Results using Tetraploid Medicago truncatula cv. Jemalong in Crosses with Alfalfa

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Introduction. The chromosome number of diploid Jemalong (below left) was doubled using colchicine to produce autotetraploid Jemalong, hereafter 4x J, (below right). This report contains pictures and research notes on plants derived from sexual crosses of 4x alfalfa X 4x J over the past few years.

The project is ongoing, and this report provides information and pictures of the unusual variation from the wide cross. The yellow flower color of Jemalong was used as a marker for transfer of 4x J genetic material, and only progeny expressing yellow were saved. We now know that there is much genome disruption associated with the cross, and some presumed self progeny that did not express yellow pigment in flowers, could have been hybrid for other 4x J traits. This possibility is being tested in a new set of progeny.

Traits segregating in advanced generations that are best explained by introgression of M. truncatula germ plasm include yellow flower color, leaf shape and serrations, plant growth habit, seed size, and pubescence.

There also are numerous morphological abnormalities segregating in advanced generations that are best explained by alfalfa chromosome rearrangements, and subsequent recombination errors. This is because plants look like monstrous alfalfa, and do not resemble 4x J, except for flower color, and sometimes leaf shape. It appears as though genomic chaos during embryogenesis, and meiotic mistakes in later generations, have a “mutagenic effect” on alfalfa. Pictures of these abnormalities are in the following files.

History of the project. In year 2000, when Medicago truncatula was becoming a model legume species for research, we made an autotetraploid out of ‘Jemalong’ (hereafter 4x J) by treating a shoot with colchicine. 4x J is a text book tetraploid with larger leaves, flowers, pollen, seed, and biomass. The goal was to transfer the 4x J leaf spot to alfalfa via the cross of 4x alfalfa X 4x J. In 2006, 4x J was used as the pollen parent in a cross with alfalfa male sterile MBms that produced a low frequency of hybrids in crosses with M. arborea (see Medicago Genetic Reports web site vol. 5, 2005). The cross of MBms X 4x J, involved only about a hundred flowers pollinated. Of three seeds and progeny produced, two were apparent self progeny, but one progeny had variegated flowers indicating that the plant possessed a yellow gene from 4x J. The plant resembled alfalfa, but was odd. Also, it was relatively infertile, but produced seeds when crossed with alfalfa. In 2008, this exercise was repeated using the special alfalfa clone M8 (*see MGR web site vol.10, 2010*). Information about clone M8 also is updated in the on line journal PLANTS, vol.2, issue 2, June 2013, p. 343-353. The results were the same, except this time, two progeny with yellow flower color were obtained. In 2009, the cross was repeated again, resulting in one hybrid. At this point in time, four hybrids had been produced based on yellow flower color.

From the start of the work, it was understood that finding the leaf spot would be difficult. The leaf spot is recessive in M. truncatula, and expected to be recessive to one or more effective factors in alfalfa. Also, at the tetraploid level, with tetrasomic inheritance, large populations must be screened. In addition to all this, we could not rule out the possibility of preferential pairing of M. truncatula chromosomes, which would potentially prevent segregation. Hence, we started a project to breakup potential preferential pairing termed the s-t breakup project**.**

The s-t breakup project. Yellow-flowered progeny from crosses of 4x alfalfa X 4x J were backcrossed to a cream-flowered alfalfa, and then advanced by sib mating. The tetraploid material is currently in the third generation of advance. Why backcross? If there should happen to be two homologous 4x J chromosomes, or segments of chromosome in a plant, the backcross eliminates one of the homologues. This prevents preferential pairing (if it is occurring), increases the chances of recombination with alfalfa **(the breakup),** and potentially enables segregation of recessive traits of M. truncatula (sometimes abbreviated M.t. in the following). These are the potential benefits of backcrossing to alfalfa. The disadvantage is that the one dose of recessive M.t. genetic material, such as the one allele for the Jemalong leaf spot, will not segregate until the dose builds up to at least duplex, which will have 1/6th gametes with two doses. And, there are not many duplexes in the random mating population. Then, two of these gametes must be involved in fertilization, 1/6 X 1/6, to produce a homozygous progeny. Moreover, we cannot select for dosage until we see the target trait segregate. Considering the probabilities, we need to look at a thousand plants to have a chance of observing one plant with the recessive leaf spot.

