# SOMATIC CELL SELECTION CRITERIA FOR WATER USE EFFICIENCY USING GENETICALLY DIFFERENTIAL ALFALFAS

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ABSTRACT

Development of crops that are water use efficient would make an important contribution to the conservation of this critical resource. Laboratory methods of genetic engineering, such as somatic cell selection, promise to be faster and more precise in the development of water use efficient crops compared to conventional breeding, provided that appropriate selection criteria can be identified. This project attempted to define somatic cell selection criteria by using a series of genetically related alfalfa populations bred by conventional methods to be increasingly more water use efficient.

The preliminary data generated by this project lend support to the following conclusions: (1) cell survival under osmotic stress was not an adequate cell selection criterion for genetic improvement of water use efficiency; and (2) titers of polyamines in cell cultures and whole-plants under stress corresponded to the degree of water use efficiency known to exist in the respective parental populations. If polyamine titers represent biochemical indices of water use efficiency, they could be used to devise an appropriate whole-plant or cell selection strategy.

Key words: water conservation, alfalfa, crops, plant breeding, genetics, plant tissues, culture

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# INTRODUCTION AND LITERATURE REVIEW

It is well known that most--about 85 percent--of the available water in states such as New Mexico is diverted to irrigated agriculture. An important way to conserve our limited water resources is to utilize crop varieties that will make reduced demands on irrigation water supplies. However, it is difficult and time consuming to breed crop varieties with improved water use efficiency. Plant tissue and cell culture systems and other laboratory based tools of genetic engineering offer the unrealized potential to speed the efficient development of new crop varieties. Somatic cell selection procedures exploit the potential advantage of screening millions of cells grown in a small laboratory space for short periods of time (weeks), compared to conventional breeding programs where thousands of plants require an acre of land for an entire growing season to perform a single cycle of selection. Plants regenerated from selected cells then offer potential usefulness in breeding programs. The goal of this research project was to evaluate the criteria needed to establish a somatic cell selection procedure aimed at the development of water use efficient crop varieties.

The first well documented effort to evaluate cultured plant cells under simulated water deficit stress utilized increasing levels of high molecular weight polyethylene glycol (PEG) as a selection pressure (Bressan et al. 1981). Cherry tomato cells placed in culture under stress conditions exhibited a 14-fold increase in growth following selection at -11 bars PEG, compared

to cells never previously exposed to PEG. The increased resistance was stable under sustained stress, but was lost upon transfer back to non-stress conditions, suggesting that the change was epigenetic rather than genetic in nature. These selected cell lines displayed a large capacity for osmotic adjustment under stress, showing elevated levels of reducing sugars, counterions, quaternary ammonium compounds, and proline (S. Handa et al. 1983). However, no plants could be regenerated from these cells selected for water deficit stress tolerance. This approach remains unvalidated as a means for genetic improvement of crop plants (A.K. Handa et al. 1983, Hasegawa et al. 1984).

This group of investigators could not determine whether true selection of genetically variant cells within the initial population of cells, or physiological adaptation of the cells to the simulated water deficit stress, had occurred. They suggested that a combination of these two mechanisms were likely to be involved. In a review of environmental stress tolerance in cultured plant cells, these investigators concluded that "...selection by exposure to [PEG induced water] stress is unable to discriminate between the innately tolerant and intolerant cell types within the population..." using current somatic cell selection procedures (Handa et al. 1982). They go on to state "...that understanding the nature of the selection mechanisms is particularly germaine in obtaining variant cells which possess agriculturally useful traits." They did not indicate how this could be accomplished, but they suggested that biochemical assays

of key metabolites or enzymes may be a better measure of tolerance than simple growth under stress conditions. Indeed, the most successful cases of plant somatic cell selection have been based on specific biochemical pathways (Flick 1983, Maliga 1980). The question now becomes: which biochemical pathways distinguish water use efficient from inefficient plants?

