WATER STRESS AND PROTEIN AND AMINO ACID METABOLISM IN BARLEY AND WHEAT

by

TARAK NATH SINGH, M.Sc.(Ag.)

Department of Plant Physiology
Waite Agricultural Research Institute
University of Adelaide
Australia

Thesis submitted for the
Degree of Doctor of Philosophy
1970

STATEMENT

I hereby declare that the thesis here presented is my own work, that it contains no material previously published, except where due reference is made in the text, and that no part of it has been submitted for any other degree.

(Tarak Nath Singh)

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SUMMARY

The effects of water stress on protein and amino acid metabolism in barley (*Hordeum distichum*) and wheat (*Triticum aestivum*) were investigated. The changes in these biochemical parameters, as a consequence of water stress, were accompanied by parallel measurement of the changes in water potential of the plant tissues to make the findings more quantitative and comparative.

Water stress, imposed either by withholding the supply of water or supplying polyethylene glycol solutions, inhibited net protein synthesis in the leaves and roots, although the latter was less susceptible to water stress. Coincident with the inhibition of net protein synthesis produced by water stress, a large accumulation of free proline (15 to 70-fold) was observed, with relatively slight changes in other amino acids. Proline was found to accumulate in every part of the intact plant in response to water stress but the efficiency of acquisition by various organs was different. Leaves accumulated the greatest amount followed by leaf sheaths, shoot apices, and roots. The accumulated proline disappeared rapidly, depending upon the degree of stress, when the stress was relieved, which suggested that the reaction to water stress was reversible. Proline did not accumulate in excised shoot apices or roots, whereas leaves and leaf sheaths

were still capable of accumulating proline when floated on osmotic solutions.

Aeration of the root medium during water stress greatly affected the distribution of proline in various parts of the plant. The termination of aeration stopped the accumulation of proline in the root without affecting the total amount of proline in the whole plant. This observation coupled with others reported in this thesis suggested that proline is translocated around the plant during water stress. Thus, the continued gain in the dry weight of the root during stress may possibly be attributed to the translocation of proline into roots from elsewhere. The need for soluble carbohydrate and nitrogenous compounds for proline biosynthesis was demonstrated with etiolated barley leaves. These leaves failed to accumulate proline during water stress but when preincubated with either sucrose or glutamic acid solutions, a pronounced increase in free proline was observed. This also showed that etiolated leaves were capable of synthesizing proline provided they were supplied with adequate precursors.

Since proline accumulation was found to be a water stress-triggered phenomenon, it was of interest to investigate the effects of those factors on proline accumulation which are known to affect the plant's response

to water stress. Seedling hardening, growth regulating compounds such as (2-chloroethyl)-trimethyl ammonium chloride (CCC), gibberellic acid (GA_3), abscisic acid (ABA), and genetical factors were all found to affect the accumulation of proline without affecting the water potential of the plant. The factors which are known to increase drought resistance of crop plants, such as seedling hardening and CCC, were found to increase proline accumulation during water stress. On the other hand, GA_3, which has been reported to decrease drought resistance decreased the accumulation of proline. Genetic variability in the capacity to accumulate proline was also revealed among 14 varieties of barley subjected to water stress. Proline accumulation was highly significantly correlated with the stability of the variety, i.e., the more stable in yielding capacity a variety was, the greater was the accumulation of proline during a period of drought. Thus, proline accumulation was found consistently associated with drought resistance under the influence of physical, chemical, and genetical factors. It is probable that proline performs some function in the biochemical, and hence physiological, regulation of drought resistance.

ACKNOWLEDGEMENTS

The author desires to place on record an expression of his indebtedness and deep sense of gratitude to Dr. D. Aspinall and Professor L.G. Paleg for having suggested and supervised the present project. It is because of them that this work reached the standard presented in this thesis.

The author also expresses his sincere thanks to Mr. D.H. Sparrow of the Department of Agronomy, Waite Institute for supplying barley varieties and pertinent information about them and Mr. B. Falk for the preparation of photographs. Financial support was provided by a University of Adelaide Research Grant which is fully acknowledged.

CHAPTER I

LITERATURE REVIEW

1. Introduction

A great deal has been written on the water relations of plants since the inception of plant physiology as a discipline. The subject has been the topic of many recent reviews (Levitt, 1951; Iljin, 1957; Vaadia, Raney, and Hagan, 1961; Henckel, 1964) and several books (Kramer, 1949; Levitt, 1956; Slavik, 1965; Kozlowski, 1968) and aspects of the problem have been studied in depth (Slatyer, 1967). In particular, in recent years, a much more precise understanding of the physical aspects of the water relations of plants (Gardner, 1960; Gardner and Ehlig, 1965) has been added to the earlier studies on the effects of water deficit on plant growth (Slatyer, 1957; Gates, 1955a, 1955b, 1968). So far, however, the effects of stress at the biochemical level of plant metabolism have been little studied and are far from understood. This is understandable in view of the previous confusion in assessing the physical status of water in the plant but, now that this can be precisely stated, progress in elucidating the effects of water status of plants on metabolism should be rapid.

2. The Water Status of the Plant

Water moves from the soil to the atmosphere through the plant along a pathway of decreasing potential. Thus water forms a continuous stream in plants and, except on comparatively infrequent occasions, the water status of the plant is a dynamic entity changing constantly with fluctuations in the rate of absorption from the soil or transpiration from the leaves and changes in the resistances to water movement (Milthorpe and Moorby, 1969). A reduction in the water status of the plant, with consequent wilting, can occur as a result of a decrease in available soil water or an increase in the potential rate of transpiration, on bright sunny days. Wilting can even occur with plants growing in dilute nutrient solution if a high rate of transpiration is combined with resistance to water movement through the roots (Kramer and Brix, 1965).

The dynamic nature of the water status of plants has caused difficulties in relating the physical status of plant water with the physiological effects of that status. At first, plant growth responses were related to soil water parameters (Harris, 1914; Magistad and Breazeale, 1929; Veihmeyer and Hendrickson, 1949, 1950) but it became increasingly clear that such relationships were, at least, variable (Stanhill, 1957). The next phase was the relating of growth responses to various empirical measure-

ments of plant water status, such as absolute water content (Miller, 1917) and relative turgidity (Weatherley, 1950). These measurements, although more closely related to the water status of the plant are, in fact, empirical, making comparisons between plants and situations dangerous. Finally, in recent years, it has been found possible to measure the physical status of plant water more or less directly to give an unequivocal measurement for comparison (Slatyer, 1962; Ehlig, 1962). This final phase has made it possible to clearly define the physical parameters in any study of the effects of water deficiency on plant growth or metabolism and hence makes it possible to both repeat and compare experiments.

2.1 Measurement of Plant Water Status

2.1.1 Empirical Methods

Initially, attempts were made to equate particular soil water status with physiological effects on the plant. Thus the term 'permanent wilting point' or 'wilting coefficient' (Briggs and Shantz, 1912) was intended to mark particular states of the soil water and to be relatively independent of the plant. Later it was realized that such measurements varied with independent characteristics of both the soil and the plant (Slatyer, 1957) and hence were not useful in characterizing either the soil or the plant water status. Attention was then turned to the

status of the water in the plant as this was more likely to reflect the response of the plants to water stress. When measurements of plant water content were found to vary with factors (such as dry weight) other than the water status (Livingston and Brown, 1912; Miller, 1917), Stocker (1929) introduced the concept of the water saturation deficit, or the percentage of the plant's maximum possible water content which has been lost. This was followed by the more widely used relative turgidity measurement of Weatherley (1950) and Barrs and Weatherley (1962) which expresses the plant's water content as a percentage of the maximum as shown below:

$$R = \frac{100\ (W_1 - W_2)}{(W_3 - W_2)}$$

where, R = relative turgidity

W_1 = fresh weight

W_2 = dry weight

W_3 = saturated fresh weight

Leaf discs of equal size are cut, ejected into a gas-tight weighing bottle and the fresh weight (W_1) is taken. The discs are subsequently floated on distilled water for a certain period to attain saturation and then removed, surface dried and reweighed to give the turgid weight (W_3). Finally, the samples are dried in an oven and weighed to give the dry weight (W_2). Two classes of error may be

associated with this technique. First, water uptake into the disc may continue after full turgor has apparently been attained, and second, disc dry weight may change while water uptake occurs. To overcome these shortcomings, however, Barrs and Weatherley (1962) defined precisely certain conditions in which errors associated with this technique could be minimised. Though relative turgidity measurements have been used frequently and are important from the field point of view, they do not represent the actual state of water in plants and vary from species to species (Slatyer, 1957). Other empirical methods of assessing the water status of the plant have also been proposed. Guard cells are very sensitive to water deficits and premature closure of stomata is often taken as the first visual indicator of developing water deficits (Heath, 1959; Alvim, 1966). Portable porometers may be used to rapidly assess these changes in stomatal aperture on plants growing in the field but only give an indirect assessment of plant water status. Similarly, the increase in leaf mass which occurs with an increase in water content (Boyer, 1969) can be detected by the attenuation in the flux of β-particles caused to pass through the leaf (Nederaki, 1961). Such measurements can be used to estimate leaf water content which may in turn be calibrated with leaf water status (Jarvis and Slatyer, 1966).

In general, measurements of water content, relative turgidity and other such parameters, although widely employed are only useful in so far as they reflect the physical status of water in the plant. Close relationships have been demonstrated, particularly in the case of relative turgidity (Weatherley and Slatyer, 1957), but calibrations have to be carried out in each new situation. Comparisons of relative turgidity between species, for instance, are meaningless without knowledge of the relationship between R and the physical water status for each species.

2.1.2 Free Energy Status of Plant Water

The only unequivocal measure of the water status of a plant is the Gibbs' free energy of the water in the system (Slatyer and Gardner, 1965). Using this system it is possible to compare the status of water in different plants or in different parts of the soil-plant-air system without the need to qualify the measurements used. Edlefsen (1941), Day (1942) and Broyer (1947 a,b) developed the concept of free energy in the dynamics of water movement and the physical state of water in the soil. Day (1942) coined the phrase 'chemical potential' to describe the physical status of water. This removed the confusion concerning the involvement of various forces, such as capillary forces, osmotic effects, etc., in the availabil-

ity and movement of water in the soil as all could be accommodated within the one term. This concept was extended to plants by Slatyer and Taylor (1960) and Taylor and Slatyer (1961) who introduced the term 'water potential' to describe the physical status of water in plants. Water potential is defined as the difference between the chemical potential or partial specific Gibbs' free energy of water in the system and the corresponding chemical potential for pure water at the standard reference temperature, pressure and location with respect to external force fields. It can be expressed as below:

$$Y_w = \frac{(U - U_o)}{\bar{V}}$$

where, Y_w = water potential (atmospheres or bars)

U = chemical potential of water in the system

U_o = chemical potential of pure free water

$\bar{V}$ = partial molal volume of water (cm^3 $mole^{-1}$)

Since plants, like soils, have large areas of surface which may interact with water, e.g., cell walls, particles and organelles, it has been convenient to group the forces associated with these surfaces in a single term 'matric potential'. Boyer (1967, 1969) suggested that total water potential, Y_w, of the system at equilibrium and constant temperature can be expressed as follows:

$$Y_w = Y_p + Y_g + Y_s + Y_m$$

where, p = effects of pressure

g = effects of gravity

s = effects of solutes

m = effects of matrix

2.1.3 Measurement of Water Potential

2.1.3.1 Pressure Techniques

Since the effect of pressure is thermodynamically equivalent to that of solutes and other components of the total water potential, it may be used as a measure of water potential. Measurements of water potentials are made by sealing the petiole of a leaf (or the stem of a leafy branch) in a pressure bomb so that the cut end of the petiole projects to the exterior while the leaf blade is subjected to pressure (Scholander, Hammel, Bradstreet, and Hemmingsen, 1965). As pressure is applied to the blade, the water potential of the cell sap rises until it equals that of the sap in the xylem vessels at atmospheric pressure. At this point, the xylem sap exudes at the cut surface of the petiole. The pressure required to reach this point is related to the water potential of the leaf cells by:

$$Y_w = P + Y_s \text{ xylem}$$

where, P represents the negative component of the water potential of the xylem sap measured as a positive pressure in the pressure chamber. As Y_s xylem is generally small

in relation to P (higher than -3 bars) it may be disregarded in most studies (Boyer, 1969).

2.1.3.2 Vapour Pressure Techniques

The vapour pressure of water in a small volume of air in equilibrium with the sampled plant tissue is a sensitive indicator of the water potential of that tissue. In essence, the vapour pressure is measured by sealing the tissue in a small container with a thermocouple and immersing the container in a bath maintained at 25 ± 0.001°C (Ehlig, 1962). The wet bulb depression is then measured when the system has come to equilibrium. In the Spanner psychrometer (1951) the wet bulb temperature is measured by first cooling the thermocouple junction by passing a current through it (Peltier effect) and then measuring the temperature as the condensed droplet of water evaporates. In the Richards and Ogata psychrometer (1958) a permanent water droplet is established on a specially designed ring-shaped thermocouple. Although the Spanner psychrometer requires more complex switching arrangements it avoids problems associated with the respiratory rise in temperature during equilibration (Barrs, 1965). In comparison with the pressure techniques, vapour pressure measurements require more elaborate instrumentation and are less easily adapted to field conditions. On the other hand, the method is not restricted to organs with a stem,

can use smaller pieces of tissue and can be adapted to use on intact plants (Lambert and van Schilfgaarde, 1965).

3. Water Stress

Water stress is a less precise term than the physical terminology discussed so far. It is essentially based on the growth response of the plant at a particular water potential, rather than the physical status of the water in the plant. Whenever the conditions of water are unfavourable to optimum plant growth, the plant is said to be under water stress (Milthorpe, 1960) and the degree of water stress can then be expressed in terms of the water potential of the plant. A particular water potential, however, does not necessarily produce the same level of stress in all plant species and varieties and the stress achieved may be different within the same plant at different times or stages of growth.

3.1 Water stress and Growth

3.1.1 Cell Enlargement

The relationship between water potential and cell enlargement has been frequently investigated (Broyer, 1950; Ordin, 1958, 1960; Brouwer, 1963) and a close positive relationship between enlargement and water potential has generally been demonstrated (Wadleigh and Gauch, 1948). Ordin (1958, 1960) attempted to distinguish between a

possible direct effect of water potential *per se* and the effects of the osmotic potential in the plant which decreases as water potential decreases. Using mannitol (relatively non-permeating) and sodium chloride (relatively permeating) as osmotica, he argued that, if reduction in water potential was the main factor affecting enlargement, the mannitol treatment should give the greatest suppression. The experimental results, obtained with *Avena* coleoptiles, supported this contention. The data also indicated that increased osmotic pressure did not suppress the metabolism of non-cellulosic polysaccharides measured by the incorporation of C^{14} from applied ^{14}C-glucose into cell wall fractions. Reduced water potential, however, affected both cell wall metabolism and elongation and Ordin suggested that some aspect of cellulose synthesis may have been inhibited by reduced tissue turgor. Similar data were obtained by Plaut and Ordin (1961) with entire leaves of sunflower and almond subjected to water stress by either desiccation or incubation in an osmotic medium.

In non-vacuolated meristematic tissues, such as in rapidly dividing root and shoot tips and developing fruiting bodies where growth is due to cell division in the main, effects of reduced water potential on growth are more likely to be due to inhibition of cell division than

of cell enlargement. The water potential values associated with reductions in the expansion rates of non-vacuolated or partly vacuolated tissues vary considerably, as do the values associated with the cessation of elongation (Loomis, 1934; Wilson, 1948; Slatyer, 1957). As the growth parameters involved in these responses (Slatyer, 1967) are complex, the experimental data are more difficult to interpret than the simpler cases discussed previously.

3.1.2 Cell Division

Cell division appears less influenced by water stress than cell elongation (Vaadia et al, 1961) but some exceptions have been found (Gardner and Nieman, 1964). Support for this generalization has been drawn from the observation that cell numbers are frequently similar in plants exposed to stress and in watered plants (Maximov, 1929). Such general evidence may be misleading, however, as more recent evidence (Nicholls and May, 1963; Husain and Aspinall, 1970) suggests that the growth of shoot apices, mainly by cell division, is very sensitive to water stress. Primordium formation in barley was completely inhibited by soil water potential between - 2.0 and - 2.5 bars (Nicholls and May, 1963). The influence of water stress on apical growth and development was further investigated by subjecting plants to osmotic stress using

polyethylene glycol as the osmotic agent and it was found that the formation of new primordia on the apical meristem of barley (Hordeum distichum) was inhibited at levels of soil water potential of -1 bar and less which had little or no effect on the growth of the plant (Husain and Aspinall, 1970). Primordium formation was inhibited even though apical water potential was unchanged.

Direct evidence for an effect of water potential on cell division has come from the observation that the rate of increase in deoxyribose nucleic acid (DNA) content of cotyledonary leaves of radish, incubated in mannitol solutions of different water potentials, is reduced by about 60% by a reduction in leaf water potential from 0 to -2 bars and a further 20% by a fall to -8 bars (Gardner and Nieman, 1964). As the water potential was reduced further to -16 bars, however, the rate of increase in DNA was not further affected. Since cell number is linearly related to DNA content (Nieman and Poulson, 1962) this suggests that the initial reduction in rate of DNA replication is associated with a proportional decrease in the rate of cell division. It is clear, then, that cell division is dependent on water potential but the relative sensitivities of cell enlargement and of cell division, to a fall in water potential, remain to be assessed.

3.1.3 Dry Matter

Water stress has been repeatedly found to decrease the rate of photosynthesis (Verduin and Loomis, 1944; Ashton, 1956; Brix, 1962; El-Sharkaway and Hesketh, 1964) and the reduction in plant dry weight associated with stress has been ascribed to this effect. A reduction in apparent photosynthesis has been frequently observed at -1 to -3 bars water potential (Brix, 1962) and it declines linearly with further decreases in water potential, even becoming negative at low potentials where respiration may exceed photosynthesis (Schneider and Childers, 1941; Bordeau, 1954; Brix, 1962). This reduction in photosynthesis has been attributed to stomatal closure and reduced rates of CO_2 exchange (Kramer, 1949; Penman and Schofield, 1951; Milthorpe, 1960) and a direct effect on the biochemical processes involved in photosynthesis (Boyer, 1965; Nir and Poljakoff-Mayber, 1967). Water stress-induced stomatal closure hinders not only CO_2 exchange but also O_2 (Thomas and Hill, 1949; Ashton, 1956), but profound effects on respiration have also been attributed to other biochemical changes (Schneider and Childers, 1941; Loustalot, 1945; Brix, 1962). As water stress is imposed, an initial increase in respiration rate (Stocker, 1960), followed by a reduction in rate (Heath and Meidner, 1961; Brix, 1962) as the plant adapts to

stress, has been observed. If stress is imposed gradually, the first phase may not become apparent (Slatyer, 1967), but with rapid water stress the initial increase in respiration rate can be quite marked. It is obvious, then, that water stress reduces the rate of photosynthesis and respiration and, if severe, can bring these processes to a complete halt. These intricate processes, in turn, affect other growth phenomena (Aspinall, Nicholls, and May, 1964) leading to reductions in fresh and dry matter production. The reduction in fresh weight, for example, may be proportionately much greater than the loss of dry weight (Gingrich and Russell, 1956; Hagan, Vaadia, and Russell, 1959). Severe water stress has been found to cause twice as much reduction as mild stress (Gates, 1955a, 1955b). Relatively mild stress of short duration, however, has been demonstrated initially to favour root growth over shoot growth (Wangermann, 1961; Jarvis and Jarvis, 1963). This initial stimulation in root growth which is followed by a reduction with progressive stress is difficult to explain on the basis of evidence available and the phenomenon remains to be explored.

3.2 Water Stress and Cell Metabolism

Although the effects of water stress on plant growth and development have been adequately documented, relatively little attention has been paid to the influence of

water potential on cell metabolism which underlies these growth responses. The information available has been reviewed by Vaadia et al, (1961), Henckel (1964) and Crafts (1968) but some important studies have been published since this last review.

3.2.1 Carbohydrate Metabolism

Decreases in total polysaccharides and increases in sucrose (Iljin, 1927), or in both mono- and disaccharides (Magness, Regeimbal, and Degman, 1933; Eaton and Ergle, 1948) have been found with vascular plants exposed to low water potential for long periods. However, a decrease in polysaccharides is not invariably accompanied by a concurrent increase in sugar content (Wadleigh and Ayers, 1945; Woodhams and Kozlowski, 1954). The reduction in polysaccharides has been attributed to reduced photosynthesis (Bordeau, 1954; Brix, 1962), increased polysaccharide hydrolysis (Eaton and Ergle, 1948), and/or decreased synthesis (Kozlowski, 1964). Interpretation of most of the available data is difficult, however, as they were obtained after long periods of water deficit.

The synthesis of ^{14}C-sucrose from ^{14}C-glucose was increased and the synthesis of starch decreased when Chlorella cultures were exposed to low water potentials (Greenway and Hiller, 1967). This suggested that

increased synthesis of sucrose could be partially responsible for the recovery of Chlorella after exposure for some time to low water potentials. These experiments, which involved only short-term exposures to water stress, may offer an explanation of the common observation of decreased polysaccharide synthesis in long-term studies. The reasons for these effects of stress on carbohydrate metabolism are not clear. Decreases in sugar phosphate with water stress in Trifolium subterraneum L. (Wilson and Huffaker, 1964) may have resulted from reduced carbohydrate phosphorylation consequent upon a shortage of ATP (Zholkevich and Koretskaya, 1959) or from increased phosphatase activity (Nir and Poljakoff-Mayber, 1966). If phosphorylation is reduced, a reduced level of UDPG might be the cause of the reduced starch level found in many species under water stress (Eaton and Ergle, 1948; Woodhams and Kozlowaski, 1954). However, in Chlorella, according to Greenway and Hiller (1967), the pronounced increase in sucrose synthesis, which over short periods usually exceeded the reduction in polysaccharide formation, would lead to a rapid removal of sugar phosphates needed to cope with increased sucrose synthesis. In support of this view, that the low level of sugar phosphates during water stress is due to their rapid removal rather than inhibition of their formation, Santarius and Ulrich (1967) reported

that in beet leaves the formation of ATP is reduced only at exceedingly low water potentials.

3.2.2 Protein and Amino Acid Metabolism

In the study of biochemical changes in plants under water stress, increasing attention is being paid to changes in nitrogen compounds (Petri and Wood, 1938; Mothes, 1956; Barnett and Naylor, 1966). Proteolysis and interruption of protein synthesis are generally found to result from water stress (Kemble and Macferson, 1954; Mothes, 1956; Zholkevich and Koretskaya, 1959), although both increases and decreases of protein have been found to follow each other (Chen, Kessler, and Monselise, 1964). As an example, Petri and Wood (1938) demonstrated a decreased net formation of proteins from amino acids as moisture deficit increased, while Chen et al (1964) found successively an increase, decrease, and a second increase in protein levels with increasing stress in citrus seedlings. A high ratio of non-protein nitrogen to protein nitrogen under unirrigated conditions (Yarosh, 1959) has also been reported. Recent studies (Barnett and Naylor, 1966; Routley, 1966; Thompson, Stewart, and Morris, 1966), however, have demonstrated that water stress causes inhibition of synthesis and hydrolysis of proteins. This leads to the accumulation of several amino acids, particularly proline. In Cynodon dactylon L. marked loss of protein-

bound arginine (Barnett and Naylor, 1966) was demonstrated under water stress reflecting a preferential hydrolysis of arginine-rich protein. However, the basic nuclear and ribosomal proteins as a whole are rich in lysine as well as in arginine, and no similar loss of lysine was observed. This could indicate that the loss of protein-arginine involves the degradation of some arginine-rich but lysine-poor protein. Whatever the nature of the proteins degraded during water stress the interruption of synthesis and the subsequent proteolysis of proteins lead to an increase in the amounts of soluble nitrogen compounds, such as amino acids, ammonium- and amide- compounds and soluble proteins in the plant (Todd and Basler, 1965; Stutte and Todd, 1967). An increase in nitrate has also been observed (Henrici, 1952). The details of the stress-induced inhibition of synthesis and proteolysis, however, remain to be elucidated.

More is known about the accumulation of free amino acids as a result of water stress (Kemble and Macferson, 1954; Petinov and Berko, 1965; Savitskaya, 1965). Petinov and Berko (1965) identified 13 and 16 free amino acids which accumulate in corn sap and roots respectively; proline, valine and arginine being in large concentrations. Kemble and Macferson (1954) demonstrated that during wilting of detached leaves proline increased, asparagine decreased, but glutamine remained unchanged. A direct

relationship between the initial concentration of carbohydrate and the formation of glutamine in barley (Yemm, 1949) has been demonstrated and it has been suggested that the concentration of readily available carbohydrate may influence amide formation in plants. However, water stress has been found specifically to cause a rapid and extensive accumulation of free proline in every part of the plant (Barnett and Naylor, 1966; Thompson et al, 1966) which turned over very slowly. Proline alone has been demonstrated to accumulate in amounts as much as 4380 µg/g dry weight in leaves of creosotebush (Larrea divaricata Cav.) seven days after withholding water (Saunier, Hull, and Ehrenreich, 1968) and about 15870 µg/g dry weight in bermuda grass (Cynodon dactylon L.) within the range of -18 bars to -37 bars of water potential of leaves (Barnett and Naylor, 1966). The mechanisms of proline accumulation during stress is not known. Aspartic acid may be converted to glutamic acid and glutamine (Naylor, Rabson, and Tolbert, 1958) but whether there is a direct conversion of asparagine to proline during wilting remains to be established. Accumulation of proline has also been suggested (Kemble and Macferson, 1954) to occur at the expense of other amino acids during wilting of detached leaves. The significance of this startling accumulation of free proline is obscure.

3.2.3 Nucleotide, RNA, and DNA Metabolism

Data on nucleotide metabolism in relation to water stress are scarce and both conflicting and confusing. Gates and Bonner (1959) demonstrated a decrease in both deoxyribose nucleic acid (DNA) and ribonucleic acid (RNA) in young tomato leaves subjected to water stress while Shah and Loomis (1965) observed increased soluble RNA and decreased RNA in ribosomal and other cell fractions in sugar beet. Reductions in acid-soluble nucleotides (West, 1962) and RNA (Kessler, 1961) have been frequently demonstrated after a long exposure to water deficit. However, leaves of moisture-stressed plants had the ability to incorporate labelled phosphate into RNA (Gates and Bonner, 1959) even though they did not exhibit net synthesis of the material. Osmotic pressures developed by KCl and sucrose stimulated the formation of poly-adenine from adenosine diphosphate (ADP) which, in turn, gave rise to typical changes in base composition of copolyribonucleotides synthesized from equimolar concentrations of ADP, GDP, CDP, and UDP (Kessler, Engelberg, Chen, and Greenspan, 1964). They suggested that environmental stress, through the effects of osmotic media on the activity of polynucleotide phosphorylase, may be coded into an altered composition of polynucleotides, leading to adaptive reactions serving as primers for RNA-dependant

polymerase. It is difficult, however, to draw any conclusions from these various data since they were either the results of prolonged water stress (Gates and Bonner, 1959; Kessler, 1961) or were not related to leaf water status (Shah and Loomis, 1965).

3.3 Water Stress and Cell Organelles

It is clear that mitochondria are the sites of respiratory enzymes (Hackett, 1963; Lehninger, 1964), ribosomes are the platform for protein synthesis (Tashiro and Siekevitz, 1965; Schweet and Heintz, 1966), and chloroplasts are the seat of photosynthetic enzymes (Calvin and Bassham, 1962; Bassham, 1964). Any factor which affects any of these sub-cellular bodies is likely to change the course of plant metabolism. Few investigations into the effects of water stress on these cell organelles have been carried out and conflicting views have been expressed. The effects of water potential (primarily osmotic potential) on both animal and plant mitochondria have been investigated. Lowering the water potential would be expected to depress any reaction in which water is a reactant and experimental evidence consistent with this view has been obtained for animal mitochondria (Nelson and Schubert, 1928; Taylor, 1954; Dixon and Webb, 1964). Uncoupling due to osmotic effects of sucrose on rat liver mitochondria has been demonstrated

(Cooper and Lehninger, 1957) and it has been suggested (Bernstein, 1961) that low water potentials are unlikely to impair the reactions of the tricarboxylic acid cycle. Recently, Flowers and Hanson (1969), working with isolated soybean mitochondria, however, demonstrated decreased oxygen uptake with decreasing water potential, but concluded that these effects on mitochondrial respiration were not due to the effects of low water potentials per se but to the effects of increasing solute concentrations.

Recently, Genkel, Satarova, and Tvorus (1967) demonstrated that in droughted shoots the protein content in the ribosomes decreased. With severe water stress polysomes were either absent or fragmented. They suggested that unfavourable factors such as drought stimulated ribonuclease activity causing polysome breakdown and, hence, resulting in a decrease in the intensity of protein synthesis. Nir and Poljakoff-Mayber (1967) attempted to study the behaviour of chloroplasts under water stress conditions and demonstrated a lower photochemical activity of chloroplasts isolated from leaves exposed to water stress and their ability to carry out the Hill reaction and cyclic photophosphorylation was much lower than those isolated from turgid leaves. Increased activity of enzymes such as phosphatase and ATPase (Nir and Poljakoff-Mayber, 1966) has been occasionally observed in stressed

chloroplasts but it has been suggested that the activity of the ATPase would not greatly affect ATP level. It is difficult to decide as yet as to the physiological significance of the increase in acid phosphatase in chloroplasts induced by water stress and dehydration of the leaf. Full evaluation of this effect must await further study.

Dehydration causes changes in the fine structure of mitochondria and plastids, damage to plasma membranes, and rearrangement of chromatin in the nucleus of the meristematic root cells of maize (Zea mays L.). The extent of these various changes is proportional to the degree of dehydration (Nir, Klein, and Poljakoff-Mayber, 1969); severe dehydration induces parallel arrangement of long reticular elements. These authors also investigated the effect of water stress on mitochondrial behaviour and structure of the same species and found that severe water stress caused a reduction in the respiratory rate (O_2 consumption) and an increase in the activity of mitochondrial cytochrome oxidase (Nir, Poljakoff-Mayber, and Klein, 1970). By a histochemical method they demonstrated that the mitochondrial cristae were not destroyed during stress but there was some change in their structure. The cristae are made up of structural proteins, enzymes, and lipids (Lehninger, 1964) and Nir et al (1970) suggested that water stress induced changes in the arrangement of

these compounds and a loss of the lipids. The lipid fraction has been suggested to serve as a hydrophobic medium in which electrons flow from one enzyme to another (Green and Silman, 1967), and this may explain the low O_2 uptake of tissue subjected to water stress.

3.4 Water Stress and the Physiology of Flowering

This is an area of plant physiology which is poorly understood. Results from field experience and from agricultural experiments suggest that the time of flowering is influenced by water stress, and it has been claimed that water stress keeps floral buds dormant (Nes, 1957). In Coffea trees, on the other hand, Alvim (1960) found that when he maintained soil moisture at a high level by weekly irrigation the flower buds failed to open. If he allowed soil moisture to drop to near the wilting point before irrigation, flowering occurred abundantly after each irrigation. He suggested that water stress removed a growth inhibitor responsible for dormancy and visualized a water stress requirement in Coffea similar in effect to the chilling requirement for breaking dormancy of many species in cold regions. At the pollen meiosis stage in wheat, water stress was demonstrated to cause male sterility in lower florets of each spikelet without affecting female fertility (Bingham, 1966). Although megasporogenesis was found to be less susceptible to water stress

than microsporogenesis, prolonged stress caused the female gametophyte to become infertile also (Skazkin and Lukomskaya, 1962). Young olive (Olea europaea L.) trees subjected to water stress at any stage between the appearance of floral primordia and full bloom had fewer flowers per inflorescence than trees not subjected to water stress (Hartmann and Panetsos, 1961). In these cases, water stress was affecting floral development following flower induction and little is known of any effects on the process of induction itself.

Recently, Husain (1967) demonstrated that water stress inhibited floral initiation in Lolium temulentum L. - a single long day requiring plant, and suggested that water stress has a profound effect on the translocation of the floral stimulus from the site of production to the site of action. Further, he observed that water stress did not cause the destruction of the floral stimulus since after the removal of stress flowering occurred normally.

3.5 Water Stress and Hormonal Physiology

It has been suggested on several occasions that some of the effects of water stress on plant growth are most readily explained by an effect of water status on hormonal physiology (Nicholls and May, 1963; Itai and Vaadia, 1965; Aspinall, Paleg, and Addicott, 1967; Pustovoitova, 1967). Rapid responses, such as the inhibition of

primordium production (Husain and Aspinall, 1970) and differential effects on growth rates of the various plant organs (Gates, 1955 a,b) are suggestive of hormonal control, but direct evidence of water stress effects on hormone physiology is meagre, however, and no clear picture emerges.

A study of the influence of soil drought on natural growth substances was begun by Alekseev (1951) who suggested that, as a result of the action of early soil drought, the content of substances of the auxin-type in the leaves of oats and wheat was reduced; subsequent watering somewhat increased their amount in the leaves which had completed their growth before the drought. The leaves that were in the embryonic state during drought did not show a restoration of the content of auxin-type substance after watering. Atmospheric drought also reduces the auxin content in wheat plants (Kydrev and Tyankova, 1960).

Turning towards relatively recently known plant hormones several workers have postulated effects of water stress on cytokinins within the plant. The root systems of sunflower plants subjected to water stress produced less cytokinin-like activity than normal roots (Itai and Vaadia, 1965) and similar results have been found with root systems subjected to salt stress (Ben-Zioni, Itai,

and Vaadia, 1967). The reduction in cytokinin activity was associated with a decrease in the ability to incorporate L-leucine-^{14}C into protein. This decreased potential could be partially overcome in stressed tobacco leaf discs by pre-treatment with kinetin (Itai, Richmond, and Vaadia, 1968). On the basis of these data Vaadia and co-workers postulated that root-produced cytokinins are important in controlling shoot growth and that the level of these cytokinins in the plant varies with water status. Much of the data was obtained from plants subjected to high salt concentrations in the external medium and the effects of salt toxicity should not be ignored.

In contrast to the information on cytokinin levels during water stress, nothing is known of changes in gibberellin levels during stress although there are a few reports of effects of flooding on gibberellin production (Phillips, 1964; Reid, Crozier, and Harvey, 1969). It is of interest, however, that exogenous gibberellin alone, of a range of plant growth hormones, was capable of overcoming the growth inhibition due to salinity stress in Phaseolus vulgaris L. (O'Leary and Prisco, 1968).

Both cytokinins and gibberellins are essential growth-promoting substances which might be expected to decrease during growth inhibition induced by water stress. Growth inhibiting substances, particularly abscisic acid, also

appear to play a role in the control of plant growth (Addicott and Lyon, 1969) and have been shown to inhibit stomatal opening (Little and Eidt, 1968; Mittelheuser and Steveninck, 1969), protein synthesis (Chrispeels and Varner, 1967) and flower initiation (Evans, 1966). All of these phenomena are associated with the effects of water stress and it is of considerable interest that Wright (1969) and Wright and Hiron (1969) have recently demonstrated an increase in the abscisic acid content of detached, wilting leaves. Wheat leaves were detached, rapidly wilted to a pre-determined water content by subjecting them to a heated stream of air, and then held at this water content between layers of damp chromatography paper. The abscisic acid content of the leaves increased rapidly, reaching a peak of four times the original concentration 110 minutes after wilting. Although this observation is of considerable interest it will have to be repeated with whole plants subjected to water stress by more conventional means with measured water potential before its significance can be assessed.

4. Drought Resistance in Plants

4.1 Definitions

Recently, the term 'drought resistance' has been redefined as an ability to stay alive in dry climates, and classified into (a) drought avoidance and (b) drought tolerance (Levitt, 1956; Levitt, Sullivan, and Krull, 1960). Drought avoidance is defined as the ability to prevent reduction in water content and includes (i) ephemerals (drought escaping) - plants with the ability to complete their life cycle before extreme drought, (ii) water spenders (drought evading) - plants with the ability to obtain large amounts of water during drought, and (iii) water savers (drought enduring) - plants able to reduce their water loss to a minimum. Drought tolerance is defined as the ability to survive reduction in water content, and is basically similar to terms such as desiccation resistance (Iljin, 1927), physiological drought resistance (Maximov, 1929), and protoplasmic and constitutional drought resistance (Stocker, 1960). From the agronomic and plant breeding point of view characteristics such as an ability to reduce water loss and survive during drought are useless unless these characteristics are accompanied by higher yield under drought conditions. Several attempts (Kearney and Shantz, 1911; Tumanov, 1927; Maximov, 1929; Dilman, 1931; Ashton, 1948) have been made

to find a positive correlation between characteristics such as an ability to reduce water loss and survive during drought and field performance of crops, but no such relation exists (Slatyer, 1957; Asana, 1965; Levitt, 1965). A valuable definition of drought resistance suggested by May and Milthorpe (1962b) defines 'drought resistant' as an all-embracing term to describe those varieties or species which are able to grow and yield satisfactorily in areas liable to periodic drought. They recognized three main types of drought resistance such as drought escape, drought endurance with high internal water content, and drought endurance with low internal water content. They regarded drought hardiness as including both types of drought endurance as the term is widely used agriculturally in this sense and because maintenance of a high internal water content appears to be a far more common element in hardiness than tolerance of partial drying. This definition resembles those previously suggested, but differs in attempting to remove confusion arising from equating 'drought' with 'plant water deficit' and in placing more emphasis on the ability to grow in a dry climate than on surviving a severe internal water stress. The applicability of this definition is apparent in the technique designed to measure the adaptability of crops over a wide range of environments recently suggested by Finlay and Wilkinson (1963). These workers suggested

two indices; the first of these is a measure of stability - technically the regression coefficient of yields of a variety grown at several sites representing a wide range of environments for several years on the mean yields of all varieties, and the second is the mean performance of the variety over all tests. A regression coefficient of 1.0 or less, combined with high mean performance indicates a high level of adaptability over all environments. This technique, in essence, when applied to an area where drought is the major yield determinant, fulfils the requirements of the definition suggested by May and Milthorpe (1962b).

4.2 Water Relations

Previously, drought avoidance has been assessed by empirical methods such as the water saturation deficit and the relative turgidity. But avoidance depends on the plant's ability to prevent or reduce a net movement of water to its environment. Since water moves along intensity and not capacity gradients (Levitt, 1965) and the above quantities measure water capacity, it is obvious that they cannot reveal quantitatively the plant's avoidance of drought. In fact, distinguishing between drought avoidance and drought tolerance requires information on the water potential of the plant tissues. The water potential of the tissues, measured by psychrometric or

other means, is the only criterion which allows unambiguous assessment of plant water deficit and which makes clear the distinction between the two plant attributes, tolerance and avoidance of drought.

4.3 Empirical Tests of Drought Resistance

In the important cereal growing countries of the world, wheat, barley and oats are grown over large areas under semi-arid conditions. Consequently, the breeding of drought resistant varieties has claimed considerable attention and effort. Although field experience under dry conditions has been usually relied upon to assess drought resistance, it has frequently been suggested (Ashton, 1948) that knowledge of the fundamental basis of drought resistance and character(s) associated with it might lead to successful tests of field drought resistance in laboratories, and might prove useful in breeding for this quality. Tests based on drought tolerance have been made to measure the drought resistance of whole plants by determining the survival of plants subjected to a period of stress either in the field (Tumanov, 1927), or in more carefully controlled conditions (Kenway and Peto, 1939; Dexter, 1942). In these two methods whole plants were used, and the problem of the transfer of water from other parts of the plant or from the soil during test was encountered. To overcome the problem inherent in the use

of whole plants, an osmotically graded series of solutions was adapted and leaf sections were placed in small chambers and allowed to come to equilibrium with the atmosphere directly above these solutions (Iljin, 1930). After a certain period of time, sections were removed and transferred to hypertonic sugar solutions containing neutral red and examined for living cells. In this way the relative humidity that was just sufficient to cuase drought killing was more accurately determined. In this method, although killing relative humidity was determined accurately, no difference in the degree or time of injury could be distinguished and, more importantly, drought chambers failed to yield results in agreement with field behaviour (Kenway, Peto, and Neatby, 1942; Cook, 1943; Milthorpe, 1950).

Physiological characters such as water requirements and transpiration rate (Richardson, 1923; Dillman, 1931; Bayles, Taylor, and Bartel, 1937), and anatomical and morphological characters (Haber, 1938; Ashton, 1948) have also not been found to provide a simple and practical index of drought resistance in selection work. Turning towards physico-chemical characters, there is less general agreement as to their significance in breeding investigations. Newton and Martin (1930) and Schmidt, Diwald, and Stocker (1940), for instance, considered that the

osmotic pressure of cell sap was unsatisfactory as an index of drought resistance. According to Buchinger (1936) and Ranninger (1936), however, the osmotic pressure of germinating seeds was a valuable basis of selection in respect of drought resistance. Conflicting results have been found in respect of bound water content in relation to drought resistance. In wheat varieties and grass species a reasonable positive correlation between bound water content and drought resistance (Newton and Martin, 1930) has been demonstrated, while others failed to find such a relationship (Whitman, 1941; Carroll, 1943). In view of the uncertain nature of morphological, physiological, and physico-chemical characters, as an indication of drought resistance for breeding purposes, in practice plant breeders have relied on field experience. The potential usefulness of a more rapid, simple test remains, however, and several workers (May and Milthorpe, 1962b; Asana, 1965; Levitt, 1965) have expressed the need for an understanding of the biochemical basis of drought resistance as an aid to the development of such a test.

