Plant Physiol. (1985) 79, 266–269
0032-0889/85/79/0266/04/$01.00/0

Physiological Changes in Cultured Sorghum Cells in Response to Induced Water Stress[1]

I. FREE PROLINE

Received for publication April 10, 1985

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ABSTRACT

Ten varieties of *Sorghum bicolor* (L.) Moench were grown as callus cultures under conditions of water stress, which was induced by addition of polyethylene glycol (molecular weight 8000) in the medium. Growth and free proline were estimated in the control and water-stressed cultures. In all varieties, proline levels were low in the absence of water stress and the levels increased in response to water stress. However, the magnitude of these increases were not correlated with stress tolerance of the individual varieties in culture. Thus increase in proline seems to be an incidental consequence of stress *in vitro* and not an adaptive response to combat water stress in sorghum.

Drought stress is very common in the sorghum-growing regions of the world, and several distinct varieties have evolved with varying degrees of drought tolerance (1). There are different mechanisms by which plants cope with water stress. One of the physiological responses to drought is the accumulation of intracellular solutes (16, 18). These are generally low mol wt metabolic products such as carbohydrates, amino acids, sugar phosphates, etc. The extent to which each of these plays a role in osmotic adjustment varies with the species. Accumulation of solutes in response to water deficits has been observed in leaves of sorghum (2, 8, 9). A similar phenomenon was also observed when plant cells were cultured under conditions of induced water stress using PEG (5). It was further shown that PEG itself did not contribute significantly to the osmotic adjustment (4).

There is virtually no information about the physiology of sorghum callus grown *in vitro* under osmotic stress. Recently, we have been able to show a certain degree of correlation between drought responses in the field and response to water stress in culture (manuscript in preparation). In this study, an attempt was made to analyze free proline levels in sorghum when grown as callus cultures with and without addition of PEG in the culture medium. The rationale was that this approach would enable us to look for any inherent differences at the cellular level that make some varieties better than others in terms of drought tolerance. In addition, growing tissue on different levels of PEG would indicate what metabolic changes occur as a result of water stress, and whether or not any of these changes confer any significant advantage to any variety to cope with water stress. Such knowledge is fundamental in selecting genotypes for breeding for drought tolerance.

[1] Supported by United States Agency for International Development Grant AID/DSAN/X11/G-0149. Technical Article No. 20160, Texas Agricultural Experiment Station, College Station, TX 77843.

MATERIALS AND METHODS

Callus Cultures. Callus cultures were initiated from sorghum seeds using the procedure of Smith *et al.* (14). Seeds of *Sorghum bicolor* (L.) Moench varieties B35, RTx7078, RTx7000, RTx432, BTx3197, BTx623, 1790E, RTx430, R9188, and BTx378 were used in our experiments. Callus was subcultured at 4-week intervals until enough callus material was obtained to start them on the stress media. All calli were less than 6 months old when they were subjected to osmotic stress.

Water Stress. PEG, mol wt 8000, was added to the growth medium at concentrations of 5, 10, 15, 20, and 25% (w/v) before adjusting the pH of the medium to 5.7. Twenty-five ml of medium was distributed into each culture tube and Heller supports (7), made with Whatman No. 2 filter paper, were placed on the medium. Water potential of the medium after autoclaving was −2 bars for the control, and −3.5, −5, −7.5, −11.5, and 16.2 bars for 5, 10, 15, 20, and 25% PEG, respectively.

Culture Conditions. Callus pieces were aseptically weighed, and placed on Heller supports. Initial inoculum weight was 250 mg for six varieties and 200 mg for four varieties. All cultures were grown at 28°C with a photoperiod of 16 h at light intensity of $30\mu E \cdot m^{-2}s^{-1}$. Ten replicates were used at each stress level, and growth of each callus was measured for 8 weeks by weighing aseptically every week. After 8 weeks, the callus pieces were frozen in liquid N_2 and then freeze dried. Dry weights were recorded and samples were processed for analysis of proline.

Amino Acid Analysis. Between 20 and 50 mg of the lyophilized tissue was extracted with 10 ml hot 80% ethanol, followed by two washings with 5 ml ethanol each. The pooled extract was air dried and dissolved in a 2-ml 40 mM lithium carbonate (pH 9.5). The procedure of Tapuhi *et al.* (17) was used to separate dansylated amino acids on an HPLC column, with the following modifications: the column consisted of 250 × 4.6 mm 4μm C_{18} spherisorb ODS. A linear gradient ramp was employed, where methanol was increased from 40 to 75% during a 50-min sample separation, at a constant flow rate of 1.4 ml/min. Proline was identified and quantitated using a dansylated standard.

Water Potential Measurement. A Wescor model HR 33T microvoltmeter with model C52 thermocouple psychrometer chambers was used to measure water potential of the culture medium.

