RESPONSE, ADAPTATION, AND SELECTION OF WHEAT CALLUS CELLS TO LOW WATER POTENTIALS

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INTRODUCTION

Each chapter in this thesis is a manuscript to be submitted for publication in Crop Science, a Crop Science Society of America publication.

CHAPTER I

WATER POTENTIAL MANIPULATION AND PREDICTION IN PLANT CELL CULTURES

Water Potential Manipulation and Prediction in Plant Cell Cultures

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ABSTRACT

Drought stress is a major constraint to obtaining maximum wheat yields in semiarid regions. The objective of this study was to develop methodology for subjecting wheat callus tissue to low water potentials. Experiments were conducted to establish the relationship between polyethylene glycol (PEG, m.w. 8,000) concentration and water potential of tissue culture basal media (B5, N6, and MS). Basal media with 0 to 32 mM PEG were prepared and water potentials determined using thermocouple psychrometers. Equations were derived for use in calculating the concentration of PEG required for a desired water potential between -0.5 to -2.0 MPa. Liquid media are used because high concentrations of PEG inhibited agar or other gelling agents from solidifying. Polyurethane and filter paper supports with liquid media were constructed and evaluated for growing callus cultures at various water potentials.

No significant differences were detected for prediction equations for each basal medium, therefore, a general prediction equation was derived. Wheat calli water potentials adjusted to the water potential of the liquid medium. This system will be useful in studying cellular responses of plant callus tissue to low water potentials and to recover plants from variant or mutant cell lines by use of *in vitro* selection.

INTRODUCTION

Drought stress is a major constraint to obtaining maximum wheat yields in semiarid regions. Drought stress limits wheat productivity by reducing tillering, leaf growth, seed weight, and seed number. To increase agricultural productivity, crop plants must be developed to efficiently utilize available moisture. Drought stress occurs at water potentials between -1.0 to -2.0 MPa resulting in inhibition of plant growth and development unless resistance mechanisms are present to reduce or prevent water loss (Hale and Orcutt, 1987). Tissue culture offers unique opportunities to study cellular responses to drought stress by being able to grow plant cells at defined water potentials.

To create low water potentials and simulate drought stress, polyethylene glycol (PEG, M.W. 8,000) is a commonly used osmoticum (Blum, 1988). Bressan et al. (1981) used PEG as a non-penetrating osmotic agent to lower the water potential of the medium and simulate drought stress in plant cell cultures. They found that exposure of cultured cells of tomato (*Lycopersicon esculentum* Mill.) to media containing PEG results in the appearance of a cell population with increased resistance to low water potentials (Handa et al., 1982). Previous reports (Handa et al., 1983) and our own experiments indicate that PEG inhibits agar or other gelling agents from solidifying in plant cell culture media. Smith et al. (1985) overcame this problem by using filter-paper bridges in test tubes to screen for drought tolerance in *Sorghum bicolor* L. callus. We describe a tissue culture system using filter paper on polyurethane supports, saturated with liquid medium, to overcome this problem. This system is based on the one described by Conner and Meredith (1984) for *in vitro* selection for aluminum tolerance.

Previously published equations establish the relationship between PEG concentrations and water potentials in water solutions (Michel, 1983). Because plant cell culture media have a high osmolarity, these equations are not useful in preparing media with defined water potentials. Experiments were conducted to establish the relationship between the PEG concentration and water potential of B5 (Gamborg et al., 1968), MS (Murashige and Skoog, 1962), and N6 (Chu et al., 1975) basal media. We describe the derivation of a generalized prediction equation for establishing plant cell culture media with defined water potentials and a system for growing callus tissue under these conditions.

MATERIALS AND METHODS

Water potential prediction equations. Basal media B5, MS, and N6 were used in experiments to establish the relationship between water potential and PEG concentration. In addition to the basal salts, each medium contained 4.52 μM 2,4-D, 1.5 μM thiamine-HCl, 1.1 μM asparagine and 58.5 mM sucrose. Gelled medium contained 2.5 g L^{-1} phytagel™ (Sigma Chem. Co., St. Louis, MO). Liquid basal media containing 7.50, 11.25, 15.00, 18.75, 22.50, 26.25, and 31.88 mM PEG were prepared. Water potentials were obtained for each PEG concentration using thermocouple psychrometers (Johnson et al., 1986). Determinations were replicated six to nine times. Prediction equations were established using the General Linear Model procedure (GLM) of the Statistical Analysis System (SAS, 1985). Media were prepared with 0, 19.25, 25.26, and 31.88 mM PEG. Water potentials were predicted using the general prediction equation and measured experimentally using thermocouple psychrometers as described above.

Callus culture. Filter paper-foam supports for growing callus tissue consisted of 1 cm thick polyurethane foam cut into 8.5 cm diameter circles (Fig. 1). To establish sterile conditions, polyurethane foam circles were autoclaved three times for 40 min. Sterilized Whatman No. 2 cellulose filter paper was placed on top of the foam and placed in a disposable 25 x 100 mm petri dish. Foam and filter paper were saturated with 50 ml of MS basal medium modified for wheat cell culture (Sears and Deckard, 1982). Calli were obtained from immature embryos of the wheat 'Bobwhite' as described by Sears and Deckard (1982). Three calli weighing ca. 0.25 g each were placed on filter foam supports with liquid with different water potentials. Callus cultures were grown at 25°C with a 12 h photoperiod (47 μE m^{-2} sec^{-1}). Fresh weights were recorded when transferring calli to fresh media at three week intervals for two transfer

periods. At the same time, media and ca. 0.10 g subsamples of the three calli were pooled to determine water potentials as described above.

RESULTS AND DISCUSSION

Water potential prediction equation. Although there were slight differences in the prediction equations for the three media (B5, MS, and N6), the coefficients for the individual prediction equations were not significantly different (Fig. 2). Therefore, data were pooled across media and a general prediction equation was derived (Fig. 3; Eq. [1]). Results indicated that the relationship between the PEG concentration and water potential was significantly different when using basal medium or water (Fig. 3).

$$\text{Media MPa} = -0.50 + 0.011(\text{mM PEG}) - 0.002(\text{mM PEG})^2 \quad R^2 = 0.98 \quad [1]$$

The accuracy of the prediction equation was tested by comparing the predicted values from the equation to the observed values determined by thermocouple psychrometry (Table 1). The predicted and observed water potential values were very close and support the validity of the prediction equation.

Callus culture. The filter paper-foam support system is well suited for growing wheat callus tissue. Table 2 summarizes the response of wheat callus tissue to varying water potentials. The calli water potentials adjusted rapidly to the water potential of the medium. Callus growth rates decline with decreasing media and callus water potentials. The growth rates of callus growing on phytagel gelled medium were not significantly different from callus growing on liquid medium with filter paper-foam supports in the absence of PEG.

This study indicates that the filter paper-foam support system will be useful in studying cellular responses of wheat to low water potentials. This system can be used to recover plants from variant or mutant cell lines. Plants have been regenerated from calli tolerant to -1.0 MPa using this system and are currently being characterized.

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Table 1. Comparison of predicted and observed water potentials of MS basal medium when supplemented with PEG.