4.4 Biochemical Investigations

Only a few fragmentary investigations have been carried out on the biochemical aspect of drought resistance and are primarily empirical in nature. For instance, Sisakjan and Kobjakova (1938, 1939), working with wheats

of varied drought resistance, showed that when the tissues attain a certain degree of dehydration, the protoplasmic structures lose their ability to bind enzymes (invertase and others) and, as a result, the enzymes go into solution and stimulate intensive hydrolytic decomposition. They suggested that this phenomenon was observed in drought resistant plants only under conditions of high water loss. Though this difference in enzymatic shift may be real, the observed distinction among varieties might be due simply to differences in water potential of plant tissues grown either in the field or pots subjected to water stress by withholding water. Similarly, it was thought that the stability of chlorophylls under drought conditions might serve as a useful criterion of drought resistance but conflicting results have been published. Kaloyereas (1958) and Murty and Majumder (1962), for instance, demonstrated a high positive relationship between chlorophyll stability index and field drought resistance in pine and rice respectively, while Panous (1967) failed to find such a relationship in pearl millet.

It has also been claimed that drought tolerance may be dependent upon RNA metabolism. Plants of the drought tolerant genus Olea synthesize increased amounts of guanine- and cytidine-rich RNA molecules under water stress in comparison with plants of the drought-sensitive genus

Ligustrum (Kessler and Frank-Tishel, 1962). The role of new RNA molecules remains to be established and the observed differences could be due simply to generic differences. In contrast, Stutte and Todd (1968) concluded that the use of the nucleotide composition of wheat RNA alone does not appear to be a suitable method of determining the potential resistance to water stress.

In view of the pivotal importance of nitrogenous compounds in plant metabolism, and the lack of quantitative biochemical studies in relation to the water status in the plant, the effects of water stress on protein and amino acid metabolism in barley (Hordeum distichum) and wheat (Triticum aestivum) were investigated. The study of these biochemical events was accompanied by the parallel measurement of the water potential in the plant, the only unequivocal measure of the physical state of water in the system, and forms the bulk of the work reported in this thesis.

CHAPTER II

MATERIALS AND METHODS

The general cultural practices and the design of the experiments are described here but further details of the individual experiments can be found in the Results section.

1. Materials

1.1 Barley (*Hordeum distichum*)

The cultivar Prior was used in most of the experiments; this is a 2-row early variety grown extensively in Southern Australia. A selection of thirteen other varieties was used in one experiment, covering a wide range of adaptability. These varieties were supplied by Mr. D.H. Sparrow of the Department of Agronomy, Waite Institute.

1.2 Wheat (*Triticum aestivum*)

The cultivar Gabo, a common Australian variety, was used exclusively.

2. Methods

2.1 Environment Control

Controlled environment cabinets of the Department of Plant Physiology were used for growing plants. Each cabinet has space (4' x 4') for one hundred pots (10 x 10 cm) arranged in 10 rows on the plant bed. The light

source was a bank of 32/80 watt 'cool white' fluorescent tubes (Philips TLF 80/33) supplemented with five incandescent strip tubes (60 watts). The light intensity was 2,000 foot-candles, with a 16-hour-photoperiod. Temperature was maintained at 17°C ± 1°C inside the cabinet. The relative humidity of the cabinet was not controlled.

2.2 Plant Culture

Seeds were germinated on wet filter papers (Whatman No. 3) in petri dishes in an incubator at 20°C for 24 hours before they were planted in pots. Soil or perlite was used as the growing medium. Ten germinated seeds were planted in each pot and were thinned to 5 or 6 healthy, uniform seedlings after 3 days. Each pot was irrigated every day with 50 ml of nutrient solution for the first 10 days and 100 ml thereafter, when the plants were grown in perlite. The composition of the nutrient solution is given in the following table:

Salt	ppm
$Ca(NO_3)_2 \cdot 4H_2O$	1180
KNO_3	202
KH_2PO_4	87
$MgSO_4 \cdot 7H_2O$	493
KCl	9.0 Cl

Salt	ppm
Fe-EDTA	0.5 Fe
$MnSO_4$	0.25 Mn
H_3BO_3	0.25 B
$ZnSO_4$	0.25 Zn
$CuSO_4$	0.02 Cu
Na_2MoO_4	0.02 Mo

No nutrient solution was supplied when the plants were grown in soil, but they were watered daily until ready for treatment.

2.3 Imposition of Water Stress

Water stress was imposed by withholding water or by adding 250 ml of polyethylene glycol (MW 4,000) solutions of a known osmotic potential to the rooting medium. Isolated plant organs were subjected to water stress by floating on similar solutions. Polyethylene glycol of varying molecular weight ranges has been claimed to be the most suitable osmotic agent for water stress investigations (Jarvis and Jarvis, 1963; Barrs, 1966; Husain and Aspinall, 1970), although some toxic responses to this substance have been reported (Leshem, 1966). Polyethylene glycol (Carbowax - Union Carbide Australia Ltd.) with an average molecular weight of 4,000 was used throughout the

present investigation. This molecule has been used previously in similar investigations without any evidence of toxicity (Husain, 1967; Husain and Aspinall, 1970).

2.4 Water Potential Measurement

In every experiment, except those with isolated organs and abscisic acid, the water potential of the leaves was measured with a Spanner thermocouple psychrometer (Barrs, 1965). 3 to 4 leaves were wrapped in a single layer over a copper wire mesh cylinder and gently inserted into the chamber. The chamber was then fitted with a thermocouple and placed in the bath. The chambers were allowed to come to equilibrium for approximately 2 hours before the deflection was recorded. The water potential was calculated by comparing the recorded deflection with the deflections obtained from a graded series of KCl solutions.

2.5 Plant Growth

2.5.1 Weight of Plants

Fresh plant material was placed in 2 x 1 cm specimen tubes, weighed and dried in a forced-draught oven at 90°C for 48 hours before the dry weight was measured.

2.5.2 Apical Growth

2.5.2.1 Dissection of Plants

Plants were dissected under a binocular microscope

with the help of a fine needle and forceps and the following observations were recorded:

2.5.2.2 Primordium Production

The total number of lateral appendages produced by the apex, including the fully-expanded, expanding, and differentiating (not visible before dissection) leaves, simple ridge primordia and spikelets, were counted.

2.5.2.3 Length of Apex

The lengths of the apices were measured with the aid of an ocular micrometer. The length from the tip of the apex to the base of the lowest simple ridge or spikelet primordium was recorded.

2.5.2.4 Stage of Development

The stage of apical development was judged from the development of the most advanced spikelet using the scale described by Aspinall and Paleg (1963):

Stage of Development	Score
Apex not elongating	1
Apex elongating	2
Double ridge on apex	3
Upper ridge enlarging	4
Lateral spikelets visible as simple mounds	5
Glume initials visible	6
Lemma initials visible	7
Stamen initials visible	8

Stage of Development	Score
Awn initials visible	9
Awns longer than spikelets	10
Anthesis	11
Inflorescence fully emerged	12

2.6 Preservation of Plant Material

Leaves, leaf sheaths, shoot apices and roots were excised, wrapped in aluminium foil and immediately frozen in liquid nitrogen or by dry ice. These samples were stored at -20°C until required.

2.7 Chemical procedures

2.7.1 Total Free Amino Acids

2.7.1.1 Technique

Thin-layer electrophoresis followed by chromatography in two different solvent systems, as described by Bieleski and Turner (1966), was used, except the plates after electrophoresis were not dipped into a tank of distilled water.

2.7.1.1.1 Preparation of Thin Layer

Thin layer plates 20 x 20 cm were spread (using Shandon equipment) with a 250 μ layer of mixed cellulose and silica gel H. Thoroughly washed plates were placed on the spreader, which was levelled with the aid of a spirit level. Plates were lightly wiped with soft tissue moistened in petroleum ether to remove any remaining

traces of grease. A standard spreading schedule was as below:

MN 300 cellulose, 12.5 gm + Merck Silica gel, H, 5 gm + water, 100 ml, were homogenized together in a Virtis homogenizer at 20,000 rpm for 30 seconds. The mixture was allowed to stand for 30 seconds, homogenized again for 30 seconds, and then evacuated for 5 minutes and finally spread at a uniform rate. The plates were left overnight to dry at laboratory temperatures.

2.7.1.1.2 Electrophoresis

10-50 µl of standard solutions of amino acids containing 3 to 15 µg of each amino acid, were applied to the plate near the left-botton corner as a band 2.0 cm long. Buffer (pH 2.0, 17 ml 90% formic acid + 57 ml acetic acid per litre) was then sprayed over the plate. The plate was held nearly vertical with the origin, aligned horizontally, at the top. Spraying commenced from the bottom until the origin was reached, the plate was then placed horizontally and excess buffer was removed by gently blotting with chromatography paper.

Wicks were then attached to each end. These wicks were made by slitting and folding a piece of washed dialysis tubing, presoaked in buffer, over one edge of a 20 x 4 cm strip of acid-washed chromatography paper, so that when the combined wick was laid on the plate, the

dialysis tubing prevented any direct contact of paper and layer. To ensure good electrical contact, wicks were thoroughly wetted with buffer immediately before being placed on the layer, and covered with a 20 x 1 cm strip of plate glass. These strips were held in place by an overlying 20 x 20 cm glass plate. Electrophoresis, at 2°C in a cold room, was performed for 20 minutes at 1000 V (55V/cm) and 20-30 mA, in an apparatus described by Bieleski (1965). Glycol Light (obtained from Golden Fleece, Australia) was used as a coolant to maintain plate temperature at 14-16°C. After electrophoresis the wicks were removed and the plate dried at 40°C for 20 minutes in a forced air draught. The air was blown across the plate parallel to the bands, in the direction of subsequent chromatography.

2.7.1.1.3 Chromatographic Separation

For chromatographic separation at right-angles to electrophoresis the plate was chromatographed twice in the same direction, first in methyl ethyl ketone/pyridine/water/acetic acid, 70/15/15/2 V/V (1 hr 40 min) to separate threonine and glutamic acid, and remove interfering materials, then in n-propanol/water/n-propyl acetate/acetic acid/pyridine, 120/60/20/9/1 V/V (4.5 hr) for the major separation.

2.7.1.1.4 Spot Development and Estimation

The chromatographed, dried plates were held vertical and sprayed with ninhydrin reagent: (methanol, 100 ml; acetic acid, 0.5 ml; cadmium acetate, 0.10 gm; and ninhydrin 1.0 gm). The plates were then held over NaOH flakes in a desiccator for 9 hours at 38°C to develop the ninhydrin-coloured compounds. The developed plates were coated with a film of cellulose acetate solution (6% cellulose acetate + 3% diethylene glycol + 2% camphor in acetone/propanol 3/1 V/V) by pouring on at one end and spreading over the entire surface with the aid of glass rod. After the cellulose acetate film was dried, each coloured spot was lifted and put into a centrifuge tube with 1.5 ml eluting solvent (methanol/ethyl acetate/water, 1/1/1 V/V, containing 1% acetic acid and 1% cadmium acetate). The tubes were gently shaken for 10 minutes using a glass marble as stopper and centrifuged at 1000 x g for 5 minutes. Optical densities of coloured supernatants were measured at 505 nm except for proline, which was measured at 345 nm, and standard curves for the individual amino acids were calculated.

2.7.1.2 Preparation of the Plant Extract for Free Amino Acid Estimation

2.7.1.2.1 Extraction and Estimation

Leaf tissue, 150-200 mg fresh weight, from the frozen material was placed into 2 ml of MCW (methanol/chloroform/

water, 12/5/3 V/V) and homogenized in a Duall conical glass homogenizer (Kontes) at room temperature. The homogenate was centrifuged, and the supernatant collected. The residue was re-extracted by shaking it for 5 minutes with a further 2 ml MCW, centrifuged and the second supernatant was added to the first. To the combined extract were added 1 ml chloroform and 1.5 ml water (to break a stable emulsion which otherwise formed); these were shaken, centrifuged to separate the phases and the chloroform layer was discarded. The aqueous layer was dried under reduced pressure at 35°C on a rotary evaporator. The dried extract was taken up in 10% isopropanol for amino acid separations and the amounts of the individual amino acids were determined as above.

2.7.1.3 Specific Test for Proline

The method used for proline estimation in plant tissue was developed from a technique described by Troll and Lindsley (1955) for animal sources.

2.7.1.3.1 Extraction

50-150 mg fresh weight of tissues (20-25 shoot apices) were extracted twice with 3 ml MCW. The extracts were centrifuged each time and the supernatants were pooled. To the combined supernatant were added 1 ml chloroform and 1.5 ml water to break the stable emulsion; the mixture was shaken and centrifuged. The chloroform

layer (containing chlorophyll) was discarded and the aqueous layer was preserved for proline estimation.

2.7.1.3.2 Colour Development and Estimation

The aqueous layer was taken into large test tubes and diluted with 10 ml water, to which was added 375 mg of acid washed Permutit resin (Folin Decalso washed as follows: 2% NaOH solution passed through until the effluent contains an excess of this solution, allowed to stand an hour, washed with excess distilled water then treated with 5% H_2SO_4 until acidic, again washed with distilled water until the effluent was very faintly acid, and dried.). This was shaken for 10 minutes to remove interfering amino acids. The solution was decanted into another test tube to which was added 5 ml glacial acetic acid + 5 ml ninhydrin reagent (125 mg ninhydrin dissolved by heating at 70°C in 3 ml glacial acetic acid + 2 ml 6M orthophosphoric acid). The mixture was heated in a boiling water bath for 45 minutes and then cooled to room temperature. Benzene (5 to 15 ml) was added to the mixture to extract the ninhydrin colour, the tubes were shaken, allowed to stand for 10 minutes, and the separated benzene layer was removed. The optical density of the ninhydrin product dissolved in the benzene was measured at 515 nm. The amount of proline was calculated from a standard curve.

2.7.1.3.3 Recovery of Proline

Satisfactory recovery of proline was obtained when known amounts were added to plant tissues and to a mixture of other amino acids. The absorption spectrum obtained when these materials were subjected to the extraction procedure was identical with that obtained when proline alone was used.

Substance added	Proline added µg	Proline recovered µg	Proline recovered percent
Leaves	50	49.25	98.5
	100	96.80	96.8
Roots	50	50.25	100.5
	100	97.40	97.4
Amino acids	50	50.00	100.0
	100	101.50	101.5

2.7.2 Protein Estimation

2.7.2.1 Extraction of Soluble Protein

50-150 mg fresh weight of tissue was homogenized three times with 2 ml 0.05 M phosphate buffer (Na_2HPO_4 - KH_2PO_4, pH7.3); each time it was centrifuged at 27,000 x g for 10 minutes and the supernatants were pooled. The water soluble protein of the combined supernatant was precipitated by adding an equal volume of cold 20%

trichloroacetic acid (TCA). The precipitate was allowed to settle for at least 10 minutes. The protein was centrifuged down at 27,000 x g for 10 minutes and the pellet was then resuspended in 10% TCA and recentrifuged. The supernatant was then discarded and the pellet was decolourized by twice incubating for one-half hour at 37°C with 2 ml of EEC (ethanol/ether/chloroform, 2/2/1 V/V) and centrifuging each time. The protein precipitate was finally dissolved overnight in 1 ml of 2% Na_2CO_3 (reagent A, below).

2.7.2.2 Estimation

Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) which, in brief, is as follows:

Reagent

(A) 2% Na_2CO_3 in 0.1 N NaOH

(B) 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate, add in 1N NaOH to dissolve the ppt.

(C) Mix 50 ml of A + 1 ml of B (just before use).

(D) Folin reagent.

2.7.2.3 Procedure

The protein was dissolved in reagent A, 2 ml of reagent C was added and the mixture was allowed to stand for 10 minutes. 0.2 ml of reagent D was then added and mixed thoroughly. After 30 minutes the optical density

was read at 750 nm. The amount of protein in the tissue was calculated from a calibration curve obtained from bovine serum albumin (BSA).

2.7.2.4 Estimation of Protein-Bound Proline

2.7.2.4.1 Hydrolysis of Protein

TCA-precipitated protein was made to 6N HCl in hydrolysis tubes. After evacuation of air, the tubes were sealed and heated at 110°C for 20 hours. Hydrolysates were dried on a rotary evaporator at 35°C. Water was then added to the residue and the sample was redried repeatedly to remove excess HCl. The hydrolysate was dissolved in MCW, and the proline released by protein hydrolysis was estimated according to the procedure described earlier (2.7.1.3).

2.7.3 Growth Regulators

(i) (2-chloroethyl) trimethylammonium chloride (CCC) was obtained from Cyanamid International, New Jersey, U.S.A. The preparation had 50% CCC and 50% inert ingredient and was dissolved in nutrient solution at concentrations indicated in connection with the appropriate experiment.

(ii) Gibberellic acid (GA_3) obtained from Merck and Co. Inc., New Jersey, U.S.A. (97.4% GA_3) was dissolved in 0.05% Tween-20. Controls were treated with an identical

concentration of Tween-20.

(iii) Abscisic acid (ABA), obtained from Reynolds Tobacco Company, North Carolina, U.S.A., was dissolved in 0.05M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH7.3).

CHAPTER III

RESULTS AND DISCUSSION

SECTION 1

1. AMINO ACIDS AND PROTEIN METABOLISM IN INTACT PLANTS

1.1 Water Stress and the Free Amino acid Content of Leaves

1.1.1 Methods

The influence of water stress on the composition of free amino acids in the leaves of barley plants (cv Prior) was investigated. The plants were germinated at 20°C for 24 hours and then planted into 10 cm plastic pots in an Urrbrae loam (4 parts) : Plympton sand (1 part) mixture (Nicholls and May, 1963) and irrigated daily in order to keep the soil water potential at -0.1 bar or above. Plants were subjected to water stress by withholding the supply of water from the 7th day of planting and the first leaves were excised on the 10th day for water potential and amino acid assessment.

The water potential of the leaves was measured with a Spanner thermocouple psychrometer (page 41). Four leaves were wrapped over a copper wire mesh cylinder in a single layer and gently inserted into the chamber. The chamber was then fitted with a thermocouple and placed in the bath. The chambers were allowed to come to equilibrium for approximately 2 hours before the deflection was recorded. The water potential was calculated by comparing

the recorded deflection with the deflections obtained from a graded series of KCl solutions. The water potential of the control plants at the time of sampling was -3.0 (± 0.4) bars and of the stressed plants -16.0 (± 1.2) bars.

The free amino acids of the first leaf (4 replicates, 150 - 200 mg fresh wt. each, containing 2-3 leaves) were extracted by homogenizing in a Duall conical glass homogenizer (Kontes) with 2 ml MCW (methanol/chloroform/water, 12/5/3 v/v). The extraction was repeated once, the extracts were centrifuged each time and the supernatants were pooled. To the combined supernatants 1 ml chloroform and 1.5 ml water were added to break a stable emulsion which otherwise formed. These solutions were shaken and centrifuged to separate the phases and the chloroform layer containing the chlorophyll was discarded. The aqueous layer was dried under reduced pressure at 35°C on a rotary evaporator. The dried extract was taken up in 10% isopropanol, applied on thin layer plates for separations by electrophoresis and chromatography (page 44). The chromatographed, dried plates were sprayed with ninhydrin reagent and heated to develop coloured spots. Ninhydrin-coloured spots of individual amino acids, identified by virtue of their Rf, were dissolved in eluting solvent, centrifuged for 5 minutes and the optical densities of the coloured supernatants were measured (page 46).

The amount of each individual amino acid was calculated from standard curves.

1.1.2 Results

Water stress had profound effects on the concentrations of free amino acid in the leaves (Table 1). The total free amino acid content of leaves subjected to stress was more than double that of control leaves but this increase was due almost entirely to a 15-fold difference in the level of free proline in the leaf (control 251 µg/g, stressed 3904). Proline was identified both by its position on the chromatogram and by the characteristic colour reaction with ninhydrin reagent and there was no doubt that this was the major component affected by stress.

Apart from this massive increase in the level of free proline in the tissue, the concentrations of asparagine, glutamine, glycine, ornithine and phenylalanine increased markedly and valine levels rose slightly with water stress. There was also the appearance of three free amino acids unrecorded in the control plants. These were tentatively identified, by virtue of their Rf., as B-alanine, hydroxyproline, and pipecolinic acid. In contrast to these increases in the levels of certain free amino acids in the leaves as a result of water stress, the concentrations of free alanine, aspartic acid, glutamic acid and leucine were

Table 1

Water stress and the free amino acid content of the first leaf of barley.

Amino acids identified on the basis of Rf	µg/g dry wt. (Ave. of 4 replicates) (Figures marked by asterisk are significantly different)	
	Control -3.0(± 0.4) bars	Stressed -16.0(± 1.2) bars
Alanine	185	93**
B-alanine	-	19+
Y-aminobutyric acid	22	36
Arginine	74	97
Aspartic acid	322	143***
Asparagine	209	265*
½ Cystine	199	237
Glutamic acid	569	215***
Glutamine	272	423*
Glycine	201	274*
Hydroxyproline	-	83+
Histidine	53	65
Leucine	179	102***
Lysine	48	52
Methionine	25	26
Ornithine	21	41***
Phenylalanine	20	84***
Pipecolinic acid	-	18+
Proline	251	3904***
Serine	210	184
Threonine	197	225
Tyrosine	52	65
Valine	127	152*
Total	3236	6803

* Significantly different from control value at 5% probability
** Significantly different from control value at 1% probability
*** Significantly different from control value at 0.1% probability
\+ Tentative identification and estimation.

considerably lower in the leaves of plants subjected to water stress than in the control plants. Glutamate and aspartate, which were the dominant components of the free amino acid pool in control plants, were decreased below the level of their corresponding amines.

These data confirmed for the barley plant previous reports (Kemble and Macpherson, 1954; Barnett and Naylor, 1966; Thompson et al, 1966) that proline concentrations in the plant were profoundly affected by water stress. In view of the large change in free proline and the much smaller changes in the other free amino acids, attention was subsequently directed to proline alone.

1.2 Protein Metabolism and Proline Accumulation in Water stressed Plants

1.2.1 Methods

Barley plants (cv Prior) were grown in perlite (page 39) for 10 days. They were then subjected to water stress by replacing the nutrient solution in each pot with 250 ml of a solution of polyethylene glycol (MW 4000, -20 bars osmotic potential) in nutrient solution. After 30 hours, stress was removed from some plants by washing out the osmoticum from the rooting medium with six changes of 200 ml water. The plants were sampled 0, 10, 20, 30, 40 and 50 hours after stress commenced. The water potential of the first leaves and the protein and proline contents of

the same leaves as well as the roots were assessed.

1.2.2 Results

1.2.2.1 Water Potential

The water potential of the leaves fell steeply from -2.5 bars to -14.9 bars within 10 hours of subjecting the plants to stress. The water potential continued to fall, although more slowly, for the remaining 40 hours of the experiment and had reached -25.0 bars when the last sample was taken (Figure 1). When stress was relieved the water potential increased slowly for the first 10 hours and more rapidly thereafter. The water potential of the watered plants fluctuated within the range -2.5 to -3.2 bars.

1.2.2.2 Protein Metabolism

Net protein synthesis in the leaves was greatly but not completely inhibited by water stress (Figure 2, top) during the first 20 hours. Subsequently, there was no further net protein synthesis in the stressed plants and a considerable loss of protein in the final 10 hours of the experiment. When the plants were re-watered at a stage of stress when net protein synthesis was completely inhibited, protein synthesis recommenced immediately at a rate as great or greater than in the control plants.

Net protein synthesis in the roots was also inhibited by water stress (Figure 2, top) but the inhibition was

Figure 1

Water potential of the first leaf of 10-day-old barley seedlings subjected to water stress by flooding the root medium with 250 ml polyethylene glycol solution of -20 bars osmotic potential.

× Not water stressed

○ Water stressed

△ Stress relieved, by washing out osmoticum with 6 changes of 200 ml water.

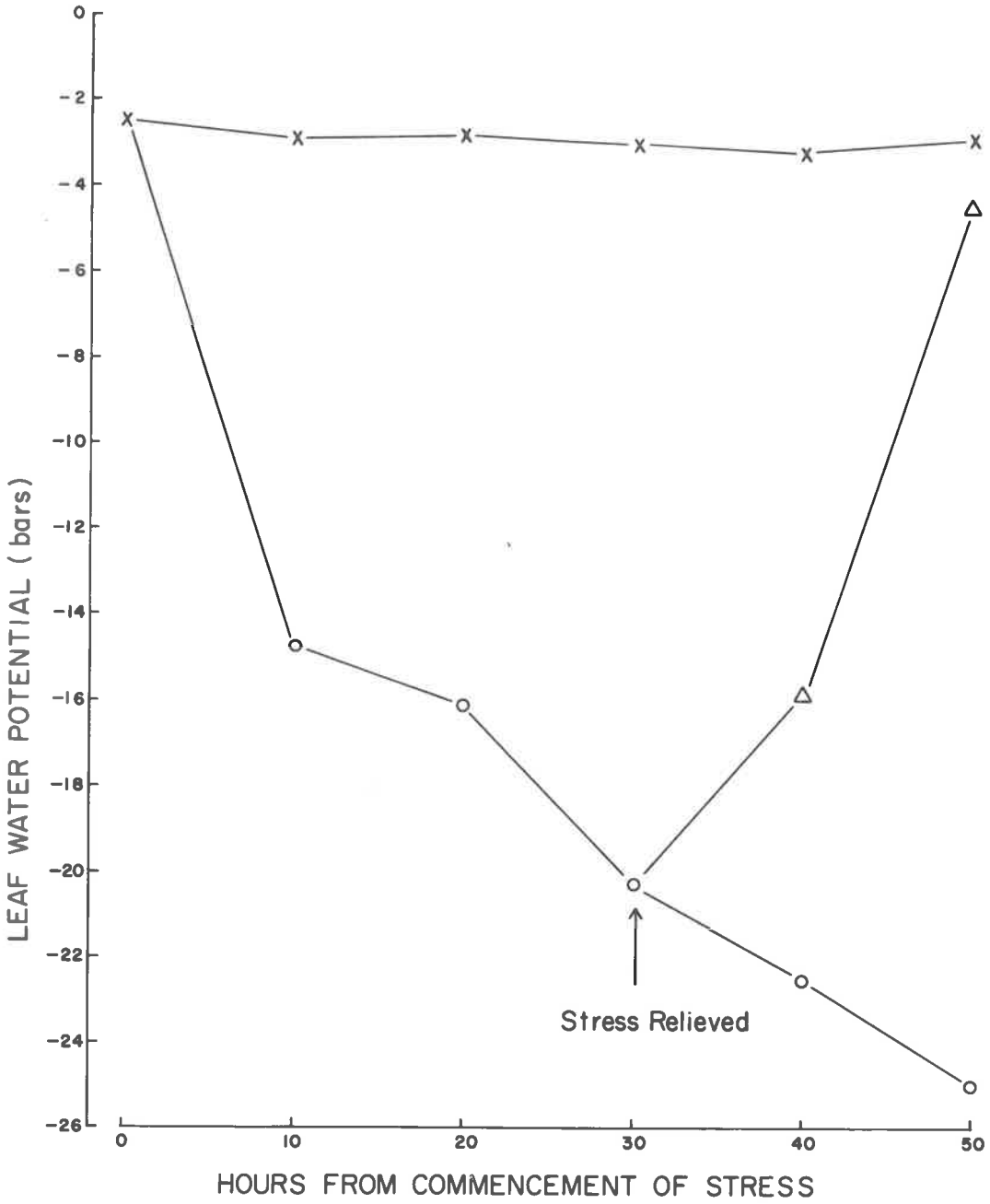

0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
LEAF WATER POTENTIAL (bars)
0
10
20
30
40
50
HOURS FROM COMMENCEMENT OF STRESS
Stress Relieved

Figure 2

Protein content (top) and protein-bound proline (bottom) in the first leaf and roots of 10-day-old barley seedlings subjected to water stress by flooding the root medium with 250 ml polyethylene glycol solution of -20 bars osmotic potential.

Leaf

× Not water stressed

○ Water stressed

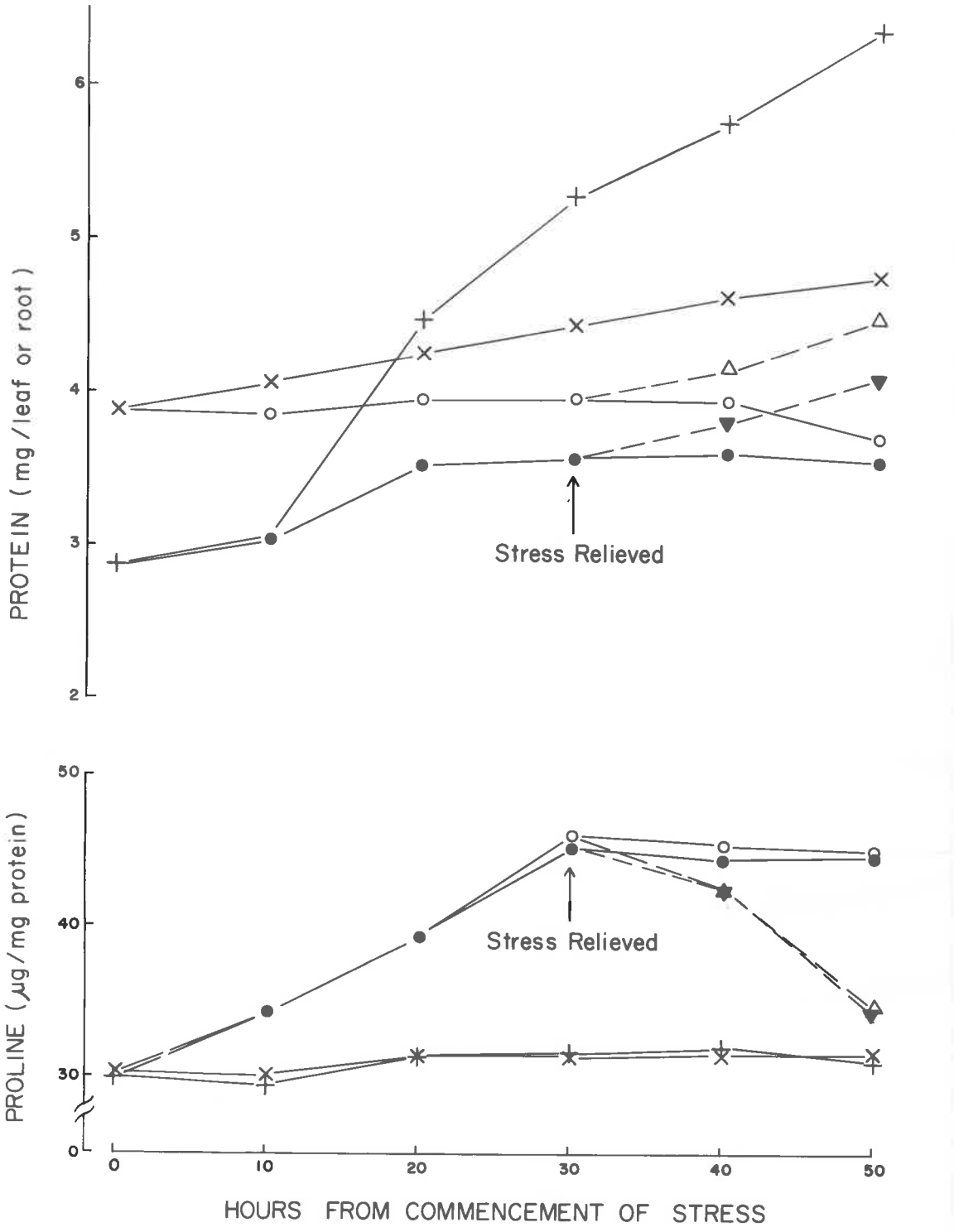
△ Stress relieved

Root

+ Not water stressed

● Water stressed

▼ Stress relieved


PROTEIN (mg /leaf or root)
6
5
4
3
2
Stress Relieved
PROLINE (µg/mg protein)
50
40
30
0
Stress Relieved
0
10
20
30
40
50
HOURS FROM COMMENCEMENT OF STRESS

slower to develop. Re-watering lead to a resumption of rapid protein synthesis in the roots also.

1.2.2.3 Proline Metabolism

1.2.2.3.1 Accumulation of Free Proline

Water stress caused a marked accumulation of proline. The rate of accumulation was slow for the first 20 hours after subjecting the plants to stress but accelerated rapidly until 40 hours after which the rate of accumulation declined once more (Figure 3). By the end of 50 hours the leaves had accumulated proline to a concentration greater than 14 mg/g dry weight. When stress was relieved, the proline content of the leaves began to fall slowly at first and then more rapidly. The free proline level of the leaves not subjected to stress averaged 259 µg/g dry weight throughout the period of the experiment.

The root tissues accumulated less proline than the leaves, reaching 4 mg/g dry weight after 50 hours stress (Figure 3). The rate of increase in proline was correspondingly slow but, unlike the leaves, the rate was relatively constant over the whole 50 hours. Again, proline disappeared from the roots within 20 hours of relieving the water stress and the level of free proline in unstressed roots was low.

Figure 3

Free proline content of the first leaf and roots of 10-day-old barley seedlings subjected to water stress by flooding the root medium with 250 ml polyethylene glycol solution of -20 bars osmotic potential.

Leaf

× Not water stressed

○ Water stressed

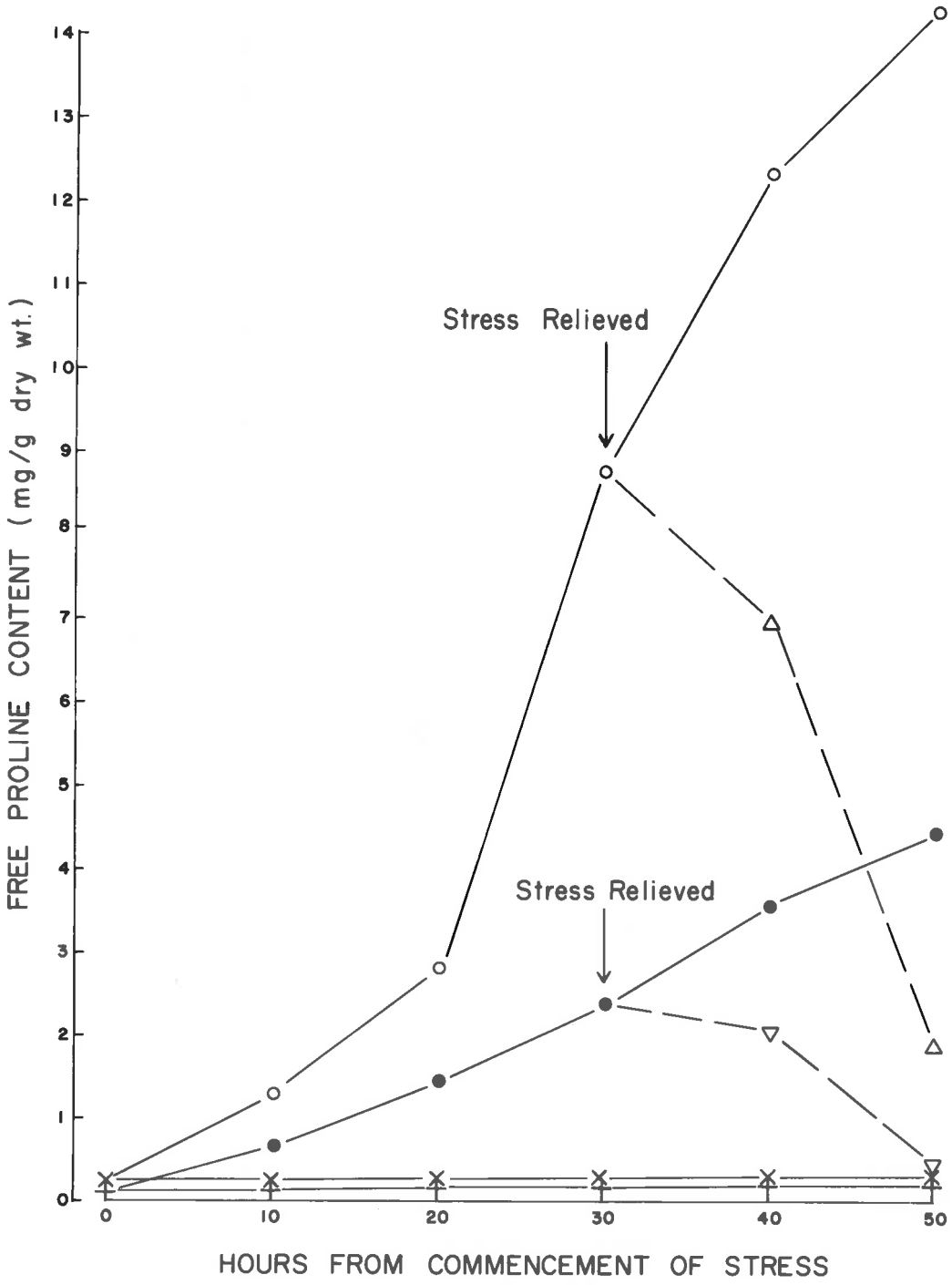
△ Stress relieved

Root

+ Not water stressed

● Water stressed

▽ Stress relieved


FREE PROLINE CONTENT (mg/g dry wt.)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Stress Relieved
Stress Relieved
0
10
20
30
40
50
HOURS FROM COMMENCEMENT OF STRESS

1.2.2.3.2 Protein-bound Proline

The protein-bound proline was also measured, and care was taken to ensure that free proline was removed completely before the bound proline was assessed. Initially, the free proline present in the plant tissues was removed by thrice extracting with MCW (page 47) and centrifuging each time. The free proline content of the residue remaining, following extraction with MCW, was measured and no evidence for the presence of free proline was obtained. The protein was then extracted from this washed residue by thrice homogenizing with 2 ml phosphate buffer (0.05M, Na_2HPO_4-KH_2PO_4, pH7.3). The extracts were centrifuged each time and the supernatants were pooled. The protein was precipitated by adding cold trichloroacetic acid (TCA) page 49). TCA-precipitated protein was hydrolysed by 6N HCl at 110°C for 20 hours. Hydrolysates were dried on a rotary evaporator at 35°C. Water was then added to the residue and the sample was redried repeatedly to remove excess HCl. The hydrolysate was dissolved in MCW and the proline content was assessed and expressed on a unit weight of protein basis.

The data suggest the preferential formation of proline-rich protein during water stress (Figure 2, bottom). The amount of proline per unit weight of protein in the first leaf increased during stress from 3.03% to 4.56% in the

first 40 hours and then remained unchanged (Figure 2, bottom). This level decreased when the stress was relieved.

The amount of proline in the root protein also increased from 3.00% to 4.50% during stress (Figure 2, bottom) and again the amount of protein-bound proline decreased when the stress was relieved.

1.3 Effect of Mild and Severe Water Stress on Apical Morphogenesis and the Accumulation and Distribution of Proline in the Barley Plant

1.3.1 Methods

The effects of two levels of water stress, -10 or -20 bars, on apical morphogenesis and the accumulation of proline in the first leaves, shoots, shoot apices, and roots were investigated. Barley plants (cv Prior) were grown in perlite for 10 days (page 39). They were then subjected to water stress by flooding the pot with 250 ml solution of polyethylene glycol (4000 Mw, -10 or -20 bars osmotic potential) in nutrient solution. After 48 hours, the stress was removed from some plants of both stress treatments by washing out the osmoticum from the rooting medium with six changes of 200 ml water. The plants were sampled 0, 7, 18, 30, 48, 56 and 72 hours after stress commenced. The main shoot of each plant was dissected under a binocular microscope and the length of shoot apices

and the lateral appendages at each node noted. The stage of floral development of the apex was assessed from the appearance of the most advanced primordium, using the scale suggested by Aspinall and Paleg (1963) (page 42). The water potential of the first leaves was determined and proline was estimated in various organs.

1.3.2 Results

1.3.2.1 Water Potential

The water potential in the leaves of plants exposed to osmotic stress declined rapidly during the first seven hours and then more slowly for the remainder of the experiment (Figure 4). After 72 hours stress the leaf water potentials were respectively -12.2 and -31.8 bars in the mild and severe stress treatments. When the osmotic stress was relieved for mildly stressed plants, the leaf water potential recovered to the control level in 24 hours. Recovery was also rapid in the severe-stress treatment although not completed in 24 hours.