Many compounds have been implicated in osmoregulation leading to drought tolerance. High concentrations of proline are found in some halophytes (Stewart and Lee 1974) and glycophytes (Routley 1966, Waldren et al. 1974, Greenway and Munns 1980) under water deficit stress. Organic compounds such as betaine (Grumet et al. 1985), malate, citrate (Stewart and Bogges 1977, S. Handa et al. 1983), putrescine, spermine, spermidine (Flores and Galston 1984), and others were found in different species under water deficit stress. But it is not clear whether any of these compounds represent causal mechanisms of stress tolerance.

Ideally, isogenic lines are used to identify causal mechanisms (or, at least, significant differences) by comparative studies. But isogenic plants differing only with respect to water use efficiency or drought tolerance are not available. However, genetically related plants are available that differ in degree of water use efficiency. These plants are a series of alfalfa plant populations including three selection cycles obtained by phenotypic recurrent selection and the original cultivar "Mesilla" (Wilson et al. 1980, Currier et al. 1982, Currier and Melton 1983). This research project represents the first attempt to utilize genetically differential plants to

investigate the causal mechanisms of water use efficiency in crops. Significant differences found among these test materials would then be used as a basis for developing somatic cell selection criteria. The efficacy of cell selection could then be compared to traditional recurrent selection for development of water use efficient crops.

The technologies are available for somatic cell culture of alfalfa (McCoy and Walker 1984). Somatic cell selection has been carried out in alfalfa to obtain salt tolerance (Croughan et al. 1978) and to achieve overproduction of methionine (Reisch et al. 1981). Plant regeneration from cultured alfalfa cells is under genetic control (Bingham et al. 1975, Reisch and Bingham 1980). Plants have been regenerated from alfalfa cells following selection (Stavarek et al. 1980, Reisch and Bingham 1981). Non-dormant and semi-dormant alfalfas adapted to the Southwest have been screened and regenerator genotypes identified (Phillips 1983). The tools and materials are therefore available to test a somatic cell selection program in alfalfa as a model species. The final steps required to validate the somatic cell selection criteria that may result from this (or a similar) research project are, first, to perform a cell selection program based on the putative criteria identified, second, to determine whether the desired trait is a heritable improvement compared to the unselected controls, and third, to compare the cell selection approach with recurrent selection breeding for relative speed and efficiency of selection.

The specific objectives of this research project were:

1. To grow the genetically related alfalfa populations exhibiting differential degrees of water use efficiency as cell cultures, and determine their growth responses under simulated water deficit stress.
2. To evaluate whether cellular growth responses under water deficit stress corresponded to the known responses of the parental populations as whole plants, and if so, whether cell growth indices might serve as suitable somatic cell selection criteria for development of water use efficient plants.
3. To evaluate biochemical indices as potential indicators of water deficit stress tolerance that could be developed into somatic cell selection criteria for improving water use efficiency, if cell growth indices did not appear to serve as suitable selection criteria under objective 2.

# METHODOLOGY

Seed of four alfalfa, *Medicago sativa* L., populations showing differential degrees of water use efficiency in the field, were provided by Drs. Bill Melton and Cliff Currier, New Mexico State University Department of Agronomy and Horticulture (Wilson et al. 1980, Currier et al. 1982, Currier and Melton 1983). These populations included the original cultivar "Mesilla" (designated MES-0, least water use efficient), and the successive three cycles obtained by phenotypic recurrent selection for improved or comparable yields and quality under limiting water, such as one-third or two-thirds as much water as needed for optimal growth. These cycles were designated MES-1 (less water use efficient), MES-2 (more water use efficient), and MES-3 (most water use efficient), respectively. Early experiments also utilized "Zia" (less water use efficient), 9-D-11A (a selection out of Zia-derived materials with more water use efficiency), and Zia-81 (first cycle obtained by phenotypic selection with more water use efficiency).

Random samples of seed lots of each population were surface sterilized and sown on media for aseptic germination following standard procedures (Phillips and Collins 1984). Epicotyls of seedlings were excised and inoculated onto standard callus induction media (Phillips 1983). Callus cultures were maintained, and cell suspension cultures established from these callus cultures, following the usual tissue culture procedures for alfalfa (McCoy and Walker 1984).