PEG	Water potential	
	Predicted†	Observed
- m*M* -	---------- MPa ----------	
0.00	-0.50	-0.54±0.05††
19.25	-1.03	-1.03±0.05
26.25	-1.59	-1.52±0.02
31.88	-2.18	-1.93±0.06

† Values obtained using the general prediction equation (Eq. [1]).

†† Standard error of mean.

Table 2. Growth rates and water potentials of wheat callus tissue grown on gelled medium and filter paper-foam supports with varying water potentials.

Media Type	T_1† Water potential		T_1 Callus growth rate	T_2 Water potential		T_2 Callus growth rate
	Medium	Callus		Medium	Callus	
	--------- MPa ------		- mg d^{-1} -	-------MPa -------		- mg d^{-1} -
Gelled	-0.50	-0.73	9	-0.40	-0.73	17
Liquid	-0.48	-0.62	13	-0.47	-0.50	18
Liquid	-1.02	-1.04	6	-0.96	-1.18	10
Liquid	-1.68	-1.68	3	-1.52	-1.92	6
Liquid	-1.96	-2.10	3	-2.03	-2.17	3
LSD (0.05)		0.39	2		0.15	8

† T = transfer period of 21 d.

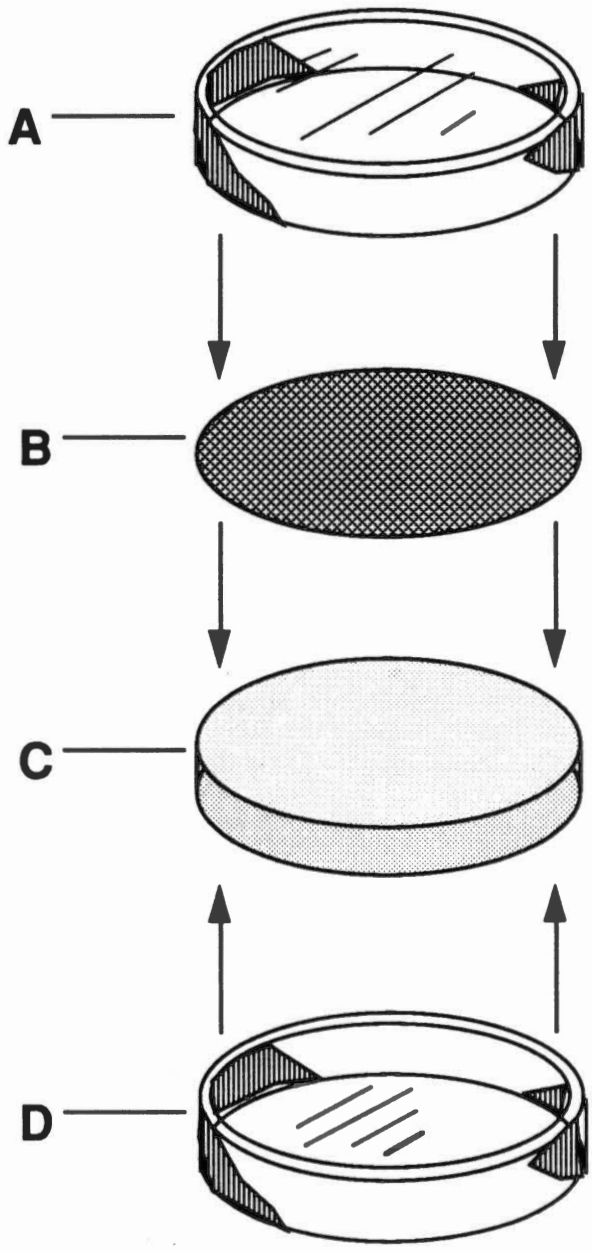

Fig. 1. The filter paper-foam support system consists of: (A) petri dish lid; (B) Whatman No. 2 cellulose filter paper; (C) polyurethane foam; and (D) petri dish bottom. Approximately 50 ml of basal medium was used to saturate the filter paper and foam.

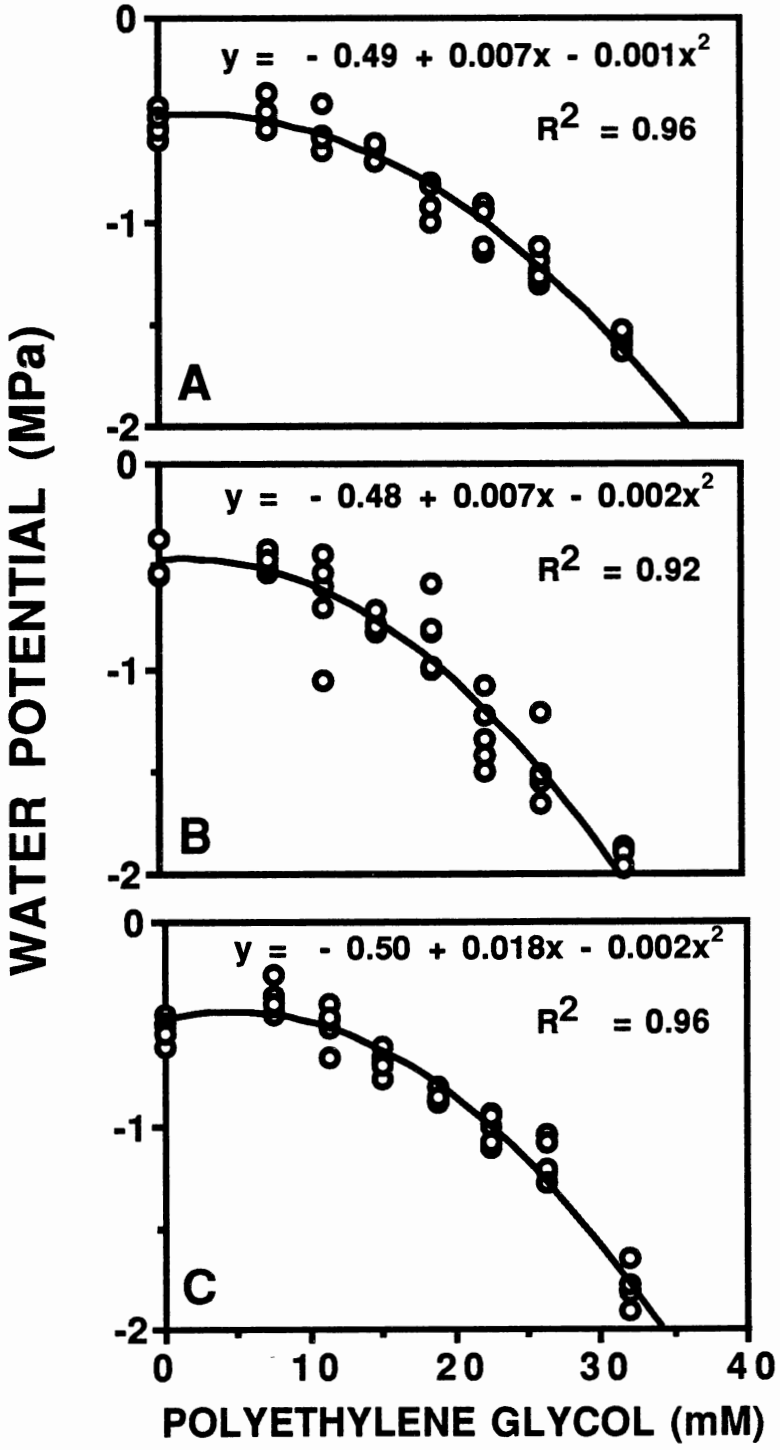

Fig. 2. Relationship between PEG concentration and water potential in B5 (A), MS (B), and N6 (C) basal medium.