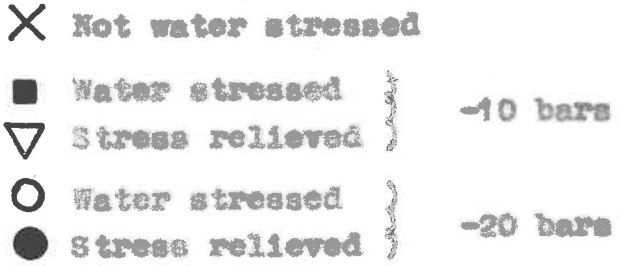
1.3.2.2 Apical Morphogenesis

1.3.2.2.1 Length of Apices

The elongation of the shoot apices was severely affected by stress. The -20 bars solution produced immediate and almost total cessation of elongation, whereas the -10 bars solution severely curtailed elongation (Figure 5).

Figure 4

Effect of mild (-10 bars) and severe (-20 bars) water stress on the water potential of the first leaf of 10-day-old barley seedlings.



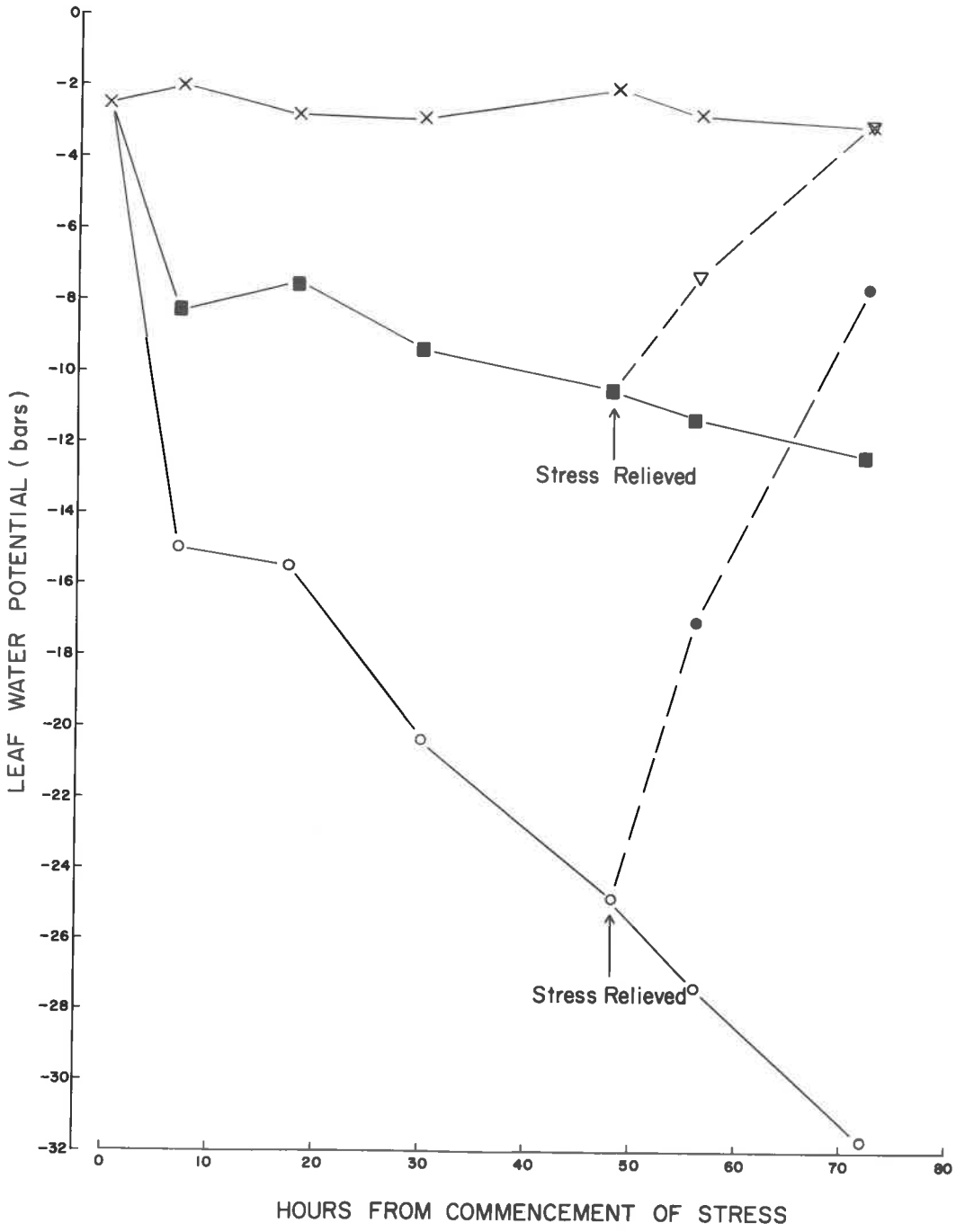
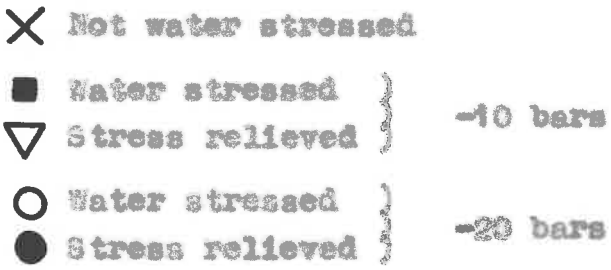
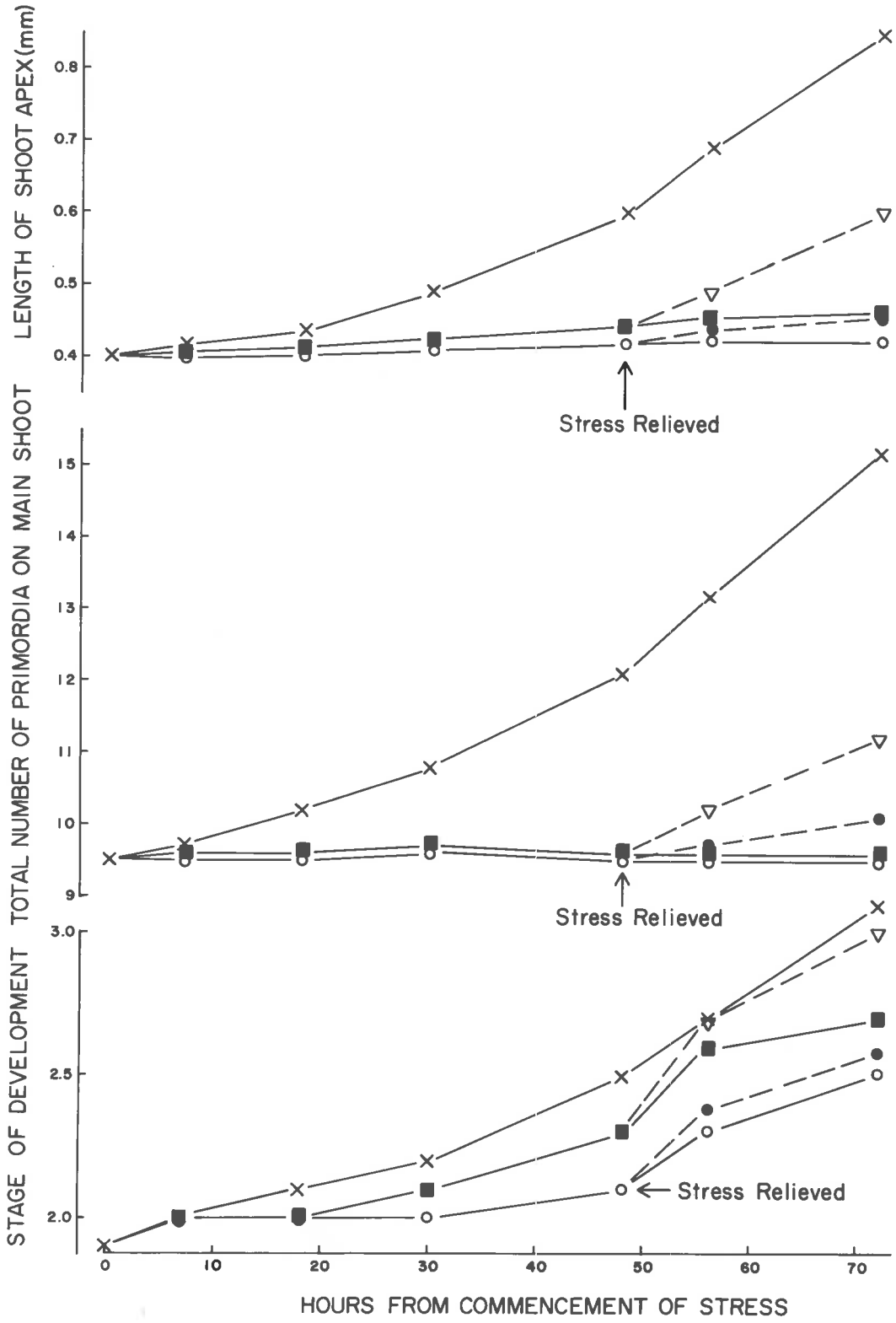

0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
-28
-30
-32
LEAF WATER POTENTIAL (bars)
Stress Relieved
Stress Relieved
0
10
20
30
40
50
60
70
80
HOURS FROM COMMENCEMENT OF STRESS

Figure 5

Effect of mild (-10 bars) and severe (-20 bars) water stress on shoot apical morphogenesis in barley seedlings.

Top - length of shoot apex.
Middle - total number of primordia on main shoot.
Bottom - stage of development.




LENGTH OF SHOOT APEX(mm)
0.8
0.7
0.6
0.5
0.4
Stress Relieved
TOTAL NUMBER OF PRIMORDIA ON MAIN SHOOT
15
14
13
12
11
10
9
Stress Relieved
STAGE OF DEVELOPMENT
3.0
2.5
2.0
Stress Relieved
0
10
20
30
40
50
60
70
HOURS FROM COMMENCEMENT OF STRESS

Removal of the osmotic solutions from the rooting zone induced a re-initiation of apex elongation but the extent of recovery was much greater in plants subjected to the less-severe stress.

1.3.2.2.2 Primordium Production

The production of new primordia at the apex was completely and immediately arrested by both mild and severe water stress (Figure 5). When the stresses were relieved there was some recovery in the production of new primordia, particularly in the plants subjected to the mild stress but the rate of primordium production was less than in plants not subjected to stress.

1.3.2.2.3 Development of Primordium

The further development of primordia already formed on the apex seemed to be less sensitive to water stress than the other parameters of apex growth already discussed (Figure 5). The shoot apices of stressed plants continued to pass from the vegetative to the reproductive phase during stress albeit at a reduced rate.

1.3.2.3 Accumulation of Proline

1.3.2.3.1 Leaf

Water stress again caused a rapid accumulation of proline in the leaves (Figure 6). The accumulation was both more rapid and more extensive in the plants subjected

to the more severe stress (Figure 6). At the end of 72 hours, the leaves had accumulated more than 4 mg/g dry weight (56 µg/g/hr) under mild stress and 17 mg/g (247 µg/g/hr) under severe stress conditions. When the stress was relieved and leaf water potential increased, the amount of proline decreased with time in both stress treatments, although the decrease was most rapid in the severe stress treatment (401 µg/g/hr).

1.3.2.3.2 Leaf sheath

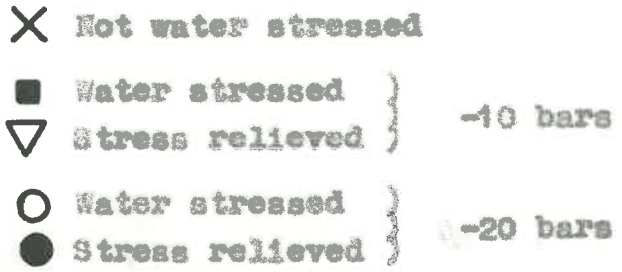
A similar pattern of proline accumulation in response to stress occurred in the leaf sheaths although the response was less than in the leaf blades (Figure 6). By the end of the experiment, leaf sheath tissues had accumulated 3 mg/g (45 µg/g/hr) under mild stress and 11 mg/g (159 µg/g/hr) under severe stress. The proline concentration also dropped when the stress was relieved (245 µg/g/hr).

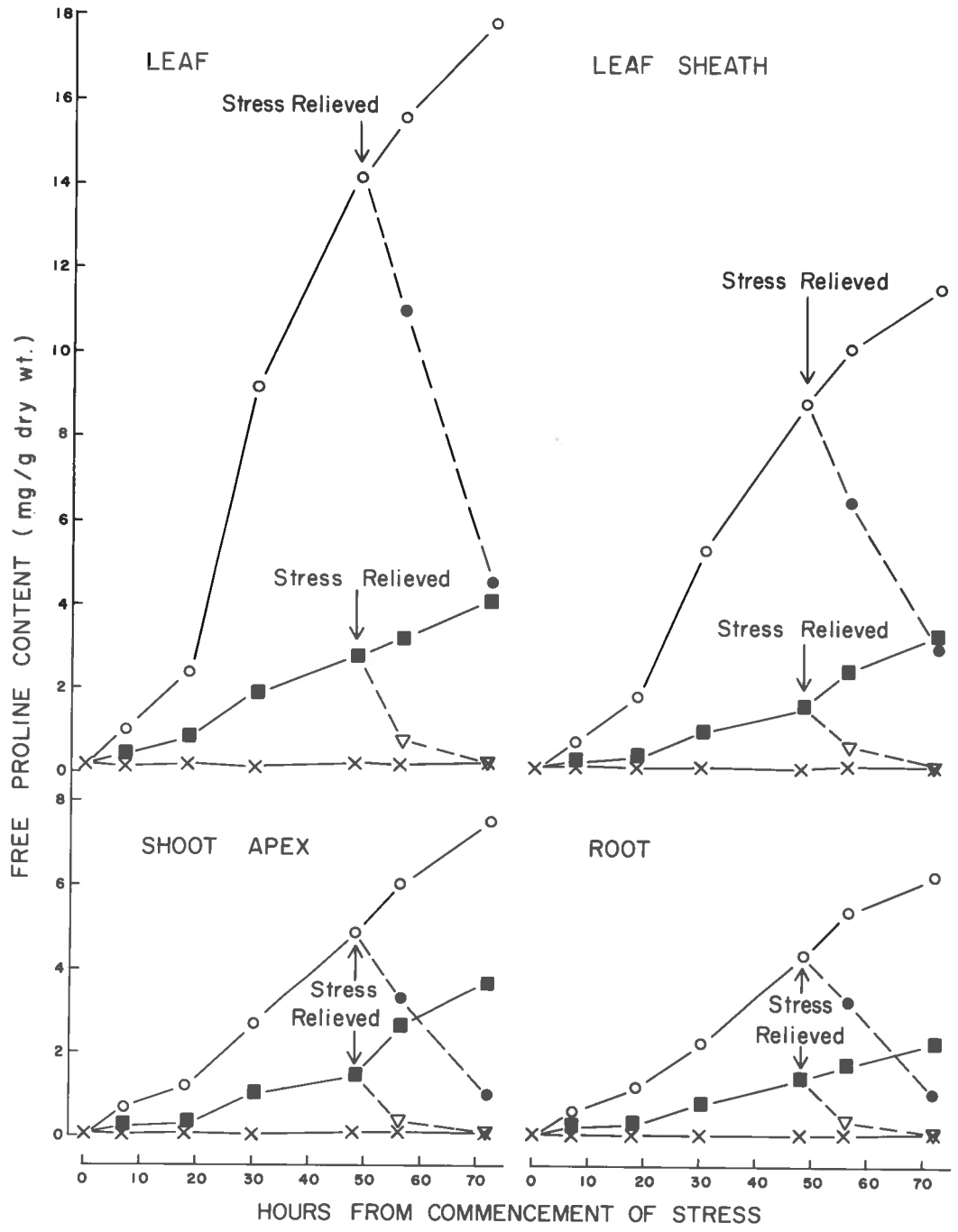
1.3.2.3.3 Shoot Apex

Shoot apices also accumulated considerable amounts of free proline during water stress (Figure 6) but it is interesting that the proline concentrations in the apices in the two stress treatments differed less from one another than did those in the leaves. Thus after 72 hours there was four times as much proline present in severely stressed leaves as in those in the -10 bar treatment but

Figure 6

Effect of mild (-10 bars) and severe (-20 bars) water stress on the accumulation of free proline in the leaf, leaf sheath, shoot apex, and root of barley seedlings.




LEAF
Stress Relieved
Stress Relieved
LEAF SHEATH
Stress Relieved
Stress Relieved
SHOOT APEX
Stress
Relieved
ROOT
Stress
Relieved
FREE PROLINE CONTENT (mg/g dry wt.)
HOURS FROM COMMENCEMENT OF STRESS
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
50
60
70

only twice as much in the apices. Again, once stress was relieved, proline disappeared.

1.3.2.3.4 Root

The roots also accumulated proline during stress, but to a lesser extent than any other organ of the plant (Figure 6). The plants in the severe stress treatment accumulated approximately three times as much free proline as did those in the mild stress treatment (6.2 as against 2.2 mg/g) and the proline level declined in both treatments when stress was relieved.

In summary, the accumulation of proline was related to the degree of stress, i.e., the lower the water potential the greater the accumulation of proline in any given time (Figure 7). Leaves were the most efficient organ for proline accumulation followed by leaf sheaths, shoot apices and finally roots. The rate of disappearance of proline was faster than the rate of its accumulation but the higher the rate of accumulation the more rapid the disappearance when stress was relieved.

1.4 Effect of Water Deficit on the Growth of Barley

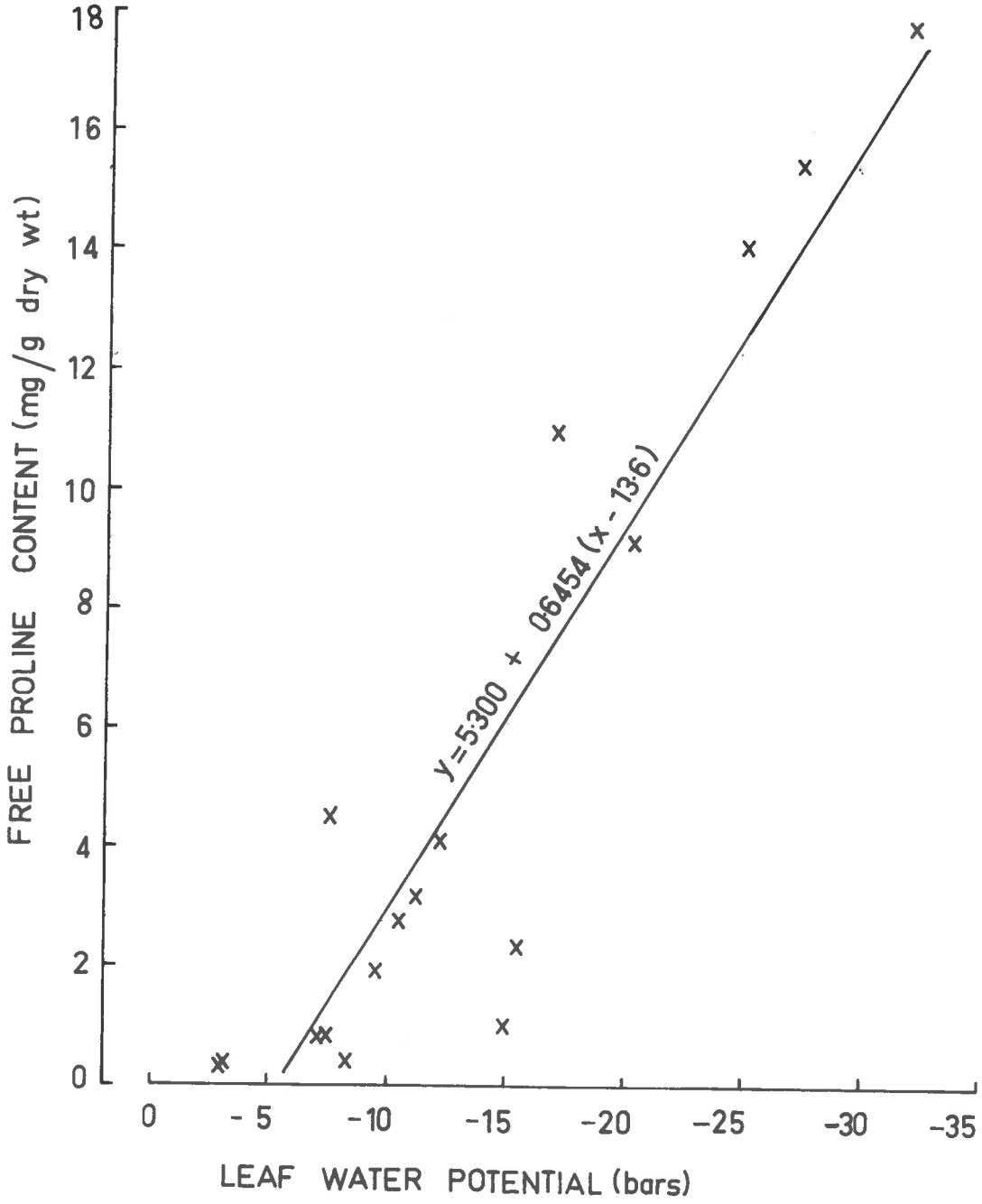
1.4.1 Methods

Barley plants (cv Prior) were grown in perlite and 10-day-old seedlings were subjected to water stress by flooding each pot with 250 ml polyethylene glycol (MW,4000)

Figure 7

Relationship between proline accumulation and leaf water potential in barley seedlings. (Values for proline content and water potential were obtained from Figures 6 (leaf) and 4, respectively.)

Correlation coefficient = 0.922


FREE PROLINE CONTENT (mg/g dry wt)
18
16
14
12
10
8
6
4
2
0
0
- 5
-10
-15
-20
-25
-30
-35
LEAF WATER POTENTIAL (bars)
y=5·300 + 06454 (x - 13·6)

solution of -10 bars osmotic potential. Plants from 5 pots, each containing 5 seedlings, from both control and stress treatments were harvested 0, 7, 14, 21, and 28 hours after the stress was imposed. Leaves and shoots were excised as quickly as possible and immediately sealed in air-tight weighing bottles to minimise the loss of water from the tissues. Roots were washed thoroughly with the tap water to remove perlite and viscous polyethylene glycol solution and then excess water from the root surfaces was removed with blotting paper. Roots were also sealed in air-tight weighing bottles and fresh weight of various organs was recorded. After recording the fresh weight these bottles were unsealed and kept at 90°C in a forced-draught oven for 48 hours and subsequently dry weight was recorded.

1.4.2 Results

1.4.2.1 Fresh Weight

Mild water stress caused significant reductions in the fresh weight of the leaves and shoots and by the end of 28 hours the weight of these organs were significantly below their corresponding fresh weights at the commencement of the stress (Figure 8, top).

The fresh weight of the roots remained constant throughout the period of water stress. This effect may have been real and due to the contrasting environments of

Figure 8

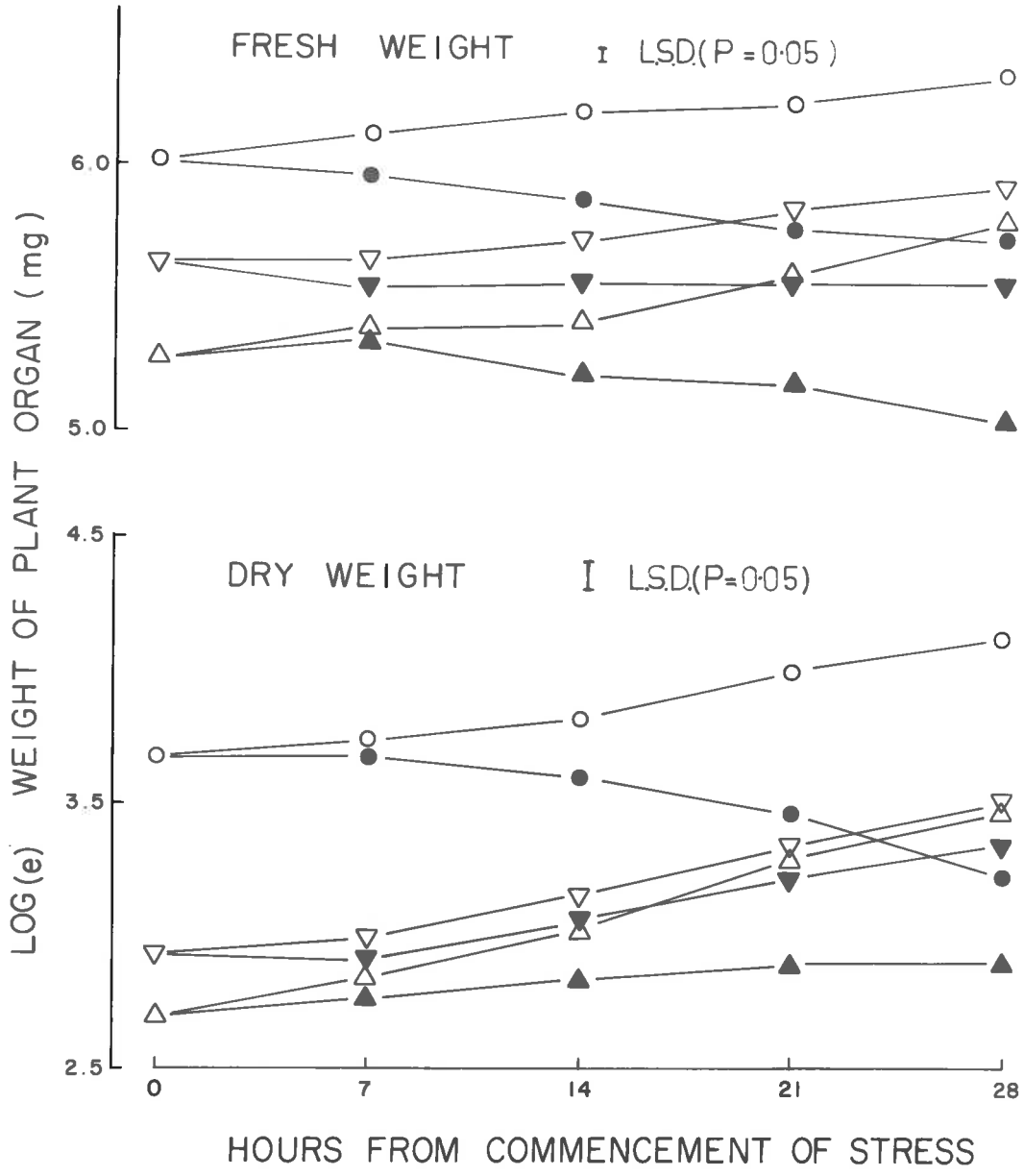
Effect of water deficit on the growth of leaves, shoot, and roots of barley plant.

(Ten-day-old seedlings were subjected to mild water stress by flooding each pot with 250 ml polyethylene glycol solution of -10 bars osmotic potential.)

○ Control
● -10 bars
} Leaves

△ Control
▲ -10 bars
} Shoot

▽ Control
▼ -10 bars
} Roots


FRESH WEIGHT
I LSD(P=0·05)
6.0
5.0
DRY WEIGHT
I LSD(P=0·05)
4.5
3.5
2.5
LOG(e) WEIGHT OF PLANT ORGAN (mg)
0
7
14
21
28
HOURS FROM COMMENCEMENT OF STRESS

the roots and tops, the tops being surrounded by an environment at a much lower water potential which would lead to rapid water loss. Alternatively, the roots may have rapidly absorbed water during the washing period, thus obliterating any loss during the period of water stress.

1.4.2.2 Dry Weight

The total dry weight of the plants was decreased due to water stress but various organs were affected differently (Figure 8, bottom). The leaves lost dry weight continuously with time under stress but the shoots and roots gained dry weight, although not at the same rate as the control plants. This continued increase in the dry weight of the roots during stress may have been due to continued normal growth but may have also been contributed to by translocation and accumulation of metabolites from elsewhere in the plant.

1.5 Effect of Aeration during Stress on the Accumulation and Distribution of Proline

1.5.1 Introduction

Free proline accumulated in appreciable amounts in every organ of an intact barley plant when it was subjected to osmotic stress (Figure 6). The roots of some plants accidentally left in the growth cabinet with their roots submerged in a beaker of unaerated polyethylene glycol

solution of -20 bars osmotic potential did not accumulate proline, however, although the leaves did. This suggested that aeration of the rooting medium might modify the distribution of the free proline accumulated during water stress. Accordingly, an experiment was designed to test this possibility.

1.5.2 Methods

Barley plants (cv Prior) were grown in perlite at 17°C ± 1°C with a 16-hour photoperiod in a growth cabinet. After 10 days growth the seedlings were removed from the pot and their roots were washed gently with water to remove the perlite. The roots of the plants were then immersed in 400 ml polyethylene glycol (MW,4000) solution of -20 bars osmotic potential contained in cylinders. The solution in half the cylinders was aerated continuously using a suction pump. After 30 hours the roots of some plants which had been aerated previously, were changed to non-aerated conditions and vice versa. Samples of 3 plants were removed from each treatment at 0, 7, 20, 30, 45 and 65 hours after the stress commenced and were immediately frozen. The free proline content of leaves, leaf sheaths including shoot and roots was measured.

1.5.3 Results

1.5.3.1 Accumulation of Proline in Roots

Aeration of the rooting medium had a pronounced effect on proline accumulation in the roots (Figure 9, bottom). In the aerated cultures, the roots accumulated proline rapidly, reaching a concentration of 5.6 mg/g dry weight (86 µg/g/hr) after 65 hours stress. In contrast, the roots in the non-aerated cultures accumulated virtually no proline, the free proline concentration changing from 120 to 259 µg/g dry weight (4 µg/g/hr) during the stress period. Proline accumulation also ceased abruptly if the roots were removed from an aerated to a non-aerated environment at 30 hours, but the proline previously accumulated remained in the roots. Conversely, roots removed from a non-aerated to an aerated environment commenced to accumulate proline at a very rapid rate (137 µg/g/hr).

1.5.3.2 Accumulation of Proline in Leaves

All the leaves of the sample plant were cut into small pieces and pooled before the proline content was determined. The accumulation of proline was also affected by aeration but the response was the reverse of that in the roots (Figure 9, top). The amount of proline in the leaves was higher when the rooting medium was not aerated (19.6 mg, 301 µg/g/hr) than when it was aerated (16.0 mg, 246 µg/g/hr). When aeration was terminated, the amount

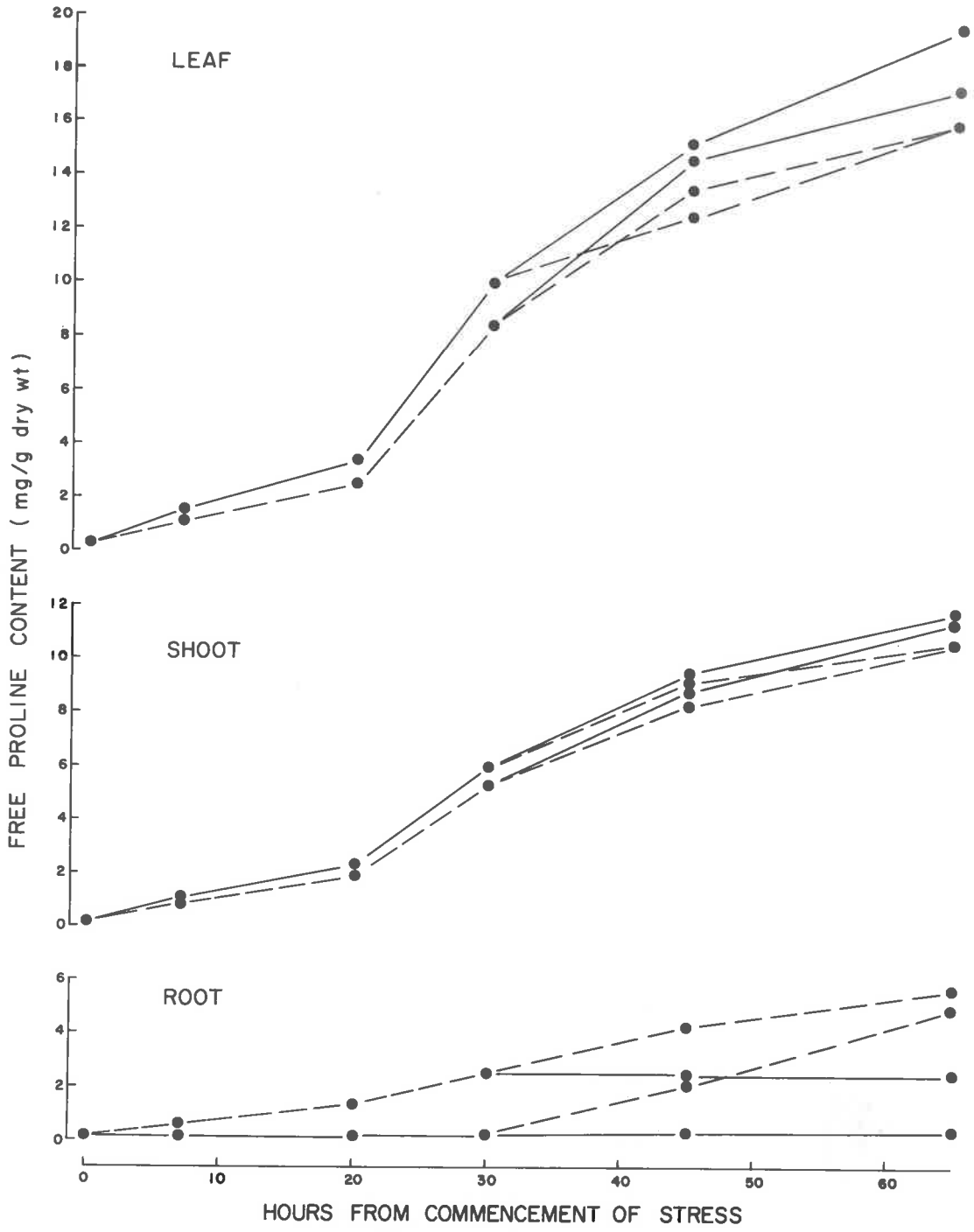
Figure 9

Effect of aeration during water stress on the accumulation and distribution of proline in various parts of intact barley plant.

(Water stress was imposed by immersing the roots of 10-day-old seedlings previously grown in perlite, into 400 ml polyethylene glycol solution of -20 bars osmotic potential contained in cylinders. The solution in the cylinders was aerated using a suction pump.)

●--● Aerated

●—● Non-aerated


LEAF
SHOOT
ROOT
FREE PROLINE CONTENT (mg/g dry wt)
HOURS FROM COMMENCEMENT OF STRESS
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
60

of proline in the leaves began to rise more rapidly.

1.5.3.3 Accumulation of Proline in the Leaf sheaths and Stem

The effects of aeration on proline accumulation in the shoot were found to follow a similar pattern to that described for the leaves (Figure 9, middle). The amount of proline was a little higher in a non-aerated culture (11.6 mg, 178 µg/g/hr) than in an aerated culture (10.5 mg, 161 µg/g/hr) after 65 hours stress.

In total, the aeration of the rooting medium during stress had virtually no effect on the total amount of free proline accumulated in the plant. The amounts of proline 65 hours after stress in tops, roots, and whole plant are given in the following table.

Table 2

Effect of aeration on the distribution of total free proline in the plant during water stress.

Treatment	Proline/plant (µg)		
	Tops	Roots	Whole plant
Aerated	827.3	220.0	1047.3
Non-aerated	1019.6	8.0	1027.6

This indicates that although the distribution of proline in the various organs was affected profoundly by

aeration of the rooting medium, the total amount accumulated in the plant was virtually unchanged.

1.6 Discussion

1.6.1 Plant Growth

There have been many studies of the weight changes in plant organs during and following episodes of water stress (Gates, 1968). The present data exhibit many of the features which have been described previously, including an apparent lack of sensitivity of the roots to the effects of stress. The fact that the roots did not lose fresh weight during stress may have been a real phenomenon, attributable to the higher water potential in those organs than the rest of the plant but is more likely an artefact induced by the brief washing which was necessary before weighing.

The total dry weight of the plant was decreased by water stress but the component organs were affected differently, for instance, the dry weight of leaves decreased whereas roots and shoot continued to grow in the presence of water stress. This differential growth response of plant organs to water stress may be attributed to three factors. Firstly, a difference in the water potential of the component organs, the leaves being at lower water potential than the roots; secondly, differences in the sensitivity of protein synthesis to lowered

water potential; thirdly, translocation of proline and other metabolites from the leaves to the roots. The evidence does not distinguish between these possibilities and each may contribute to the observed differences in growth response. Protein synthesis in the roots may be less sensitive to water stress than in the leaves (Figure 2, top). Translocation of free proline into the roots from the leaves during stress probably occurs (Figure 9 and Table 2) but free proline itself, based on the proline content of water stressed roots, could contribute at most 16 µg/root. This is insignificant when compared with the increase of 9.5 mg in dry weight which occurred in the roots during the 28 hours period. On the other hand, the major portion of the metabolites concerned in the increasing weight of the roots must have been translocated to the roots from elsewhere and it is possible that proline formed a major component, being subsequently utilised by protein synthesis in the root. The concept of enhanced translocation of free proline during water stress is supported by the fact that an anaerobic root medium halted the accumulation of proline in the roots without affecting the total amount of proline in the whole plant (Table 2). This suggests that proline can move around in the plant during an episode of water stress and may affect growth phenomenon (pages 14 and 15, 3.1.3). In this connection it may be of significance that the leaves,

which show the largest accumulation of free proline during water stress grow least whereas the roots and stems accumulate less proline but their growth is less affected.

1.6.2 Apical Morphogenesis

Many of the effects of water stress on plant growth have their origin in inhibition of development in the meristematic regions of the plant. The formation of new primordia on the shoot apex is particularly susceptible to water stress (Nicholls and May, 1963) being inhibited at water potentials which have comparatively little effect on other growth processes. This earlier observation was confirmed in the present study (Figure 5) as was the finding that floral organogenesis on the same apices is less affected by lowered water potential (Husain and Aspinall, 1970).

These effects of water stress on meristematic activity at the shoot apex could be mediated by changes in water potential in the apical tissues or by changes in metabolism elsewhere in the plant resulting in differences in the products translocated to the apex. Husain and Aspinall (1970) obtained water potential data for the apices which, although of a preliminary nature, suggested that a direct effect of water stress on apical water potential was unlikely to be involved.

It is apparent that levels of water stress which do not (apparently) result in a lowering of apical water potential do inhibit meristematic activity and result in an accumulation of free proline in the apex (Figure 6). This proline may accumulate in the apex as a result of a change in the metabolic pattern in the apical tissues or may be translocated there from elsewhere in the plant. The fact that isolated apices accumulate little free proline, even when subjected to stress (Figure 10) could be construed as evidence for the second possibility. Alternatively, however, proline accumulation in the isolated apex may be inhibited due to the absence of some essential precursor supplied by the rest of the plant in the intact situation.

1.6.3 Water Stress and Composition of Free Amino Acids

Water stress, both mild and severe, profoundly altered the relative concentrations of the free amino acids in barley plants. The most striking effect of water stress was the accumulation of large quantities of free proline in the leaves. The concentrations of other free amino acids such as asparagine, glutamine, glycine, ornithine, phenylalanine increased markedly and valine levels rose slightly with water stress. Similar changes in free amino acid composition have been reported in other species (Kemble and Macpherson, 1954; Barnett and Naylor,

1966; Routley, 1966) and it has been suggested that the increase in the amount of free amino acids during stress is accompanied by an inhibition of protein synthesis and increase in the decomposition of proteins (Gates and Bonner, 1959; Shah and Loomis, 1965; Barnett and Naylor, 1966). The concentrations of free alanine, aspartic acid, glutamic acid, leucine, and serine declined during stress, however. The decline in the content of these amino acids particularly aspartic acid and glutamic acid may have been due to their transformation into the corresponding amides (Chibnall, 1939).

The accumulation of free proline may be a consequence of the inhibition of protein synthesis which takes place during water stress (Figure 2, top). Under these circumstances, amino acid synthesis may not be initially impaired. Although the excessive accumulation of other amino acids would be inhibited by feed-back reactions, this apparently does not occur with proline which accumulates rapidly (Kemble and Macpherson, 1954; Barnett and Naylor, 1966). Alternatively, but less likely, proline biosynthesis may be specifically promoted by low tissue water potential.

Together with the large accumulation of free proline, a few other ninhydrin positive compounds appeared only in the water stressed samples (Table 1). These were tenta-

tively identified, on the basis of Rf, as B-alanine, hydroxyproline, and pipecolinic acid. As these were only present in trace amounts, no attempt was made to identify them further. Hydroxyproline is closely related structurally to proline but its existence in the plant in the free state has been disputed (Steward and Thompson, 1950). It is possible that its presence in the free state may be linked with water stress, however, as it has been found in considerable concentration in dried sandal leaves (Radhakrishnan and Giri, 1954). Pipecolinic acid is related structurally to proline and acts as a proline analogue in some systems. Fowden (1967) has suggested that the enzymes concerned with proline biosynthesis are not strictly specific and can synthesize structural analogues of proline under certain circumstances. If this is so, it is not surprising to find trace amounts of such substances when proline biosynthesis is occurring rapidly. Relatively high concentrations of proline have also been found in seeds (Jones and Pierce, 1967; Sane and Zalik, 1968; Wang, 1968a, 1968b), pollen grains (Bathurst, 1954; Virtanen and Kari, 1955; Britikov and Musatova, 1964; Britikov, Vladimirtseva, and Musatova, 1965) and dormant buds (Jaarma, 1966). Each of these tissues pass through periods of low water potential during development which may well determine the accumulation of proline.