In one experiment, whole-plants were analysed for comparison to cell cultures. Sixty plants of each of the MES populations were grown in drought boxes in the shadehouse as described previously (Salter et al. 1984). One box was maintained at field capacity moisture level (control) while the other was permitted to dry to the point of inducing wilt in all of the plants.

Osmotic stress was simulated in laboratory cultures by the addition of mannitol, PEG (MW-8000), and/or a salt mixture composed of 85% NaCl with 15% $CaSO_4$, into the standard culture media (Phillips 1983, Phillips and Collins 1984).

Dry weight analyses were obtained from aliquots of cell cultures in the usual manner (Bottino 1981). Fresh weight increases of callus masses were measured directly or estimated by volumes relative to control cultures. Amino acid analyses were performed by extracting the free fraction with 2 N HCl through a Dowex 50Wx8 cation exchange column and quantitating by standard procedures on a Durrum Model 400 Amino Acid Analyzer.

Samples were analyzed for polyamine titers by grinding at $4^{o}C$ with 10% trichloroacetic acid (70 mg tissue/ml) in a homogenizer. The supernatant solution was extracted three times with ethyl ether, and the aqueous supernatant containing the free cellular polyamines was chromatagraphed on a Dowex column. The polyamine fraction was then dansylated and quantitated using a Pharmacia automated FPLC system, PepRPC HR5/5 bonded phase column and Glenco Model MM70 Analyzer (Kremmer et al. 1985). Quantitation was achieved using a relative calibration procedure with hexanediamine as the internal standard (McNair and Bonelli 1969).

## EXPERIMENTAL RESULTS

### Seed Germination under Osmotic Stress

Seeds were germinated from each of the available test populations to provide explant tissues for initiating stock callus cultures for experimentation. As an initial indication of the responses of these test populations to artificial osmotic stress, seed samples were germinated under standard conditions and under various kinds of osmotic stress induced by PEG, mannitol, and/or $NaCl:CaSO_4$ (85:15, w:v) salt treatments. A sample size of 12 seeds for each population by treatment combination was used in this preliminary experiment.

All test populations except one germinated well under control conditions, as anticipated (table 1). All osmotic stress treatments reduced germination rates, as expected. Germinating seedlings appeared to be more sensitive to mannitol than PEG. Treatments including high salts or a combination of salts with mannitol resulted in almost total inhibition of germination. The three Mesilla derived populations exhibited the expected populational responses to the 10% PEG treatment, but the three Zia derived populations showed a response converse to that expected, based on previously determined whole-plant water use efficiency in the field. Possible interpretations of these results include: (a) sample sizes were too small to obtain accurate results; (b) seedling response did not correlate to whole-plant response; (c) none of the osmotic stress treatments adequately simulated moisture stress in the field, or moisture

Table 1

Response of alfalfa populations selected for differential degrees of water use efficiency to osmotic stress treatments applied during seed germination

| Treatment[a] | Populations | | | | | |
|---|---|---|---|---|---|---|
| | MES-0 | MES-1 | MES-2 | Zia | 9-D-11A | Zia-81 |
| | Percent Seed Germination[b] | | | | | |
| Control | 92 | 67 | 100 | 100 | 100 | 100 |
| 10% PEG | 25 | 50 | 75 | 83 | 50 | 17 |
| 15% PEG | 25 | 0 | 8 | 8 | 58 | 0 |
| 10% Mannitol | 25 | 0 | 8 | 33 | 17 | 50 |
| 15% Mannitol | 0 | 0 | 0 | 0 | 8 | 0 |
| 1% Salts | 25 | 0 | 8 | 50 | 42 | 25 |
| 2.5% Salts | 0 | 0 | 8 | 0 | 0 | 0 |
| 10% Mannitol + 1% Salts | 0 | 0 | 0 | 0 | 0 | 0 |
| 10% Mannitol + 2.5% Salts | 0 | 0 | 0 | 0 | 0 | 0 |
| 15% Mannitol + 2.5% Salts | 0 | 0 | 0 | 0 | 0 | 0 |

[a]All treatments were based on the use of seed germination media (Phillips and Collins 1984).