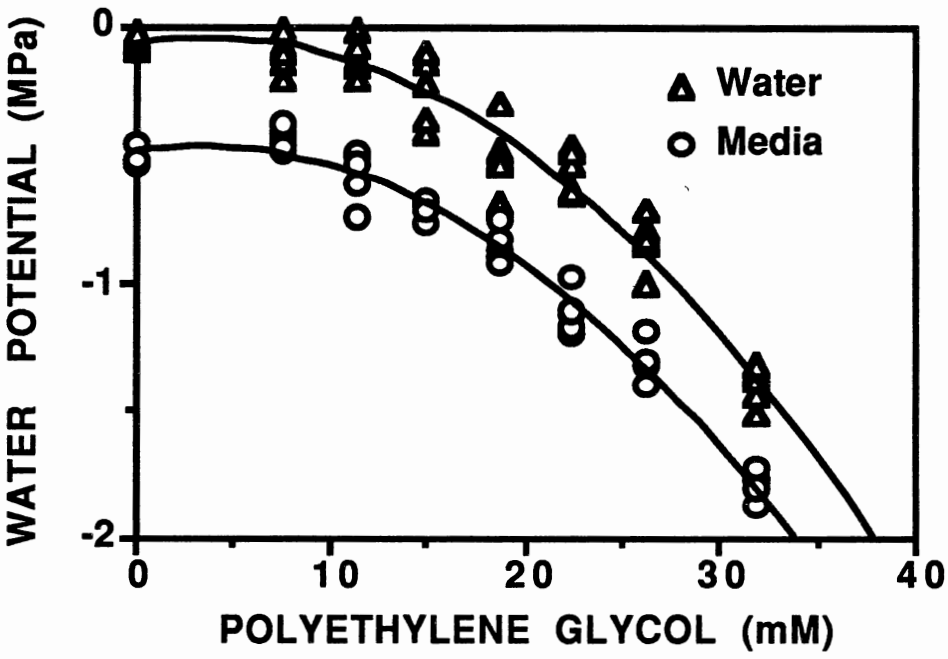

Fig. 3. Relationship between PEG concentration and water potential for distilled water and combined data from three basal media.

CHAPTER II

ADAPTATION OF WHEAT (*Triticum aestivum* L. em. Thell.) CALLUS TO LOW WATER POTENTIALS AND *IN VITRO* SELECTION FOR INCREASED TOLERANCE

Adaptation of Wheat (*Triticum aestivum* L. em. Thell.) Callus to Low Water Potentials and *In Vitro* Selection for Increased Tolerance

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ABSTRACT

Cellular adaptation to low water potentials involves a vast number of response mechanisms. Wheat callus cultures growing at varying water potentials (-0.5, -1.0 and -1.5 MPa) were examined to investigate metabolic changes that are associated with adaptation. Wheat callus adapted to -1.0 MPa showed a 3-fold increase in the concentration of free proline and a 6-fold increase in abscisic acid (ABA). At the stress of -1.5 MPa, a 6-fold and 8-fold increase in the cellular concentration for free proline and ABA, respectively, were observed. An *in vitro* selection strategy was implemented to regenerate plants from callus adapted to -1.0 MPa. Selfed seed was obtained from one plant regenerated from callus tissue tolerant to -1.0 MPa. Results from this study indicate that callus response is similar to whole plant response in the accumulation of proline and ABA with decreasing water potentials. Since cellular responses are similar to well documented whole plant responses, *in vitro* selection strategies may be applied to recover mutations influencing adaptation to low water potentials.

INTRODUCTION

Drought stress is a major constraint for wheat (*Triticum aestivum* L. em. Thell.) productivity world wide (Gusta and Chen, 1987). Johnson and Kanemasu (1982) reported that fall droughts can reduce tillering, leaf growth, and potential grain yield in winter wheat. Drought in the spring reduces seed weight and number. Plants respond to drought stress by exhibiting various morphological and biochemical changes which are involved in cellular survival and adaptation.

One response to drought stress is osmotic adjustment (Rhodes, 1987). It involves the reduction of cell water potential through intracellular solute accumulation (carbohydrates, amino acids, sugar phosphates), allowing plant adaptation to water deficits (Morgan, 1984). Schobert (1977) also suggested that solutes which accumulate, such as free amino acids, can associate with hydrophobic groups of macromolecules thereby improving their stability under drought stress conditions. The most profound alteration in free amino acid pools, in response to drought stress, is the biosynthesis and accumulation of proline (Aspinall and Paleg, 1981).

Considerable research investigating proline accumulation in response to drought stress has been conducted on whole plants (Naidu, 1990; Pesci, 1988; Navari-Izzo et al., 1990). Tan and Halloran (1982) observed accumulation of free proline in drought stressed wheat leaves. The proline accumulation in drought stressed plants is due to a combination of stimulated synthesis due to loss of feedback inhibition, inhibited oxidation, and impaired protein synthesis (Stewart and Hanson, 1980). Tissue culture has been utilized to study proline accumulation by limiting the complex interactions between tissues and organs of intact plants. It provides more precise control over environmental parameters and provides uniform plant

cell response. Recent investigations have used tissue culture as an approach to study metabolic changes at the cellular level involving proline accumulation in response to drought stress (Bhaskaran et al., 1985; Corcuera et al., 1989). Rhodes et al. (1986) reported a 300-fold increase in free proline in drought stress adapted tomato suspension cells. Amino acid accumulations observed in tissue culture are similar to those documented at the whole plant level in response to drought stress for tomato. The free proline concentration of adapted carrot cell suspension cultures increased up to 40-fold, and similar responses were shown for other amino acids including alanine, histidine and arginine (Fallon and Phillips, 1989).

Alteration of plant hormone levels is another mechanism by which plants respond to drought stress (Zeevaart and Creelman, 1988). Wright and Hiron (1969) classify abscisic acid (ABA) as a "stress hormone" because of its involvement in regulating metabolic and physiological changes in plants subjected to drought. It is well established that ABA concentration increases in response to drought (Davies and Mansfield, 1983) and its accumulation in leaves of whole plants causes stomatal closure (Walton, 1980). Recent studies at the molecular level have shown that ABA regulates gene expression in response to water deficits (Singh et al., 1989; Wilson et al., 1990; Morris et al., 1991). These studies established the relationship between ABA concentration and synthesis of specific mRNAs and polypeptides. Cohen and Bray (1990) found that endogenous ABA regulates the accumulation of three mRNAs in tomato leaves which represent genes expressed in response to drought stress.

In recent years major advances in plant cell biology have allowed for the use of *in vitro* selection strategies to recover mutants useful in plant improvement programs (Larkin et al., 1985). Much of the genetic variability created by tissue culture is believed to be adaptive responses which allow cells

to survive the intense selection pressure (Gonzales and Widholm, 1985; Roy, 1985; Hungta et al., 1987). Galibra et al. (1989) investigated the adaptation responses of different wheat cultivars *in vitro* to osmotic stress. They observed decreased growth and protein content in callus of drought sensitive cultivars compared to drought resistant cultivars. The screening of cell cultures to osmotic stress has been attempted in tomato (Handa et al., 1983) and *Nicotiana* species (Wong and Sussex, 1980). Both reports showed the ability of tissue culture cells to adapt to osmotic stress.