1.6.4 Synthesis of Proline-rich Protein during Water Stress

There was some evidence that, apart from accumulating large quantities of free proline during water stress, proline was preferentially incorporated into protein during stress. As the protein was isolated from plant organs containing abundant free proline, stringent precautions were taken to remove all free proline before determining bound proline (page 63, 1.2.2.3.2). The data indicated that the proline content of the isolated protein increased during a period of stress both in the leaves and the roots (Figure 2, bottom). This suggested that a new kind of protein was synthesized which was specifically rich in proline. Both protein synthesis and degradation occur in plants during stress (Gates and Bonner, 1959) and the present evidence suggests that different proteins are synthesized under these conditions. The reason for the synthesis of a protein with altered composition is not known and it is possible that a looser binding of the proline molecule to protein may be involved. The presence of proline, which accumulates during stress, itself may have exerted some regulatory effect on both ribonucleic acid (RNA) and protein synthesis and perhaps influenced the composition of RNA which resulted in synthesis of proline-rich protein (Kessler, 1961). The precise

mechanism of this phenomenon is difficult to pinpoint, however, in order to synthesise this kind of protein changes must occur in some of the four possible triplets, such as CCU, CCC, CCA, CCG, which have been assigned for the incorporation of proline into protein.

SECTION 2

2. ISOLATED ORGANS

2.1 Accumulation of Proline in Isolated Organs

2.1.1 Introduction

Proline accumulates in every organ of the intact barley plant during water stress, but the rate of accumulation varies widely between the various organs of the plant. Since aeration profoundly affects the accumulation of proline in the roots and causes complementary changes in the free proline content of the shoot, it was postulated (page 79) that the proline which accumulates in the root is synthesized in the leaves. As the evidence for this hypothesis was circumstantial, it was decided to examine the accumulation of proline in excised organs subjected to an osmotic stress.

2.1.2 Methods

Barley plants (cv Prior) were grown in perlite with nutrient solution for 10 days. The plants were subsequently removed from the pots and the roots were washed thoroughly with tap water. The roots were excised intact but the first leaves and leaf sheaths were cut into 2 cm sections after excision. Finally, the shoot apices were removed from the shoot remnants using a binocular microscope. The plant tissues were floated on water as soon as they were excised. One sample of each tissue was

immediately frozen and kept at -20°C as 0 hour sample. Sixty leaf sections, forty leaf sheath sections, eighty shoot apices, and eight root systems (from 8 plants) were transferred into separate beakers each containing either 200 ml polyethylene glycol (MW,4000) solution of -20 bars osmotic potential or 200 ml of water. Replication was 3-fold. The tissue was then incubated at 20°C in the dark and aerated by forcing air through the solutions. Samples of fifteen leaf sections, ten leaf sheath sections, twenty shoot apices or two roots were taken from each beaker 4, 16, 32, and 48 hours after the commencement of the treatments. The samples were washed thoroughly, frozen in dry ice and maintained at -20°C until the proline content and sample dry weight were determined.

2.1.3 Results

Leaf sections floated on either an osmotic solution or water accumulated free proline but leaves subjected to stress accumulated the greater amount (Figure 10). Proline increased continuously at a steady rate in leaf sections subjected to water stress. There appeared to be an initial lag period of some 16 hours with leaves floated on water in which little or no proline accumulation occurred. Subsequently proline accumulated in these sections also at a relatively rapid rate.

Figure 10

Accumulation of proline in excised leaf, leaf sheath, shoot apex, and root of 10-day-old barley plants.

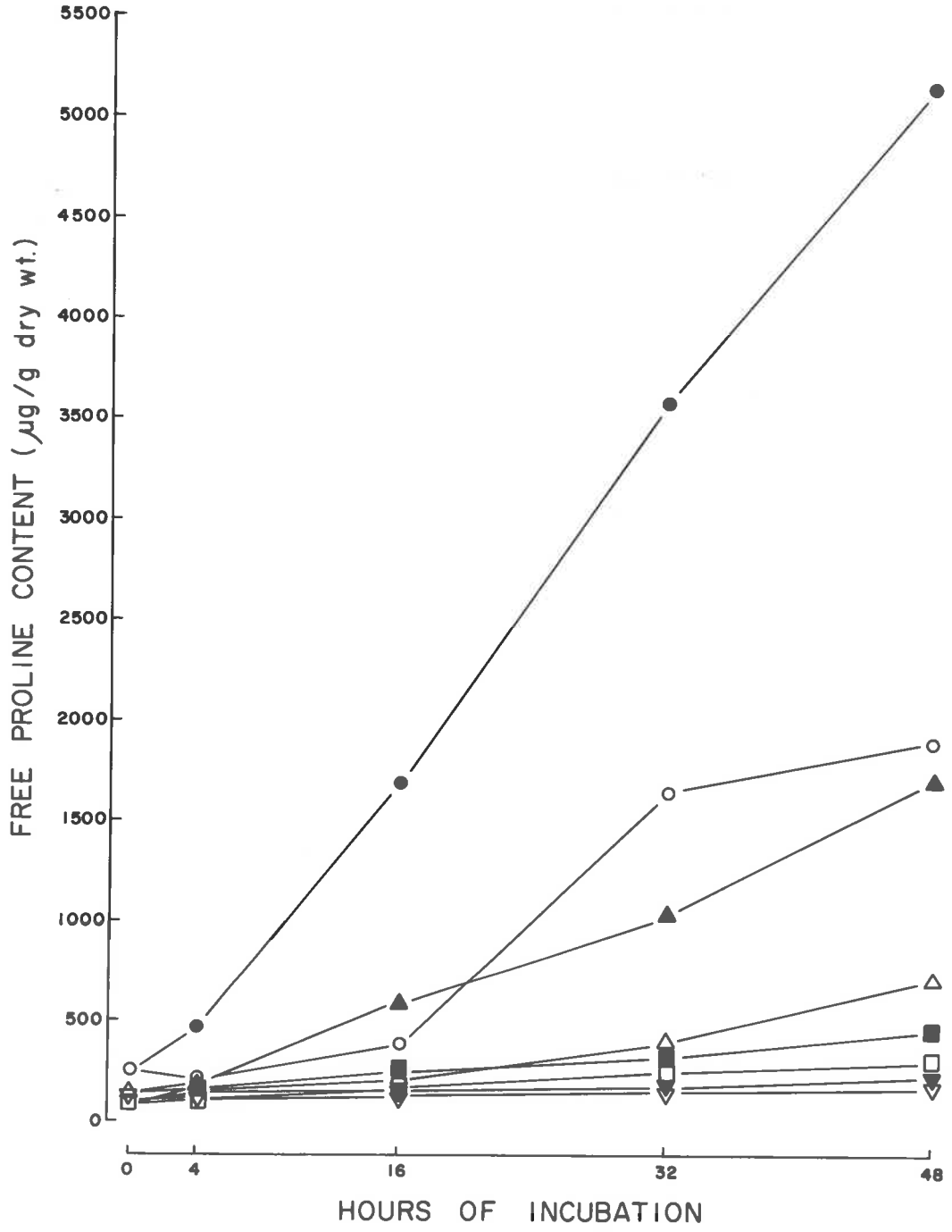
(2 cm sections of leaf and leaf sheath, shoot apex and root as such, were floated either on polyethylene glycol solution of -20 bars osmotic potential or water.)

○ Water
● -20 bars } Leaf

△ Water
▲ -20 bars } Leaf sheath

□ Water
■ -20 bars } Shoot apex

▽ Water
▼ -20 bars } Root


FREE PROLINE CONTENT (µg/g dry wt.)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
4
16
32
48
HOURS OF INCUBATION

The isolated leaf sheaths subjected to stress also commenced to accumulate proline as soon as they were placed in the osmotic solution. Leaf sheath sections floated on water accumulated proline less rapidly. In both cases, the rate of accumulation (µg proline/g dry weight of tissue/hour) was considerably less than the corresponding rate in the isolated leaf tissue.

There was negligible accumulation of free proline in the apices floating on water and only a slight increase with time in those floating on the osmotic solution. Isolated roots were incapable of accumulating any proline, even when subjected to an osmotic stress.

Apparently the isolated organs of the barley plant have varying potentials to accumulate proline, with leaf tissue at one end of the scale accumulating proline rapidly and roots, at the other, not accumulating any. The rates of accumulation were less than those of the corresponding organs on the intact plant (Figure 6) although they were also stimulated by water stress. In contrast to the intact plant, leaf and leaf sheath tissue accumulated free proline even when not subjected to stress suggesting that excision itself triggers the accumulation mechanism.

2.2 Accumulation of Proline in Etiolated Leaves

2.2.1 Introduction

In the previous experiment it was found that excised leaves and leaf sheaths accumulated a large amount of proline when floated on an osmotic solution. On the other hand, the shoot apices showed only a slight increase in free proline and isolated roots were incapable of accumulating proline (Figure 10). Since the shoot apices contain only a small amount of chlorophyll and the roots are completely devoid of it, there may be a relationship between the presence of chlorophyll and the capacity to accumulate proline. The present experiment was designed to investigate this possibility.

2.2.2 Methods

Barley plants (cv Prior), were grown for 10 days in perlite with nutrient solution either in complete darkness at 25°C or in continuous light at the same temperature.

The first leaves of the etiolated and the green plants were then excised, cut into 2 cm sections and pooled on water. Eighty sections of etiolated and of green leaves were transferred into beakers containing 200 ml of either water or a polyethylene glycol solution of -20 bars osmotic potential. Again, replication was 3-fold. The leaf tissues were then incubated at 20°C in the dark with continuous aeration. Twenty leaf sections from each

beaker were removed 4, 16, 32, and 48 hours after the treatment began, washed with water, and immediately frozen in dry ice. The samples were stored at -20°C until the proline and dry weight were determined.

2.2.3 Results

Light-grown leaves floated either on an osmotic solution or on water accumulated proline, the rate being considerably higher in leaf tissue subjected to stress (Figure 11). The results were very similar to those obtained in the previous experiment (Figure 10). In contrast the etiolated leaves had a slightly higher initial free proline level (335 µg/g dry weight compared to 270 µg/g dry weight) but accumulated very little proline. The initial proline content had approximately doubled at the end of 48 hours in etiolated leaves subjected to stress, whereas the proline content of stressed green leaves increased sevenfold in the same period. Very little proline accumulated in excised etiolated leaves floated on water.

2.3 Effect of Sucrose and Glutamic Acid on the Accumulation of Proline in Etiolated Leaves

2.3.1 Introduction

Etiolated leaves accumulated very little free proline in response to osmotic stress whereas green leaves accumulated a substantial amount of proline (Figure 11).

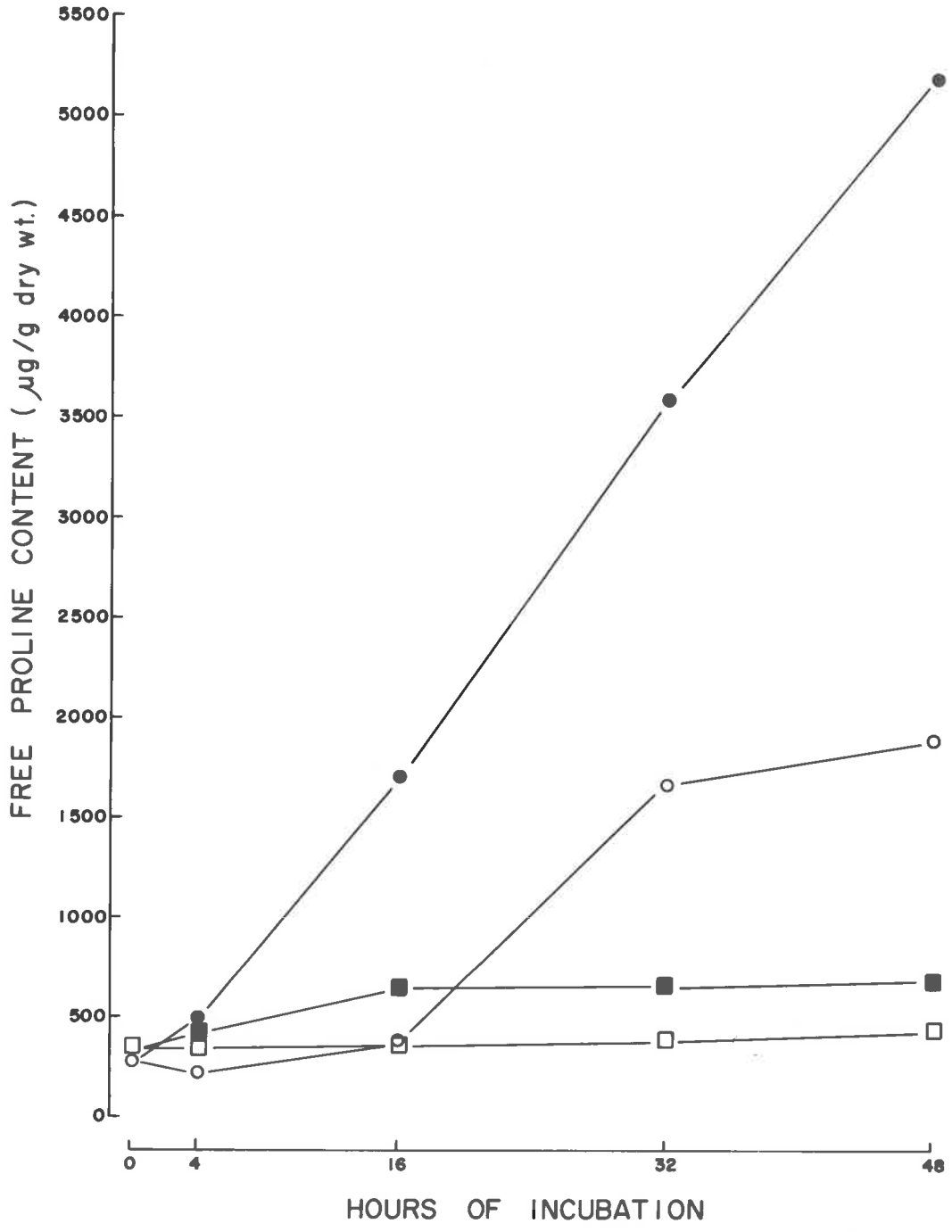
Figure 11

Accumulation of proline in excised etiolated and green leaves of 10-day-old barley plants.

(Two-centimeter-long sections of leaves were floated either on polyethylene glycol solution of -20 bars osmotic potential or water.)

○ Water
● -20 bars
} Green leaves

□ Water
■ -20 bars
} Etiolated leaves


FREE PROLINE CONTENT (µg/g dry wt.)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
4
16
32
48
HOURS OF INCUBATION

This may have been linked to the absence of chlorophyll in etiolated leaves but such tissues contain only small reserves of soluble carbohydrates and amino acids (Chibnall, 1939). This may well have been the reason for the lack of proline accumulation following osmotic stress. The aim of the present experiment was to explore this possibility by supplying sucrose and glutamic acid to etiolated leaves.

2.3.2 Methods

Barley plants were again grown in complete darkness at 25°C for 10 days. The first leaves were excised and cut into 2 cm sections as quickly as possible. Two hundred and forty leaf sections were incubated in the dark at 20°C for 2 hours and 30 minutes in beakers containing 400 ml of 0.05M sucrose (S_1), 0.10M sucrose (S_2), 250 µg/ml glutamic monochloride (G_1), 500 µg/ml glutamic monochloride (G_2), $S_1 + G_1$, $S_1 + G_2$, $S_2 + G_1$, $S_2 + G_2$, or water. The leaf sections were gently stirred thrice at intervals of 30 minutes during incubation. Following incubation, the leaf sections were transferred to beakers containing 200 ml of a polyethylene glycol solution of -20 bars osmotic potential. Replication was 3-fold. The tissues were maintained at 20°C in the dark and aerated continuously. Twenty leaf sections from each replication of the treatments were taken out 4, 16, 32, and 48 hours

after subjecting to osmotic stress, washed with water, frozen in dry ice and kept at -20°C. At the end of the experiment the proline content was determined and results were expressed on a dry weight basis.

2.3.3 Results

Sucrose and glutamic acid both stimulated proline accumulation in etiolated leaves (Figure 12). Leaves fed with sucrose or glutamic acid alone accumulated approximately double the amount of proline produced in 48 hours by leaves incubated on water. However, the effect of these compounds was not proportional to their concentrations, the higher concentrations being only slightly more effective than the lower in promoting proline accumulation (0.05M and 0.10M sucrose - 28 µg and 31 µg/g dry wt/hour respectively whereas 250 and 500 ppm glutamic acid - 26 µg and 29 µg/g dry wt/hour respectively). When both metabolites were supplied, however, there was a synergistic response in proline accumulation at the higher concentration (75 µg/g dry wt/hour) but not at the lower (37 µg/g dry wt/hour). The relative responses to different concentrations of the two metabolites suggested that, although both were essential for maximum proline accumulation, glutamic acid supply was more likely to limit accumulation than sucrose. An increase in the concentration of glutamic acid supplied had a larger effect than an increase in sucrose supply.

Figure 12

Effect of sucrose and glutamic acid on the accumulation of proline in etiolated barley leaves.

(The leaf sections were incubated in the dark at 20°C for 2 hours and 30 minutes in beakers containing solutions of either sucrose or glutamic monochloride or combinations thereof before they were transferred to polyethylene glycol solution of -20 bars osmotic potential for varying periods of time.)

Control; ● Without sucrose and glutamic monochloride

A. Sucrose; ▲ 0.10M, × 0.05M

B. Glutamic monochloride; ▲ 500 µg/ml, × 250 µg/ml

C. Sucrose + glutamic monochloride

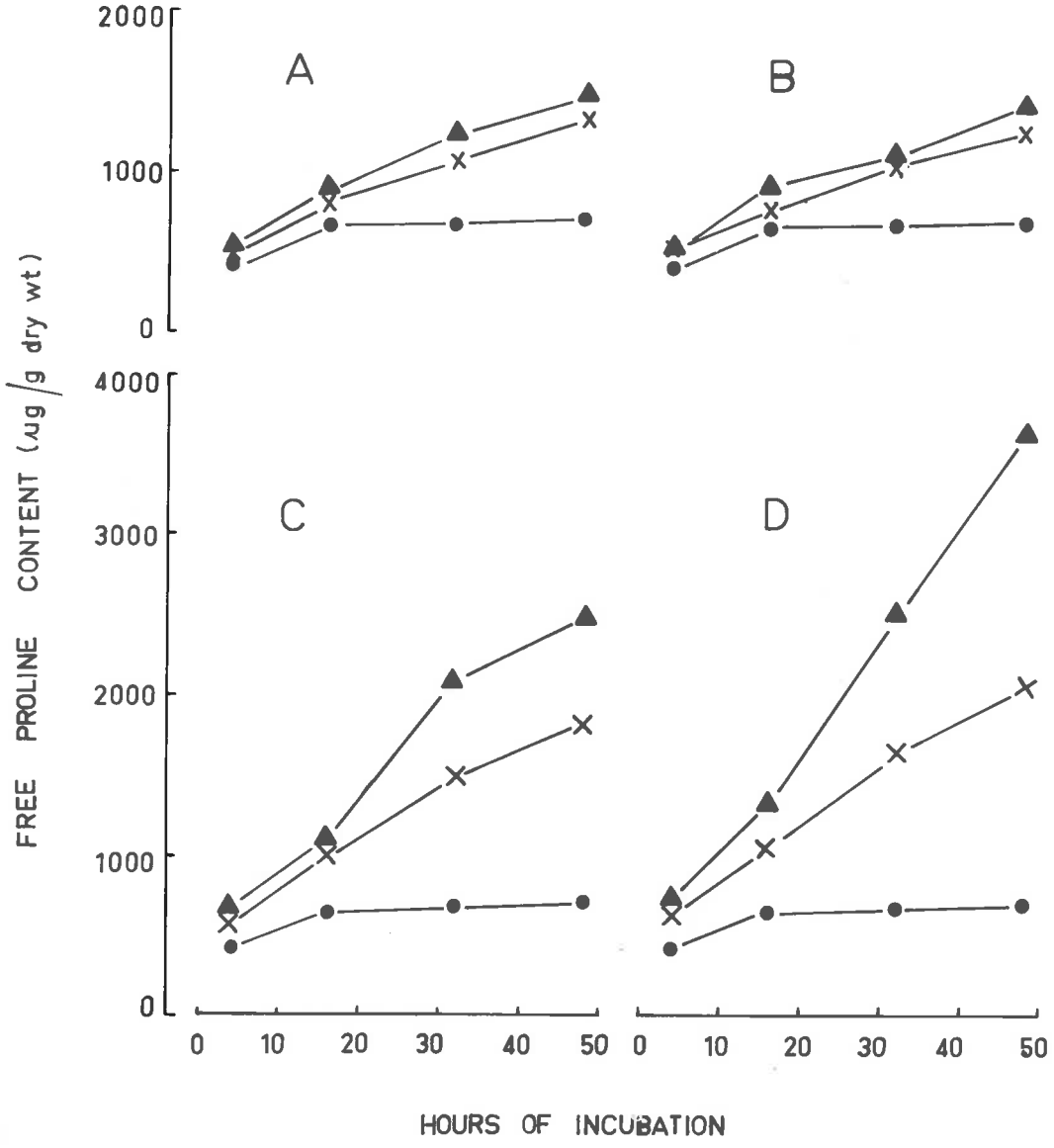
▲ 0.05M sucrose + 500 µg/ml glutamic monochloride

× 0.05M sucrose + 250 µg/ml glutamic monochloride

D. Sucrose + glutamic monochloride

▲ 0.10M sucrose + 500 µg/ml glutamic monochloride

× 0.10M sucrose + 250 µg/ml glutamic monochloride


A
B
C
D
2000
1000
0
4000
3000
2000
1000
0
0
10
20
30
40
50
FREE PROLINE CONTENT (µg/g dry wt)
HOURS OF INCUBATION

2.4 Discussion

Results obtained with excised organs of green barley plant clearly showed that leaves and leaf sheaths accumulated an appreciable amount of free proline when subjected to an osmotic stress whereas shoot apices did so only slightly and roots not at all (Figure 10). This demonstrated that only the leaves and leaf sheaths were organs with the potential for proline synthesis during stress. The reason for the inability of excised shoot apices and roots to accumulate proline is not known. It could be that proline accumulation is somehow related to chlorophyll or confined to chlorophyll-containing organs because leaves and young leaf sheaths are the main chlorophyllous organs. Stewart, Morris and Thompson (1966) also found that proline did not accumulate in dehydrated discs of storage roots from potato, carrot, and turnip or dehydrated excised roots from corn seedlings. Observations reported here coupled with those of Stewart et al (1966), therefore, tend to indicate that the phenomenon of proline accumulation is restricted to green tissues. This contention is further supported by the fact that etiolated leaves of barley did not accumulate proline (Figure 11). Alternatively, excised shoot apices and roots may not accumulate proline due to an inadequate supply of precursors or some other chemical messenger. In the intact plant these

substances may be transported from the leaves and leaf sheaths to the shoot apices and roots. Alternatively, it could be that proline biosynthesis is controlled by a feed-back mechanism in the roots and shoot apices, hence no proline is accumulated during an osmotic stress. If this is true, it must mean, consequently, that the feed-back mechanism does not operate as efficiently in the leaves as it does in roots. It is difficult, however, to pin-point which of the above hypotheses is correct.

The inability of etiolated leaves to accumulate proline (Figure 11) suggested that a supply of carbohydrates and other precursors for proline biosynthesis was necessary (Chibnall, 1939; Naylor and Tolbert, 1956). Glutamic acid plays a central role in the nitrogen metabolism of plants and is a precursor for arginine and proline synthesis (Fowden, 1964).

The present data with etiolated leaves also suggests that glutamic acid supply is closely linked with proline biosynthesis during water stress (Figure 12). A combination of sucrose and glutamic acid produced a synergistic effect on proline accumulation. Etiolated leaves obviously have the potentiality for proline accumulation, provided they are supplied with appropriate substrates such as sucrose and glutamic acid. The biosynthesis of proline from ^{14}C-glutamic acid has been shown in partially

wilted leaves of Cynodon dactylon (Barnett and Naylor, 1966) and non-stressed etiolated barley leaves (Naylor and Tolbert, 1956). A role of carbohydrates in proline accumulation during stress was suggested by Routley (1966) but definitive data were not presented. Subsequently, Stewart et al (1966) determined the role of sugar in proline accumulation by incubating wilted leaves of radish (Raphnus sativus) with different levels of carbohydrate. Their results also showed that proline accumulation was greater and more prolonged in wilted leaves with higher sugar and starch content. More specifically, the prevention of proline accumulation by either anaerobiosis or certain inhibitors of oxidative sugar metabolism indicated that the carbon skeleton for proline biosynthesis is supplied by sugar oxidation (Stewart et al, 1966). Thus, it appears that the supply of sucrose and glutamic acid is essential for proline synthesis in etiolated leaves. Alternatively, although less likely, it can be postulated that sucrose and glutamic acid, apart from their function as precursors, also trigger the production or activation of some chemical messenger which leads to the accumulation of proline during osmotic stress.

SECTION 3

3. EFFECT OF ABSCISIC ACID ON PROLINE ACCUMULATION

3.1 Introduction

It has been demonstrated that wilting induces a marked increase in the concentration of the plant growth inhibitor (±) cis-trans abscisic acid (ABA) in excised wheat leaves (Wright, 1969; Wright and Hiron, 1969). The authors speculated that this accumulation of a growth inhibitor during stress may cause some of the physiological effects associated with a water deficit, e.g. stomatal closure. It has been shown that ABA is concerned in the normal endogenous control of plant growth (Addicott and Lyon, 1969). Applied ABA inhibits stomatal opening (Little and Eidt, 1968; Mittelheuser and Steveninck, 1969) and protein synthesis (Chrispeels and Varner, 1967) and in several systems these effects are counteracted by GA_3. Similar responses occur during water stress and Wright and Hiron's (1969) suggestion that ABA may be the causative agent is plausible. In view of this evidence it was decided to explore the effects of ABA on proline accumulation, the most marked metabolic consequence of water deficit.

3.2 Methods

3.2.1 Isolated Organs

Barley plants (cv Prior) were grown in standard conditions for 2 weeks. Plants were then taken from the pots and the roots were thoroughly washed with tap water to remove adhering perlite. Roots and the first leaves were excised and the leaves were cut into 1 cm sections. Both the roots and the leaf sections were pooled on water until sufficient sections had been cut. Seventy five leaf sections and equivalent amounts of root tissue were floated separately on water, polyethylene glycol solutions of -10 or -20 bars osmotic potential or ABA solutions of 0.5 µg or 5.0 µg/ml. In each case the tissues were floated on 20 ml of the appropriate solution contained in a beaker and continuously aerated at 20°C. There were four beakers for each treatment/plant organ combination and these were sampled after 0, 4, 16 and 32 hours incubation. On each occasion 25 leaf sections or an appropriate amount of root tissue was removed from each beaker. The free proline content of each sample was determined and expressed on a tissue dry weight basis.

3.2.2 Intact Plants

Barley plants (cv Prior) were grown for 14 days in standard conditions, with 5 plants per pot. At the end of this period the plants in 8 pots were subjected to mild

water stress by flooding the pots with polyethylene glycol solution of -10 bars osmotic potential. At the same time plants in the same number of pots were sprayed with solutions containing 0, 0.5 or 5.0 µg/ml abscisic acid. In this case the ABA was dissolved in water and sprayed on the plants to run-off. Samples of the first leaves and entire root-systems were collected 0, 4, 16 and 32 hours after the treatments were applied. At each time 3 samples were taken from each treatment. The proline concentration (mg/g dry weight) in these samples was subsequently determined.

3.3 Results

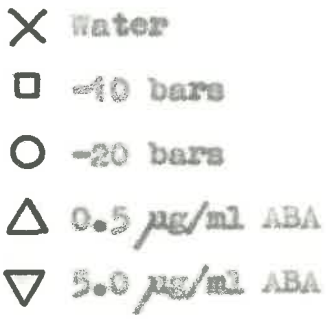
3.3.1 Excised Organs

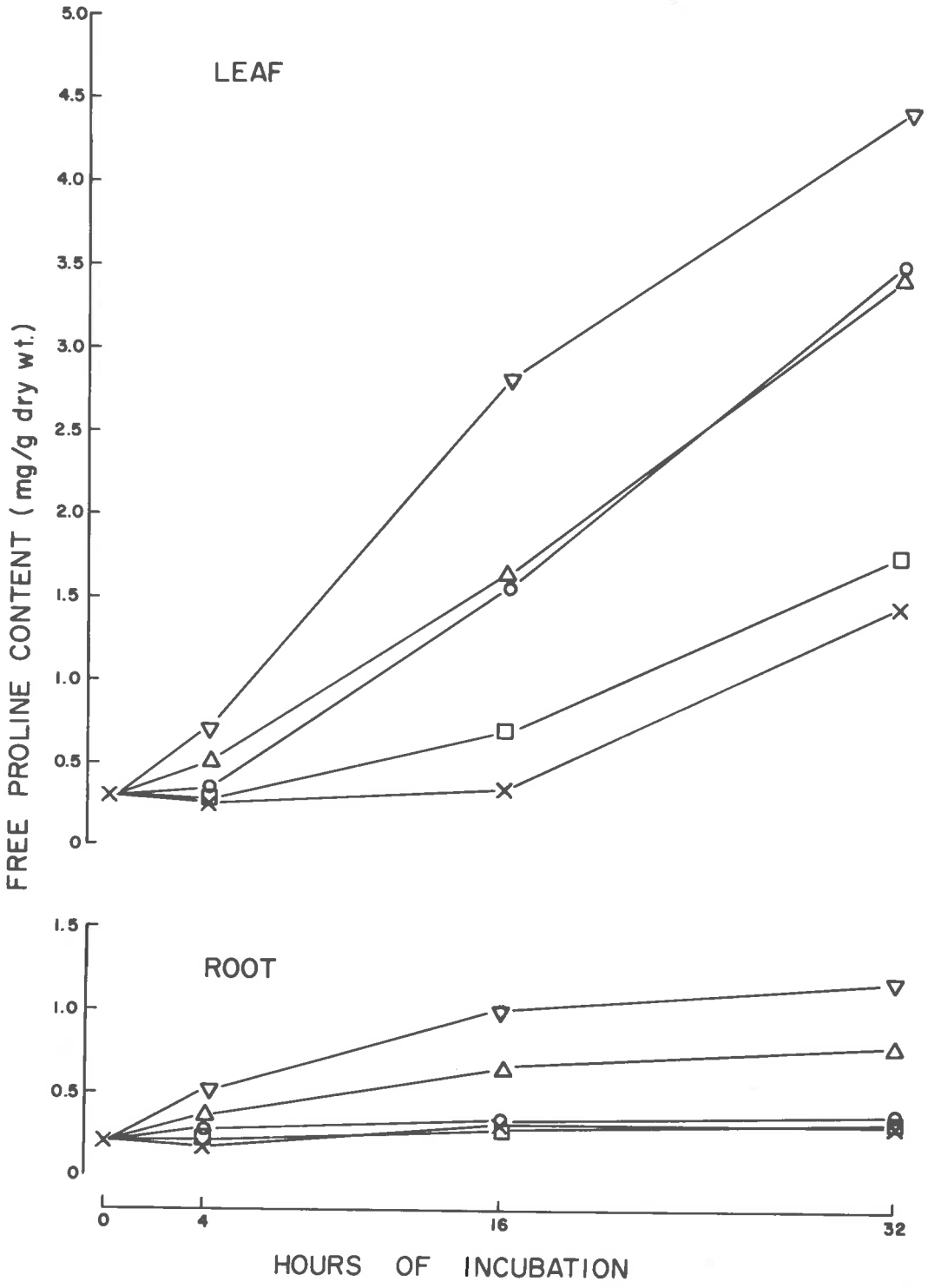
The leaf sections accumulated a considerable amount of free proline when incubated in ABA solutions, the higher concentration being more effective (Figure 13). The induction of proline accumulation by ABA was detectable at 4 hours and the rate of accumulation was promoted by ABA to the end of the experiment. The lower concentration of ABA had a similar effect on proline accumulation (3.3 mg/g dry weight) as a polyethylene glycol solution of -20 bars osmotic potential (3.5 mg/g dry weight) whereas the higher concentration of ABA produced more proline (4.4 mg/g dry weight). As in the previous experiments with excised leaf sections (Figure 10) the free

Figure 13

Effect of abscisic acid (ABA) and osmotic stress on proline accumulation in excised leaf (top) and root (bottom) of barley plant.

(Leaf sections and roots were floated separately on water, polyethylene glycol solutions of -10 or -20 bars osmotic potential or ABA solutions of 0.5 µg or 5.0 µg/ml concentration.)




LEAF
ROOT
FREE PROLINE CONTENT (mg/g dry wt.)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
1.5
1.0
0.5
0
0
4
16
32
HOURS OF INCUBATION

proline level in sections floated on water also increased later in the experiment. Sections floated on water did not accumulate proline up to 16 hours but a rapid increase was observed thereafter.

As in the previous experiments (Figure 10), root tissues floated on osmotic solutions of polyethylene glycol failed to accumulate free proline. In contrast, root tissues floated on ABA solutions accumulated some free proline, but far less than the amount accumulated by the isolated leaves. In this case, therefore, the response of isolated roots to ABA differed from their response to a water deficit. It may be significant, however, that roots on intact plants accumulate free proline in response to water deficit (Figure 6).

3.3.2 Intact Plants

The application of ABA to intact plants also increased the concentration of free proline in the first leaves (Figure 14). In contrast to the excised tissues, however, ABA had a much smaller effect on the concentration of free proline in the roots of intact plants. The rate of accumulation of free proline in the leaves was dependent on the concentration of ABA applied but both concentrations of ABA produced a greater accumulation of free proline than did the mild osmotic stress. The accumulation of free proline in response to ABA was measurable within 4

Figure 14

Effect of abscisic acid (ABA) and osmotic stress on proline accumulation in intact leaf (top) and root (bottom) of barley plant.

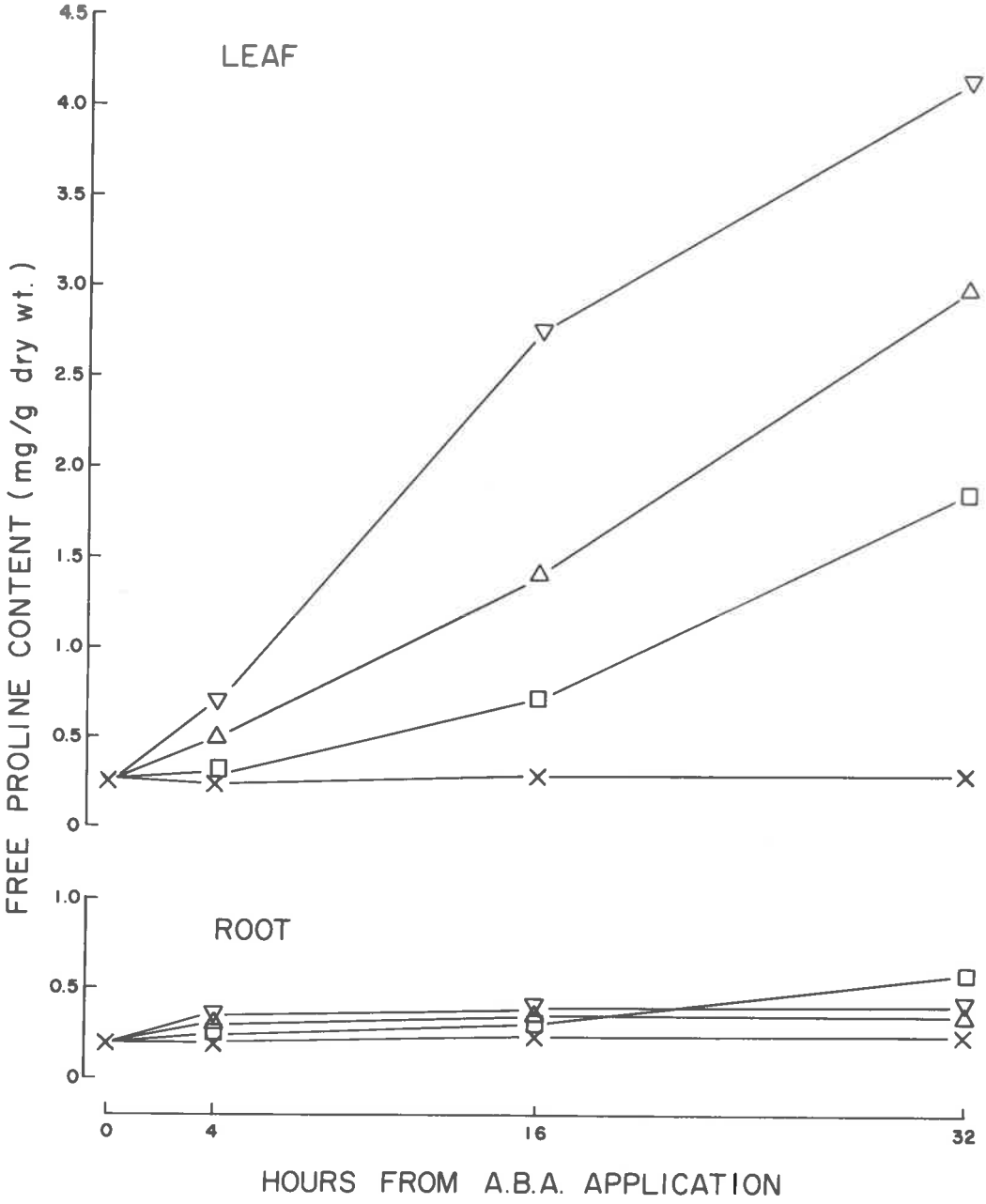
(Two-week-old seedlings were either sprayed with solutions containing 0, 0.5 or 5.0 µg/ml ABA or subjected to mild water stress (-10 bars) by flooding the pot with polyethylene glycol solution.)

× Not water stressed

□ -10 bars

△ 0.5 µg/ml ABA

▽ 5.0 µg/ml ABA


LEAF
ROOT
FREE PROLINE CONTENT (mg/g dry wt.)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
1.0
0.5
0
0
4
16
32
HOURS FROM A.B.A. APPLICATION

hours of treatment and at the end of 32 hours the leaves had accumulated 3.0 (0.5 µg ml ABA) or 4.1 mg/g dry weight (5.0 µg/ml ABA). Water stress produced a small, but significant increase in the free proline concentration in the roots on the last sampling occasion.

3.4 Discussion

Abscisic acid (ABA) is a naturally occurring plant growth inhibitor which affects several physiological and biochemical phenomena in plants (Addicott and Lyon, 1969). Inhibition of stomatal opening (Mittelheuser and Steveninck, 1969) and of protein synthesis (Chrispeels and Varner, 1967) due to applied ABA has been reported. The accumulation of free proline in response to applied ABA which was detectable within four hours (Figures 13, 14), may have been due to such an inhibition of protein synthesis, possibly accompanied by protein degradation under the influence of ABA. It is of interest that this ABA-induced accumulation of proline was comparable with the accumulation induced by water stress. Since water stress has been reported to increase tissue ABA concentration (Wright and Hiron, 1969) it is possible that water stress-induced accumulation of proline may be due to an increased ABA concentration. Again, the roots did not accumulate significant quantities of proline either when treated with ABA or when osmotically stressed although

there was a slight increase in the proline concentration in excised roots treated with a high concentration of ABA. This further suggests that proline biosynthesis in roots may be regulated by feed-back reactions.

SECTION 4

4.1 EFFECT OF CCC ON ACCUMULATION OF PROLINE, GROWTH AND APICAL MORPHOGENESIS IN WHEAT

4.1.1 Introduction

Of the several known growth retarding chemicals, (2-chloroethyl) trimethylammonium chloride (CCC) appears to be the most potent on cereals, particularly wheat, inducing height reductions, lodging resistance and yield increases (Pinthus and Halevy, 1965; Humphries, 1968a). It has also been suggested that plants treated with this chemical are less susceptible to water stress (Halevy and Kessler, 1963; Plaut and Halevy, 1966). The mode of action of CCC in inducing drought resistance is not yet known since none of the characters such as transpiration, stomatal opening, osmotic potential, water saturation deficit, or morphological and anatomical features showed a consistent correlation with the effects of this chemical on drought resistance (Plaut, Halevy, and Shmueli, 1964). The aim of the present experiment was to investigate the effect of CCC on proline metabolism, and growth and apical morphogenesis in the water stressed wheat plant.

