Variability in the Accumulation of Amino Acids and Glycinebetaine in Wheat and Barley under Environmental Stress

by

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Dedicated to my parents

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SUMMARY

This investigation aimed to study (1) changes in amino acid and glycinebetaine content in response to the nature of stress imposition under laboratory conditions and (2) possible effects of environmental factors on variability in the content of proline and glycinebetaine in barley and wheat seedlings. Proline, asparagine, glutamine, glycine, valine, γ-amino butyric acid, and glycinebetaine accumulated in response to a reduction in leaf water status whereas glutamic acid, aspartic acid and alanine levels declined; the overall effect was a net increase in amino acid content. However, the concentrations of accumulated amino acids varied markedly with the nature and rapidity of water stress. A rapid water stress imposed by withholding water or by PEG application at normal or high temperature resulted in the accumulation of amides to a level comparable to or more than that of proline. Progressive water stress resulted in the accumulation of proline and glycinebetaine while other amino acids accumulated to a lesser extent. The relief of a moderate water stress resulted in complete disappearance of the accumulated proline within 1 day. Most stress induced metabolic changes returned to normal upon water stress relief with some exceptions, such as the metabolism of the accumulated glycinebetaine. Changes in metabolism induced by low temperature were independent of changes in RWC, Ψ, and Ψ_p, but resembled those induced by water stress except for the accumulation of aspartic acid and alanine.

Barley seedlings with different temperature histories showed different abilities to accumulate proline and glycinebetaine during subsequent water stress at a common temperature (20°C). The investigation to find the cause for this response revealed that both compounds respond to low temperature whereas glycinebetaine alone responded to high temperature in the absence of changes in leaf water status. The critical temperature required for the accumulation of glycinebetaine fell between 25 and 30°C. The rate of increase in glycinebetaine content was more than that for proline content with increase in temperature during water stress.

Wheat seedlings from two cultivars grown from seed matured at cooler temperatures generally accumulated more solute than seedlings grown from seed matured at a warmer temperature. Seed size also varied with parent temperature, and elimination of seed size differences by selection of similar size ranges eliminated the previously observed differences in proline content. The glycinebetaine content of the two wheat cultivars showed a residual effect of parent temperature, however. The proline and glycinebetaine content of 3 barley cultivars also varied with parent seed size. Excelsior seedlings grown from small seed accumulated more proline than Proctor grown from seeds of the same size but the opposite was true when the cultivars were grown from large seed. The glycinebetaine content of these cultivars showed no reversal in response with seed size.

Water stressed seedlings grown from two seed sources of barley cultivar Norbert, obtained directly from Canada (CN) or grown for two generations and subjected to selection pressure in Australia (AN*) showed differences in the ability to accumulate proline, but not glycinebetaine. This difference in response was the result of genetic differences due to selection pressure, in the absence of such selection no differences in proline content were found between the two seed sources.

A high VPD during seedling growth or water stress resulted in the accumulation of more proline and glycinebetaine. These effects of VPD during plant growth were independent of changes in leaf water status, an effect similar to 'hardening', but the effect of VPD during water stress may have been a result of the rate of water loss. Four barley cultivars grown at a high or low VPD and subsequently water stressed at a common VPD regime had different abilities to accumulate proline and glycinebetaine, such that the proline accumulating capacities of Excelsior and Proctor were in the reverse order in the two VPD regimes.

These results demonstrated the effects of experimental conditions on the metabolism of amino acids and glycinebetaine and offer an explanation for the conflicting responses of the two barley cultivars, Excelsior and Proctor, in proline accumulation when studied by two different groups (Singh *et al.*, 1972; Hanson *et al.*, 1977).

STATEMENT

I hereby declare that the thesis here presented contains no material which has been accepted for the award of any other degree or diploma in any University and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

(BODAPATI PURUSHOTHAMA NAIDU)

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B.P. Naidu

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CHAPTER I
General Introduction

Crop plants of semi-arid and arid regions of the world often fail to attain full genetic potential for yield because of limitations imposed by environmental stresses, especially lack of adequate soil moisture, salinity and unfavourable temperature. All these stresses influence a wide variety of physiological and biochemical processes in crop plants, notable among which are changes in leaf water relations and the accumulation of low molecular weight nitrogenous compatible solutes such as proline and betaines. Much attention has been paid to the physiology and biochemistry of proline accumulation in response to environmental stresses (Aspinall and Paleg, 1981) since the first report of its acumulation in wilted plant tissue (Kemble and Mac Pherson, 1954).

The accumulation of various betaines is now considered to be physiologically important in a number of crop plants, glycinebetaine being the compound which has received most attention. Considerable information is available concerning its accumulation in conditions of water and salt stress (Wyn Jones and Storey, 1981), but none on its response to unfavourable temperature.

With the increasing need to develop suitable cultivars for dryland farming areas, suggestions have been made to select cultivars on morphological, physiological and biochemical parameters. One such suggestion was the use of higher proline accumulating ability as a metabolic measure of grain yield stability in dry areas (Singh *et al.*, 1972). This suggestion has been challenged by Hanson *et al.* (1977) who found different response to that of Singh *et al.* (1972) from the same cultivars during water stress, and formed the conclusion that proline accumulation is a senescence phenomenon which can be related to stress-induced damage.

Glycinebetaine has been hypothesised to accumulate as a non toxic cytoplasmic osmoticum (Wyn Jones *et al.*, 1977) and recently this hypothesis has been supported by Grumet and Hanson (1986) with evidence that glycinebetaine accumulation is genetically controlled and is a component of osmoregulation in salinised barley plants. Grumet and Hanson(1986) considered glycinebetaine to be a very useful metabolite although Hanson and Hitz (1982) concluded that proline was a breakdown product of stress metabolism. However, both compounds accumulate in a similar manner in barley subjected to water or

salt stress (Hanson and Nelson,1978; Storey and Wyn Jones, 1977). Several protective roles at biochemical and organelle level have been suggested for both proline and glycinebetaine during stress (Stewart and Lee, 1974; Storey and Wyn Jones, 1977, 1978a, 1978b; Pollard and Wyn Jones, 1979; Paleg *et al.*, 1981, 1984, 1985; Nash *et al.*, 1982; Jolivet *et al.*, 1982,1983; Jagels, 1983).

The work reported in this thesis is an attempt to find the causes which contribute in part, to the variability in the accumulation of proline and glycinebetaine, and to extend this analysis to barley cultivars of known yield performance in dry areas. The nature and rapidity of stress imposition on the amino acid composition, temperature and relative humidity during seedling growth and water stress, and seed sizes and seed sources have been tested as potential factors causing variability in solute accumulation, since some of these factors are known to have differed in the work of different groups. This examination was intended not only to explain the observed discrepancies between the results of different workers (Singh *et al.*, 1972 ; Hanson *et al.*, 1977) but also to further explore the use of metabolic factors in varietal selection for drought resistance.

CHAPTER II
Literature Review

1 Environmental Stresses

1.1 Introduction

Drought, chilling, freezing, heat, salinity, flooding, heavy wind, shade, nutrient deficiency, air pollution and radiation constitute the environmental stresses to which plants may be exposed (Levitt, 1980). Of the major climatic variables water and temperature are the most unpredictable (Christiansen, 1979). Considerable attention has been paid to the physiology and biochemistry of crop plants in the process of developing reliable cultivars for these unpredictable environments.

1.2 Definition

It has now been accepted by many workers that biological stress (caused by environment) can be defined in terms analogous to physical stress and strain as proposed by Levitt (1972). However, biological stresses differ from physical stresses in two main ways. First, since the plant is able to erect barriers between its living matter and the environmental stresses, the stress must be measured not in units of force but in units of energy. Second, the term in biology always has a connotation of possible injury i.e., of irreversible or plastic strain. Levitt (1972) defined a biological stress as "Any environmental factor capable of inducing a potentially injurious strain in living organisms". It is commonly understood that stress caused by water-deficiency is called water (moisture) stress. It is not necessary that physical definitions should apply to the biological systems; Taylor (1968) defined water stress without consideration of stress and strain theory of physics - " Whenever the conditions of water are unfavourable to optimum plant growth, the plant is said to be under water stress". These definitions can be extended to cold stress - "The number of degrees that the environmental temperature is below optimum (only cool enough to cause injury but not cool enough to freeze the plant) for the plant activity being measured" (Levitt, 1980). High temperature or heat stress for any organism increases with temperature above the lowest one that imposes a stress (Levitt,1980).

Drought, cold or heat stress can each result in tissue water deficit but, as well,

each stress may have an effect on specific plant processes in addition to this dehydration effect (Levitt, 1972, 1980).

2 The physical status of plant tissues during environmental stress

2.1 Water status

Water is an essential component of plant life. It comprises approximately 85 to 90% of the total fresh weight in physiologically active herbaceous plants. If the water content in most species falls below a threshold level, many of the physiological activities are impaired. Hence, the quantitative measurement of the water status of plants is of great importance (Kramer, 1980).

2.1.1 *Measurement of the water status of plants*

The two basic parameters which describe the degree of unsaturation, i.e. the plant water deficit, are (i) the water content and (ii) the energy status of water in plants.

2.1.1.1 The water content

The acceptable method of measuring water content is as a function of water content at full turgidity i.e. the relative water content (RWC).

$$RWC = \frac{\text{(fresh weight - dry weight)}}{\text{(turgid weight - dry weight)}} \times 100$$ (Weatherley, 1965).

Water saturation deficit (WSD) is measured in a similar way to that of RWC but is numerically different and is defined as: WSD = 100-RWC.

2.1.1.2 The energy status

The energy status of the water in plants is expressed as the total water potential (ψ). It is best understood as the capacity of the water to do work, i.e. to move from a higher to a lower potential energy (Taylor and Slatyer, 1961; Kramer, 1969). Water potential (ψ) is defined as the difference in the chemical potential of water in a system and

pure free water at atmospheric pressure and the same temperature (Taylor and Slatyer, 1961). Since a difference in temperature has a complicated effect on ψ, temperature variation should be avoided during measurements (Taylor, 1968). ψ is quoted in pressure units (megapascals, MPa or bars).

ψ can be analysed in terms of its component potentials (Taylor and Slatyer, 1961; Boyer, 1969).

$$\psi = \psi_g + \psi_s + \psi_m + \psi_p$$

ψ_g - gravitational potential,

ψ_s - solute (osmotic) potential,

ψ_m - matric potential,

ψ_p - turgor potential.

As ψ_g is only 0.01 MPa m^{-1}, it can be neglected in herbaceous plants (Boyer, 1969), although, it is important in tall trees (Conner *et al.*, 1977). Both osmotic and matric forces reduce potential and are negative. Distinguishing between ψ_s due to dissolved solutes and ψ_m due to adsorption and surface tension is difficult.

ψ_p is normally equivalent to the positive hydrostatic pressure in the cell. As water becomes limiting and ψ falls, ψ_p also falls rapidly. Wilting is a visible sign of low ψ_p in the plant (Slatyer, 1969).

The measurement of ψ of plant tissue has now become routine with the development in the 1950's and 1960's of two basic instruments for its measurement, viz. (i) the thermocouple psychrometer: the Peltier psychrometer (Spanner, 1951) and wet loop psychrometer (Richards and Ogata, 1958) and (ii) the pressure chamber (Dixon, 1914; Hains, 1935; Scholander *et al.*, 1965).

2.1.2 *Effect of water shortage*

Living cells need to be more or less saturated with water to function maximally but are not usually in this condition. Plant water deficits can be caused by an excessive rate of water loss or too slow absorption or both. When plants of most species suffer a water deficit a common response is a reduction in the water status of the tissue accompanied by a decline in ψ. This may be due primarily to a decrease in ψ_s or to a decrease in ψ_p or,

more commonly, to a combination of both (Chu *et al.*, 1974, 1976).

In any part of the plant cell under equilibrium conditions ψ is the sum of ψ_s, ψ_p and ψ_m (as indicated in the section 2.1.1.2 of this chapter); however, the components of ψ may differ. In the vacuole ψ is determined by osmotic and turgor forces, whereas in the wall, it arises largely from matric forces and to a small degree from osmotic forces. Thus the total water potential of the plant cell is given by:

$$\psi = \psi_s + \psi_p \quad = \psi_s + \psi_p + \psi_m \quad = \psi_s + \psi_m$$

in the vacuole — in the cytoplasm — in the wall

In practice, ψ_m in the cytoplasm can be resolved into either a pressure term or osmotic term and ψ inside the plasmalemma can be considered to arise essentially from osmotic and pressure components (Passioura, 1980). Hence, the general equilibrium water relation of plant cell can be written as:

$$\psi = \psi_s + \psi_p$$

With fully turgid tissue, a decrease in tissue water content causes a decrease in ψ. In this phase of dehydration, decreases in ψ_p are usually much more marked than decreases in ψ_s and account for the major part of the diminution of ψ. After more water is lost and ψ_p falls to a negligible level, decreases in ψ_s alone account for the further decrease in ψ (Gardner and Ehlig, 1965). It has been generally assumed (Slatyer, 1967) that the wilting point corresponds to zero ψ_p in the leaves when $\psi = \psi_s$. However, this has been found to be other than zero and to vary from species to species, -3.2 MPa for barley (Miller *et al.*, 1970), -1.9 MPa for cotton and -2.24 MPa for sunflower (Gardner and Ehlig, 1965). It may also vary within the species depending on differences in osmotic adjustment (Henson *et al.*, 1982; Morgan *et al.*, 1986) (discussed in section 4.1.1 of this chapter). Some dicotyledons show wilting even at a ψ_p of 0.2 or 0.3 MPa (Gardner and Ehlig, 1965) and rice at approximately 0.3 MPa (Tomor and Ghildyal, 1973). In the latter case wilting was associated with a marked change in the elastic properties of the leaf tissue.

Partly because of the importance of the total ψ on the driving force for water movement, and partly because of relative ease of measurement, ψ and RWC have gained

prominence as measures of plant water status in the past. However, there is no proof that ψ has any direct effect on physiological processes. Indeed, it is increasingly evident that it is the turgor and osmotic components of ψ that are physiologically active (Wiebe, 1972; Oertli, 1976; Hsiao *et al.*, 1976) during water stress.Turgor may not always be important as plant growth in barley was limited in spite of adequate turgor maintenance (Termaat *et al.*, 1985). Flower and Ludlow (1986) suggested that measurement of RWC is important to measure dehydration tolerance rather than ψ or ψ_p since pigeonpea leaves with different rates of osmotic adjustment died at water potentials between -3.4 MPa and -6.3 MPa, but all leaves died at the same RWC (32%). Due to the perceived lack of relationship between leaf water status (eg. ψ_p) and many physiological processes, Turner (1986), in a recent review, has raised the possibility that biological processes are more closely correlated with soil water potential or content rather than ψ or ψ_p of the leaf (Turner *et al.*, 1985; Gollan *et al.*, 1986). Here, a role for roots has been proposed as the sensing organs for water deficit and for phytohormones as messengers which control physiological responses in the shoot.

2.1.3 *Effects of temperature extremes*

2.1.3.1 High temperature

Plant response to supra-optimal temperature is frequently associated with water stress. The work of Lahiri and Singh (1969) and of Mattas and Pauli (1965) shows that when plants are exposed to supra-optimal temperatures the plant water status remains constant for an initial period and then declines sharply. This is due to direct effects of temperature on the diffusion constant of water, the steepening of the vapour pressure gradient between the leaf and atmosphere (Levitt, 1980) or effects on stomatal aperture (Barbaltchuk and Tcherniavskaya, 1974). All these processes increase transpiration and result in water deficit. Additionally, root temperatures above 30°C have been found to reduce water absorption by lemon (Bialoglowski, 1936) and grapefruit (Haas, 1936) also contribute to leaf water deficit during high temperature stress. However, growing plants in conditions of high humidity could, presumably, allow investigation of the effects of

high temperature uncomplicated by tissue water deficit. When Petinov and Razmaev (1962) and Chu *et al.* (1974) investigated the effects of a brief exposure to high temperature (up to 40°C) under conditions of low (<0.74 KPa) or high (3.68 KPa) vapour pressure deficit (VPD), there were no substantial changes in leaf water status at low VPD.

2.1.3.2 Low temperature

Sachs (1864; see Molisch, 1896) reported for the first time that tobacco and cucumber began to wilt due to the development of secondary water stress if their roots were cooled to just above zero. Sugarcane is more sensitive and wilts if its root temperature drops to 15°C. This may lead, eventually, to death by desiccation. Wheat plants wilt at 10°C due to low leaf water potential (Frank *et al.*, 1973) and cotton roots were unable to absorb water at temperatures below 10°-12°C (St. John and Christiansen, 1976). Molisch (1896) concluded from his experiments that the seat of injury was the roots which cannot absorb water sufficiently rapidly to keep up with transpiration loss even though this is also decreased.

Kramer (1942) found that low temperature decreased water absorption more in chilling-sensitive than in chilling-resistant crops. He concluded that this decreased absorption was due to a chilling-induced decrease in root permeability. Evidence in favour of this interpretation has now been produced by Kaufmann (1975). In cold sensitive citrus plants, cooling markedly lowered the permeability of roots to water which then became limiting; but no such lowering was found in the roots of the cold-tolerant spruce. In contrast to root permeability, increased permeability of leaf cell membranes has been reported in response to chilling (Lyons, 1973) and this increased permeability has been related to greater water loss from the cells which may lead to a situation in which symptoms of drought would occur (Wright and Simon, 1973). However, the possibility of a reduction in ψ due to low temperature was ruled out in barley and radish (Chu *et al.*, 1974) and in alfalfa seedlings (Parameshwara, 1984).

2.2 Tissue temperature

Changes in plant tissue temperature have been noted in plants exposed to drought and temperatures extreme. Lack of transpirational cooling due to stomatal closure causes the rise in tissue temperature of droughted plants (Smith, 1978). The temperature of *Xanthium strumarium* leaves in which closed stomata caused reduced transpiration, was 6-10°C above the temperature of similar leaves that had open stomata and were transpiring normally (Drake and Salisbury, 1972). Tanner (1963) reported that the temperature difference between well-watered and water stressed potatoes was 1.5 to 3.0°C and that the temperature of potatoes rose 11.5°C above air temperature as a result of water stress on stomatal control of transpiration. Ehrler *et al.* (1978b) reported that droughted wheat had up to 7°C greater leaf to air temperature difference than the well irrigated plots. However, in many cases the rise in leaf temperature accompanying substantial reduction in transpiration has been measured or calculated to be only a few degrees (Gale and Hagan, 1966; Poljakoff-Mayber and Gale, 1972). Hence, Hsiao (1973) concluded that elevation in leaf temperature does not play an important role in water stress effects. However, it seems likely that the importance of higher temperature will depend on the individual plant species and on the circumstances or plant organ.

2.2.1 *Measurement*

Microthermocouples can be used successfully to measure leaf temperature on a small scale; such instrumentation is limited to ground use and individual leaves (Ehrler *et al,* 1978a). Infrared (IR) thermometers have also been very useful for this. IR was first developed for use in plant communities by Fuchs and Tanner (1966), but several modifications and developments have taken place subsequently. The currently available IR thermometers are portable, hand operated at ground level, or can be installed in an aircraft or satellite to be used from great distances; temperature measurements can be made with a 0.5°C accurracy. Blum *et al.* (1978) measured leaf water deficit or leaf temperature indirectly using aerial IR photography, based on the principle that leaf reflectance in the near IR spectrum is affected, among other factors, by leaf water status.

3 Biological responses to stress

There are very few biological processes which do not change when the plant undergoes environmental stress (Hsiao, 1973; Hsiao *et al.*, 1976; Aspinall, 1986). However, the sensitivity of each response to mild or moderate stress varies.

3.1 The accumulation of solutes

Notable among the metabolic changes in plants under various environmental stresses is the accumulation of low molecular weight solutes such as amino acids, betaines or sugars and polyols depending on the organism. Amino acids and other soluble nitrogenous compounds play essential roles in plant metabolism being the primary products of inorganic nitrogen assimilation and precursors of proteins and nucleic acids and, hence, there has been much interest in the influence of environmental stresses on their metabolism and accumulation.

3.1.1 *Amino acids*

3.1.1.1 Proline

Water stress. Proline was first noted to accumulate in wilted plant tissue by Kemble and MacPherson (1954) in experiments with excised perennial rye-grass. Proline is also known to accumulate in bacteria (Tempest *et al.*, 1970; Measures, 1975), algae (Besnier *et al.*, 1969; Brown and Hellebust, 1978) and in higher plants (e.g. Palfi *et al.*, 1973). In higher plants proline accumulation has been demonstrated in many different species. Proline content was reported as high as 10% of leaf dry weight of halophytes (Stewart and Lee, 1974) and to undergo a 10-100 fold increase in water stressed *Cyanodon dactylon* shoots (Barnett and Naylor, 1966). Proline accumulation has been reported for many crop plants: rice (Mali and Mehta, 1976), barley (Singh *et al.*, 1973a; Joyce *et al.*, 1984; Riazi *et al.*, 1985), wheat (Rajagopal *et al.*, 1977; Munns *et al.*, 1979; Quarrie, 1980; Karmanos *et al.*, 1983; Monneveux and Nammer, 1986), maize (Carcellar and Fraschina, 1980), sorghum (Blum and Ebercon, 1976; Parameshwara and Krishnasastry, 1980), radish (Chu *et al.*, 1974), and soybean (Singh and Gupta, 1983).

More than 50 species of plants from 14 families have been catalogued to accumulate proline (see review by Aspinall and Paleg, 1981). However, proline accumulation is not a universal response to environmental stress.

The accumulation of proline in these various microorganisms and higher plants has been thought to be initiated by the reduction in the water potential of the cell or tissue. In barley the content of proline in the leaves at any time is correlated with the length of exposure to water deficit, the water potential of the leaves, and the amount transported from leaves to other tissue (Singh *et al.*, 1973a). The lower limit of the threshold water potential for the response has been estimated as -0.7 MPa (see Aspinall and Paleg, 1981) while for cotton it was -1.0 MPa (McMichael and Elmore, 1977). However, recent experimental evidence suggests that cell turgor or volume, or osmotic potential, signals the initiation of proline accumulation rather than the bulk water potential. Greenway *et al.* (1982) concluded from their experiments on the fresh water alga *Chlorella emersonii* that very small changes in turgor or cell volume may elicit maximum activation of enzymes involved in the synthesis of proline. When the cell penetrating but non-metabolisable osmoticum, 3-*O*-methyl glucose, is fed to salinised algal cells to maintain tugor, the accumulated proline was depleted. However, work on salinised barley plants (Chu *et al.*, 1976) and on water stressed tomato cell cultures (Handa *et al.*, 1986) disagree with these findings. Initiation of proline accumulation in these higher plants appears to depend on the osmotic potential of the tissue rather than turgor. For instance, transfer of cultured tomato tissue to a lower water potential environment resulted in the initiation of proline accumulation accompanied by a drop in turgor, but proline levels continued to increase even after turgor was regained during osmotic adjustment , supporting the belief that osmotic potential controls the accumulation of proline (Handa *et al.*, 1986).

In addition to such changes in cell water relations, Pesci and Beffagna (1985) and Goring and Plescher (1986) have suggested that proline accumulation could be considered as a metabolic response to cell wall-penetrating weak acid-induced (ABA, isobutyric acid, acetic acid, or indole acetic acid) acidification of the cell sap. Water stress has indeed been shown to lower cytoplasmic pH (Goring and Zoglauer, 1979; Goring, 1981) possibly due

to the accumulation of ABA (Aspinall *et al.*, 1973; Aspinall, 1980; Parameshwara, 1984; Stewart and Voetburg, 1985). However, the recent work of Stewart and Voetburg (1987) demonstrates that ABA accumulation is not a *per se* pre-requisite for proline accumulation in wilted leaves. When barley seedlings were grown with and without fluridon, a potent inhibitor of ABA accumulation, treated as well as non- treated plants accumulated proline when subjected to stress. Similarly, both wilty tomato (with no ability to accumulate ABA during drought) and wild type (which accumulates ABA) accumulated proline in response to drought. Even if acidification of cytoplasm is the triggering factor for proline accumulation (Pesci andBeffagna 1985; Goring and Plescher, 1986) then ABA alone is unlikely to be the mediator (Stewart and Voetburg, 1987).

Proline accumulates in all organs of the intact plant, although the accumulation is more rapid and extensive in leaves (Barnett and Naylor, 1965; Singh *et al.*, 1973b). Isolated organs of barley showed maximum proline accumulation in leaves, some in leaf sheaths and none in roots or shoot apices (Singh *et al.*, 1973b). Such findings led to the suggestion that proline accumulated in the roots of stressed intact barley plant had been translocated from the leaves. This was supported by the observation that supply of precursors of proline synthesis to excised and stressed barley roots could not induce proline accumulation, although a similar supply to etiolated or albino leaves did initiate proline accumulation in barley (Singh *et al.*, 1973b). Similar results were obtained with isolated, dehydrated organs of wheat, paprika and sunflower where there was no proline accumulation in roots and stems, but accumulation to a very high concentration in the lamina (Palfi, 1971). In contrast to these findings the work of Parameshwara (1984) shows extensive accumulation of proline in response to environmental stress in nodulated alfalfa roots suggesting that nodules were a major contributing factor in proline accumulation in nodulating plants, although the site of synthesis has not been clearly worked out.

Temperature extremes. Proline accumulates not only in response to water deficit but also in response to temperature extremes (Chu *et al.*, 1974). When plants were subjected to

high temperature (35°C) under otherwise normal environmental conditions, plants accumulated proline; but this accumulation was accompanied by a fall in leaf water potential. If this concomitant change in leaf water potential was prevented by maintaining plants in a saturated atmosphere, no proline accumulated at this temperature (Chu *et al.*, 1974). This suggests that high temperature *per se* may have no effect on the accumulation of proline.

Various studies have shown that the soluble nitrogen content of plants increases at low temperature (Zech and Pauli, 1960), such an increase is confined to specific components, such as proline, rather than being a general increase in all fractions. Chilling temperatures resultd in the accumulation of proline in barley and radish (Chu *et al.*, 1974, 1978), sunflower, bean and paprika (Palfi and Juhasz, 1970), wheat, oat (Kinbacher, 1960), stylo (Gates *et al.*, 1971), alfalfa seedlings (Parameshwara, 1984) and maize callus (Duncan and Widholm, 1987). Accumulation of proline has been reported to occur in *Citrus* during cold hardening (Yelenosky, 1979) and in *Nothofagus dombeyi* during the cold season of the year (Meza-Basso *et al.*, 1986).

In contrast to both water and high temperature stress, the initiation of proline accumulation in response to cold stress cannot be ascribed to any change in plant water status (Chu *et al.*, 1974; Parameshwara, 1984) not withstanding a high water status. It is clear that proline accumulates below 8°C in barley (Chu *et al.*, 1974, 1978) and at 5°C in alfalfa seedlings (Parameshwara, 1984). Low temperature-induced proline accumulation also differs from water stress-induced accumulation, since it is completely light dependent and not immediately reversible (Chu *et al.*, 1974). However, Aspinall and Paleg (1981) were of the opinion that, despite these differences in response, it is possible that a common accumulation mechanism exists. The relationship between water and macromolecules, and the hydration of membranes are similarly responsive to both a net reduction in hydration and a lowering of tissue temperature (Aspinall and Paleg, 1981). Such changes may not be observed by measurements of bulk tissue water parameters.

3.1.1.2 Other amino acids

Water stress. Although much attention has been paid to proline accumulation in water stressed plants, changes occur in other amino acids. In some species these are quantitatively more important than those occuring in proline. Many water stressed plants accumulate asparagine (Thompson *et al.*, 1966; Barnett and Naylor, 1966; Singh *et al.*, 1973a; McMichael and Elmore, 1977; Munns *et al.*, 1979; Thakur and Rai, 1982; Drossopoulos *et al.*, 1985) and in some species the other amide, glutamine, accumulates (Singh *et al.*, 1973a; Munns *et al.* 1979; Drossopoulos *et al.*, 1985). The level to which amides accumulate is, in some species, similar to that observed for proline. In terms of plant nitrogen such accumulation may deserve equal importance (McMichael and Elmore, 1977).

Other amino acids generally increase in water-stressed plants to a lesser extent than proline or the amides. Such reports often concern detached wilted leaves and may be be associated with an inhibition of protein synthesis and an increase in proteolysis (Hsiao, 1973). However, the accumulation of compounds like pipecolic acid (Barnett and Naylor, 1966; Palfi *et al.*, 1974), ornithine (Singh *et al.*, 1973a) and ß-alanine (Barnett and Naylor, 1966) under water deficit cannot be a direct result of proteolysis, since these are non-protein amino acids. In water-stressed barley (Singh *et al.*, 1973a), turnip (Thompson *et al.*, 1966) and Bermuda grass (Barnett and Naylor, 1966), a very pronounced decrease in aspartic and glutamic acids has been reported. These decreases could result from substrate depletion, glutamate being metabolised to proline and aspartate to asparagine. In many water stressed plants alanine has also been reported to decline (Barnett and Naylor, 1966; Singh *et al.*, 1973a). However, Mokronosov *et al.* (1973), Munns *et al.* (1979) and Drosopoulos *et al.* (1985) have reported an increased level of alanine in response to water stress, and Pulich, (1986) reports that alanine accumulates in *Halophila engelmanni* Aschers, a tropical sea grass, in response to salinity.

Temperature extremes. The concentrations of other amino acids, besides proline, increase in plants grown at low temperatures. In *Lolium perenne*, serine, glycine, and

glutamine increased (Draper, 1972) while in barley and radish the only amino acid to show any marked increase was alanine. Asparagine, valine, α–aminobutyric acid and isoleucine showed a slight increase whilst glutamic acid, aspartic acid and methionine decreased (Chu *et al.*, 1974). Wallace *et al.* (1984) demonstrated very rapid accumulation of γ-aminobutyric acid and alanine in several plant species, including barley, in response to cold stress. Alanine and glutamine have been reported to accumulate in alfalfa seedlings at low temperature (Wilding *et al.*, 1960). In passionfruit, alanine increased in the initial 10 h of chilling. Accumulation continued beyond that time only in chilling-sensitive species, while in chilling-resistant species the levels returned to normal levels in 46 h (Patterson *et al.*, 1981). Glutamate levels declined in these plants.

3.1.2 *Betaines*

In the past decade there has been increasing interest in the physiology and biochemistry of betaines in relation to drought and salinity stress. Betaines are fully N-methyl substituted amino acids. Individual betaines are named by reference to their parent amino acid (e.g. N,N',N''-trimethyl glycine, glycinebetaine), except for those compounds produced by methylation of the ring nitrogen of pyridine where there is no root amino acid (e.g. trigonelline). Thirteen betaines have been listed and their taxonomic distribution reviewed by Wyn Jones and Storey (1981). Of the wide variety of betaines found in plants, glycinebetaine was the first to be isolated and studied extensively . Since wheat and barley are known to accumulate glycinebetaine alone, although containing a low concentration of choline which does not respond to stress (Storey and Wyn Jones, 1977), this literature review is restricted to glycinebetaine.

Glycinebetaine was first isolated by Husemann and Marme (1863) (for reference see Karrer, 1958) from the arid zone shrub, *Lycium barbarum*. It is structurally the simplest of the betaines and often occurs in large quantities. Glycinebetaine seems to occur universally in chenopods (Wyn Jones and Storey, 1981; Poljakoff-Mayber *et al.*, 1987) and is common in some tribes of grasses (e.g. Hordeae); although plants in the tribe Maydae accumulate very low quantities (Hitz and Hanson, 1980). Glycinebetaine

has been reported to accumulate in response to water deficit or salinity in barley (Storey and Wyn Jones, 1978a; Hanson and Nelson, 1978), wheat and oats (Storey and Wyn Jones, 1977), corn, sorghum, millet and rice (Hitz and Hanson, 1980).

Glycinebetaine is found in both shoots and roots; root concentrations are usually lower than shoot concentrations in mature plants but young plant roots contain high glycinebetaine concentrations (Storey and Wyn Jones, 1977; Jefferies *et al.*, 1979; Hanson and Wyse, 1982). Cromwell and Rennie (1953) showed that the glycinebetaine content of *Beta vulgaris* leaves generally increases with age, reaching a maximum at flowering, even though the root level declines. Wheat anthers and pollen contain high concentrations of glycinebetaine and its precursor choline (Pearce *et al.*, 1976). Glycinebetaine also occurs in seeds, for example, cotton seed (Pollock and Stevens, 1965). Very high concentrations occur in mature wheat aleurone and embryo tissues but not in the starchy endosperm . The glycinebetaine content of the aleurone layer was much higher than the total content of amino acids. The amount of glycinebetaine did not change during the first few days of germination but both it and the amino acid pool decreased dramatically with gibberellic acid treatment after 4 days of germination (Chittenden *et al.*, 1978).

Glycinebetaine accumulates to about five fold in response to water deficit and several fold in response to salinity in leaf tissues of grasses and chenopods (Storey and Wyn Jones, 1977; Wyn Jones and Storey, 1978a; Hanson and Scott, 1980; Hitz and Hanson, 1980; Pan *et al.*, 1981; Grattan and Grieve, 1985; Grumet and Hanson, 1986). Glycinebetaine has been reported to accumulate to 100-200 µmol (11.7-23.4 mg) g dry wt^{-1} in water stressed barley leaves (Wyn Jones and Storey, 1978a; Hitz *et al.*, 1981) at the rate of 0.4-0.7 µmol g dry wt^{-1} h^{-1}. Increase in glycinebetaine content has only been recorded following atleast 24 h exposure to stress (Wyn Jones and Storey, 1981) whereas proline accumulates within tens of minutes (Singh *et al.*, 1973a). No threshold leaf water potential has been computed for glycinebetaine accumulation. In salinised barley, however, glycinebetaine accumulates at a leaf osmotic potential of -1.1 MPa (Grumet and Hanson, 1986).

3.1.3 *Sugars and polyols*

Sugars and polyols accumulate in some stressed lower organisms and higher plants, in addition to or in place of nitrogenous compatible solutes. Since this subject matter is beyond the scope of this thesis, the accumulation of carbohydrates has been reviewed only briefly.

An increase in the sugar content of leaves, at the expense of the starch content, has been reported during stress (Iljin, 1957; Stewart, 1971; Munns *et al.*, 1979; Munns and Wire, 1982; Delane *et al.*, 1982; Munns and Termaat, 1986; Timpa *et al.*, 1986). Glucose is the principal sugar in expanding leaves and sucrose in matured leaves of wheat (Munns and Weir, 1981). Such a stress-induced increase in sugar content has been attributed to either amylase activity (Spoehr and Malner, 1939) or decreased invertase activity (Maranville and Paulsen, 1970). Hsiao (1973) disputed the former explanation on the grounds that increased amylase activity would lead to the accumulation of equal amounts of glucose and fructose, but in many cases glucose alone or glucose and sucrose were the sugars found.

Apart from simple sugars, polyols, or sugar alcohols, are known to accumulate in various organisms undergoing stress. The term polyol refers to a group of carbohydrate-derived compounds containing 3 or more hydroxyl groups and include glycerol, alditols (sorbitol and mannitol) and sterioisomers of inositol (Stacey, 1974). The reducing group (CHO or CO) of a monosaccharide is replaced by an alcohol group in the formation of a simple polyol. Accumulation of glycerol and alditols is common in microorganisms and lower plants (Borowitzka, 1981) but, a higher plant halophyte, *Plantago maritima,* has also been reported to accumulate sorbitol under saline conditions (Ahmad *et al.*, 1979). Accumulation of various inositols in higher plants has been reported by several workers (Loewus and Loewus, 1983). Ford and Wilson (1981) noted a threefold increase in pinitol (1D-3-*O*-methyl-Chiro-inositol) in water stressed *Macroptilium atropurpureum*, a tropical legume. Extension of these results to *Vigna* species indicated that all the species surveyed accumulated *O*-methyl-inositols during water stress (Ford, 1982). Pinitol has been most frequently reported (*O*-methyl-inositol) (Ford, 1984; Gorham *et al.*, 1984) but,

ononitol (1D-4-*0*-methyl-myo-inositol) and *0*-methyl-scyllo-inositol have also been reported in a few legume species (Ueno *et al.*, 1973; Ford, 1984). It has been suggested that the accumulation of inositols is a general characteristic of legumes during water stress and that the species accumulating pinitol were more resistant to a low leaf water potential than were legumes accumulating other inositols (Ford, 1984). However, the osmotic role of carbohydrates and polyols in higher plants is not clear because of the lack of compartmentation of these compounds (Wyn Jones and Gorham, 1983; Gorham *et al.*, 1984), although sucrose has this function in *Chlorella emersonii* (Greenway and Setter, 1979).

3.2 The metabolism of proline

The physiology and biochemistry of L-proline (pyrroline-α-carboxylic acid) has been studied intensively in the past 10 to 15 years due to the unique properties that ensure for it a special role in the vital activity of plants. An ability to accumulate proline is apparently the general response to stress of many different species of plants, this response finds reflection in the characteristics of the biosynthesis and metabolism of this compound. Knowledge in this area is essential in an understanding of the contribution made by proline to the stress resistance of the species which accumulate it.

3.2.1 *Biosynthesis*

The two pathways of proline biosynthesis known to be present in higher plants are named from the precursor amino acids, viz., (i) the glutamate pathway (Fig. 1a) and (ii) the arginine and ornithine pathway (Fig. 1b). The differences between them lie in reactions that precede the formation of common key intermediate products, i.e., glutamate L-γ-semialdehyde and the products of its spontaneous cyclization, 1-pyrroline-5-carboxylate.

3.2.1.1 The glutamic acid pathway

Proline biosynthesis proceeds from glutamic acid via the intermediate Δ'-pyrroline-5-carboxylic acid (Fig. 1a). Initially, glutamate kinase (EC 2.7.2.11) converts glutamate to L-γ-glutamyl phosphate in the presence of a phosphate donor such as ATP. Glutamate kinase has been purified from bacteria (Boshinaga *et al.*, 1967; Baich, 1969; Krishna and Lesinger, 1979; Smith *et al.*, 1984). Glutamyl phosphate reductase (EC 1.2.1.41) then catalyses the oxidation of L-γ-glutamyl phosphate to glutamate- L-γ-semialdehyde, this step requiring an electron donor such as NADH or NADPH (Baich, 1971). These two reactions are well documented in microorganisms but no cell-free preparation has been obtained from higher plants that catalyses this reaction alone. The glutamate semialdehyde formed undergoes non-enzymic cyclization to form Δ'-pyrroline-5-carboxylic acid (P5C). These two forms are in equilibrium, the cyclic form being the more stable (Strecker, 1960). The final step in the formation of proline in plant tissue is the conversion of P5C to proline in the presence of NADH or NADPH as the electron donor (Thompson *et al.*, 1966; Boggess *et al.*, 1976b). The enzyme involved, Δ'-pyrroline-5-carboxylate reductase (EC 1.5.1.2) has been demonstrated in several plant extracts including barley (Noguchi *et al.*, 1966; Miler and Stewart, 1976; Treichel, 1986).

3.2.1.2 The arginine and ornithine pathway

Biosynthesis of proline in this path starts either directly from ornithine or from arginine (Fig. 1b) which is converted to ornithine by hydrolysis catalysed by arginase (EC 3.5.3.1) producing urea as the end product (Thompson, 1980). Ornithine has been shown to serve as a precursor for proline synthesis in peanut cotyledons (Mazelis and Fowden, 1969) and bean leaves (Stewart and Boggess, 1977). Ornithine can be converted to proline by two possible pathways depending on the amine group of ornithine transaminated (either α or δ), followed by cyclization and reduction. If the ornithine is deaminated at the δ-position, glutamyl-γ-semialdehyde will be formed and the reaction is mediated by L-ornithine-oxoacid aminotransferase (EC 2.6.1.13). The glutamyl-γ-semi-

Fig. 1 Pathways of proline metabolism.

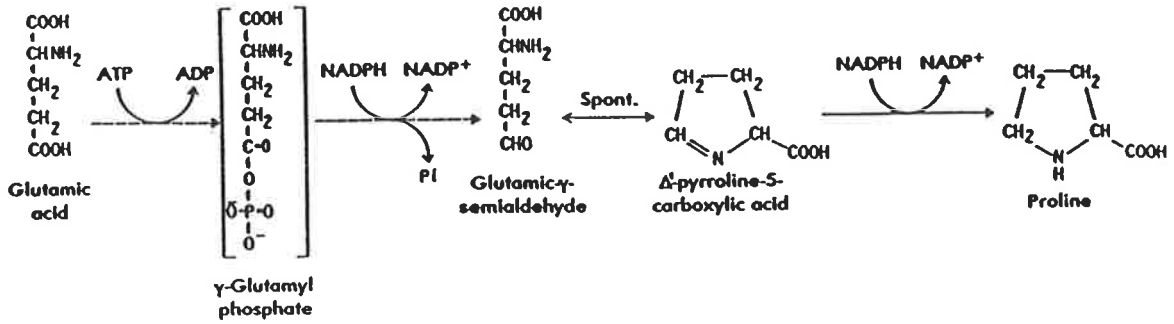
A. The glutamic acid pathway of proline biosynthesis.