The experiments reported in this paper were conducted to evaluate the effects of low water potentials on the accumulation of amino acids and ABA in wheat callus tissue. In addition, an *in vitro* selection strategy was implemented to regenerate plants from cell lines tolerant to low water potentials.

MATERIALS AND METHODS

Callus Culture. Calli were obtained from immature embryos of *Triticum aestivum* L. em. Thell. c.v. Bobwhite as described by Sears and Deckard (1982). Calli were maintained on filter paper-foam supports (Conner and Meredith, 1984) saturated with M.S. (Murashige and Skoog, 1962) medium modified for wheat cell culture (Sears and Deckard, 1982). Calli cultures were grown at 25°C with 12 h photoperiod (47 $\mu E\ m^{-2}\ sec^{-1}$). Fresh weights were recorded when transferring calli to fresh media at four week intervals. Relative growth rates were determined as described by Singer and McDaniel (1986). For experimental treatments, unadapted calli were maintained on control medium without polyethylene glycol (PEG, m.w. 8,000). Stress treatments consisted of callus maintained on medium containing 19.25 mM and 26.25 mM PEG to create water potentials of -1.0 and -1.5 MPa, respectively. The adaptation period was for a minimum of three transfer periods (90 d). Water potential for all media and calli were determined by peltier-cooled thermocouple psychrometers (Johnson et al., 1986). All treatments were replicated four times in a completely random design and water potential determinations were repeated six times.

Free Amino Acid and ABA Analysis. Two cell lines adapted to -1.0 and -1.5 MPa water potential were sampled and tissue was stored at -70°C. Small samples of callus (ca. 0.2 g fresh wt.) were allowed to reach room temperature prior to free pool amino acid and ABA determinations. Free pool amino acids were extracted using the method of Bieleski and Turner (1966). An internal standard of 25,000 pmol norleucine was added to correct for experimental error during extraction and quantification. Additional purification (Anderson and Hibberd, 1985) was obtained by cation exchange chromatography (Bond Elut, SCX, Analytichem International) and 10,000

m.w. ultrafiltration (Millipore, Ultrafree-MC). Partial hydrolysis of amino acids was performed with 2N HCl, under nitrogen, for 2 h at 100°C to convert asparagine to aspartic acid (Asx) and glutamine to glutamic acid (Glx). Amino acid analyses were performed as phenylisothiocyanate derivatives separated by reverse-phase high performance liquid chromatography (Henderson and Meredith, 1983). Free amino acid determinations were replicated three times.

Methods used for extraction and quantification of ABA from callus tissue were that of Tahara et al. (1991) with modification of the extraction solvent to 80:15:5 (v/v/v) methanol:water:acetic acid. All assays were replicated three times. Determinations for free amino acid and ABA analysis were analyzed using standard analysis of variance procedures.

***In vitro* selection and plant regeneration.** One hundred and sixty calli were maintained, as described above, and grown in medium containing a water potential of -1.0 MPa (19.25 mM PEG) for a period of three months. Evaluation and selection of tolerant calli was performed on relative growth rates of individual calli using a prediction interval described by Steel and Torrie (1980):

$$P\left[\bar{Y} - t_{0.025}\sqrt{s^2\frac{(n+1)}{n}} \le Y \le \bar{Y} + t_{0.025}\sqrt{s^2\frac{(n+1)}{n}}\right] = 0.95$$

Prediction intervals (P) were calculated based on relative growth rates for each transfer period. Y is the predicted sample mean, $\bar{Y}$ is the sample mean, $t_{0.025}$ is the value of two-tailed Student's t such that the probability of a larger positive value is 0.025, n is the number of obervations, and s^2 is the sample variance. Selected tolerant calli were placed on regeneration medium and subsequent

shoots transferred to rooting medium. Small plantlets were then transferred to soil and allowed to produce seed in a greenhouse.

RESULTS

Callus growth. The effects of water deficit on the growth of wheat callus were examined after three months of exposure to -1.0 and -1.5 MPa water potential media (Table 1). Only a few calli adapted and grew well on the filter paper-foam support system at each water potential. Adapted calli were selected and increased for biochemical evalutions. Comparison of callus relative growth rates revealed no change with decreasing water potentials from -1.0 to -1.5 MPa.

Free amino acid and ABA. The total free amino acids increased 3-fold at -1.0 MPa and decreased at -1.5 MPa when compared to -0.5 MPa water potential (Table 2). The cellular concentration of free proline increased significantly in callus cells growing in media with decreasing water potential (Table 2). Proline concentration increased 3-fold in callus cells adapted to -1.0 MPa and 6-fold in cells adapted to -1.5 MPa when compared to the control (-0.5 MPa). Proline was the second most abundant amino acid in cells adapted to -1.5 MPa. Although the total amount of amino acids decreased at -1.5 MPa, the mole percentage of proline significantly increased from 4% at -0.5 MPa to 29% at -1.5 MPa.

Considerable variation was observed in the response of other amino acids between the different water potential treatments. At -1.0 MPa, 13 of the amino acids showed an increase in concentration while 3 showed a decrease in concentration in comparison with the control. With the exception of a few amino acids (Asx, leucine, tyrosine, proline), a decrease in concentration was generally found with severe stress (-1.5 MPa). Leucine, tyrosine, and Asx concentrations increased slightly compared to the dramatic increase of proline.

The cellular ABA concentration increased approximately 6-fold in callus cells growing at -1.0 MPa when compared to -0.5 MPa control media (Table 1). An 8-fold increase in ABA was observed for cells growing in the -1.5 MPa media.

***In vitro* selection and plant regeneration.** At the end of the third transfer, growth of two calli adapted to -1.0 MPa water potential exceeded the upper prediction interval (Fig. 1) and were selected for plant regeneration. Although plants were regenerated from both drought tolerant calli, only one plant reached maturity and set seed. Seed from the regenerated plant are being increased for use in future experiments.

DISCUSSION

To fully understand cellular adaptation to low water potentials, a number of responses must be characterized. These responses include solute concentration (sugars, amino and organic acids, inorganic solutes, betaines, polyamines), hormone levels (ABA, ethylene, auxins, gibberellins, cytokinins), and protein metabolism. This study describes several important responses of wheat callus during adaptation to low water potentials. Our results indicate that calli were able to adapt to a low water potential medium and maintain growth rates similar to the control (Table 1). This may be partially achieved by cellular responses of osmotic adjustment and ABA accumulation in response to decreasing water potentials.

It has been well documented (Aspinall and Paleg, 1981) that proline accumulation is a component of osmoregulation. Although the increase in proline content in our study were not as dramatic as those reported in other culture cell systems (Fallon and Phillips, 1989; Rhodes et al., 1986), the increased proline accumulation is in agreement. Additional experimentation is necessary to verify the exact role that proline plays in cellular adaptation during water stress conditions.