[b]Seed germination rates were based on sample sizes of 12 seeds; germination was scored after 30 days.

stress was unrelated to water use efficiency; and (d) a combination of these factors or others were involved.

Callus Growth under Osmotic Stress

Additional seedlings were germinated under control conditions as described above to provide explant tissues for callus initiation. Fifteen callus lines from each test population were established. Duplicate callus pieces from each line representing each population were inoculated onto standard callus culture media with comparable osmotic stress treatments as for the seed germination experiment above. The callus lines representing MES-2 were lost to microbial contamination before data could be collected.

Callus appeared to be less sensitive to osmotic stress treatments (table 2) compared to seedlings (table 1). Some populations, e.g. MES-0, responded with reduced growth under more severe stress, as expected (table 2). Some lines, e.g. MES-1, were more sensitive to PEG as an osmotic stress agent, while others, e.g. MES-0, were more sensitive to mannitol as an osmotic stress agent. The three Zia derived populations responded in the predicted differential manner under 10% PEG treatment, but few other correlations of callus to previous whole-plant responses were observed. Similarly as with the seed germination experiment, the results of this preliminary experiment with callus cultures were equivocal. However, it was decided to utilize a treatment of 10% PEG to simulate osmotic stress in the further experimentation. This treatment is comparable to that

Table 2

Response of callus cultures of alfalfa populations selected for differential degrees of water use efficiency to osmotic stress treatments

| Treatment[a] | Populations | | | | |
|---|---|---|---|---|---|
| | MES-0 | MES-1 | Zia | 9-D-11A | Zia-81 |
| | Percent Callus Volume[b] | | | | |
| Control | 100 | 100 | 100 | 100 | 100 |
| 10% PEG | 83 | 30 | 60 | 64 | 75 |
| 15% PEG | 33 | 20 | 48 | 36 | 50 |
| 10% Mannitol | 67 | 55 | 92 | 60 | 75 |
| 15% Mannitol | 33 | 13 | 80 | 31 | 50 |
| 1% Salts | 67 | 70 | 40 | 67 | 25 |
| 2.5% Salts | 67 | 50 | 60 | 48 | 40 |
| 10% Mannitol + 1% Salts | 33 | 20 | 48 | missing | 40 |
| 10% Mannitol + 2.5% Salts | 33 | 5 | 20 | 24 | 50 |
| 15% Mannitol + 2.5% Salts | 17 | 0 | 8 | 12 | 0 |

[a]All treatments were based on standard callus media (Phillips 1983, Phillips and Collins 1984).

[b]Callus volumes were visually estimated relative to the control for each population; growth was scored after 30 days. Estimates were based on an composite score for 15 lines representing each population. The relative growth of each population based on a scale of 0=non-viable to 5=most prolific was: MES-0=3, MES-1=4, Zia=2.5, 9-D-11A=4.2, Zia-81=2.

utilized in prior reports on plant cell selection for water deficit stress tolerance (Bressan et al. 1981). It was also decided to limit further experimentation to the Mesilla derived populations.

Another observation made during this experiment was that each population was represented by callus lines (genotypes) which, under virtually all of the stress treatments, showed the extremes of response. In other words, some callus lines of each population were completely susceptible to any stress treatment, while a few showed nearly complete tolerance to most stress treatments. This observation has two important implications. First, it is likely that every alfalfa population contains both susceptible and tolerant genotypes. Indeed, this likelihood is probably the basis of the capacity of conventional breeders to improve alfalfa cultivars for virtually any trait (Dr. Bill Melton, New Mexico State University, personal communication). Second, the observation that each population is represented by callus lines that exhibit the extremes of growth response suggests that the variation within populations covers a similar range of response as among populations. Therefore, the variances of quantitative growth measurements would be so large that statistical differences would be difficult to obtain. This issue was addressed in the next experiment.