B. The arginine and ornithine pathway of proline biosynthesis.

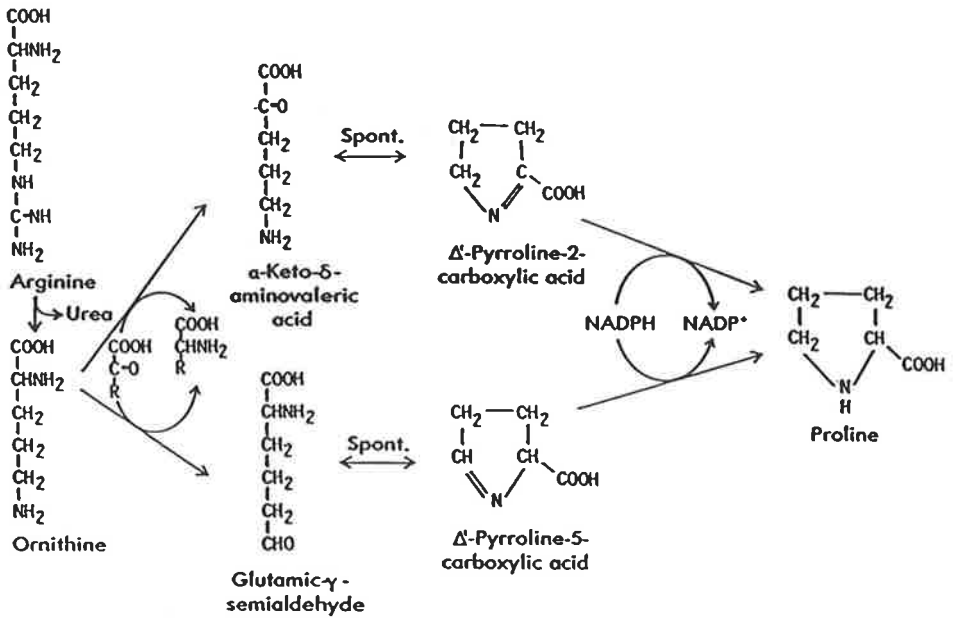
C. The pathway of proline oxidation

See the text for enzymes involved.

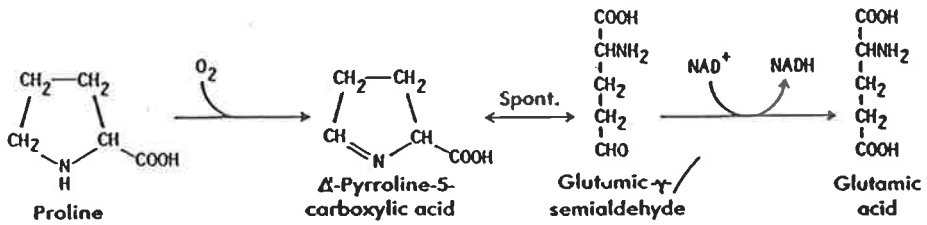
(From Stewart, 1981)



A —— The proline biosynthetic pathway. Solid arrows represent documented reactions. Dotted arrows refer to postulated reactions.



B —— Pathways of conversion of arginine and ornithine to proline.



C —— The pathway of proline oxidation.

aldehyde formed subsequently is cyclized to P5C and reduced to proline by P5C reductase as in the glutamic acid pathway. Much of the literature has assumed that δ-transamination is the route of proline synthesis from ornithine, but without absolute proof (Mazelis and Fowden, 1969; Lu and Mazelis, 1975; Miler and Stewart, 1976).

On the other hand, if the α-amino group of ornithine is transaminated by ornithine-α-transaminase, then α-keto-δ-aminovaleric acid will be formed which then cyclizes to Δ'-pyrroline-2-carboxylic acid. Pyrroline-2-carboyxlate reductase (EC 1.5.1.1) reduces P2C to proline.

The universal operation of δ-transamination of ornithine in higher plants has been questioned by Mestichelli *et al.* (1979) in a study with ^{3}H-ornithine labelled in the 2 or 5 position, and 5-^{14}C-ornithine. For *Nicotiana, Datura* and *Lupinus*, the authors concluded that α-deamination of ornithine is the pathway that converts ornithine to proline. However, several technical and interpretational shortcomings in the work of Mestichelli and co-workers have been pointed out (Adams and Frank,1980). Adams and Frank (1980) and Treichel (1986) suggested instead that formation of P5C would occur in the ornithine pathway, as it is a very important intermediate, rather than P2C which has fewer metabolic roles.

3.2.2 *Utilization of proline in turgid tissue*

Plants have the capacity to oxidize (Fig. 1c) proline (Oaks *et al.*, 1970; Stewart, 1972a; Rena and Splittstoesser, 1974), the oxidation resulting in carbon being fed into the Krebs cycle and eventually respired to CO_2 (Stewart, 1972c). The pathway involves conversion to glutamic acid via the intermediate P5C. P5C is formed either by the action of proline dehydrogenase in the cytoplasm (Mazelis and Fowden, 1971; McNamer and Stewart, 1974) or through proline oxidase in mitochondria (Strecker, 1971; Boggess *et al.*, 1978; Huang and Cavalieri, 1979; Elthon and Stewart, 1981; Xiao-Nan *et al.*, 1986). Once P5C is formed, the second step is spontaneous, identical but opposite to the reaction in proline bisoynthesis. The final step is an oxidative one in which P5C or the open chain form, glutamic-γ-semialdehyde, is oxidized to glutamic acid. The enzyme

involved, Δ'-pyrroline-5-carboxylase dehydrogenase, has been measured in mitochondrial preparations of pea, corn, castor bean and pumpkin seedlings (Stewart and Lai, 1974) and in barley leaves (Boggess *et al.*, 1975).

The other major metabolic fate of proline is in the synthesis of protein (Stewart, 1972a). Proline is incorporated into protein fractions of both cytoplasm and cell walls. The former fraction contains proline as such, whereas in the cell wall proline is first incorporated into protein (extensin) and then converted to hydroxyproline by the action of the enzyme prolyl hydroxylase (Adams and Frank, 1980).

3.2.3 *Regulation of proline levels*

Under normal turgid conditions the intracellular proline level is maintained at a low level but during stress it increases to higher concentrations. Possible metabolic regulation is discussed in this section.

3.2.3.1 In turgid tissue

Microorganisms and higher plants have been shown to exhibit feedback inhibition of proline synthesis at the first reaction step, ie. the conversion of glutamate to glutamyl-γ-aldedehyde. Krishna and Leisinger (1979) have shown a 40% inhibition by 5 mM proline of the enzymes responsible for conversion of glutamate to glutamyl-γ-aldehyde, and complete inhibition by 30 mM proline in *Pseudomonas aeruginosa*. Other reports of feedback inhibition of this first step include diatoms (Liu and Hellebust, 1976), maize roots (Oaks *et al.*, 1970) and barley and tobacco leaves (Boggess *et al.*, 1976a). Similar feedback inhibition of the first step in the ornithine pathway of proline biosynthesis has been discussed in the review of Shevyakova (1983). The second step in the formation of proline, the conversion of glutamyl-γ-semialdehyde to proline proceeds unrestricted in microorganisms (Baich and Pierson, 1965; Krishna *et al.*, 1979) and tobacco (Noguchi *et al.*, 1966). Thus, the only rate controlling step in the biosynthesis of proline in turgid tissue is the conversion of glutamate to glutamyl-γ-semialdehyde.

A stimulation of proline oxidation in the presence of added proline has been

reported in bean leaves (Stewart, 1972a), maize root tips (Oaks *et al.*, 1970), tobacco leaf discs (Boggess *et al.*, 1976a) and barley leaf discs (Boggess *et al.* 1976a; Stewart *et al.* 1977). Proline oxidase has been shown to have increased activity with increased proline concentration, reaching a maximum at 20 to 30 mM, in plant mitochondria (Boggess *et al.*, 1978) . The second enzyme in the oxidative step, P5C dehydrogenase, was not inhibited significantly by 19 amino acids, including proline and glutamate (Lundgren and Ogur, 1973). Thus, Stewart *et al.* (1977) concluded that the proline oxidizing system of barley leaves was not saturated by normal to high proline concentrations. Proline oxidation may therefore serve a regulatory function, acting in concert with the control of synthesis, in maintaining free proline at low levels in turgid tissue .

In normal turgid tissue the incorporation of proline into protein represents the major metabolic fate (Stewart, 1972a) keeping the proline levels low, in addition to the regulation of its synthesis and oxidation.

3.2.3.2 In stressed tissue

Feedback inhibition of the first step of proline synthesis is lost in wilted leaves; in fact proline synthesis from glutamate is stimulated in bean leaves although proline levels are increasing under drought (Stewart, 1972c). Boggess *et al.* (1976b) showed that feeding radioactive ornithine or P5C did not increase proline synthesis unless proline levels were high as a result of previous water stress. Only by feeding ^{14}C-glutamate did they recover labelled free proline. This led to the conclusion that the stimulation of proline synthesis during stress must be through P5C formation rather than its reduction to proline as suggested by Huber (1974). Similar observations have been made with tobacco leaves (Noguchi *et al ., 1968*) and *Eschericia coli* (Baich and Pierson, 1965). However, Treichel (1986) suggested that *de novo* synthesis of P5C reductase occurs in salinized halophytes and that the regulation of this enzyme may also be important in achieving elevated proline levels during stress.

Proline oxidation in water stressed leaves seems to differ from that in turgid tissue. Stewart *et al.* (1977) observed an inhibition of proline oxidation in wilted leaves

after 2 h of wilting which approached 100% (Stewart and Boggess, 1978). This may be due to a loss of compartmentation of the products of proline oxidation so that they leak from mitochondria to the cytoplasm where they may subsequently be converted back to proline. However, the work of Sells and Koeppe (1980) demonstrates that isolated corn mitochondria from water stressed shoots showed a 70% inhibition of proline oxidation compared to control mitochondria. This suggests that mitochondria are also temporarily loosing the ability to oxidise proline in addition to the suggested loss of compartmentation (Stewart and Boggess, 1978). Stewart *et al.* (1977) calculated that if proline oxidation were not inhibited in stressed tissue, proline would only attain half the normally observed levels. Thus, it is clear that inhibition of proline oxidation is a significant contributory factor to proline accumulation under drought stress.

Drought has been shown to inhibit the incorporation of proline into protein (Stewart, 1972c; Hsiao, 1973; Stewart *et al.*, 1977) and the notion that proline accumulation is a consequence of impaired protein synthesis has been explored (Boggess and Stewart, 1980) by the use of protein synthesis inhibitors. It was concluded that inhibition of protein synthesis alone was not sufficient to cause proline to accumulate. In barley, inhibition of protein synthesis could account for 20% of the accumulated proline (Stewart *et al.*, 1977) but in bean the contribution has been shown to be 70% (Stewart, 1972c); however, bean accumulates 10 to 15 fold less proline than barley.

In summary, the increase in tissue proline levels during drought involves (1) a stimulation of synthesis from glutamate involving loss of feedback inhibition and loss of some subcellular compartmentation, (2) an inhibition of proline oxidation and (3) an impairment of protein synthesis.

3.2.3.3 During stress relief

Several reports indicate that proline accumulated during an episode of water stress is rapidly lost, principally by oxidation to glutamate, once the water deficit is eliminated (Stewart, 1972b; Singh *et al.*, 1973b, 1973c; Blum and Ebercon, 1976; Parameshwara and Krishnasastry, 1980; Xiao-Nan *et al.*, 1986). The rate and extent of the lowering of

proline levels has been shown to be variable depending on crop species and the severity of the water stress. In barley, proline concentration returned to the control level within 48 h (Singh *et al.*, 1973b). In sorghum, however, the recovery was not as complete, and tissue proline concentration remained above the non-stressed concentration for several days (Blum and Ebercon, 1976). This has been attributed to a delay in the recovery of leaf water status (Wample and Bewley, 1975). Even in barley Hanson *et al.* (1977) failed to show complete recovery of tissue proline levels and Lewin (1980) found that proline continued to increase for 5 days after rewatering. In both cases the plants were severely stressed, possibly causing damage to cell membranes, drying of part of the leaf, or damage to the vascular system restricting translocation, all of which may have contributed to the lack of recovery. This is supported by Riazi *et al.* (1985) who found that the proline accumulated in the basal or central area of the barley leaf blade disappeared within 5 h of stress relief whereas even 24 h after stress relief proline concentration remained high in the leaf tip which had dried as a result of the stress treatment.

3.3 The metabolism of glycinebetaine

3.3.1 *Biosynthesis*

Bregoff and Delwiche (1955) and Delwiche and Bregoff (1958) first established with unstressed beet leaf tissue that glycinebetaine synthesis proceeds by a circuitous series of reactions involving serine, ethanolamine, and choline as intermediates rather than by direct methylation of glycine to glycinebetaine. This also seems to be the likely pathway in animals (Paxton and Mayr, 1962) and in several higher plants including wheat (Bowman and Rohringer, 1970), barley (Hanson and Nelson, 1978) and chenopods (Delwiche and Bregoff, 1958; Hanson and Wyse, 1982; Coughlan and Wyn Jones, 1982). The information on the enzymes of the pathway is limited in higher plants, however, and there appears to be minor differences between different families of plants and other organisms.

3.3.1.1 The pathway of glycinebetaine biosynthesis

The biosynthesis of glycinebetaine (Fig. 2) starts with serine derived from the condensation of glycine and formate or formaldehyde (Sakami, 1948), glycine serving as a source of the ß-carbon of serine (Elwyn *et al.*, 1955; Delwiche and Bregoff, 1958). Ethanolamine is derived as a unit from serine molecules in the presence of the enzyme serine decarboxylase. This was confirmed by the inability of ^{14}C-formate and 1-^{14}C-glycine to contribute label to ethanolamine, and by the ability of uniformly labelled serine and 2-^{14}C-glycine to form ethanolamine (Delwiche and Bregoff, 1958).

In mammalian liver, in microorganisms, and in plants, the substrates for the methylation steps leading to the formation of choline have been considered to be the phosphatidyl derivatives of serine and the ethanolamines (Greenberg, 1969; Kates and Marshall, 1975). In that case turnover of these phospholipids must be involved in net choline or glycinebetaine synthesis (Hanson and Hitz, 1982). However, radiotracer studies on water stressed barley (Hanson and Hitz, 1981; Hitz *et al.*, 1981), salinised sugar beet (Hanson and Hitz, 1981; Hanson and Wyse, 1982), and spinach (Coughlan and Wyn Jones, 1982) all indicate that the stepwise methylation of ethanolamine involves water-soluble intermediates, probably phosphate esters of the bases (phosphorylmonomethylethanolamine, phosphoryldimethylethanolamine and phosphorylcholine). Grasses and chenopods process the product of the methylation sequence, phosphorylcholine, in different ways. In barley phosphorylcholine is first incorporated into the phospholipid, phosphatidylcholine (PC), before free choline is released by a phospholipase-D reaction; whereas, in sugar beet, phosphorylcholine is hydrolysed directly to free choline (Hitz *et al.*, 1981; Hanson and Rhodes, 1983). The choline thus formed is oxidised to glycinebetaine aldehyde, the reaction being catalysed by choline dehydrogenase. This enzyme has been shown to be located in chloroplasts (Hanson and Grumet, 1985) as compared to its location in mitochondria or peroxisomes in animal tissue (Nagasawa *et al.*, 1975; Tsuge *et al.*, 1980). Glycinebetaine aldehyde is oxidised to glycinebetaine, catalysed by the enzyme glycinebetaine aldehyde dehydrogenase (Pan *et al.*, 1981; Weigel *et al.*, 1986).

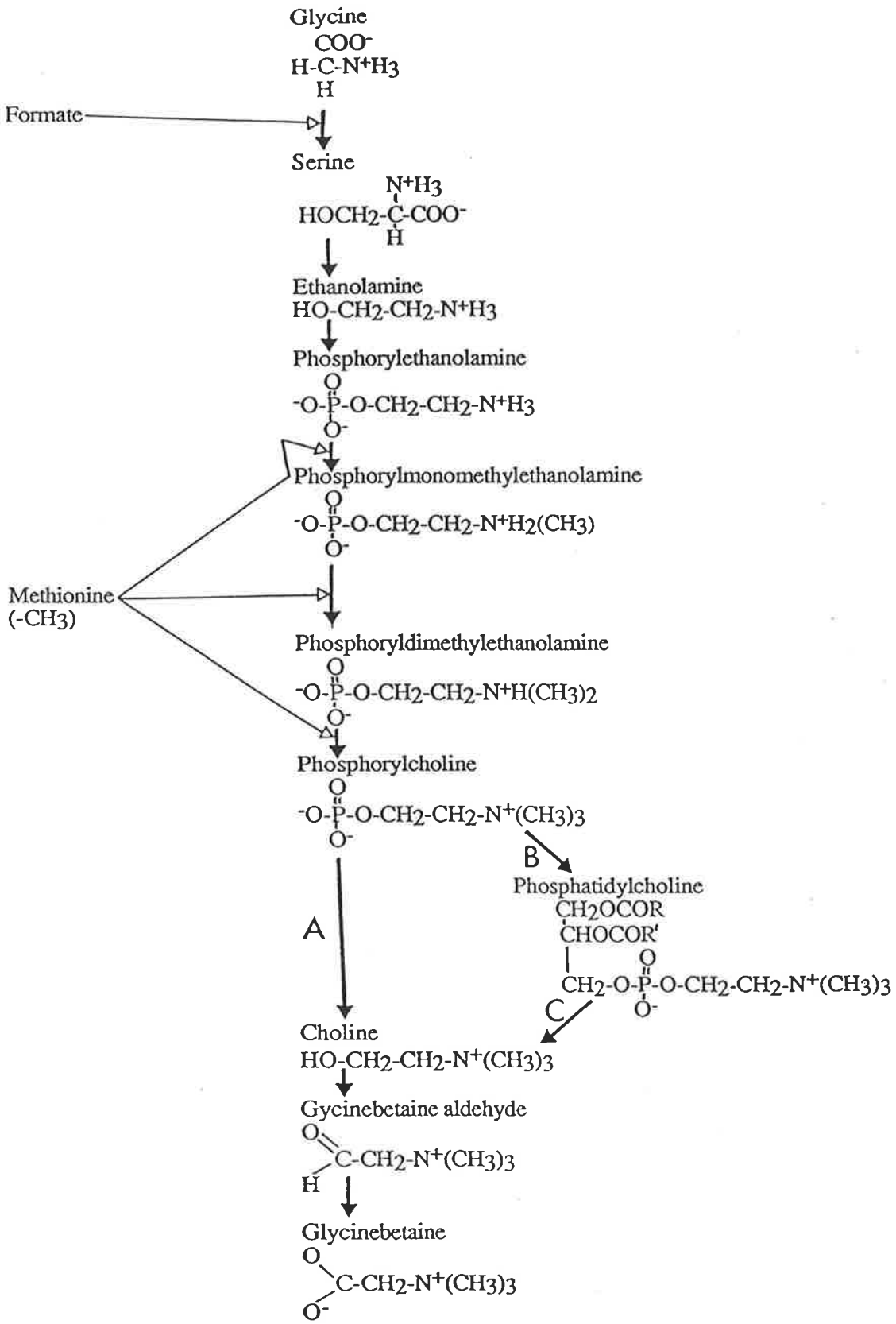
Fig. 2 The biosynthetic pathway of glycinebetaine.

Step A occurs in chenopods.

Steps B and C occur in grasses.

See the text for enzymes involved.

(Adapted from Wyn Jones and Storey, 1981; Hanson and Grumet, 1985)


Glycine
COO⁻
H-C-N⁺H3
H
Formate
Serine
N⁺H3
HOCH2-C-COO⁻
H
Ethanolamine
HO-CH2-CH2-N⁺H3
Phosphorylethanolamine
O
-O-P-O-CH2-CH2-N⁺H3
O⁻
Phosphorylmonomethylethanolamine
O
-O-P-O-CH2-CH2-N⁺H2(CH3)
O⁻
Methionine
(-CH3)
Phosphoryldimethylethanolamine
O
-O-P-O-CH2-CH2-N⁺H(CH3)2
O⁻
Phosphorylcholine
O
-O-P-O-CH2-CH2-N⁺(CH3)3
O⁻
A
B
Phosphatidylcholine
CH2OCOR
CHOCOR'
O
CH2-O-P-O-CH2-CH2-N⁺(CH3)3
O⁻
C
Choline
HO-CH2-CH2-N⁺(CH3)3
Gycinebetaine aldehyde
O
C-CH2-N⁺(CH3)3
H
Glycinebetaine
O
C-CH2-N⁺(CH3)3
O⁻

3.3.2 *Regulation of glycinebetaine levels*

3.3.2.1 In turgid tissue

In turgid leaves of barley and other crop plants glycinebetaine levels are usually 10 times lower than in stressed leaves and, provided the plant does not experience stress, the levels remain stable. The mechanism(s) by which these levels are maintained has not been thoroughly explored . However, radiotracer work with barley (Hanson and Scott, 1980) and chenopods (Hanson and Rhodes, 1983) gives some indication of how the glycinebetaine levels in turgid and water stressed tissue are regulated.

From computer modelling of their experimental data, Hanson and Rhodes (1983) suggest that the regulatory step in glycinebetaine biosynthesis in turgid sugarbeet leaves lies at the phosphorylcholine – choline step. The data also suggest that, if the unlabelled phosphorylcholine level in the storage pool in turgid tissue is raised, then synthesis of labelled phosphorylcholine from the precursor is reduced dramatically. This indicates that phosphorylcholine controls its own biosynthesis by feedback inhibition. Once availability of free choline from phosphorylcholine is limited, further steps leading to the formation of glycinebetaine have been shown to be reduced. Activity in the two oxidative steps, from choline to glycinebetaine aldehyde (Coughlan and Wyn Jones, 1982) and from glycinebetaine aldehyde to glycinebetaine (Hanson and Scott, 1980) has been shown to be high in turgid tissue and equal to that found in stressed tissue. Hence these steps have no regulatory role in the maintenance of glycinebetaine levels in unstressed leaves.

Unlike proline oxidation, the rate of glycinebetaine degradation is unlikely to be a major mechanism by which betaine levels are regulated in turgid tissue, since glycinebetaine behaves as an inert end product in plants (Hanson and Nelson, 1978; Ahmad and Wyn Jones, 1979; Ladyman *et al.*, 1980). However, some glycinebetaine may be lost in the process of methyl donation (Sribney and Kirkwood, 1954; Byerrum *et al.*, 1956).

3.3.2.2 In stressed tissue

When a glycinebetaine-accumulating plant species undergoes water or salinity stress the level of glycinebetaine increases (discussed in section 3.1.2 of this chapter). The glycinebetaine thus accumulated originates from stimulated *de novo* synthesis from 2-C and 1-C precursors. The conversion to glycinebetaine of pre-existing pools of potential quaternary ammonium compound precursors such as free choline, phosphorylcholine, and phosphatidylcholine is a negligible factor (Chetal *et al.*, 1980; Hitz *et al.*, 1981; Coughlan and Wyn Jones, 1982).

Coughlan and Wyn Jones (1982) reported that, in spinach, only the conversion of ^{14}C-serine to ethanolamine increased (by 2.5 fold), the rest of the biosynthetic pathway showing no stimulation due to stress. Hence, they concluded that the increased accumulation of glycinebetaine in spinach is due to the activation of serine decarboxylation to form ethanolamine. However, the radiotracer data of Hanson and Scott (1980) show that, in water stressed barley, the labelling of glycinebetaine from ^{14}C-serine, ^{14}C-ethanolamine, and ^{14}C-choline was about 7- to 10-fold greater in leaves wilted for 2 days than in turgid leaves, and label from ^{14}C-glycinebetaine aldehyde appeared in glycinebetaine at about the same rate in both wilted and turgid leaves. Moreover, the activity of the 1-C pathway leading from formate to methyl groups (of nitrogen) certainly remains high in both stressed barley and sugarbeet leaves (Hanson and Nelson, 1978; Hanson and Wyse, 1982) and may actually increase during stress (Hanson and Nelson, 1978). Based on these observations, Hanson and his co-workers suggested that glycinebetaine accumulates in barley because of the activation of all the biosynthetic steps except the oxidation of glycinebetaine aldehyde to glycinebetaine. This oxidative step could be very important and might limit glycinebetaine accumulation in barley. Varietal differences in the capacity to accumulate glycinebetaine (Ladyman *et al.*, 1983) may reflect differences in the activity of this oxidation step in the biosynthetic pathway.

As catabolism of glycinebetaine is negligible (Wyn Jones and Storey, 1981; Hanson and Hitz, 1982; Grattan and Grieve, 1985) changes in activity of possible catabolic pathways by stress (Byerrum *et al.*, 1956) will not be significant in the

regulation of gycinebetaine levels.

3.3.2.3 During stress relief

Sribney and Kirkwood (1954) and Byerrum *et al.* (1956) suggested that glycinebetaine may represent a storage pool for methyl groups available for transmethylation reactions. However, in both healthy and rust-affected wheat plants (Bowman and Rohringer, 1970) supplied with $^{14}CH_3$-glycinebetaine, the ^{14}C was retained 91 - 99% after incubation for 22 h in the dark. A lack of glycinebetaine utilization has been observed in adult wheat plants for as much as 10 days after injection of labelled glycinebetaine (Bowman and Rohringer, 1970). Hanson and Nelson (1978) reported that 5 days after rewatering only 25% of the glycinebetaine that had accumulated during water stress remained in a viable leaf blade. However, these authors did not monitor the level in the whole plant. When this is done (for barley) the glycinebetaine level per shoot was found to remain almost constant at the elevated level during the post stress period(Ahmad and Wyn Jones, 1979). In this experiment, the maintenance of an almost constant shoot glycinebetaine concentration for 6 days after stress, in spite of an increase in shoot fresh weight, suggests continued accumulation of the compound in the shoot even during stress relief. A similar lack of betaine degradation, in cycles of stress and rewatering, has been reported for barley (Ladyman *et al.*, 1980) and recent work of Grattan and Grieve (1985) demonstrates that wheat plants lack the ability to utilize accumulated glycinebetaine as a source of N even when they are subjected to a N deficiency during transient salinity stress.

The reported decline in the level of accumulated glycinebetaine in the mature leaves following stress relief (Hanson and Nelson, 1978; Ladyman *et al.*, 1980; Grattan and Grieve, 1985) has been shown to be due to translocation of glycinebetaine to younger leaves or growing areas in both barley (Ladyman *et al.*, 1980) and sugarbeet (Hanson and Wyse, 1982) and not to net metabolism. It is evident that the compound is phloem-mobile. This lack of net utilization of glycinebetaine upon re-watering contrasts with the fate of accumulated proline which is metabolised completely by viable leaf tissue.

4 Evaluation of the significance of selected biological responses to stress in relation to plant resistance to stress

Evaluation of metabolic traits for their adaptive significance is a general goal of ecological biochemistry, i.e. the investigation of biochemical adaptations of organisms to the environment (Harborne, 1977). The identification of specific adaptive metabolic traits in mesophytic crop plants has specific importance because such traits might be exploited in plant breeding for drought resistance, particularly by modern genetic engineering approach because metabolic traits are usually controlled by few genes (Hanson and Grumet, 1986).

Three putative adaptive metabolic responses to water stress have been studied with plant breeding and crop improvement in view: the accumulation of proline (Singh *et al.*, 1972; Hanson *et al.*, 1977; Aspinall and Paleg, 1981; Hanson and Hitz, 1982), of glycinebetaine (Wyn Jones and Storey, 1981; Hanson and Hitz, 1982; Hanson and Grumet, 1985; Grumet and Hanson, 1986; Wyn Jones and Gorham, 1986), and of ABA (Aspinall, 1980; Milborrow, 1981; Henson *et al.*, 1981; Austin *et al.*, 1982; Henson, 1983). The significance of the accumulation of proline and glycinebetaine are discussed in this thesis.

4.1 Drought stress

Drought triggers the accumulation of proline and glycinebetaine in several crop plant species (see section 3 of this chapter) and it is necessary to consider whether plants possessing this mechanism have a physiological advantage during drought. This approach concentrates upon possible functional aspects of proline and glycinebetaine accumulation rather than treating the accumulation of these solutes as a consequential and physiologically neutral, or even detrimental effect of stress. Several possible positive roles have been suggested for proline and glycinebetaine accumulation in stress metabolism, and is worthwhile considering the evidence for these before discussing the relationship between solute accumulation and resistance to stress.

4.1.1 *Osmotic adjustment (osmoregulation or turgor maintenance)*

Progressive plant-water deficit in the field or laboratory has been shown to lower the osmotic potential (at full-turgor) in many species and cultivars and thereby contribute to the maintenance of turgor as ψ decreases (Hsiao *et al.*, 1976; Turner and Jones, 1980; Morgan, 1984). This process, known as osmotic adjustment or osmoregulation, is accomplished by net solute accumulation; however,the lowering of ψ_S through an increase in the concentration of cell sap consequent upon water loss during stress is not considered as an osmotic adjustment. Turner and Jones (1980) suggested that "osmotic adjustment" be used only for the accumulation of solutes in higher plants in response to water deficit and that "osmoregulation" and "turgor regulation" be retained for use in relation to lower plants and microorganisms, or the change in osmotic potential of higher plants in response to salinity. The solutes involved in osmotic adjustment in higher plants include proline, glycinebetaine, sugars and sugar alcohols (Jones *et al.*, 1980; Munns and Weir, 1981; Wyn Jones, 1984).

The extensive accumulation of proline and glycinebetaine is aided by their outstanding solubility in water. A high concentration of these solutes in plant tissue would be expected to exert a lower ψ_S but, in several plant species when the contribution of these solutes to osmotic adjustment was assessed on a total cell water basis, it was found to be negligible (Ford and Wilson, 1981; Riazi *et al.*, 1985). This observation led Wyn Jones *et al.* (1977) to propose that the cells of saline-tolerant plants have the capacity for "solute compartmentation", in that if absorbed toxic inorganic ions (such as Na^+) are accumulated in the vacuole, the osmotic balance between the cytoplasm and vacuole is maintained by the accumulation of non-toxic organic solutes in the cytoplasm (these are called "compatible solutes" the term first used by Brown and Simpson, 1972). Evidence in favour of this hypothesis was advanced by Hall *et al.* (1978) with salinised *Suaeda maritima* where use of a technique based on the formation of an iodoplatinate precipitate and transmission analytical electron microscopy revealed accumulation of glycinebetaine in the cytoplasm but not in the vacuole. The work of Leigh *et al.* (1981) also showed a concentration of glycinebetaine and proline in the cytoplasm, but failed to demonstrate

complete localisation of these solutes to the cytoplasm. The apparent presence of these solutes in the vacuole in this study might have resulted from the technique used which could have caused leakage of solutes into the vacuoles. Recent work of Robinson and Jones (1986) in spinach using an NMR technique, and of Matoh *et al.*, (1987) in *Atriplex gmelini* using isolated protoplasts and vacuoles clearly demonstrated that glycinebetaine is localised in the cytoplasm and more specifically in chloroplasts of salt stressed spinach leaves (Robinson and Jones, 1986). It is possible that such compartmentation is operating in water stress also, and accumulated solutes could then contribute a substantial fall in osmotic potential. In some water-stressed plants the solutes which accumulate in vacuole are proposed to be Na^+ or K^+ since these are seen to increase during water deficit (Ford and Wilson, 1981). If the organic solutes are confined to the cytoplasm, occupying 10% of the total cell volume, measured osmotic potential and that calculated from solute concentrations assumed to result from compartmentation are in good agreement. When cytoplasmic volume was assumed to be 5%, proline alone accounted for osmotic adjustment in salinised barley (Voetberg and Stewart, 1984).

Osmotic adjustment has been studied extensively in both field and laboratory with many crop plants and has been observed to result in partial or full turgor maintenance (Turner and Jones, 1980). A greater degree of osmotic adjustment was observed in the apex and expanding leaves than in older leaves of wheat (Munns *et al.*, 1979; Morgan, 1980). The extent of osmotic adjustment depends on the rapidity of stress imposition (Turner and Jones, 1980), a slower development of water stress producing greater osmotic adjustment than rapid stress (Flower and Ludlow, 1986).

Osmotic adjustment has been suggested as a potential selection criterion for crop plants in dry areas (Morgan, 1983). Morgan *et al.* (1986) studied the association between osmotic adjustment and grain yield of wheat genotypes under laboratory and field conditions. The yields of genotypes with a high capacity for osmotic adjustment were 11-17% higher in bread wheats and 7% higher in durum wheats than those with a low capacity for osmotic adjustment. Blum and Sullivan (1986) also have demonstrated that landraces of sorghum and millet from drier regions of the world have a greater capacity

for osmotic adjustment. In these cases osmotic adjustment, an indirect measure of the solute accumulating ability, has been chosen to evaluate drought resistance.

4.1.2 *Protection of biopolymers or enzymes*

Aspinall and Paleg (1981) were of the opinion that linking the phenomenon of solute accumulation solely to the maintenance of cell turgor (Kauss, 1977), was an over-simplification. High levels of cytosolutes have been shown to have no deleterious effects on the biochemical functions of the cell (Stewart and Lee, 1974) and, hence, the water environment of cell membranes, and the stability of enzymes and macromolecules may also be important considerations.

Proline in solution has been shown to enhance the solubility of various proteins and to protect bovine albumin from denaturation by $(NH_4)_2SO_4$ or ethanol (Schobert and Tschesche, 1978). Similarly, glycinebetaine and its sulphur analogue, dimethylsulfonioacetate, protect the respiratory activity of bacteria from salt damage (Shkedy-Vinkler and Avi-Dor, 1975). The protective action diminished as the quaternary nitrogen group was selectively demethylated. A similar effect was observed by Pollard and Wyn Jones (1979) in the effect of salt on the activity of malic dehyodrogenase in barley. Paleg *et al.* (1984) showed that the precipitation of barley glutamine synthetase by PEG was reduced by both proline and glycinebetaine in a concentration-dependent manner. The PEG-induced precipitation of the enzyme was pH dependent, and because of differences in effectiveness of proline and glycinebetaine at high and low pH values, these authors suggested that a plant able to accumulate both proline and glycinebetaine would be better able to cope with a range of intracellular pH values than a plant able to accumulate only one of the solutes. Proline was also demonstrated to reduce the PEG-induced precipitation of alfalfa mosaic virus and of an ^{3}H-testosterone/antiserum complex (Paleg *et al.*, 1985).

The work of Manetas *et al.* (1986) demonstrated that glycinebetaine and proline protects phosphoenolpyruvate carboxylase (PEPCase) extracted from two members of Poaceae which have the ability to accumulate these solutes during stress. In contrast, only

glycinebetaine protected PEPCase extracted from a chenopod (*Salsola soda*), and proline accelerated NaCl-induced inhibition of enzyme activity. *S. soda* did not accumulate proline during stress. These authors suggested the possibility of a co-evolution of PEPCase properties and the ability to accumulate the appropriate osmoticum. A similar inhibitory effect of proline, but not glycinebetaine, on ribulose-1,5-bisphosphate carboxylase/oxygenase activity from *Aphanothece halophytica*, a halophilic cyanobacterium, has been reported and, again, this organism has the capacity to accumulate glycinebetaine alone (Incharoensakdi *et al.*, 1986).

It seems clear that compatible solutes protect enzymes (proteins) *in vivo,* at least in plants that have the ability to accumulate that molecule during stress. The next consideration is the mechanism by which these solutes protect the macromolecules. Schobert (1977) proposed a regulatory function for these accumulated substances through two mechanisms other than osmotic regulation. It was assumed that the regulatory pathways concern with hydrophobic groups on biopolymers in the cytoplasm. The alternatives proposed were: (1) Polyols can replace water molecules through their water-like OH-groups and participate in the hydrophobically-enforced water structure. (2) Proline is postulated to associate, via its hydrophobic region, with hydrophobic side chains of proteins thereby converting them into hydrophilic groups by exposure of the carboxyl and amino group of proline to the bulk solution . This would increase the solubility of the protein and maintain a more intact hydration sphere around the biopolymers. ^{1}H-NMR studies showed a strong hydrogen bonding of water in proline solution and it was concluded that proline forms aggregates by the stepwise stacking of water on to the protein-proline complex, thus increasing the hydration of biopolymers during drought (Schobert and Tschesche, 1978). However, Paleg *et al.* (1984) disagree with this mechanism. It was reasoned (Paleg *et al.*, 1984)that, in experiments involving the protection of protein against PEG precipitation by proline, at intermediate concentrations of both compounds, that if either proline or PEG brought about their effects through binding to the protein, an increase in the amount of protein should lead to a dilution of the interaction and a decreased effect. The absence of any effect of protein

concentration strongly supports the view that there is no such specific binding of either PEG or proline to the protein. These authors concluded that the influence of both PEG and proline on the solvation of protein is dependent on interactions between solutes and water molecules, rather than between solutes and proteins, as suggested by Schobert and Tschesche (1978).

4.1.3 *Alternate roles*

In addition to roles as an osmoticum or protein protectant, proline may also help the plant (i) to conserve energy and amino groups and (ii) as a sink for soluble nitrogen and reducing power.

4.1.3.1 The conservation of energy and amino groups

Proline has been suggested as having a role as a storage compound, which would be rapidly utilized upon the relief of stress (Barnett and Naylor, 1966; Stewart *et al.*, 1966). Proline could function as an effective reserve for nitrogen and carbon skeletons by virtue of its close proximity to glutamate and ready conversion to that key compound in amino acid metabolism. Proline is rapidly accumulated in young leaves (Singh *et al.*, 1973a) and disappears upon rehydration (Jager and Meyer, 1977). It is rapidly oxidized to glutamate in turgid leaves (Stewart *et al.*, 1977), even though there is no marked increase in the concentration of either glutamate or α-aminobutyrate. In sorghum, an increase in tissue ammonia concentration was observed after water stress relief and it can be presumed that proline was deaminated with the carbon skeleton entering the Kreb's cycle, a conclusion supported by the simultaneous increase in dark respiration (Blum and Ebercon, 1976). In *Myxomycetes*, cell division was correlated with proline concentration (Threlfall and Thomas, 1979). The high concentration of proline accumulated during the formation of pollen , which is rapidly reduced during pollen tube growth, has also been proposed to be a readily accessible energy source (Dashek and Harwood, 1974). Holden (1973) suggested a similar energy-source role for proline in the cockroach. It is thus conceivable that proline may play a major role as a storage compound, being rapidly

metabolised with the onset of favourable conditions to yield energy for biological processes. No such role can be ascribed to glycinebetaine, because the energy trapped in the molecule does not seem to be readily utilized after stress relief (Hanson and Nelson, 1978; Ahmad and Wyn Jones, 1979), nor even in nitrogen deficient cereals (Grattan and Grieve, 1985).

4.1.3.2 The sink for soluble nitrogen

During an episode of water stress there is the potential for an accumulation of low molecular-weight soluble nitrogen-containing compounds, such as the ammonium ion, which are toxic other than in very low concentration. These soluble and deleterious nitrogenous compounds could result from protein hydrolysis (Petrie and Wood, 1958; Shah and Loomis, 1965) coupled with inhibited protein synthesis. Proline has been proposed to detoxify the liberated toxic nitrogenous compounds (Savitskaya, 1976).

Glycinebetaine accumulation might also perform such a detoxifying role. For example, high levels of choline were found to inhibit the activity of heated plant enzymes *in vitro* (Nash *et al.*, 1982; Paleg and Keech, unpublished) and hence the accumulation of glycinebetaine could serve to maintain choline at a physiologically optimum level.

4.1.4 *Solute accumulation and resistance to water stress*

The accumulation of proline in relation to drought resistance, and glycinebetaine in relation to salinity tolerance has been studied extensively. More recently there have also appeared a few reports on glycinebetaine accumulation and drought resistance. Further, it has been suggested that mechanisms giving tolerance to one form of stress may also give tolerance to others. Associations between drought, heat, and freezing tolerance, in particular, have been considered (Parker, 1968; Levitt, 1972, 1980) as each may cause internal water deficit. Evidence for this suggestion has been provided in studies which demonstrate the ability of drought resistant wheat cultivars to resist salinity (Kirkham, 1984; Mozafar and Goodin, 1986).