4.1.2 Methods

The effect of the growth retarding chemical (2-chloroethyl) trimethylammonium chloride (CCC) on proline metabolism, plant growth and apical morphogenesis

in wheat was investigated. Wheat plants (cv Gabo) were grown in perlite with nutrient solution for one week and were then treated with 100 ml of a solution containing 40 mg CCC per pot as a soil drench. The plants were subsequently subjected to water stress commencing ten days after the CCC treatment. This time interval was arbitrarily chosen so that any effects of CCC on morphological characters could be seen before water stress was imposed. The pots were flooded with 250 ml polyethylene glycol (MW,4000) solution of 0, -10 or -20 bars osmotic potential. Some plants were relieved from water stress after 48 hours by washing out the osmoticum from the rooting medium with 6 changes of 200 ml of water. The plants were sampled 0, 7, 18, 30, 48, 56 and 72 hours after stress commenced. Water potential of the second leaves, and the amounts of proline in the second leaves, leaf-sheaths, shoot apices and roots were assessed. Plants were dissected under a binocular microscope and the growth of the shoot apices, particularly primordium production and length was assessed. The height of the plants was also measured. This presents difficulty in a grass plant but the distance from the point of root initiation to the base of the leaf blade of the last fully-emerged leaf was utilised as a convenient and reproducible measure. Dry weight of the plants and area of leaves were measured only at the time of commencement of stress. Leaf area

was recorded by use of an electronic planimeter, Paton Industries, South Australia.

4.1.3 Results

4.1.3.1 Water Potential

The water potential of the leaves of plants not subjected to stress varied within the range -2.0 to -3.4 bars whether they were treated with CCC or not (Figure 15). Similarly, CCC had no effect on the water potential of the leaves on plants subjected to stress. In both stress treatments, the water potential fell rapidly initially and then more slowly, although the level of leaf water potential was much lower in plants subjected to a -20 bars osmotic potential than in those subjected to -10 bars. This pattern of response was also observed in earlier experiments (Figure 4).

4.1.3.2 Growth of Plants

4.1.3.2.1 Dry Weight

Total dry weight was reduced significantly in CCC-treated plants (Table 3) but various organs were differently affected. Reduction in dry weight was comparatively more apparent in shoots (Control-24.9%, treated-20.9%) than in leaves (Control-50.0%, treated-48.9%). Conversely, treated roots appeared to have gained dry weight (Control-25.0%, treated-30.0%).

Figure 15

Effect of CCC and water stress on the water potential of the second leaf of wheat plants.

(One week old seedlings were treated with CCC and the plants were subsequently subjected to mild (-10 bars) and severe (-20 bars) water stress commencing 10 days after the CCC treatment.)

- CCC	+ CCC	
○	●	Control
△	▲	-10 bars
⬡	⬢	Stress relieved
▽	▼	-20 bars
□	■	Stress relieved

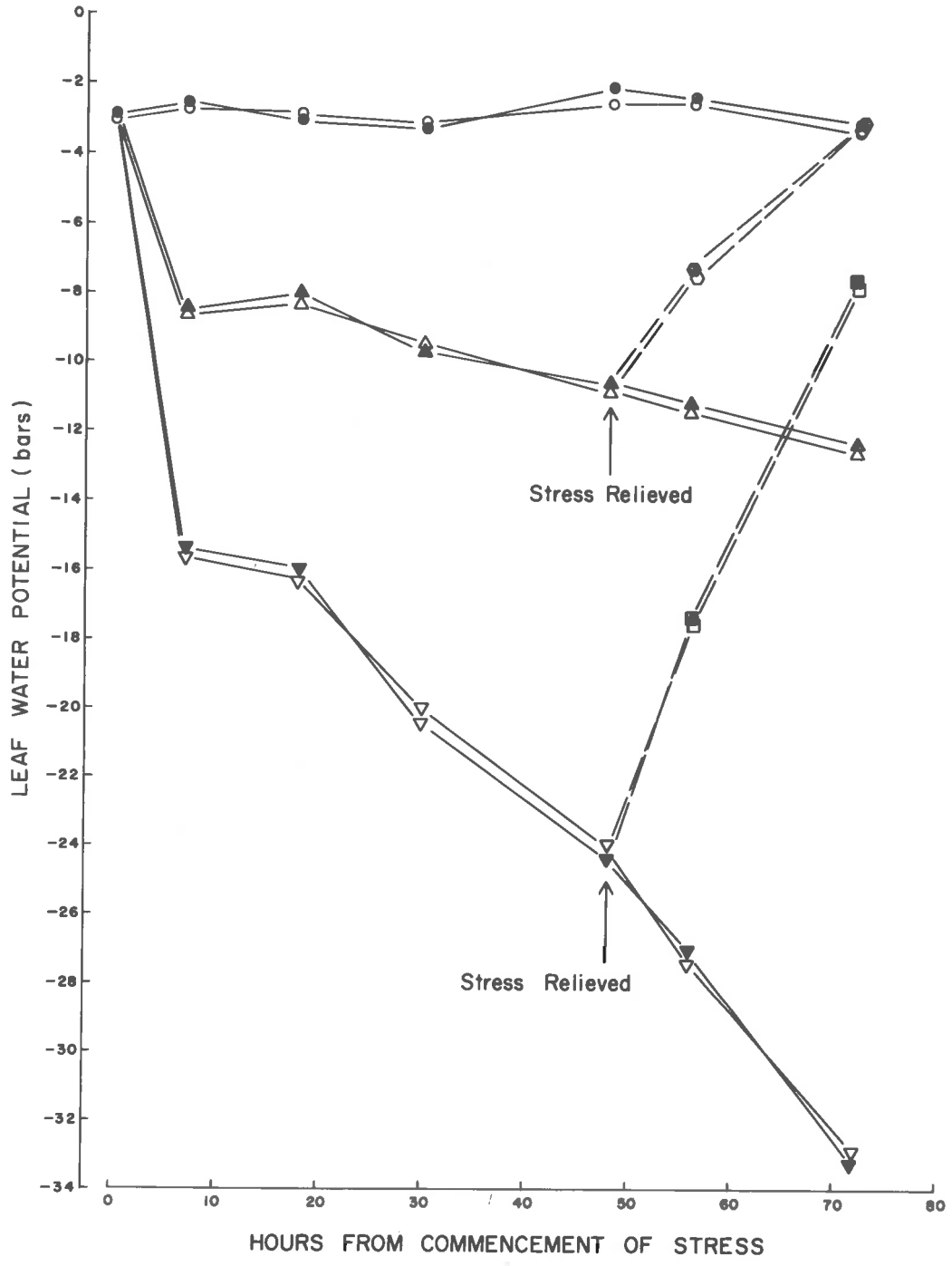

LEAF WATER POTENTIAL (bars)
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
-28
-30
-32
-34
0
10
20
30
40
50
60
70
80
HOURS FROM COMMENCEMENT OF STRESS
Stress Relieved
Stress Relieved

Table 3

Effect of (2-chloroethyl) trimethyl ammonium chloride (CCC) on the dry weight of wheat plants (cv Gabo).

Concentration of CCC (mg/pot)	Plant organ	Dry Weight (g/plant) 10 days after treatment
0	Leaf	0.1183 (50.00%)
	Shoot	0.0590 (24.93%)
	Root	0.0593 (25.06%)
	Total	0.2366
40	Leaf	0.0768 (48.94%)
	Shoot	0.0329 (20.96%)
	Root	0.0472 (30.08%)
	Total	0.1569

4.1.3.2.2 Height

Treatment with CCC drastically reduced the height of the plant (Figure 16, top). Ten days after treatment with CCC the height of untreated plants was nearly double the height of treated plants. Since the rate of elongation was greater in the untreated plants the inhibitory effects of water stress on plant height were more apparent in the untreated plants than in the treated ones.

4.1.3.2.3 Leaf Area

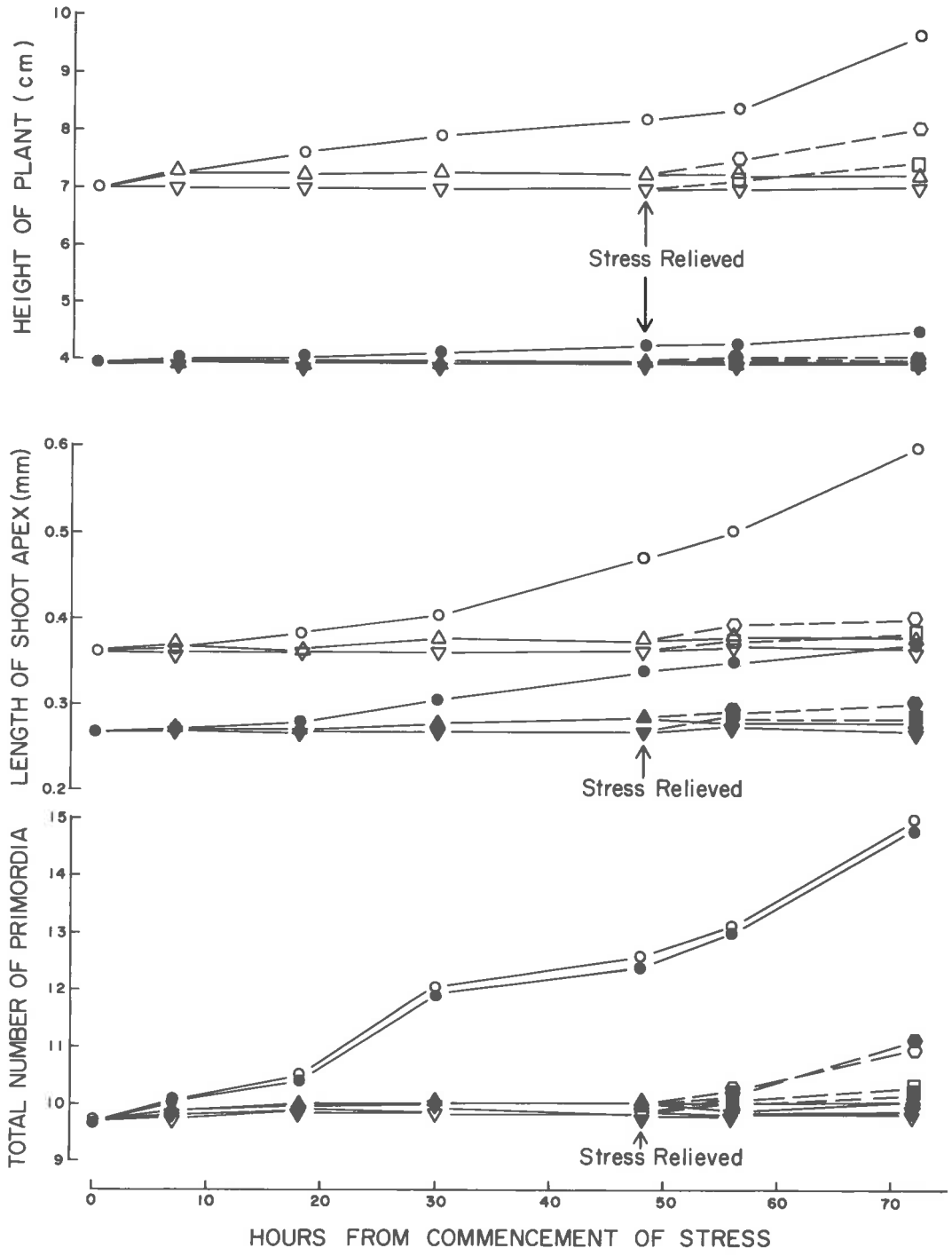
A great reduction in leaf area was another morphological effect of CCC. Ten days after CCC-treatment,

Figure 16

Effect of CCC and water stress on height of plant (top), length of shoot apex (middle), and primordium production (bottom) in wheat.

(One week old seedlings were treated with CCC and the plants were subsequently subjected to mild (-10 bars) and severe (-20 bars) water stress commencing 10 days after the CCC treatment.)

- CCC	+ CCC	
○	●	Control
△	▲	-10 bars
⬡	⬢	Stress relieved
▽	▼	-20 bars
□	■	Stress relieved


HEIGHT OF PLANT (cm)
10
9
8
7
6
5
4
Stress Relieved
LENGTH OF SHOOT APEX (mm)
0.6
0.5
0.4
0.3
0.2
Stress Relieved
TOTAL NUMBER OF PRIMORDIA
15
14
13
12
11
10
9
Stress Relieved
0
10
20
30
40
50
60
70
HOURS FROM COMMENCEMENT OF STRESS

treated plants had a leaf area of 23.6 sq. cm whereas untreated ones had 36.4 sq. cm.

4.1.3.3 Apical Morphogenesis

4.1.3.3.1 Length of Shoot Apices

Elongation of shoot apices was affected in a similar manner by CCC as the height of the plant (Figure 16, middle). Water stress, both mild as well as severe, terminated the further growth of apices in CCC-treated and untreated plants alike.

4.1.3.3.2 Primordium Production

Unlike other morphological characters such as height, leaf area; primordium production (Figure 16, bottom) was unaffected by CCC treatment in spite of the fact that length of apices was greatly reduced. Water stress inhibited primordium production very severely and the effect was identical in treated and untreated plants.

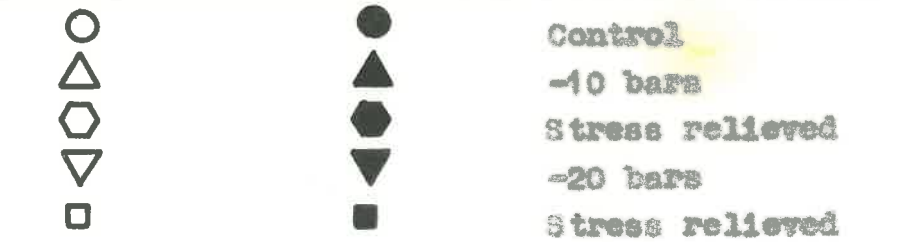
4.1.3.4 Accumulation of Proline

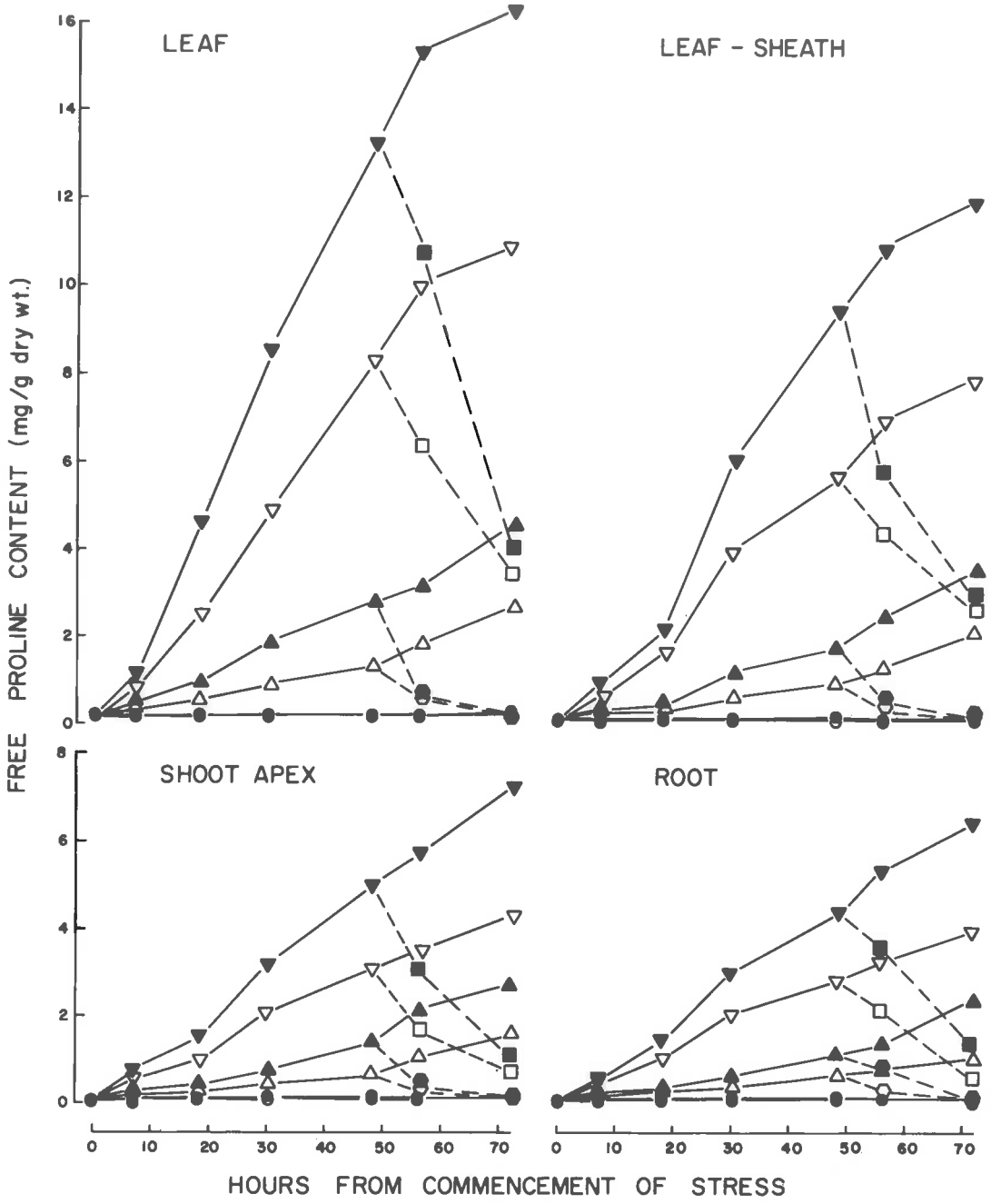
Water stress caused a rapid accumulation of proline in the leaves of the wheat plant (Figure 17) comparable to that observed in the barley plant (Figure 6). CCC had no effect on proline levels in the control plants but increased the capacity of the second leaves to accumulate proline during stress. The accumulation was both more rapid and more extensive in the plants subjected to the

Figure 17

Effect of CCC and water stress on the accumulation of free proline in the leaf, leaf sheath, shoot apex, and root of wheat plant.

(One week old seedlings were treated with CCC and the plants were subsequently subjected to mild (-10 bars) and severe (-20 bars) water stress commencing 10 days after the CCC treatment.)




LEAF
LEAF - SHEATH
SHOOT APEX
ROOT
FREE PROLINE CONTENT (mg/g dry wt.)
HOURS FROM COMMENCEMENT OF STRESS
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60
70

severe stress. At the end of 72 hours, the second leaves of CCC-treated plants had accumulated more than 4.4 mg/g dry weight (61 µg/g/hour) under mild stress and 16.3 mg/g dry weight (227 µg/g/hour) under severe stress whereas the same leaves of untreated plants had accumulated only 2.8 mg/g dry weight (39 µg/g/hour) and 10.9 mg/g dry weight (151 µg/g/hour) under mild and severe stress respectively. This represents an approximately 50% increase in capacity to accumulate proline, due to CCC pre-treatment, in both stress situations. When the stress was relieved, the water potential increased rapidly (Figure 15) and the amount of proline in the second leaf decreased with time in both stress treatments. The rate of decrease was slightly greater in plants pre-treated with CCC. Some proline remained in the leaves 24 hours after removal of the osmoticum from the rooting medium in the -20 bars treatment.

The effects of CCC on proline accumulation in the leaf sheaths, apices and roots were also assessed (Figure 17). In each case, pre-treatment with CCC increased the capacity of the particular tissue to accumulate proline when subjected to water stress. In every case the capacity to accumulate proline was increased by approximately 50-60% so that the potential proline accumulation of the various organs relative to each other

was unaffected. Over all, proline accumulation, as in barley (Figure 7), was correlated with the water potential of the leaf (Figure 18); the lower the leaf water potential, the higher was the accumulation of proline, but the rate of proline accumulation as a function of water potential was significantly higher in CCC-treated plants. In spite of a large difference between the rates of proline accumulation, there was no difference in the total amount of proline accumulated during stress when calculated on the basis of per plant.

4.2 THE INTERACTION OF CCC AND GA_3 ON THE ACCUMULATION OF PROLINE, GROWTH AND APICAL MORPHOGENESIS IN WHEAT

4.2.1 Introduction

In the previous experiment, CCC was found to increase the capacity of wheat plants to accumulate proline during water stress. Many of the effects of growth retardants, such as CCC, are reversed by gibberellic acid (GA_3) and it was of interest to discover whether this was also the case with the accumulation of proline.

In this experiment, the effects of two levels of CCC, two levels of GA_3, and one level of stress on the water potential of the second leaves, proline accumulation in the same leaves, growth in height and apical morphogenesis in wheat (cv Gabo) were investigated.

Figure 18

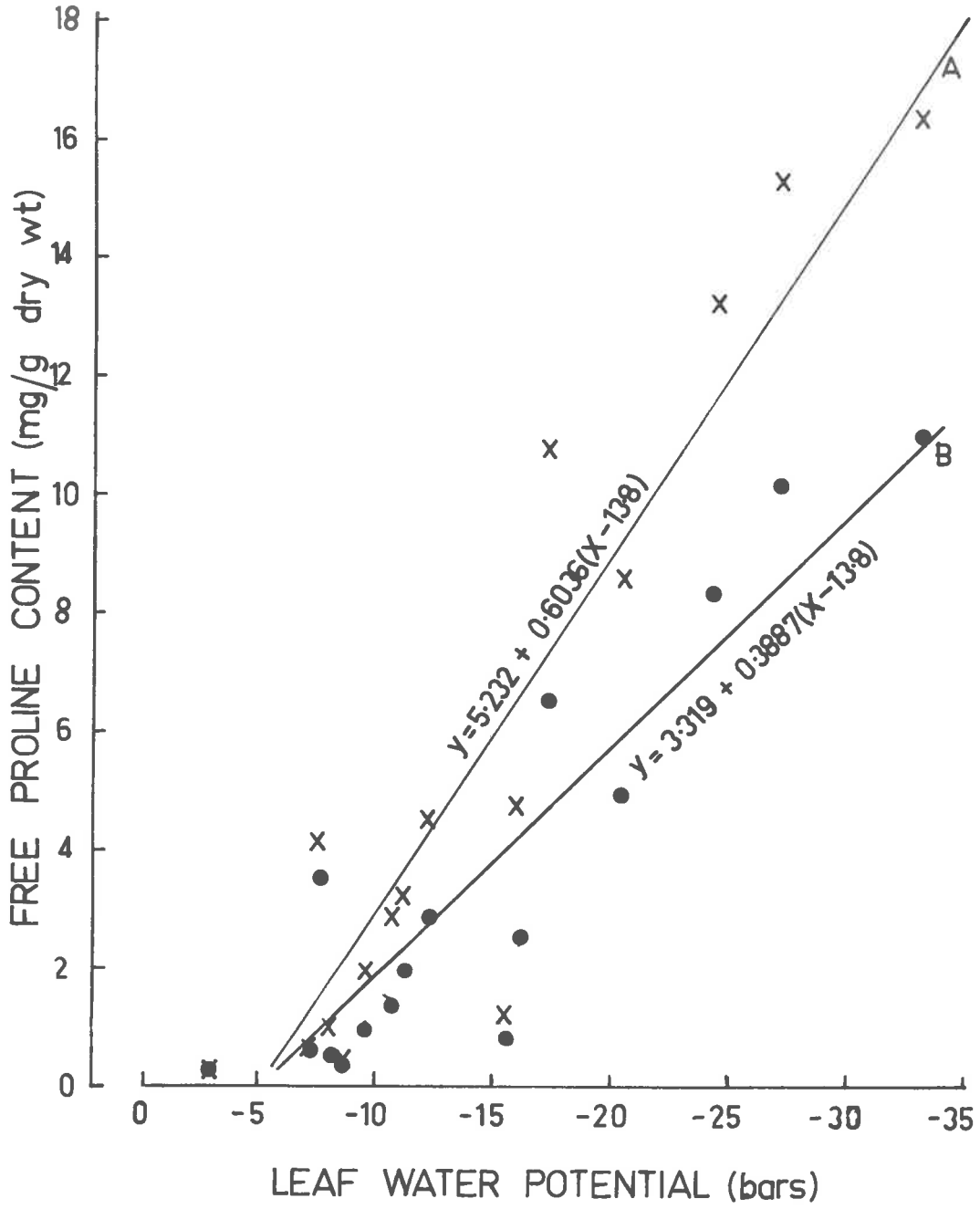
Relationship between proline accumulation and water potential in CCC-treated and untreated plant.

(Values plotted on this graph were adopted from Fig. 15 for water potential and Fig. 17 (leaf) for proline content.)

A. (+ CCC) regression line ×

B. (- CCC) regression line ●

Regression coefficients of these two lines are significantly different at 1% level of probability.


FREE PROLINE CONTENT (mg/g dry wt)
LEAF WATER POTENTIAL (bars)
18
16
14
12
10
8
6
4
2
0
0
-5
-10
-15
-20
-25
-30
-35
A
B
y=5·232 + 0·6036(X-138)
y=3·319 + 0·3887(X-138)

4.2.2 Methods

Plants (6 per pot) were grown in perlite for 8 days and then treated with 100 ml solution containing 20 or 40 mg CCC/pot. Ten days after treatment with CCC, the plants were subjected to water stress by flooding each pot with 250 ml of polyethylene glycol (MW,4000) solution of -20 bars osmotic potential. Twenty-four hours after commencing the osmotic stress treatment the plants were treated with 0.04 ml of a solution containing 0, 10 or 100 µg of GA_3 dissolved in 0.05% Tween-20. The solutions were applied to the axil of the second leaf with a dropper. Plants were sampled 0, 24, 27, 30, 33 and 36 hours after the stress commenced.

4.2.3 Results

4.2.3.1 Water Potential

Neither CCC nor GA_3 had any effect on the water potential of the second leaves (Table 4). The water potential of plants subjected to stress had fallen to -21 - -22 bars at the end of the experiment whereas the water potential of control plants fluctuated between -2 and -3 bars throughout.

4.2.3.2 Plant Height

As in the previous experiment, both CCC and water stress reduced plant height (Table 5). In the case of water stress continued elongation of the shoot system was

Table 4

Water potential of the second leaf of wheat plants (cv Gabo) treated with (2-chloroethyl)-trimethyl ammonium chloride (CCC) at time 0 and gibberellic acid (GA_3) 24 hours later.

Osmotic potential (bars)	Concentration of CCC (mg/pot)	Concentration of GA_3 (µg/plant)	Time (hours) +24	+27	+30	+33	+36
			Mean water potential (bars)				
		0	-2.7	-2.9	-2.9	-2.6	-2.8
	0	10	-	-2.7	-2.9	-2.7	-2.8
		100	-	-2.8	-2.9	-2.7	-2.7
		0	-2.6	-2.6	-3.0	-2.8	-2.8
0	20	10	-	-2.7	-3.0	-2.8	-2.9
		100	-	-2.8	-2.8	-2.8	-2.9
		0	-2.7	-2.9	-3.0	-2.9	-2.9
	40	10	-	-2.8	-3.0	-2.7	-3.0
		100	-	-2.8	-2.9	-3.0	-2.8
		0	-18.4	-19.1	-21.0	-21.3	-21.7
	0	10	-	-18.2	-20.6	-20.4	-21.5
		100	-	-18.5	-20.7	-20.7	-21.1
		0	-18.7	-19.0	-20.6	-21.2	-22.1
-20	20	10	-	-19.0	-20.9	-21.3	-21.8
		100	-	-18.7	-20.8	-21.8	-21.6
		0	-18.6	-18.9	-21.2	-21.5	-21.9
	40	10	-	-18.5	-21.2	-21.8	-21.9
		100	-	-18.8	-20.6	-21.7	-21.7
Least Significant Difference (P=0.05)				0.8	1.0	0.8	0.8

Table 5

Height of wheat plants (cv Gabo) treated with (2-chloroethyl)-trimethyl ammonium chloride (CCC) at time 0 and gibberellic acid (GA_3) 24 hours later.

Osmotic potential (bars)	Concentration of CCC (mg/pot)	Concentration of GA_3 (µg/plant)	Time (hours) +24	+27	+30	+33	+36
			Mean height of plant (cm)				
		0	7.94	7.99	8.02	8.28	8.51
	0	10	-	8.02	8.51	8.97	9.51
		100	-	8.12	8.85	9.41	10.21
		0	5.39	5.40	5.50	5.59	5.60
0	20	10	-	5.50	5.80	6.10	6.64
		100	-	5.57	6.00	6.65	7.23
		0	4.00	4.01	4.10	4.20	4.29
	40	10	-	4.10	4.29	4.58	4.92
		100	-	4.31	4.60	5.10	5.57
		0	7.39	7.42	7.41	7.40	7.38
	0	10	-	7.40	7.60	7.84	8.13
		100	-	7.50	7.79	8.14	8.75
		0	5.04	5.01	5.12	5.13	5.10
-20	20	10	-	5.19	5.31	5.37	5.57
		100	-	5.20	5.48	5.80	6.23
		0	3.80	3.80	3.83	3.89	3.89
	40	10	-	3.89	4.01	4.21	4.42
		100	-	4.10	4.30	4.54	4.83
Least significant difference (P=0.05)			0.16				

completely suppressed soon after the imposition of stress, whether the plants were pre-treated with CCC or not. In all situations, the application of GA_3 to the plants induced renewed elongation although the response was of the greatest magnitude in the absence of both CCC and water stress.

4.2.3.3 Apex growth

Similarly, GA_3 promoted both apex elongation (Table 6) and primordium production (Table 7) under all conditions of water stress and CCC. Again, primordium production was not affected by CCC although apex elongation was considerably reduced. GA_3 increased primordium production equally with or without CCC and the response to GA_3 was unaffected by water stress although the production of primordia was almost completely inhibited by water stress before the application of GA_3.

4.2.3.4 Proline accumulation

CCC, again, substantially increased the amount of free proline accumulated in the leaves during stress (Figure 19). This response in the first 24 hours after stress commenced was dependent upon the concentration of CCC used. Plants treated with the high concentration of CCC (40 mg/pot) accumulated proline at the rate of 278 µg/g dry weight/hour, those treated with 20 mg CCC/pot at the rate of 213 µg/g dry weight/hour and those not treated

Table 6

Length of shoot apices of wheat plants (cv Cabo) treated with (2-chloroethyl)-trimethyl ammonium chloride (CCC) at time 0 and gibberellic acid (GA_3) 24 hours later.

Osmotic potential (bars)	Concentration of CCC (mg/pot)	Concentration of GA_3 (µg/plant)	Time (hours) +24	+27	+30	+33	+36
			Mean length of shoot apices (mm)				
		0	0.400	0.422	0.428	0.442	0.450
	0	10	-	0.424	0.448	0.502	0.598
		100	-	0.428	0.484	0.612	0.706
		0	0.352	0.372	0.374	0.384	0.390
0	20	10	-	0.370	0.400	0.460	0.532
		100	-	0.384	0.434	0.510	0.642
		0	0.302	0.310	0.320	0.332	0.340
	40	10	-	0.326	0.350	0.418	0.494
		100	-	0.342	0.396	0.500	0.622
		0	0.368	0.368	0.364	0.368	0.370
	0	10	-	0.372	0.390	0.424	0.470
		100	-	0.382	0.426	0.486	0.564
		0	0.322	0.286	0.288	0.290	0.290
-20	20	10	-	0.342	0.358	0.380	0.420
		100	-	0.370	0.416	0.452	0.500
		0	0.279	0.272	0.276	0.274	0.278
	40	10	-	0.290	0.314	0.332	0.362
		100	-	0.320	0.350	0.396	0.436

Least significant difference (P=0.05) 0.080

Table 7

Primordium production in wheat plants (cv Gabo) treated with (2-chloroethyl)-trimethyl ammonium chloride (CCC) at time 0 and gibberellic acid (GA_3) 24 hours later.

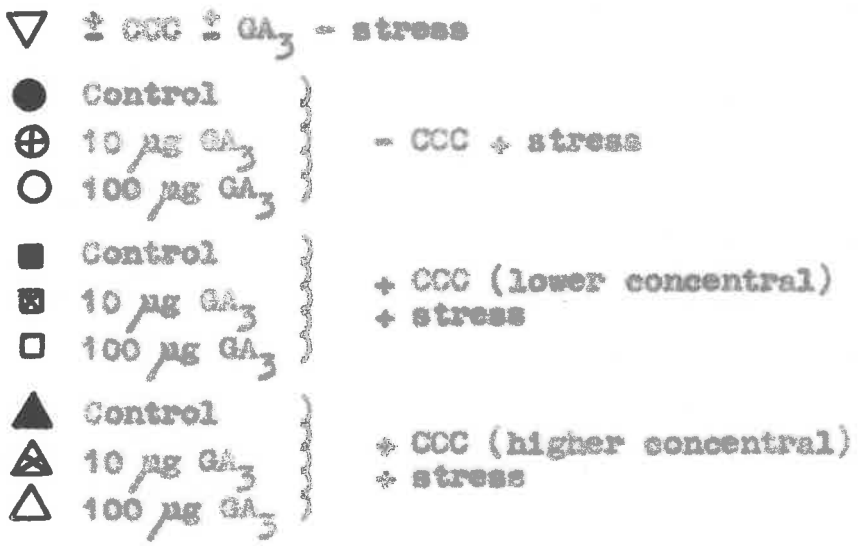
Osmotic potential (bars)	Concentration of CCC (mg/pot)	Concentration of GA_3 (µg/plant)	Time (hours) +24	+27	+30	+33	+36
			Total number of primordia/main shoot				
		0	11.5	11.7	12.1	12.4	12.8
	0	10	-	12.0	12.4	13.2	14.2
		100	-	12.2	12.8	13.8	15.9
		0	11.5	11.8	12.2	12.5	12.8
0	20	10	-	12.1	12.4	13.3	14.1
		100	-	12.2	12.8	13.7	15.9
		0	11.4	11.7	12.2	12.4	12.8
	40	10	-	12.0	12.4	13.3	14.1
		100	-	12.2	12.9	13.9	15.8
		0	9.8	9.9	9.8	9.8	9.8
	0	10	-	10.0	10.4	10.8	11.5
		100	-	10.5	11.2	12.0	12.9
		0	9.8	9.7	9.8	9.8	9.8
-20	20	10	-	9.9	10.2	10.8	11.4
		100	-	10.5	11.1	12.0	12.9
		0	9.7	9.7	9.8	9.8	9.8
	40	10	-	9.9	10.3	10.9	11.6
		100	-	10.4	11.1	12.1	12.9

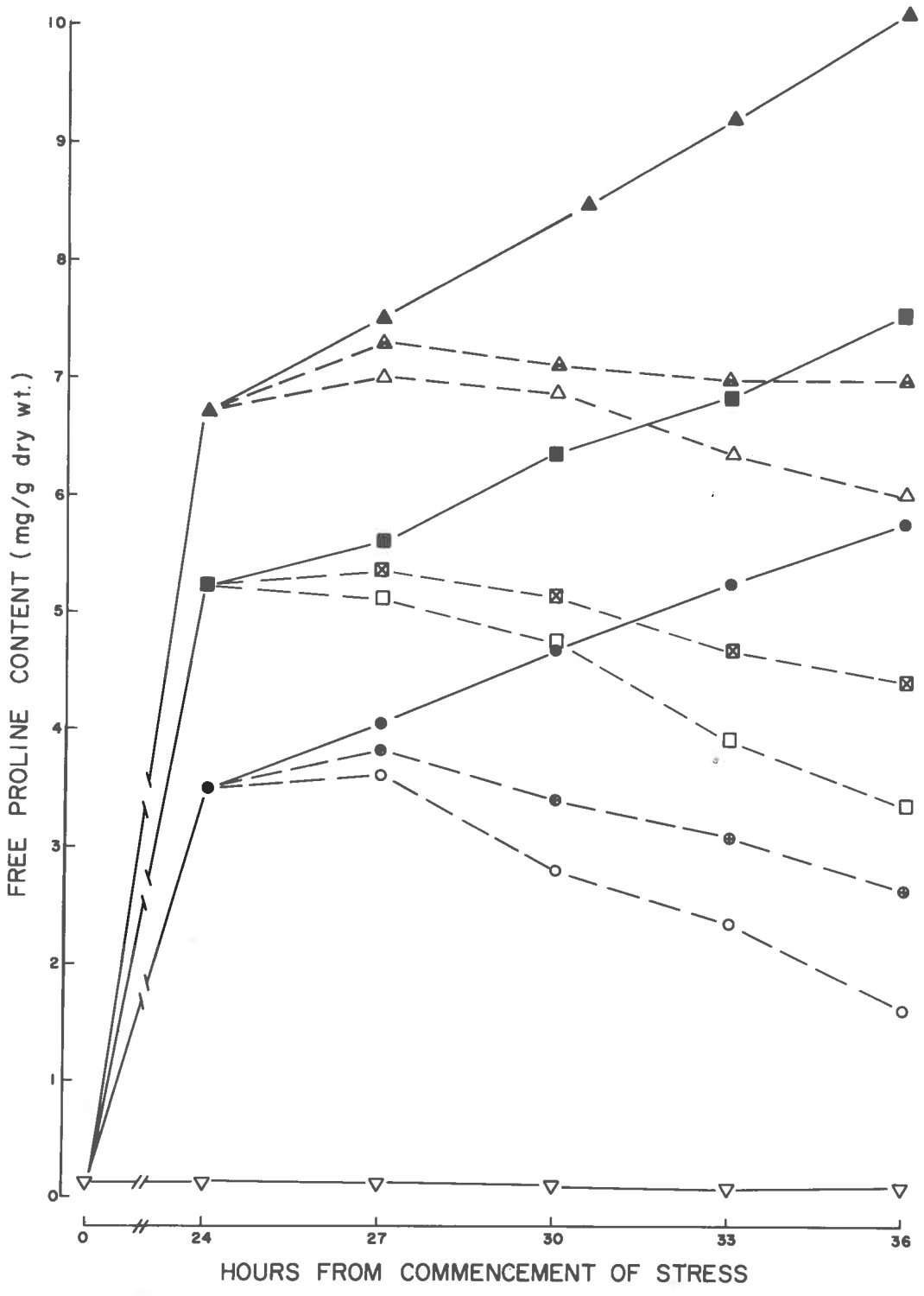
Least significant difference (P=0.05) 0.55

Figure 19

Effect of gibberellic acid (GA_3) on proline accumulation in the second leaf of CCC-treated and untreated wheat plants during water stress.

(Eight-day-old seedlings were treated with CCC and the plants were subjected to water stress (-20 bars) 10 days after the CCC treatment. GA_3 was applied 24 hours after the commencement of stress.)




FREE PROLINE CONTENT (mg/g dry wt.)
0
1
2
3
4
5
6
7
8
9
10
0
24
27
30
33
36
HOURS FROM COMMENCEMENT OF STRESS

with CCC at the rate of 144 µg/g dry weight/hour. The concentration of proline in the tissues began to decline soon after the plants were treated with GA_3 and had declined considerably by the end of the experiment. In contrast, plants not treated with GA_3 continued to accumulate proline. In this case, therefore, GA_3 initiated a decline in the amount of proline in the tissue despite a continuing decline in the water potential of the leaves. In this, as in other cases of growth response (Cathey, 1964) the effects of GA_3 and CCC were opposite.

4.3 DISCUSSION

4.3.1 Effect of CCC

Effects of quaternary-ammonium compounds, particularly CCC, on morphogenesis in cereals have been extensively reported (Cathey, 1964; Humphries, 1968a). Many of these observations have been repeated in the present investigation results where the height of the plant (Figure 16, top) and the total leaf area (page 113, 4.1.3.2.3) were greatly reduced by treatment with CCC. The dry weight of the component parts of the plant were also generally reduced and the distribution of total dry matter between the various organs was also affected. The comparative weight of the roots was increased by CCC treatment while that of the stem decreased (Table 3). Reduction in the total dry weight of CCC-treated plants

has been considered to be due partially to the reduced photosynthetic area and partially to the inhibition of stem elongation which would reduce the size of potential sinks for assimilate (Humphries, 1968b). These general effects of the growth retardant on plant growth, which have been described before, are probably traceable to effects on organogenesis. Retardant effects on organogenesis have not been described previously, however, and it is of interest that although primordium production at the shoot apex was not affected by CCC, the growth of the shoot apex in length was greatly reduced (Figure 16, middle). This suggests that the process of internode extension in the apex itself is inhibited by the growth retardant.