## Estimation of Standard Errors in Callus Growth Rates

Seeds of the three Mesilla derived populations were again germinated and callus established as before. During the second

and third months of culture, fresh weights of each of ten callus lines were obtained to represent each population under standard and stress conditions (table 3). Standard errors were large, but each population showed proportionately similar variation. Although the growth rate of MES-2 could be distinguished from the growth rates of the other two populations under standard conditions, none of the growth rates could be distinguished under stress conditions. These results could be interpreted either as a failure to establish a correlation of growth rates between callus cultures under osmotic stress and corresponding whole-plant capacities for water use efficiency, or that the callus sampling procedure was inadequate to represent growth differences that may actually exist among these populations.

Amino Acid Profiles

From the previous set of materials, a single callus line was chosen to be representative of water use efficient MES-2 based on tolerance to osmotic stress. This callus line was established as a cell suspension culture. The cell culture was exposed to 10% PEG stress and sampled for amino acid analysis at the time of stress induction (control) and after 6 and 12 hours of stress (table 4). The intent of this preliminary experiment was to ascertain whether it would be worthwhile assaying amino acid titers as a biochemical indicator of stress tolerance. Certain trends were apparent: most of the amino acids exhibited a general increase over time during stress development, with transient increases at 6 hours for most of the rest of the amino

Table 3

Estimation of alfalfa callus fresh weight growth rates by generating means and standard errors from ten lines representing each population

| Treatment[a] | Populations | | |
|---|---|---|---|
| | MES-0 | MES-1 | MES-2 |
| | Fresh Weight Increase (mg/mg of inoculant) Mean $\pm$ Standard Error[b] | | |
| Control | 3.83 $\pm$ 1.84 | 4.90 $\pm$ 1.86 | 7.04 $\pm$ 2.53 |
| 10% PEG | 2.68 $\pm$ 2.06 | 2.70 $\pm$ 2.10 | 5.28 $\pm$ 3.59 |

[a] All treatments were based on standard callus media (Phillips 1983, Phillips and Collins 1984).

[b] Means and standard errors were based on samples of ten callus lines representing each population, each scored twice after 30 days growth (at the ends of the second and third months of incubation).

Table 4

Amino acid profiles under development of osmotic stress of a tolerant line of alfalfa grown as a cell suspension culture

| Amino Acid | Retention Time | Hours after Stress Induction 0 | 6 | 12 |
|---|---|---|---|---|
| | | Integrated Area x $10^3$ | | |
| Aspartic acid | 298-300 | 374 | 442 | 462 |
| Threonine | 324-326 | 370 | 437 | 474 |
| Serine | 342-344 | 1405 | 1743 | 1948 |
| Glutamic acid | 408-410 | 1174 | 916 | 797 |
| Glycine | 496-497 | 247 | 288 | 332 |
| Alanine | 513-518 | 1211 | 1553 | 1704 |
| Valine | 567-568 | 496 | 591 | 644 |
| Isoleucine | 647-648 | 181 | 222 | 264 |
| Leucine | 671 | 182 | 231 | 277 |
| Tyrosine | 752-753 | 372 | 415 | 389 |
| Phenylalanine | 781 | missing | 227 | 328 |
| Lysine | 875 | 121 | 165 | 121 |
| Histidine | 976-977 | 215 | 246 | 214 |
| Gamma-Aminobutyric acid | 815 | 763 | 1225 | 764 |

acids, with only glutamic acid showing a general decrease in titer during stress development. These trends were compatible with the interpretation that, generally, the plant undergoes degeneration during stress with increases in amino acid titers due to protein breakdown. There was no single amino acid or group of amino acids that showed a large change that would indicate a relationship to a tolerance mechanism. However, the profiles lacked data on proline, which had been implicated with stress tolerance in the literature; and arginine, which was implicated in the polyamine biosynthesis literature. There had been difficulties in getting reliable and complete analyses from the local amino acid analyzer during this period of time, also; therefore, it was decided to abandon further experimentation on amino acid profiles.