Proline accumulation although not universal, is widely distributed in a variety of

families (Aspinall and Paleg, 1981); however, glycinebetaine accumulation is restricted to a few families (Wyn Jones and Storey, 1981).

The occurrence of proline accumulation in response to water deficit in a wide range of living organisms has been suggested as evidence that this reaction is a primitive regulatory response (Measures, 1975). If this is so, proline and, possibly, glycinebetaine accumulation may be a characteristic of plants growing in drier habitats. To explore this possibility, Poljakoff-Mayber *et al.* (1987) studied solute accumulation in plants from various ecological habitats. This study shows clearly that neither proline nor glycinebetaine accumulation is a universal characteristic of plants of drier habitats; however, the large majority of plants accumulated these solutes. Lack of a general occurrence of proline accumulation in plants of drier habitats has also been observed by Hubac and Guerrier (1972) and Pourrat and Hubac (1974). Two desert plants, *Artemisia herba-alba* and *Carex pachystylis*, were extremely drought resistant in their natural habitat but their mechanisms of drought resistance seemed to differ. *C. pachystylis* accumulated a high concentration of proline during drought, while in *A. herba-alba* physiological adaptations were found which helped to avoid dehydration (reduced transpiration and well developed root system, etc.). Similarly, when diverse stress resistant species of marsh halophytes were compared (two C_3 species, three C_4 grass species and three succulents) the C_3 grasses showed massive proline accumulation commencing at a leaf water potential of -1.0 MPa (0.25 M NaCl in rooting medium), the C_4 grasses showed less proline with a lower leaf water potential threshold (-2.0 MPa) at 0.5 M salinity in the rooting medium, and the succulents only accumulated proline at a very high salinity (0.75 M NaCl) with a leaf water potential of -2.5 MPa (Cavalieri and Huang, 1979). As similar responses were obtained with PEG treatment the authors concluded that proline accumulation had no adaptive value in C_4 succulents. Stewart and Lee (1974) have also reported a lack of massive proline accumulation in all halophytes studied. Although some plant spp. didn't accumulate proline, for example, a few tropical legumes (Gorham *et al.*, 1984; Ford, 1984), halophytic chenopods (Storey and Wyn Jones, 1979; Cavalieri and Huang, 1979), and *Melaleuca* spp. (Poljakoff-Mayber *et al.*, 1987; Naidu *et al.*, 1987) other

nitrogenous solutes or polyols were found to accumulate in these plants.

Storey and Wyn Jones (1977) studied 14 plant species from a wide range of salt resistance capacity including halophytes, semi-resistant glycophytes and sensitive glycophytes. Since imposed salinity caused an increased accumulation of glycinebetaine in halophytes and semi-resistant glycophytes but not in sensitive glycophytes, these authors concluded that plants accumulate glycinebetaine which balances the low osmotic potential resulting from the uptake of salt. Work of Storey *et al.* (1977) and a review of the distribution of glycinebetaine in various taxonomic groups (Wyn Jones and Storey, 1981) suggests that the accumulation of glycinebetaine is a characteristic of drought resistant and saline tolerant plants. Guy *et al.* (1984) reported a correlation between glycinebetaine accumulation and soil salinity in the field. In related study it was found that although glycinebetaine accumulation occured in plants growing in sub-humid, semi-arid, arid and saline habitats, the compound was predominant in plants from a saline habitat (Poljakoff-Mayber *et al.*, 1987). Interspecific comparisons within the family gramineae (Hitz and Hanson, 1980) do not show a clear relationship between glycinebetaine accumulation and drought resistance. The more drought resistant sorghum and millet accumulate less glycinebetaine than barley and wheat. However, corn and rice accumulate little glycinebetaine when stressed, which appears to correlate with the drought sensitivity of these species. Clearly, glycinebetaine accumulating ability is not the sole determinant of drought resistance.

These reports suggest that comparisons between taxonomically unrelated groups of plants, even at the same ecological site, might lead to confusion and various interpretations of the validity of the relationship between solute accumulation and drought resistance. Such discrepancies are to be expected, as strategies for survival in unfavourable conditions are different in different plant species. Hence, intra-specific variation might be more meaningful in assessing the value of solute accumulation in drought resistance.

4.1.4.1 Intra-specific variation in solute accumulation and drought resistance

The first indication that proline accumulation might be related to drought resistance in higher plants came from the work of Barnett and Naylor (1966). These authors found that bermuda grass (*Cynodon dactylon*) clones which came from drier habitats were able to accumulate more proline than those from wetter areas when subjected to water stress. A similar response was found in *Carex* sp. (Hubac *et al.*, 1969).

Subsequently, Palfi and co-workers suggested that drought resistant crop varieties could be selected by screening for the ability to accumulate proline. They presented very little critical evidence for this proposal other than showing differences in the ability to accumulate proline under stress (Palfi and Juhasz, 1971; Palfi *et al.*, 1973). Singh and co-workers supported this proposal with an investigation of proline accumulation and varietal adaptability to drought in barley (Singh *et al.*, 1972, 1973c). Singh *et al.* (1972) reported a high negative correlation (r = -0.89**) between the amount of proline accumulated in the first leaves of 3 week old barley genotypes water stressed for 60 h by applying PEG to the rooting medium and the stability index calculated for these genotypes in field trials by Finlay and Wilkinson (1963). The yield stability index for each variety was calculated as the regression coefficient of the relationship between the log yield of that variety and the mean log yield of all varieties at each site in each season. A stability index of less than 1.0 then indicated a relatively stable yield in varying environments and, in this context a desirable degree of drought resistance since water stress was the major field variable. Indeed, the use of log yield in the analysis emphasized the performance of varieties in the lower yielding drier sites (Knight, 1970).

Further work of Singh *et al.* (1973c) showed that the amount of proline accumulated by five barley genotypes stressed osmotically was related to their recovery when the stress was relieved. The cultivars with the higher relative growth rate for the first 4 days after stress relief were those that had accumulated most proline during the stress period and also had the least leaf senescence.

Blum and Ebercon (1976) worked with grain sorghum genotypes and found that free proline commenced to accumulate at leaf water potentials of -1.4 to -1.6 MPa in

plants stressed by withholding water. The genotypes differed with respect to the amount of proline accumulated by the end of the stress period. The ability to recover after relief from stress was also highly correlated with the amount of proline accumulated during the stress period, but not with the resistance of isolated tissue to dessication. Proline accumulation has been found to be correlated with drought resistance in safflower (Reddy and Sastry, 1977), rice seedlings (Mali and Mehta, 1977) and *Brassica* (Richards, 1978; Richards and Thurling, 1979). In this latter study the correlation was more apparent in *Brassica napus* than in *B. campestris*. with yield performance being measured under dry field conditions and proline estimated on glasshouse-grown seedlings.

Such correlations between yield performance and proline accumulation are not confined to seedlings. Field grown wheat cultivars also exhibit differences in proline accumulation during the reproductive phase. The drought resistant variety Generoso accumulated more proline than the susceptible variety, Yecora (Karamanos *et al.*, 1983). Monneveux and Nammar (1986) reported that field grown durum wheats accumulated more proline than bread wheats, but that within each group the varieties accumulating more proline showed greater foliar resistance to transpiration. Proline accumulation was considered to be an important biochemical mechanism of resistance to water stress and these workers suggested that proline accumulating ability should be used in selecting resistant parents for plant breeding .

There is less information on the correlation between glycinebetaine accumulation and drought resistance as the potential significance of the accumulation of glycinebetaine has been realised only recently. Ladyman *et al.* (1983) conducted an extensive survey of 339 genotypes of wild and cultivated barley for the ability to accumulate glycinebetaine. There were significant differences between genotypes in the level of glycinebetaine present both with and without water stress. The level of the solute present in stressed plants was correlated with the non-stressed level in the same genotype. When 13 cultivated barley species were grown under simulated dry land field conditions, the glycinebetaine level varied significantly at the ear emergence stage independent of leaf water and osmotic potentials. It was suggested that genotypic variation in metabolism

of glycinebetaine, independent of leaf water potential, is an adaptive character. Grumet *et al.* (1985) investigated the inheritance of glycinebetaine-accumulating potential using genotypes varying in glycinebetaine content. They found that glycinebetaine-accumulating potential is a nuclear, predominantly additive trait of relatively high narrow-sense heritability. Recent work of Grumet and Hanson (1986) provides genetic evidence for an osmoregulatory function of glycinebetaine accumulation. These workers developed F_4 isopopulations with different mean glycinebetaine levels. The parents and isopopulations also differed for ψ_s. The high glycinebetaine isopopulations and parents maintained 0.1 MPa lower ψ_s than low glycinebetaine genotypes at all salt levels. In both populations and parents, glycinebetaine level was linearly related to ψ_s ($r^2 \geq 0.97$). Based on these observations it was suggested that glycinebetaine accumulation is a mandatory component of osmoregulation in barley.

Drought resistance has been increased by the exogenous application of proline and glycinebetaine to tissue. When 500 μM proline was added to the rooting medium of wheat plants there was a greater maintenance of green colour and also quicker resumption of growth after re-watering (Tyankova, 1966). A similar increase in resistance was observed in *Brassica compestris* treated with a 3% proline solution (Hubac, 1967) and in drought sensitive *Carex certifolia* (Hubac and Guerrier, 1972). Also, red pepper plants sprayed with 1.5% proline lost turgor more slowly in response to stress than those sprayed with water (Palfi *et al.*, 1972). Itai and Paleg (1982) reported an increased growth during recovery from water-stress of barley plants treated with 25 mM proline, 25 mM glycinebetaine or a mixture of 12.5 mM each; however there was no effect on growth of during water stress. Recently, Handa *et al.* (1986) reported that treatment of tomato cell cultures with 10 mM proline enhanced the water stress tolerance of the tissue by maintaining growth under stress.

4.1.5 *Criticisms of the postulated role of solute accumulation in stress resistance*

As there is a close relationship between leaf water potential, length of stress period and consequent proline accumulation, it is important to ensure that the water status of the cultivars to be compared is similar. Inherent differences in rates of transpiration and water uptake render it difficult to achieve such an ideal comparision, however, Singh *et al.* (1972) claimed that 10 barley cultivars differed in proline content despite identical leaf ψ level and that this accumulation correlated with yield stability of genotypes. This relationship has been qeustioned by many workers. Lewin and Sparrow (1975) suggested that the apparent drought resistance of the barley cultivars used was strongly correlated with their time of maturity in the field. Relatively early flowering cultivars accumulated most proline and were apparently the most drought resistant, but this was due more to drought evasion than to resistance. This criticism can only be resolved by comparing genotypes of similar maturity but different drought resistance, a task which has not been undertaken so far.

The use of proline accumulating ability as a metabolic index of drought resistance was criticised by Hanson and co-workers. Hanson *et al.* (1977) studied the proline accumulation and drought resistance of two contrasting barley cultivars also included in the range of varieties compared by Singh *et al.* (1972). These were Excelsior, a variety with greater stability in yield performance and Proctor, a variety with less stability in yield performance in dry areas (Finlay and Wilkinson, 1963). They found that there was a more rapid rate of proline accumulation in Proctor correlated with a more rapid decline in water status, and a more severe leaf firing (death). In both varieties proline reached the highest concentration as leaf kill became severe, and much was localised in the non-viable parts of the leaf. These workers were of opinion that massive proline accumulation was a symptom of severe internal water deficit and apparently had no survival value. When an empirical plot was made of proline accumulation against leaf ψ for Excelsior, Proctor and wild barley, there were no apparent differences in the "threshold" ψ at which proline accumulation began nor in proline accumulation at any given ψ (Hanson *et al.*, 1979). Crosses between Excelsior and Proctor revealed heritable differences in proline

accumulation in the F_3 population. Among segregating individual plants of the F_3 generation there was a positive association between the amount of free proline accumulated during stress and the severity of leaf firing. Based on these results, Stewart and Hanson (1980) argued that selecting genotypes for high proline accumulating potential would tend to produce genotypes whose water status would fall more rapidly during stress and result in increased susceptibility rather than resistance to drought.

The physiological and metabolic roles attributed to the accumulation of proline has also been criticised (Hanson, 1980; Stewart and Hanson, 1980; Hanson and Hitz, 1982). These authors were of the opinion that proline accumulation (maximum of 50 μmol/g fresh weight in crop plants) cannot contribute to the osmotic potential unless proline is compartmented in small fractions of the total cell volume. Proline accumulation and its possible roles in nitrogen and energy metabolism has been evaluated (Hanson and Nelson, 1978; Tulley *et al.*, 1979). Proline did not begin to accumulate rapidly until the second and subsequent days of stress (Tulley *et al.*, 1979), at which time much of the accumulated proline was found to be sequestered in leaf portions that were irreversibly wilted or "fired" (Hanson *et al.*, 1977). Commencement of the fast phase of proline accumulation coincided with cessation of N export from the leaves (Tulley *et al.*, 1979). Thus, little proline accumulated during early stress when the leaf was still actively exporting N and capable of full recovery from wilting. Though it was probable that much of the free proline synthesised at this time was exported, it was not a major contributor to N movement from the stressed leaf and accounted for only 13% of N movement. Although proline came eventually to dominate the free amino acid pool, it represented a small quantity of N relative both to the amount of N translocated from the wilted leaf and to the amount of N that was retained in the leaf. Further work of Tulley and Hanson (1979) supported this idea in that they found the proline level in phloem exudates from stressed plants to be low compared to the levels of other amino acids. Since these authors found much of the proline in drought-killed leaf tips, it was clearly unavailable for use upon relief of stress. Hence, Stewart and Hanson (1980) disputed the idea of a major adaptive role for proline as either an N transport form or an N reserve.

The suggestion that proline accumulation is involved in the detoxification of free ammonia liberated during stress was investigated (Hanson and Tulley, 1979). When tracer $^{13}NH_3$ was fed both as gas and as a droplet of $^{13}NH_4^+$ solution, ^{13}N label was assimilated by both turgid and stressed leaves under light. After incubation times of up to 30 min, however, the major ^{13}N-labelled products were always glutamine and glutamate. Proline was essentially unlabelled in both turgid and stressed leaves. Turgid blades exported 1 to 3% of the assimilated ^{13}N to sheaths. Calculations on mass transfer rates of glutamate, glutamine and proline indicated that glutamate carried 18 μgN, glutamine 58 μg N, and proline 9 μg N per day per leaf blade. Based on these observations Hanson and Tulley (1979) concluded that proline synthesis during water stress neither replaces nor substantially supplements amide formation as a pathway for the rapid disposal of ammonia.

Stewart *et al.* (1966) suggested that proline could act as a stable store of reducing power useful for the plant during recovery. *A priori*, a similar argument could be advanced for glycinebetaine acumulation. However, Hanson and Nelson (1978), from observed rates of glycienbetaine and proline accumulation calculated that proline synthesis required 4 μmol of NAD(P)H/leaf/day and glycinebetaine 2 μmol of NAD(P)H/leaf/day, i.e. a total of 6 μmol per day per leaf. This amount of reducing power can form only a very small component of the energy budget of an illuminated leaf, even if the capacity for photosynthetic electron transport is diminished by 90% during stress. Thus, roles for accumulated proline and glycinebetaine as reductant storage pools appeared very unlikely to Hanson and Nelson (1978). In the case of glycinebetaine, a slow rate of metabolism would presumably vitiate its value as a source of reducing power upon stress relief, and both proline and glycinebetaine sequestered in dead tissue could not be salvaged following stress relief. A major fraction of excess reducing power may be consumed in photorespiratory reactions and only a small proportion diverted to the synthesis of these solutes. These authors suggested that the accumulation of both solutes was purely incidental to the onset of moderate water stress, without any particular adaptive value.

As there is a negligible or at most a very slow rate of metabolism of accumulated glycinebetaine (Bowman and Rohringer, 1970) upon stress relief, Hanson and Nelson (1978) suggested that the glycinebetaine content could be utilized as a cumulative water stress indicator. For example, in a nursery trial in a dry site genotypes which had experienced prolonged or severe internal water deficit might contain more glycinebetaine than less affected genotypes. They suggested that genotypes with high glycinebetaine content should be discarded in selection for adaptation to dry conditions. Such a view denies the existance of ψ-independent genotypic variation in glycinebetaine content.

It is interesting to note that, in later work, Hanson and co-workers (Ladyman *et al.*, 1983; Hanson and Grumet,1985; Grumet *et al.*, 1985; Grumet and Hanson,1986; Hanson *et al.*, 1986) postulated an important adaptive role for glycinebetaine in both drought and salinity resistance. Nevertheles, these authors maintain that proline is merely a metabolic breakdown product (Hanson and Hitz, 1982).

4.2 Temperature stress

4.2.1 *Protective roles of solutes*

Reduced transpiration during water stress alone, or in association with high ambient temperature, can cause leaf temperature to increase due to reduced transpirational cooling (see section 2.2 of this chapter). However, lower growth temperatures in colder regions will also be a detrimental environmental factor in growth. Under these conditions the activity of at least some enzymes will be jeopardized (Smith, 1978).

The accumulation of proline and glycinebetaine has been shown to protect plant processes under unfavourable temperature situations. Heber *et al.* (1971) reported that freezing spinach chloroplasts for 4 h at -25°C inactivated the photophosphorylation of thylakoids by irreversibly altering essential membrane properties. However, thylakoids frozen in the presence of proline were protected against such damage. Photophosphorylation upon thawing was proportional to proline concentration in the freezing medium and, with high proline concentrations, thawed samples had the same activity as those not subjected to freezing. This increased stability was suggested to be

due to protection of membranes by proline against toxic organic and inorganic cellular substances concentrated during stress. Freezing maize cells in the presence of a 10% proline solution similarly conferred an increased post-thaw viability and growth potential on the cells (Withers and King, 1979). *Nicotiana tabacum* callus cells caused to accumulate proline as a result of cold hardening or ABA treatment showed 50% less cellular leakage on thawing, the callus surviving sub-zero temperatures and regenerating when thawed (Bornman and Jansson, 1980). Bokarev and Ivanova (1971) studied the frost resistance of *Solanum tuberosum*, a cultivated and frost susceptible potato, and of *S. schreiteri*, a wild and frost resistant potato, after treatment with glycinebetaine. These authors suggested that glycinebetaine increased the frost resistance of both species indirectly by increasing the total amino acid content, particularly proline, serine, and glycine. It is also reasonable to suggest that glycinebetaine had a more direct effect on frost tolerance.

Protection of membranes by glycinebetaine against the adverse effects of high temperature have been examined (Jolivet *et al.*, 1982, 1983) with respect to high temperature. High temperature-alone- or high temperature and oxalate-induced membrane leakage of betacyanine from beet root cells is inhibited when glycinebetaine is added to the medium. However, the exact mechanism of membrane protection is unknown. Proline and glycinebetaine also protect organelle integrity during high temperature stress and maintain the activity of cytoplasmic, mitochondrial and chloroplastic enzymes (Paleg *et al.*, 1981; Nash *et al.*, 1982). α-amylase, glucose-6-phosphate dehydrogenase, hexokinase, and pyruvate carboxylase derived from various sources have been protected by proline and glycinebetaine from heat inactivation (Paleg *et al.*, 1981). These authors concluded that as the solutes protected enzymes from different sources (yeast, wheat and chicken), the response, in terms of effector and receptor molecules, could be universal.

Nash *et al.* (1982) demonstrated that when isolated plant mitochondria were heated, isocitrate dehydrogenase, malate dehydrogenase and fumarase lose activity at different rates. The loss of activity of each enzyme was reduced if the mitochondria were heated in the presence of proline, glycinebetaine or other solutes at 0.5 M. Since these

solutes protected enzymes both in organelles and as dissolved enzymes (the dissolved enzymes being protected more completely) it was concluded that the solutes protect the enzymes by affecting the intra-mitochondrial environment and not through an osmotic effect.

4.2.2 *Resistance to temperature stress*

Barley accumulates proline in response to a reduced temperature (Chu *et al.*, 1974) and cultivars showed more than twofold difference in the concentration of proline accumulated (Chu *et al.*, 1978). When this proline-accumulating ability of cultivars in response to cold stress was compared with accumulation in response to water stress (Singh *et al.*, 1972) the cultivars ranked differently (Chu *et al.*, 1978).

In three alfalfa varieties, there was a 20% increase in total amino acid content in the roots at low temperatures, most hardy varieties accumulating proline (Wilding *et al.*, 1960). Similarly, the most cold resistant varieties have been reported to accumulate most proline in apple (Benko, 1968), potato (Bokarev and Ivanova, 1971) and grape (Bozhinova, 1972). Proline accumulation was generally greater in *Citrus* root stocks rated cold hardy than those rated cold tender (Yelenosky, 1979). Seasonal fluctuations in the freezing resistance of *Nothofagus dombeyi* is matched by changes in proline concentrations (Meza-basso *et al.*, 1986) and recent work of Duncan and Widholm (1987) suggests that the accumulation of proline is related to the cold tolerance of regenerable maize callus.

There are no reports of increased resistance to cold or heat stress associated with glycinebetaine accumulation.

5 Conclusions

It is clear that water, in respect of deficiency (drought) or quality (salinity), is a major environmental factor limiting crop productivity (Hanson and Nelson, 1980). Temperature extremes are also added to the problem of water stress in some regions. Plant water status is measured in terms of the components of leaf water potential.

Several physiological processes are altered during drought and attempts have been made to correlate these changes with drought resistance. The accumulation of low molecular weight solutes such as proline and glycinebetaine has been studied with the objective of improving drought resistance of plants. These biochemical changes, if proven to have any adaptive value, are suitable for manipulation by genetic engineering (Le Rudulier *et al.*, 1984; Hanson and Grumet, 1985; Wyn Jones and Gorham, 1986).

The accumulated low molecular weight solutes have been shown to play several protective roles while providing osmotic adjustment and functioning as metabolically very useful compounds. Several workers found the correlation between the accumulation of these solutes with drought resistance of cultivars under field and laboratory conditions. However, because of conflicting responses of two barley cultivars (Singh *et al.*, 1972; Hanson *et al.*, 1977) Hanson and Hitz (1982) disagreed with the idea that proline has an adaptive value (Aspinall and Paleg, 1981). Considering the proven useful roles of proline and glycinebetaine during stress situation, the work presented in this thesis examined the possible causes for variability in the capacity to accumulate proline and glycinebetaine. Possible variability in the capacity to accumulate glycinebetaine is also studied in this study, although glycinebetaine does not suffer from a criticism similar to proline. The knowledge in this area is thought to be useful in cultivar evaluation programmes.

CHAPTER III
Materials and Methods

1. Material

Barley (*Hordeum vulgare*) seed of cvs. Clipper, Excelsior, Proctor, and one source of Norbert, grown at the Waite Agricultural Research Institute's farm were obtained from Dr. D.H.B. Sparrow of the Department of Agronomy. The second source of Norbert (Metcalfe and Bendelow, 1981), matured in Canada, was obtained from Dr. D.R. Metcalfe through Dr. D.H.B. Sparrow. Phytotron-grown material of wheat (*Triticum aestivum*), varieties Kalyansona and Banks were supplied by Dr. I.F. Wardlaw, C.S.I.R.O. Division of Plant Industry, Canberra, A.C.T., Australia.

2. General Methods

2.1 Environmental control

2.1.1 *Growth conditions*

Plants were grown in growth cabinets under standard controlled conditions. The photoperiod in all experiments was 16 h with a constant day and night temperature of 20 ± 1°C. The photon flux density at pot level in any one experiment was maintained constant; however, photon flux densitiy varied between experiments from 200 to 500 μE m^{-2} s^{-1}, as indicated for each experiment. The light source was either a bank of 32/80 watt cool white fluorescent tubes (Phillips TLF 80/33) giving photon flux density of upto 250 μE m^{-2} s^{-1} or four high power sodium lamps of 400 watts (Lucalox Lamp, GE, U.S.A.) supplemented with fluorescent lights giving photon flux density of upto 500 μE m^{-2} s^{-1}. The position of the pots within the cabinets was changed at least once a day to minimise gradient effects of light, temperature and wind movements across the cabinet. A photon flux density of 500 μE m^{-2} s^{-1} with 16 h photoperiod and 20°C day and night temperature is referred to in this thesis as "standard environmental conditions".

2.1.2 *VPD control*

Growth cabinets were specially fitted with automatic RH control facilities (supplied by Johnson Controls, Australia) to maintain high or low VPD. Low VPD was achieved by electric steam generators (Devellis Steam Generator, 220 V) installed at the return-air passage of the cabinets. The steam generators were connected to a

continuous supply of de-ionised water with a low concentration of sodium bicarbonate added to increase the efficiency by increasing the conductivity. The RH sensors installed in the cabinet at the plant leaf level regulated the precise amount of steam to be generated to achieve the required VPD. High VPD was maintained in a second cabinet with a similar facility when ambient VPD was higher than the one required. However, when the ambient VPD was lower than the required, high VPD was maintained by a refrigeration and re-heating technique. A refregeration condenser (4 fins/ inch) installed in the air-flow duct was activated by RH sensors through relays and condensed excess moisture in the air. This de-humidified air was then passed through a heating coil (1200 W) to raise the temperature to the required level before returning the cabinet. The steam generators, condensor and heating-coil were pre-programmed and automatically controlled by RH sensors.

2.2 Plant culture

Seeds were germinated on wet filter paper in closed petri dishes for 24 h at 20°C in the dark before they were planted in 15 cm plastic pots. Twenty-two such seeds were planted in each pot and 20 uniform plants were grown on after an initial thinning soon after emergence. The plants were grown in river sand, well washed with de-ionised water prior to sowing. The pots were watered daily with de-ionised water for the first 3 days, the fourth day with 1/4 strength Hoagland's solution (Hoagland and Arnon, 1938) and the fifth and subsequent days with 1/2 strength Hoagland's solution. The nutrient solution was applied carefully to the rooting medium through a 1 cm diameter rubber tube, by siphoning action, so as to avoid contact between the nutrient solution and leaf surfaces which might have led to scorching. The volume supplied was in excess of the field capacity of the sand so as to ensure against the buildup in concentration of any ions.

2.3 Imposition of water stress

Water stress was imposed either by withholding water or by applying polyethylene glycol (PEG, M. W. 4000, BDH, Australia) solution to the rooting medium, depending on the nature of the experiment. PEG was dissolved in distilled water by heating at 80°C for 30 minutes. The cooled PEG solution was diluted with nutrient stock solution to bring the nutrient content equal to half strength Hoagland's solution and the osmotic pressure to -1.5MPa. Control plants were supplied with half strength Hoagland's solution. Each water stressed pot was supplied with 3 applications of 100 ml each of PEG on the first day and one application of 100 ml on the second day. PEG has been claimed to be the most suitable osmoticum for water stress investigations (Barrs, 1966; Singh, 1970). Although it has been suggested that contaminants can produce toxic effects (Leshem, 1966; Plaut and Federman, 1985; Munns and Termaat, 1986), no evidence for such toxicity was observed with the PEG used during these experiments or in previous investigations in this department (Husain and Aspinall, 1970; Singh *et al.*, 1973a; Joyce *et al.*, 1984).

2.4 Imposition of cold stress

Cold stress was imposed by maintaining the potted plants in a cold room at 4 + 1°C for 5 days in continuous light provided by a bank of fluorescent tubes providing an irradiance of 300 $\mu E\ m^{-2}\ s^{-1}$ at the leaf level. The plants were watered with 1/2 strength Hoagland's solution maintained at the same temperature.

2.5 Harvesting the tissue

Plant tissue in all experiments was harvested immediately after the end of each treatment. The whole of the first true leaf was cut into a glass vial using a pair of scissors and quickly frozen with liquid nitrogen to stop any further biochemical changes. Frozen samples were freeze-dried for 48 h. The dried samples were then weighed after equilibration in a desiccator at room temperature, and stored at -20°C in capped vials until required for chemical analysis.

2.6 Measurement of water status of plants

2.6.1 *Water potential*

Leaf ψ was measured with a Spanner thermocouple psychrometer (Barrs, 1968). The first leaf from the centre of each pot was selected, excised, immediately put into the psychrometer chamber, and covered with parafilm until taken to the psychrometer room (within 10 minutes after excision). The leaves in the chamber were sealed with the thermocouple assembly and allowed to equilibrate in a water bath at 25°C in a constant temperature room (24°C) for 4 h before recording the thermocouple output. The water potential was calculated by comparing the readings with those obtained from a graded series of NaCl solutions.

2.6.2 *Osmotic potential*

ψ_S was measured on the same leaves that were used for the measurement of ψ. After the ψ measurement, the leaf was removed from the thermocouple chamber and blotted with filter paper to remove any water droplets that may have fallen on it during removal from the thermocouple assembly. The leaf was then either sealed in the chamber with a rubber stopper or sealed in an air tight glass vial, and frozen in liquid nitrogen. Freezing the leaf in a sealed container minimised the any condensation of atmospheric moisture on to the sample. The frozen leaf was allowed to thaw in the sealed container at room temperature for about 2 h before the leaf was used for the measurement of ψ_S. Equilibration and other operations were as for ψ measurements.

2.6.3 *Turgor potential*

$\psi_P$was estimated from the measurement of ψ and ψ_S, assuming the contribution from $\psi_m$was negligible.

$$\psi_P = \psi_S - \psi$$

The components of leaf water potential are given in Mega Pascals (MPa).

2.6.4 *Relative water content*

The relative water content (RWC) was measured by the method of Barrs and Weatherley (1962) with the modifications suggested by Turner (1981). The first leaves were cut into 2 cm segments and weighed to obtain the fresh weight (FW). The segments were then floated on distilled water in petri dishes in a constant temperature room for 24 h under a photon flux density of 10-20 $\mu E\ m^{-2}\ s^{-1}$. The leaf segments were removed from the distilled water, surface blotted to remove moisture and weighed to obtain the turgid weight (TW). The segments were then dried in a pre-heated oven at 80°C for 36 h and their dry weight recorded (DW). The relative water content was calculated using the formula:

$$\text{RWC (\%)} = \frac{\text{FW-DW}}{\text{TW-DW}} \times 100$$

2.7 Estimation of solutes

2.7.1 *Extraction*

The extraction of solutes was carried out as described by Jones *et al.* (1986) which is a modification of the procedure of Singh *et al.* (1973a).

Freeze dried leaf material of about 250 mg was homogenised in a centrifuge tube, using an Ultraturrax, in 10 ml methanol:chloroform:water (M:C:W, 12:5:3) until the plant material was completely broken down. During the homogenisisation the centrifuge tube was maintained in dry ice to minimise heating which would otherwise acidify the homogenate. The grinding head was washed with 10 ml water which was added to the homogenate to break the emulsion formed during extraction. The mixture was thoroughly shaken on a Vortex test tube mixer and centrifuged at 3.5 K rpm for 15 min at 20°C. The volume of the clear supernatant was measured before storage in a sealed conical flask . The chloroform-containing pellet was re-extracted with the Ultraturrax using 10 ml of M:W (12:3), the grinding head was washed with 10 ml of water, and the remaining procedure was repeated as in the first extraction. The methanol-water phases were pooled, the total volume noted, and the extract stored at 4°C or below.

2.7.2 *Determination of amino acids : high performance liquid chromatography (HPLC)*

The amino acids present in the extracts were separated (as shown in Fig. 3) on a Waters Associates amino acid column in the lithium form (Waters part no. 80002) maintained at 43°C. The column was used with a Varian 54 Automated Liquid Chromatograph and Series 8000 autosampler. The autosampler injected 10 µl of the millipore-filtered leaf extract. The flow rate was 0.4 $ml.min^{-1}$. The compositions of the eluting solutions are shown in Table 1 and the gradient used in Table 2.

The separated amino acids were reacted with *o*-phthalaldehyde reagent (Roth and Hampai,1973; Pfeifer and Hill, 1983) in a Waters-Millipore post-column derivitization unit (Pfeifer and Hill, 1983) operated at 40°C. The reagent flow was 0.3 $ml.min^{-1}$. The amounts of derivatives formed were measured in a Varian fluorichrom fluorescence detector. Proline, hydroxyproline and secondary amines do not react with *o*-phthalaldehyde. These compounds, however, formed fluorescent derivatives with *o*-phthalaldehyde after reaction with hypochlorite (Bohlen and Mellet, 1979;Pfeifer and Hill, 1983).

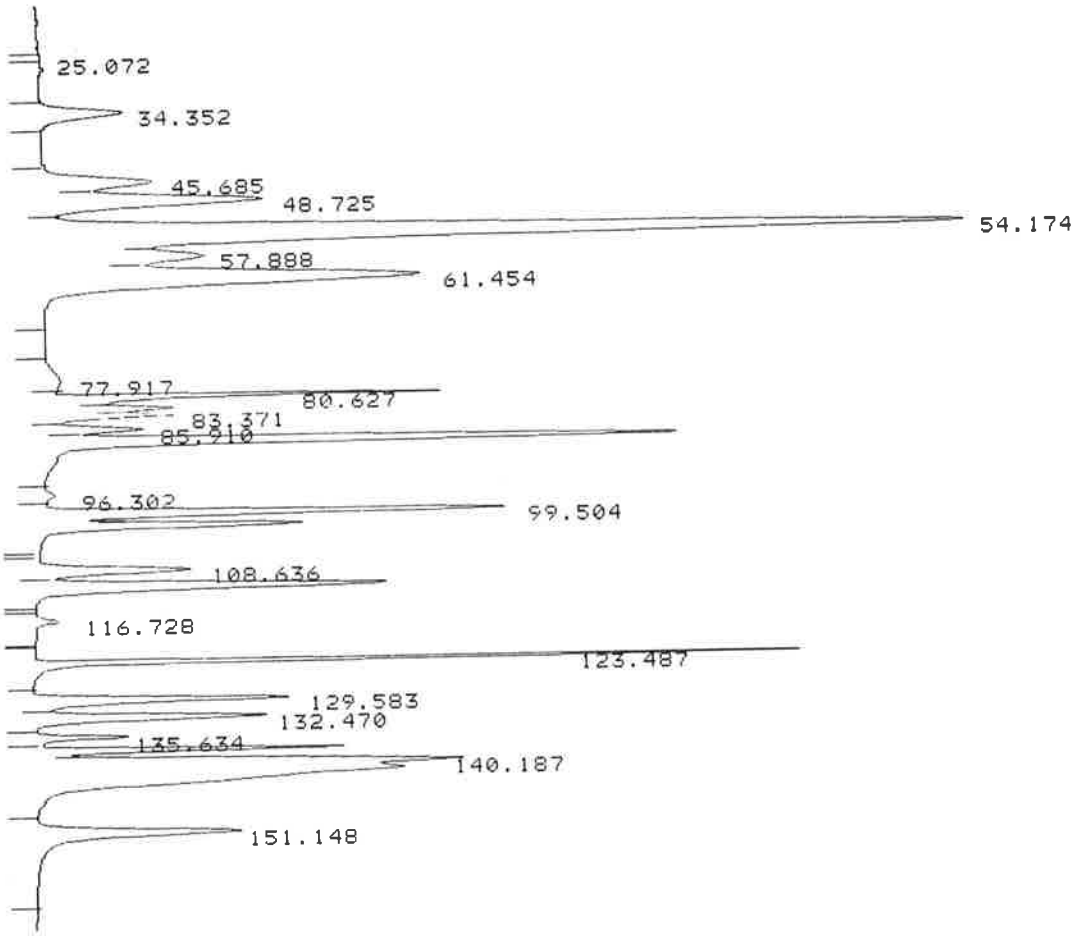
The proline contents of the leaf extracts obtained in one experiment were determined by treating the column effluent with a solution of sodium hypochlorite, flowing at a rate of 0.3 $ml.min^{-1}$, in the Waters-Millipore post-column derivatization unit at 40°C before the reaction with *o*-phthalaldehyde (Pfeifer and Hill, 1983).

2.7.2.1 Standard solution of amino acids

A solution of amino acids and related compounds was prepared to contain 1000 pmoles of each compound/10 µl of the standard solution injected.

Fig. 3 The elution profile of amino acids from a stressed wheat leaf extract using the HPLC system.

Elution time (min)	*Amino Acid*
25.072	Taurine (Tau)
34.352	Aspartic acid (Asp)
45.685	Threonine (Thr)
48.725	Serine (Ser)
54.174	Asparagine (Asn)
57.888	Glutamic acid (Glu)
61.454	Glutamine (Gln)
77.917	Unknown
80.627	Peak due to reagent blank
82.659	Glycine (Gly)
83.371	Alanine (Ala)
85.910	Citrulline (Cit)
87.876	Valine (Val)
96.302	Methionine (Met)
99.504	Isoleucine (Ile)
101.434	Leucine (Leu)
108.636	Tyrosine (Tyr)
111.319	Phenylalanine (Phe)
116.728	ß-alanine (ß-Ala)
123.487	γ-aminobutyric acid (GABA)
129.583	Histidine (His)
132.470	Tryptophan (Try)
135.634	Ornithine (Orn)
137.822	Lysine (Lys)
140.187	Peak due to reagent blank
151.148	Arginine (Arg)


25.072
34.352
45.685
48.725
54.174
57.888
61.454
77.917
80.627
83.371
85.910
96.302
99.504
108.636
116.728
123.487
129.583
132.470
135.634
140.187
151.148

2.7.2.2 Preparation and Composition of the reagents and standard solution

Solution A - The composition of eluting solution A is shown in Table 1 and was prepared by gently boiling a 0.01 M lithium hydroxide solution for 30 min. When cool, all reagents were added, together with sufficient trilithium citrate to raise the pH to about 1.5. Dowex 50 (H^+ form) resin (10-12 $g.l^{-1}$) was added and the suspension was stirred for 2 h, allowed to settle, and the solution was carefully decanted and the volume measured. Further trilithium citrate was added to adjust the pH to 2.55. The solution was filtered through a MF-millipore type GS, 0.22 μm filter before use.

Solution B - A 0.19 M lithium hydroxide solution was boiled until the volume decreased to the extent that the solution was 0.20 N when cold. It was filtered through a Millipore Durapore, type GV, 0.22 μm filter before use.

o-phthalaldehyde reagent - For each litre of reagent, 700 mg of *o*-phthalaldehyde was dissolved in 20 ml of methanol and then mixed with 1.0 M potassium borate buffer (pH 10.5) (Pfeifer and Hill, 1983). The solution was filtered as for solution B and 4 ml of mercaptoethanol was added. This reagent was usable for at least 10 days, but with a slow loss of activity necessitating the running of standards at regular intervals so that correction factors could be calculated.

Sodium hypochlorite reagent- A solution of sodium hypochlorite was prepared in 1M potassium borate buffer (pH 11.5), containing 0.08g of available chlorine per litre.

Table 1

The composition of the eluting solutions

Solution A - (pH 2.55)

(i)	trilithium citrate	0.058 M (≡ 0.175 M Li)*
(ii)	boric acid	0.035 M
(iii)	formic acid	0.064 M
(iv)	Brj 35	0.06%
(v)	*iso*-propanol	2% (v/v)
(vi)	hydrochloric acid	0.123 M

Solution B -

(i)	lithium hydroxide	0.20 N

*actual concentration was about 0.185 M Li - see solution A preparation

Table 2

The Elution Gradient

Time (min)	solution A	solution B	pH*
0	98	2	2.65
40	96	4	2.73
65	81	19	3.45
105	57	43	5.40
110	48	52	10.90
120	46	54	11.60
130	43	57	12.00
152	43	57	12.00
155	98	2	2.65
175	98	2	2.65
175	end of elution and regeneration cycle		

*nominal pH derived from a titration curve.

2.7.3 *Proline estimation*

Proline was measured using a rapid method developed by Singh *et al.* (1973a) based on the method of Troll and Lindsley (1955) for animal tissue.

The major interfering amino acids, such as ornithine, lysine, and pipecolic acid, were eliminated from the plant extracts by shaking with a weak cation exchange resin. Zerolit 225 (a product of Diamond Shamrock (Polymers Ltd.) supplied by Hopkin and William, England) or 'Amberlite' resin 1R-120 (Na) 14-52 mesh (BDH Chemicals Ltd., Poole, England) were used. Both were equally effective for the purpose; however, the latter needed regeneration with 1M NaOH prior to use. One volume of resin was treated with 4 volumes of NaOH on a column and the resin was finally washed with distilled water until the effluent reached a pH of about 8.