Table I. *Field Ratings of Ten Varieties of Sorghum and Relative Growth of Callus Under Simulated Drought Stress in Culture for 8 Weeks*
Field ratings were provided by Dr. Fred Miller, Professor, Sorghum Breeding, Texas A & M University, College Station.

Variety		BTx623	RTx7000	RTx7078	RTx430	RTx432	1790E	B35	BTx3197	BTx378	R9-188
Field plants, early drought		Very good	Very good	Very good	Very good	Good	Fair	Very poor	Intermediate susceptible	Very susceptible	Poor
Field plants, late drought		Poor	Poor	Fair	Good	Good	Very good	Excellent			Good
Callus on	0%	138	146	166	775	203	167	85	265	89	270
Polyethylene	5%	166	115	195	315	105	−0.1	0.8	0.1	51	98
Glycol	10%	71	107	60	235	56	17	8	1.6	58	6.7
(Relative growth)	15%	26	52	−5	69	10	39	−23	−29	−17	−44
	20%	7	−11	−12	27	−50	−29	−25	−50	−28	−52
	25%	−19	−28	−44	4.5	−50	−32	−53	−52	−43	−44

Relative growth = (Final fresh wt − initial fresh wt/Initial fresh wt) × 100.
Initial inoculum fresh weight, 200 mg to 250 mg.

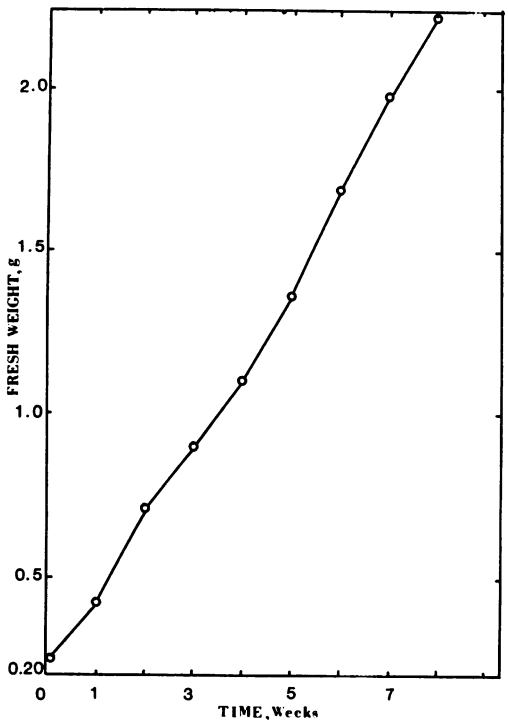

FIG. 1. Growth curve of RTx430.

RESULTS AND DISCUSSION

The field ratings of ten varieties of sorghum to drought stress and the growth response of their corresponding callus tissue to induced water stress in culture are given in Table I. Remarkable differences are observed among varieties with respect to their growth characteristics in culture. The water potential of −2 bars for the control medium was due to the presence of salts and sucrose. Relative growth based on fresh weight (RG) ranged from a low value of 85 in B35 to 775 in RTx430 in these cultures. RTx430 gained from an initial inoculum weight of 250 mg to a final weight of 2.22 g in 8 weeks, and growth was linear for 8 weeks (Fig. 1). Thus, nutrient depletion in the culture medium can be ruled out as a possible cause for differences in relative growth among varieties. They may be due to intrinsic differences among varieties when grown in culture. Statistical analysis of the growth response over a period of 8 weeks is shown in Table II.

RTx430 stands out as the sole variety that can take the highest stress without losing weight for 8 weeks. Statistically, RTx430, RTx7078, RTx7000, and BTx623 fared better than the others on 20% PEG, based on 5 weeks growth data (Table II). These four varieties can tolerate drought in the early growing season, under field conditions. This suggests that there may be some cellular-level mechanisms for early drought tolerance which are also expressed when they are grown as callus cultures.

This type of correlation between whole plants and callus cultures is significant, because this allows one to use callus cultures to analyze for stress tolerance mechanisms at the cellular level under controlled experimental conditions. Proline has been known as an osmoregulator in many organisms under water stress. Accumulation of proline was observed in leaves of sorghum in response to water stress (2) as well as in cultured tomato cells adapted to water stress (5). Many salt-tolerant plants also accumulate high concentrations of proline (18). Proline analysis in cultures which differed in their response to water stress thus provides an excellent opportunity to evaluate the role of proline in water stress. Results of this analysis are given in Figures 2 and 3. The levels of proline in nonstressed callus range from 0.1 to 0.5 $\mu g\ mg^{-1}$ dry weight. Considering the dramatic increases in proline content in response to water stress, these values are very low in the nonstressed tissue. This rules out any intrinsic differences between the varieties in terms of proline content. Proline increased significantly in cultures in response to stress. Highest net increases in proline content were in RTx432, B35, BTx3197, RTx7000, BTx623, and BTx378. RTx7078, 1790E, and RTx430 showed only moderate increases in proline as a result of stress. The general trend of increase in proline as a consequence of stress gives one *prima facie* evidence that it is more or less a universal phenomenon in water stress. This has been documented in prokaryotes where proline overproducing mutants have been isolated which are osmotically tolerant (3, 11). In such mutants, γ-glutamyl kinase is less sensitive to feedback inhibition by proline (11). It is therefore suggested that osmotic stress does induce a change in the genetic regulation coding for the enzymes which causes an overproduction of proline which in turn acts as an osmoprotectant.