Growing Alfalfa Cell Cultures as a Combination of Lines

Plant tissue and cell cultures are normally grown as cultures derived from individual genotypes or explants. Replication is then achieved by duplication of an individual line, or sampling of several genotypes within the cultivar or species as multiple cultures. This is the approach taken so far in this project. However, this approach is parallel to a representation of a cultivar or population as monoculture plots of whole-plants with multiple plots averaged to represent the population. Whole-plant experimentation is not conducted in this manner with alfalfa or other cross-pollinated crops. Whole-plant research with cross-pollinating crops is normally conducted by

representing numerous genotypes within a plot, and then replicating the plots. The question for the cell culturist becomes: how does one represent a population of plants within a single cell culture flask, such that cell cultures can be replicated in flasks in a manner parallel to plot replication in the field? A review of plant cell culture literature did not reveal a ready answer to this question.

In the previous callus induction experiments conducted for this project, it had been observed that about 10-15% of the cultures derived from each population were responsible for the extremes in callus grown rates under standard conditions. About 85-90% of the callus cultures exhibited similar growth rates. It was speculated that a combination of equal volumes of callus from a large number of genotypes or lines might be feasible for the establishment of a cell culture that represented the parent population in a manner parallel to a whole-plant plot. The alfalfa breeders recommended that a minimum of 60 genotypes were needed to adequately represent the genetic variation present in any given population (Dr. Bill Melton and Dr. Cliff Currier, New Mexico State University, personal communication).

With these considerations, 120 seedlings of each of the four Mesilla derived populations were germinated. About 110 seedlings of each line established callus successfully. Each population was then represented by 80-100 callus lines that showed somewhat comparable callus growth rates. Equal volumes (about 0.5 g fresh weight) of each of these callus line were combined into single flasks (500 ml of culture media in 1 liter flasks) to represent

each population. These cell cultures were maintained as stock cultures for each population. It was predicted that each stock culture should maintain a reasonable balance of at least 60 genotypes for periods up to 6 months without significant drift to small numbers of genotypes within a stock culture, based on observed growth rates.

Over a period of months, aliquots of these stock cultures were inoculated onto control and PEG containing media, incubated for two weeks, and dry weight analyses performed (table 5). MES-0 showed sensitivity to all levels of osmotic stress, with virtually complete inhibition of growth at levels of 8% PEG or higher. MES-1 and MES-2 actually showed an increase in growth under moderate levels of stress. MES-1 exhibited growth inhibition at levels of 24% PEG or higher, while MES-2 showed growth inhibition at levels of 28% PEG or higher. MES-3 showed relatively constant growth rates except under the highest levels of stress. Standard errors were large; however, there was a clear difference in growth rates under high levels of stress between the less water use efficient populations, MES-0 and MES-1, and the more water use efficient populations, MES-2 and MES-3.

In general, the four populations exhibited growth patterns under osmotic stress that could be said to be correlated to the predicted patterns based on known performance of these populations under field conditions. However, these data could also be used to argue that growth was not adequate as a criterion to distinguish water use efficient from inefficient genotypes,

Table 5

Dry weight analyses of alfalfa cell cultures grown as combinations of large numbers of genotypes under conditions of osmotic stress

| Treatment[a] | Populations | | | |
|---|---|---|---|---|
| | MES-0 | MES-1 | MES-2 | MES-3 |
| | Dry Weight (mg/5 ml Cell Culture)[b] | | | |
| Control | 9.2 ± 4.1 | 21.1 ± 10.0 | 84.9 ± 7.7 | 67.7 ± 13.9 |
| 4% PEG | 3.8 ± 1.3 | 21.6 ± 8.2 | 94.7 ± 6.2 | 52.4 ± 8.1 |
| 8% PEG | 1.6 ± 0.4 | 72.9 ± 33.1 | 97.2 ± 10.6 | 66.5 ± 11.1 |
| 12% PEG | 1.4 ± 0.6 | 74.9 ± 35.0 | 135.5 ± 16.8 | 63.3 ± 17.9 |
| 16% PEG | 1.7 ± 0.8 | 54.0 ± 20.4 | 111.2 ± 15.6 | 63.8 ± 17.4 |
| 20% PEG | 2.2 ± 0.4 | 29.8 ± 10.9 | 125.9 ± 34.7 | 50.8 ± 18.4 |
| 24% PEG | 1.3 ± 0.6 | 8.8 ± 1.6 | 92.8 ± 14.1 | 37.4 ± 10.7 |
| 28% PEG | 1.3 ± 0.6 | 6.2 ± 0.6 | 33.6 ± 5.2 | 25.0 ± 6.9 |
| 32% PEG | 1.1 ± 0.7 | 3.7 ± 1.0 | 14.8 ± 7.0 | 17.2 ± 9.0 |