The general effects of growth retardants on plant growth were found by several workers to be apparently accompanied by an increase in the ability of the plant to resist the effects of water stress (Halevy and Kessler, 1963; Plaut and Halevy, 1966). Plants treated with growth retardants were generally greener and sturdier than untreated plants. Subsequent investigations indicated that untreated plants, when water was withheld, desiccated and died about two weeks earlier than those treated with CCC or Phosphon D (Halevy and Kessler, 1963). Transpiration rate, stomatal opening, osmotic potential, water saturation deficit, and anatomical and morphological

features were not consistently correlated with the effects of these chemicals on drought resistance (Plaut et al, 1964). The present data showed that CCC treatment did not affect the water potential in the leaves of plants subjected to water stress (Figure 15). This confirms that CCC does not change the ability of the plant to gain or lose water. Any effect on the response of the plant to water stress must be due to CCC-induced changes in the growth and metabolism of the plant. Some related effects of growth retardants have been reported although their significance in drought resistance is not clear. Growth retardants delay the senescence of detached bean leaves (Halevy, 1967) and this effect and the increased drought tolerance may be due to effects of CCC on nucleic acid and protein metabolism (Halevy, 1967). The stimulation of proline synthesis during water stress due to CCC treatment may be another consequence of this response (Figure 17) although the underlying cause is not known. An increased protein content in CCC-treated plants has been reported (Stoddart, 1965). If this is due to increased protein synthesis then inhibition of this increased rate by water stress could conceivably lead to enhanced proline accumulation. Alternatively, a preferential conversion of carbohydrates to amino acids (Stoddart, 1965) may be responsible or CCC may have changed the endogenous hormone

balance which in turn exerted some effect on proline accumulation during water stress. Clearly, the mechanism by which CCC brings about an increased potential for proline accumulation is unknown but it is of interest that a treatment reputed to increase water stress resistance of the plant also affects the accumulation of proline.

4.3.2 Effect of GA_3

Studies on the action of CCC and related chemicals have shown that the growth responses induced are opposite to those resulting from gibberellin applications (Cathey, 1964). It has also been suggested that the growth retardants might be used to protect plants from extreme environmental conditions such as drought (Plaut and Halevy, 1966; Halevy, 1967). It has already been demonstrated (Figure 17) that CCC increases the potential for proline accumulation during water stress. This increase is inhibited by applied gibberellin (Figure 19) and, as with CCC alone, the water potential of the plant is unaffected (Table 4). Plants not treated with GA_3 continued to accumulate proline during stress but the level of proline began to fall when the GA_3 was applied. In view of other opposite effects of these two categories of growth regulators, it is noteworthy that CCC, which is reputed to increase drought resistance (Plaut *et al*, 1964), also increased proline accumulation potential, whereas

GA_3, which probably decreases drought resistance (Sitnikova, 1966), decreased proline accumulation. The metabolic effects of these two substances, as demonstrated by proline accumulation, were paralleled by growth effects. Increase in height, length of the shoot apex, and primordium production were all promoted by GA_3 application in both stressed and non-stressed plants whether they had been previously treated with CCC or not (Tables 5, 6, 7). Apparently, this GA_3-stimulated growth during an episode of water stress was accompanied by the disappearance of accumulated proline. It is possible that protein synthesis was promoted by the gibberellin supplied which in turn utilized the accumulated proline for growth. A similar effect of exogenous GA_3 on growth during stress has been reported for beans where GA_3 application restored shoot growth in osmotically stressed plants (O'Leary and Prisco, 1968).

SECTION 5

5.1 REPEATED CYCLES OF WATER STRESS AND THE ACCUMULATION OF PROLINE

5.1.1 Introduction

It has been frequently stated (May and Milthorpe, 1962a, 1962b) that subjecting plants to one episode of water stress 'hardens' them against the effects of subsequent stress. It was of interest, therefore, to explore the effects of such a pre-treatment on the capacity of the plant to produce proline. The present experiment was designed to investigate the effects of repeated cycles of water stress on water potential, proline accumulation, and chlorophyll content in barley.

5.1.2 Methods

Barley plants (cv Prior) were grown in soil for 10 days before any treatments were imposed. At this time, half of the plants were subjected to stress by withholding water for 4 days, the remaining plants being irrigated daily. Plants were sampled just before re-watering and when the plants had fully recovered 2 days later, following daily irrigation. Half of the plants which had not been sampled were then subjected to a further 3-day cycle of water stress and were sampled on two occasions as before. This gave three treatments at this stage, plants subjected to two cycles of stress, plants subjected to one

and plants irrigated daily throughout. Finally, surviving plants were subjected to a 2-day cycle of stress following 2 days of recovery. These plants were also sampled immediately before re-watering and following 2 days of recovery. In this case there were four treatments, plants subjected to 3, 2, 1 or no cycles of stress. The period over which water was withheld in each successive episode of stress was reduced by one day in an effort to compensate for the growth of the plants which resulted in more rapid transpiration. This provision was partially successful as the leaf water potentials at the time of re-watering were very similar on each occasion.

Separate samples of the first and second leaves were taken on each occasion for water potential, proline and chlorophyll determinations. Replication was three-fold for water potential and proline but only 2-fold for chlorophyll estimation.

5.1.3 Results

5.1.3.1 Water Potential

The water potential of both the first and second leaves of irrigated plants fluctuated between -2.5 and -3.0 bars throughout the experiment with no apparent differences between the leaves (Figure 20). The water potential of leaves subjected to a water deficit returned to this level upon re-watering on each occasion. The

Figure 20

Effect of repeated cycles of water stress on water potential of the first (top) and second (bottom) leaf of barley plant.

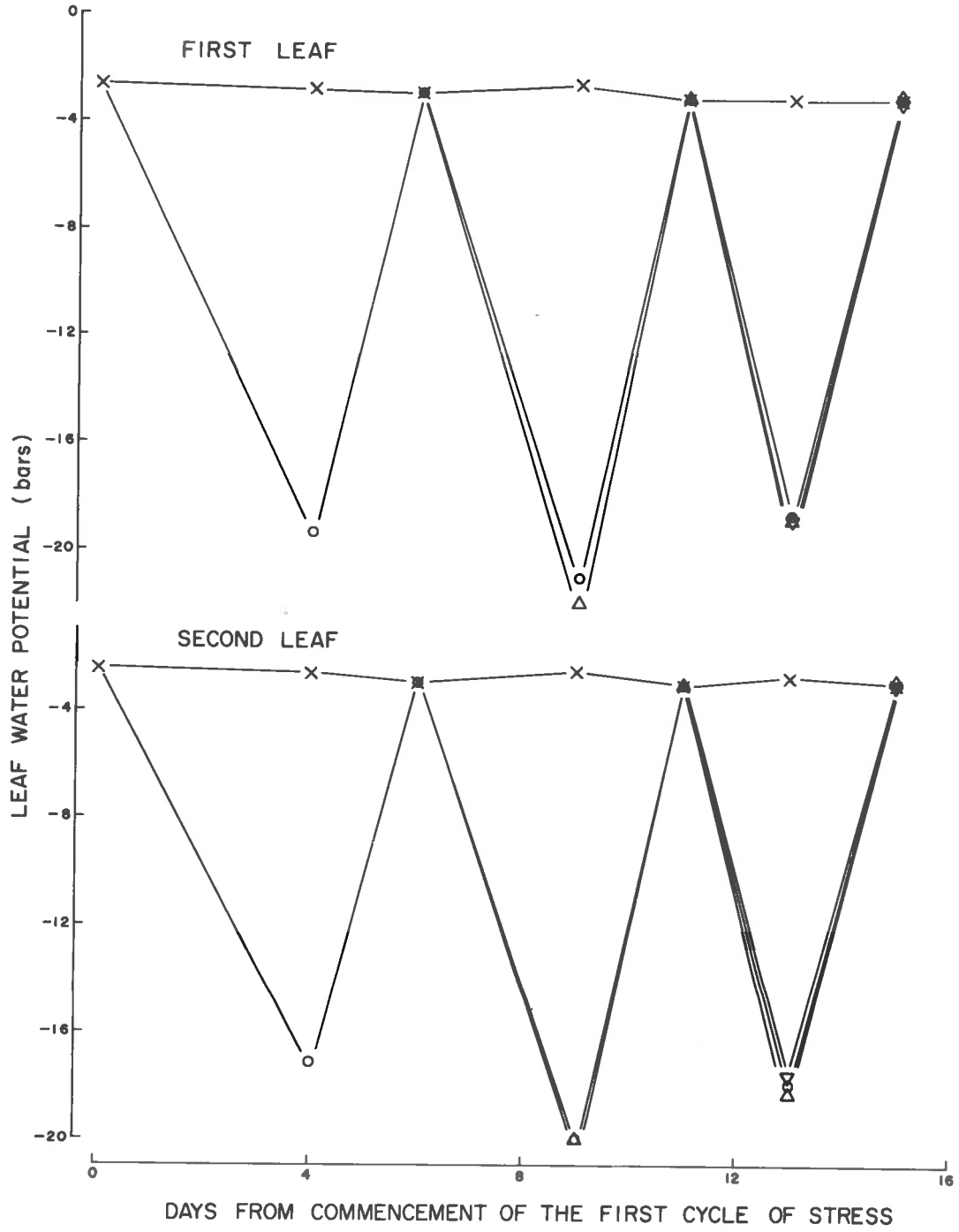
(Plants were grown in soil and watered daily for 10 days before the first cycle of stress commenced. Stress was imposed by withholding the supply of water. The first cycle of stress lasted for 4 days, the second for 3 days, and the third for 2 days. Plants were irrigated and allowed to recover fully following each cycle of water stress.)

× Not water stressed

▽ One cycle of water stress

△ Two cycles of water stress

○ Three cycles of water stress


FIRST LEAF
SECOND LEAF
LEAF WATER POTENTIAL (bars)
0
-4
-8
-12
-16
-20
0
4
8
12
16
DAYS FROM COMMENCEMENT OF THE FIRST CYCLE OF STRESS

water potential of the first leaves fell to -19.4 bars during the first episode of stress, -21 to -22 during the second and -18.5 to -19.0 during the third. The water potential of the second leaves were similar but slightly higher on each occasion. There was no evidence of any effect of the previous water stress history of the plants on the water potential of the leaves either during or following stress.

5.1.3.2 Accumulation of Proline

Previous exposure to a water deficit increased the rate of proline accumulation in the 1st and 2nd leaves (Figure 21). During the third episode of stress, for instance, plants subjected to 2 previous episodes of stress accumulated more proline in the first leaves (14.3 mg/g) than those subjected to only 1 previous episode (11.7 mg/g) and considerably more than those subjected to stress for the first time (9.0 mg/g). These changes in the capacity to accumulate proline were not accompanied by any differences in the water potential of the leaves between the treatments. A similar response in both first and second leaves was also discernible during the second episode of stress.

Between each period of water stress the plants appeared to recover fully, the water potential increased to the control level and the proline content of the leaves

Figure 21

Effect of repeated cycles of water stress on proline accumulation in the first (top) and second (bottom) leaf of barley plant.

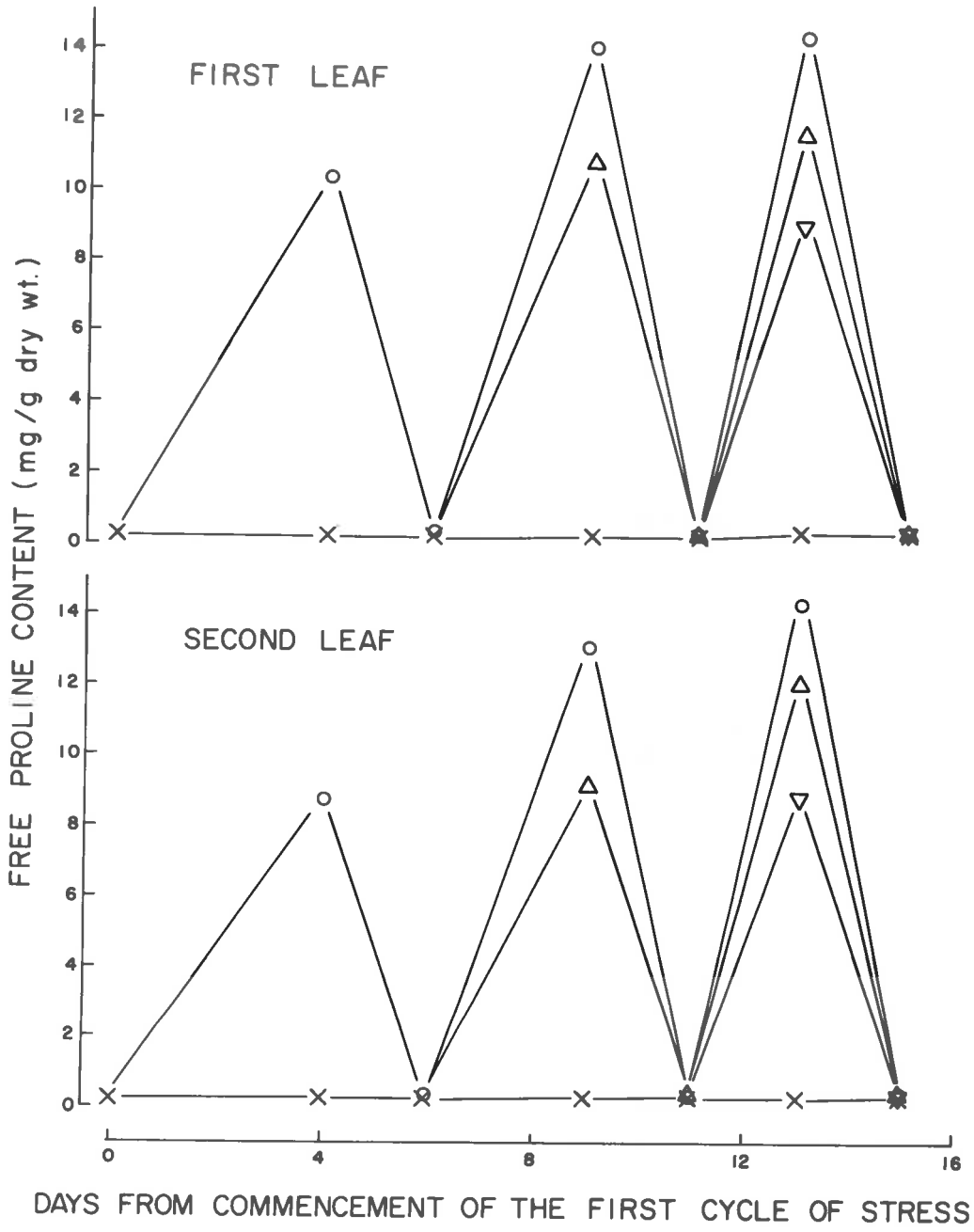
(Plants were grown in soil and watered daily for 10 days before the first cycle of stress commenced. Stress was imposed by withholding the supply of water. The first cycle of stress lasted for 4 days, the second for 3 days, and the third for 2 days. Plants were irrigated and allowed to recover fully following each cycle of water stress.)

× Not water stressed

▽ One cycle of water stress

△ Two cycles of water stress

○ Three cycles of water stress


FIRST LEAF
SECOND LEAF
FREE PROLINE CONTENT (mg/g dry wt.)
0
2
4
6
8
10
12
14
0
4
8
12
16
DAYS FROM COMMENCEMENT OF THE FIRST CYCLE OF STRESS

decreased to the level of watered plants. This indicated that the increase in proline accumulation during subsequent stress cycles was not due to a carry-over of residual proline in the leaves. Apparently a previous episode of water deficit in the tissues had an effect on the metabolism of the leaf which led to a more rapid accumulation of proline during subsequent stress.

5.1.3.3 Chlorophyll Content

Water stress caused a considerable degradation of chlorophyll but this effect was reduced where the plants had been previously exposed to a water deficit (Figure 22). During the third cycle of stress, for instance, the chlorophyll content of the 1st leaves of plants subjected to 2 previous episodes of water stress fell to 7.1 mg/g dry weight, in comparison those subjected to only one previous episode fell to 6.3 mg/g and those stressed for the first time reached 5.1 mg/g. In all cases, once the stress was relieved the chlorophyll content of the leaves increased but not to the level present in irrigated plants. The concentration of chlorophyll in the leaves of the irrigated plants increased with time, although at a much lower rate than occurred upon the re-watering of stressed plants. Very similar patterns of changes in the leaf chlorophyll concentration in response to stress also occurred in the 2nd leaves (Figure 22).

Figure 22

Effect of repeated cycles of water stress on chlorophyll content in the first (top) and second (bottom) leaf of barley plant.

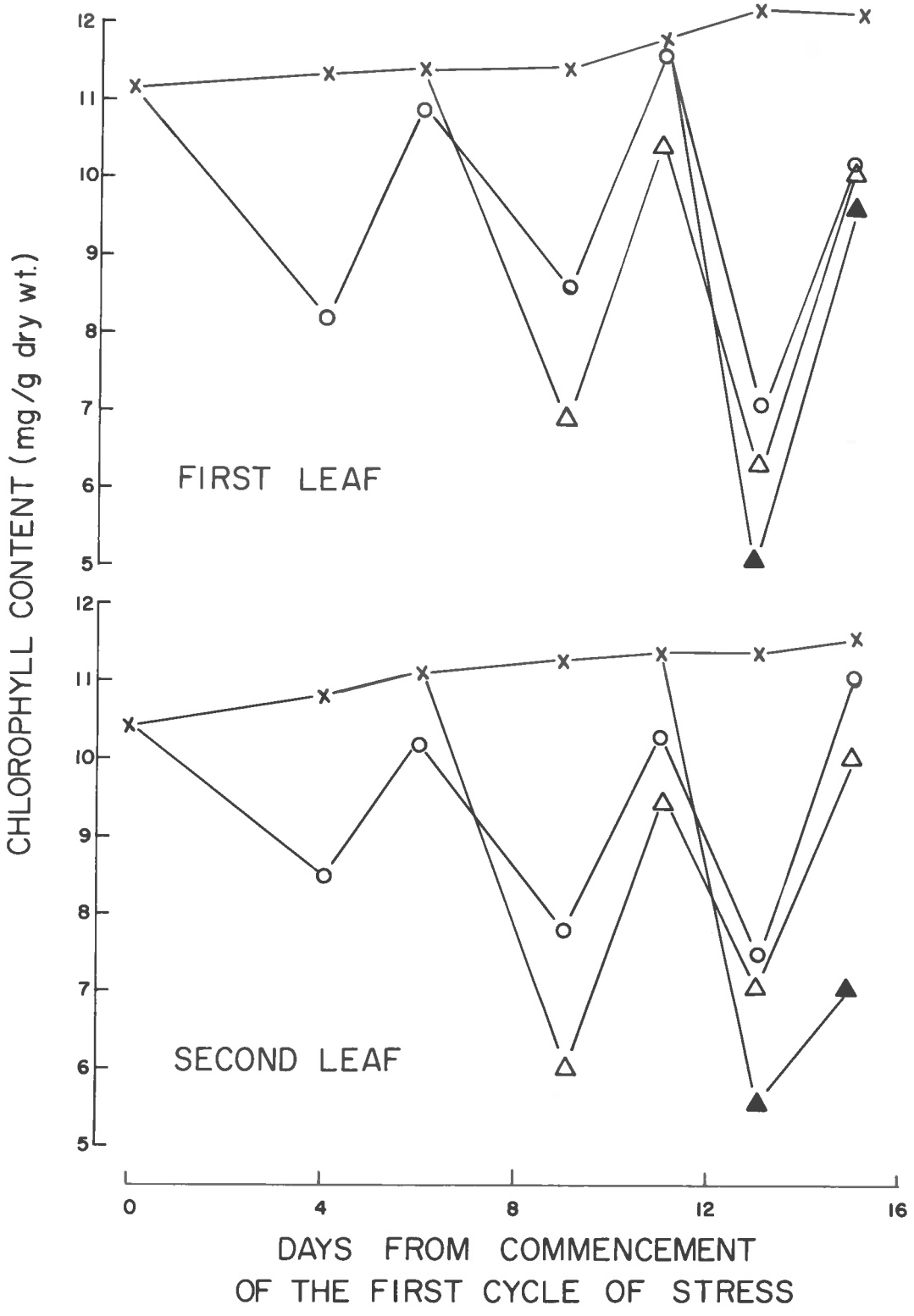
(Plants were grown in soil and watered daily for 10 days before the first cycle of stress commenced. Stress was imposed by withholding the supply of water. The first cycle of stress lasted for 4 days, the second for 3 days, and the third for 2 days. Plants were irrigated and allowed to recover fully following each cycle of water stress.)

× Not water stressed

▽ One cycle of water stress

△ Two cycles of water stress

○ Three cycles of water stress


CHLOROPHYLL CONTENT (mg/g dry wt.)
12
11
10
9
8
7
6
5
FIRST LEAF
SECOND LEAF
0
4
8
12
16
DAYS FROM COMMENCEMENT
OF THE FIRST CYCLE OF STRESS

5.2 GENETIC VARIABILITY IN THE CAPACITY TO ACCUMULATE PROLINE IN RESPONSE TO WATER STRESS IN BARLEY

5.2.1 Experiment I

5.2.1.1 Introduction

The evidence so far discussed in this thesis demonstrates that water stress induces the accumulation of free proline in various parts of the plant (page 68, 1.3.2.3). The accumulated proline disappears when the stress is relieved and the plants recommence normal growth. This accumulation and subsequent disappearance of proline during and after stress, resulted in both qualitative as well as quantitative changes in the metabolism of the plant (page 57, 1.2).

In view of this relationship between proline metabolism and water stress it was possible that proline accumulation would be affected by factors influencing plant resistance to water deficit. Several pieces of evidence supporting such a relationship have already been discussed. Thus treatment with CCC, (2-chloroethyl) trimethyl ammonium chloride, which increases stress resistance (Halevy and Kessler, 1963) increases the rate of proline accumulation in water-stressed wheat (page 115, 4.1.3.4) whereas GA_3 treatment, which reduces resistance (Sitnikova, 1966) also reduces proline accumulation (page 123, 4.2.3.4). Furthermore 'hardening' plants by subjecting them to

repeated stress cycles also increases their ability to accumulate proline (page 135, 5.1.3.2).

These experiments strongly suggest that the ability to accumulate proline and to resist the effects of water stress are closely linked. This correlation was further explored by examining the ability of barley varieties, differing in their relative drought resistance, to accumulate proline during stress.

5.2.1.2 Methods

Five varieties of barley namely, Prior, W.I.2137, C.I.3576, C.I.5611 and Asahi-2 were grown in perlite for 3 weeks (Table 8).

Table 8

Five Australian and exotic varieties of barley and their origin and flowering behaviour.

Variety	Origin and Flowering Behaviour
Prior	South Australian standard variety, believed to be a farmer's selection from Chevallier type.
W.I.2137	Very early, Waite Institute selection now released as Ketch.
C.I.3576	Early, ex U.S.D.A., believed of Egyptian origin, parentage unknown.
C.I.5611	Late, ex U.S.D.A., believed of Turkish origin, parentage unknown.
Asahi-2	Early, Japanese.

The plants were then subjected to water stress by flooding each pot with 250 ml polyethylene glycol solution of -20 bars osmotic potential. Plant samples for water potential, leaf viability and proline estimation were collected 0, 7, 25, 50, 73, and 97 hours after the stress commenced. The osmotic stress was relieved for some plants 50, 73 or 97 hours after the commencement of stress and these plants were harvested 4 days after relieving the stress to assess plant recovery.

The objective measurement of the drought resistance of different varieties is difficult (Milthorpe, 1950; Asana, 1961, 1965; Levitt, 1965) but some measure was required to compare with proline accumulation. Four techniques were used: 1. assessment of plant water status, 2. leaf viability, 3. recovery of leaves and 4. relative growth rate following rewatering.

The water status of the first leaves of both watered and stressed plants of the various varieties was assessed by the psychrometric method of measuring water potential.

The viability of the cells of the first leaves were assessed using triphenyl tetrazolium chloride (TTC). Previously this test has been used as a qualitative measure, and if the red formazan derivative was produced the tissue was considered to be viable (Parker, 1953; Parcell and Young, 1963). However, such measurements are

essentially subjective and differences have been reported as dark pink, pale pink, streaked pink, or pink (Parker, 1956). In this work an attempt has been made to place this test on a quantitative basis to compare with the other quantitative data collected.

Two first leaves for each determination were cut into 1 cm pieces, mixed thoroughly and equal amounts (approximately 20 mg dry weight) from each variety were transferred into glass vials (5.0 x 2.5 cm) containing 6 ml of 0.6% TTC solution prepared in 0.05M Na_2HPO_4-KH_2PO_4 (pH7.3) with 0.05% wetting agent (Tween-20). The solution was applied to the leaf sections by vacuum infiltration for 3 minutes, and the vials were then capped and incubated at 30°C for 15 hours. Penetration of the solution into the leaf sections was confirmed by examination of sample sections microscopically. After incubation, the solutions were gently decanted and the leaf sections were twice homogenized in a Duall conical glass homogenizer (Kontes) with 4 ml 95% ethanol. The extracts were centrifuged each time and the supernatants were pooled. The optical density of the pooled supernatant was recorded at 530 nm and the results were expressed in relation to the dry weight of the leaf tissue used.

Leaf recovery was assessed from the proportion of the 1st, 2nd and 3rd leaves which were found to survive 4 days

after stress relief. Barley leaves, in common with the leaves of other cereals, tend to senesce back from the tip during water stress and survival was assessed simply from the length of leaf tissue remaining green and turgid compared with total leaf length.

The relative growth rates were calculated for those plants relieved from stress at 50 hours over the subsequent period of 4 days when the plants were watered daily. The relative growth rate was hence assessed only during the phase of recovery from the effects of a water deficit.

The proline content of the 1st, 2nd, and 3rd leaves of each variety was determined for each sample and the data were expressed on a dry weight basis.

5.2.1.3 Results

5.2.1.3.1 Water Potential

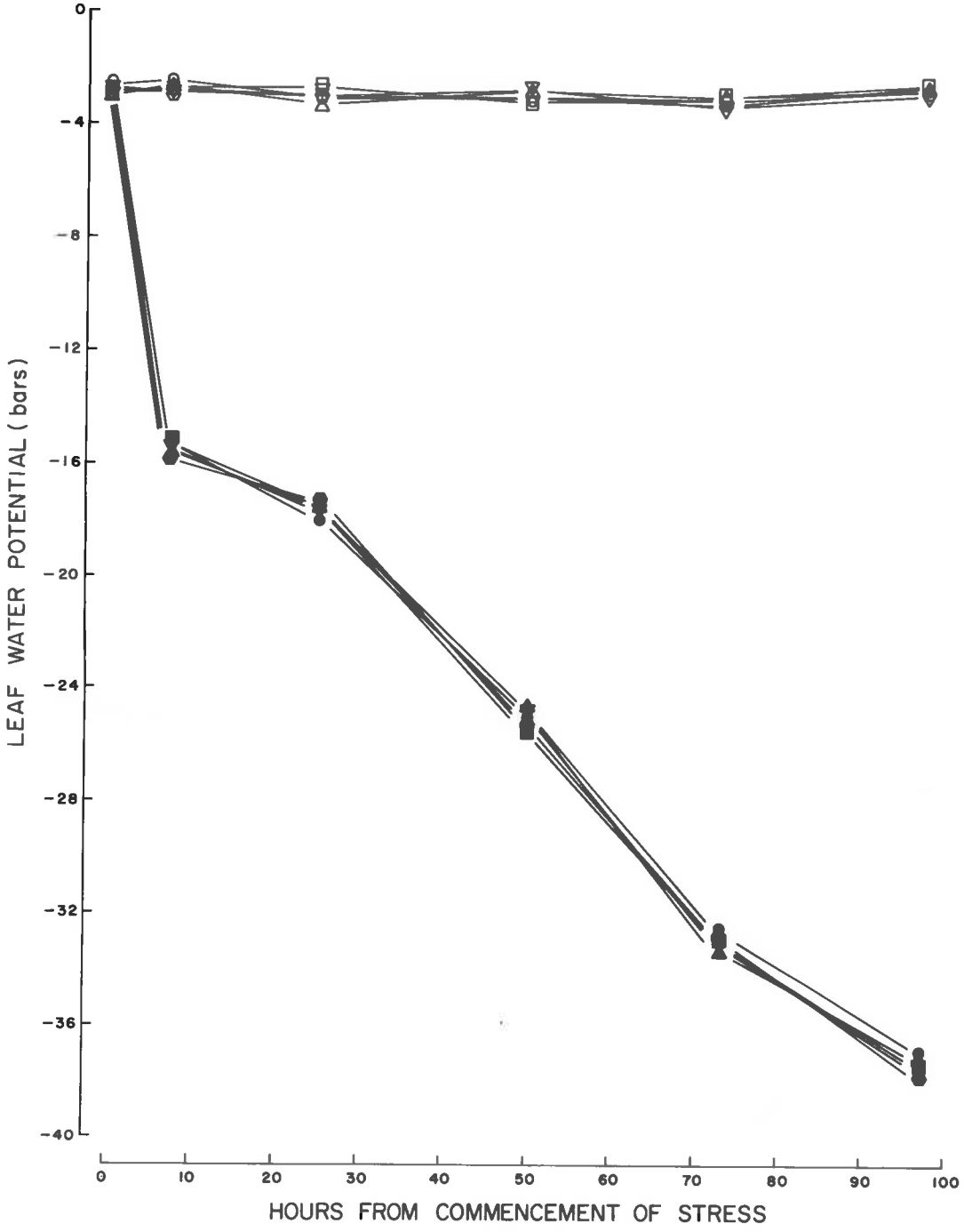
No varietal differences in the water potential of the first leaves were observed at any time when the plants of the five cultivars were subjected to the same osmotic potential (Figure 23). The water potential fell rapidly initially and then more slowly reaching -37.0 to -37.6 bars at the end of 97 hours. The water potential of the watered plants fluctuated between -2.0 and -3.4 bars throughout.

Figure 23

Effect of water stress on water potential of the first leaf of 5 varieties of barley.

(Three-week-old seedlings were subjected to water stress by flooding the pot with 250 ml polyethylene glycol solution of -20 bars osmotic potential.)

Control	Stressed	Variety
○	●	Prior
△	▲	C.I.5611
▽	▼	W.I.2137
□	■	C.I.3576
⬡	⬢	Asahi-2


0
-4
-8
-12
-16
-20
-24
-28
-32
-36
-40
LEAF WATER POTENTIAL (bars)
0
10
20
30
40
50
60
70
80
90
100
HOURS FROM COMMENCEMENT OF STRESS

5.2.1.3.2 Leaf-cell Viability

The activity of the dehydrogenases which catalyze the reduction of triphenyl tetrazolium compounds to formazan derivatives in the leaf tissues, decreased steadily with decreasing water potential but no varietal differences in dehydrogenase activity was obtained (Figure 24). A positive correlation between dehydrogenase activity and water potential can be seen (Figure 25).

5.2.1.3.3 Survival from Water Deficit

The five varieties differed in their ability to recover after they were subjected to stress for varying periods of time (Table 9). The first leaves of Asahi-2 and C.I.5611 did not recover when subjected to stress for 97 hours. The leaves of Prior showed the greatest amount of recovery following stress whereas W.I.2137 and C.I.3576 were intermediate. Very similar recovery patterns were also found for the second and the third leaves.

5.2.1.3.4 Relative Growth Rate

The relative growth rate was calculated for the period of 4 days at each occasion when the stress was relieved 50 hours after the stress commenced. The relative growth rate was highest in cv Prior and least in cv Asahi-2 (Table 10) and the differences between the varieties were in the same order as the differences in leaf recovery.

Figure 24

Effect of water stress on the ability of 5 varieties of barley in relation with tetrazolium reduction.

(Two first leaves from every variety, both stressed (-20 bars) and non-stressed plants, were cut into 1 cm pieces, mixed thoroughly, and equal amounts from each variety were transferred into glass vials containing 6 ml of 0.6% triphenyl tetrazolium chloride solution prepared in 0.05M Na_2HPO_4-KH_2PO_4, pH7.3 buffer with 0.05% Tween-20. The solution was infiltrated in vacuum for 3 minutes and then incubated at 30°C for 15 hours before the formazan derivative was extracted with 95% ethanol.)

Control	Stressed	Variety
○	●	Prior
△	▲	C.I.5611
▽	▼	C.I.2137
□	■	C.I.3576
⬡	⬢	Asahi-2

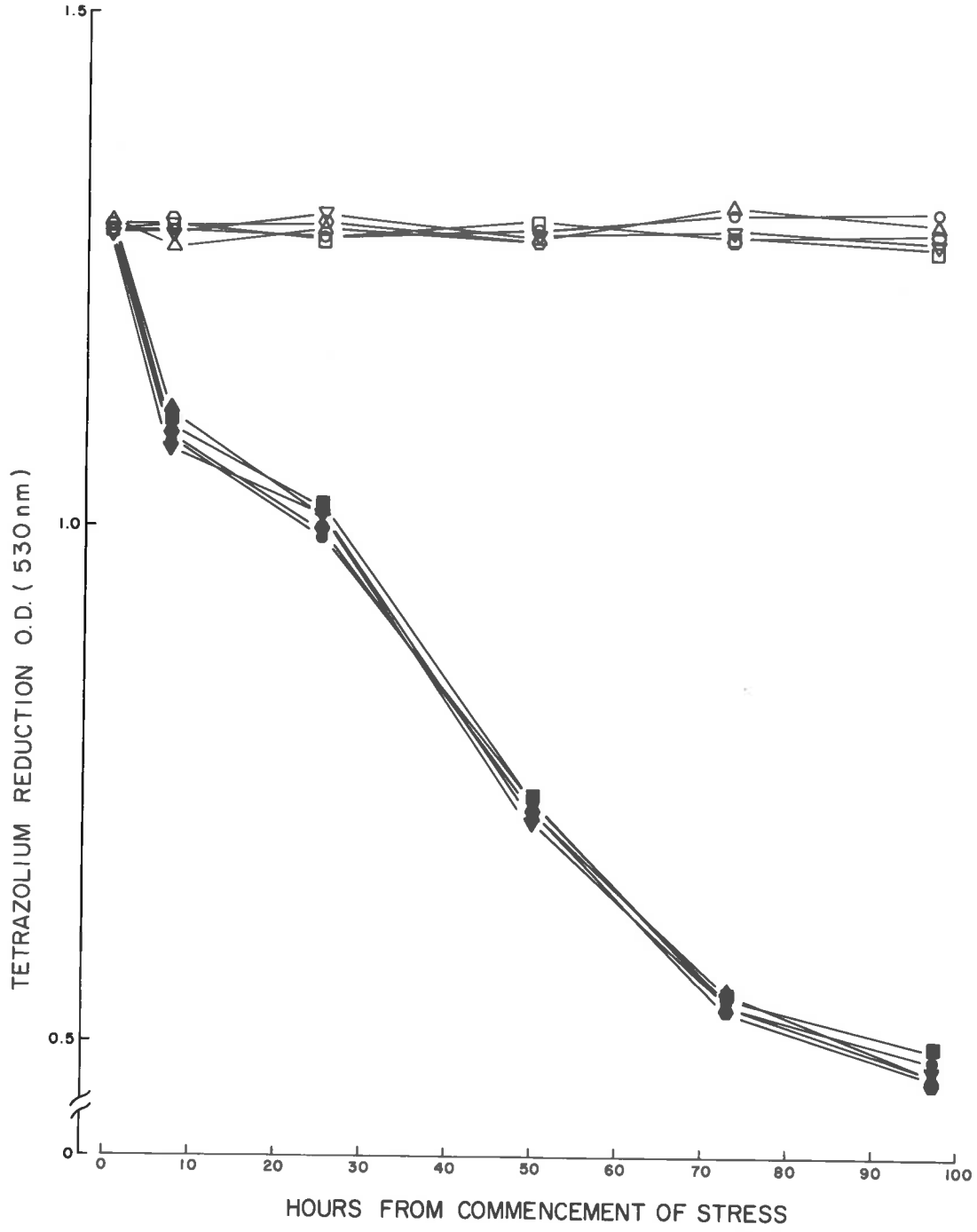

1.5
1.0
0.5
0
TETRAZOLIUM REDUCTION O.D. (530 nm)
0
10
20
30
40
50
60
70
80
90
100
HOURS FROM COMMENCEMENT OF STRESS

Figure 25

Relationship between tetrazolium reduction and water potential of the leaf.

(Values, mean of 5, for this graph were obtained from Fig. 23 for water potential and Fig. 24 for tetrazolium reduction.)

Correlation coefficient = 0.983

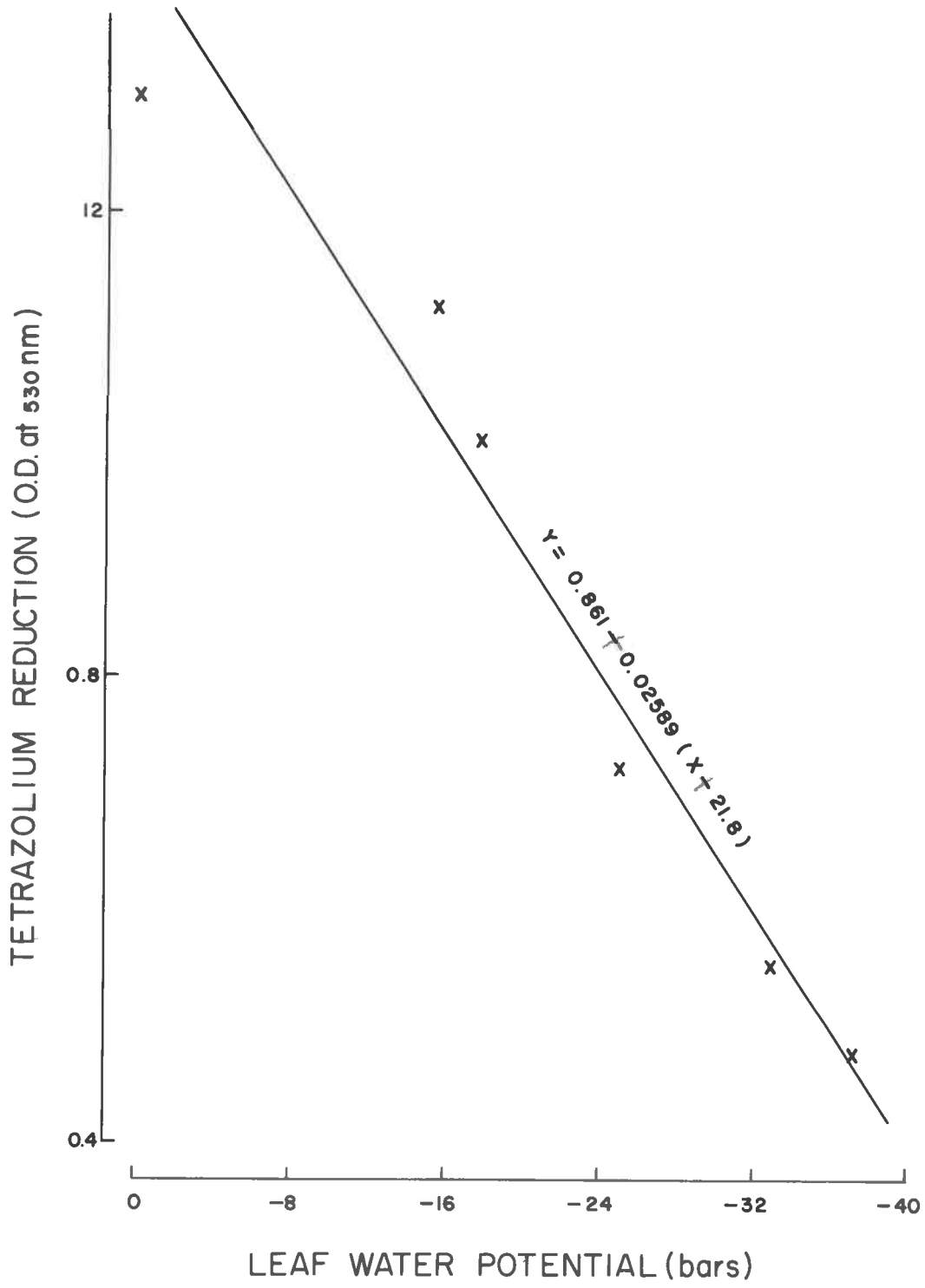

TETRAZOLIUM REDUCTION (O.D. at 530 nm)
1.2
0.8
0.4
0
-8
-16
-24
-32
-40
LEAF WATER POTENTIAL (bars)
Y = 0.861 + 0.0259 (X + 21.8)

Table 9

Portions of leaves, remaining green (% of initial length), surviving water deficit for varying periods of time after full recovery.

Cultivars	Total duration of stress (hours)								
	50			73			97		
	% of leaf length remaining green								
	Leaf			Leaf			Leaf		
	1	2	3	1	2	3	1	2	3
Prior	72.7	78.0	83.9	54.0	65.1	70.9	30.3	53.3	58.2
C.I.5611	39.5	46.6	48.9	26.8	27.8	42.0	4.2	19.2	22.2
W.I.2137	47.7	66.1	72.4	41.3	45.0	50.5	21.1	30.6	40.1
C.I.3576	45.3	58.8	63.4	35.5	42.8	44.7	13.0	29.3	32.0
Asahi-2	30.2	28.5	38.2	16.1	16.2	22.5	2.9	7.0	13.8

The relative growth rate was also related to the ability of the leaves to recover.