It is tempting to speculate a similar role for proline in sorghum. However, close scrutiny of the data reveals that cultures that showed the highest increases in proline in response to water stress are not necessarily the ones that grew better under stress. RTx430 is found to be the best in terms of growth in response to stress. Proline increases in RTx430 is not nearly as high as in B35 or BTx3197, two varieties that grow poorly under stress. The highest proline levels (>10 $\mu g\ mg^{-1}$ dry weight) are found in B35, BTx3197, and RTx7000 at −16 bars and BTx623 and BTx378 at −11.5 bars. It needs to be mentioned that at these stress levels, all cultures had ceased growth. This is indicated by the decrease

Table II. *Statistical Evaluation Using the Scott-Knott Procedure at p = 0.01 of the 10 Sorghum Varieties Over a Period of 8 Weeks in Culture under Induced Water Stress*

The 0 through 9 are numerical codes used for the varieties for statistical analysis. 0 = R9188, 1 = RTx430, 2 = 1790E, 3 = B35, 4 = RTx7078, 5 = RTx7000, 6 = RTx432, 7 = BTx3197, 8 = BTx623, 9 = BTx378. Fresh weight increases were analyzed at weekly intervals for 8 weeks for each level of PEG. Significant differences between varieties are marked by slashes. Thus all varieties that are grouped together within slashes grew better than the group following them and poorer than the group preceding them.

	PEG, %					
	0	5	10	15	20	25
Week						
1	1/527493/086	1/4583/9207	15/4/938/2670	1/542/8396/07	1/45/3829/760	1/85293/4607
2	1/527/409836	1/4859/32067	1/54/98632/70	1452/839670	1/45/8293/760	1/825943607
3	1/5/72/4098/63	1/4598/02637	1/54/9862307	1542/869307	145/8293670	1/852943067
4	1/57024/986/3	1/458/906237	1/54/9862037	1542/869307	1458/239670	1852493076
5	1/574862093	1/458/690/237	1/54/9862037	1542/896307	1458/293670	1852493067
6	1/574982063	1/4/85960/327	1/54/9862037	15248/96307	1458923670	1582943067
7	1/750426983	1/4/58609/327	1/54/9086237	152/4869370	1458329670	1528943067
8	1/072456/893	1/4/58609/327	1/54986237 (No 0)	152/8469370	154839276 (No 0)	152894367 (No 0)

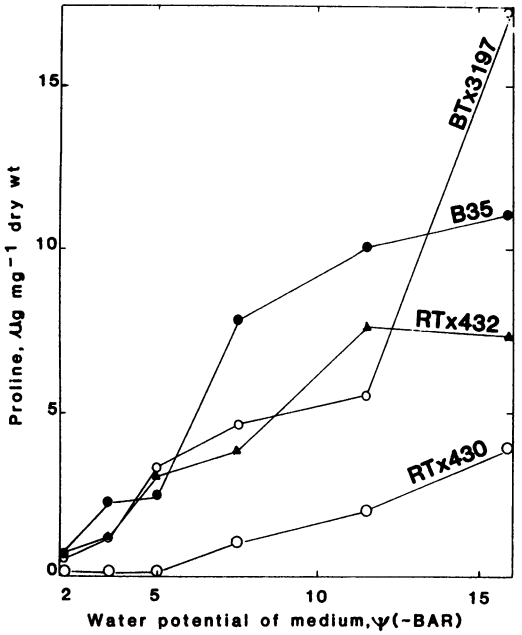

FIG. 2. Free proline in RTx430, RTx432, B35, and BTx3197 cultures in response to water stress.