500 mg of the resin was placed in a test tube and to this was added 2 ml of extract from non-water stressed tissue or 0.25 to 0.5 ml of extract from water stressed tissue. The volume in the test tube was adjusted to 3 ml in all cases and thoroughly shaken for 15 min. The supernatant and subsequent three washings of resin, each with 2.5 ml of water were transferred into a boiling tube containing a few glass beads, to prevent bumping. A glass marble covered the mouth of the tube to prevent evaporation. Five ml of ninhydrin solution (125 mg of ninhydrin was dissolved in a mixture of 3 ml of glacial acetic acid and 2 ml of 6 M orthophosphoric acid by heating at 70°C) and 5 ml of glacial acetic acid were added to the boiling tube. The tubes were thoroughly mixed, boiled in a water bath for 45 min, then cooled to room temperature and shaken with a known volume of toluene. The optical density (OD) of the toluene-extracted ninydrin product was measured at 520 nm using a Brinkmann PC/600 Colorimeter with a probe attachment. The proline content was calculated from a regression curve obtained from standard proline solutions run at the same time as the samples.

2.7.4 *Glycinebetaine estimation*

The glycinebetaine content of the leaf extracts was estimated using the method developed at this laboratory (Jones *et al.*, 1986). This method involves an initial purification of the extract on cation exchange columns followed by quantitation with proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.

2.7.4.1 Purification on columns

The original methanol-water phase of the leaf extract (see section 2.7.1 of this chapter) contained amino acids, quaternary ammonium compounds, sugars and a trace of dissolved pigments. Use of such a sample for quantitation by NMR resulted in broadening of peaks and inaccurate quantitation of the compounds of interest, so it was necessary to purify the extract on a strongly cationic exchange column.

The ion-exchange column consisted of a 30 x 1 cm glass column with its tapered end plugged with glass wool and a screw provision for the control of fluid flow. Five grams of Dowex-50W resin, hydrogen form, 2% cross linked with a mesh size of 50-100 (Sigma Chemical Company, U.S.A.) was placed into the column in the form of slurry. Initially, the resin was washed with large quantities of water to eliminate all contaminants. Then the resin was converted to the hydrogen form by passing 25 ml of 8 N HCl through it and washed with distilled water until the eluant reached pH 5-6.

A known volume of the leaf extract after pH adjustment to 6-7 (if necessary) was loaded onto the column with minimum disturbance to the resin bed. An approximate flow rate of 5-10 ml.min^{-1} was maintained and, after the extract passed through the resin, the column was washed with 100-150 ml of distilled water (10-20 ml.min^{-1}). Glycinebetaine, together with amino acids and choline, was eluted from the column with 100-125 ml of 4 N HCl (10 ml.min^{-1}). The acid eluent was dried *in vacuo* at 50-60°C. The residue was dissolved in 5 ml ethanol and redried to eliminate HCl), redissolved in 5 ml distilled water and again dried *in vacuo* to eliminate traces of ethanol. The elimination of most of the HCl and ethanol was necessary as a lowered pH induces shifts in peak positions; the presence of ethanol makes it difficult to interpret the spectra,

and the presence of moisture in the sample broadens the peaks. The last traces of water in the sample were removed by drying the flasks in a pre-heated oven (60-70°C) for 15 min and then drying under a steady steam of dry nitrogen for 5 min. The residue was then dissolved in 0.8 ml of D_2O and 0.5 ml of this solution was transferred to a 5 mm NMR tube to which was added 2 µmol t-butanol (as 10 µl of solution in D_2O) as an internal standard. The final pH of the sample was in the range of 1-3.

The cation exchange resin was discarded after 5-6 runs. Before each run the resin was regenerated with 25 ml 8 N HCl and washed until the pH of the eluant reached 5-6.

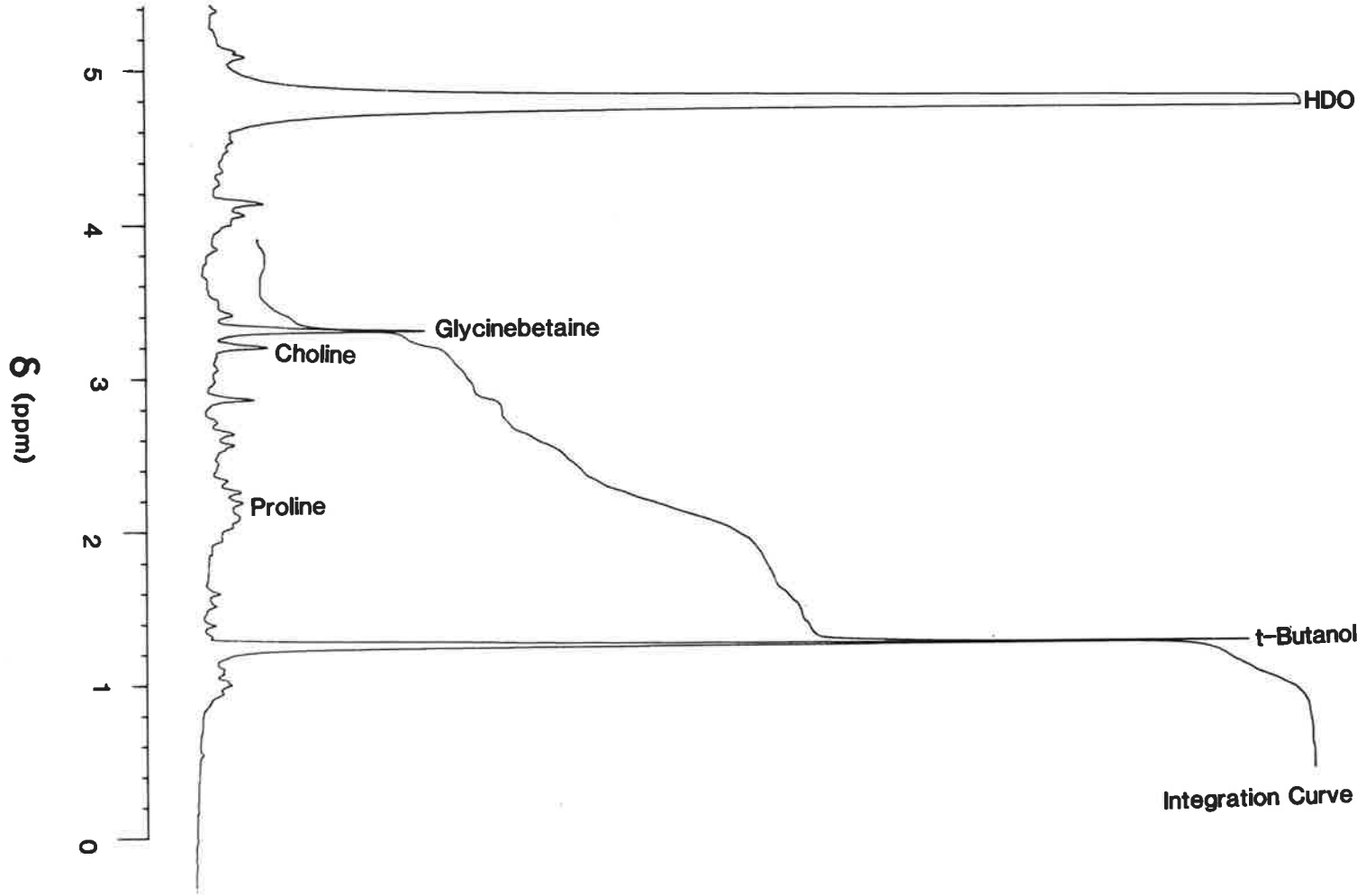
2.7.4.2 NMR measurements

'H NMR spectra were obtained on a JEOL FX 90Q Fourier Transform NMR spectrometer operating at a frequency of 90 MHz at an ambient probe temperature of 24°C. Routinely, 32 aquisitions into 8 K memory addresses, using a spectral width of 1000 Hz and 15 µsec (45°) pulse width with a recycle time of approximately 4.2 sec were accumulated. Peak identities were confirmed by the addition of authentic standards. A typical NMR spectrum of a water-stressed barley leaf extract is shown in Fig. 4. Quantitation of glycinebetaine was achieved by comparing integrated peak intensities (the peak area/t-butanol area) against a standard curve of peak intensities for varying concentrations of glycinebetaine with 2 µmol t-butanol. The peak areas were measured digitally using the NMR spectrometer's integration software.

2.8 Statistical approach

All the treatments in the experiments reported in this thesis were replicated 3 times. Chemical analyses and water status were all measured on each of the 3 replicates. A split plot design with factorial concept was followed except in experiments 1.4 an 1.5, where a completely randomised design was followed. Analysis of variance was carried out using the "Genstat" statistical programme on Cyber or Vax computer system. Wherever F-test was not significant (NS) at 0.05 level of probability (P), the least significant difference (LSD) is not shown.

Fig. 4 ^{1}H NMR spectrum of water stressed barley leaf extract. The peak positions or chemical shifts (δ) were measured relative to sodium trimethylsilylpropionate (TSP) using t-butanol as an internal reference with $\delta_{(t\text{-butanol})} = \delta_{(TSP)} + 1.245$ parts per million (ppm). Peak positions for t-butanol, proline, choline, glycinebetaine, and water (HDO) are marked and integration curve included.


HDO
Glycinebetaine
Choline
Proline
t-Butanol
Integration Curve
δ (ppm)
5
4
3
2
1
0

CHAPTER IV
Results and Discussion

Section 1 : Variability in the amino acid accumulation pattern

1.1 Introduction

Plants are able to adapt better to drought conditions by physiological changes when the rate of stress development is slower than when it is rapid (Turner and Jones, 1980; Flower and Ludlow, 1986). Some of the stress-induced physiological changes have been found to be differently sensitive to the rate of development of leaf water deficit (Jones and Rawson, 1979). Such a differential response has also been observed in the accumulation of proline and glycinebetaine, the quantitative relationship between these two solutes varying with gradual or abrupt osmotic shock (Wyn Jones and Storey, 1978). No similar evidence is available on the effects of rapidity of water stress imposition on the metabolism of other amino acids. However, under laboratory situations water stress is often imposed as an abrupt osmotic shock by the application of PEG (Hanson *et al.*, 1977; Hanson and Tulley, 1979; Tulley *et al.*, 1979; Lewin, 1980) to evaluate the adaptive value of proline accumulation in the resistance to drought. Under these circumstances proline failed to fulfill some of the metabolic standards set by these workers. Notable among these was that proline was not the major amino acid in ammonia turnover during stress, compared to amides (Hanson and Tulley, 1979). Further, failure of complete metabolic conversion of proline upon stress relief (Hanson *et al.*, 1977; Lewin, 1980) led to the conclusion that the accumulation of proline has no metabolic adaptive role during water stress or its relief (Stewart and Hanson, 1980; Hanson and Hitz, 1982).

The experiments reported in this section of the thesis were designed to examine the possibility that the nature and rapidity of water stress imposition determines the level of proline in relation to other amino acids, and utilization of the accumulated amino acids during stress relief.

1.2 High temperature during water stress and amino acid accumulation in wheat

1.2.1 *Methods*

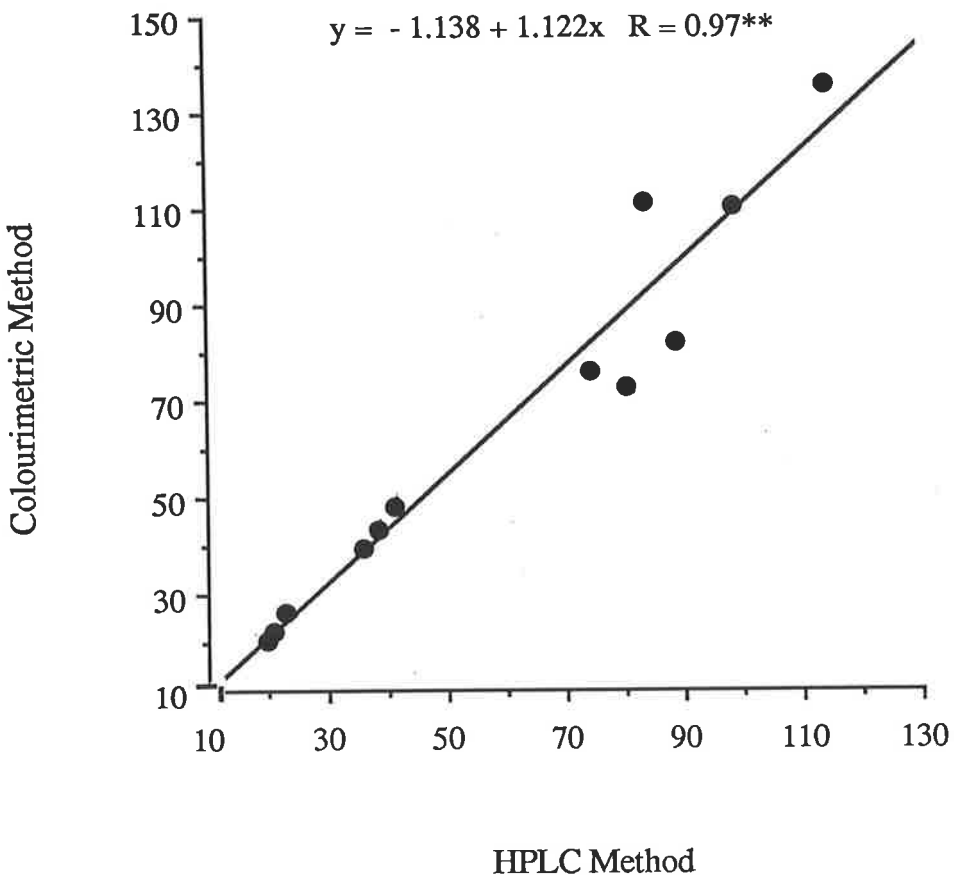
Wheat plants (cv. Banks) were grown in pots filled with sand under constant growth cabinet conditions of 20°C with a photoperiod of 16h and a photon flux density of 500 $\mu E\ m^2\ s^{-1}$. Ten day old seedlings were moved to two cabinets; one at 20°C and the other at 35°C with the same light environment. They were then water stressed by applying -1.5 MPa PEG to the rooting medium with well watered control plants maintained at each temperature. Care was taken to apply PEG or nutrient solution to the soil of plants which had been maintained at the same temperature as the treatment. Both control and stress treatments were replicated thrice.

First leaves of water stressed and control plants at both temperatures were harvested at the end of the 1st and 2nd day of the treatment and immediately frozen in liquid nitrogen for solute estimation. RWC, ψ, ψ_s, and ψ_p were measured on freshly cut first leaves.

Individual amino acids from wheat leaf extracts were estimated with the HPLC system, except for proline which was estimated both by HPLC and by the colourimetric method of Singh *et al.* (1973a) (Table 3; HPLC values are in parentheses). While the other amino acids were readily determined by HPLC, proline did not react with the *o*-phthalaldehyde reagent unless it was first made to react with sodium hypochlorite (see the Materials and Methods, section 2.7.2), which required a separate run on HPLC. Hypochlorite destroyed proline in concentrations up to 100 pmol/10 µl of the injected standard solution, equalent to 2 $\mu mol.\ g^{-1}$ dw of proline in leaf extracts, before it gaving an estimate of proline. This complication made it difficult to get accurate estimates of proline even at 3 times the concentration in control leaf extracts. The colourimetric method, which did not have such a high threshold level, was suitable for estimating proline in extracts with low levels of proline. Estimates obtained with stressed leaf extracts using the two methods agreed well (Fig. 5). Considering this limitation of the HPLC method for proline estimation, it was decided to adopt the colourimetric method for

Fig. 5 Relationship between proline (μmol g^{-1} dw) estimated by HPLC and the colourimetric method.

**, R value significant at 1%.


y = - 1.138 + 1.122x R = 0.97**
150
130
110
90
70
50
30
10
10
30
50
70
90
110
130
Colourimetric Method
HPLC Method

this purpose in all experiments.

Glycinebetaine was estimated by the NMR method of Jones *et al.* (1986). Details of extraction and solute estimation are described in the Materials and Methods (section 2.7). All solute concentrations are expressed as μmol g^{-1} dw of leaves.

1.2.2 *Results*

1.2.2.1 Water status

The RWC of the well watered control plants did not change with exposure to the higher temperature either on the 1st or 2nd day and ranged from 98.2 to 95.3% at 20 or 35°C. However, when the plants were water stressed, RWC declined significantly with both time and increase in temperature (Fig. 6a). The lowest RWC (56.8%) resulted from the exposure of water stressed plants to high temperature (35°C).

ψ (Fig. 6b) responded in a similar way to RWC, declining as a result of water stress and more so at the higher temperature. The increased transpirational demand is a likely cause of the sharp decline in both RWC and ψ in water stressed plants at the high temperature.

Heat stress alone (well watered plants exposed to 35°C compared to 20°C) did not influence ψ_S (Fig. 6c). Water stress lowered ψ_S depending on time and temperature. The higher temperature significantly lowered ψ_S of water stressed plants on both days of observation.

The control plants at both temperatures maintained a positive ψ_P which ranged from 0.50 to 0.28 MPa (Fig. 6d). Water stress resulted in significant loss of turgor, and the combination of heat and water stress resulted in negative ψ_P. ψ_P showed no interaction between the effects of water stress, temperature and time; however, temperature by stress was significant.

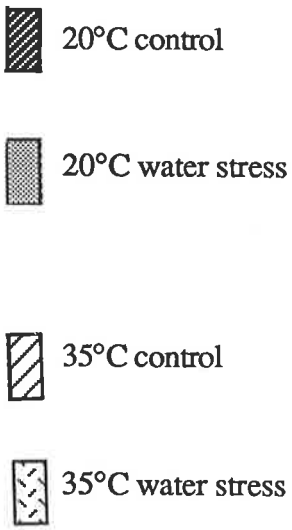
Fig. 6 Leaf water relations of water and heat stressed wheat (cv. Banks) seedlings.

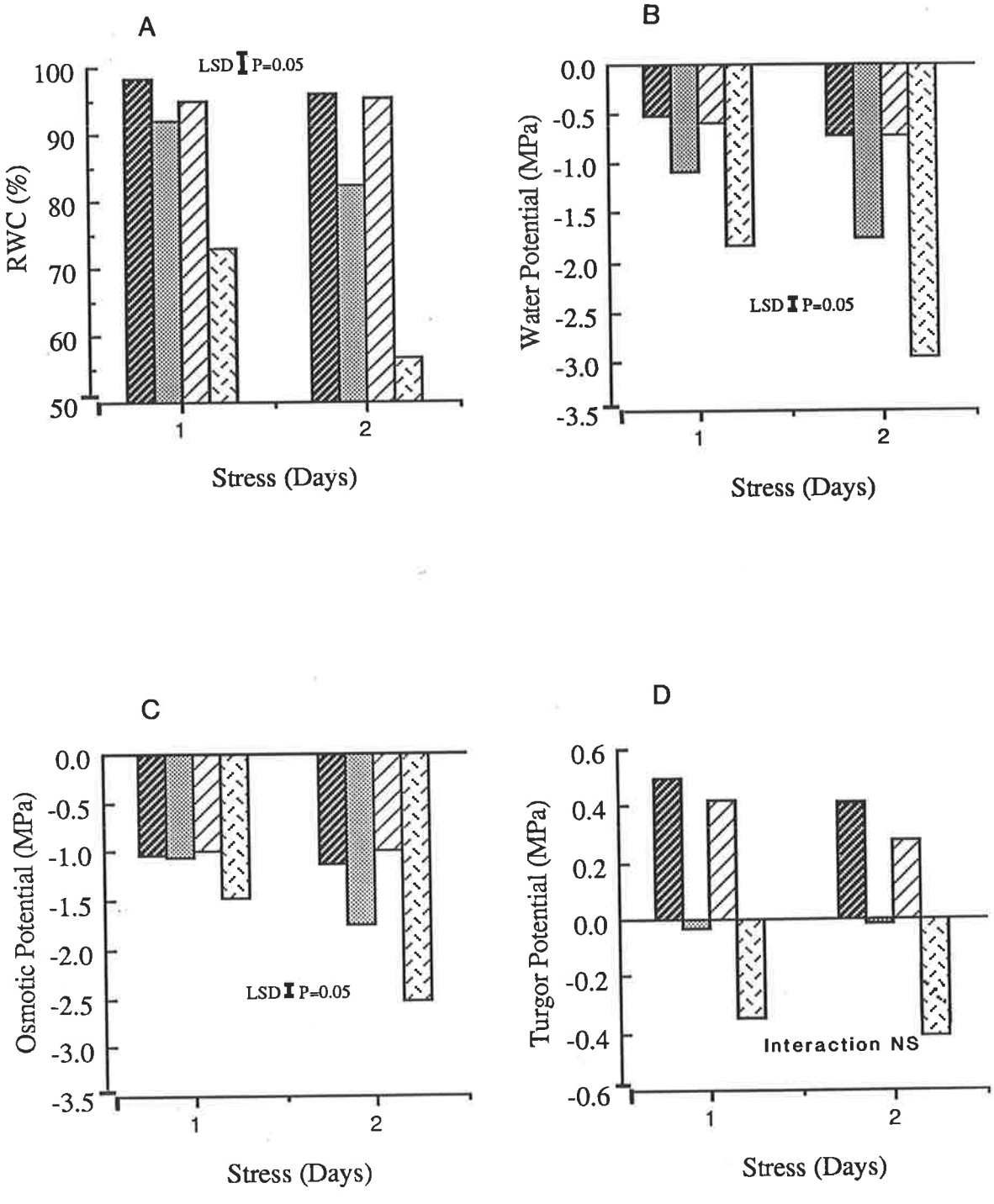
A. Relative water content

B. Water potential

C. Osmotic potential

D. Turgor potential




A
LSD P=0.05
RWC (%)
100
90
80
70
60
50
1
2
Stress (Days)
B
Water Potential (MPa)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
LSD P=0.05
1
2
Stress (Days)
C
Osmotic Potential (MPa)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
LSD P=0.05
1
2
Stress (Days)
D
Turgor Potential (MPa)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
Interaction NS
1
2
Stress (Days)

1.2.2.2 Amino acid and glycinebetaine content

Total amino acid content increased (Table 3) by more than 2 fold over the control at 20°C on the first day of water stress and the level did not change greatly on the second day. Water stress at 35°C resulted in 3.9 and 6 fold increases in amino acid content over the control level on the first and second days, respectively.

Quantitatively minor increases were recorded for taurine, citrulline, methionine, ß-alanine, and ornithine. Moderate increases in level were exhibited with threonine, serine, glycine, isoleucine, leucine, tyrosine, phenylalanine, γ-aminobutyric acid, histidine, tryptophan, lysine, and arginine. Most of these amino acids increased with water stress, time, and temperature during water stress, except serine which declined on the first day of water stress at 20°C and showed an increase in level on the second day.

The concentrations of asparagine, glutamine, valine, proline and glycinebetaine rose dramatically with water stress. When water stressed at 20°C, the greatest increase (more than 75 times over control plants) was recorded for proline followed by glutamine, asparagine, glycinebetaine, valine, and glycine. The quantitative relationship between these amino acids was changed by high temperature (35°C) during water stress; for example, the highest level (188.8 $\mu mol\ g^{-1}$ dw) was recorded for asparagine, followed by proline (119.8 $\mu mol\ g^{-1}$ dw), glutamine (100.9 $\mu mol\ g^{-1}$ dw), and valine and glycinebetaine (each about 50 $\mu mol\ g^{-1}$ dw) which differs from the pattern observed at 20°C.

In contrast to the amino acids that increased in concentration in response to water stress, aspartate, glutamate, and alanine showed significant reductions in levels at both temperatures. Only aspartate and glutamate declined significantly (on the second day) in well watered plants exposed to the higher temperature (35°C).

High temperature resulted in a reductions in level of serine, glutamine, glycine, and citrulline in well watered plants, although the concentration of these amino acids increased with water stress. In contrast, the levels of valine, isoleucine, γ-aminobutyric acid, lysine, and glycinebetaine in well watered plants increased in response to high temperature however, valine and lysine levels returned to normal on the second day.

Table 3: Amino acid and glycinebetaine content (µmol g^{-1} dw) in wheat leaves (cv. Banks) under water (PEG) and heat stress

	1 Day after stress				2 Days after stress				LSD
	20-C	20-S	35-C	35-S	20-C	20-S	35-C	35-S	P = 0.05
Tau	0.07	0.13	0.12	0.85	0.06	0.16	0.15	1.27	0.11
Asp	19.80	6.03	18.88	10.17	19.39	6.36	15.66	10.70	NS
Thr	5.62	10.42	5.22	28.98	5.66	16.59	4.92	19.89	0.69
Ser	36.00	33.28	27.18	37.88	38.35	44.58	24.12	37.56	2.52
Asn	1.77	11.99	2.34	128.10	1.80	25.38	1.90	188.83	4.74
Glu	53.97	46.30	52.27	40.13	53.53	39.63	36.82	25.10	2.65
Gln	14.23	9.22	8.11	68.68	15.05	37.17	7.78	100.91	NS
Gly	6.31	22.50	4.92	24.02	6.68	21.04	3.81	15.27	1.96
Ala	17.58	8.99	18.62	10.55	16.44	5.82	16.59	7.91	NS
Cit	1.42	1.18	0.08	7.63	1.43	3.96	0.13	7.19	0.38
Val	3.70	10.11	5.65	43.68	4.56	22.06	5.17	49.21	0.96
Met	0.38	0.88	0.47	0.79	0.58	0.28	0.51	0.7	0.07
Ile	0.85	5.92	2.70	33.31	0.96	13.89	1.69	32.94	0.62
Leu	1.15	6.68	2.42	37.96	1.45	15.10	1.61	20.27	1.40
Tyr	0.63	3.21	0.86	20.22	0.65	5.16	0.38	22.59	NS
Phe	0.62	5.22	0.91	27.71	0.60	11.97	0.50	33.17	NS
ß-Ala	0.16	0.86	0.23	1.62	1.20	0.63	0.09	2.12	0.26
GABA	5.06	7.56	6.64	12.83	5.06	12.58	7.68	22.44	1.29
His	0.53	2.82	1.30	14.20	0.35	6.47	0.61	21.47	0.76
Try	0.07	1.49	0.71	11.33	0.08	4.75	0.12	15.21	NS
Orn	0.35	0.74	0.94	4.46	0.44	1.61	0.47	5.30	1.07
Lys	0.40	2.44	2.43	14.72	0.40	4.58	0.82	16.68	0.63
Arg	0.30	2.79	1.67	20.49	0.19	6.50	0.34	24.07	NS
Pro	1.03 (ND)	23.45 (21.83)	1.01 (ND)	44.20 (38.61)	1.01 (ND)	77.37 (81.11)	1.15 (ND)	119.81 (98.83)	10.25
Total	172.51	401.99	165.69	644.32	175.92	383.64	133.02	800.61	
Glb	5.27	14.19	7.40	27.38	5.28	25.33	8.14	49.53	1.94

20C and 35C: controls at 20° and 35°C respectively; 20S and 35S: stressed at 20° and 35°C respectively; NS: interaction between temperature and water stress non significant Proline (**Pro**) values in parentheses are determined by the HPLC method. ND = Not detectable, Sec. 1.2.1 of this section. **Glb** = Glycinebetaine

1.3 Amino acid changes during water stress and stress relief

1.3.1 *Introduction*

The amino acid accumulation pattern in the previous experiment with wheat differed from those reported for Bermuda grass (Barnett and Naylor, 1966) and barley (Singh *et al.*, 1973a). In particular there were differences in the extent of accumulation of asparagine, glutamine, and valine. This could be due to the nature of water stress imposition (by PEG) which was differed from that in the other studies, in addition to the fact that the crop species was also different. The effects of high temperature and water stress have not been studied together and so a series of experiments were conducted to evaluate the various additional experimental factors that may influence the type and quantity of amino acids that accumulate in response to water stress.

The present experiment was conducted to determine whether the amino acid accumulation pattern was different when water stress was applied by withholding the water supply rather than by applying PEG. At the same time the opportunity was taken to observe changes in the spectrum of amino acids consequent upon stress relief as there is little information on this aspect (McMichael and Elmore, 1977) and there is also a minor controversy concerning the disappearance of proline upon stress relief (Hanson *et al.*, 1977; Lewin 1980).

1.3.2 *Methods*

Wheat seedlings (cv. Banks) were grown on sand for 10 days with a light intensity of 400 μE m^2 s^{-1} and 20/20°C temperature. Water stress was applied by withholding water for 4 days while the control plants were watered daily with the nutrient solution. After 4 days of water stress one set of plants were harvested with the controls, and the rest of the water stressed plants, with controls, were watered twice daily for a further period of 3 days. The quantity of nutrient solution applied at each time was in excess of field capacity of the sand and resulted in solution draining from the pot. Leaf tissue was harvested at 1/2, 1, and 3 days after re-watering. All the treatments were replicated thrice. RWC, ψ, ψ_S, and

ψ_p were measured on freshly cut first leaves at each observation while the bulk of the remaining leaf tissue was frozen in liquid nitrogen for amino acid and glycinebetaine analyses.

1.3.3 *Results*

1.3.3.1 Water status

Water stress created by withholding the water supply for 4 days lowered RWC by 30% (Fig. 7a). However, upon re-watering, RWC of leaves returned to the control level within 1/2 day and no changes occurred thereafter. There was a significant reduction in ψ, ψ_S, and ψ_p in response to water stress (Figs. 7b, c and d) and, when plants were re-watered, leaf ψ and ψ_S did not return to the control level as rapidly as RWC. Complete recovery of leaf ψ and ψ_S occurred only by the third day of observation. Plants lost all turgor in response to water stress but recovered within 1/2 day from re-watering as with RWC. The degree of water stress achieved by withholding water (Figs. 7 a and 7b) and by PEG application at 20°C (Figs. 6a and 6b) were comparable although the duration of the stress was different.

1.3.3.2 Amino acid and glycinebetaine content

Although the basic patterns of amino acid accumulation during water stress treatments induced by PEG application (Table 3) or by withholding nutrient solution (Table 4) were the same, the quantitative changes in some of the amino acids were different.

Proline concentration was higher than the concentration of any other amino acid in both stresses, but asparagine, glutamine, and valine also increased to a comparable level when stressed with PEG (Table 3). These amino acids did not respond to the same extent when water was withheld (Table 4). In addition to proline, there was an accumulation of glutamine, valine, γ-aminobutyric acid, and glycinebetaine. Slight to moderate, but significant, increases in threonine, asparagine, glycine, isoleucine, leucine, tyrosine,

Fig. 7 Leaf water relations of wheat (cv. Banks) seedlings during water stress relief.

A. Relative water content

B. Water potential

C. Osmotic potential

D. Turgor potential

□ Control

■ Stress relieved

0 day of stress relief following 4 days water stress.

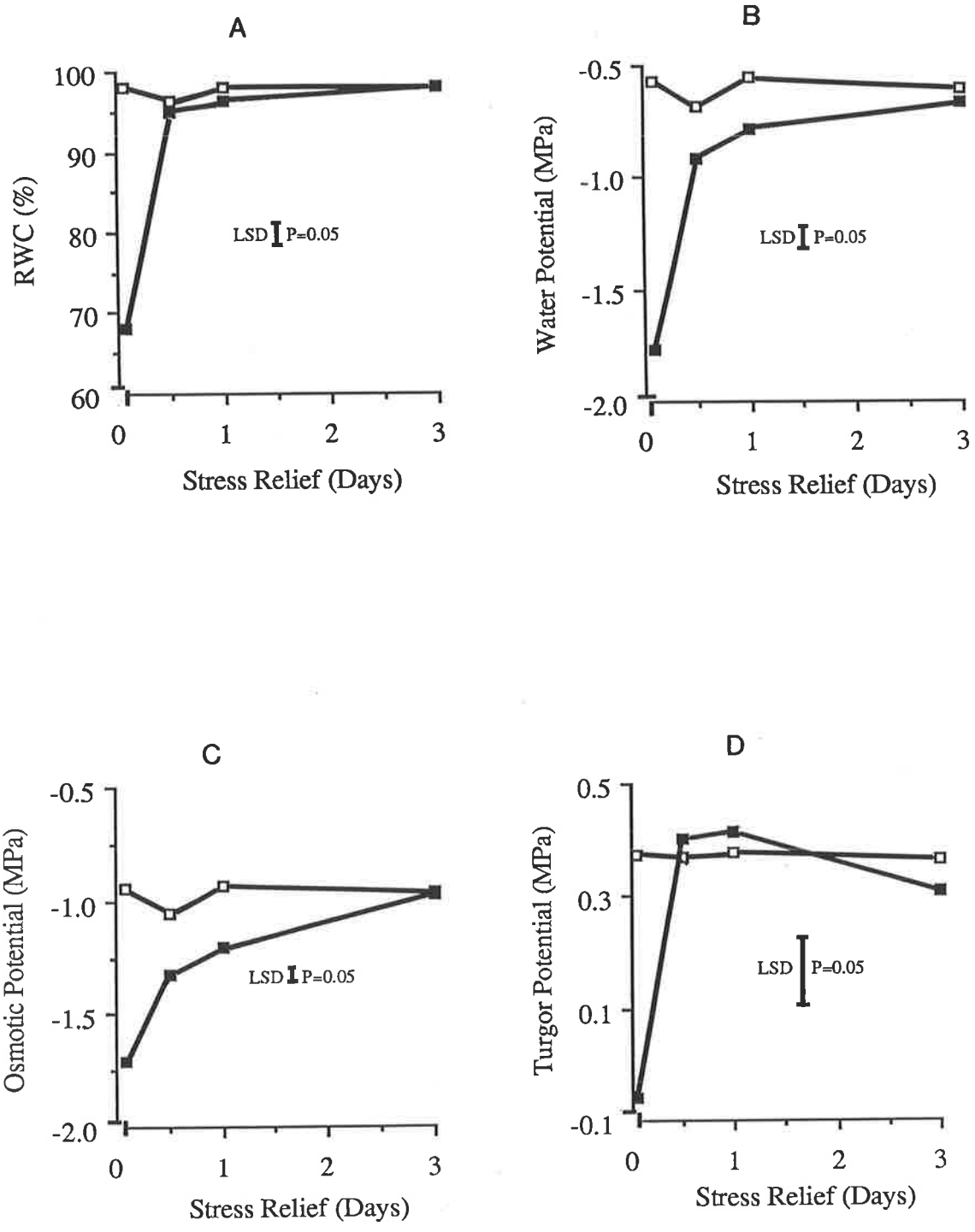

A
100
90
80
70
60
RWC (%)
LSD P=0.05
0
1
2
3
Stress Relief (Days)
B
-0.5
-1.0
-1.5
-2.0
Water Potential (MPa)
LSD P=0.05
0
1
2
3
Stress Relief (Days)
C
-0.5
-1.0
-1.5
-2.0
Osmotic Potential (MPa)
LSD P=0.05
0
1
2
3
Stress Relief (Days)
D
0.5
0.3
0.1
-0.1
Turgor Potential (MPa)
LSD P=0.05
0
1
2
3
Stress Relief (Days)

Table 4: Changes in amino acid and glycinebetaine content (µmol g^{-1} dw) of wheat leaves (cv. Banks) upon water stress relief

	0 day		1/2 day		1 day		3 days		LSD
	C	S	C	SR	C	SR	C	SR	P = 0.05
Asp	22.30	7.51	22.67	9.17	22.83	12.51	23.73	18.17	1.24
Thr	4.88	6.47	4.77	7.44	4.90	9.43	5.20	6.40	0.66
Ser	23.47	19.10	26.20	25.43	24.13	25.70	24.67	27.20	0.92
Asn	0.52	3.06	0.64	8.20	0.57	18.07	0.56	2.30	0.50
Glu	49.53	25.00	48.57	20.73	49.47	20.50	49.37	33.50	2.07
Gln	8.53	13.67	8.33	21.35	8.44	25.43	9.00	12.13	1.56
Gly	5.09	10.27	5.30	8.63	4.60	10.33	3.97	11.80	0.64
Ala	17.50	8.30	17.90	12.47	17.77	16.60	17.53	18.23	0.62
Val	4.48	12.40	4.50	15.70	4.67	17.37	4.73	12.50	0.80
Met	0.34	0.10	0.38	0.12	0.38	0.11	0.38	0.20	NS
Ile	0.80	2.67	0.81	5.13	0.89	3.80	0.90	2.24	0.34
Leu	0.93	3.20	0.96	2.72	0.96	1.84	1.06	1.60	0.26
Tyr	0.59	1.92	0.69	1.87	0.60	1.60	0.69	0.97	0.31
Phe	0.36	3.10	0.35	1.37	0.40	1.65	0.40	1.30	0.54
GABA	8.10	26.27	8.23	20.50	8.10	15.33	8.73	11.83	1.10
His	0.65	0.64	0.55	0.70	0.68	0.71	0.53	0.60	NS
Try	0.21	0.33	0.20	2.37	0.25	2.56	0.31	2.50	0.24
Orn	0.60	1.74	0.58	0.89	0.49	1.32	0.62	0.70	0.23
Lys	0.82	2.02	0.85	1.58	0.83	1.42	0.79	0.90	0.34
Arg	0.60	1.48	0.61	1.30	0.63	1.10	0.57	0.90	0.26
Pro	0.95	27.03	1.09	5.52	0.91	1.93	0.96	1.17	1.18
Total	151.25	176.28	154.18	173.19	152.50	189.31	154.7	167.14	
Glb	4.67	20.70	7.53	21.73	6.70	22.17	7.70	12.93	2.26

C = control

SR = stress relieved

NS = Interaction non significant

0 day = 4 days water stress and beginning of stress relief (SR)

phenylalanine, ornithine, lysine, and arginine were also recorded (Table 4).

Histidine, and tryptophan did not respond when plants were stressed by withholding water even though they accumulated significantly when PEG was used. Serine, aspartic acid, glutamic acid, alanine, and methionine, showed significant reductions in level as a response to withholding water.

Re-watering resulted in changes in amino acid composition to varying degrees in three days of observation. In addition to the increase in the concentrations of asparagine, glutamine, and valine during stress, the concentrations of these amino acids increased for a further day after re-watering, but had declined significantly by the 3rd day of stress relief. A similar trend, but with lesser change, was noted for threonine. Glycinebetaine showed no significant change in concentration within a day of stress relief but declined by the 3rd day. Glycine declined within 1/2 day of stress relief but again increased to higher level 1 and 3 days after stress relief. Tryptophan did not respond to stress, but increased rapidly within 1/2 day of stress relief and remained almost constant thereafter. The levels of tyrosine, phenylalanine, γ-aminobutyric acid, ornithine, lysine, and arginine gradually declined upon re-watering but, only ornithine and lysine returned to the control level.

The most interesting feature of the metabolic change during stress relief was proline (Table 4). The proline level declined rapidly (within 1/2 day) after re-watering and returned to the control level 1 day after rewatering.

Another metabolic change during water stress relief was that aspartic acid, serine, glutamic acid, and alanine which decreased with water stress, increased during stress relief. The alanine concentration returned to the control level in 3 days, but aspartic acid did not. Serine reached the control level within 1/2 day and the concentration was higher than the control from then onwards. Glutamic acid, however, declined 1/2 day after re-watering and increased on the third day only.

1.4 Rapidity of water stress imposition and amino acid changes in wheat

1.4.1 *Introduction*

The accumulation patterns for individual amino acids, particularly the responses of asparagine, glutamine, and valine, were different depending on the way that the water stress was imposed (see Sections 1.1 and 1.2 of this chapter). These differences could be due to differences in the relative rapidity with which stress was imposed with either with PEG (an osmotic shock) or by withholding water (slower than PEG), or to some other characteristic of PEG.

The experiments reported in this section were designed to examine the effects of different rates of water stress imposition, through application of PEG or through withholding water on amino acid accumulation in wheat seedlings.

1.4.2 *Methods*

Wheat seedlings (cv. Banks) were grown in sand-filled pots under "standard conditions". Two different rates of water stress were imposed with PEG. When stress was imposed at the slower rate, PEG was supplied at an initial ψ_S of -0.3 MPa and decreased by daily increments of 0.3 MPa (three changes of 100 ml each) reaching a final ψ_S of -1.5 MPa on the fifth day. The plants were harvested on the sixth day. The rapid PEG stress (osmotic shock) was imposed by applying 300 ml of -1.5 MPa PEG (an excess) to the rooting medium each day, the plants being harvested 2 days after the imposition of stress. The osmotic shock treatment was commenced on the fourth day of the incremental stress treatment so that seedlings of both stress treatments, and the control plants which received 1/2 strength Hoagland's solution, were of the same age when harvested.