5.2.1.3.5 Accumulation of Proline

The five varieties differed in their ability to accumulate proline in the first leaves when they were subjected to varying periods of stress (Figure 26). The first leaves of Prior accumulated the greatest amount of proline (21.1 mg/g) at 97 hours and Asahi-2 the least (8.7 mg/g). The other three cultivars, W.I.2137, C.I.3576 and C.I.5611 also differed in proline accumulation, but were intermediate between the two extremes noted

for Prior and Asahi-2. Similar patterns of proline accumulation were also found for the second (Figure 27, top) and the third leaves (Figure 27, bottom) but the rate of proline accumulation was reduced.

Table 10

Relative growth rates of plants (mg/mg/day) over the subsequent period of 4 days when the plants were relieved from stress at 50 hours and watered daily thereafter.

Cultivars	Treatment	Period (days from relief of osmotic stress) 0-1	1-2	2-3	3-4
		(mg/mg/day)			
Prior	Control	0.148	0.144	0.138	0.132
	Relieved	0.074	0.132	0.160	0.155
C.I.5611	Control	0.155	0.148	0.148	0.134
	Relieved	0.045	0.080	0.112	0.110
W.I.2137	Control	0.145	0.143	0.137	0.130
	Relieved	0.065	0.120	0.149	0.140
C.I.3576	Control	0.142	0.140	0.137	0.129
	Relieved	0.059	0.100	0.134	0.128
Asahi-2	Control	0.147	0.144	0.139	0.132
	Relieved	0.034	0.060	0.092	0.090

Figure 26

Effect of water stress on proline accumulation in the first leaf of 5 varieties of barley.

(Three-week-old seedlings were subjected to water stress by flooding the pot with 250 ml polyethylene glycol solution of -20 bars osmotic potential.)

⊠ All varieties with no stress

● Prior with stress

▼ N.I.2137 " "

■ C.I.3576 " "

▲ C.I.5611 " "

✳ Asahi-2 " "

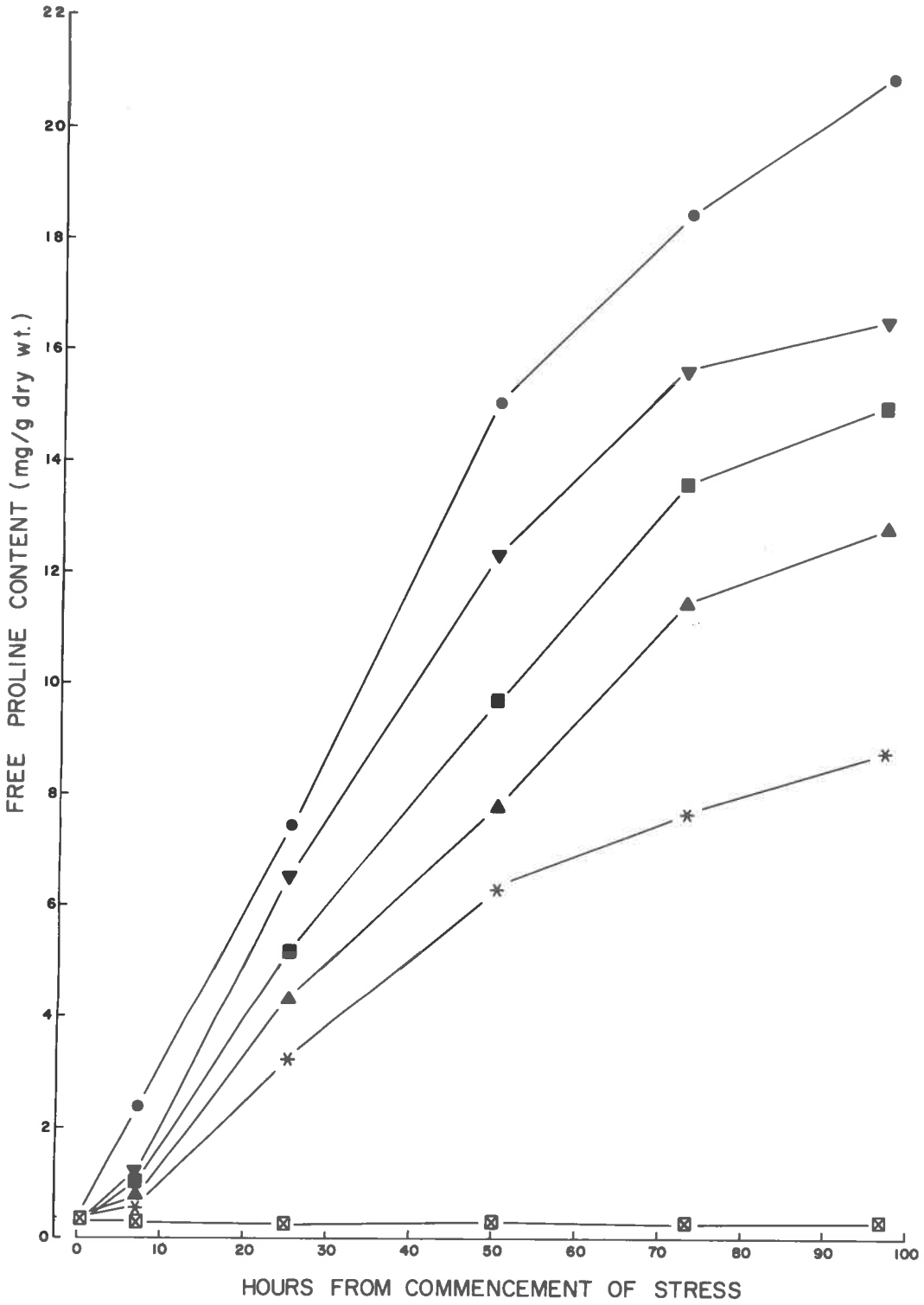

FREE PROLINE CONTENT (mg/g dry wt.)
0
2
4
6
8
10
12
14
16
18
20
22
0
10
20
30
40
50
60
70
80
90
100
HOURS FROM COMMENCEMENT OF STRESS

Figure 27

Effect of water stress on proline accumulation in the second (top) and third (bottom) leaf of 5 varieties of barley.

(Three-week-old seedlings were subjected to water stress by flooding the pot with 250 ml polyethylene glycol solution of -20 bars osmotic potential.)

All varieties with no stress

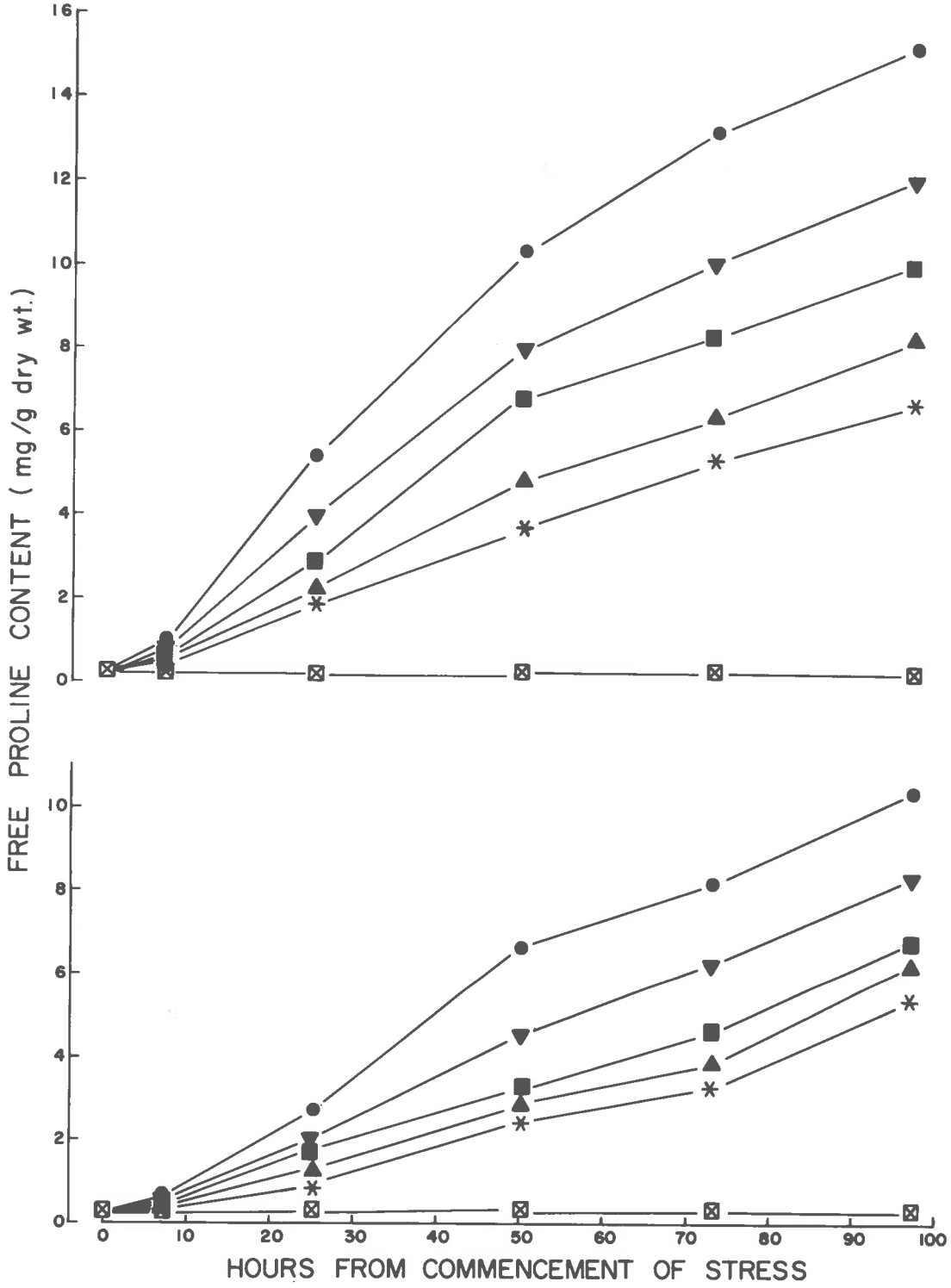
Prior with stress

W.I.2137 " "

C.I.3576 " "

C.I.5611 " "

Asahi-2 " "


FREE PROLINE CONTENT (mg/g dry wt.)
HOURS FROM COMMENCEMENT OF STRESS
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60
70
80
90
100

In summary, no differences in water potential or dehydrogenase activity (TTC) were found between the five varieties of barley during osmotic stress but they differed in proline accumulation, leaf survival and relative growth rate. A close positive relation existed between proline accumulation and both leaf survival (Figure 28) and plant relative growth rate (Figure 29). Prior, with the highest leaf survival and relative growth rate during recovery was found to have accumulated the greatest amount of proline. In contrast, Asahi-2 with the lowest leaf survival and relative growth rate accumulated the least amount of proline.

5.2.2 Experiment II

5.2.2.1 Introduction

The data obtained from the previous experiment with 5 barley varieties, suggested that extension of the investigation to include further varieties of barley would be worthwhile. Accordingly, the water potential, chlorophyll content and proline accumulation of 10 barley varieties of known yield stability was assessed. Chlorophyll content was here used as a measure of the effect of stress on plant function rather than the various measures used in the previous experiment.

Figure 28

Relationship between leaf survival from water deficit and proline content.

(Three-week-old seedlings of 5 varieties of barley were subjected to water stress by flooding the pot with 250 ml polyethylene glycol solution of -20 bars osmotic potential. Some plants were relieved from water stress 50, 73, and 97 hours after the commencement of stress and allowed to recover for 4 days at each occasion with daily watering. The portions of leaves surviving water deficit were recorded and expressed as % of initial length. The five points on each line represent 5 varieties.)

□ Third leaf

○ Second leaf

× First leaf

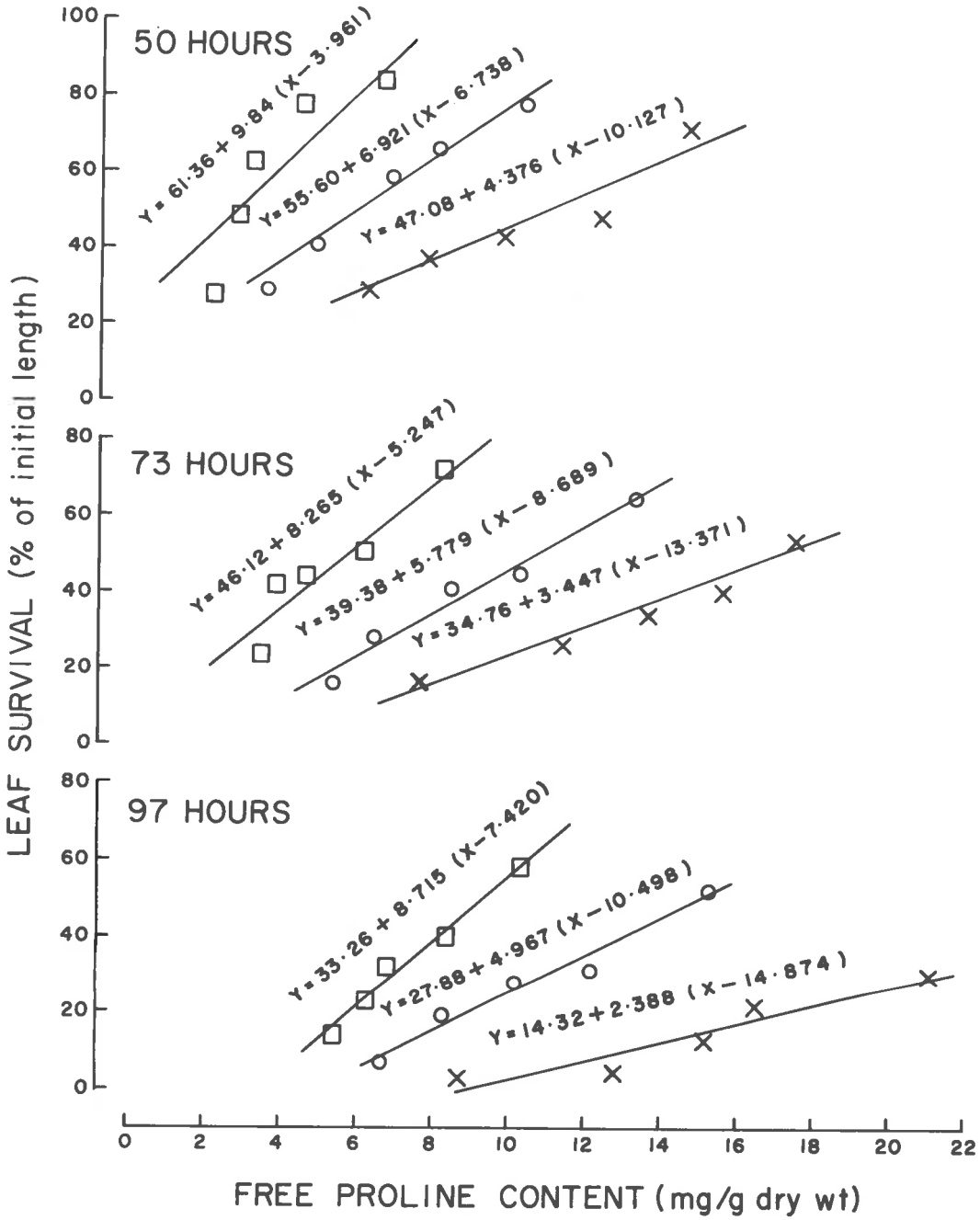

50 HOURS
Y = 61·36 + 9·84 (X − 3·961)
Y = 55·60 + 6·921 (X − 6·738)
Y = 47·08 + 4·376 (X − 10·127)
73 HOURS
Y = 46·12 + 8·265 (X − 5·247)
Y = 39·38 + 5·779 (X − 8·689)
Y = 34·76 + 3·447 (X − 13·371)
97 HOURS
Y = 33·26 + 8·715 (X − 7·420)
Y = 27·88 + 4·967 (X − 10·498)
Y = 14·32 + 2·388 (X − 14·874)
LEAF SURVIVAL (% of initial length)
FREE PROLINE CONTENT (mg/g dry wt)
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
18
20
22

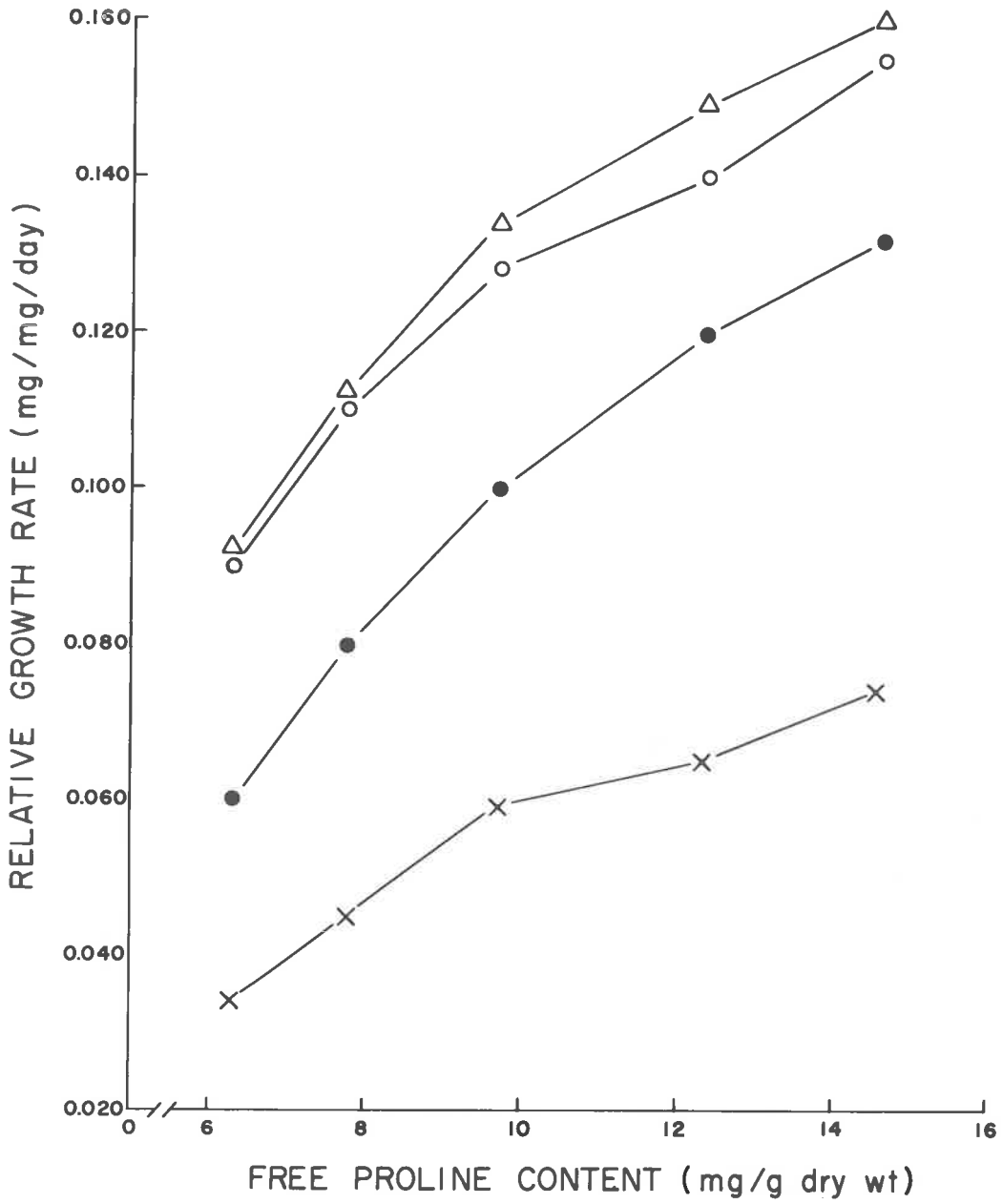
Figure 29

Relationship between relative growth rate of plant and proline content.

(Three-week-old seedlings of 5 varieties of barley were subjected to water stress by flooding the pot with 250 ml polyethylene glycol solution of -20 bars osmotic potential. Some plants from each variety were relieved from water stress 50 hours after the commencement of stress and the relative growth rate of plants was calculated over the subsequent period of 4 days. Each point on a curve represents a variety.)

Period (days from relief of osmotic stress)

×	0-1
●	1-2
△	2-3
○	3-4


0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0
6
8
10
12
14
16
RELATIVE GROWTH RATE (mg/mg/day)
FREE PROLINE CONTENT (mg/g dry wt)

5.2.2.2 Methods

Ten varieties of barley namely, Excelsior, Bankuti Korai, Prior A, Princess, Arivat, Velvon II, C.I.3576, B.R.1239, Maraini, and Proctor were grown in perlite with nutrient solution for 3 weeks (Table 11). The plants were then subjected to water stress by replacing the nutrient solution in each pot with 250 ml of polyethylene glycol solution of -20 bars osmotic potential.

Table 11

Ten Australian and exotic varieties of barley with their comparative yield and yield stability tested in a wide range of climatic conditions in South Australia.

Varieties	Heading date	Comparative Yield (g/plot)	Stability index*
Excelsior	Late	177	0.47 (very stable)
Bankuti Korai	Extremely early	102	0.04 (extremely stable)
Prior A	Mid-late	357	1.06 (average)
Princess	Very late	225	1.10 (average)
Arivat	Mid-early	499	0.85 (average)
Velvon II	Late	227	0.90 (average)
C.I.3576	Early	434	0.90 (average)
B.R.1239	Early	102	0.84 (average)
Maraini	Extremely late	277	1.80 (unstable)
Proctor	Very late	306	1.80 (unstable)

*Stability index is regression coefficient of a given variety when its yields from separate sites are plotted against the mean yields of several varieties under trial (Finlay and Wilkinson, 1963).

The polyethylene glycol solutions were washed out of the pots 60 hours after the stress commenced and leaf samples were collected 0, 60, 84 and 108 hours after the treatment began. Water potential, chlorophyll content, and free proline content were measured in the first, second, and third leaves of all varieties.

Chlorophyll was extracted in 80% acetone and measured by the method of MacKinney (1941). Two leaves of the same age were excised, cut into small pieces and mixed thoroughly for each determination. Equal fresh amounts (approximately 15 mg dry weight) from each variety were twice homogenized in a Duall conical glass homogenizer (Kontes) with 12.5 ml 80% acetone. The extracts were centrifuged each time and the supernatants were pooled. The optical density of the pooled supernatant was recorded at 663 nm (chlorophyll a) and 645 nm (chlorophyll b), respectively and the amount of chlorophyll was presented as sum of chlorophyll a and b.

5.2.2.3 Results

5.2.2.3.1 Water Potential

The water potential of the first leaves of all cultivars decreased to between -27.0 and -28.7 bars 60 hours after the stress commenced (Table 12) as in the previous experiment there was no evidence for varietal differences in water potential. The water potential of

Table 12

Water potential of the first leaf of barley varieties subjected to osmotic stress 21 days from sowing (0 hour) and relieved 60 hours later (+ 60 hours).

Variety	Osmotic potential (bars)	Time (hours) + 60	+ 84	+ 108
		Water potential (bars)		
Excelsior	0	-2.7	-2.8	-2.6
	-20	-28.7	-24.1	-18.5
Bankuti Korai	0	-2.8	-2.7	-2.7
	-20	-27.7	-23.5	-18.8
Prior A	0	-2.6	-2.9	-2.8
	-20	-28.2	-24.5	-18.6
Princess	0	-2.4	-2.8	-2.7
	-20	-28.8	-23.9	-18.4
Arivat	0	-3.1	-2.9	-2.8
	-20	-28.0	-24.1	-18.9
Velvon II	0	-2.9	-3.0	-2.8
	-20	-28.0	-24.0	-18.8
C.I.3576	0	-2.9	-2.9	-2.8
	-20	-28.6	-25.0	-18.5
B.R.1239	0	-2.9	-3.1	-2.8
	-20	-28.0	-23.5	-18.9
Maraini	0	-2.7	-2.8	-3.0
	-20	-28.3	-24.4	-20.0
Proctor	0	-2.9	-2.9	-2.8
	-20	-28.5	-24.4	-20.2
L.S.D. (P=0.05)	0	-0.5	-0.6	-0.5
	-20	-1.8	-2.1	-1.8

the leaves increased slowly when the stress was relieved and was still low (-18 to -20 bars) some 48 hours after relief of the stress. A similar pattern of change in the water potential of the second (Table 13) and the third leaves (Table 14) was also found but the fall in water potential with stress was less in the second and least in the third leaves. The water potential of these leaves increased relatively more rapidly when the stress was relieved.

5.2.2.3.2 Chlorophyll Content

The chlorophyll content of the first leaves was considerably reduced due to stress but this reduction was of the same magnitude in all varieties tested (Table 15). The chlorophyll content did not significantly vary between varieties either in watered or stressed plants, although minor differences occurred. The second and the third leaves of all varieties when watered had less chlorophyll than the first leaves but the rate of increase with time was somewhat higher (Table 16, 17). Water stress also reduced the chlorophyll content of these leaves.

5.2.2.3.3 Proline Accumulation

A large difference in the proline accumulation by the first, second, and third leaves of the various varieties occurred in response to water stress (Table 18). Bankuti Korai and Excelsior accumulated as much as 24.0 and 18.4 mg

Table 13

Water potential of the second leaf of barley varieties subjected to osmotic stress 21 days from sowing (0 hour) and relieved 60 hours later (+ 60 hours).

Variety	Osmotic potential (bars)	Time (hours) + 60	+ 84	+ 108
		Water potential (bars)		
Excelsior	0	-2.9	-3.0	-3.0
	-20	-22.6	-15.5	-7.5
Bankuti Korai	0	-2.9	-3.0	-3.0
	-20	-22.8	-15.5	-7.9
Prior A	0	-2.8	-3.1	-3.2
	-20	-22.6	-15.3	-7.7
Princess	0	-2.9	-2.9	-2.8
	-20	-23.8	-15.5	-7.5
Arivat	0	-2.7	-2.9	-3.2
	-20	-22.6	-15.7	-7.3
Velvon II	0	-2.8	-2.7	-3.0
	-20	-23.2	-15.8	-7.8
C.I.3576	0	-2.9	-3.0	-2.9
	-20	-22.1	-15.1	-7.6
B.R.1239	0	-2.9	-2.9	-3.0
	-20	-22.8	-15.8	-7.5
Maraini	0	-2.9	-2.7	-2.9
	-20	-23.0	-15.7	-7.7
Proctor	0	-2.7	-3.0	-2.9
	-20	-22.6	-15.8	-7.6
L.S.D. (P=0.05)	0	-0.7	-0.5	-0.5
	-20	-1.6	-1.2	-1.0

Table 14

Water potential of the third leaf of barley varieties subjected to osmotic stress 21 days from sowing (0 hour) and relieved 60 hours later (+ 60 hours).

Variety	Osmotic potential (bars)	Time (hours) + 60	+ 84	+ 108
		Water potential (bars)		
Excelsior	0	-3.0	-2.9	-2.9
	-20	-19.0	-11.0	-4.6
Bankuti Korai	0	-3.2	-2.8	-3.0
	-20	-18.3	-11.8	-5.2
Prior A	0	-2.8	-3.1	-2.8
	-20	-18.8	-11.7	-5.2
Princess	0	-3.1	-3.0	-2.7
	-20	-18.3	-11.4	-5.1
Arivat	0	-2.8	-3.1	-2.9
	-20	-18.3	-11.5	-5.6
Velvon II	0	-2.9	-2.9	-3.1
	-20	-18.9	-11.6	-5.2
C.I.3576	0	-2.9	-3.1	-3.1
	-20	-19.0	-12.2	-5.3
B.R.1239	0	-2.9	-3.2	-2.9
	-20	-18.2	-12.0	-5.3
Maraini	0	-2.9	-3.1	-2.9
	-20	-18.7	-11.9	-5.5
Proctor	0	-3.0	-3.1	-2.9
	-20	-19.3	-11.9	-5.0
L.S.D. (P=0.05)	0	-0.6	-0.6	-0.6
	-20	-1.4	-1.4	-1.2

Table 15

Chlorophyll content of the first leaf of barley varieties subjected to osmotic stress 21 days from sowing (0 hour) and relieved 60 hours later (+ 60 hours).

Variety	Osmotic potential (bars)	Time (hours) + 60	+ 84	+ 108
		Chlorophyll content (mg/g dry wt)		
Excelsior	0	12.1	11.9	11.4
	-20	6.1	6.5	8.0
Dankuti Korai	0	12.2	12.0	11.5
	-20	6.1	6.3	8.1
Prior A	0	12.1	11.9	11.2
	-20	6.1	6.5	8.2
Princess	0	12.0	12.0	11.3
	-20	6.0	6.4	8.0
Arivat	0	12.2	12.1	11.3
	-20	5.9	6.4	8.1
Velvon II	0	12.0	11.9	11.4
	-20	6.1	6.5	7.9
C.I.3576	0	12.1	12.0	11.5
	-20	6.0	6.3	8.0
B.R.1239	0	11.9	12.1	11.5
	-20	6.1	6.4	8.1
Maraini	0	12.2	12.0	11.5
	-20	6.2	6.5	8.0
Proctor	0	12.0	12.1	11.2
	-20	5.9	6.3	8.0
Least Significant Difference (P=0.05)		0.6	0.6	0.6

Table 16

Chlorophyll content of the second leaf of barley varieties subjected to osmotic stress 21 days from sowing (0 hour) and relieved 60 hours later (+ 60 hours).

Variety	Osmotic potential (bars)	Time (hours) + 60	+ 84	+ 108
		Chlorophyll content (mg/g dry wt)		
Excelsior	0	9.3	9.9	10.6
	-20	6.1	7.2	8.5
Bankuti Korai	0	9.4	10.0	10.6
	-20	6.0	7.4	8.7
Prior A	0	9.3	9.8	10.5
	-20	5.8	7.2	8.6
Princess	0	9.2	10.0	10.8
	-20	5.9	7.4	8.6
Arivat	0	9.4	9.8	10.4
	-20	5.9	7.2	8.7
Velvon II	0	9.3	9.8	10.5
	-20	6.1	7.3	8.4
C.I.3576	0	9.4	10.1	10.7
	-20	6.1	7.1	8.7
B.R.1239	0	9.2	10.1	10.6
	-20	5.9	7.1	8.6
Maraini	0	9.4	9.9	10.4
	-20	6.1	7.2	8.5
Proctor	0	9.4	9.8	10.5
	-20	5.9	7.4	8.4
Least Significant Difference (P=0.05)		0.6	0.6	0.5

Table 17

Chlorophyll content of the third leaf of barley varieties subjected to osmotic stress 21 days from sowing (0 hour) and relieved 60 hours later (+ 60 hours).

Variety	Osmotic potential (bars)	Time (hours) + 60	+ 84	+ 108
		Chlorophyll content (mg/g dry wt.)		
Excelsior	0	9.2	9.6	10.2
	-20	7.0	7.7	9.0
Bankuti Korai	0	9.3	9.6	10.3
	-20	6.8	7.6	8.9
Prior A	0	9.2	9.7	10.2
	-20	6.8	7.6	8.9
Princess	0	9.3	9.6	10.1
	-20	6.9	7.7	9.0
Arivat	0	9.4	9.7	10.3
	-20	6.9	7.7	9.1
Velvon II	0	9.4	9.8	10.3
	-20	6.8	7.9	8.8
C.I.3576	0	9.2	9.8	10.1
	-20	7.0	7.8	8.9
E.R.1239	0	9.3	9.7	10.2
	-20	6.9	7.6	8.8
Maraini	0	9.4	9.8	10.1
	-20	6.8	7.7	9.0
Proctor	0	9.3	9.8	10.2
	-20	6.7	7.8	8.9
Least Significant Difference (P=0.05)		0.5	0.6	0.6

Table 18

Free proline accumulated in the first leaf of barley varieties subjected to osmotic stress 21 days from sowing (0 hour) and relieved 60 hours later (+ 60 houre).

Variety	Osmotic potential (bars)	Time (hours) + 60	+ 84	+ 108
		Proline content (mg/g dry wt.)		
Excelsior	0	0.25	0.28	0.32
	-20	18.50	16.73	12.63
Bankuti Korai	0	0.27	0.30	0.34
	-20	24.05	20.78	16.22
Prior A	0	0.32	0.33	0.35
	-20	15.66	13.57	10.20
Princess	0	0.32	0.34	0.36
	-20	13.27	12.35	9.53
Arivat	0	0.22	0.25	0.26
	-20	12.90	12.01	9.43
Velvon II	0	0.27	0.30	0.33
	-20	12.34	11.48	9.44
C.I.3576	0	0.37	0.40	0.41
	-20	11.12	10.53	8.84
B.R.1239	0	0.25	0.28	0.30
	-20	14.14	12.77	9.72
Maraini	0	0.30	0.34	0.35
	-20	9.11	8.41	7.19
Proctor	0	0.28	0.30	0.32
	-20	9.00	8.30	7.29

proline/g dry weight, respectively, in the first leaves whereas Naraini and Proctor accumulated only 9.0 mg/g dry weight in response to stress. The response of the remaining 6 varieties was intermediate. The level of proline decreased slightly in the first leaves when the stress was relieved. The amounts of proline in the second (Table 19) and the third leaves (Table 20) in all varieties was less than in the first leaves and the free proline concentration decreased rapidly in these leaves when the stress was relieved. These data suggest that in this experiment the stress applied was sufficient to injure the first leaf and impair its recovery whereas the other leaves were not as adversely affected.

In comparing the proline accumulation potential of these varieties it is apparent that the more stable (see definitions, pages 30, 31, 32) a variety the more proline was accumulated during water stress. A good correlation was found between the stability index of yield and the accumulation of proline in the first leaf (Figure 30). A similar relationship exists between the stability index and proline accumulation in the second and third leaves also (Table 21).

Table 19

Free proline accumulated in the second leaf of barley varieties subjected to osmotic stress 21 days from sowing (0 hour) and relieved 60 hours later (+ 60 hours).

Variety	Osmotic potential (bars)	Time (hours) + 60	+ 84	+ 108
		Proline content (mg/g dry wt.)		
Excelsior	0	0.27	0.30	0.35
	-20	14.74	11.52	7.06
Bankuti Korai	0	0.28	0.32	0.37
	-20	18.16	14.67	10.50
Prior A	0	0.27	0.28	0.29
	-20	11.13	8.59	4.86
Princess	0	0.34	0.37	0.41
	-20	8.93	7.12	4.11
Arivat	0	0.23	0.26	0.28
	-20	8.69	6.60	3.56
Velvon II	0	0.30	0.35	0.38
	-20	8.02	6.00	3.44
C.I.3576	0	0.28	0.30	0.32
	-20	7.05	5.36	3.19
B.R.1239	0	0.27	0.28	0.29
	-20	9.60	7.87	4.42
Maraini	0	0.35	0.38	0.38
	-20	6.13	4.79	2.75
Proctor	0	0.29	0.33	0.34
	-20	6.07	4.51	2.66

Table 20

Free proline accumulated in the third leaf of barley varieties subjected to osmotic stress 21 days from sowing (0 hour) and relieved 60 hours later (+ 60 hours).

Variety	Osmotic potential (bars)	Time (hours) + 60	+ 84	+ 108
		Proline content (mg/g dry wt.)		
Excelsior	0	0.28	0.37	0.39
	-20	12.21	8.15	2.92
Bankuti Korai	0	0.32	0.39	0.40
	-20	16.04	11.10	4.11
Prior A	0	0.27	0.29	0.31
	-20	9.50	6.34	2.34
Princess	0	0.38	0.41	0.43
	-20	7.16	5.01	2.14
Arivat	0	0.26	0.29	0.31
	-20	6.68	4.31	1.95
Velvon II	0	0.34	0.38	0.38
	-20	6.11	3.73	1.79
C.I.3576	0	0.34	0.36	0.33
	-20	5.93	3.52	1.55
B.R.1239	0	0.29	0.32	0.33
	-20	7.39	5.65	2.46
Maraini	0	0.39	0.41	0.45
	-20	4.84	3.55	1.48
Proctor	0	0.32	0.34	0.36
	-20	4.45	3.22	1.37

Figure 30

Relationship between stability index and proline content in 10 varieties of barley.

(Three-week-old seedlings of 10 varieties of barley were subjected to water stress by flooding the pot with 250 ml polyethylene glycol solution of -20 bars osmotic potential.)

Correlation coefficient = 0.885

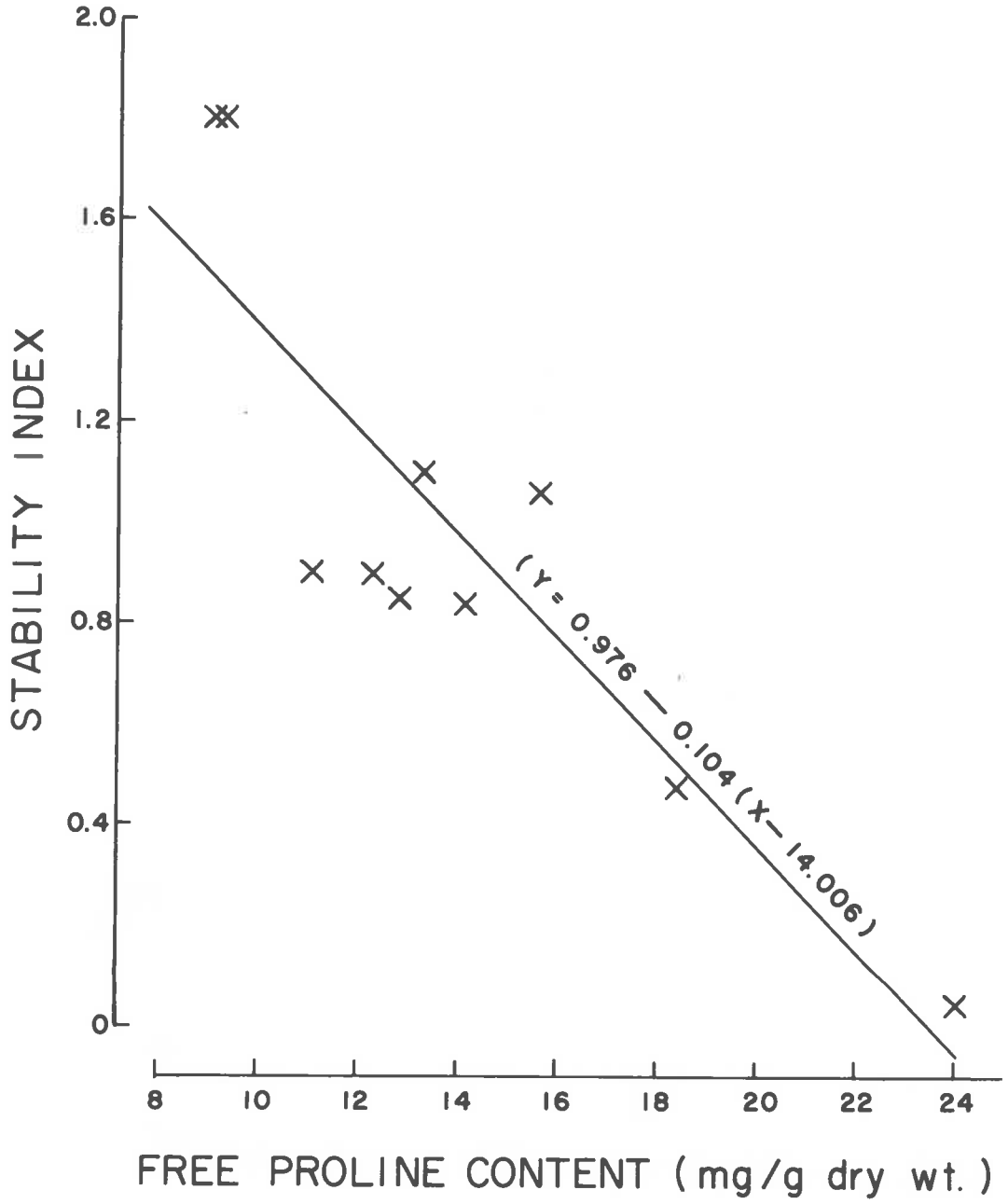

2.0
1.6
1.2
0.8
0.4
0
STABILITY INDEX
(Y = 0.976 − 0.104 (X − 14.006)
8
10
12
14
16
18
20
22
24
FREE PROLINE CONTENT (mg/g dry wt.)

Table 21

Relationship between stability index and proline accumulation in 10 varieties of barley during water stress.