It has been shown that water deficit increases the biosynthesis of ABA (Wright and Hiron, 1969) and ABA is involved in drought resistance. Cohen and Bray (1990) suggest that ABA may coordinate drought induced changes in gene expression (ABA responsive genes). Results of this study revealed significant increases in ABA content at the -1.0 MPa (6-fold increase) and -1.5 MPa (8-fold increase) stress treatments relative to the control. Comparable increases in ABA content have been found in drought-stressed maize roots (Lachno and Baker, 1986) and tobacco cell cultures (Wong and Sussex, 1980). Wright (1978) induced drought stress in wheat plant roots using PEG. He

observed a 23 to 171 mg kg^{-1} fresh weight ABA concentration increase within the leaf after 4 h. Wheat seedlings treated with 20% PEG (-1.60 MPa) were shown to have significant increases in levels of shoot ABA after 6 d (Larsson et al., 1989). It appears that ABA responses observed in our experiments are similar to those widely reported for both cell and whole plant systems subjected to low water potentials.

A general decrease in population mean for relative growth rates of calli grown on media of -1.0 MPa was observed over time (Fig. 1). However, two calli from the 90 d transfer period exceeded the upper prediction interval and maintained growth rates similar to that of control calli. These calli were classified as tolerant to -1.0 MPa water potential. Since cellular responses are similar to that at the whole plant level, it appears feasible that an *in vitro* selection strategy would recover mutants influencing adaptation to low water potentials. Smith et al. (1985) suggested that cellular-level screening on PEG could be used to select for tolerant cells and subsequent regeneration of plants that might express enhanced tolerance to drought stress.

The study demonstrated that osmotic adjustment of callus cells necessary to compensate for a low water potential environment is associated with the accumulation of proline and ABA. Further studies on the characterization and evaluation of plants from the cell line tolerant to -1.0 MPa are in progress.

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Table 1. Water potentials, relative growth rate and ABA content of adapted wheat callus tissue with varying water potentials.

	Water Potential		Callus Relative	
PEG	Medium	Callus	Growth Rate	ABA
--mM--	----------MPa----------			pmol g^{-1}
0.00	-0.5	-0.53	0.018	2.90
19.25	-1.0	-1.25	0.015	18.63
26.25	-1.5	-1.78	0.016	23.47
LSD(0.05)	-	0.11	-	0.90

Table 2. Comparison of free amino acid† content of wheat callus tissue adapted to three water potentials.

Amino Acid	Water Potential (MPa)						LSD(0.05)
	-0.5		-1.0		-1.5		
	pmol mg^{-1}	mol%‡	pmol mg^{-1}	mol%	pmol mg^{-1}	mol%	pmol mg^{-1}
Asx§	2024	3.8	14380	9.6	3195	8.4	7978
Glx¶	4929	9.1	27794	18.7	1136	3.0	13197
Serine	1435	2.6	8642	5.6	413	1.1	7301
Histidine	1017	1.9	2249	1.3	905	2.4	NSD
Glycine	2280	4.2	5315	3.8	1291	3.4	1822
Arginine	24369	44.7	39155	26.4	13873	37.1	17377
Threonine	647	1.2	2934	2.1	316	0.8	1439
Alanine	9981	18.2	24242	16.0	3325	8.9	17788
Proline	2297	4.2	17289	11.7	10755	28.6	7273
Tyrosine	143	0.3	251	1.2	195	0.6	20
Valine	746	1.4	3865	2.7	733	2.0	524
Methionine	580	1.1	259	0.2	176	0.5	57
Cysteine	109	0.2	59	0.1	64	0.2	NSD
Isoleucine	203	0.4	572	0.4	144	0.4	39
Leucine	337	0.6	520	0.4	339	0.9	107
Phenylalanine	230	0.4	326	0.2	102	0.3	32
Lysine	3109	5.7	1124	0.7	518	1.4	776
Total	54434		149332		37479		80266

† Tryptophan not quantified.

‡ mol% = (pmol mg^{-1}/pmol mg^{-1}total amino acids)*100

§ Asx = pooled value for Aspartic acid and Asparagine

¶ Glx = pooled value for Glutamic acid and Glutamine

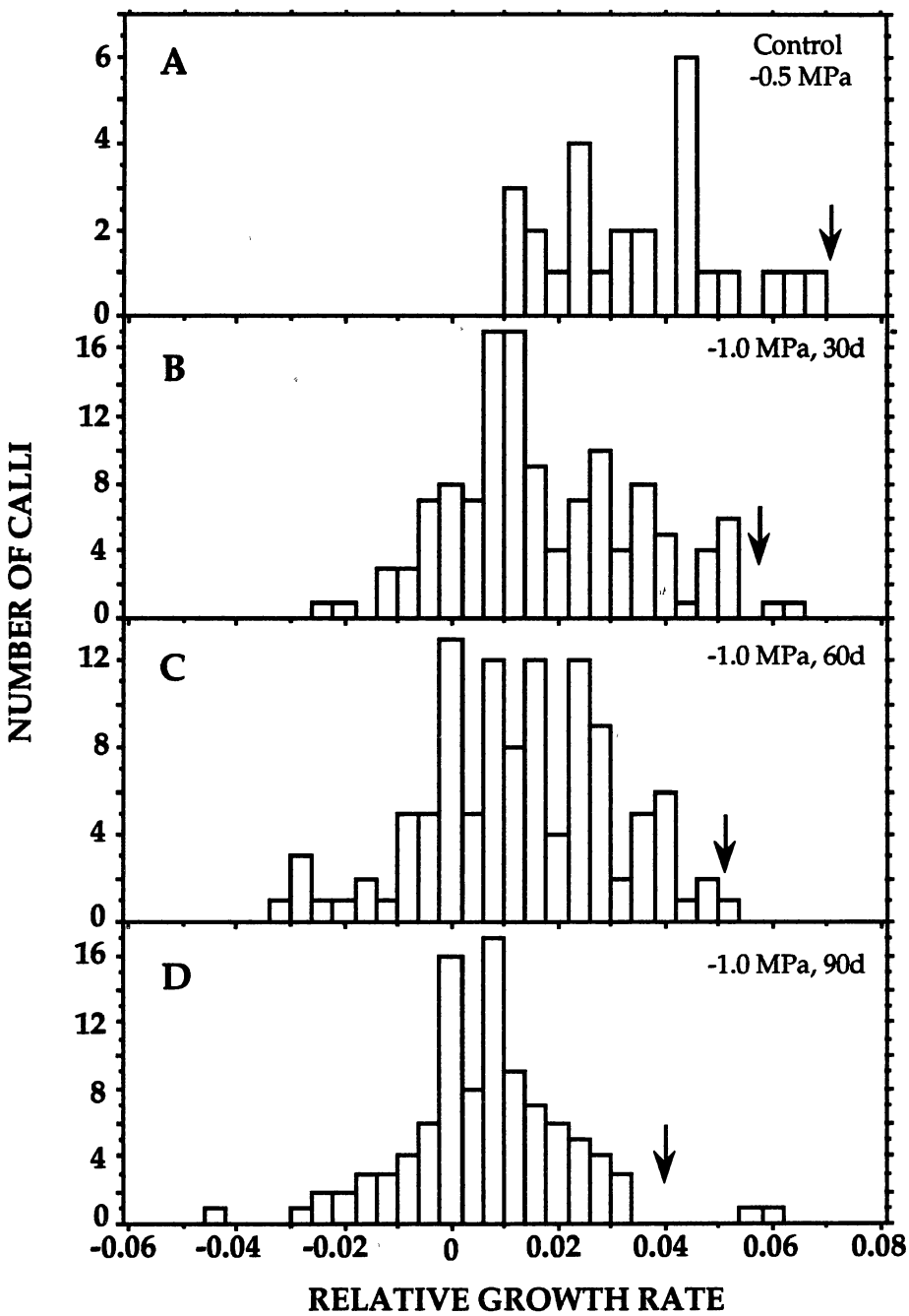

Fig.1. Histograms of relative growth rates for control calli (A) and calli growing on medium with a water potential of -1.0 MPa for three transfer periods; (B) = 30 days, (C) = 60 days, and (D) = 90 days. Bars to the right of arrow indicate calli with growth rates exceeding the upper prediction interval.