[a]All treatments were based on standard cell suspension culture media (Phillips 1983, Phillips and Collins 1984).

[b]Means and standard errors were based on four replications of each population incubated for two weeks. Each population was represented by a cell culture established from a combination of 80-100 callus lines into a single flask; replications were inoculated into different flasks.

because growth under high stress was not completely inhibited in the less efficient population, MES-1. Because the standard errors were so large, it was unclear whether the more efficient populations, MES-2 and MES-3, could be distinguished from the less efficient population, MES-1, based on growth rate alone under high stress--even though the growth differential may provide an eventual advantage for the more efficient populations. Another observation of importance was that these data were generated as the means from four replications, but five replications were actually carried out: one of the five replications in each case consisted of zeroes, which indicated that sometimes no growth was obtained at all, due to unidentified factors beyond the control of the operator. These observations are relevant to the arguments presented previously in the literature (Handa et al. 1982).

Polyamines as Indicators of Cellular Osmotic Stress Tolerance

A leading hypothesis of biochemical mediation of plant water deficit stress tolerance concerns the polyamines (Flores and Galston 1984). In particular, it was hypothesized that the biosynthesis of spermidine (and possibly other higher molecular weight polyamines) from putrescine may represent the key protective factor during stress. Samples taken from the control cultures and the 16% PEG stress treatment from three of the Mesilla derived populations in the above experiment were prepared for polyamine analyses (samples from the other population were lost during preparation). Samples were analyzed for titers of

putrescine and spermine (table 6). These preliminary data indicated that the more water use efficient populations contain greater amounts of both putrescine and spermidine under stress conditions, but less of each under control conditions. These data indicated that the synthesis of polyamines may be induced in tolerant genotypes under stress conditions. Because specific suicide inhibitors of the polyamine biosynthetic enzymes are available (Flores and Galston 1984), it is possible to design somatic cell selection schemes for overproduction of polyamines based on selection for tolerance to the suicide inhibitors, which should result in altered (more efficient) forms of the biosynthetic enzymes. However, additional data are needed to corroborate these preliminary polyamine titer observations at the cellular level.

Polyamines as Indicators of Plant Water Deficit Stress Tolerance

In order to obtain some preliminary indication that polyamine titers in whole-plants corresponded to the trends observed in cell cultures, plants of the four Mesilla derived populations were grown in drought boxes, one maintained with optimum moisture levels and the other permitted to dry down for induction of full wilt in the plants. Shoot tips were then collected for polyamine analyses (table 7). The four populations were virtually indistinguishable with respect to putrescine, spermidine or total polyamine contents under control conditions. However, the two more water use efficient populations, MES-2 and MES-3, contained more spermine than did the less efficient

Table 6

Polyamine titers in control and osmotically stressed cell cultures of alfalfa populations selected for differential degrees of water use efficiency

| Treatment[a] | Populations MES-1 | MES-2 | MES-3 |
|---|---|---|---|
| | Putrescine (nmoles/g dry weight)[b] | | |
| Control | 74,099 | 33,930 | 34,839 |
| 16% PEG | 12,059 | 28,950 | 46,922 |
| | Spermidine (nmoles/g dry weight) | | |
| Control | 25,684 | 15,232 | 10,450 |
| 16% PEG | 7,710 | 10,210 | 11,592 |
| | Total Polyamine Contents (nmoles/g dry weight) | | |
| Control | 99,783 | 49,162 | 45,289 |
| 16% PEG | 19,769 | 39,160 | 58,514 |

[a]All treatments were based on standard cell suspension culture media (Phillips 1983, Phillips and Collins 1984).