Two rates of water stress were also imposed by withholding nutrient solution. Plants grown in a similar way to those in the PEG experiment were transferred to two cabinets. The slower rate was imposed by withholding water for six days in a cabinet ventilated normally . Rapid water stress was imposed in the second cabinet by ventilating only at the plant level so that the plants were subjected to a rapidly moving current of air

(1.45 m s^{-1}, against normal wind velocity of 0.3 m s^{-1}). Control, well watered plants were maintained in each cabinet. With this rapid wind movement severe leaf water deficit was achieved within 2 days. However, the slow water stress treatment was prolonged for 4 extra days (a total of 6 days) to achieve a comparable degree of water stress. Leaf water potential was monitored daily with a pressure bomb, and at the end of each treatment ψ and ψ_s were measured with the psychrometer. Proline, amino acid and glycinebetaine contents were estimated on first leaf extracts.

1.4.3 *Results*

1.4.3.1 Water status

PEG shock resulted in a lower RWC than the incremental PEG stress (Table 5). Leaf water potential showed no significant difference between the two PEG stresses, although, ψ_s was significantly lower when PEG was applied incrementally than when it was used as a shock. Osmotic shock with PEG resulted in a significantly lower turgor (reached a negative value) than the incremental PEG application in which plants showed positive ψ_p, although this was considerably lower than in well watered plants.

When plants were water stressed at two different rates by withholding water, the faster rate of water stress resulted in a significantly lower RWC than the slower rate of water stress, although ψ and ψ_s did not differ (Table 6). Both rates of water stress resulted in a significant loss of ψ_p (negative), but rapid water stress produced a significantly lower ψ_p than the slower stress.

Although the two rates of water stress imposition resulting from the application of PEG or from withholding water resulted in different degrees of RWC and loss of turgor, ψ and to some extent ψ_s was comparable in the various treatments.

1.4.3.2 Amino acid and glycinebetaine content

The general patterns of amino acid accumulation (Tables 5 and 6) resemble those in the previous two experiments (Tables 3 and 4); however, there were considerable differences when water stress was imposed in different ways and at different rates. When

Table 5: Water status and solute content (μmol g^{-1} dw) of wheat (cv. Banks) leaves as influenced by PEG stress rapidity*

	Control	PEG incremental	PEG shock	LSD (P = 0.05)
RWC (%)	97.50	63.50	47.20	8.95
ψ (-MPa)	0.43	3.01	2.95	0.20
ψ_S (-MPa)	0.79	3.10	2.76	0.21
ψ_p (MPa)	0.36	0.09	-0.19	0.09
Asp	17.83	6.81	9.01	1.19
Thr	3.38	9.55	8.50	0.99
Ser	23.13	34.22	18.51	1.20
Asn	0.72	9.28	3.07	1.10
Glu	43.63	32.19	20.55	4.90
Gln	6.46	69.02	37.90	6.08
Gly	3.21	12.38	9.55	1.06
Ala	12.59	6.15	8.07	1.35
Val	3.17	20.65	14.42	1.43
Met	0.70	2.71	2.69	0.66
Ile	0.76	11.97	7.07	1.13
Leu	1.76	14.38	10.77	2.09
Tyr	0.72	2.96	1.97	0.32
Phe	1.63	14.58	10.41	1.91
GABA	4.48	16.50	30.87	5.76
His	0.62	9.45	7.50	0.96
Try	0.51	6.79	8.10	0.46
Orn	0.38	5.86	6.81	2.06
Lys	1.00	2.92	5.85	0.85
Arg	1.97	1.44	1.64	0.60
Pro	0.20	119.70	71.40	9.78
Total	128.85	409.51	294.59	
Glb	6.68	43.83	14.31	3.05

*Measurements made at the end of stress period (see Methods 1.4.2 of this section)

Table 6: Water status and solute content (μmol g^{-1} dw) of wheat (cv. Banks) leaves in response to rapidity of water stress imposition by withholding water*

	Control	Progressive water stress	Control	Rapid water stress	LSD (P = 0.05)
RWC (%)	97.53	50.05	94.80	21.25	2.13
ψ (-MPa)	0.44	3.10	0.44	3.25	NS
ψ_S (-MPa)	0.75	3.02	0.77	3.01	NS
ψ_P (MPa)	0.31	-0.08	0.33	-0.24	0.15
Asp	18.08	8.31	13.36	10.16	1.00
Thr	3.25	11.33	5.21	10.26	1.90
Ser	20.39	33.27	30.83	31.44	3.89
Asn	0.86	4.04	1.22	7.20	2.01
Glu	46.17	41.73	20.92	25.71	2.04
Gln	4.80	14.97	6.43	29.48	4.42
Gly	2.10	9.39	3.61	7.21	1.85
Ala	19.21	7.96	18.68	11.77	2.19
Val	2.97	11.60	4.08	15.09	3.02
Met	0.44	0.60	0.61	1.40	NS
Ile	0.71	8.03	0.58	9.17	0.88
Leu	1.21	8.64	0.85	11.91	1.62
Tyr	0.63	3.59	0.65	3.69	NS
Phe	1.45	6.86	1.25	10.99	1.78
GABA	4.73	14.90	14.23	32.67	1.35
His	0.61	6.58	1.17	5.32	1.25
Try	0.31	7.75	0.24	6.12	NS
Orn	0.45	4.18	0.27	2.94	0.58
Lys	1.57	6.80	0.18	3.29	1.25
Arg	0.25	1.98	0.25	0.81	0.18
Pro	0.12	97.08	0.12	80.75	3.03
Total	130.31	309.59	124.74	317.38	
Glb	6.86	31.13	10.53	22.68	3.42

NS, Interaction non significant
*Measurements made at the end of stress period (see Methods 1.4.2 of this section)

water stress was imposed at the slower rate with PEG, the total amino acid content was higher than when a PEG shock was given, whereas the rate of water stress imposition when water was withheld had no effect on total solute content. The concentrations of individual amino acids were influenced by both the nature and the rapidity of water stress imposition.

Daily incremental application of PEG resulted in higher final concentrations of threonine, asparagine, glutamine, glycine, valine, histidine, isoleucine, leucine, tyrosine, phenylalanine, histidine, proline and glycinebetaine than when plants were exposed to an osmotic shock with PEG. In this case, the greater period available for accumulation in the incremental treatment may have determined the amount. However, γ-aminobutyric acid and lysine increased more with shock than with incremental application of PEG (Table 5). Serine declined significantly in response to the PEG shock but accumulated when the stress was imposed gradually.

Amino acids found previously to decline during stress showed differences in concentration as a consequence of stress rapidity. Aspartic acid and alanine levels declined more in response to the incremental PEG stress, again, presumably, due to the greater time under stress, involving their conversion into other compounds (eg. aspartic acid to asparagine) . In contrast glutamic acid declined more rapidly in response to PEG shock (Table 5).

Rapid water stress imposed by a high wind velocity, when compared with a progressive water stress at a lower transpiration rate, led to the accumulation of less threonine, glycine, histidine, proline, and glycinebetaine. These changes compare closely with those resulting from rapid stress imposition with PEG and presumably can, be attributed to the same cause. Most of the other amino acids, particularly asparagine, glutamine, valine, and γ-aminobutyric acid, accumulated in response to the rapid water stress. The pattern of decline in levels of aspartic acid, glutamic acid, and alanine in response to stress rapidity was the same as when water stressed by PEG application.

Even the well watered control plants maintained in a cabinet with high wind velocity showed significant changes in levels of some of the amino acids independent of

changes in leaf water relations (Table 6). The levels of threonine, serine, and γ-aminobutyric acid increased, whereas aspartate and glutamate declined significantly.

1.5 Cold stress and amino acid accumulation in wheat seedlings

1.5.1 *Introduction*

It is well established that proline accumulates in various plant species undergoing cold stress (Chu *et al.*, 1974, 1978; Aiyar, 1981; Parameshwara, 1984; Meza-Basso *et al.*, 1986); however, other amino acids have also been reported to accumulate in various plants. These include alanine and glutamine in alfalfa (Wilding *et al.*, 1960); alanine in barley and radish (Chu *et al.*, 1974) and valine, lysine, histidine, serine and alanine in *Nothofagus dombeyi* (Meza-Basso *et al.*, 1986). Wallace *et al.* (1984) claimed that there was a very rapid accumulation of γ-aminobutyric acid and alanine in various plant species including barley, which was not found in the data of Chu *et al.* (1974).

Glycinebetaine responds to heat stress (Table 3); however, no information is available on the response to cold stress. This experiment was designed to examine the changes in amino acid and glycinebetaine content in response to cold stress in wheat seedlings.

1.5.2 *Methods*

Wheat seedlings (cv. Banks), grown for 10 days as described in Materials and Methods chapter (section 2.1 and 2.2), were subjected to cold stress (4°C ± 1°) with continuous light provided by a bank of fluorescent tubes giving a photon flux density of 300 $\mu E\ m^{-2}\ s^{-1}$. The cold stressed plants were watered with nutrient solution maintained at the same temperature as the plants. The corresponding control plants were maintained at 20°C in a similar light environment. Ten seedlings were subjected to cold stress for five days with three replications .

At the end of the period of exposure to low temperature first leaves were collected for the measurement of water status and for estimation of amino acids and glycinebetaine.

The osmotic contribution of the accumulated solutes was calculated based on the factor that 1M solution of organic solutes exerts ψ_s of -2.48 MPa (Nobel, 1974).

1.5.3 *Results*

1.5.3.1 Water status

Leaf RWC, ψ and ψ_p of cold stressed plants did not differ significantly from that in plants at 20°C (Table 7). ψ_S, however, was significantly lower in cold stressed plants than in the control plants, with a drop of 0.19 MPa.

1.5.3.2 Amino acid and glycinebetaine content

The total amino acid content of cold stressed wheat leaves increased 3.8 fold (Table 7) over plants grown at 20°C during the same period. Minor contributions to this increase were made by threonine, glutamate, glycine, valine, and isoleucine. Glycinebetaine concentration increased by more than 2 fold. Moderate to high contributions to the total amino acid increase were made by aspartic acid, asparagine, glutamine, alanine, γ-aminobutyric acid, and proline. Aspartic acid and alanine showed consistent declines in response to water deficit; however, these accumulated significantly in response to cold stress. Most of the other amino acids showed no significant change in response to cold stress.

Among the accumulated amino acids the highest contribution made to the increase in amino acid content was by glutamine (51.3%), followed by proline (10.3%), γ-aminobutyric acid (9.6%), alanine (8.2%), aspartic acid (8%), asparagine (5.5%), glycine (3.6%), and glycinebetaine (2.2%). However, the imino acid, proline, responded to cold stress by increasing more than 52 fold over the control, followed by the amide glutamine (25.3 fold). The accumulated solutes contributed to a drop of 0.17 MPa in ψ_S when calculated on an assumption that the solutes were distributed uniformly without any compartmentation.

Table 7: Water status and solute content (μmol g^{-1} dw) of wheat (cv. Banks) leaves exposed to cold stress for 5 days

	Control (20°C)	Cold stressed (4°C)	LSD (P = 0.05)
RWC (%)	98.33	96.27	NS
ψ (-MPa)	0.59	0.72	NS
ψ_S (-MPa)	0.96	1.15	0.06
ψ_P (MPa)	0.38	0.44	NS
Asp	24.97	61.30	6.18
Thr	5.13	8.37	3.15
Ser	26.37	27.90	NS
Asn	0.73	25.90	4.36
Glu	50.03	38.50	6.50
Gln	9.60	242.70	52.58
Gly	4.20	20.49	4.29
Ala	18.83	56.30	3.81
Val	5.20	12.67	2.38
Met	0.67	1.40	NS
Ile	0.95	2.00	1.02
Leu	1.00	1.83	NS
Tyr	0.80	1.23	NS
Phe	0.50	1.10	NS
GABA	9.00	52.50	4.47
His	0.60	0.67	NS
Try	0.47	0.60	NS
Orn	0.76	1.10	NS
Lys	0.75	0.93	NS
Arn	0.67	1.33	NS
Pro	0.90	47.60	6.80
Total	162.13	616.42	
Glb	7.87	17.93	3.50

NS = non significant

1.6 Discussion

In the present investigation, rapid water stress imposition by the application of PEG or by withholding water resulted in negative ψ_p (Tables 5 and 6) and this was more severe when plants were water stressed at high temperature (Fig. 6d). The existence of negative ψ_p has been questioned and dismissed as an artifact resulting from the dilution of symplastic water with apoplastic water when the cell membranes were broken by freezing and thawing (Tyree, 1976). Such mixing of apoplastic and symplastic water could have contributed, at least in part, to the apparent negative ψ_p observed in the present study but recent evidence supports the case that negative ψ_p in plants is a real phenomenon (Oertli, 1985, 1986a, 1986b).

Among the amino acids, proline is the only one to consistently accumulate to a very high level in a range of species when water stressed (Aspinall and Paleg, 1981). However, the results reported in this section demonstrate that the quantitative relationship between proline and other amino acids is altered by the nature of stress. Proline dominated the amino acid pool when plants were water stressed at 20°C, with other amino acids such as glutamine, asparagine, valine, and glycine also accumulating but to a lesser extent (Table 3). The maintenance of high temperature during water stress resulted in a different pattern of accumulation, in which asparagine was the dominant amino acid followed by proline, glutamine and valine.

The accumulation of the amide form of amino acids and amide domination of the amino acid pool (Table 3) in response to rapid water stress in the present investigation is similar to the composition of the phloem sap of water stressed plants in the work of Hanson and Tulley (1979). These workers found that amides were synthesised in preference to proline when labelled ammonia was fed to water stressed leaves. This led to the conclusion that proline is not involved in the detoxification of ammonia liberated during water stress. This conclusion has been questioned by Yamada and Fukutoku (1983) on the basis of the results of Kato (1980) who showed that externally fed labelled ammonia was incorporated into proline, and that accumulation of proline could serve as a

potential ammonia disposal mechanism. In the present investigation, a massive accumulation of amides was not observed when water stress was imposed by withholding water (Table 4) where plants were subjected to slower development of leaf water deficit (a reduction of 0.4-0.5 MPa per day compared to 1.5 MPa reduction when osmotically shocked by PEG).

Although no radio active material was used in this investigation, the tissue amino acid concentrations found here and in earlier studies (Barnett and Naylor, 1966; Singh *et al.*, 1973a; Hanson and Tulley, 1979; Tulley and Hanson 1979; Kato,1980) suggest that the differences in tissue amino acid composition and the differences in the presence of label in various amino acids, particularly in glutamine (Hanson and Tulley, 1979) is due to the manner of water stress imposition. In the work of Barnett and Naylor (1966), Singh *et al.* (1973a), and the present investigation, when water stress was imposed by withholding water or by drying the plant material in air, proline alone accumulated. Similarly, in plants stressed in the same manner proline alone was labelled from ^{15}N-labelled ammonia (Kato, 1980). However, when plants were exposed to an osmotic shock (Table 3) which was further enhanced by exposure to high temperature, a situation similar to that observed by Hanson and Tulley (1979) was recorded. These observations suggest that abrupt water stress imposition (Hanson and Tulley, 1979 applied -1.9 MPa PEG abruptly) is responsible for glutamine appearing as the dominant amino acid in the phloem (Hanson and Tulley, 1979). A further reason to question the conclusion of Hanson and Tulley (1979) arises from the work of Kato (1980) who found that labelled ammonia was incorporated into glutamine during the initial 1 h and, following this lag period, proline was synthesised from ammonia and became the major labelled compound. Hanson and Tulley (1979) measured the amino acid composition of phloem 30 min after feeding labelled ammonia and did not present the time course of incorporation. It would seem that their obsevations were too early to record any rapid proline synthesis from the supplied ammonia, even if the stress which they imposed would have eventually resulted in the synthesis of proline from labelled ammonia.

A further potential consideration is that, PEG may have a specific effect on amino acid metabolism unrelated to the rate of stress imposition. Both the shock PEG application and the incremental treatment induced the accumulation of glutamine, asparagine, and valine. A similar response was observed when plants were rapidly stressed by withholding water at a high ventilation rate but not when water was imposed slowly in the absence of PEG. These results suggests that both PEG, however supplied, and rapid stress favours the accumulation of these compounds. As PEG application produces some differences in response in amino acid accumulation it should be used with caution. Further water stress imposition as an osmotic shock and PEG is a poor combination for simulating field stress responses. Nevertheless, in the present investigation, due to the over-riding concern to examine the differences in the responses recorded by these two groups, this experimental techinique was adopted.

Both proline and glycinebetaine showed a greater response to water stress (by PEG or withholding water) when it developed slowly than rapidly. This contrasts with the conclusions of Wyn Jones and Storey (1978) who stated that proline accumulated most rapidly in abrupt water or salinity stress whereas glycinebetaine reached higher concentrations with slower stress imposition. As a consequence, they concluded that proline accumulation is correlated with tissue dehydration and glycinebetaine with osmotic adjustment (Wyn Jones and Storey, 1981).

In this study, rapid ventilation induced accumulation of γ-aminobutyric acid in well watered wheat plants. Further, this amino acid accumulated mostly in response to rapid water stress. It has been suggested that γ-aminobutyric acid accumulates in aging leaves (Lahdesmaki, 1968), in response to wounding (Selman and Cooper, 1978), and following leaf detachment or leaf rolling (Wallace *et al.*, 1984). It is possible that leaf movement caused by wind, damaged cells or cell membranes resulting the accumulation of γ-aminobutyric acid.

The elevated proline concentration during stress rapidly returned to the control concentration after re-watering (Table 4). A similar rapid loss of proline has been observed many times in both monocots and dicots (Stewart *et al.*, 1972b; Singh *et al.*,

1973a; McMichael and Elmore, 1977; Parameshwara and Krishnasastry, 1980; Riazi *et al.*, 1985; Xiao-nan *et al.*, 1986) but differs from the results of Hanson *et al.* (1977) who reported a lack of complete reduction in proline content and of Lewin (1980) who found a continuing increase in proline concentration up to 5 days after re-watering. In the experiments of both these authors the plants were severely water stressed leading to death of part of the leaves. The unutilized proline in the plants of Hanson *et al.* (1977) was most certainly in dead cells which would undoubtedly have restricted both the translocation and further metabolism of proline. The proline which was lost from the first leaves of the wheat seedlings in the current work was probably oxidized to glutamate (Stewart, 1972b) and served as an energy source for the recovering plant (Barnett and Naylor, 1966; Blum and Ebercon, 1976). This is evident from the increase in glutamate level during stress relief. It would appear that, in the absence of tissue death, any accumulated proline is readily available for metabolism following stress relief.

The glycinebetaine concentration in the previously stressed leaves did not decline until the third day after relief of stress and even this decline may have been due to translocation to growing areas and not to metabolism of the compound (Ahmad and Wyn Jones, 1979; Ladyman *et al.*, 1980; Grattan and Grieve, 1985). It has been suggested by Bowman and Rohringer (1970) that plants have no ability to metabolise glycinebetaine, as most of the externally fed radio labelled glycinebetaine has been unutilised even after several days in wheat plants.

The concentrations of amides, asparagine and glutamine (together with valine) increased during the first day after stress relief, possibly due to increased synthesis during the detoxification of ammonia originating from the rapid catabolism of proline (Blum and Ebercon, 1976) and other amino acids. The subsequent decline in the level of amides in the leaf by the third day may have been due to translocation, as well known N carriers, to the growing areas of the wheat seedlings (Lea and Miflin, 1980). Valine may have a similar function in wheat seedlings during the stress recovery period.

The continued increase in the concentrations of glycine after re-watering, and the partial reduction in aromatic amino acids, tryptophan, phenylalanine, and tyrosine

concentrations differ from the responses found by of McMichael and Elmore (1977) where there was a complete return of these compounds to control concentrations in cotton plants following stress relief. This difference in response suggests that wheat and cotton may possess some differences in metabolism during stress relief. In addition, high levels of aromatic amino acids (eg. phenylalanine) has been found to be detrimental to the enzyme activity (Paleg and Keech, unpublished). These amino acids, threfore, may have been sequestered into vacuoles and hence immediate metabolism was not apparent upon stress relief. γ-aminobutyric acid, in this investigation, also failed to return to the control level within 3 days of water stress relief, showing a distinct difference from its response during cold stress recovery (Wallace *et al.*, 1984). Initiation of a partial recovery of glutamate, aspartate, and alanine indicates that the stress-induced response of these amino acids is reversible.

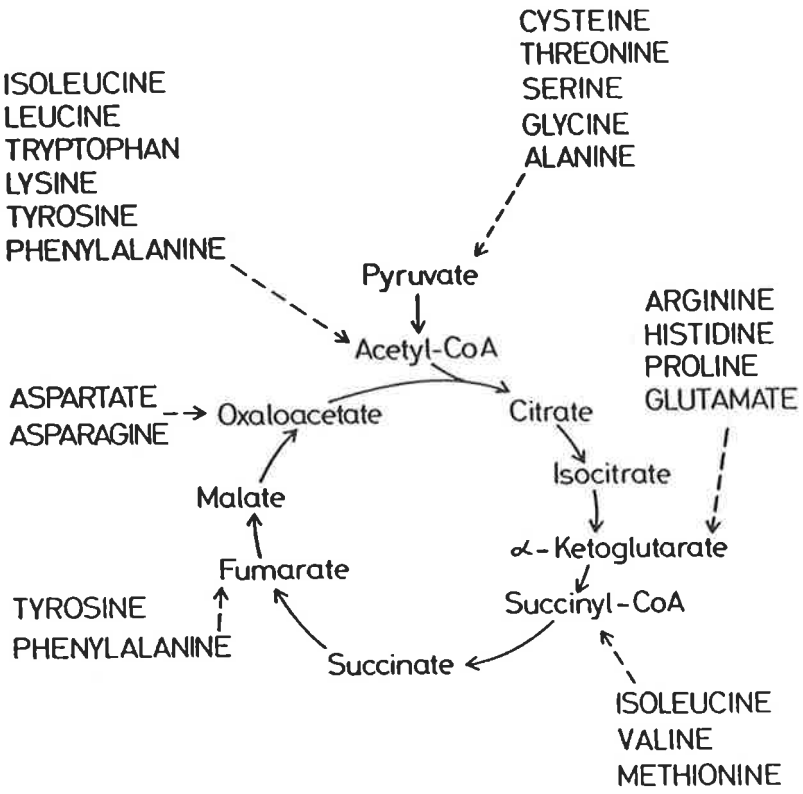
There were more similarities than differences in the spectrum of amino acid changes in response to cold and water stress. There was an accumulation of threonine, asparagine, glutamine, glycine, valine, isoleucine, γ-aminobutyric acid, proline and glycinebetaine (see Tables 4 and 7). However, cold stressed plants accumulated aspartate and alanine which were always depleted during water stress. Hence, it is possible that the derivation of at least some of these amino acids in response to cold stress might be different to that in water stress. Amino acid accumulation cannot be related to a change in leaf water status in cold stressed wheat leaves as there were no differences in RWC, ψ, or ψ_p as was previously noted by both Chu *et al.* (1974, 1978) and Parameshwara (1984). Cold stressed plants in the present experiment showed a significant reduction in leaf ψ_s. Calculated osmotic contribution of accumulated amino acids and glycinebetaine was almost equal to the measured reduction in ψ_s. This occured without taking into consideration the contribution of sugars and polyamines and even when an assumption was made that no compartmentation occured. The assumption of compartmentation would result in an over estimation of osmotic contribution in cold stress. Similar condition was encountered by Ford and Wilson (1981) with water stressed pasture plants accumulating proline and glycinebetaine. It seems likely that compartmentation of solutes

is not occuring in cold stressed plants as in salinised plants (Wyn Jones, 1984) or the compartmented solutes are in some way partially osmotically inactive. For example, Schobert (1977) suggested that proline and other low molecular weight solutes which accumulate in response to water stress, may become associated with, and increase the water binding capacity of the plant cell walls. However, the accumulated solutes in cold stress may be involved in membrane protection as in heat stress (Jolivet *et al.,* 1982, 1983) and thus may be inactive osmotically. In cold stress, accumulation of amino acids may be a consequence of the reduction in respiratory activity at low temperature (Raison, 1980). When respiratory activity is reduced, several compounds involved in the TCA cycle tend to accumulate, from which specific amino acids may be synthesised. Such compounds include α-ketoglutarate, and pyruvate (Murata, 1969). Alternatively, the entry of amino acids into the respiratory processes may be inhibited by low temperature leading to accumulation. The accumulated amino acids in cold stressed wheat seedlings can be classified into several groups based on the key compound from which they are synthesised, or through which they would be oxidised in the TCA cycle (Fig. 8). Glutamine, glutamate, and proline are metabolically related to α-ketoglutarate in the TCA cycle; glycine, alanine, and serine to phosphoenolpyruvate, or pyruvate; aspartate, and asparagine to oxaloacetate; isoleucine, and valine to acetyl CoA (ap Rees, 1980; Mazelis, 1980) and γ-aminobutyrate to glutamate (Wallace *et al.*, 1984).

Glycinebetaine accumulated in response to both high (Table 3) and low temperature (Table 7) in the absence of leaf water deficit. This is the first report of its accumulation in response to high temperature and contrasts with proline which accumulates in cold but not heat stress (Chu *et al.*, 1974, 1978). A detailed study of the response of glycinebetaine to temperature will follow in the next section of the thesis.

Fig. 8 Possible routes of amino acid catabolism.

(adapted from ap Rees, 1980)


CYSTEINE
THREONINE
SERINE
GLYCINE
ALANINE
ISOLEUCINE
LEUCINE
TRYPTOPHAN
LYSINE
TYROSINE
PHENYLALANINE
Pyruvate
ARGININE
HISTIDINE
PROLINE
GLUTAMATE
Acetyl-CoA
ASPARTATE
ASPARAGINE
Oxaloacetate
Citrate
Isocitrate
Malate
α-Ketoglutarate
Fumarate
Succinyl-CoA
TYROSINE
PHENYLALANINE
Succinate
ISOLEUCINE
VALINE
METHIONINE

Section 2: Variability in the accumulation of proline and glycinebetaine associated with temperature during barley seedling growth environment

2.1 Introduction

Proline and glycinebetaine-accumulating abilities of barley cultivars have been found to be genetically controlled (Hanson *et al.*, 1979; Grumet *et al.*, 1985), but the conflict between the responses of Excelsior and Proctor cultivars of barley in the studies of Singh *et al.* (1972) and Hanson *et al.* (1977) suggest that accumulation is subject to modification. Differences in growth conditions during the experiments of Singh *et al.* (1972, 1973c) and Hanson *et al.* (1977) may be responsible for the differences in response. Hanson *et al.* (1977) grew their barley seedlings in a glasshouse. The performance of such plants has been found to differ from that of plants grown with strict environmental control and temperature has been thought to be responsible for the difference (Waldron, 1941).

The experiments reported in this section examine the effects of temperature during seedling growth on solute accumulation when the seedlings were subsequently water stressed. The experiments were extended to study the effects of temperature on the accumulation of these solutes in both water stressed and well-watered plants.

2.2 Temperature during seedling growth and the accumulation of proline and glycinebetaine during subsequent water stress.

2.2.1 *Methods*

Barley seedlings (cv. Clipper) were grown with a photon flux density of 500 μE m^{-2} s^{-1}, 16h photoperiod and 20° ± 1°C temperature in a growth cabinet. At eight days one group of seedlings was transferred for 4 days to 10°C, at 9 days a further group was transferred to 15°C for 3 days, and at 10 days the remaining seedlings were transferred to 20, 25, 30, or 35°C for 2 days. These varying length of treatment at different

temperatures were designed to equalise the physiological age of all seedlings at the end of the period in the various temperatures. After this pre-stress period, seedlings were returned to a growth cabinet at 20°C. The photon flux density during pre-stress and the subsequent water stress period was only 100 $\mu E\ m^{-2}\ s^{-1}$, plants being illuminated continuously with light from 5 incandescent lights (Philips, SL*25; 25W/1200 lumen/240V-50 Hz). A low photon flux density has been shown to be effective in inducing proline accumulation during water stress if plants are previously illuminated with bright light (Joyce *et al.*, 1984). Three pots from each of the pre-stress temperature regimes were water stressed for 1 day by flooding the rooting medium with PEG (-1.5 MPa) and another 3 well-watered pots from each of the pre-stress temperature regimes served as controls. Proline and glycinebetaine contents were estimated from the freeze dried first leaves.

2.2.2 *Results*

2.2.2.1 Proline content

The proline content of both control and water stressed plants showed a general decline with increasing temperature during the pre-stress period (Fig. 9a). This occured despite the fact that all plants were returned to 20°C for 24h before being sampled for proline content. Exposure to water-stress during this 24h increased the proline content; however, proline content was dependent on the temperature history of the seedlings. A significantly higher proline content was found in plants that experienced 10 or 15°C prior to water stress than in the rest.

2.2.2.2 Glycinebetaine content

The glycinebetaine content of plants exposed to different temperatures in the pre-stress period also varied (Fig. 9b). As with proline accumulation, water stress did not vary the pattern of accumulation associated with the pre-stress temperature although it increased accumulation throughout. In contrast to proline accumulation, however, glycinebetaine accumulation was stimulated both by low (10°C) and by high (30 - 35°C)

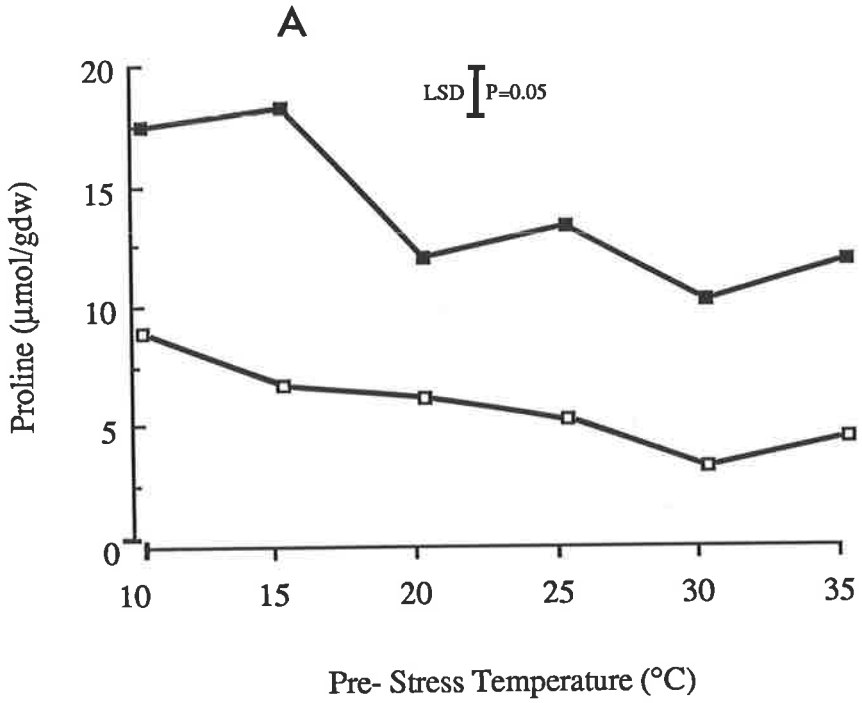
Fig. 9 Leaf proline and glycinebetaine content (μmol g^{-1} dw) of barley seedlings grown prior to water stress at various temperatures.

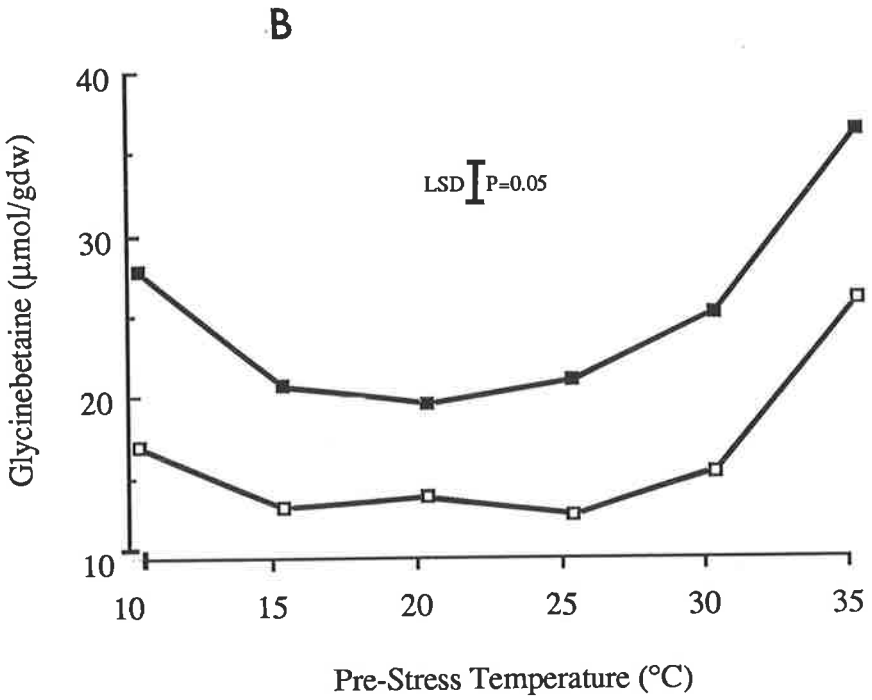
A. Proline

B. Glycinebetaine

□ Control

■ Water stressed


A
LSD P=0.05
Proline (µmol/gdw)
20
15
10
5
0
10
15
20
25
30
35
Pre- Stress Temperature (°C)


B
LSD P=0.05
Glycinebetaine (µmol/gdw)
40
30
20
10
10
15
20
25
30
35
Pre-Stress Temperature (°C)

temperatures, with accumulation being at a minimum over the range of 15 to 25°C. However, pre-treatment with high temperature was more effective than low temperature in increasing glycinebetaine levels.

2.3 Temperature during water stress and the time course of proline and glycinebetaine accumulation

2.3.1 *Introduction*

Proline and glycinebetaine content (Figs. 9a and 9b) varied with the temperature during seedling growth, eventhough plants were exposed to water stress at one temperature. To understand these effects, knowledge of the responses of proline and glycinebetaine to temperature was thought to be useful. This experiment was to further explore the effect of temperature on accumulation by subjecting plants to different temperatures during water stress and measuring the course of accumulation of glycinebetaine and proline. No information is available of the response of glycinebetaine content to temperature extremes although, the response of proline is documented (Chu *et al.*, 1974,1978).

2.3.2 *Methods*

Barley seedlings (cv. Clipper) were maintained under standard environmental conditions for 11 days and then transferred to 25 or 35°C and water stressed 4 hours after the beginning of the photoperiod by flooding the rooting medium with -1.5 MPa PEG. Well-watered control plants were maintained at both temperatures. The PEG and nutrient solutions used were maintained at cabinet temperature. The light environment during water stress was the same as in the previous experiment (2.2). Both control and water stressed plants were sampled 0, 2, 4, 8, 12, 18, 24, 36 and 48 hours after the commencement of stress. All treatments were replicated three times. ψ was measured on first leaves at each sampling time, and freeze-dried first leaf samples were used to estimate the contents of proline and glycinebetaine.

2.3.3 *Results*

2.3.3.1 Water status

Well watered plants at 35°C had a significantly lower ψ than similar plants maintained at 25°C on most sampling occasions (Fig. 10). There was a slight decline in ψ of the control plants at 25°C (until 8 h) which may have been due to the early phase of the normal diurnal pattern of transpiration, however, ψ stabilised thereafter. Plants water stressed at 25°C showed a continuous decline in ψ over the whole 48 h, showing significant differences from control at 12 h and falling to -1.7 MPa at 48 h after the inception of stress. Water stress at the higher temperature (35°C) resulted in a more rapid decline in ψ, the differences from well-watered plants being apparent as early as 4 h after stress inception. The rate of decline in ψ seemed to increase at 18 h and ψ reached -3.0 MPa, at 48 h.

2.3.3.2 Proline content

Exposure of well watered plants to the higher temperature (35°C) produced no significant change in leaf proline content (Fig. 11a) over 48 h despite the slight drop in ψ. Proline accumulated continuously over the whole 48 h period when plants were water stressed. The proline content of stressed leaves was higher than the control levels within 12 h of the inception of stress at both temperatures. The rate of net proline accumulation was higher at 35 than at 25°C (0.84 and 0.44 μmol g^{-1} dw h^{-1} at 35 and 25°C, respectively). At the end of 48 h, proline concentration had reached 22.8 μmol g^{-1} dw at 25°C and 42.3 μmol g^{-1} dw at 35°C with no evidence of a slowing in the rate of accumulation at either temperature.

2.3.3.3 Glycinebetaine content

The glycinebetaine content of well watered plants maintained at 25°C did not change in the course of the experiment (Fig. 11b). However, as in the previous experiment, the glycinebetaine content of the plants increased with time at 35°C even when they were not exposed to water stress. Water stress caused glycinebetaine

Fig. 10 Changes in leaf water potential (-MPa) of water stressed (-1.5 MPa PEG) barley seedlings at 25 or 35°C over a period of 48 h.

△ Control at 25°C

▲ Stressed at 25°C

□ Control at 35°C

■ Stressed at 35°C

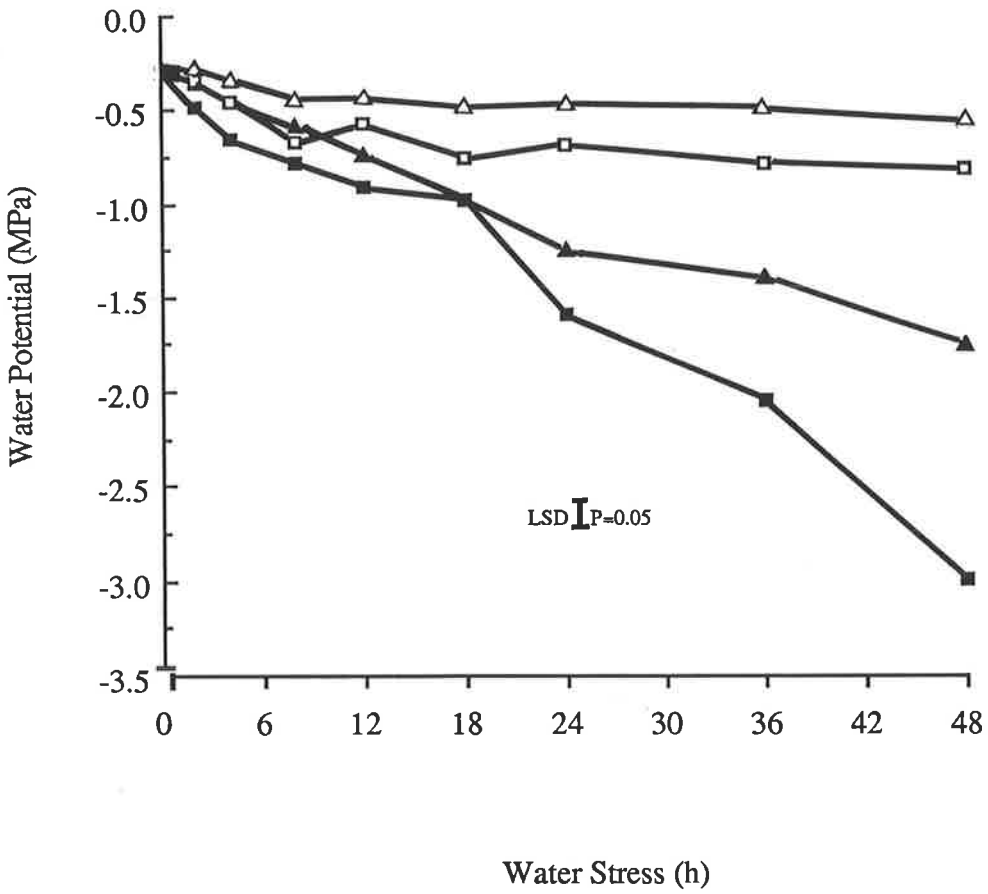

0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
Water Potential (MPa)
LSD P=0.05
0
6
12
18
24
30
36
42
48
Water Stress (h)

Fig. 11 Leaf proline and glycinebetaine content (μmol g^{-1} dw) of water stressed (-1.5 MPa PEG) barley seedlings at 25 or 35°C over a period of 48 h.