Variety	Stability index	Proline content (mg/g dry wt) at time + 60 hours		
		1st leaf	2nd leaf	3rd leaf
Excelsior	0.47	18.473	14.743	12.207
Bankuti Korai	0.84	24.049	18.158	16.043
Prior A	1.06	15.660	11.131	9.503
Princess	1.10	13.269	8.935	7.163
Arivat	0.85	12.901	8.692	6.679
Velvon II	0.90	12.346	8.019	6.110
C.I.3576	0.90	11.115	7.053	5.931
B.R.1239	0.84	14.136	9.598	7.382
Naraini	1.80	9.113	6.132	4.838
Proctor	1.80	9.000	6.069	4.450

5.3 DISCUSSION

5.3.1 Effect of Repeated Cycles of Water Stress

It has been frequently noted that subjecting a plant to water stress increases its ability to withstand subsequent exposure to stress (May and Milthorpe, 1962b). Various parameters of cellular metabolism and plant growth such as protoplasmic viscosity, bound water content, cell sap osmotic pressure, photosynthetic rate, respiration rate, xeromorphic characteristics and root production have been shown to alter under such a regime and several

explanations for the phenomenon have been advanced (Henckel, 1964). The present evidence suggests that the plant's capacity to control the loss of water is not affected, as similar water potentials developed in plants subjected to stress whether they had been previously hardened or not (Figure 20). This suggests that the parameter which is modified by previous water stress is the response of the plant to a given water potential.

In seeking a modification in the metabolic response of the plant to low water potential brought about by previous exposure to water stress, two parameters were considered. In the case of leaf chlorophyll content, previous exposure to water stress reduced the loss in chlorophyll during subsequent episodes of low leaf water potential. With free proline content, on the other hand, the increase which occurs during water stress was accentuated by a previous history of stress. There is no evidence that these two phenomena are related, although a biochemical route for chlorophyll synthesis from proline has been suggested (Breyhan, Heilinger, and Fischnich, 1959).

The increase in the potential for proline synthesis brought about by exposure to water stress may have any of a number of explanations. Although free proline itself largely disappears between episodes of stress, the

biosynthetic pathway which was active during the period of low water potential may remain intact, although largely inoperative. This potential pathway may owe its activity to the activation of certain enzymes, enhanced synthesis of specific enzymes or increased substrate availability. All of these factors may be potentiated by an episode of stress such that the synthesis of proline is more rapidly and effectively stimulated by any subsequent fall in tissue water potential. It is possible that the factors which result in these changes in the proline biosynthetic pathway may be the changes in the physical state of the cytoplasm described by Henckel (1964). On the other hand, both parameters may result from changes in the growth hormones, gibberellins, auxins, cytokinins or inhibitors, as a result of stress.

5.3.2 Genetic Variability in Proline Accumulation

Many attempts have been made to correlate the ability of plant varieties to yield during adverse drought conditions with some easily measured parameter of seedling growth which could be used as an index (Ashton, 1948; May and Milthorpe, 1962b). The general failure of this approach has been due both to the complex nature of the drought response under field conditions and the arbitrary character of most of the proposed test indices. These and other problems in relating varietal performance to

specific aspects of the field environment led Finlay and Wilkinson (1963) to use the mean yield performance of a large group of varieties as an index of the field environment and to test individual varietal yielding capacity against that of the whole population. A variety with a yield which varied less from season to season and site to site than the mean of the whole population could then be said to be better adapted to the environment (or region) than the population as a whole. Conversely, a variety which performed worse than average in poor situations but as well or better than average in good situations would be poorly adapted. In the context of South Australia, the major yield determinant of cereal crops both between seasons and sites is water stress. Thus the yield stability index of Finlay and Wilkinson (1963) when measured in this geographical region, is largely, but not wholly, an index of varietal response to water stress.

In the present study, a group of varieties were chosen which were known to differ in this stability index, measured in South Australia, and therefore, presumably, in their reaction to field water stress. As there was no difference in the rate of water loss, as measured by leaf water potential, these varieties did not appear to possess different means of controlling their water balance during an episode of water stress (Figure 23; Table 12, 13, 14).

Similarly, the degree of tetrazolium reduction (indicating dehydrogenase activity) (Figure 24), and the chlorophyll content of the leaves (Table 15, 16, 17) did not differ between varieties although both decreased with increasing period of water stress. It has been suggested that the ability to retain chlorophyll during an episode of water stress might serve as an index of varietal drought resistance (Kaloyereas, 1958). This suggestion is not supported by the present data nor by that of Panous (1967).

In contrast to these characteristics which showed no evidence of genotypic variation, the survival of leaves following a period of water stress, plant relative growth rate immediately following such a stress, and the leaf free proline content during stress all differed between varieties. All three of these parameters were correlated both with each other (Figure 28, 29) and with the stability index of the variety (Figure 30). A variety which, from its field performance, might be expected to withstand the effects of water stress better than most other varieties would tend to accumulate more free proline during stress, have more leaf tissue surviving a severe stress and grow more rapidly immediately following stress than the majority of varieties. The relationship between leaf survival and subsequent relative growth rate may well be direct but the relationship between both

parameters and the ability to accumulate free proline is less obvious.

In the past, one important problem in assessing both the criteria for selection of drought resistant varieties and the performance of the varieties themselves has been the lack of a suitable criterion of water stress effect in the field. Reliance has frequently been placed on subjective assessments and the consequent lack of correlation between objective and arbitrary measurements (such as chlorophyll content) and field performance has not been surprising. Finlay's (1969) stability index, although necessarily a crude measurement, does give an objective measurement of comparative field performance and when carried out under varying drought situations, an objective measure of field resistance to water stress. The close relationship between this index and three plant parameters, measured during a short period of water stress applied to the seedling plant in the limited selection of varieties sampled, suggests that the three parameters could be profitably investigated as the basis of a seedling test for drought resistance. Of the three parameters, proline content is both the most accurately measurable and simplest to perform. If the present relationship between the ability to accumulate proline and field performance in

adverse conditions should be maintained for a wider group of genotypes then it may be profitable to include this assessment as a selection criterion in choosing varieties or breeding new varieties for areas subject to drought.

CHAPTER IV

GENERAL DISCUSSION

One of the most important aspects of plant-water relationships is the measurement and expression of the water status of a plant. Several methods of measuring water status have been proposed from time to time (Kramer and Brix, 1965; Barrs, 1968) but the only unequivocal measure of water status of a system is Gibbs' free energy of the water in that system (Slatyer and Gardner, 1965). Using this measure it has become possible to compare the status of water in different plants or in different parts of the soil-plant-air system without the need to qualify the measurement. This concept was extended to plants by Slatyer and Taylor (1960) who proposed the term 'water potential' to describe the physical status of water in plants. In view of the previous confusion in assessing the physical status of water in the plant, very little progress in understanding the effects of stress at the biochemical level of plant metabolism has been achieved. In the present work, protein and amino acid metabolism were investigated at varying levels of water potential in the plant.

Recently, several Russian workers demonstrated the presence of large quantities of free proline in crop plants in the absence of sufficient water supply (Petinc

and Berko, 1965; Savitskaya, 1965, 1967; Vlasyuk, Shmat'ko, and Rubanyuk, 1968; Protsenko, Shmat'ko, and Rubanyuk, 1968; Vlasyuk, Rubanyuk, and Shmat'ko, 1969) but these authors did not measure the water potential of their plants. Barnett and Naylor (1966), however, measuring both water potential and levels of amino acids also found an accumulation of free proline in burmuda grass (Cynodon dactylon L.) at known water potentials and established an apparent correlation between the two parameters. The present work confirmed the above findings for barley and wheat plants when, in a preliminary experiment, a 15-fold increase in free proline and only slight changes in other amino acids were observed. This led to a thorough investigation of proline accumulation in response to varying levels of water stress and proline was found to accumulate in every part of the intact barley plant, though at different rates in the different organs. The accumulated proline rapidly disappeared when the stress was relieved which suggested that the effect of water stress on proline accumulation was reversible.

In attempting to find an explanation for the efficiency of different organs of the plant to accumulate proline, excised organs such as leaves, leaf sheaths, shoot apices, and roots, were floated on osmotica and it

was found that the shoot apex and the root failed to accumulate proline. The reason for the inability of the shoot apex and root to accumulate proline is unknown and, at least three explanations can be put forward. Firstly, proline accumulation may somehow be related to chlorophyll, or confined to only chlorophyll-containing organs, such as the leaf and leaf sheath. Very little information is available concerning this possibility and it is not yet able to be properly evaluated. Secondly, excision itself may have stopped the supply of a precursor or some other chemical messenger to the shoot apex and root from elsewhere. In fact, the essentiality of precursors, such as sucrose and glutamic acid, for proline biosynthesis was clearly shown in etiolated leaves. Etiolated barley leaf sections preincubated with either sucrose and glutamic acid showed a marked increase in proline level when subjected to osmotic stress as compared with non-precursor supplied controls. The biosynthesis of proline from ^{14}C-glutamic acid has also been reported in partially wilted leaves of burmuda grass (Barnett and Naylor, 1966) and non-stressed etiolated barley leaves (Naylor and Tolbert, 1956). A role for carbohydrates in proline accumulation was also suggested by Routley (1966), and subsequently elaborated by Stewart et al (1966), who demonstrated the prevention of proline accumulation by either anaerobiosis or certain inhibitors of oxidative

sugar metabolism. The findings of these authors indicated that the carbon skeleton for proline synthesis was supplied by sugar oxidation.

Thirdly, proline biosynthesis may be under the control of feed-back reactions in the shoot apex and the root. As a point of interest, more recently Barnard and Oaks (1970) demonstrated that proline synthesis in maize root tips is strictly controlled by a feed-back mechanism. However, on the basis of the present evidence it is difficult to tell which one or how many of the above explanations is operative.

Proline is an imino acid, one of the 22 amino acids found in animal and plant proteins. Its biosynthetic pathway in bacteria has been fully described (Fowden, 1964) but there is little rigorous proof that a similar pathway also exists in higher plants. Much of the work on the biosynthesis of proline and its analogues in higher plants has come from Fowden's laboratory and it has been suggested that the enzymes involved are not strictly specific and can synthesize structural analogues of proline under certain circumstances (Fowden, 1967). It is too early to trace the reason and mechanism of proline accumulation during water stress with certainty, though it could be due either to increased synthesis or decreased decomposition, or both. Since, however, treatment with abscisic acid

(which is an inhibitor of protein synthesis (Chrispeels and Varner, 1967)) can also lead to proline accumulation comparable to that induced by water stress (page 101), it is of importance that wilting has been reported to induce the production of abscisic acid in wheat leaves (Wright and Hiron, 1969). If this is a general phenomenon, occurring in intact plants also, then abscisic acid may be the causative agent in the accumulation of proline. The synthesis of protein can be inhibited by water stress at several recognized locations within the cell, such as nucleus, cytoplasm, mitochondria and chloroplasts. The question of which one of the above systems is interrupted first, is difficult to presume and far from clear. However, water potential is a dynamic property and therefore it appears unlikely that these systems will be affected differently. The precise mechanism of the inhibition of protein synthesis and, possibly, stimulation of protein decomposition, by low water potentials is unknown, although Genkel et al (1967) demonstrated the fragmentation of polysomes due to drought and suggested that water stress stimulated ribonuclease activity causing polysome breakdown and, hence, a decrease in protein synthesis. Increased activity of other enzymes such as phosphatase and ATPase in stressed chloroplasts has also been reported which might affect ATP levels (Nir and Poljakoff-Mayber, 1966). More recent evidence

in favour of a disruption of energy supply comes from the works of Nir et al (1969) who demonstrated that water stress changed the fine structure of mitochondria, plastids, plasma membranes, chromatin in the nucleus, and brought about a reduction in the respiratory rate, as well as an increase in the activity of mitochondrial cytochrome oxidase (Nir et al, 1970).

Since proline accumulation is a water stress-triggered phenomenon, it was of interest to investigate the effects of those factors upon proline accumulation which are known to influence the response of plants to water stress. In 1963, Halevy and Kessler reported that the growth retardants, CCC and Phosphon D, increased the drought tolerance of bean plants. Shortly after it was found that none of the usual characters associated with drought resistance, such as transpiration rate, stomatal opening, osmotic potential, water saturation deficit, and anatomical and morphological features were related to the retardant-induced increased desiccation resistance (Plaut et al, 1964). The present investigation also showed that CCC did not affect the water potential of leaves of wheat plant during an episode of water stress and thus confirmed that CCC does not change the ability of plant to gain or lose water. It is clear, therefore, that the increased drought tolerance by CCC must be due to CCC-induced changes in the growth and metabolism of the plant. It is

difficult to pinpoint the reason for the increased drought tolerance due to CCC treatment. Growth retardants delay the senescence of detached bean leaves and this effect and increased drought tolerance may be due to the effects of CCC on nucleic acid and protein metabolism (Halevy, 1967). The stimulation of proline synthesis during water stress due to CCC treatment may be another consequence of this effect since an increased protein content following CCC application has been reported (Stoddart, 1965). If this is due to increased protein synthesis then inhibition of this increased rate by water stress could eventually lead to enhanced proline accumulation. Alternatively, a preferential conversion of carbohydrates to amino acids may be responsible (Stoddart, 1965). CCC may also have changed the endogenous hormone balance which, in turn, exerted an effect on proline accumulation during water stress. Obviously, the mechanism underlying the increased capacity for proline accumulation brought about by CCC is uncertain but it is significant that a treatment reputed to increase water stress resistance of the plant, also affected the accumulation of proline without affecting the water potential of leaves. Here, the relationship between water potential and proline accumulation was offset indicating that one parameter can be varied without varying the other.

The increase in proline accumulation was reversed by applied gibberellic acid but, again, as in the case with CCC, the water potential of the plant was unaffected. Plants not treated with GA_3 continued to accumulate proline during water stress, but the level of proline fell and growth was stimulated when the GA_3 was applied. In this case also, evidence was obtained that proline accumulation can be varied without varying the water potential of plant. It has been reported that CCC inhibits gibberellin biosynthesis and the growth responses induced by CCC are opposite to those of resulting from gibberellin applications (Cathey, 1964; Paleg, 1965). In view of other opposite effects of these two categories of growth substances, it is noteworthy that CCC, which increases drought resistance (Halevy and Kessler, 1963; Plaut et al, 1964), also increased proline accumulation, whereas GA_3, which probably decreases drought resistance (Sitnikova, 1966), decreased proline accumulation. The metabolic effects of these two growth substances, as demonstrated by proline accumulation, were paralleled by growth effects where applied GA_3 increased the height of plant, length of the shoot apex, and primordia production in both stressed and non-stressed plants. Since the GA_3-stimulated growth during water stress was accompanied by the disappearance of accumulated proline, it is probable that protein synthesis was revived by the applied

gibberellin. Plant growth, which is almost always inhibited by water stress, thus seems to be more directly dependent upon metabolism rather than on the water potential of the plant.

Repeated episodes of water stress also influenced the metabolic response of the plant, as clearly demonstrated by proline accumulation. The capacity to accumulate proline was stimulated at a given water potential by repeated cycles of stress, and the level of proline decreased to the level of that in control plants when the stress was relieved. This suggests that the increase in proline concentration was not due to a carry-over of free proline from the previous cycle of stress. The question of whether newly synthesized proline-rich protein, which takes place during water stress (Figure 2), also decreases to the level of that in control plants upon the relief of stress will depend upon the turn-over of the protein(s). It has often been found that subjecting a plant to water stress increases its ability to withstand subsequent exposure to stress (May and Milthorpe, 1962b) and this has now been shown to be accompanied by an increased proline accumulation potential. It is possible that changes in the physical state of cytoplasm, as described by Henckel (1964), may be important in this respect. On the other hand, changes in these parameters may result from changes in the concentration and/or spectrum of growth hormones

such as gibberellins, auxins, cytokinins or abscisins as a result of stress.

Several attempts have been made to quantitate drought resistance of crop plants but so far no single definitive measure has emerged which seems valid for all instances (Ashton, 1948). The methods used so far measure either avoidance of drought, or some easily measured parameter of seedling growth (Asana, 1961, 1965; May and Milthorpe, 1962b; Levitt, 1965). Recently, however, a statistical, though necessarily an indirect, approach to this problem has been taken which, in essence, measures the yield stability of a variety in a wide range of environmental conditions (Finlay and Wilkinson, 1963). This approach, in fact, fulfils the requirements of the definition of drought resistance suggested by May and Milthorpe (1962b), which defines 'drought resistant' as an all-embracing term to describe those varieties or species which are able to grow and yield satisfactorily in areas liable to periodic drought. In view of the crude, laborious, and time-consuming nature of the above statistical approach of measuring drought resistance (adaptability ?), a quick and reasonable laboratory test of drought resistance was highly necessary. The findings of the present investigation suggest that the assessment of proline accumulation may eventually be of significant utility as a criterion of drought resistance.

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APPENDIX

STATISTICAL TREATMENT OF THE DATA

(Analysis of variance, regression analysis, and test of homogeneity of regression coefficients.)

The following abbreviations have been used:

D.F.	Degrees of freedom
M.S.	Mean sum of squares
F	Calculated variance ratio
*	Significant at 5% level of probability
**	Significant at 1% level of probability
***	Significant at 0.1% level of probability
L.S.D. (P=0.05)	Least significant difference at 5% level of probability

SECTION 1 (page 53)

PROTEIN METABOLISM AND PROLINE ACCUMULATION IN WATER STRESSED PLANTS (1.2)

Water Potential (Figure 1)

		Time (hours)					
		10	20	30		40	50
Source of variation	D.F.	M.S.	M.S.	M.S.	D.F.	M.S.	M.S.
Treatment	1	214.24***	264.02***	446.69***	2	869.86***	454.59***
Residual	4	0.13	0.21	0.38	6	0.25	0.27
L.S.D. (P=0.05)		0.8	1.0	1.4		1.0	1.0

Protein (Figure 2, top)

		Time (hours)					
Leaf		10	20	30		40	50
Source of variation	D.F.	M.S.	M.S.	M.S.	D.F.	M.S.	M.S.
Treatment	1	0.060***	0.1330***	0.2100***	2	0.6240***	0.8955***
Residual	4	0.002	0.0005	0.0022	6	0.0028	0.0021
L.S.D. (P=0.05)		0.099	0.047	0.105		0.105	0.090
Root							
Treatment	1	0.0003	1.4080***	4.403***	2	4.241***	6.5405
Residual	4	0.0061	0.0025	0.003	6	0.002	0.0016
L.S.D. (P=0.05)		0.174	0.111	0.122		0.026	0.078

Protein-bound Proline (Figure 2, bottom)

	Time (hours)			
Leaf	10 to 30		40 to 50	
Source of variation	D.F.	M.S.	D.F.	M.S.
Treatment	1	401.39***	2	287.22***
Time	2	77.39**	1	34.72*
Treatment x Time	2	53.05**	2	30.59*
Residual	12	6.83	12	4.66
L.S.D. (P=0.05)		2.68		2.71

		Time (hours)		
Root	10 to 30		40 to 50	
Source of variation	D.F.	M.S.	D.F.	M.S.
Treatment	1	346.73***	2	260.16***
Time	2	63.39*	1	32.00*
Treatment x Time	2	31.04	2	36.16*
Residual	12	8.50	12	6.19
L.S.D. (P=0.05)		4.74		2.55

Free Proline (Log. transformed data, Figure 3)

		Time (hours)		
Leaf	10 to 30		40 to 50	
Source of variation	D.F.	M.S.	D.F.	M.S.
Treatment	1	5.3330***	2	4.41800***
Time	2	0.2805***	1	0.12300***
Treatment x Time	2	0.2280***	2	0.18500***
Residual	12	0.0033	12	0.00025
Root				
Treatment	1	3.9320***	2	2.7505***
Time	2	0.1535***	1	0.1500***
Treatment x Time	2	0.0805***	2	0.2540***
Residual	12	0.0004	12	0.0009

EFFECT OF MILD AND SEVERE WATER STRESS ON APICAL MORPHOGENESIS AND THE ACCUMULATION AND DISTRIBUTION OF PROLINE IN BARLEY PLANT (1.3)

Water Potential (Figure 4)

	Time (hours)											
	7		18		30		48		56		72	
Source of variation	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.
Treatment	2	126.940***	2	122.590***	2	234.125***	2	397.420***	4	272.715***	4	433.057***
Residual	6	0.175	6	0.065	6	0.063	6	0.115	10	0.151	10	0.163
L.S.D. (P=0.05)		0.8		0.5		0.5		0.7		0.7		0.7

Length of Sheet Apex (Figure 5, top)

Source of variation	Time (hours) 7 to 48 D.F.	M.S.	56 to 72 D.F.	M.S.
Treatment	2	0.0655000***	4	0.417275***
Time	3	0.0400000***	1	0.094300***
Treatment x Time	6	0.0166666***	4	0.024875***
Residual	108	0.0000055	90	0.000031
L.S.D. (P=0.05)		0.001		0.003

Total Number of Primordia (Figure 5, middle)

Source of variation	Time (hours) 7 to 48 D.F.	M.S.	56 to 72 D.F.	M.S.
Treatment	2	17.85***	4	77.54***
Time	3	3.63***	1	11.56***
Treatment x Time	6	3.27***	4	3.56***
Residual	108	0.40	90	0·37
L.S.D. (P=0.05)		0.28		0.38

Stage of Development (Figure 5, bottom)

Source of variation	Time (hours) 7 to 48 D.F.	M.S.	56 to 72 D.F.	M.S.
Treatment	2	0.3095	4	0.690
Time	3	0.5413***	1	1.440*
Treatment x Time	6	0.0580	4	0.190
Residual	108	0.0824	90	0.208

Accumulation of Proline (log. transformed data, Figure 6)

Leaf	Time (hours) 7 to 48		56 to 72	
Source of variation	D.F.	M.S.	D.F.	M.S.
Treatment	2	4.74250***	4	3.6242***
Time	3	0.81533***	1	0.1510***
Treatment x Time	6	0.21066***	4	0.1270***
Residual	24	0.00012	20	0.0001

Leaf Sheath	Time (hours) 7 to 48		56 to 72	
Source of variation	D.F.	M.S.	D.F.	M.S.
Treatment	2	4.8335***	4	3.56720***
Time	3	0.6773***	1	0.20800***
Treatment x Time	6	0.1768***	4	0.15550***
Residual	24	0.0036	20	0.00015

Shoot Apex	Time (hours) 7 to 48		56 to 72	
Source of variation	D.F.	M.S.	D.F.	M.S.
Treatment	2	5.8940***	4	3.7660***
Time	3	0.5443***	1	0.3140***
Treatment x Time	6	0.1480***	4	0.2545***
Residual	24	0.0006	20	0.0006

Root	Time (hours) 7 to 48		56 to 72	
Source of variation	D.F.	M.S.	D.F.	M.S.
Treatment	2	4.27700***	4	2.84025***
Time	3	0.56766***	1	1.11500***
Treatment x Time	6	0.12150***	4	0.72500***
Residual	24	0.00016	20	0.00075

Relationship between Proline Accumulation and Water Potential in Barley Leaves (Figure 7)

	Regression Analysis	
Source of variation	D.F.	M.S.
Regression	1	470.116***
Residual	15	5.510
Correlation coefficient		0.922

EFFECT OF WATER DEFICIT ON THE GROWTH OF BARLEY (1.4)

Weight of Plant (Figure 8)

		Fresh weight	Dry weight
Source of variation	D.F.	M.S.	M.S.
Organ	2	4.12400***	5.32950***
Stress	1	3.10000***	2.33200***
Time	3	0.02166***	0.44800***
Organ x stress	2	0.09350***	0.23350***
Organ x time	6	0.01050***	0.12700***
Stress x time	3	0.30033***	0.33566***
Organ x stress x time	6	0.02200***	0.06850***
Residual	96	0.00158	0.00731
L.S.D. (P=0.05)		0.050	0.108

EFFECT OF ABRATION ON THE ACCUMULATION AND DISTRIBUTION OF PROLINE (1.5)

Accumulation of Proline (Log. transformed data, Figure 9)

	Time (hours)			
Leaf	7 to 30		45 to 65	
Source of variation	D.F.	M.S.	D.F.	M.S.
Treatment	1	0.30900***	3	0.00900***
Time	2	0.99800***	1	0.04900***
Treatment x time	2	0.04100***	3	0.00100***
Residual	12	0.00033	16	0.00006
Leaf sheath and shoot				
Treatment	1	0.0270***	3	0.0070***
Time	2	0.9195***	1	0.0850***
Treatment x time	2	0.0065***	3	0.0003
Residual	12	0.0005	16	0.0003
Root				
Treatment	1	3.6270***	3	1.6143***
Time	2	0.1900***	1	0.0440
Treatment x time	2	0.1080***	3	0.2326***
Residual	12	0.0008	16	0.0213

SECTION 2 (page 88)

ISOLATED ORGANS (2)

Excised organ (Figure 10)

Source of variation	D.F.	Time (hours) 4 M.S.	16 M.S.	32 M.S.	48 M.S.
Organ	3	64130.0***	655788.0***	7729569.3***	14002204.0***
Stress	1	42336.0***	1233971.0***	2598674.0***	7315104.0***
Organ x stress	3	21691.3***	885406.3***	1166438.6***	3278669.6***
Residual	16	209.7	3176.2	5341.7	9484.6
Etiolated leaves (Figure 11)					
Organ	1	2436.0***	876420.0***	13385856.0***	26388502.0***
Stress	1	99918.0***	1943270.0***	3616812.0***	9492744.0***
Organ x stress	1	25670.0***	765581.0***	1976409.0***	7036477.0***
Residual	8	916.6	3767.5	8143.7	12222.2
Sucrose and Glutamic acid (Figure 12)					
Sucrose	2	137830.5***	226603.0***	1784286.5***	4027989.0***
Glutamic acid	2	57793.0***	258096.5***	1718574.0***	3732933.5***
Sucrose x glutamic acid	4	4007.0*	9125.2	1541540.5***	526876.7***
Residual	18	1294.4	3170.7	4375.1	3840.6

SECTION 3 (page 101)

EFFECT OF ABSCISIC ACID ON PROLINE ACCUMULATION (3)

Excised organs (Figure 13)

		Time (hours)		
Leaf		4	16	32
Source of variation	D.F.	M.S.	M.S.	M.S.
Treatment	4	0.1452***	3.6560***	6.3520***
Residual	15	0.0021	0.0540	0.2040
L.S.D.(P=0.05)		0.068	0.351	0.681
Root				
Treatment	4	0.0712***	0.3605***	0.5987***
Residual	15	0.0020	0.0070	0.0028
L.S.D.(P=0.05)		0.068	0.123	0.149

Intact Organs (Figure 14)

Leaf				
Treatment	3	0.1363***	3.5370***	8.1290***
Residual	8	0.0007	0.0123	0.0405
L.S.D.(P=0.05)		0.046	0.207	0.368
Root				
Treatment	3	0.0083**	0.0176**	0.1666***
Residual	8	0.0006	0.0008	0.0027
L.S.D.(P=0.05)		0.046	0.050	0.096

SECTION 4 (page 109)

EFFECT OF CCC ON ACCUMULATION OF PROLINE, GROWTH, AND APICAL MORPHOGENESIS IN WHEAT (4.1)

	Dry Weight (Table 3)		Leaf Area (4.1.3.2.3)	
Source of variation	D.F.	M.S.	D.F.	M.S.
CCC	1	0.0158483***	1	1244.330***
Organ	5	0.0093589***	-	-
CCC x Organ	5	0.0006508	-	-
Residual	78	0.0000101	28	0.154

Water Potential (Figure 15)

		Time (hours) 7 to 48		56 to 72
Source of variation	D.F.	M.S.	D.F.	M.S.
CCC	1	0.010	1	0.060
Stress	2	1604.245***	4	1385.997***
Time	3	49.840***	1	23.560***
CCC x stress	2	0.025	4	0.012
CCC x time	3	0.030	1	0.020
Stress x time	6	28.670***	4	108.730***
CCC x stress x time	6	0.056	4	0.027
Residual	48	0.124	40	0.058

Height of Plant (Figure 16, top)

Source of variation	Time (hours) 7 to 48 D.F.	M.S.	56 to 72 D.F.	M.S.
CCC	1	996.340***	1	1009.430***
Stress	2	6.275***	4	14.925***
Time	3	0.886***	1	5.040***
CCC x stress	2	2.400***	4	5.372***
CCC x time	3	0.450***	1	2.750***
Stress x time	6	0.623***	4	1.315***
CCC x stress x time	6	0.205***	4	0.672***
Residual	336	0.028	280	0.026

Length of Shoot Apex (Figure 16, middle)

CCC	1	0.8350***	1	1.0090***
Stress	2	0.0465***	4	0.1930***
Time	3	0.0250***	1	0.0140*
CCC x stress	2	0.0025	4	0.0275***
CCC x time	3	0.0006	1	0.0070
Stress x time	6	0.0125***	4	0.0140***
CCC x stress x time	6	0.0006	4	0.0002
Residual	336	0.0022	280	0.0024

Total number of primordia (Figure 16, bottom)

CCC	1	0.040	1	0.010
Stress	2	67.800***	4	167.545***
Time	3	18.326***	1	27.000***
CCC x stress	2	0.375	4	0.155
CCC x time	3	0.010	1	0.010
Stress x time	6	12.106***	4	8.490***
CCC x stress x time	6	0.063	4	0.090
Residual	336	0.263	280	0.341

Free Proline (Log. transformed data, Figure 17)

Leaf	Time (hours) 7 to 48		56 to 72	
Source of variation	D.F.	M.S.	D.F.	M.S.
CCC	1	0.49700***	1	0.22100***
Stress	2	9.56300***	4	6.81950***
Time	3	1.14533***	1	0.23600***
CCC x stress	2	0.09500***	4	0.01875***
CCC x time	3	0.01000***	1	0.00300***
Stress x time	6	0.29083***	4	0.23825***
CCC x stress x time	6	0.00383***	4	0.24200***
Residual	48	0.00017	40	0.00010
Leaf sheath				
CCC	1	0.32500***	1	0.21900***
Stress	2	8.96450***	4	6.36000***
Time	3	1.27433***	1	0.11100***
CCC x stress	2	0.07150***	4	0.02250***
CCC x time	3	0.01000***	1	0.00700***
Stress x time	6	0.29150***	4	0.17225***
CCC x stress x time	6	0.04666***	4	0.00325***
Residual	48	0.00012	40	0.00007
Shoot Apex				
CCC	1	0.35500***	1	0.39600***
Stress	2	10.82750***	4	6.22575***
Time	3	0.95366***	1	0.29200***
CCC x stress	2	0.06700***	4	0.03150***
CCC x time	3	0.00766***	1	0.00300**
Stress x time	6	0.14516***	4	0.28900***
CCC x stress x time	6	0.00183***	4	0.00150**
Residual	48	0.00029	40	0.00032

Root	Time (hours) 7 to 48		56 to 72	
Source of variation	D.F.	M.S.	D.F.	M.S.
CCC	1	0.2110***	1	0.7000***
Stress	2	9.4700***	4	5.5280***
Time	3	0.9996***	1	0.4150***
CCC x stress	2	0.0245	4	0.0320***
CCC x time	3	0.0096	1	0.0130***
Stress x time	6	0.2090***	4	0.4095***
CCC x stress x time	6	0.0080	4	0.4422***
Residual	48	0.0114	40	0.0007

Relationship Between Proline Accumulation and Water Potential in Wheat Leaves (Figure 18)

Test of homogeneity of regression coefficients

Treatment	Regression D.F.	Regression M.S.	Residual D.F.	Residual M.S.	F
+ CCC	1	415.388***	15	4.281	8.143**
- CCC	1	171.493***	15	2.153	

THE INTERACTION OF CCC AND GA_3 ON THE ACCUMULATION OF PROLINE, GROWTH, AND APICAL MORPHOGENESIS IN WHEAT (4.2)

Water Potential (Table 4)

Source of variation	D.F.	Time (hours) 27 M.S.	30 M.S.	33 M.S.	36 M.S.
CCC	2	0.030	0.130	1.340**	0.425
Stress	1	3440.020***	4329.110***	4618.530***	4822.330***
GA_3	2	0.270	0.095	0.155	0.265
CCC x stress	2	0.215	0.040	0.579	0.110
CCC x GA_3	4	0.147	0.107	0.142	0.045
Stress x GA_3	2	0.270	0.035	0.045	0.240
CCC x stress x GA_3	4	0.090	0.095	0.237	0.027
Residual	36	0.244	0.364	0.217	0.261
L.S.D. (P=0.05)		0.8	1.0	0.8	0.8

CCC + GA_3 continued (Table 5, 6, 7, and Figure 19)

		Plant Height	Shoot Apex Length	Primordia number		Free Proline (Log. values)
Source of variation	D.F.	M.S.	M.S.	M.S.	D.F.	M.S.
CCC	2	936.965***	0.5435***	0.050	2	0.65350***
Stress	1	74.620***	0.9890***	938.500***	1	105.30400***
GA_3	2	31.505***	0.8570***	157.000***	2	0.43800***
Time	3	21.156***	0.4630***	111.110***	3	0.02900***
CCC x stress	2	5.655***	0.0015	0.050	2	0.39050***
CCC x GA_3	4	0.127**	0.0030	0.017	4	0.01100***
CCC x time	6	0.410***	0.0015	0.038	6	0.00583***
Stress x GA_3	2	0.905***	0.0015	3.080***	2	0.04450***
Stress x time	3	2.240***	0.0573***	7.693***	3	0.00033
GA_3 x time	6	3.665***	0.1071***	18.250***	6	0.05600***
CCC x stress x GA_3	4	0.065	0.0077	0.067	4	0.00900***
CCC x stress x time	6	0.106**	0.0010	0.076	6	0.00216***
Stress x GA_3 x time	6	0.118**	0.0093	0.938*	6	0.00666***
CCC x GA_3 x time	12	0.102***	0.0007	0.038	12	0.00216***
CCC x stress x GA_3 x time	12	0.031	0.0006	0.008	12	0.00166***
Residual	648	0.034	0.0083	0.406	144	0.00020
L.S.D. (P=0.05)		0.16	0.080	0.55		

SECTION 5 (page 132)

REPEATED CYCLES OF WATER STRESS AND THE ACCUMULATION OF PROLINE (5.1)

Water Potential (Figure 20)

First Leaf

		Time (days) 4		6		9		11		13		15	
Source of variation	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.
Treatment	1	414.669***	1	0.003	2	357.722***	2	0.007	3	186.161***	3	0.098
Residual	4	0.424	4	0.082	6	0.483	6	0.028	8	0.397	8	0.745
L.S.D. (P=0.05)		1.5				1.4				1.2		

Second Leaf

Treatment	1	320.616***	1	0.004	2	307.105***	2	0.033	3	171.893***	3	0.007
Residual	4	0.358	4	0.023	6	0.199	6	0.035	8	0.337	8	0.061
L.S.D. (P=0.05)		1.4				0.9				1.1		

Proline Content (Figure 21)

First Leaf

Treatment	1	152.409***	1	0.0024*	2	156.396***	2	0.0010*	3	110.810***	3	0.0003
Residual	4	0.015	4	0.0003	6	0.003	6	0.0001	8	0.003	8	0.0003
L.S.D. (P=0.05)		0.277		0.038		0.107		0.004		0.101		

Proline Content (Continued)

Second Leaf

Source of variation	Time (days) 4 D.F.	M.S.	6 D.F.	M.S.	9 D.F.	M.S.	11 D.F.	M.S.	13 D.F.	M.S.	15 D.F.	M.S.
Treatment	1	107.070***	1	0.0006	2	129.505***	2	0.0020	3	114.836***	3	0.0006
Residual	4	0.003	4	0.0003	6	0.003	6	0.0004	8	0.005	8	0.0003
L.S.D. (P=0.05)		0.122				0.107				0.131		

Chlorophyll Content (Figure 22)

First Leaf

Treatment	1	9.688***	1	0.2930***	2	12.361***	2	0.973*	3	19.138***	3	2.399**
Residual	2	0.002	2	0.0015	3	0.011	3	0.092	4	0.005	4	0.092
L.S.D. (P=0.05)		0.18		0.16		0.33		0.96		0.06		0.84

Second Leaf

Treatment	1	5.1400***	1	0.7560***	2	12.139**	2	1.751**	3	12.222***	3	6.313**
Residual	2	0.0035	2	0.0005	3	0.141	3	0.002	4	0.004	4	0.097
L.S.D. (P=0.05)		0.25		0.09		1.12		0.14		0.06		0.85

GENETIC VARIABILITY IN THE CAPACITY TO ACCUMULATE PROLINE IN RESPONSE TO WATER STRESS IN BARLEY (5.2)

Water Potential (Figure 23)

Osmotic potential (bars)	Source of variation	D.F.	Time (hours) 7	25	50	73	97
			M.S.	M.S.	M.S.	M.S.	M.S.
0	Variety	4	0.082	0.015	0.092	0.015	0.017
	Residual	10	0.205	0.066	0.188	0.077	0.048
-20	Variety	4	0.105	0.092	0.152	0.247	0.075
	Residual	10	0.232	0.174	0.578	0.607	1.116

Leaf Viability (Figure 24)

Source of variation	D.F.	Time (hours) 7	25	50	73	97
		M.S.	M.S.	M.S.	M.S.	M.S.
Variety	4	0.0005	0.0002	0.0002	0.0007	0.0002
Stress	1	0.2440***	0.6800***	2.5110***	4.2990***	4.9810***
Variety x stress	4	0.0010	0.0010	0.0002	0.0005	0.0007
Residual	20	0.0021	0.0011	0.0006	0.0006	0.0010

Relationship Between Tetrazolium Reduction and Water Potential (Figure 25)

Source of variation	D.F.	M.S.
Regression	1	0.532***
Residual	4	0.005

Correlation coefficient = 0.983

Free Proline (Log. transformed data, Figure 26 and 27)

Source of variation	D.F.	First Leaf M.S.	Second Leaf M.S.	Third Leaf M.S.
Variety	4	0.0825***	0.1037***	0.0595***
Stress	1	62.1550***	51.3870***	32.4910***
Time	4	2.0740***	2.0992***	1.9461***
Variety x stress	4	0.2840***	0.0465***	0.2987***
Variety x time	16	0.0011***	0.0014***	0.0031***
Stress x time	4	1.4260***	1.4262***	1.3850***
Variety x stress x time	16	0.0230***	0.0660***	0.0068***
Residual	100	0.0003	0.0002	0.0002

Survival and Proline (Figure 28)

Time (hours)	Source of variation	D.F.	First Leaf M.S.	Second Leaf M.S.	Third Leaf M.S.
50	Regression	1	875.62*	1352.23**	1099.09*
	Residual	3	42.24	28.47	75.23
	Correlation coefficient		0.934	0.969	0.917
73	Regression	1	808.19***	1322.90**	1056.27*
	Residual	3	4.41	17.77	31.28
	Correlation coefficient		0.964	0.980	0.934
97	Regression	1	484.62*	1113.16**	1147.42**
	Residual	3	16.42	17.91	7.99
	Correlation coefficient		0.962	0.987	0.989

Water Potential (Table 12, 13, and 14)

Osmotic potential (bars)	Source of variation	D.F.	First Leaf +60 M.S.	First Leaf +84 M.S.	First Leaf +108 M.S.	Second Leaf +60 M.S.	Second Leaf +84 M.S.	Second Leaf +108 M.S.	Third Leaf +60 M.S.	Third Leaf +84 M.S.	Third Leaf +108 M.S.
0	Variety	9	0.124	0.035	0.025	0.038	0.042	0.050	0.046	0.043	0.044
	Residual	20	0.107	0.127	0.104	0.158	0.079	0.079	0.130	0.066	0.128
-20	Variety	9	0.390	0.673	1.908	1.000	0.164	0.058	0.613	0.314	0.228
	Residual	20	1.079	1.500	1.130	0.876	0.510	0.366	0.667	0.688	0.523

Chlorophyll Content (Table 15, 16, and 17)

Source of variation	D.F.	First Leaf +60 M.S.	First Leaf +84 M.S.	First Leaf +108 M.S.	Second Leaf +60 M.S.	Second Leaf +84 M.S.	Second Leaf +108 M.S.	Third Leaf +60 M.S.	Third Leaf +84 M.S.	Third Leaf +108 M.S.
Variety	9	0.023	0.90	0.027	0.060	0.075	0.064	0.018	0.046	0.013
Stress	1	541.591***	466.968***	165.981***	167.585***	103.822***	59.892***	88.042***	60.170***	23.075***
Variety x stress	9	0.023	0.031	0.042	0.031	0.091	0.044	0.050	0.008	0.022
Residual	40	0.120	0.133	0.130	0.143	0.152	0.084	0.083	0.125	0.125

Free Proline (Log. transformed data, Table 18, 19, and 20)

Source of variation	D.F.	First leaf M.S.	Second leaf M.S.	Third leaf M.S.
Variety	9	0.06540***	0.1230***	0.1284***
Stress	1	112.4710***	78.7050***	53.9780***
Time	2	0.0050*	0.2855***	0.9630***
Variety x stress	9	0.0910***	0.1576***	0.1471***
Variety x time	18	0.0023	0.0018	0.0037
Stress x time	2	0.1905***	0.5510***	1.4650
Variety x stress x time	18	0.0030*	0.0128***	0.0024***
Residual	120	0.0015	0.0017	0.0020

Relationship Between Stability Index and Proline Accumulation (Table 21)

Source of Variation	D.F.	First leaf M.S.	Second leaf M.S.	Third leaf M.S.
Regression	1	2.0042***	1.8580**	1.8110**
Residual	8	0.0693	0.0870	0.0930
Correlation coefficient		0.885	0.852	0.840