CHAPTER III

PROTEIN SYNTHESIS IN WHEAT CALLUS IN RESPONSE TO LOW WATER POTENTIALS

Protein Synthesis in Wheat Callus in Response to Low Water Potentials

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ABSTRACT

Altered protein synthesis is an important component of cellular adaptation to low water potentials. The effect of low water potentials on protein synthesis in wheat cell culture was investigated by using two dimensional polyacrylamide gel electrophoresis (2D-PAGE) of proteins labeled *in vivo* with ^{35}S-methionine and cysteine. Changes in protein synthesis were examined in callus tissue subjected to acute (28 d) and chronic stress (90 d) of -1.0 MPa water potentials. In response to chronic stress, an altered protein profile was observed in adapted callus cells. The most dramatic changes observed were decreased levels of 24 proteins and the disappearance of 39 proteins. Other changes included increased levels of several proteins and the expression of four new proteins (25.5 kD, 26 kD, and two at 28.3 kD). Less dramatic changes were observed in the protein pattern of unadapted callus cells exposed to the acute stress treatment. The most prominent changes were the synthesis of seven new proteins (25.5 kD, 26 kD, the 28.3 kD pair, 29 kD, 29.1 kD, and 29.3 kD). The results indicate that both quantitative and qualitative changes in protein synthesis occur when wheat cell cultures are exposed to acute and chronic stress treatments of -1.0 MPa water potential.

INTRODUCTION

Plants subjected to drought stress undergo numerous metabolic responses which are involved in adaptation. These responses include osmotic adjustment (Fallon and Phillips, 1989), alterations in plant hormone levels (Cohen and Bray, 1990) and changes in gene expression and protein synthesis (Bray, 1990). One approach to understanding the ability of plants to tolerate environmental stresses is to investigate stress-induced changes in protein synthesis.

The best characterized response in the pattern of protein synthesis to environmental stresses have been observed for heat-shock (Cooper and Ho, 1983; Key et al., 1981). Although not as well characterized, altered protein synthesis responses have also been observed for drought stress (Bray, 1990; Navari-Izzo, 1990; Vartanian et al., 1987) and salinity stress (Ramagopal, 1987a; Hurkman, 1988). Both drought and salt stresses induce similar plant responses by lowering the osmotic potential of the plant cell environment (Greenway and Munns, 1980; Morgan, 1984).

Cultured plant cells are a convenient tool for investigating mechanisms operating at the cellular level by eliminating complex phenotypic responses observed in plant adaptation to stress. In addition, ease of establishing, growing, and manipulating cultured plant cells adapted to defined levels of stress can be achieved in tissue culture systems (Hasegawa et al., 1984). Considerable information has been obtained on the effect of salt stress on protein synthesis using suspension cell cultures. In a series of studies on cultured tobacco cells adapted to medium containing high levels of NaCl, Singh et al. (1985) found qualitative and quantitative changes in protein synthesis. Synthesis of a 26-kilodalton (kD) protein was suggested to be involved in the adaptation of cultured tobacco cells to high levels of NaCl.

The 26-kD protein is named 'osmotin' and constitutes approximately 10% of the total cellular protein in adapted tobacco cells (Singh et al., 1987a). Osmotin synthesis is also induced by treating the cells with abscisic acid (ABA) (Singh et al., 1987b). With the isolation of a cDNA encoding osmotin, Singh et al. (1989) confirmed that ABA induces the synthesis of mRNA for osmotin and a posttranscriptional event allowed the enhanced accumulation of osmotin in adapted cells. Also shown was the comparison of the amino acid sequence of osmotin with four homologous proteins.

Ramagopal (1986) reported three new proteins and increased or decreased levels of several other proteins could be found in maize (*Zea mays* L.) callus upon exposure to media containing different levels of NaCl. To date, only a few studies on the effect of low water potentials on protein synthesis *in vitro* have been reported (Singh et al., 1985; Ramogopal, 1986).

In this study we describe the effect of low water potentials on protein synthesis in wheat callus tissue using two-dimensional gel electrophoresis following *in vivo* incorporation of radiolabeled amino acids. Changes in protein synthesis were detected by comparing the profile of nonstressed callus to 1) adapted callus growing at a low water potential for > 90 d (chronic stress), and 2) nonadapted callus transferred to a low water potential media (acute stress).

MATERIALS AND METHODS

Callus Culture. Calli were obtained from immature embryos of *Triticum aestivum* L. em. Thell. cv. Bobwhite as described by Sears and Deckard (1982). Calli were maintained on filter paper-foam supports (Conner and Meredith, 1984) saturated with MS (Murashige and Skoog, 1962) medium modified for wheat cell culture (Sears and Deckard, 1982). Callus cultures were grown at 25°C with 12 h photoperiod (47 $\mu E\ m^{-2}\ sec^{-1}$). Fresh weights were recorded while transferring calli to fresh media at four week intervals. Relative growth rates were determined as described by Singer and McDaniel (1986). Treatments consisted of 1) non-stressed unadapted callus (control), 2) stress adapted callus (chronic stress), 3) stressed, unadapted callus (acute stress). Control callus was maintained on MS control medium (-0.5 MPa water potential) without polyethylene glycol (PEG, M.W. 8,000). Stress adapted callus was obtained by selecting callus which grew as well as control callus after three months on MS stress medium (-1.0 MPa water potential) containing 19.25 mM PEG (chronic stress). Unadapted callus was maintained on MS control medium and then transferred to the MS stress medium and maintained for 28 d (acute stress). Water potentials for all media and callus tissue were determined with peltier-cooled thermocouple psychrometers (Johnson et al., 1986). Water potential determinations were replicated six times.

***In Vivo* Labeling.** Calli for each treatment (ca. 900 mg each) were transferred to filter paper-foam support petri dishes containing a radiolabeling medium with the desired water potential. The media contained 400 μCi of Trans-[^{35}S]-labeled TM amino acids (c. 70% L-methionine, 15% L-cysteine; 1,082 Ci/mmol; ICN Radiochemicals, Irvine, CA). Calli were maintained on the radiolabeling media for 28 d as described above.