A. Proline

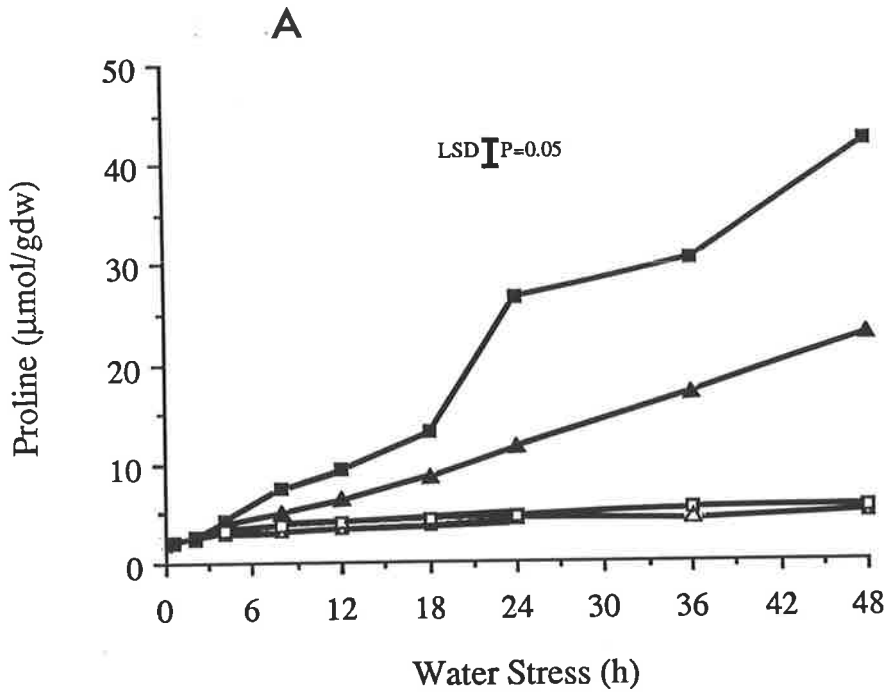
B. Glycinebetaine

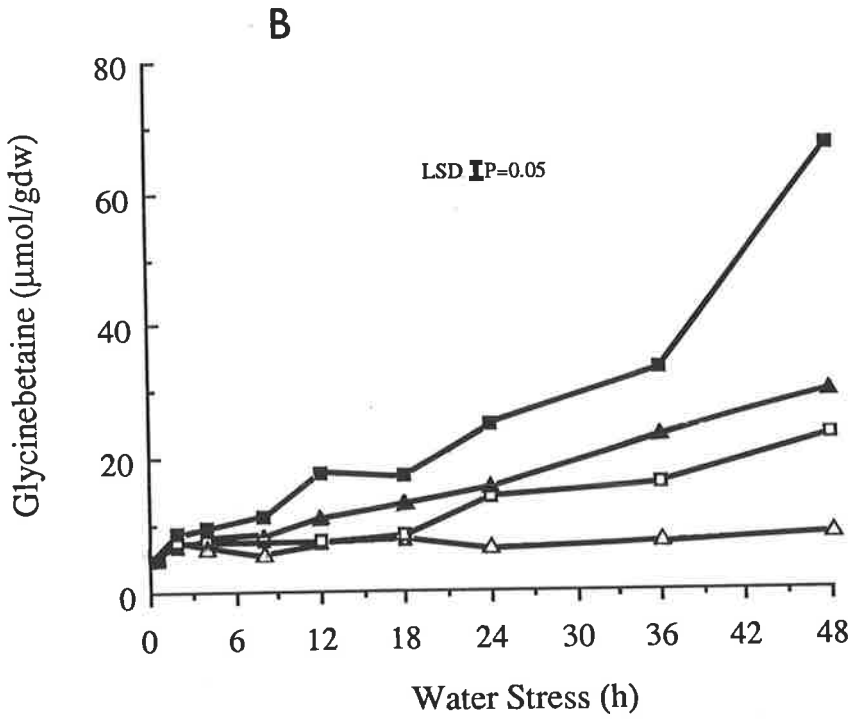
△ Control at 25°C

▲ Stressed at 25°C

□ Control at 35°C

■ Stressed at 35°C


A
Proline (µmol/gdw)
LSD P=0.05
0
10
20
30
40
50
0
6
12
18
24
30
36
42
48
Water Stress (h)


B
Glycinebetaine (µmol/gdw)
LSD P=0.05
0
20
40
60
80
0
6
12
18
24
30
36
42
48
Water Stress (h)

accumulation at both temperatures and the glycinebetaine content of water stressed plants was significantly higher than in the well-watered controls at 12 h and 8 h after stress initiation at 25 and 35°C, respectively. The mean net accumulation rates for glycinebetaine at 25 and 35°C were similar to those for proline at the same temperatures, at least for the first 36 h, but thereafter there was an apparent increase in the rate of glycinebetaine accumulation at 35°C.

2.4 Proline and glycinebetaine accumulation during water stress at a range of lower temperatures

2.4.1 *Introduction*

The results of the previous experiment (2.3) showed that the time required for the significant accumulation and subsequent rate of increase of proline and glycinebetaine in response to rapid water (PEG) stress varied with temperature. The objective of this experiment was to examine the effects of lower temperatures on accumulation rates and the time required for the significant accumulation of these two solutes.

2.4.2 *Methods*

Barley plants (cv. Clipper) were grown for 11 days under standard environmental conditions and water stressed (-1.5 MPa PEG) at 5, 15 and 25°C. Corresponding well-watered controls were maintained. Plants were sampled 0, 6, 12, 18 and 24 h after water stress imposition. The treatments were replicated three times The ψ measurements and estimation of proline and glycinebetaine were carried out on the first leaves.

2.4.3 *Results*

2.4.3.1 Water status

Well watered plants showed no differences in ψ with time at 15 or 5°C, but there was a drop in ψ by 12h at 25°C, possibly due to peak diurnal transpiration (Fig. 12). At all temperatures, leaf ψ of water stressed plants differed significantly from the well

Fig. 12 Changes in leaf water potential (-MPa) of water stressed (-1.5 MPa) barley seedlings at 5, 15 or 25°C over a period of 24 h.

○ Control at 5°C

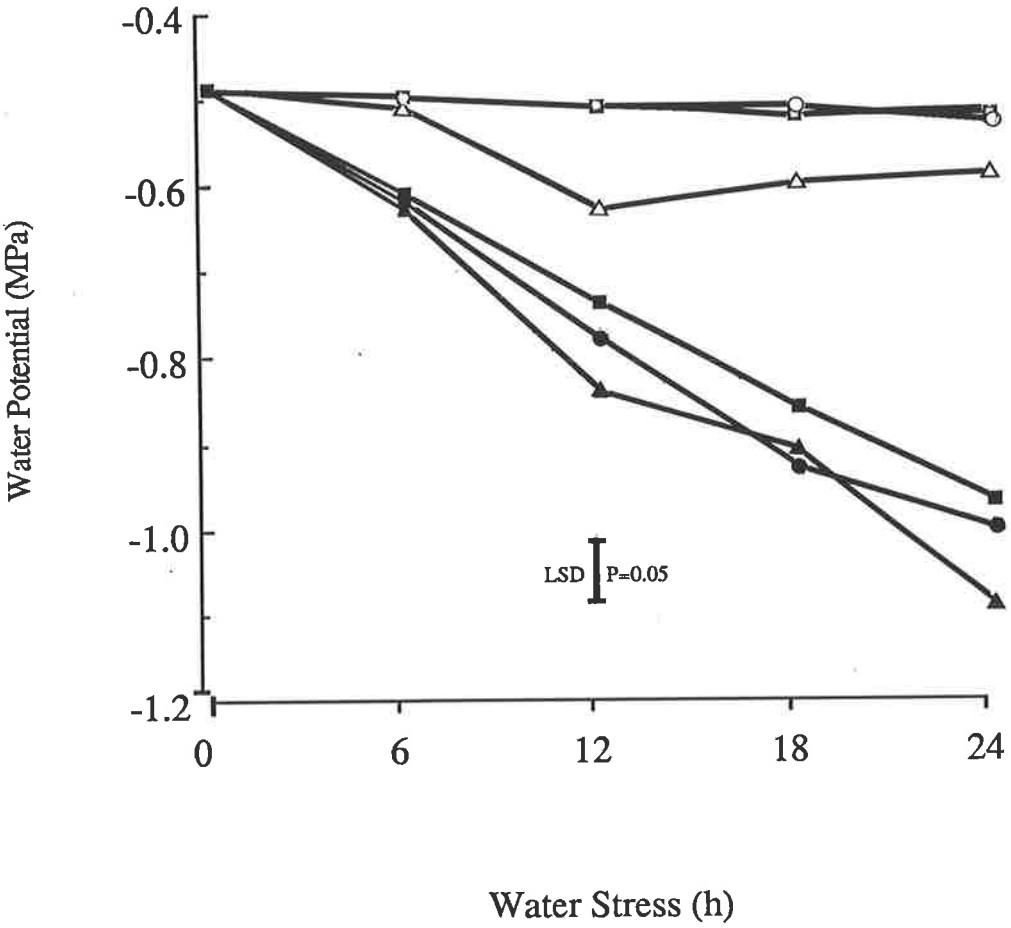
● Stressed at 5°C

□ Control at 15°C

■ Stressed at 15°C

△ Control at 25°C

▲ Stressed at 25°C


-0.4
-0.6
-0.8
-1.0
-1.2
Water Potential (MPa)
LSD P=0.05
0
6
12
18
24
Water Stress (h)

watered plants 12 h after the inception of stress and showed a continuous decline with time. The lowest ψ reached was -1.1 MPa at 25°C at 24 h which was the only occasion on which ψ at this temperature diverged significantly from that at lower temperatures. However, ψ at 15 and 5°C did not differ from one another (Fig. 12).

2.4.3.2 Proline content

The proline contents of well-watered plants did not vary over the 24 h of the experiment at 15 and 25°C but the plants accumulated some proline at 5°C (Fig. 13a). Water stressed plants at all temperatures contained a higher proline content than the corresponding control plants 12 h after stress inception (Fig. 13a). The rates of proline accumulation in water stressed plants at the 3 temperature regimes did not differ.

2.4.3.3 Glycinebetaine content

Glycinebetaine, like proline, accumulated in well watered plants at 5°C but not at 15 or 25°C. Water stressed plants accumulated significant amounts of glycinebetaine 12 h after the inception of stress and the highest rate of accumulation (0.61 μmol g^{-1} dw h^{-1}; assuming the mean accumulation rate is linear) was at 25°C (Fig. 13b). The lowest rate of accumulation was in the plants water-stressed at 15°C, and an intermediate rate was found with those stressed at 5°C.

Fig. 13 Leaf proline and glycinebetaine content (μmol g^{-1} dw) of water stressed (-1.5 MPa PEG) barley seedlings at 5, 15 or 25°C over a period of 24 h.

A. Proline

B. Glycinebetaine

○ Control at 5°C

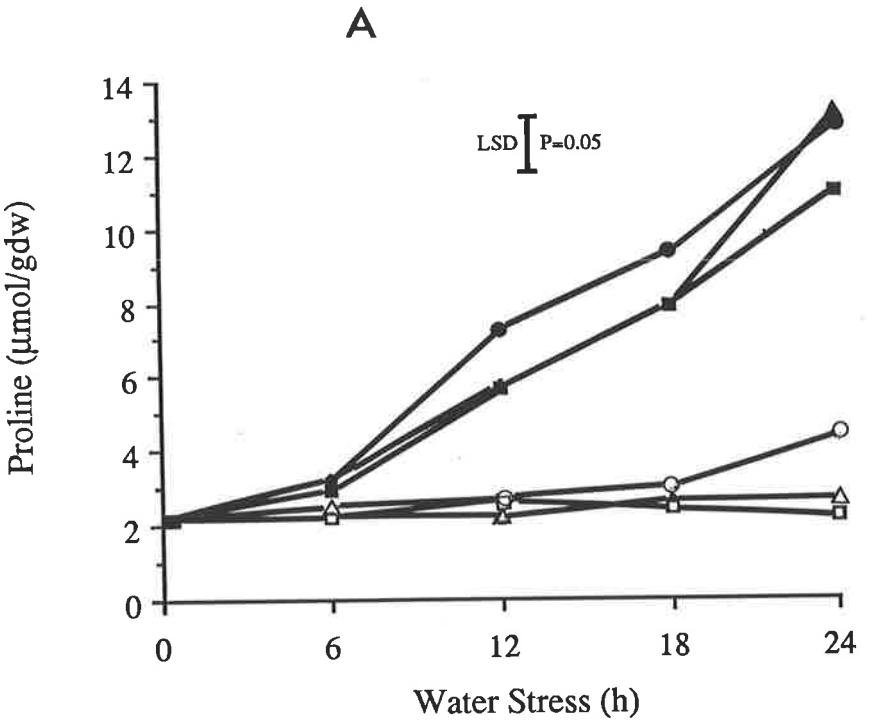
● Stressed at 5°C

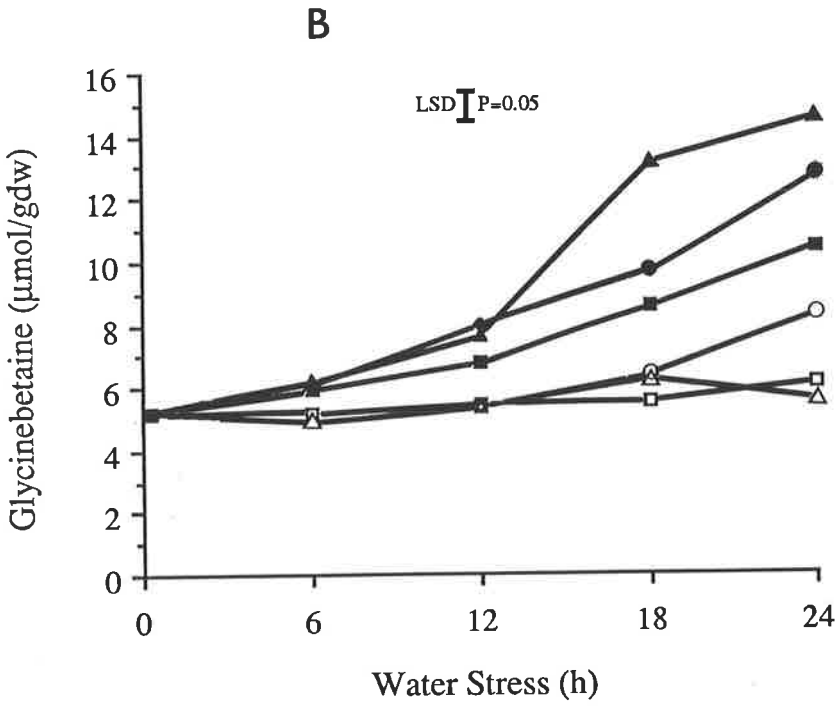
□ Control at 15°C

■ Stressed at 15°C

△ Control at 25°C

▲ Stressed at 25°C


A
Proline (µmol/gdw)
LSD P=0.05
0
2
4
6
8
10
12
14
0
6
12
18
24
Water Stress (h)


B
Glycinebetaine (µmol/gdw)
LSD P=0.05
0
2
4
6
8
10
12
14
16
0
6
12
18
24
Water Stress (h)

2.5 Proline and glycinebetaine accumulation during heat stress at high or low vapour pressure deficit in well watered seedlings

2.5.1 *Introduction*

The increase in glycinebetaine content of well-watered plants in response to heat stress in the earlier experiments could possibly be attributed to the accompanying fall in leaf water status. The objective of this experiment was to examine the heat stress response of glycinebetaine in the absence of any concomitant leaf water deficit.

2.5.2 *Methods*

Barley seedlings (cv. Clipper) were grown under standard environmental conditions for 11 days and were then transferred to different growth cabinets with a photon flux density of 250 $\mu E\ m^{-2}\ s^{-1}$ (fluorescent lights) and 16 h photoperiod at either 20 or 35°C. At each temperature low and high vapour pressure deficit (VPD) regimes were maintained to differentiate between the effects of temperature *per se* (at a low VPD) and temperature plus a temperature-induced leaf water deficit (at high VPD). All treatments were replicated thrice. A low VPD of 0.2 KPa was maintained at both temperatures by adjusting the RH of the cabinets. The high VPD differed between 20 and 35°C, however, with a VPD of 1.2 KPa at 20°C and 2.7 KPa at 35°C. All plants were watered twice daily to minimise the development of water deficits. Half strength Hoagland's solution maintained at the temperature of the treatment was used. ~~The treatments were replicated three times~~. The first leaf was sampled 4 h after the initiation of photoperiod, before watering 1, 3, 6 and 10 days after exposing the plants to high temperature.

2.5.3 *Results*

2.5.3.1 Water status

There were no significant differences in mean ψ (over all days of sampling) between VPD regimes when the temperature was 20°C (Fig. 14a). With an increase in

Fig. 14 Leaf water relations of well-watered barley seedlings exposed to 20 or 35°C at a low or high atmospheric vapour pressure deficit (VPD). Each value is an average over 10 days.

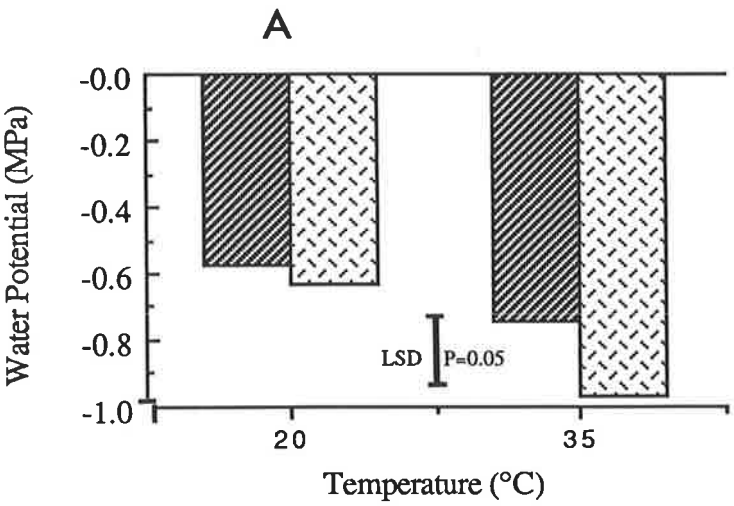
A. Water potential

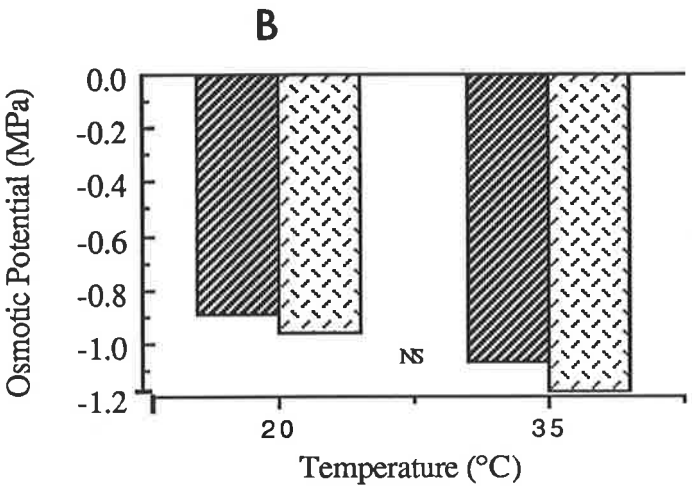
B. Osmotic potential

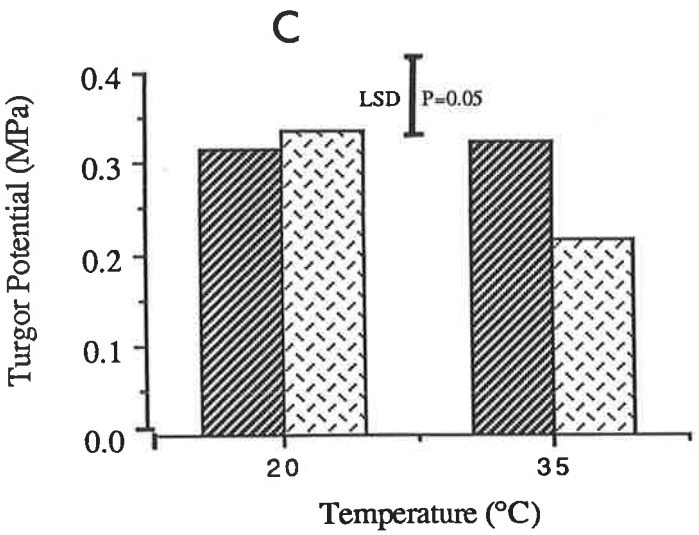
C. Turgor potential

Low VPD

High VPD


A
Water Potential (MPa)
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
LSD P=0.05
20
35
Temperature (°C)


B
Osmotic Potential (MPa)
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
NS
20
35
Temperature (°C)


C
Turgor Potential (MPa)
0.4
0.3
0.2
0.1
0.0
LSD P=0.05
20
35
Temperature (°C)

temperature to 35°C, ψ fell considerably. This fall in ψ was greater when the VPD was high than when it was low. Even maintenance of a low VPD could not completely prevent a fall in ψ at 35°C from the value at 20°C. The ψ_s of the leaf showed a similar trend to that of ψ (Fig. 14b) but, differences were not significant. As a result, seedlings grown at the high temperature and the high VPD had the lowest ψ_p (Fig. 14c), there being no other significant differences in ψ_p between treatments.

2.5.3.2 Proline content

Exposure of plants to 20 or 35°C resulted in no significant variation in the proline content over the period of 10 days at either VPD regimes (Fig. 15a). This was despite the fall in ψ of 0.3 MPa at the high VPD at 35°C.

2.5.3.3 Glycinebetaine content

The glycinebetaine content of leaves maintained at 20°C did not vary with VPD regime and there was no accumulation of glycinebetaine with time (Fig. 15b). However, the glycinebetaine content of the leaves of plants at 35°C was higher than that at 20°C. Moreover, the glycinebetaine content of the leaves differed between VPD regimes at 35°C. After the first day, the plants at a high VPD had a higher glycinebetaine content than did the plants at low VPD.

Fig. 15 Leaf proline and glycinebetaine content (μmol g^{-1} dw) of well-watered barley seedlings exposed to 20 or 35°C for a period of 10 days at low or high atmospheric VPD.

A. Proline

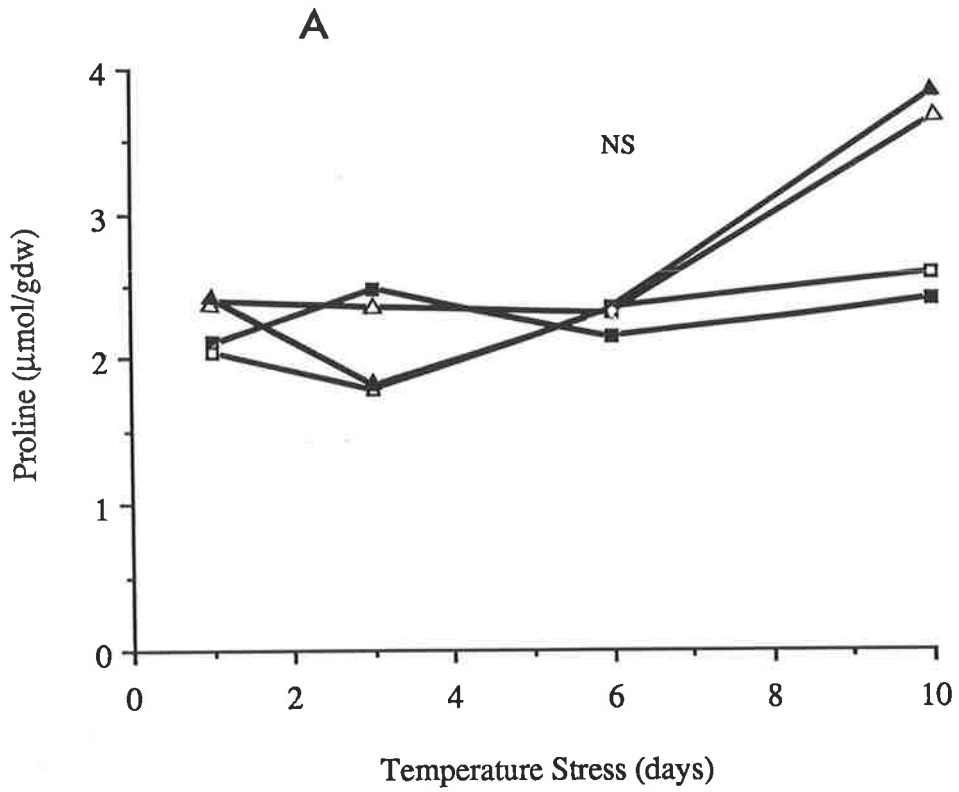
B. Glycinebetaine

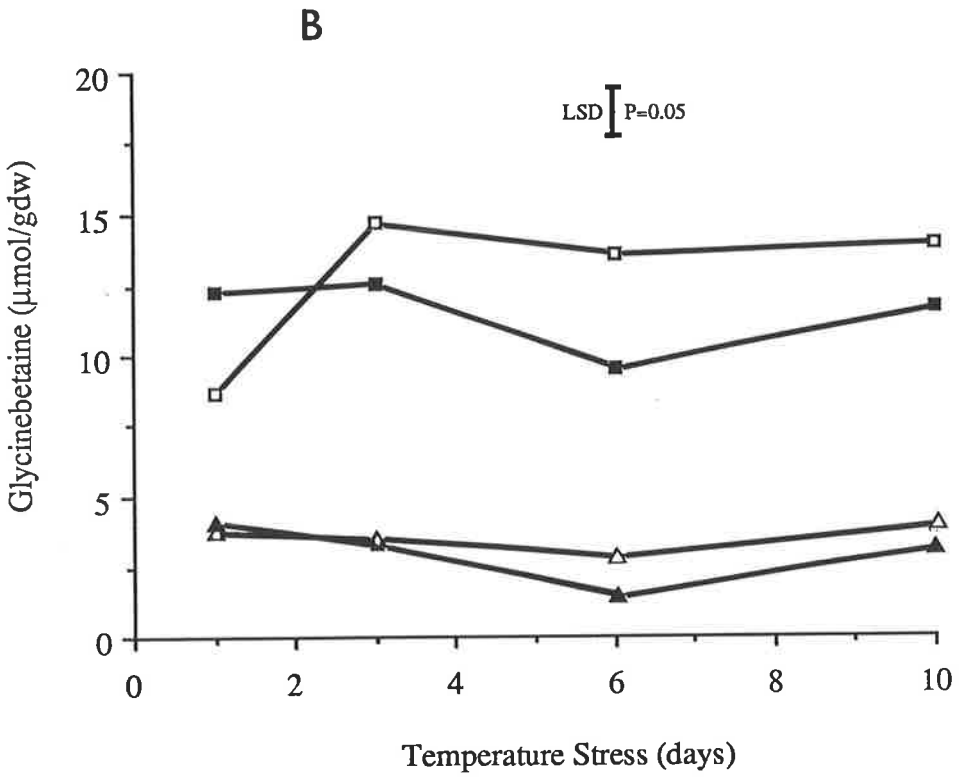
▲ Low VPD at 20°C

△ High VPD at 20°C

■ Low VPD at 35°C

□ High VPD at 35°C


A
NS
Proline (µmol/gdw)
0
1
2
3
4
0
2
4
6
8
10
Temperature Stress (days)


B
LSD P=0.05
Glycinebetaine (µmol/gdw)
0
5
10
15
20
0
2
4
6
8
10
Temperature Stress (days)

2.6 Proline and glycinebetaine accumulation during heat stress at high or low VPD in water stressed seedlings

2.6.1 *Introduction*

The glycinebetaine content of well-watered plants increased in response to temperature *per se* (see section 2.5). This response was also examined in water stressed plants with two levels of VPD.

2.6.2 *Methods*

Barley (cv. Clipper) seedlings were grown and stressed in similar environmental conditions to those in the experiment 2.4. Seedlings were water stressed by flooding the rooting medium with PEG (-1.5 MPa) at 20 or 35°C with similar VPD regimes to the previous experiment. Plants were water stressed for 1 day with well watered plants as controls and all treatments were replicated thrice. ψ, ψ_S, ψ_P, proline, and glycinebetaine were measured with the first leaves.

2.6.3 *Results*

2.6.3.1 Water status

Again well watered plants showed a significant reduction in ψ (Table 8a) in response to heat stress (35°C) and this was more pronounced at high VPD than at low VPD. Similar levels of ψ were found to those in the previous experiment. Water stress resulted in a lowering of ψ at both temperatures and both VPD regimes. When VPD was low, the water stressed plants had a similar ψ at 20 and 35°C. However, water stress at high VPD resulted in a lower ψ when the temperature of the environment was high (35°C). Plants at this temperature and VPD had a lower ψ than plants in all other treatments. ψ_S of the leaves (Table 8b) varied in a similar manner to ψ. Water stressed plants at 20°C had a significantly lower ψ_S when VPD was high than when it was low. At the low VPD there was a lower ψ_S when plants were water stressed at a high rather than a low temperature.

Table 8: Leaf water relations of water- and heat-stressed barley (cv. Clipper) seedlings exposed to low or high VPD for 1 day.

A: Water potential (-MPa)

		Low VPD	High VPD	LSD (P=0.05)
20°C	Control	0.60	0.69	
	Stress	1.04	1.17	
35°C	Control	0.76	0.97	
	Stress	1.17	2.10	0.17

B: Osmotic potential (-MPa)

		Low VPD	High VPD	LSD (P=0.05)
20°C	Control	0.95	0.98	
	Stress	1.07	1.17	
35°C	Control	1.01	1.14	
	Stress	1.30	1.79	0.094

C: Turgor potential (MPa)

		Low VPD	High VPD	LSD (P=0.05)
20°C	Control	0.35	0.29	
	Stress	0.03	0.00	
35°C	Control	0.25	0.17	
	Stress	0.13	-0.31	0.14

Plants lost turgor in response to water stress at both temperatures and both VPD regimes (Table 8c). The lowest ψ_p resulted from a combination of water stress, high temperature and high VPD.

2.6.3.2 Proline content

Variations in temperature and VPD regime alone had no effect on the proline content of well watered plants (Table 9a). When water stressed, however, plants at the high VPD accumulated significantly more proline than ones at the low VPD at 20 or 35°C although the effect was more pronounced at 35°C. High temperature at both VPD regimes resulted in significantly more proline accumulation than occurred in corresponding water stressed plants at 20°C. The proline content of the leaves was highly correlated with changes in ψ, ψ_s, and ψ_p of those leaves (Figs. 16 a, b, c).

2.6.2.3 Glycinebetaine content

Glycinebetaine accumulated in well watered plants in response to heat stress in both VPD regimes, however, low VPD resulted in a higher glycinebetaine content in leaves than when the VPD was high (Table 9b). When water stressed at 20°C, plants at the high VPD accumulated significantly more glycinebetaine than plants at the low VPD, as with the response of proline. However, when water stressed at the higher temperature, more glycinebetaine accumulated when VPD was low than when it was high. In this case, as ψ was higher at low than at high VPD, a relationship between glycinebetaine accumulation and leaf ψ was not maintained (Fig. 16a). In fact, glycinebetaine accumulation was significantly correlated with ψ_s alone, and that correlation was not close (Fig. 16b).

Table 9: Proline and glycinebetaine content (μmol g^{-1} dw) of water- and heat-stressed barley (cv. Clipper) seedlings exposed to low or high VPD for 1 day.

A: Proline

		Low VPD	High VPD	LSD (P=0.05)
20°C	Control	2.43	2.41	
	Stress	22.69	45.11	
35°C	Control	2.13	2.05	
	Stress	113.16	190.15	9.78

B: Glycinebetaine

		Low VPD	High VPD	LSD (P=0.05)
20°C	Control	4.09	3.78	
	Stress	7.60	10.34	
35°C	Control	12.36	8.71	
	Stress	55.53	39.96	2.41

Fig. 16 Relationship between components of leaf water potential and the proline and glycinebetaine contents of water stressed barley seedlings at 20 or 35°C at a low or high atmospheric VPD. (Data obtained from Tables 8 and 9.)

A. Proline or glycinebetaine content vs water potential

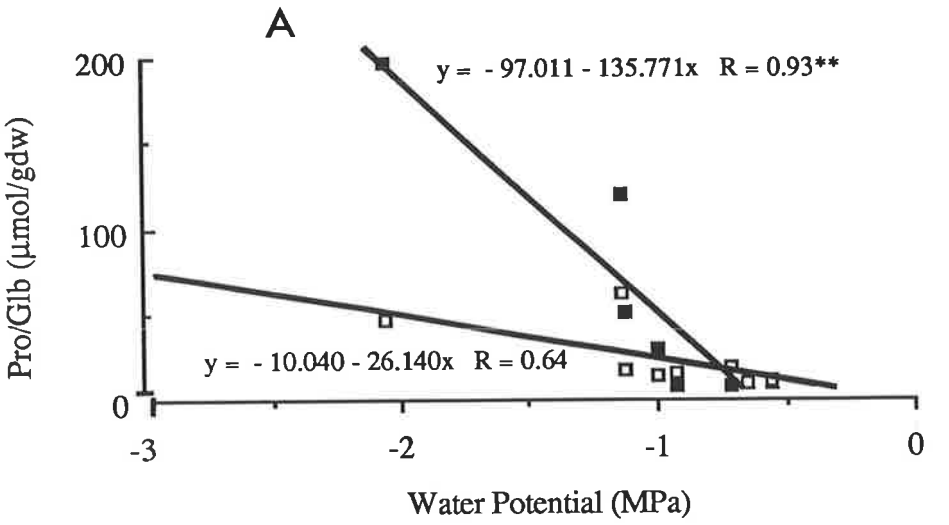
B. Proline or glycinebetaine content vs osmotic potential

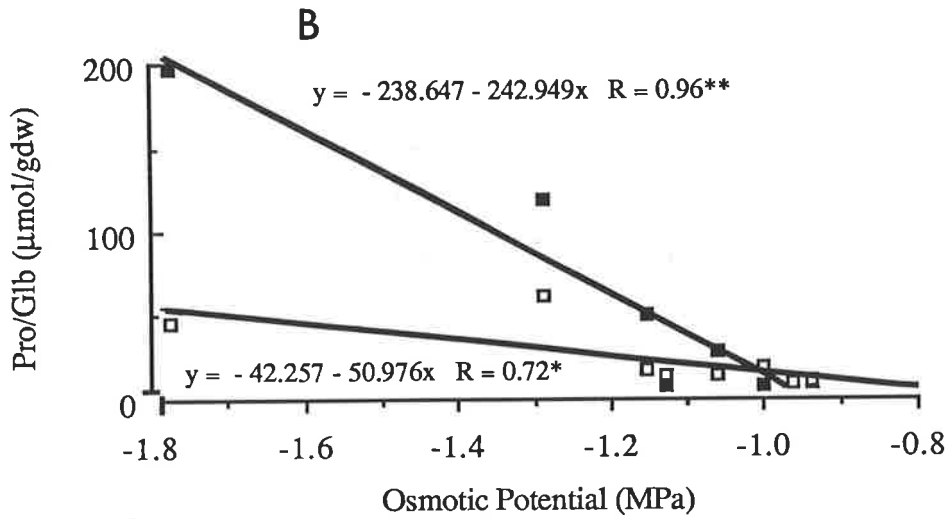
C. Proline or glycinebetaine content vs turgor potential

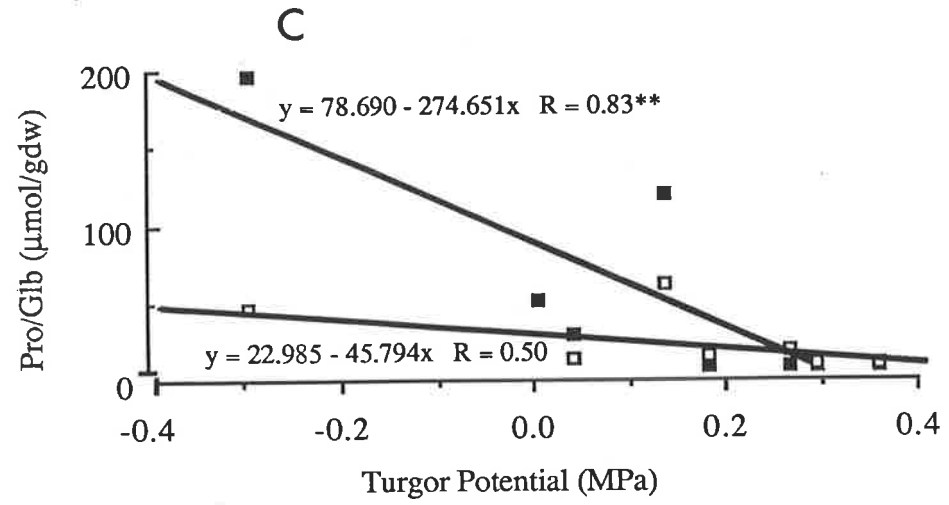
■ Proline

□ Glycinebetaine

*, ** R values significant at 5 and 1%, respectively


A
y = - 97.011 - 135.771x R = 0.93**
y = - 10.040 - 26.140x R = 0.64
Pro/Glb (µmol/gdw)
200
100
0
-3
-2
-1
0
Water Potential (MPa)


B
y = - 238.647 - 242.949x R = 0.96**
y = - 42.257 - 50.976x R = 0.72*
Pro/Glb (µmol/gdw)
200
100
0
-1.8
-1.6
-1.4
-1.2
-1.0
-0.8
Osmotic Potential (MPa)


C
y = 78.690 - 274.651x R = 0.83**
y = 22.985 - 45.794x R = 0.50
Pro/Glb (µmol/gdw)
200
100
0
-0.4
-0.2
0.0
0.2
0.4
Turgor Potential (MPa)

2.7 Proline and glycinebetaine accumulation at a range of temperatures at low VPD in well watered seedlings

2.7.1 *Introduction*

The results reported above show that proline and glycinebetaine are differentially responsive to temperature extremes and particularly to high temperature. The objective of this experiment was to find the critical temperature which induces glycinebetaine accumulation.

2.7.2 *Methods*

Barley seedlings (cv. Clipper) were grown under the standard environmental conditions for 11days and then exposed for 1 or 5 days to 5, 20, 25, 30 and 35°C with a photon flux density of 250 $\mu E\ m^{-2}\ s^{-1}$. A VPD of 0.2 KPa was maintained at each temperature except at 5°C where VPD was not controlled at 5°C. Plants were watered twice daily with 1/2 strength Hoagland's solution maintained at the temperature of each treatment. The treatments were replicated three times. At the end of 1 or 5 days first leaves were harvested for the measurement of ψ, ψ_S, and ψ_p, and proline and glycinebetaine content.

2.7.3 *Results*

2.7.3.1 Water status

Leaf ψ showed significant differences between days of sampling and temperature regimes, however, there was no interaction between temperature and time (Fig. 17a). On the first day of sampling, ψ_S was significantly lower at 5°C than at 20°C (Fig. 17b). This difference had increased by the fifth day with ψ_S decreasing by some 0.2 MPa at 5°C but increasing at higher temperatures. ψ_p was the same at 5°C on the first day but by the fifth day, significantly more positive turgor was maintained at 5°C than at 20°C (Fig. 17c), at this time ψ_p was significantly lower at temperatures above 20°C.

Fig. 17 Leaf water relations of well-watered barley seedlings exposed to a range of temperatures (5° to 35°C) for a period of 1 or 5 days.

