Preface to the following report. It was written for the Alfalfa Genome Conference in 1999, but not included because Joe Saunders decided he wanted to lengthen it and submit it to a journal. Shortly thereafter Joe was diagnosed with brain cancer. Joe lost the battle against cancer April 7, 2002. We sorely miss him. To our knowledge the report was not published, at least in this form. Joe's insights about early tissue culture events based on his many years of experience make for fresh and exciting reading.

ALFALFA SHOOTS AND SOMATIC EMBRYOS FROM CALLUS

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In vitro culture of alfalfa anthers to obtain haploids began in the Bingham lab in 1969, inspired by the published success in tobacco, Datura and rice. Using 10-20 greenhouse ramets (vegetative cutting copies) of tetraploid clone S-4 from 'Saranac', within a year we had callus from anthers, ironically on a Blaydes medium formulation (our code BII) identical to that effective in the rice in vitro androgenesis (Niitzeki and Oono, 1968). Very small green structures were seen on some calli, especially after early transfer to fresh BII, but transfer to some similar media without 2,4-D led easily to shoots, a response which was most intense on the Blaydes formulation without growth regulators but with included inositol and yeast extract (our code B0i2Y). Shoots were rooted in liquid medium on curious cardboard wick bridges (Saunders and Bingham, 1972) for transfer to greenhouse and field. Most of the resultant plants were very similar to the source tetraploid clone, and 5 of 166 were octoploids, conspicuously different in appearance. However, no haploids were found. Histological work indicated that the callus arose from interlocular connective tissue in the anthers.

Copious callus could also be obtained with BII medium from cotyledons, hypocotyls, internodes, and immature ovaries. Callus from octoploid and a seed-derived haploid plant from S-4 regenerated shoots nearly as well as S-4. But using seedlings and the simple media sequence BII-B0i2Y, we found that only 4 of 34 'Saranac' individuals and 1 of 26 'Vernal' plants produced shoots from callus (Saunders and Bingham, 1972). Overshadowed by the failure to obtain haploids and by the recovery of octoploids from S-4, the production of an unthrifty dwarf and several albinos from S-4 callus was noted (Saunders and Bingham, 1972) but went unrecognized as a harbinger of the coming general interest in somaclonal variation.

That original publication on recovery of alfalfa plants from callus started the first avalanche of tissue culture research on a major crop species (other than tobacco), despite the failure of obvious followup publications from some other labs to cite it. Although unfortunately the title and abstract of that first seminal paper didn't mention them, somatic embryos were described and depicted in that paper as one route leading to shoots. A mass of secondary embryos on the

hypocotyl of a primary somatic embryo was recorded in one of the photos. We had two later experiences with somatic embryos, although at the time we seriously underestimated their value and subsequent interest in them. The first unpublished experiences involved our only attempts to produced suspension cultures. Several grams of S-4 callus from BII medium were placed in each of several one-liter Erlenmeyer flasks containing 500 ml of liquid B0i2Y medium. The flasks were shaken on a machine in an unused out-of-the-way lab that discouraged frequent observation. After a month I visited the cultures to find in each flask a marvelous large bright opaque green mass of primary and secondary somatic embryos, with very little other living tissue in the flask. It was extremely esthetic.

Years later I started to understand what process took place in those flasks. With the absence of a strong auxn in B0i2Y, callus growth soon ceased. Growth of primordia into shoots was denied, probably by the submerged conditions. Embryos were the only growth outlet for the biological production potential pent up in the medium, yet embryo development could not progress into germination, again perhaps because of submersion. The only remaining developmental outlet available for further biomass production (growth) by the culture then became secondary embryo production from the hypocotyls regions of the primary embryos. We used some of these embryos for a novel mutagenic effort mentioned in Bingham and Saunders (1974). Since it was likely that secondary embryos arose from epidermal or at least sub- epidermal cells on the hypocotyl of the primary embryo, we treated a number of somatic embryos of S-4 with ethidium bromide, which at that time was being used as a mitochondrial genome mutagen by fungal geneticists. The treated embryos were washed and placed on semi-solid B0i2Y medium, where they produced a number of secondary embryos which we grew out into plants. One had leaflets with irregular margins, epinastic curvature, and one lateral leaflet missing in some leaves (Bingham and Saunders, 1974). I moved on in my career at that point, and lost track of the many regenerant plants that I had left behind.