[b]Single samples were analyzed.

Table 7

Polyamine titers in plants of alfalfa selected for differential degrees of water use efficiency grown under optimum moisture or fully wilted

| Treatment[a] | Populations MES-0 | MES-1 | MES-2 | MES-3 |
|---|---|---|---|---|
| | Putrescine (nmoles/g wet weight)[b] | | | |
| Control | 1,000 | 1,260 | 940 | 813 |
| Wilted | 86 | -0- | -0- | 607 |
| | Spermidine (nmoles/g wet weight) | | | |
| Control | 475 | 481 | 497 | 401 |
| Wilted | 138 | 333 | 212 | 589 |
| | Spermine (nmoles/g wet weight) | | | |
| Control | -0- | -0- | 249 | 107 |
| Wilted | -0- | -0- | 249 | 345 |
| | Total Polyamine Contents (nmoles/g wet weight) | | | |
| Control | 1,475 | 1,741 | 1,686 | 1,321 |
| Wilted | 224 | 333 | 461 | 1,541 |

[a]Treatments were established in drought boxes (Salter et al. 1984). The "control" box was maintained at optimum moisture level while the "wilted" box was permitted to dry to the point of incipient wilt of all plants.

[b]Single samples were analyzed, composed of shoot-tips combined from 15 plants. A "-0-" denotes that amines were not detected by the analytical quantitation method employed.

populations, both under control and stress conditions. Under stress conditions, the most water use efficient population, MES-3, clearly contained significantly more putrescine, spermidine, spermine, and total polyamines than did any other population.

By comparing the results of this whole-plant experiment (table 7) with the results of the cell culture experiment (table 6), the polyamine titer trends indicate a correspondence of response. This correspondence provides encouragement for utilizing polyamine titers, especially of the higher molecular weight spermidine and spermine, as indicators of water use efficiency both in whole-plants and in cell cultures. Further experimentation is needed to confirm these preliminary results and to ascertain a cause and effect relationship between polyamine titers and water use efficiency. If these results are confirmed, polyamine titers could be used as a screening method for more effective breeding of plants for water use efficiency, as well as to develop cell selection criteria for improvement of crop plants for water use efficiency. The fact that the yield and quality of these alfalfas are comparable supports the view that the trait of water use efficiency in these plants has agronomic value for selection or screening (Wilson et al. 1980, Currier et al. 1982, Currier and Melton 1983).

## SUMMARY AND CONCLUSIONS

This research project was exploratory, seeking to establish a basis for somatic cell selection criteria to be used to genetically engineer plants for improvement of water use efficiency. The experimental approach was to establish a correspondence of response between cultured cells and the known whole-plant response among a series of genetically related alfalfa populations selected for differential degrees of water use efficiency.

Growth rates of cell cultures or callus cultures derived from small numbers of genotypes within populations did not appear to be adequate as selection criteria because of large variances within populations. However, growing cell cultures as combinations of 80-100 genotypes representing each population appeared to yield growth curves under osmotic stress conditions that corresponded generally with predicted growth responses based on known whole-plant water use efficiencies.

Polyamine titers in cultured cells grown under osmotic stress corresponded well with polyamine titers of whole-plants from the same genetic populations grown under wilt conditions. Polyamine contents, especially of the higher molecular weight spermine or spermidine, were higher in the more water use efficient populations under stress conditions compared to the less water use efficient populations. These preliminary results are suggestive of an important component of the stress tolerance (water use efficiency) mechanism. If these results are

confirmed, polyamine assays could be developed into indices for breeding purposes, and the use of suicide inhibitors of the polyamine biosynthetic enzymes could be useful in a somatic cell selection scheme.

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