Johnson and Hanneman. The EBN concept for successful seed development appears to apply in many species, and there are reports from McCoy's laboratory of a tetraploid that permitted wide crossing, and had a haploid content. A permissive EBN could be could explain the results. Knowledge of these subjects has helped us to keep screening for special genotypes in alfalfa. These references are available on request, and McCoy and Bingham, 1988, reviewed some of this literature.

It is important to note that Nenz, et al. 1996, reported producing somatic hybrids of *M. sativa* and *M. arborea* by electrofusion.. They documented chromosome elimination in their hybrids that were from fusing two tetraploids. The hybrids in their study were near the octoploid level and reportedly did not flower. Please consult their paper for other details.

We recently learned about *M. citrina* that reportedly had a higher plant leaf area, and was more drought tolerant than *M. arborea (Lefi et al. 2004a,b).* The authors concluded that M. citrina would have greater potential then *M. arborea* as a forage resource and erosion barrier crop for semi-arid conditions. Now, we want to take a look at *M. citrina.* Is it a close relative of *M. arborea*, for example? And, who can tell us about *M. strasseri*?

At the time of this writing, November 2004, the field season is coming to a close and we will not learn anymore about the materials for a few months. Hence, the following observations are offered at this time.

- *M. arborea* does not have contractile growth during seedling establishment, and four of the sac progeny had very little contractile

growth and limited crown development. It appears to be a quantitative trait and is not easy to classify.

- *M. arborea* grows more slowly and flowers later than *M. sativa*, and so have the sac progeny and their progeny in crosses with each other and with *M. sativa* testers. In fact only about half of the testcross progeny flowered in the past growing season of about 150 days. Such general responses suggest that there may be more gene transfer than we are lead to believe by the qualitative markers.

- In 150 days of sustained growth without cutting, *M. arborea*, *M. sativa*, and *M. falcata* all produce a woody central stem approaching one centimeter in diameter. Interestingly, *M. arborea* produces only about half the total biomass in this period of the other two species. *M. arborea* has more genotypes with stems at or near one centimeter, but stems this large can still be found in the other two species. There may be literature on this. Can anyone provide references to such information?

- Test-cross segregations at the P locus were studied in three variegated plants, and the progeny of three more did not flower before the season ended. Each of the three that could be studied behaved differently. One had a very low frequency of the recessive class either due to preferential chromosome pairing or segregation distortion. Another had a higher than expected segregation of recessives suggesting loss of a *M. sativa* chromosome carrying P, or segregation distortion. The last one did not segregate as a seed parent, but had a low frequency of segregation as a pollen parent. This was one phenotypic trait; imagine what might be seen with DNA markers covering more of the genome.

- In the initial grow-out of the 12 seeds in the spring of 2004, four plants were considered to be haploids early on. They were small plants with terete-shaped leaflets rather than obovate-shaped leaflets, which is a good sign of haploidy. When the plants flowered, however, three of them were variegated and could not be haploid. Only one was haploid. This now makes us wonder about the origin of haploids in general in alfalfa. Perhaps haploids sometimes arise by elimination of the male chromosomes in a wide cross. This phenomenon is used to obtain haploids of barley, for example. The variegated plants are all showing signs of chromosome loss, including reduced fertility, seedling lethality, and distorted segregation at the P locus.

- About 25 years ago, we saw evidence of partial hybridization in a cross of 6-4ms X *M. glomerata.* There was a plant with 16 chromosomes that looked like a haploid of 6-4ms except that it had variegated flower color. And, there were some near progeny with near 32 chromosomes, which

had maternal flower color, but also had restored male fertility indicating some genetic material was transferred. We knew that the material was interesting, but we were busy with funded research, and put the material on the shelf, so to speak. This cross also should be repeated, and research done on the phenomenon. In retrospect it appears to be similar to the phenomenon that we are dealing with in the cross involving *M. arborea*.

- The main trait of interest to us is large seeds, and one of the sac progeny has seeds almost twice as large as the average for alfalfa. But, the aneuploidy, the late flowering, and the lack of mature seed in our field nurseries have prevented us from screening effectively thus far. It seems unlikely that the full seed size of *M. arborea* will be transferable to alfalfa without undesirable linkages, but we will see just how much gain can be made.

- Several reproductive and growth habit abnormalities were observed in the few progeny of sac X sac crosses that we were able to obtain in 2004. These will be reported at a later date. They fall into the category of outbreeding depression, and they are typical of what Allard (1999) indicates is expected when different genetic complexes are hybridized and then undergo genetic segregation.

- We close with a question: If a trait of potential breeding value is found, but it contributes to outbreeding depression in an advanced generation, is there any way to exploit it in a breeding product?

- Additional information and pictures from the *M. arborea* project are in a following report.

- New information will be posted as it is obtained.

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