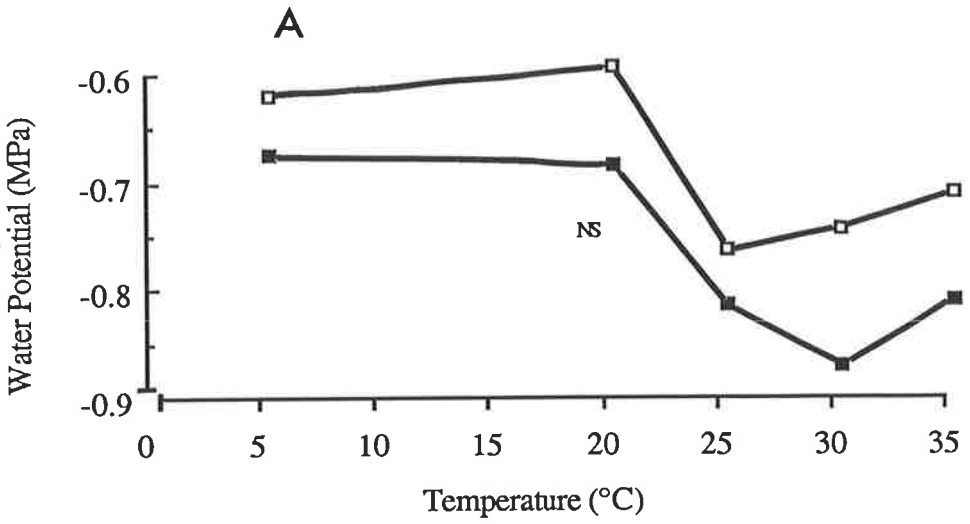
A. Water potential

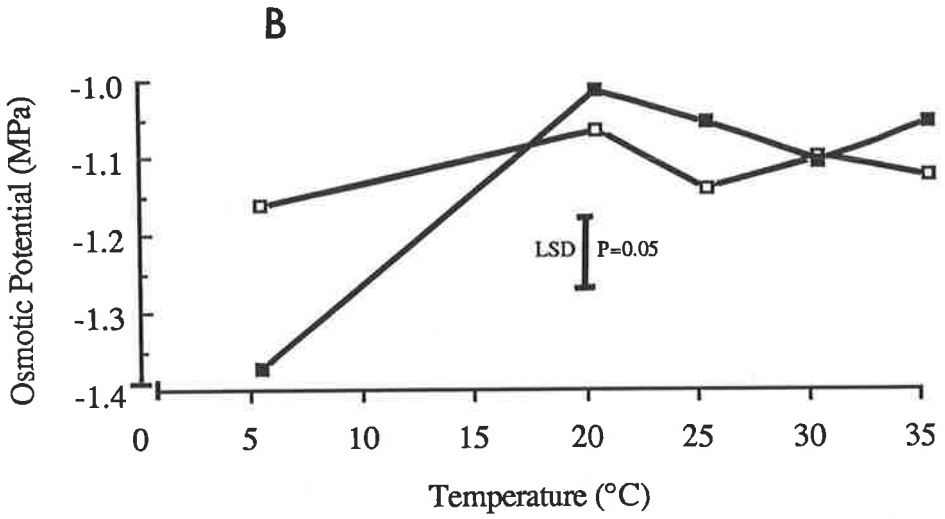
B. Osmotic potential

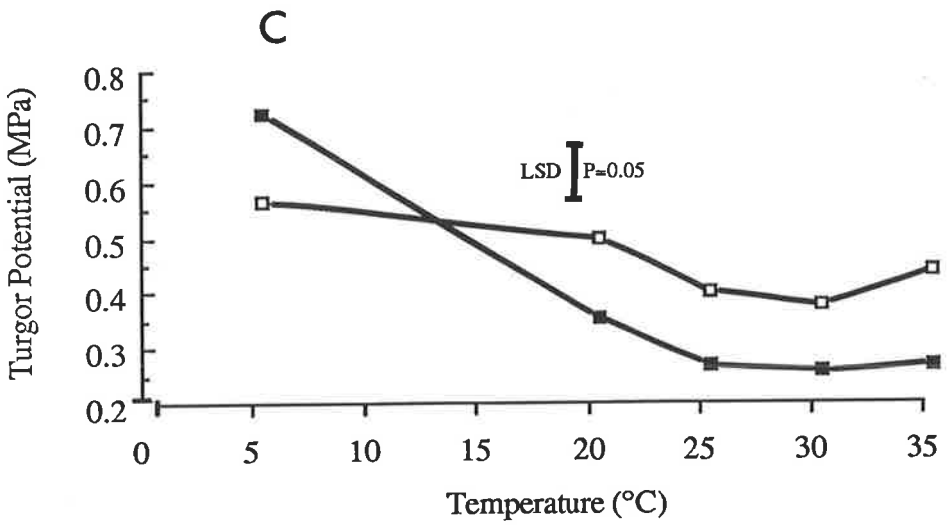
C. Turgor potential

□ 1 Day after exposure

■ 5 Days after exposure


A
Water Potential (MPa)
-0.6
-0.7
-0.8
-0.9
NS
0
5
10
15
20
25
30
35
Temperature (°C)


B
Osmotic Potential (MPa)
-1.0
-1.1
-1.2
-1.3
-1.4
LSD P=0.05
0
5
10
15
20
25
30
35
Temperature (°C)


C
Turgor Potential (MPa)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
LSD P=0.05
0
5
10
15
20
25
30
35
Temperature (°C)

2.7.3.2 Proline content

Proline content did not vary with temperature on the first day (Fig. 18a) but proline accumulation had taken place at 5°C by the fifth day, to a concentration of 23.5 μmol g^{-1} dw. No accumulation occured at any other temperature; rather, some proline was lost in the four days between the first and second samplings.

2.7.3.3 Glycinebetaine content

The glycinebetaine content of the leaves increased in response to both temperature extremes compared to its level at 20°C (Fig. 18b). There was no accumulation at 5°C on the first day, but significant accumulation had occured by the fifth day at 5°C. At 20 and 25°C there was a significant loss of glycinebetaine from the leaf tissue in the interval between the first and second samplings. Above 25°C glycinebetaine content increased with temperature (Figs.18b and 19), but did not change significantly with time. This suggests that glycinebetaine accumulated relatively rapidly in the leaves when they were first exposed to high temperature but did not vary thereafter. The critical temperature required to induce glycinebetaine accumulation lay between 25 and 30°C.

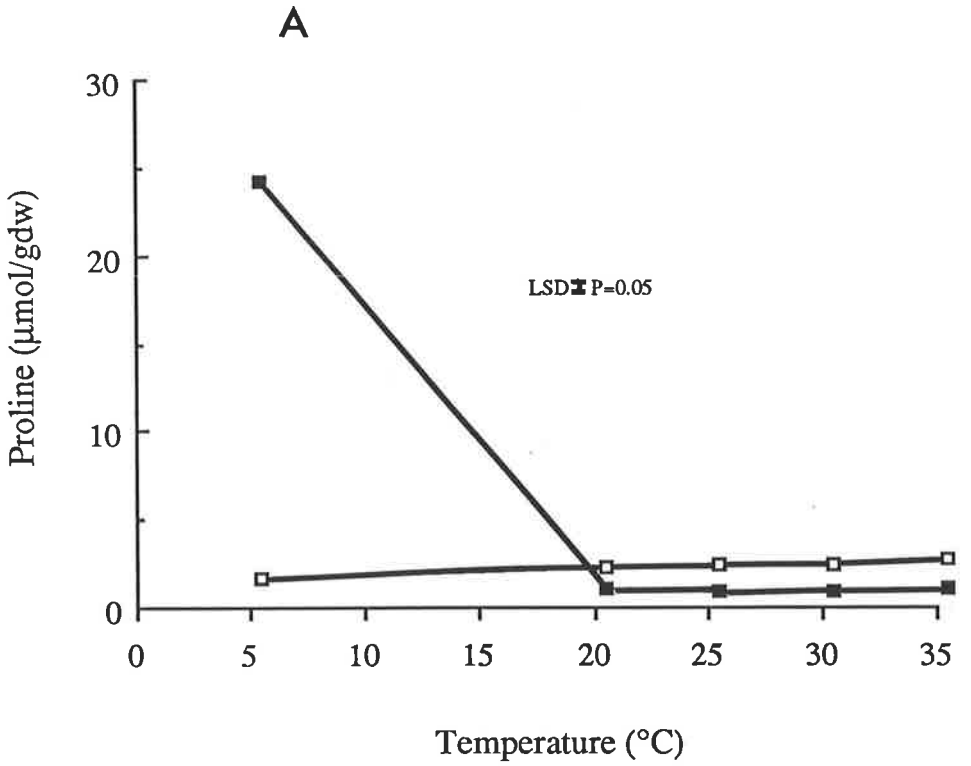
Fig. 18 Leaf proline and glycinebetaine content (μmol g^{-1} dw) of well-watered seedlings exposed to a range of temperatures (5 to 35°C) for a period of 1 or 5 days.

A. Proline

B. Glycinebetaine

□ 1 Day after exposure

■ 5 Days after exposure


A
Proline (µmol/gdw)
30
20
10
0
LSD P=0.05
0
5
10
15
20
25
30
35
Temperature (°C)

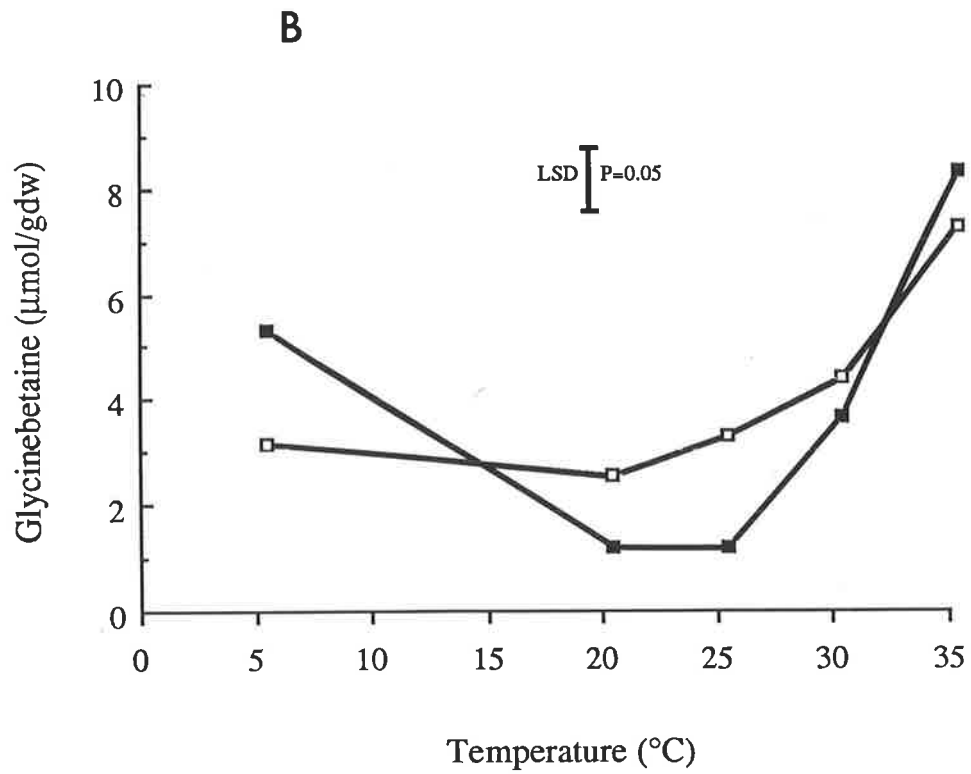
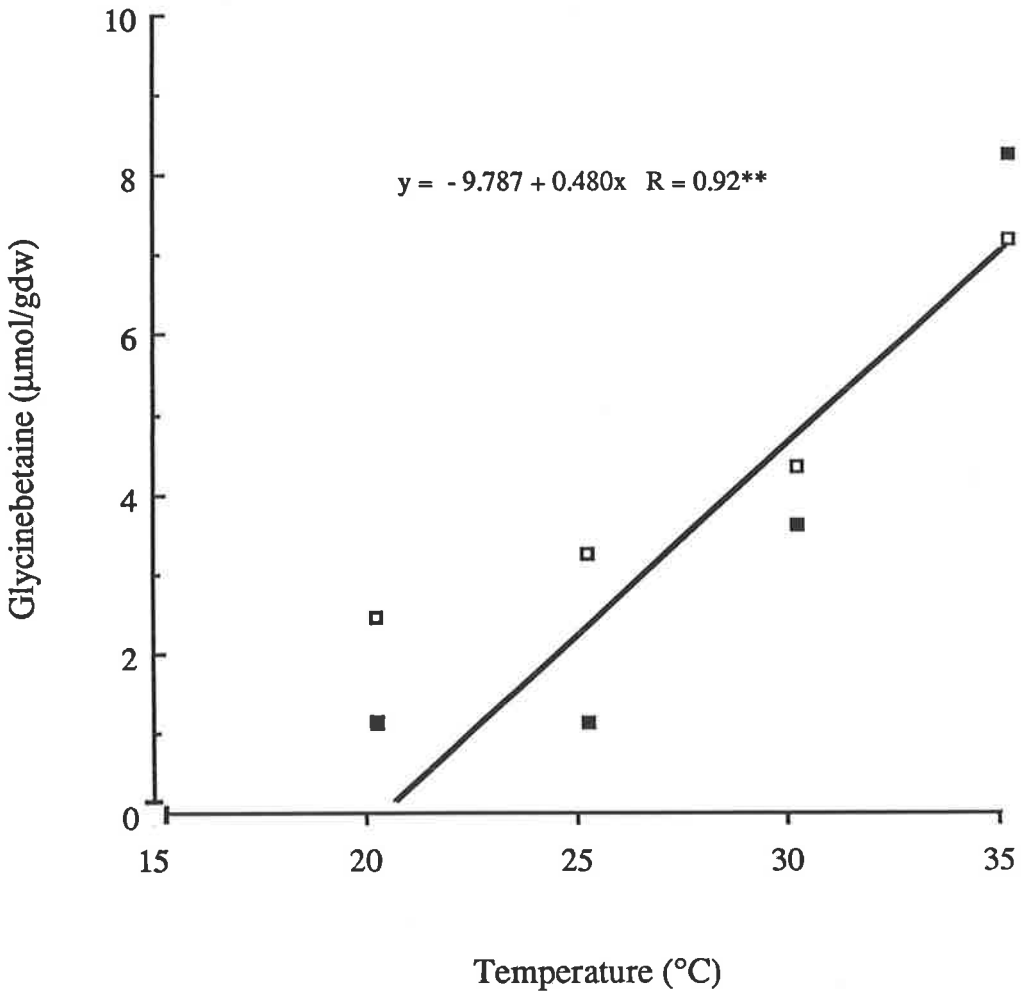

B
Glycinebetaine (µmol/gdw)
10
8
6
4
2
0
LSD P=0.05
0
5
10
15
20
25
30
35
Temperature (°C)

Fig. 19 Relationship between leaf glycinebetaine content and temperature. Data obtained from Fig. 18b.

□ 1 Day after exposure

■ 5 Days after exposure

** R value significant at 1%


y = - 9.787 + 0.480x R = 0.92**
Glycinebetaine (µmol/gdw)
Temperature (°C)
0
2
4
6
8
10
15
20
25
30
35

2.8 Discussion

Water stressed barley seedlings with different growth temperature histories showed variations in the accumulation of proline and glycinebetaine, although the temperature during water stress was maintained at the same level (20°C) (Figs. 9a,b). Temperature seems to have pre-conditioned the plants to accumulate various quantities of these solutes. Hence, it is possible that seedlings grown in environments with poor temperature control, such as in a glasshouse (Hanson *et al.*, 1977), might accumulate varying quantities of proline and glycinebetaine, compared to the situation with strict environmental control (Singh *et al.*, 1972). Plants occasionally exposed to a relatively low temperature would tend to accumulate more proline than plants at a normal temperature (20°C). On the other hand plants exposed to warmer temperatures tend to accumulate more glycinebetaine than plants grown at 20°C. This shows that exposure of plants to different temperatures may have differentially potentiated the biosynthetic pathways for proline and glycinebetaine production so that, when the plants were subsequently water-stressed, they accumulated different amounts of the solutes even at a common temperature. These results suggest that there could be differences in the response of these two solutes to temperature extremes in well watered and water stressed plants and any such differences might explain the differential responses obtained with temperature pre-stress treatment.

Although these solutes differed in their response to water stress after a pre-stress treatment, they commenced significant accumulation within the same period (less than 12 h) together with the associated changes in ψ (Fig. 10; 11a,b; 12; 13a,b). These observations conflict with the findings of Wyn Jones and Storey (1981) who reported that glycinebetaine does not accumulate before 24 h after osmotic or NaCl shock. This apparent lack of early accumulation of glycinebetaine could well be due to their use of the periodide method for glycinebetaine estimation (Storey and Wyn Jones, 1977), since this is not specific for glycinebetaine alone and would require higher levels for determination. The NMR spectroscopy used in this investigation, however, is both specific and sensitive (Jones *et al.*, 1986).

Glycinebetaine was found to accumulate at high temperature (35°C) in well watered plants (Fig. 11b). To test whether this was due to a change in leaf water status, barley seedlings were exposed to 20 or 35°C at low or high VPD. The maintenance of a low VPD resulted in the maintenance of a high ψ, even though at 35°C ψ was significantly lower than at 20°C. This reduction in ψ was not great (approximately 0.12 MPa, Fig. 14a) and hence the metabolic effects at low VPD and high temperature can reasonably be related to temperature alone. The accumulation of glycinebetaine in these conditions confirms that it is a response to high temperature and not solely to leaf ψ (Fig. 15b). At low VPD, due to reduced transpirational cooling, leaf temperature tends to be higher (Smith, 1978). This may account for the higher glycinebetaine content found initially at 35°C at low rather than at high VPD. Continuing heat stress at a high VPD eventually resulted in the accumulation of more glycinebetaine than at low VPD (Fig. 15b), possibly due to combined effects of heat stress and heat-induced leaf water deficit. The glycinebetaine content of well watered plants is highly correlated with increase in temperature above 25°C (Fig. 19) and the critical temperature inducing accumulation of glycinebetaine falls between 25 and 30°C. The apparent heat stress response of glycinebetaine was observed even in water stressed seedlings at low VPD, where increases in glycinebetaine levels were not correlated with reductions in ψ or ψ_p (Figs. 16a, b). The positive correlation between ψ_s and glycinebetaine content may be due to the osmotic contribution of the increased glycinebetaine content (Grumet and Hanson, 1986).

Glycinebetaine accumulated at both temperature extremes (35 or 5°C) in the absence of water stress, thus differing from proline which accumulates in cold (5°C) stressed plants only (Figs. 18a, b). This distinct difference between the solutes may indicate fundamental difference in their accumulation mechanisms or in their sites of synthesis. The accumulation of proline in cold stress, as discussed in Section 1 of this chapter, may be due to reduced utilization of proline (ap Rees, 1980). On the other hand the lack of its acumulation during heat stress may be due to accelerated respiration which depletes the level of potential precurors such as glutamate and carbohydrates (Stewart,

1972c). The heat and cold stress response of glycinebetaine may be related to the membrane-bound intermediate, phosphatidylcholine (PC) (Hitz et al., 1981; Hanson and Rhodes, 1983) and its sensitivity to temperature. Changes in membrane lipid composition (increases in PC content) have been shown to occur in cold- (Christiansen, 1984) and heat- stressed plants (Levitt, 1980), resulting in the greater availability of PC or its greater turnover rate in the membranes, thus leading to the accumulation of glycinebetaine.

The results reported in this section showed that the temperature history of barley seedlings during growth can cause variability in the accumulation of proline and glycinebetaine. The extent the differences in the accumulation of the solutes depends on the temperature at which the seedlings were grown. This is because proline increases more during cold stress than did glycinebetaine, and because only glycinebetaine levels respond to heat stress (Figs. 18a, b). It is difficult to assess the extent of contribution of possible temperature differences in the experiments of the two groups (Singh *et al.*, 1972; Hanson *et al.*, 1977) to the difference in results as the range of temperature in the glass house experiments of Hanson *et al.* (1977) is not known. However, unless there was an extreme variation in temperature, it is unlikely that this factor was a major cause for the differences in results as there is a broad minimum around 20°C for the response of these compounds.

Section 3: Variations in proline and glycinebetaine accumulation associated with parent seed maturity temperature and seed size

3.1 Introduction

The work of Highkin (1958) demonstrated with peas that a genetically controlled phenotypic character such as growth rate or plant height can be altered by unfavourable temperature during the growth and seed maturation of the previous generation. The growth inhibitory effects of continuous exposure to a constant unfavourable (lower) temperature was cumulative from generation to generation producing the greatest growth inhibition in the fifth generation. When seed from such less vigorous plants were re-exposed to unfavourable temperature after two generations had been raised at a favourable temperature, less vigorous growth was immediately apparent. Exposure to the favourable temperature for at least three generations was required before the cumulative ill-effects of growing previous generations at an unfavourable temperature was lost.

Based on these results, Highkin (1958) suggested that the expression of a character may be dependent on the genetic heritage together with both the present environment and that in which the parents were raised. The temperature during the growth of barley seedlings has been shown to cause appreciable variation in the content of both proline and glycinebetaine although the two solutes respond differently to temperature extremes (see section 2 of this chapter). The experiments reported in this section of the thesis examine the effect of temperature during seed maturation on the water-stress induced accumulation of proline and glycinebetaine by seedlings raised from that seed. Such a response in barley cultivars may account, at least in part, for the observed discrepencies between the results of Singh *et al.* (1972) and Hanson *et al.* (1977)

3.2 Temperature during parent seed maturation and proline, and glycinebetaine accumulation in wheat seedlings

3.2.1 *Methods*

Wheat seed (cvs. Banks and Kalyansona) grown in the Canberra phytotron and exposed to various temperature regimes (33/28, 24/19, 15/10°C day/night) from anthesis to seed maturity was used. Seedlings of both cultivars matured in the three temperature regimes were grown under standard conditions for 11 days and subjected to -1.5 MPa PEG stress for 1 day at 20 or 35°C with well-watered plants as controls. The treatments were replicated three times. Leaf water potential and proline, and glycinebetaine contents were measured on first leaves. The data for Banks and Kalyansona are presented separately since these two cultivars were subjected to water stress at different times although, in a similar environment. A photon flux density of 500 and 100 $\mu Em^{-2}s^{-1}$, respectively, was maintained during the growth and stress periods.

3.2.2 *Results*

3.2.2.1 Water status

Seedlings of Banks and Kalyansona grown from seed at different temperatures showed no differences in Ψ when water stressed (Table 10a). Both cultivars had a significantly lower Ψ when water stressed at 35°C than at 20°C (Table 10b). Kalyansona always had a higher Ψ than Banks when water stressed at either temperature which may have been due to the inherent capacity of these two cutlivars or to the fact that they were water stressed at different times. The interaction of parent seed temperature, stress temperature and water stress was not significant for either cultivar.

3.2.2.2 Proline content

Well-watered seedlings of Banks and Kalyansona raised from seed matured at different temperatures showed no differences in leaf proline content (Table 11b). However, when water stressed, seedlings with different histories showed differences in

Table 10: Water potential (-MPa) of water and heat stressed wheat (cvs. Banks and Kalyansona) seedlings grown from seed with 15/10, 24/19, and 33/28°C parent temperatures.

A:

	Parent temperature (°C)			LSD
	15/10	24/19	33/28	(P = 0.05)
Banks	1.11	1.15	1.13	NS
Kalyansona	0.75	0.73	0.71	NS

B:

	Banks			Kalyansona		
Temperature (°C)	Control	Stress	LSD (P = 0.05)	Control	Stress	LSD (P = 0.05)
20	0.72	1.00	0.13	0.40	0.80	0.08
35	0.57	2.24		0.55	1.17	

Table 11: Proline content (μmol g^{-1} dw) of water and heat stressed wheat (cv. Kalyansona) seedlings grown from seed with 15/10, 24/19, and 33/28°C parent temperatures.

A:

Temperature (°C)	Control	Water Stress	LSD (P = 0.05)
20	2.07	5.36	1.52
35	3.10	10.37	

B:

	Parent temperature (°C)			LSD (P = 0.05)
	15/10	24/19	33/28	
Control	2.09	2.89	2.78	1.78
Water stress	9.18	7.55	6.88	

proline content (Fig. 20a and Table 11b). In Banks, seedlings from seed matured at 33/28°C accumulated most proline and the least was found in seedlings from seeds matured at 15/10°C, when they were water stressed at 20°C. However, when seedlings were water stressed at 35°C, the pattern of proline accumulation was different, most proline accumulated in seedlings with 15/10°C parent temperature, least in seedlings with a history of 24/19°C parent temperature and seedlings with 33/28°C parent temperature accumulated intermediate amounts of proline (Fig. 20a).

Kalyansona exhibited a different response to that of Banks. Water stressed Kalyansona seedlings accumulated decreasing amounts of proline with increase in parent temperature (Table 11b). Parent temperature, water stress and stress temperature showed no interaction (Fig. 20b).

3.2.2.3 Glycinebetaine content

Parent temperature had no effect on the glycinebetaine content of well-watered plants of both varieties at either 20 or 35°C (Figs. 21a and b). In Banks, water stressed seedlings at both 20 and 35°C showed the lowest glycinebetaine content with a parent temperature history of 33/28°C and the content increased with decreasing parent temperature. Seedlings of Kalyansona also exhibited an increasing glycinebetaine content with decreasing parent temperature (Table 12), however, the interaction of parent temperature, water stress and stress temperature was not significant (Fig. 21b).

3.2.2.4 Relationship between parent seed maturation temperature and seed weight

One possible explanation for the effect of parent seed maturation on solute accumulation by the seedlings is that the temperature during seed maturation affects seed weight which in turn affects seedling potential to accumulate solute. There was in fact, a negative relationship between grain weight and maturation temperature (Fig. 22).

Table 12: Glycinebetaine content (μmol g^{-1} dw) of water and heat stressed wheat (cv. Kalyansona) seedlings grown from seed with 15/10, 24/19, and 33/28°C parent temperatures.

Stress temperature (°C)			
20	35		LSD (P = 0.05)
8.16	16.6		1.39
Control	Stress		
7.61	17.17		0.87
Parent temperature (°C)			
15/10	24/19	33/28	
14.57	11.47	11.13	1.08

Fig. 20 Leaf proline content (μmol g^{-1} dw) of wheat seedlings grown from seed matured at a range of temperatures and water stressed for 1 day.
(Parent plants grown at 15/10, 24/19 or 33/28°C, seedlings water stressed [1 day, PEG, -1.5 MPa] at 20 or 35°C)

A. cv. Banks

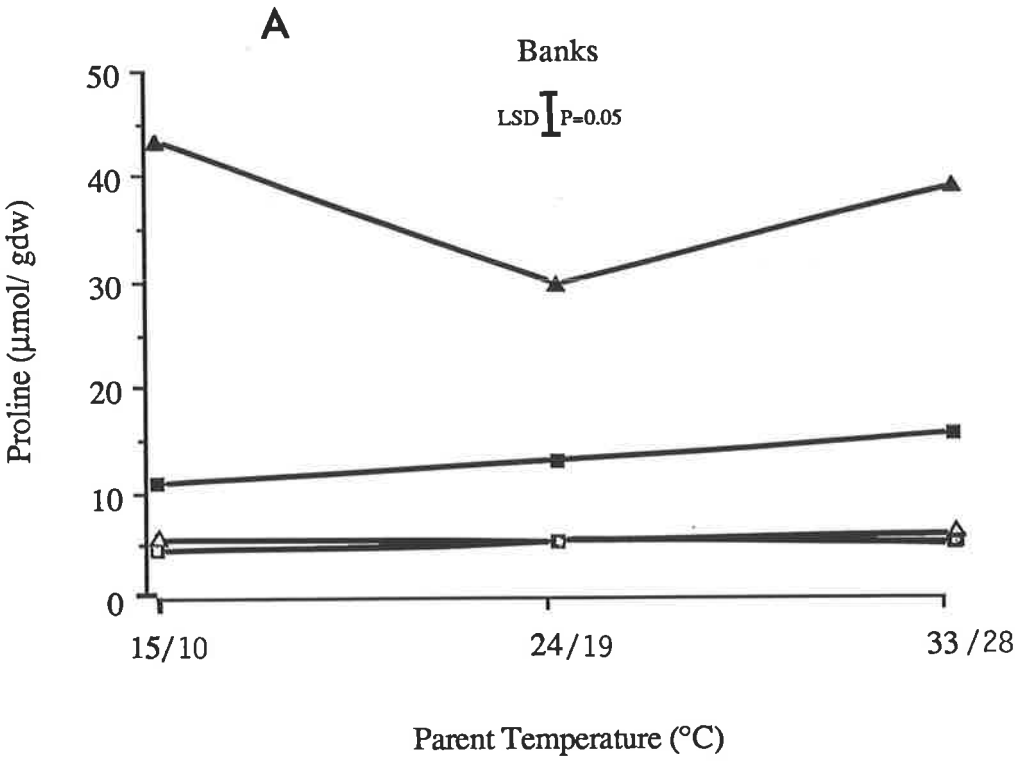
B. cv. Kalyansona

□ Control, 20°C

■ Stressed, 20°C

△ Control, 35°C

▲ Stressed, 35°C


A
Banks
LSD P=0.05
50
40
30
20
10
0
Proline (µmol/ gdw)
15/10
24/19
33 /28
Parent Temperature (°C)

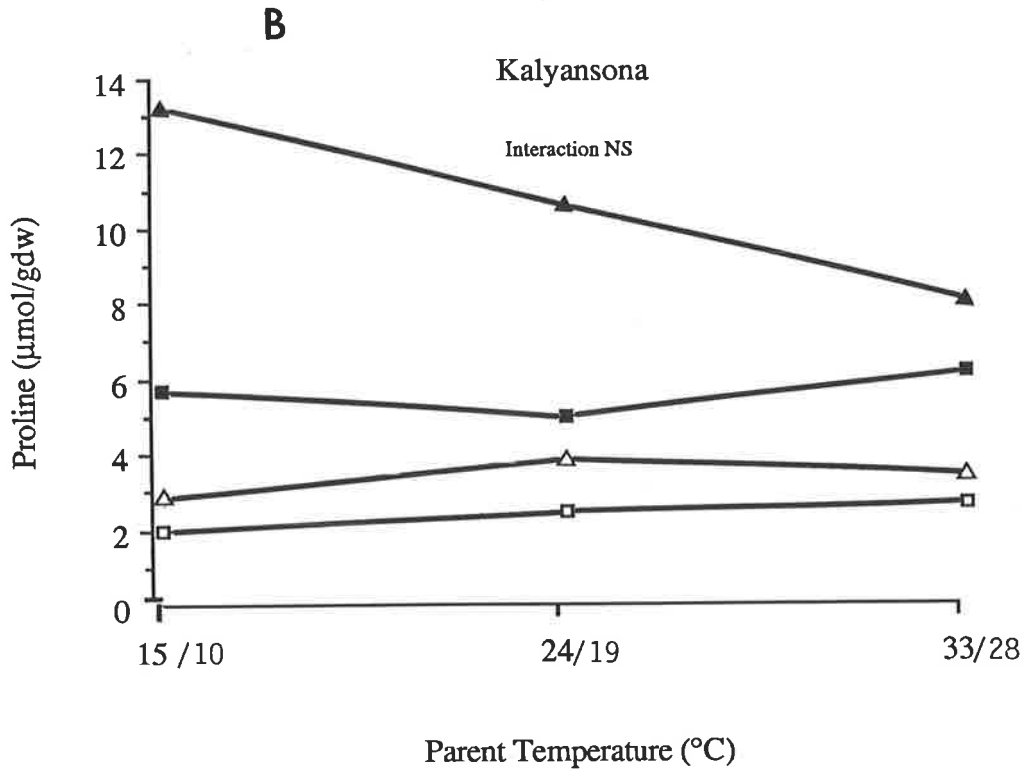

B
Kalyansona
Interaction NS
14
12
10
8
6
4
2
0
Proline (µmol/gdw)
15 /10
24/19
33/28
Parent Temperature (°C)

Fig. 21 Leaf glycinebetaine content (μmol g^{-1} dw) of wheat seedlings grown from seed matured at a range of temperatures and water stress for 1 day. (Parent plants grown at 15/10, 24/19 or 33/28°C, seedlings were water stressed [1 day, PEG, -1.5 MPa] at 20 or 35°C.)

A. cv. Banks

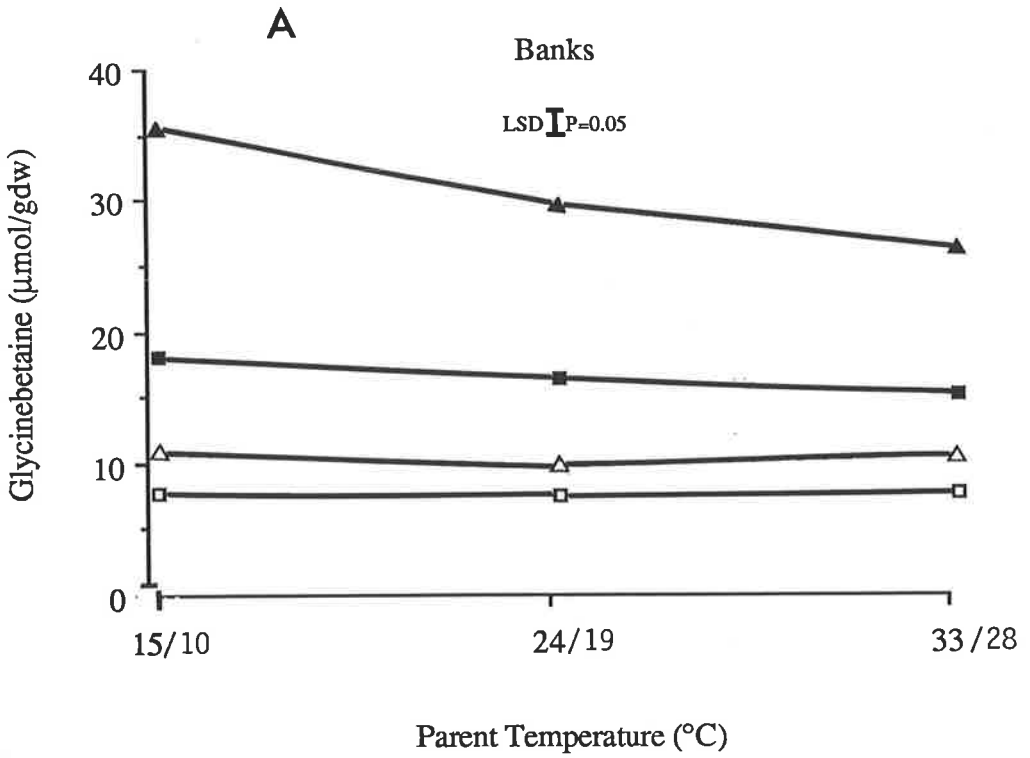
B. cv. Kalyansona

□ Control, 20°C

■ Stressed, 20°C

△ Control, 35°C

▲ Stressed, 35°C


A
Banks
LSD P=0.05
40
30
20
10
0
Glycinebetaine (µmol/gdw)
15/10
24/19
33/28
Parent Temperature (°C)

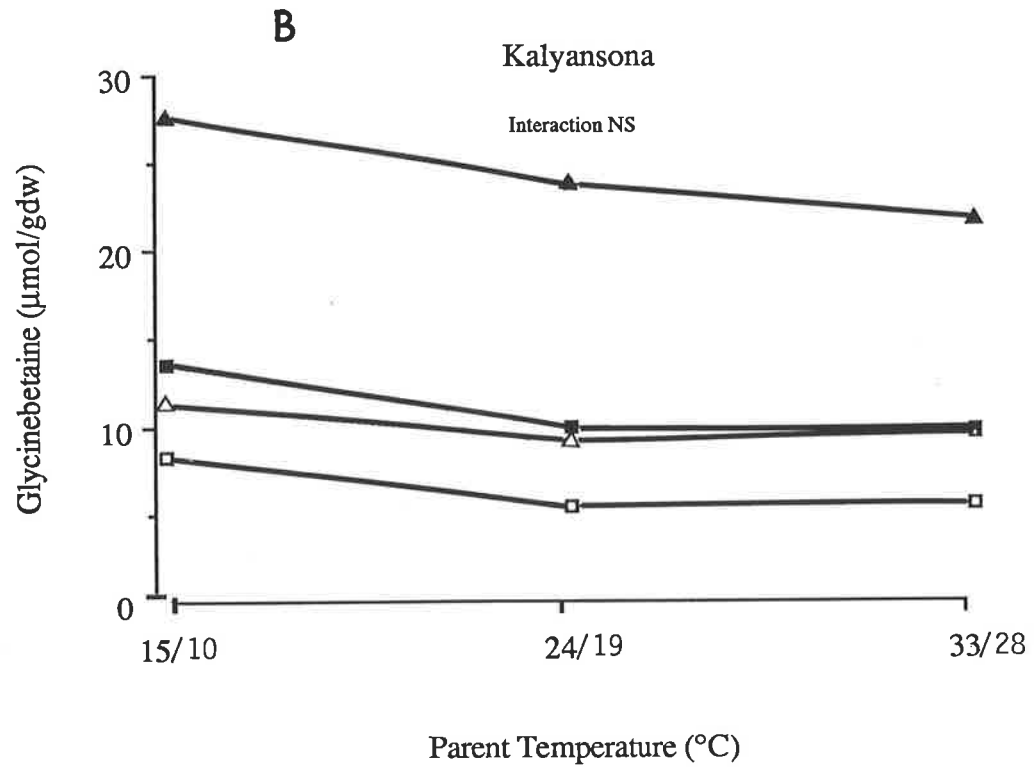
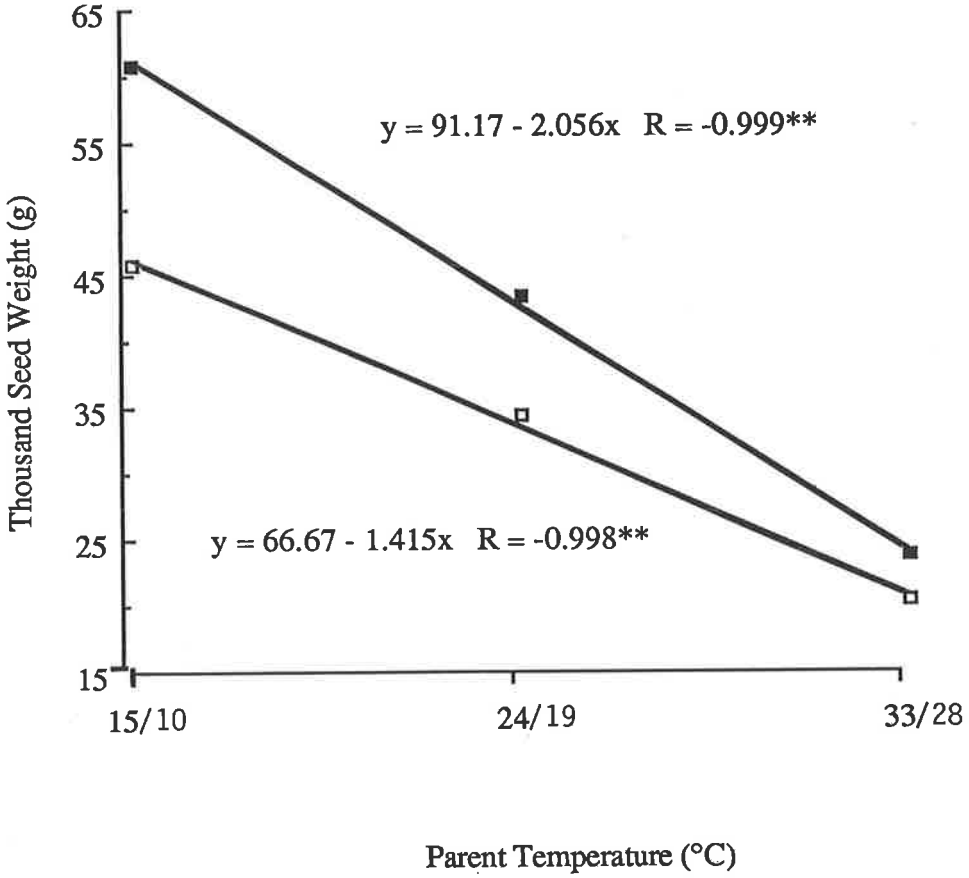

B
Kalyansona
Interaction NS
30
20
10
0
Glycinebetaine (µmol/gdw)
15/10
24/19
33/28
Parent Temperature (°C)

Fig. 22 Relationship between parent temperature and thousand seed weight of wheat cultivars (Banks and Kalyansona).

■ Kalyansona

□ Banks

**, R value significant at 1%


65
55
45
35
25
15
Thousand Seed Weight (g)
y = 91.17 - 2.056x R = -0.999**
y = 66.67 - 1.415x R = -0.998**
15/10
24/19
33/28
Parent Temperature (°C)

3.3 Elimination of seed size as the source of the response to parent seed maturity temperature

3.3.1 *Introduction*

Parent seed temperature had an effect on the accumulation of proline and glycinebetaine when seedlings grown from the seed were subjected to water- and heat-stress (see Section 3.2 of this chapter) but this may have been due solely to differences in seed size generated by the different maturation regimes.

In this experiment seed size difference was eliminated with the objective of examining any possible carry-over effects of parent seed temperature, independent of seed size, on the accumulation of proline and glycinebetaine.

3.3.2 *Methods*

From the population of seeds from each maturation temperature, seed was hand sorted and then weighed to obtain samples of uniform seed weight from each maturation temperature (Table 13).

Table 13. The uniform 100 seed weight (g) of wheat cultivars from 3 parent temperatures

Parent temperature (°C)	Banks	Kalyansona
15/10	2.27	2.69
24/19	2.26	2.55
33/28	2.12	2.48

Only a few seeds were obtained from each maturation temperature since only the large seeds from the 33/28°C maturation temperature and small seed from the 15/10°C maturation temperature could be chosen to obtain a uniform seed size. Even so it was difficult to obtain samples of exactly similar seed weights from the three maturation

temperatures (Table 13).