Protein Extraction and Electrophoresis. Proteins were extracted and denatured as described by Ramagopal (1987b). Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) was performed according to the method described by Damerval et al. (1986) with slight modifications. IEF gels contained 2.4% Servalyt 3 to 10 and 1.6% Servalyt 5 to 7. The IEF gels were 13.5 cm long with a diameter of 1mm. IEF was performed for 16,200 volt x hours in 0.1 N NaOH catholyte and 0.06% H_3PO_4 anolyte electrode solutions. IEF gels were equilibrated and then transferred to a 12% acrylamide gel for separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Damerval et al. (1986). Slab gels were 20 cm x 20 cm x 1 mm and electrophoresed at 24 mA gel^{-1}. The migration buffer used is described by Laemmli (1970). Visualization of proteins was done by fluorography. Measurement of uptake and incorporation of label were determined by trichloractic acid (TCA) precititable counts (Mans and Novelli, 1961). Equal counts (500,000 cpm) of TCA-precipitable [^{35}S]-label protein were loaded onto each gel. Gels were fixed and fluorographed as described by Porter et al. (1989). Dried gels were exposed to Kodak X-Omat x-ray film with an X-Omatic cassette intensifying screen (Kodak). The exposure time of the fluorograph at -70°C was adjusted to a standard of 1×10^6 cpm day^{-1} gel^{-1}.

RESULTS

Callus Growth and Incorporation of Radiolabeled Amino Acids. The effects of low water potential on the growth of wheat callus cultures were examined for a period of three months. Calli adapted to -1.0 MPa had a relative growth rate of 0.026 compared to 0.029 for the control (Table 1). The ability of cells to take up and incorporate ^{35}S-labeled amino acids varied between the two stress treatments (Table 1). Higher percent incorporation was observed for the control callus compared to callus for both the chronic and acute stress treatments. The difference of percent incorporation between the control and chronic stress callus was more dramatic than the difference observed between control and acute stress callus.

Protein Synthesis in Response to Low Water Potentials. At least 450 *in vivo* ^{35}S-labeled proteins were identified in fluorographs from gels of control tissue. Major changes in the pattern of protein synthesis were observed in callus subjected to chronic stress (Fig. 1). Comparison between fluorographs from control and chronic stress callus tissue showed both quantitative and qualitative differences in protein synthesis. The most dramatic changes were indicated by decreased level of synthesis (ca. 24) and disappearance (ca. 39) of proteins (Fig. 1B). In addition, synthesis of some proteins were increased, and four new proteins (25.5 kD, 26 kD, and two at 28.3 kD) were detected in the chronic stressed callus.

Fluorographs of proteins from the acute stress treatment revealed only a small number of changes in the protein pattern as compared to the control (Fig. 2). The most striking changes detected were the synthesis of seven new proteins (25.5 kD, 26 kD, two at 28.3 kD, 29 kD, 29.1 kD, and 29.3 kD). Further analysis revealed that certain proteins (25.5 kD, 26 kD and the 28.3 kD pair)

were synthesized in response to both acute and chronic stress treatments (Table 2).

DISCUSSION

These results demonstrate that relative levels of synthesis of specific proteins were altered when cultured wheat calli cells were exposed to low water potentials. Comparable growth rates of nonstressed calli and calli adapted to chronic stress indicate that wheat cells adapted, allowing cell division and growth at -1.0 MPa water potential.

Decreases in synthesis and disappearance of some proteins were observed in cells adapted to -1.0 MPa medium (chronic stress treatment). The reduction in proteins detected in the chronic stress treatment was reflected by a reduction in the amount of incorporation of radiolabeled amino acids. The lower percent incorporation observed in the chronically stressed callus may be explained by the decreased number and levels of proteins synthesized. These results inidicate that four new proteins are synthesized during the chronic stress treatment. We found only small differences in the protein patterns of control callus exposed to the acute stress. The levels of radiolabel incorporation for the control and acute stress treatments were similar, indicating similar rates of protein synthesis.

The synthesis of four new proteins (25.5 kD, 26 kD, and the 28.3 kD-pair) were common to both the chronic and acute stress treatments. The level of synthesis of the two new 28.3 kD proteins was higher in the chronic stress treatment. These new proteins are of interest because their synthesis is induced in the early stages of adaptation and became more prevalent in later stages of adaptation. Three new proteins (29 kD, 29.1 kD, and 29.3 kD) observed in the acute stress treatment were not present in the chronic stress treatment. Bray (1990) speculated that proteins which are synthesized and accumulate to high levels early in response to drought stress and then

decrease are probably injury responses or are required only in the early stages of adaptation.

We conclude that several new proteins are associated in the cellular adaptation of wheat callus to -1.0 MPa water potential. However, further research will have to be performed to determine if the observed changes in protein patterns are the result of unique gene regulation, preferential translation of mRNA, post-translational processing, or stability of mRNA and protein. Although it is not possible from 2D-PAGE radiolabeling studies alone to determine if the same proteins accumulate in different species (Bray, 1990), we observed similar proteins (26 kD and 28.3 kD) described by Ramogopal (1986) in maize callus exposed to low water potentials.

Future research will determine if the 26 kD low water potential-induced proteins observed in our experiments are immunological cross reactive with the osmotin (Singh et al., 1987). This study and ongoing research will provide information on gene expression and protein synthesis during cellular adaptation to low water potentials in wheat.

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Table 1. Uptake and incorporation of Trans-[^{35}S]-label in wheat callus cultures in the presence of low water potential.†

Treatment	Water Potential	Relative Growth Rates	Uptake‡	Incorp.§	Incorp.¶	Specific Activity
	---MPa---		– cpm mg^{-1} fresh wt –		-%-	-cpm µg^{-1} protein-
Control	-0.5	0.029	3,921	1,696	43	7,165
Chronic Stress	-1.0	0.026	2,693	339	12	1,406
Control	-0.5	----	2,269	724	32	3,466
Acute Stress	-1.0	----	3,608	964	27	7,111

† Calli were labeled and cpm quantitated as described in "Materials and Methods."

‡ Cpm in total homogenate.

§ Cpm in TCA-precipitable material.

¶ Incorp. = Incorporation

Table 2. Expression of callus proteins affected by -1.0 MPa water potential under chronic and acute stress when compared to control tissue.

Expression of Proteins	Chronic Stress	Acute Stress	Common in Both Stresses	Total
Appear	7	4	4	11
Increase	17	3	1	20
Disappear	39	-	-	39
Decrease	24	2	2	26