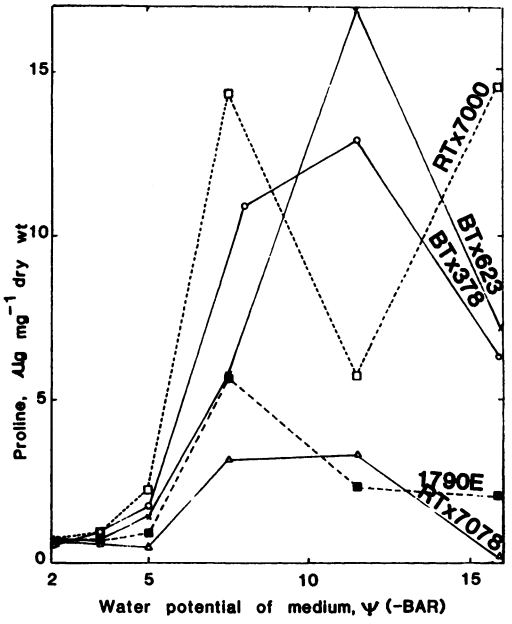

FIG. 3. Free proline in BTx623, BTx378, RTx7078, RTx7000, and 1790E cultures in response to water stress.

in both dry weight and fresh weight (Fig. 4). The steady increase in dry weight to fresh weight ratio thus is only an indication of the steady loss of water from the callus. If proline were to act as an osmoprotectant it should have been evident at a time when the cultures were actively growing, *i.e.* when they were accumulating dry matter in the presence of stress. This occurred between −2 bars and −5 bars in most cultures. Proline increase at these stress levels was, however, not as pronounced as at higher stress levels. In RTx430, proline level remained virtually constant up to a stress of −5 bars. Blum and Ebercon (2) found changes in free proline content in sorghum leaves only when leaf water potential reached −14 to −16 bars. They further observed significant differences in the amount of accumulated proline among cultivars at the peak of desiccation but established that drought tolerance was not correlated with accumulated proline. Our data on callus cultures are also in general agreement with their observations.

The role of proline as an osmoprotectant in sorghum as well as in other cereal crops (6) thus seems questionable. A correlation between increasing stress and an accumulation of solute is not proof that the solute is beneficial. Proline can accumulate for several reasons: (a) stimulated synthesis from its precursors, (b) low rates of proline oxidation (15), (c) slow incorporation into protein due to impaired protein synthesis, or (d) accelerated protein breakdown. A stimulated synthesis might indicate a useful role of proline as an osmoprotectant. A low rate of proline oxidation may indicate a secondary effect of stress. Decreased use in growth can also cause proline accumulation (10). Water stress may also cause increased proteolysis (12), thus increasing the pool of free amino acids, including proline. Further experiments are needed to identify which of the above factors contribute to the increase in proline in stressed tissue. Accumulation of significant amounts of proline at a time when metabolic processes are going downhill as indicated by decrease in both fresh and dry weights rules out an active role for proline in combating water stress. It would be very useful to examine the metabolism of proline in cultured tissue under water stress. Physiological adaptations to water stress certainly do exist in plants, and there is a definite need to distinguish those that are of adaptive value from

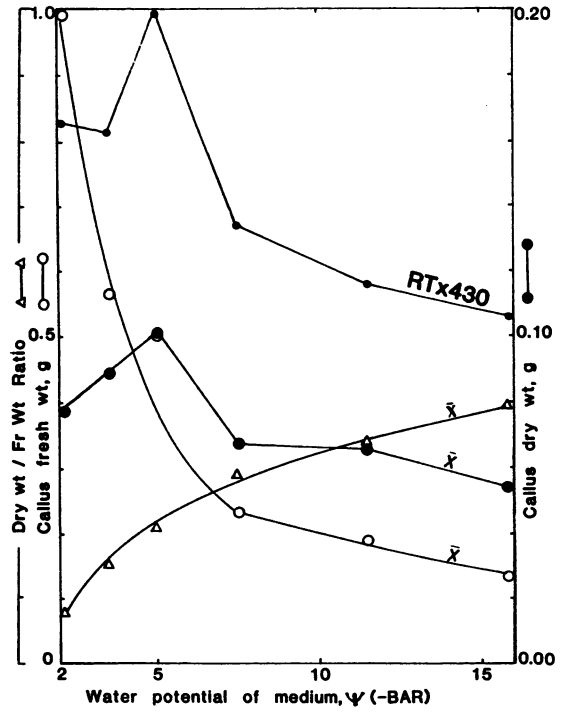

FIG. 4. Mean fresh and dry weights of callus, and dry weight to fresh weight ratios as a function of water stress. $\bar{x}$ – mean of 10 varieties. Dry weight of RTx430 is also known separately. Note the significantly higher dry weight of RTx430.

for proline as an osmoprotectant. Proline as a useful selection criterion for drought tolerance has not stood the test in plant breeding programs in barley (6) and rapeseed (13). Accumulation of proline in water-stressed leaves of sorghum was found to be related to the ability of the plant to recover upon relief of stress (2), which is an encouraging observation.

Acknowledgment—The authors are indebted to Dr. Fred Miller for the field ratings of the sorghum as well as for the supply of seeds for tissue culture.

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