To make matters worse, we do not know how much M. truncatula genetic material may have been lost due to chromosome elimination during embryogenesis and seed formation. Finally, there could be additional losses at the plant level as evidenced by sectoring. Hence, the chance of finding the recessive leaf spot in the segregating material is very small. Nonetheless, yellow flower color was transferred, and 4x J leaf shape and serrations are segregating in advanced generations indicate that some genetic material from M. truncatula has been transferred and retained. The advanced generations also are segregating for unusual variation that we have never seen before in any alfalfa or alfalfa hybrid material involving M. falcata or M. arborea. And, the variation is more bizarre than somaclonal variation we observed over many years of regenerating plants from tissue culture.

Pictures and Comments about Variation

Variation in s-t populations ranges from subtle to remarkable. About one forth of s-t plants have remarkable variation, and frequencies will be reported later.

Below are pictures of s-t plants with yellow flower color pigment that was not in alfalfa parents.

Above: Typical yellow in F1 generation (left); and, a weaker yellow in an advanced generation, with threshold development of pigment (right). Eventually all petals will be yellow on this plant, except for occasional sectoring due to chromosome loss. The timing of yellow expression is very late in petal development in this material.

Below: Serrations on leaflets of a plant in the s-t breakup population, similar to the serrations on M. truncatula leaves.

Note the larger, deeper serrations that are halfway down the margins typical of M. truncatula. Typical alfalfa serrations are shallower, and only on the upper one-third of margin.

The multifoliolate conditions shown below on both 2x and 4x M. truncatula and on 4x alfalfa are essentially unnerving to me. I am unnerved because I have never seen this type of multifoliolate in alfalfa. In M. truncatula, it was only observed on the first or second trifoliolate, after which the plants were trifoliolate.

Note the unusual extra leaflet on one side of terminal leaflet, observed only on the first or second trifoliolate leaves. It was not seen on later leaves. Observed in GH in May 2011, on one of five A17 plants, and one of five 4x Jemalong, in spring 2011.
The leaf below is from an ‘alfalfa-like’ plant segregating in the ‘s-t breakup’ population.

Once again, I have never seen this type of multifoliolate leaf in alfalfa. This plant continued to produce this specific multifoliolate leaf for a few nodes, and then stopped (as it did in M. truncatula). Note the upper large leaflet. It has a darker green sector indicating that this plant is sectoring. The serrations on this plant are abnormal, and the petiolule of the upper leaflet is very long, as it is on M. truncatula. This plant has yellow flowers.

The below picture showing a flower with one yellow wing petal, and cream in the other petals looks like sectoring due to loss of the yellow gene, but it also could be differential threshold – expression. Plants expressing sectored flowers usually continue to sector in random locations. Frequency of sectoring varies with the genotype.

The below pictures illustrate the slow development of yellow pigment in a flower petal that has lost purple. Anthocyanin is the first pigment expressed in Medicago species, with carotinoids/xyanthophylls second, but the delay of yellow expression is extreme in these materials. Timing of expression is unusual in the s-t breakup population.


Above: Same raceme; day 0 (left) with cream sector; day 2 (right) with yellow expression. This plant is likely simplex for the purple gene (P), and the chromosome, or segment of chromosome carrying P was lost to produce the sector.

Dendritic cercospora leaf spot (below) got our attention. The first time we observed it was in 2011 in the field. However, more of it was observed in 2012 on alfalfa as well as this material. Hence, it is a disease, and probably has nothing to do with the leaf spot on M. truncatula cv. Jemalong.

This file was created in 2011, and we were not paying attention to truncatula leaf shape and serration pattern. In looking at the pictures in this file in 2012, it is interesting that most of the leaves in the pictures resemble M. truncatula in shape and serrations.


Section on Anthocyanin Expression in Leaves

Below: M. truncatula A17. Field grown, Fall 2012, cool days, light frosts. Anthocyanin expression is intense on all Medicagos in fall. Picture (left) is top side of leaf.

Above: Picture (right) is bottom side of leaf. About 40% of A17 leaves show some pigment on bottom side under these cool fall conditions. (The same is true for 4x J) This much pigment on the bottom side in not seen under greenhouse conditions, or in summer. This plant was transplanted to the GH on 10-15-12, and on 12-12-12 had weak anthocyanin expression, and many leaflets showed no top mark on 12-12-12. No leaflets showed marks on the bottom side.

Note the detached upper leaflet in both pictures. The detached leaflet when held up to sun light in the following picture, indicates that the anthocyanin pigment is in the epidermis of the leaf. The top side is toward the sun, and bottom side is photographed. The top and bottom spots are both visible. It appears that the mesophyll contains no anthocyanin. It was necessary to know this because there are many anthocyanin variants in the s-t populations, and some appear to involve the mesophyll.