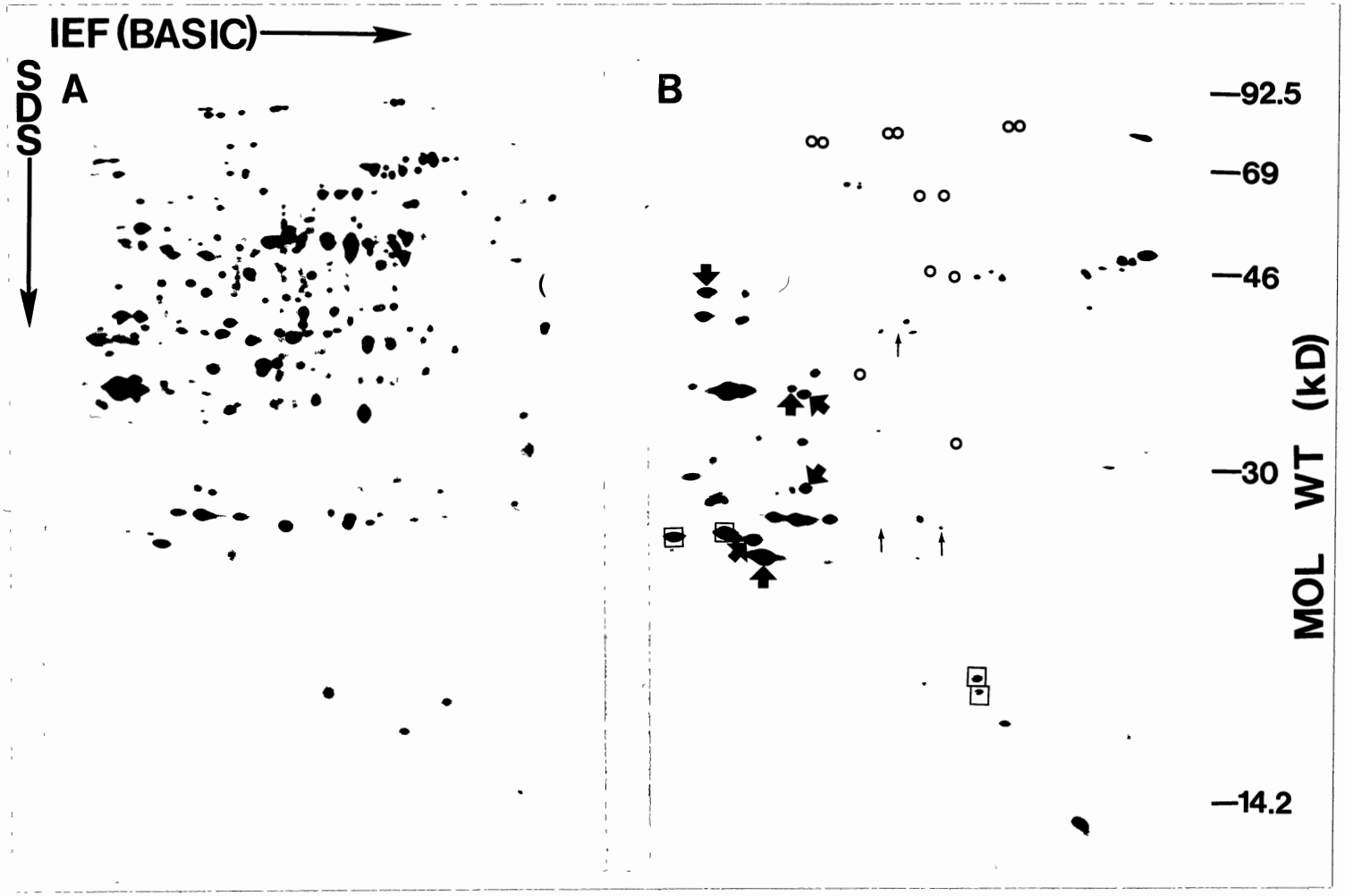

Fig. 1. Fluorographs of ^{35}S-labeled proteins of wheat callus tissue resolved by 2D PAGE showing patterns of protein synthesis for control callus (A) and chronic stress callus (B). Selected proteins have been highlighted which increase (⬆), decrease (⬇), appear (□), or disappear (○).

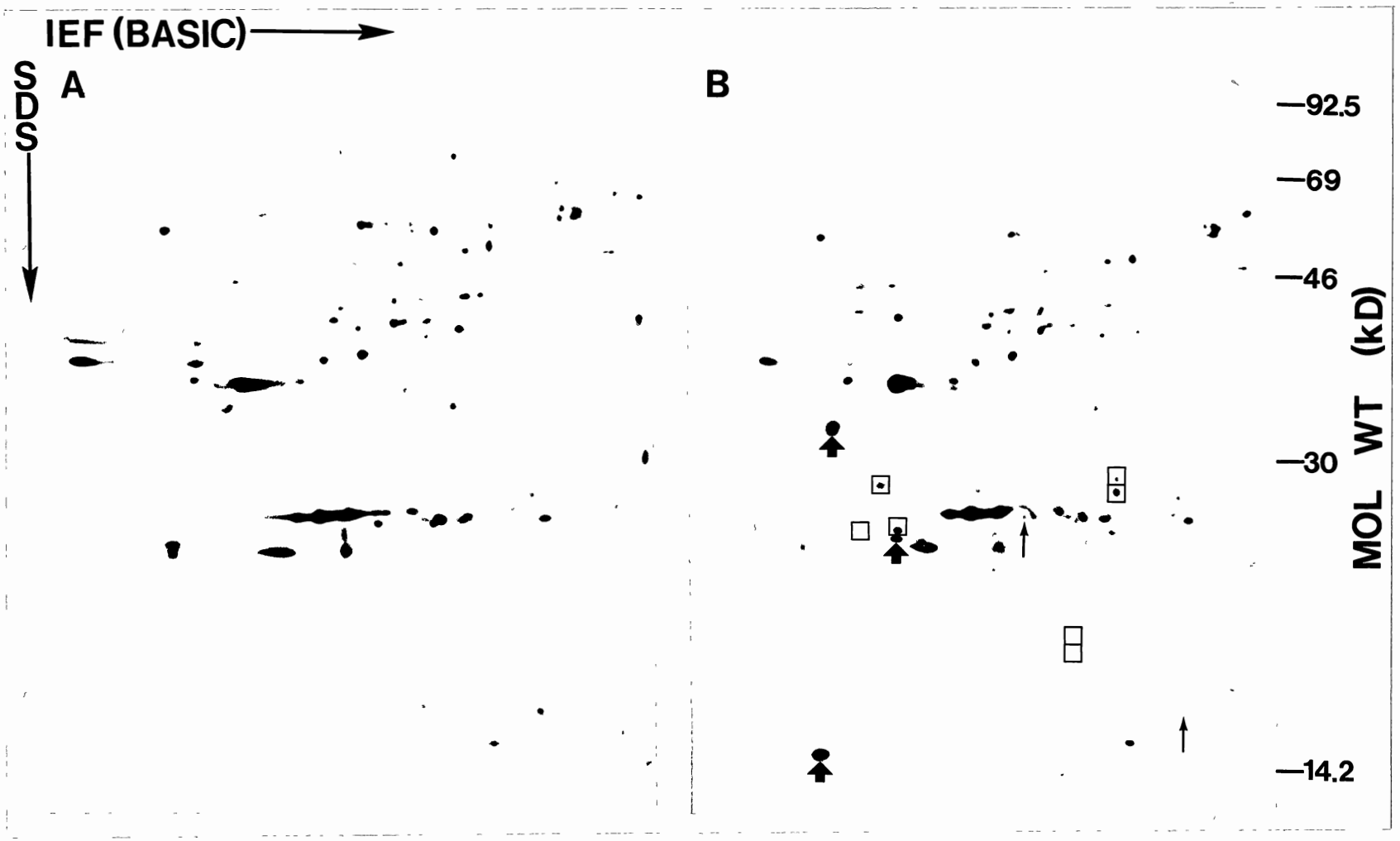

Fig. 2. Fluorographs of ^{35}S-labeled proteins of wheat callus tissue resolved by 2D PAGE showing patterns of protein synthesis for control callus (A) and acute stress callus (B). Selected proteins have been highlighted which increase (⬆), decrease (↑) or appear (□).

VITA

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