S-4 was used for a more detailed study of the effects of the growth regulators (2, 4-D, NAA, and kinetin) used in BII on conditioning the initial callus for subsequent shoot regeneration on semi-solid B0i2Y. It was a huge 252 treatment 6x6x7 factorial experiment, which produced a positive amazingly straight line major effect of 2, 4-D concentration on subsequent shoot regeneration. NAA, the other auxin in the experiment after 2, 4-D, had a negligible effect, and the cytokinin kinetin had a minor effect (Saunders and Bingham, 1975). Based on that information, it was possible to design an experiment wherein S-4 and several other 'Saranac' clones could be phenotypically characterized two-dimensionally for their response intensity and breadth over a wide range of initial 2, 4-D concentrations (Saunders and Bingham, 1975).

After we had established the repeatability of shoot regeneration from callus, Bingham charged me, as a doctoral candidate, to come up with applications for that phenomenon. Initial efforts to propagate callus from root nodules, and to regenerate octoploids and who knows what else from such callus, were stymied

by failure to obtain uncontaminated cultures. My next idea was to try to obtain hexaploids from endosperm of immature S1 seed of S-4. The simplest approach was to place excised immature seeds onto the callus-inducing BII medium. Some response was obtained from 3 days post-(self)pollination immature seeds taken from the greenhouse in warm weather, but immature seeds taken up to ten days post-(self)pollination were responsive in highest frequency. The response was generally a slow but continued swelling of the young seed, followed by the transient appearance of an opaque green body (apparently the zygotic embryo) nearly filling the seed. After the green body reached its maximum size, the clear seed covering split open, and callus spilled out as the green body then lost its entire integrity while growing into a mass of largely colorless callus.

Transfer of this callus in standard fashion to medium B0i2Y for shoot regeneration and plant recovery yielded tetraploid plants collectively resembling S1 plants from S-4. This indicated that most of the callus originated from the zygotic embryos, rather than endosperm or even maternal (integumental) tissue. This work was prematurely terminated by my departure from Madison, and a subsequent manuscript was turned down by Crop Science because we didn't have convincing evidence for the origin of the callus. However, the ability of BII medium to first foster what appeared to be embryonic growth, prior to the proliferation of callus, within the developing seed, is worthy of investigation relative to interest in salvaging progeny of interploidy, interspecific or intergeneric crosses. Was it a coincidence that BII was a medium capable of both initiating only the earliest steps of somatic embryogenesis in callus, and of nurturing (apparently) early to intermediate stages of zygotic embryogenesis in ovulo?

S-4 and three other 'Saranac' clones identified as shoot regenerators from callus in the first screening of 'Saranac' were intercrossed with a regenerator clone from 'DuPuits' at Madison after I left. This led to the public release of 'Regen-S' as a tissue culture amenable alfalfa germplasm for genetic manipulations (Bingham et al., 1975), and inspired my own later development and release of popular analogous sugarbeet germplasms REL-1 and REL-2 (Saunders, 1998). An aspect of the development of 'Regen-S' that arose later was whether regenerant propagules of any of the genotypes selected for their regenerability were used in sexually producing the succeeding generation. This question of whether somaclonal variation for greater regenerability, and unconscious selection for such cells in the production of any regenerant later used in developing 'Regen-S', could not be unambiguously resolved by reading the original publication, written in the mid-1970's when most regenerants from callus were generally viewed as identical copies of the source genotype, at least in our minds.

As I look back, I see several important oversights that were probably excusable at the time. One was my failure to maintain distinct identities for every callus and regenerant plant. Only by such care can credible statements about the frequencies of various independently occurring somaclonal variations (including different ploidies) be made. However, one notices in the literature that such care in describing the origins and meaningful frequencies of somaclonal variants is

rather uncommon. Secondly, there was a period of time in the 1970's when it was presumed that regenerant plants with a doubled chromosome number had been cleanly polyploidized, with the doubling of chromosome number being the only genetic change, and that these could be used in place of colchicine-doubled genotypes. But as we know today, most likely other much less noticeable genetic alterations could also have been found if we had looked or had the techniques to closely look. In closing, let me share with you the lasting graphic image of regenerant alfalfa that stays in my mind: the downcast facial geometry of a forlorn captive trifoliolate alfalfa leaf pressed up against the inside wall of a glass culture vial: high cheeks formed by the two lateral leaflets, forehead by the terminal leaflet, and narrow eyes outlined by the linear gaps between the terminal and lateral leaflets.

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