Below is an anthocyanin variant in the s-t population in mid October. The frequency of such variants in the s-t material is higher and more extreme than in alfalfa populations at this time of year. Note the pattern of anthocyanin expression in this variant. The anthocyanin is around the outer edges of the leaflets, and the mid-veins are green.

Above: Left picture is the upper side of the leaflets. Right picture is of the lower side. The anthocyanin pigmentation is more intense on the lower side, and appears to be in the epidermis.

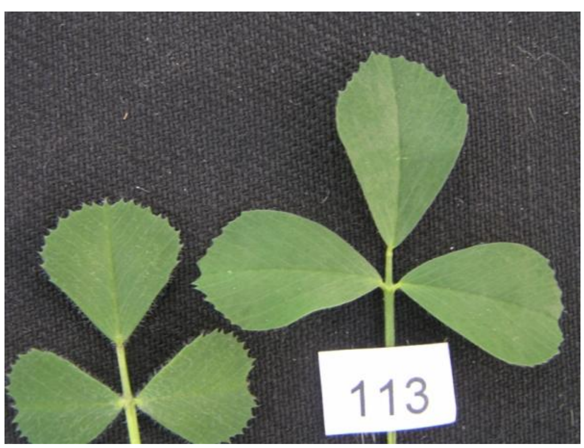
Note in the pictures below that the pattern of pigmentation on two other s-t plants, radiates from the mid-vein, and the edges are green. Pigmentation appears to be in the mesophyll. I have never seen either of these patterns before in any Medicago species, or any hybrid population.

The picture below of leaflets collected in the field, fall 2012, bears on at least three issues found in the s-t material. Firstly, the anthocyanin pigmentation in the mesophyll seems to radiate out from the mid-vein, in an alfalfa-type leaf on the left, and in an M.truncatula-type leaf in the center. Secondly, the leaflet shape and serrations on the center and right leaflet resemble those of M.truncatula. Note that the serrations are in the upper half of the leaflets. Thirdly, the leaflet on the right expresses a pattern in the mesophyll, but no anthocyanin. This causes me to wonder if the leaf spot in M.t. Jemalong might actually have two levels of genetic control. Level one could be the position/shape, and level two could be production of anthocyanin. I wonder if there is literature on this in M. truncatula ? The leaflet on the right looks like a water mark. These traits that were expressed in the field, but were not expressed in the greenhouse.

The M. truncatula leaf shape/serration condition in the s-t material is quite common, 15-20% of the plants. Anthocyanin variation is also relatively common, 10-15%. This is higher than in alfalfa check populations. Also, we have not seen the pattern variants in check populations at this time of the year (or any other time).

In summary, we see variation in leaf shape/serrations that could come from M. truncatula. And, the anthocyanin expression system seems upset, which could happen in a wide hybrid. However, we have not seen an M.t. anthocyanin spot in the epidermis in the s-t material, yet. The above observations are from a population of about 2000 s-t F3 plants that survived out of about 5,000 seeds sown in the field in mid August 2012, and carefully managed. Close to 80% of the seeds germinated, but there was much lethality beginning soon after emergence. When we noticed this, we “pickled” a jar of seedlings and small abnormal plants that were obviously not going to survive. There are several types of gross developmental abnormalities including some green growths with no structure. This makes me wonder about the genetic material that is still being lost in the F3, as evidenced by seedling lethality, and the various sectors observed in flowers, leaves, and plant body. When will the material stabilize? What are the mechanisms producing the variation?

Variation Recorded in the Green House in 2013 on F3 Plants



Above: 4x J (left), and 4x M. sativa X truncatula (s-t) derivative 113 (right). Plant 113 leaflet shape and serrations resemble M. truncatula.

Above: Abnormal fasciated shoot on a plant number 2 from a cross of plants derived from crosses of alfalfa X 4x J.

The above plant from an F3 s-t population lacks a crown. Such plants redevelop after cutting from above-ground nodes. About 5% of F3s lack a crown. This trait has not been observed in alfalfa populations.

Pictures of flowers with sectors follow. This probably reflects differences in timing of cell division, occasionally resulting in loss of a chromosome, or piece of chromosome carrying a pigment gene. We assume most, if not all other chromosomes are involved in occasional losses, but the effects are not easily observed.

Above: An example of loss of the chromosome carrying the ‘P’ allele for purple flower color. This lets us observe expression of yellow. Although this material is tetraploid, some plants are simplex, i.e. one P allele, one Y allele, etc., and losses of one chromosome are easily observed.

Above: Loss of yellow expression; but, is it loss of timing, or loss of pigment production?

Above: Loss of purple in the lower half of a wing petal (left) in a plant with no genes for yellow. There is much evidence like this that indicates the wing petals are a fusion product in evolution, and each half of the fusion product is under separate control on different chromosomes. Plant 1134 above right, has loss of purple in the upper half of the wing.

Above: Loss of purple in half of the standard petal, permitting us to observe the yellow in this plant. Two shades of purple are also evident in the wing petals of this plant.

Above: Plant 111 in the F3 s-t population with multiple abnormalities, none of which appear derived from 4x J. Our interpretation at the moment is that the M. sativa genome was very disrupted by wide hybridization, and much or all of the M. truncatula material has been eliminated. Left to right: a shoot with odd multifoliolate leaves, terminating in an aborted flowering structure. The stem on the center shoot is flat due to fasciation. Shoot on the right has normal leaves, but abnormal racemes. This plant produced about a dozen racemes with a few abnormal flowers on each raceme. Nonetheless, about ten F4 seeds were produced by intercrossing with other F3 plants.

Below: Abnormalities of plant 111 continued. Close-up of three abnormal, self-tripped flowers.

Above: Another picture of plant 111 showing several of its abnormalities. The horizontal upper shoot has only two leaves showing in the picture; an abnormal simple leaf near the apex, and an abnormal trifoliolate near the middle. Note that the terminal leaflet on the trifoliolate leaf has a chlorophyll deficient sector, and the lateral leaflets are smaller and one is misshapen. This plant is “falling apart”.

Abnormalities on other plants continued.

Above: Plant 113 has many leaves with the shape and serrations of M. truncatula leaves, as discussed in an earlier picture. This picture shows aborted racemes on this plant. Raceme development is on a threshold in this plant, and although most racemes abort before flower development, occasional side branches have normal flowers with variegated flower color. Fortunately, this plant produced F4 seed, and produced seed in various test crosses

Above: Plant 114 has multiple abnormalities. Note that the serrations extend almost all the way around the leaflets, and some leaflets are smaller than normal. Also, leaflets are elliptical instead of normal obovate shape. This leaf condition does not resemble M. truncatula, M. sativa, or any Medicago that we have observed.

Below: Multifoliolate leaves are common in s-t populations.

Above: All of the primary leaves on plant 119 were multifoliolate, although the secondary leaves developing in the leaf axils are trifoliolate. Pebbles are holding down leaves of 119 for the photograph. Note that a puff of wind blew the top leaf of 116 onto the top leaf of 115.

Above: Plant 1110 has abnormal racemes with leaves and flowers, as well as abnormal flowers. Fortunately this plant produced seed in crosses with other s-t plants.

Above: Abnormal pod coiling on plant 1118. The plant had only two or three pods per raceme, but they contained seed.

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Above: Two cases of monstrous flower development where fusion of two flowers results in radial symmetry.

Above: Monstrous plant 1135 that had abnormal leaves, stems, racemes, flowers, and was sterile. The white arrow points to a raceme with simple leaves, i.e. one leaflet.

Above: A plant with extremely long racemes and irregularly spaced flowers. Note on the left, the variegated flower color with co-expression of purple and yellow pigments.

Above: Plant 1128 with variegated flowers, some of which are abnormal.

Above: Plant 1125 from the s-t population was cross fertile, and produced a few self seeds. Most s-t plants produce little or no pollen. About one in ten s-t plants have enough pollen to use in crosses with other s-t plants and advance the generations. About one in twenty five s-t plants produce a little self seed. Hence, plant 1125 is special. The extreme size variation of self progeny is shown in the picture. Less than half of the self seeds germinate and produce a seedling, and about one third of the seedlings are small and do not survive. The six plants in the picture are the survivors of twenty seeds planted in Jiffy-7 peat moss pellets in the greenhouse.

Above: A very unusual Medicago plant. It segregated in the s-t population. It is labeled: 1/1000, because it was the only plant of its kind in one thousand. No tap root, extremely fiborous roots, a prolifically branched crown, and yet it had a strong central shoot, represented by the long cut stem in the top of the picture. In the field, summer 2013, clonal propagules of this plant are semiprostrate similar to 4x J, whereas the alfalfa parents of the s-t population were upright.

Thus far, focus has been on variation that can be seen with the naked eye. How much biochemical and physiological variation is there that cannot be detected by eye?