The seedlings of both cultivars were grown under standard environmental conditions for 11 days and then water stressed for 1 day by applying PEG (-1.5 MPa) to the rooting medium at 20 or 35°C with well-watered plants as controls at each temperature. The light environment was the same as in experiment 3.2. The treatments were replicated three times. Leaf water status, proline and glycinebetaine were measured on the first leaves at the end of 1 day of water stress.

3.3.3 *Results*

3.3.3.1 Water status

Ψ of neither Banks nor Kalyansona showed any difference due to parent seed temperature (Table 14a). Kalyansona had a higher Ψ which confirms that observed when seed size was variable (see experiment 3.2). The interaction of parent temperature, water stress, temperature, and varieties was not significant (Table 14b).

3.3.3.2 Proline content

The differences in proline content in both cultivars apparently due to parent seed temperature (see experiment 3.2) disappeared when seed size differences were eliminated (Table 15), however the proline content of Banks was two to three fold higher than that of Kalyansona. The four factor interaction involving the effects of parent temperature, water stress, temperature and varieties was not significant (Table 16a).

3.3.3.3 Glycinebetaine contnet

Differences in glycinebetaine content due to seed maturation temperature were expressed even after uniform sized seed were used in the experiments (Table 15), however, the pattern of glycinebetaine content was different to that observed before the elimination of seed size differences (Fig. 21 and Table 12). Most glycinebetaine was found in seedlings grown from seed matured at 33/28°C. However, the four factor interaction was not significant (Table 16b).

Table 14: Leaf water potential (-MPa) of water- and heat-stressed wheat seedlings (cvs. Banks and Kalyansona) grown from seed with vairous parent temperatures after the elimination of seed size differences*.

A:

Parent temperature (°C)	Banks	Kalyansona
15/10	1.27	1.05
24/19	1.25	1.05
33/28	1.23	1.02

B:

	BANKS			KALYANSONA		
	Parent temperature (°C)			Parent temperature (°C)		
	15/10	24/19	33/28	15/10	24/19	33/28
20°C control	0.67	0.63	0.54	0.58	0.61	0.52
20°C stress	1.30	1.36	1.45	1.14	1.10	1.17
35°C control	0.81	0.69	0.76	0.66	0.55	5.83
35°C stressed	2.14	2.32	2.34	1.80	1.94	1.81

*There were no significant differences in Ψ, except between control and stress.

Table 15: Proline and glycinebetaine content (μmol g^{-1} dw) of wheat seedlings grown from 15/10, 24/19, and 33/28°C parent temperatures.

	Parent temperature (°C)			
	15/10	24/19	33/28	LSD (P = 0.05)
Proline	12.19	12.31	12.02	NS
Glycinebetaine	11.24	11.77	12.55	1.03

Table 16: Proline and glycinebetaine content (µmol g^{-1} dw) of water- and heat-stressed wheat seedlings (cvs. Banks and Kalyansona) grown from seed with various parent temperatures after the elimination of seed size differences

A: Proline

	BANKS			KALYANSONA		
	Parent temperature (°C)			Parent temperature (°C)		
	15/10	24/19	33/28	15/10	24/19	33/28
20°C control	1.34	2.41	2.01	1.20	1.36	1.04
20°C stress	18.03	23.58	21.55	10.48	11.72	10.25
35°C control	3.60	3.61	4.68	2.08	2.31	2.34
35°C stress	44.47	36.38	34.68	16.27	17.06	19.61

B: Glycinebetaine

	BANKS			KALYANSONA		
	Parent temperature (°C)			Parent temperature (°C)		
	15/10	24/19	33/28	15/10	24/19	33/28
20°C control	5.78	6.62	7.07	4.78	4.13	4.54
20°C stress	11.99	12.54	11.90	10.34	9.38	9.71
35°C control	8.72	11.66	11.82	8.45	6.83	6.97
35°C stress	22.62	25.18	27.02	19.67	17.82	21.34

3.4 Proline and glycinebetaine accumulating capacity associated with parent seed weight in barley cultivars

3.4.1 *Introduction*

It is clear from the previous two experiments (3.2 and 3.3) that seed matured at different temperatures had different capacities to accumulate proline and glycinebetaine. Since this response was mediated through seed size in the case of proline, and modified by seed size in the case of glycinebetaine the effect of seed size on solute accumulating capacity of barley cultivars (Excelsior, Proctor, and Clipper) was studied in the present experiment. The first 2 cultivars were included in the studies of Singh *et al.* (1972) and Hanson *et al.* (1977). Since neither group presented seed weights, this experiment was of considerable interest in examining possibile reasons for the different responses recorded by the two groups.

3.4.2 *Methods*

Large and small barley seeds (Table 17) were hand sorted from field grown seed lots of Excelsior, Proctor and Clipper. There was a slight but unavoidable difference between cultivars in the seed weight within each seed size fraction (Table 17).

Table 17. Thousand seed weight (g) of large or small seeds of barley cultivars

Cultivar	Large	Small
Excelsior	47.28	26.51
Proctor	42.83	23.46
Clipper	50.38	25.32

Seedlings from each seed size of each cultivar were grown under standard environmental conditions for 11 days and water stressed for 2 days by applying PEG (-1.5 MPa) to the rooting medium. The treatments were replicated three times. Leaf water

status, and proline and glycinebetaine contents were measured on the first leaves.

3.4.3 *Results*

3.4.3.1 Water status

Water stress resulted in a significant reduction in leaf RWC, Ψ, and Ψs of all cultivars. Water stressed seedlings of Proctor grown from small seed showed a lower RWC, Ψ, and Ψs than seedlings grown and stressed from large or small seed of the other 2 cultivars (Figs. 23a, b, c) when comparisions were made within a cultivar. Excelsior seedlings grown from small seed showed significantly lower RWC and Ψs (Figs. 23ab) than seedlings grown from the large seed, however, the other two cultivars showed no change in leaf water status due to seed size.

3.4.3.2 Proline content

There was no effect of cultivar or seed size on proline content in non-stressed seedlings (Fig. 24). However, water stressed seedlings showed significant differences in proline content in response to seed size and cultivar. Seedlings grown from large seed accumulated more proline in Proctor and Clipper but less in Excelsior. On the other hand, if seedlings were grown from small seeds, Excelsior accumulated most proline. So it is possible that the apparent proline accumulating capacity of cultivars differs if seeds of different sizes are compared. See appendix 1a for the analysis of variance.

3.4.3.3 Glycinebetaine content

The level of glycinebetaine in control plants showed no significant variation in response to seed size or cultivar. (Fig. 24b). However, the mean glycinebetaine content of water stressed and well-watered seedlings differed significantly (Fig. 25). When seedlings were grown from small seeds, the glycinebetaine content showed no significant difference between cultivars, but use of large seed resulted in higher glycinebetaine content in Excelsior and Proctor than in Clipper. When the effect of seed size was examined in each cultivar, only Excelsior showed significant variation in glycinebetaine

Fig 23 Leaf water status of barley cultivars Excelsior, Proctor and Clipper grown from small or large seed and water stressed. Each value is a mean from control and water stressed plants (6 plants in total).

(Plants were water stressed for 2 days with PEG, -1.5 MPa but as there was no interaction between the effects of stress and seed size the data from control and stressed plants have been pooled.)

A. RWC

B. Water potential

C. Osmotic potential

Seedlings grown from small seed

Seedlings grown from large seed

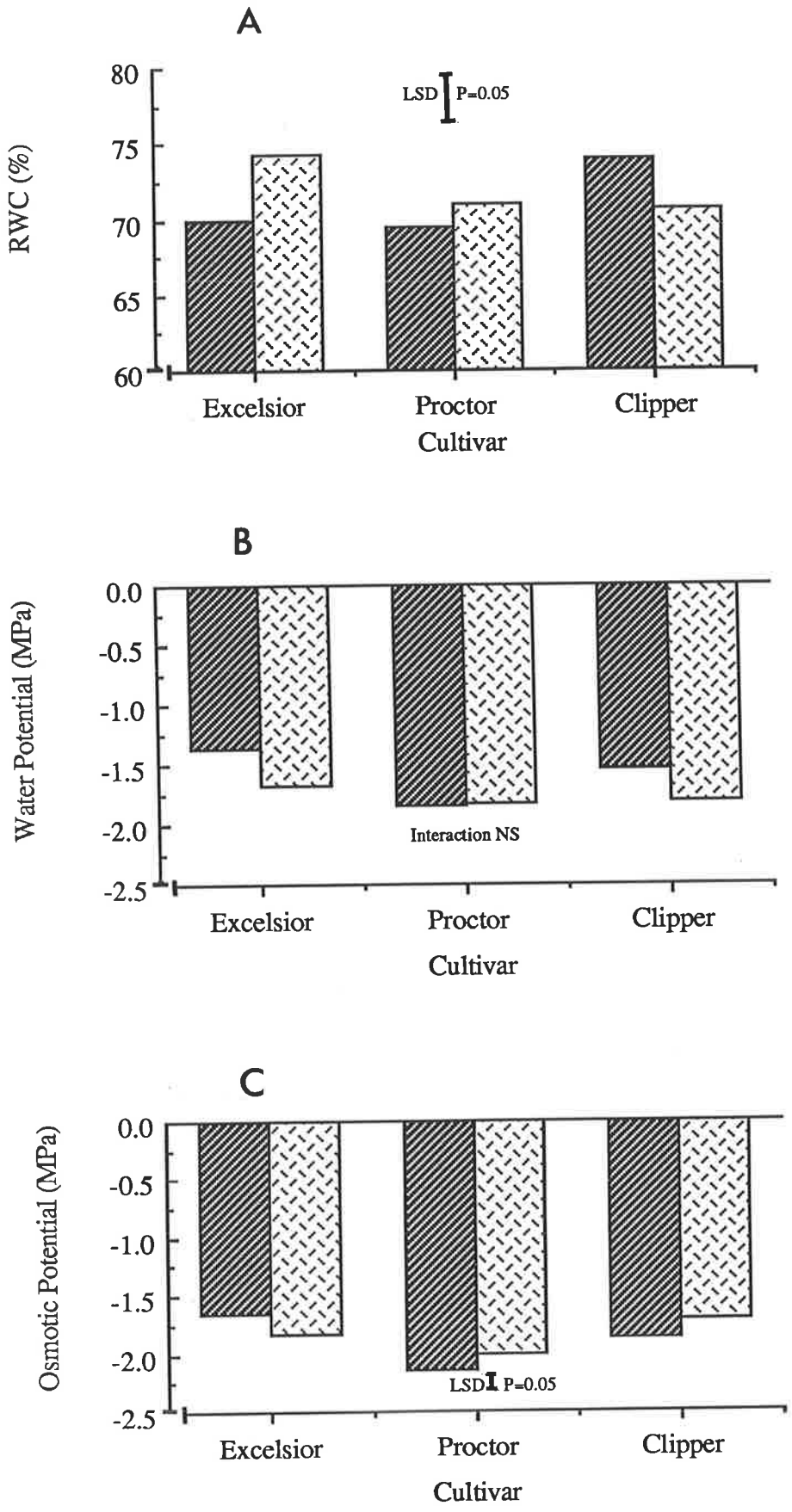
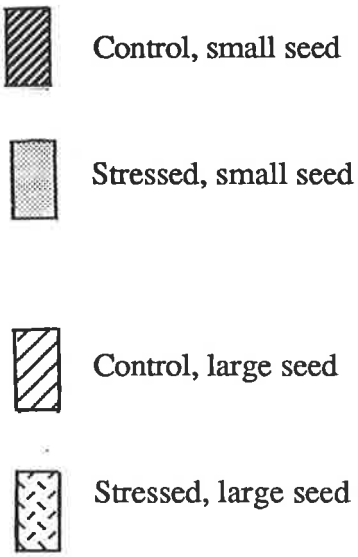
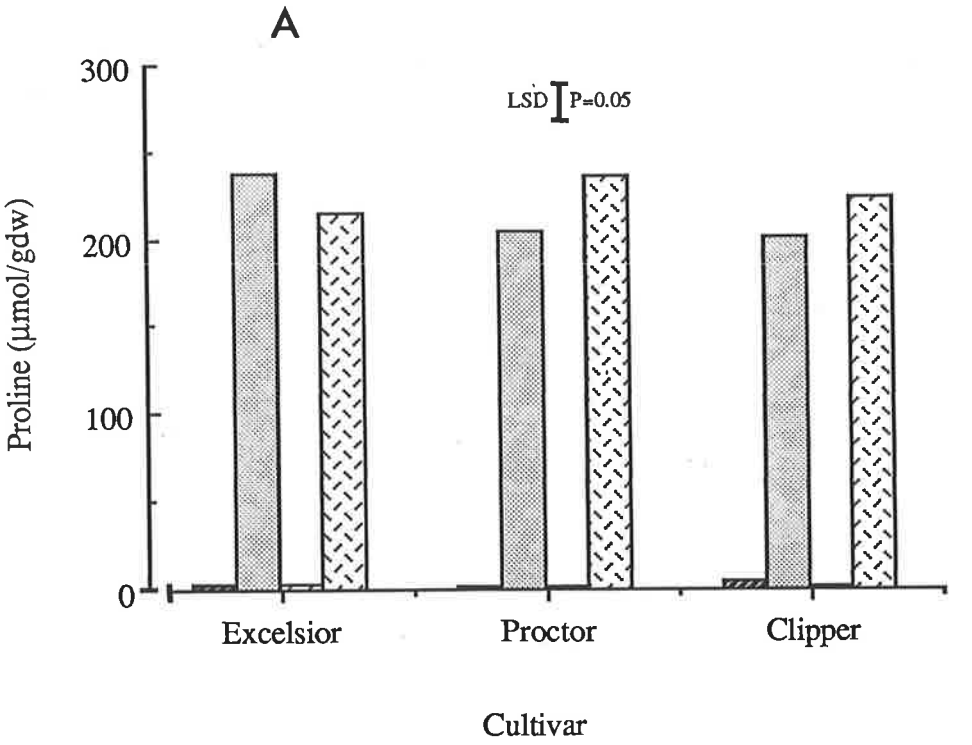

A
RWC (%)
80
75
70
65
60
LSD P=0.05
Excelsior
Proctor
Clipper
Cultivar
B
Water Potential (MPa)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Interaction NS
Excelsior
Proctor
Clipper
Cultivar
C
Osmotic Potential (MPa)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
LSD P=0.05
Excelsior
Proctor
Clipper
Cultivar

Fig. 24 Leaf proline and glycinebetaine content of barley cultivars Excelsior, Proctor and Clipper grown from small or large seed and water stressed for 2 days.

A. Proline

B. Glycinebetaine




A
300
200
100
0
Proline (µmol/gdw)
LSD P=0.05
Excelsior
Proctor
Clipper
Cultivar

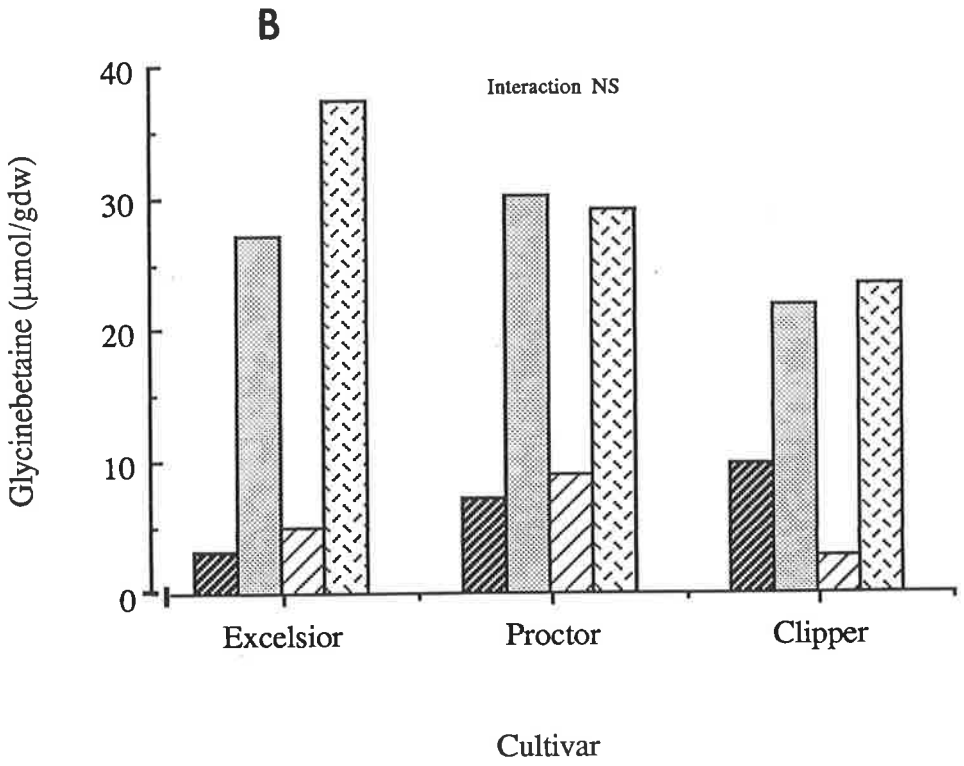
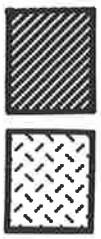
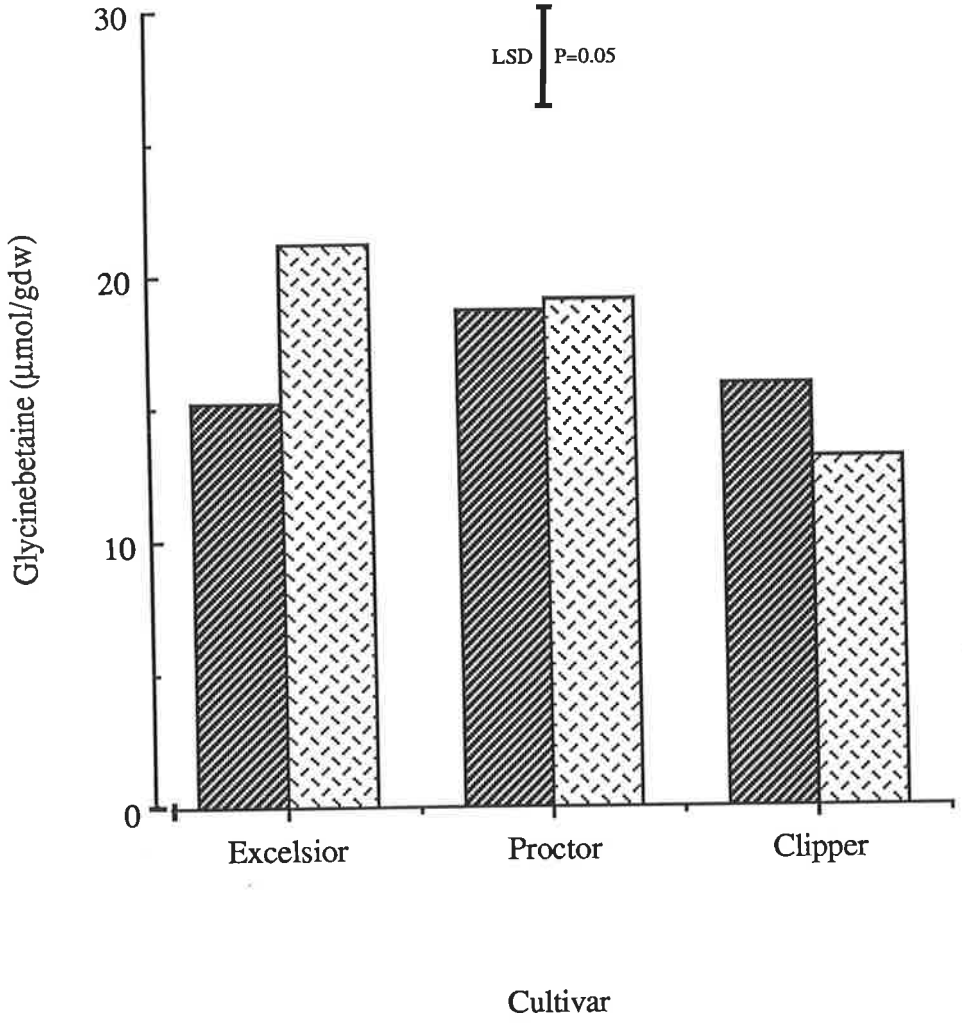

B
40
30
20
10
0
Glycinebetaine (µmol/gdw)
Interaction NS
Excelsior
Proctor
Clipper
Cultivar

Fig. 25 Leaf glycinebetaine content of barley cultivars Excelsior, Proctor and Clipper grown from small or large seed and water stressed for 2 days. Each value is a mean from control and water stressed plants (6 plants in total). (Plants were water stressed for 2 days with PEG, -1.5 MPa but as there was no interaction between the effects of stress and seed size the data from control and stressed plants have been pooled.)




30
20
10
0
LSD
P=0.05
Glycinebetaine (µmol/gdw)
Excelsior
Proctor
Clipper
Cultivar

content (Fig. 25). The interaction of seed size, water stress and cultivars was not significant, (Fig. 24b).

3.5 Discussion

The experiments reported in this section show that the proline and glycinebetaine accumulating capacity of cultivars was altered by seed related factors. These can potentially explain at least some of the observed differences between the results of Singh *et al.* (1972) and those of Hanson *et al.* (1977).

Water stressed wheat seedlings of cultivars Banks and Kalyansona showed considerable differences in proline and glycinebetaine contents (Figs. 20a,b & 21a,b) in response to their parent seed maturity temperatures of 15/10, 24/19 or 33/28°C. The pattern of proline accumulation in relation to the seed maturity temperature was markedly different between cultivars (Figs. 20a,b), but the pattern of glycinebetaine accumulation was similar (Figs. 21a,b). The carry-over effects of temperature during plant growth, and seed maturation and the resultant variation in growth and vigour in the plants of subsequent generations have been studied by Highkin (1958). The variation in the stress metabolism of proline and glycinebetaine due to parent seed maturity temperatures in wheat cultivars gives an additional evidence to the results of Highkin (1958) and also shows that such a variation occurs even in the plant stress metabolism. The reasons for variations in the content of proline and glycinebetaine could be explained in two ways: Since in the present case there was a striking reduction in the seed size (weight) of cultivars with increase in parent temperature (Fig. 22), the observed variation in solute content could be related to seed size differences associated with the maturity temperatures. The effects of parent seed size on proline and glycinebetaine content during water stress has not been reported previously. However, seed size has been extensively studied in relation to its effects on crop yield performance. Wheat seedlings grown from large seeds are more vigorous (Lafond and Baker, 1986) and accumulate more dry matter (Brenchley, 1923; Evans and Bhatt, 1977) resulting in significantly higher yields (Kiesselbach, 1924) than seedlings grown from smaller seed. This variation in solute

content could be due to temperature-induced chemical defects or imbalances of some essential metabolites such as vitamins (Ketellapper, 1963), during parent seed development at unfavourable temperatures which may have been carried over into the seedlings causing the differences. However, any of the carry-over effects of parent seed maturation temperature were confounded with seed size differences. Therefore, seed size variation between any two parent temperatures and also between the cultivars was eliminated by careful choosing of uniform seed size (Table 13).

In the absence of seed size differences, the parent temperature dependent variation in proline content of cultivars disappeared (Table14), suggesting the earlier observed differences in proline were mere reflections of seed size. The presence of variation in glycinebetaine content, although of a different pattern, which is independent of seed size differences indicate that this may have been due to residual or carry-over effects of seed maturation tempetature. However, the precise nature of the residual effect carried through the seed is not known.

Since seed weight resulted in the variation of the accumulation of both proline and glycinebetaine in wheat cultivars, the possibility that seed weight differences was responsible for the difference between the results of Singh *et al.* (1972) and Hanson *et al.* (1977) was tested by growing seedlings of Excelsior, Proctor and Clipper from large or small seed and subsequently subjecting them to water stress. The proline content varied with seed size and cultivar (Fig. 24a) and such results suggest an explanation for at least part of the difference between the two sets of results. The use of small seed resulted in more proline being accumulated in Excelsior than in Proctor (Fig. 24a). It is possible that the seed used by Singh *et al.* (1972) in Australia may have been small because of the frequent occurence of drought and high temperature during the seed filling and maturation period (see Gentilli, 1971). The use of such seed would lead to Excelsior being seem to accumulate more proline than Proctor, as was reported by Singh *et al.* (1972). On the other hand the use of large seed in the present experiment resulted in Proctor accumulating more proline than Excelsior (Fig. 25). Hanson *et al.* (1977) multiplied seed for these two cultivars in glasshouse and subsequently in field. The glasshouse grown

seed would normally be large (Waldron, 1941) and subsequent multiplication of that seed in the field plots in the absence of drought (Bryson and Hare, 1974) would have been again conducive for large seed production. Use of such large seed would lead Proctor to accumulate more proline than Excelsior, as was reported by Hanson *et al.* (1977).

The glycinebetaine content of cultivars was also influenced by seed weight, but the pattern was different from the one observed for proline (see Figs. 24a,b). The existing differences betwen the glycinebetaine content of Excelsior and Clipper grown from large seed disappeared when the seedlings were grown from small seed, but this did not result in the reversal of the response as in the case of proline accumulation.

Even 11 days after germination, there was a general tendency for barley seedlings grown from large seed to accumulate greater amounts of proline and glycinebetaine than those grown from small seeds (with an exception of proline accumulation in Excelsior). The effects of seed size on solute accumulation could be due to similar causes to the effects on growth and yield observed by Evans and Bhatt (1977). The beneficial effects of growth from large seeds have been attributed to the larger amount of seed protein available (Evans and Bhatt, 1977) or to total seed nitrogen content (Bulisani and Warner, 1980). However, in the present experiments the seedlings of Excelsior grown from large seed did not accumulate greater amount proline than that in seedlings grown from small seed, suggesting that N content varies with cultivar and also seed size (Phillips and Schlesinger, 1974). This may be responsible for the differences in the cultivar abilitiy to accumulate solute when seed size was a variable.

The environment during parent seed development together with factors which alter the seed size might alter the relationship between cultivars with repect to the proline or glycinebetaine content. It is possible that seedlings grown from seed sources of different origins might also differ, this possibility is tested in the next section of the thesis.

Section 4. Variability in the accumulation of proline and glycinebetaine associated with seed sources in barley

4.1 Introduction

As early as 1905 it was realised that the use of seed of wheat from different sources resulted in differences in crop performance (Lyon and Keyser, 1905) leading to variation in crop yield (Quinby, 1962). The use of seed from different sources results in variability in several other plant capabilities, in addition to yield. Suneson and Peltier (1936) found highly significant winter hardiness differences within the same varieties when different seed sources were compared. Holton and Heald (1936) with wheat, and Tervet (1944) with oats, found that the incidence of ear smut disease in a variety depended on the environment of the previous growing season.

The discrepancy between the results of Singh *et al.* (1972) and Hanson *et al.* (1977) may have been a result of the differences in seed sources. Singh *et al.* (1972) used seed that was grown at the Waite Agricultural Research Institute, South Australia, whereas Hanson *et al.* (1977) obtained seed of Excelsior and Proctor from various sources (see the materials and methods in Hanson *et al.*, 1977). Seed of Excelsior was obtained from the USDA small grains collection (CI 11509) and also from the Waite Institute, South Australia (WI 278). Both of these sources of seed were selections made by the institutions from the original parent (CI 11248). Similarly, seed of Proctor was obtained by Hanson *et al.* (1977) from various sources. The seed from all the sources was multiplied in glasshouse and then in the field before being used in laboratory stress evaluation experiments.

The possibility that use of different seed sources causes variability in the accumulation of proline and glycinebetaine is tested in the experiments reported in this section.

4.2 Seed sources and proline, and glycinebetaine content in barley seedlings

4.2.1 *Introduction:*

Hanson *et al.* (1977) used seed of Excelsior and Proctor from various seed sources, also, the seed of Excelsior used by these workers was a selection from the original parent (CI 11248). The possiblity that such a seed source difference was responsible for the discrepancy in the results from those of Singh *et al.* (1972) was examined with two seed sources of barley (cv. Norbert) grown under two different field environments followed by selection in plants from one of the seed sources.

4.2.2. *Methods*

Barley seed (cv. Norbert) grown and matured in Canada (CN) (Metcalfe and Bendelow, 1981) or in Australia (AN*) (from Dr. D. H. Sparrow, the Waite Institute) was obtained. CN originated from the cross complex C.I. 5791/Parkland/ Betzes/3/Betzes/Piroline/4/Akka/5/Centennial/6/Klages. The AN* sample was, obtained originally from Canada (the same seed as in Metcalfe and Bendelow, 1981) and was grown at the Waite Institute under field conditions for two generations with selection for shattering resistance being carried out in the second generation. The seed weights of AN* and CN were 3.96 and 4.43 g/100 seed, respectively. Seedlings were grown from these two seed samples, AN* and CN, in standard controlled conditions (20°C) for 11 days and were then subjected to water stress for 1 day by flooding the rooting medium with -1.5 MPa PEG at 5, 15, 25, or 35°C. Well-watered control plants of both AN* and CN were grown at each temperature. RH was not controlled either during seedling growth or water stress. Ψ, proline, and glycinebetaine were measured in the first leaves at the end of 1 day of water stress.

4.2.3 *Results*

4.2.3.1 Water status

Ψ of the first leaves fell significantly with water stress at all temperatures (Table 18). Water stressed plants showed a decrease in Ψ with increase in temperature during water stress, Ψ being lowest at 35°C in one day. However, there were no differences in Ψ between the two seed sources, AN* and CN, when water stressed at any temperature (Table 18).

4.2.3.2 Proline content

Water stress for 1 day resulted in a significant accumulation of proline at all temperatures (Fig. 26a). The two seed sources of Norbert, AN* and CN, showed no significant differences in proline content in well-watered or water stressed plants at 5 or 15°C. However, the proline content of seedlings grown from the two seed sources differed significantly at 25 and 35°C. Norbert obtained from Canada, CN, contained more proline than AN* at both these temperatures, despite similar Ψ in these seedlings (Table 18).

4.2.3.3 Glycinebetaine content

The glycinebetaine content of well-watered seedlings increased significantly with temperature between 25 and 35°C, confirming this response to temperature (see section 2). Water stress increased glycinebetaine content at 25 and 35°C but not at the lower temperatures (Fig. 26b). The glycinebetaine content of seedlings grown from the two seed sources did not differ from each other.

Table 18: Leaf water potential (-MPa) of barley (cv. Norbert) either originated from Canada (CN) or grown in Australia (AN*) for 2 generations. There being no significant difference between these seed sources, the values in the body of the table are the means of the two samples.

Stress Temperature (°C)	Control	Water stress	LSD (P = 0.05)
5	0.78	0.93	
15	0.68	1.04	
25	0.70	1.11	
35	0.86	1.44	0.21
Seed source	AN*	CN	LSD (P = 0.05)
	0.96	0.93	NS

Fig. 26 Leaf proline and glycinebetaine content of barley (cv. Norbert) seedlings raised from seed grown either in Canada (CN) or Australia for two generations (AN*). Seedlings were water stressed (-1.5 MPa PEG) for 1 day at 5, 15, 25 or 35°C.

A. Proline

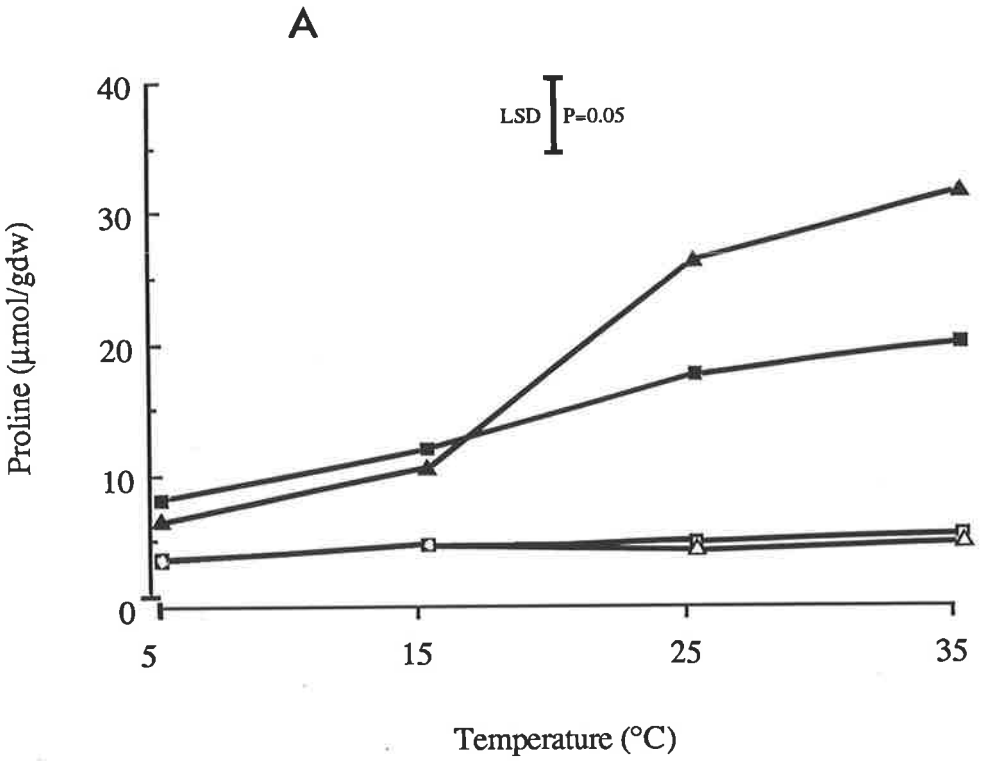
B. Glycinebetaine

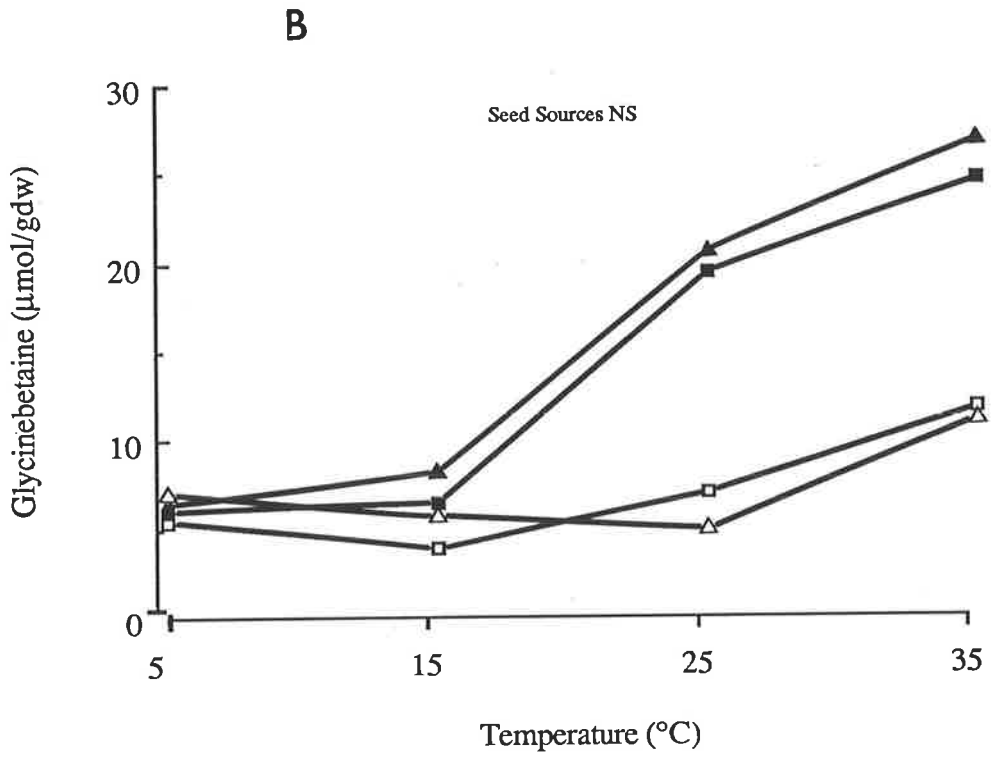
□ AN* control

■ AN* stressed

△ CN control

▲ CN stressed


A
LSD P=0.05
Proline (µmol/gdw)
40
30
20
10
0
5
15
25
35
Temperature (°C)


B
Seed Sources NS
Glycinebetaine (µmol/gdw)
30
20
10
0
5
15
25
35
Temperature (°C)

4.3 Seed sources and proline, and glycinebetaine content, in the absence of selection

4.3.1 *Introduction*

In the previous experiment, barley seedlings grown from two sources of seed showed differences in the accumulation of proline in response to stress (experiment 4.2). However, as the genetic composition of the two seed sources may not have been identical, due to the limited selection applied to AN*, it cannot be determined whether the differences in response was due to genetic factors or to the environment during parent seed growth and maturation. This experiment was designed to eliminate this uncertainty by using grain of identical genetic background, but grown in different environments.

4.3.2 *Methods*

Barley seed (cv. Norbert) was obtained from Canada (CN) (Metcalfe and Bendelow, 1981) and grown to maturity at the Waite Institute under field conditions for 1 generation without any deliberate selection. This seed source was designated Australian Norbert (AN). The two sources of Norbert seed, AN and CN were used to grow seedlings under controlled standard environmental conditions for 11 days. The seedlings were then water stressed for 2 days at 20°C by flooding the rooting medium with PEG (-1.5 MPa). The control plants were well-watered during the water stress period. Leaf water status, proline, and glycinebetaine were measured on the first leaves at the end of 2 days water stress.

4.3.3 *Results*

4.3.3.1 Water status

Water stress resulted in a significant drop in Ψ of both AN and CN, however, seedlings grown from the two seed sources did not differ from each other (Table 19).

Table 19: Leaf water potential, proline and glycinebetaine content of barley (cv. Norbert) grown in Australia (AN) and Canada (CN).

		AN	CN
Ψ (-MPa)	control	0.50	0.46
	stress	2.06	2.09
Proline	control	0.9	1.1
(μmol g^{-1} dw)	stress	119.9	116.7
Glycinebetaine	control	4.8	5.0
(μmol g^{-1} dw)	stress	26.5	26.6

4.3.3.2 Proline content

The proline content of leaves increased by more than 100 fold as a result of water stress (Table 19). However, the earlier observed differences in proline content between the seed sources (Fig. 26a), did not appear in the present case (Table 19) in the absence of seed selection in one of the seed sources. Although the duration of stress was different between these two experiments (4.2 and 4.3), it is unlikely that this difference would have caused the disappearence of any differences in proline content between the seed sources.

4.3.3.3 Glycinebetaine content

The glycinebetaine content of leaves increased by only 5 fold (Table 19) in response to 2 days water stress. The glycinebetaine content of seedlings from the two seed sources, AN and CN, was the same (Table 19).

4.4 Test of genetic similarity of different barley seed sources

4.4.1 *Introduction*

The work reported in experiments 4.2 and 4.3 suggests that the two seed sources of barley (cv. Norbert) differed in their capacity to accumulate proline only when one parent had been subjected to limited selection pressure. One suggestion which can be made to account for this difference is that the selection pressure for shattering resistance was sufficient to cause a genetic difference between the populations, sufficient to account for the effects on proline content. This could not be tested directly, so evidence for a genetic shift in the population was sought by examining hordeins (seed storage proteins) patterns. The hordeins of barley cultivars or biotypes within a cultivar differ and can be distinguished by gel electrophoresis (Marchylo and Laberge, 1981; Gebre *et al.*, 1986; Heisel *et al.*, 1986). The objective of this experiment was to seek genetic similarities and differences between AN* and CN, and AN and CN using one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1-D SDS-PAGE) of hordeins.

4.4.2 *Methods*

The 3 barley seed sources cv. Norbert; AN*, CN and AN were used for SDS-PAGE studies. The reduced proteins from the barley endosperm were extracted as described by Lawrence and Shepherd (1980). 150 µl of extraction buffer was prepared by freshly mixing equal volumes of the stock solution (0.125 M tris (hydroxymethyl) aminomethane (Tris), 8% (w/v) SDS, 20% (v/v) glycerol, 0.002% (w/v) bromophenol blue made to pH 6.8 with HCl) and distilled water. The crushed pieces of barley endosperm were treated in the presence of 2% 2-mercapto-ethanol for about 16 h at 40°C.

The discontinuous 1-D SDS-PAGE used was based on the method of Laemmli (1970) as modified by Lawrence and Shepherd (1980). Gels (145 x 100 x 1.6 mm) were poured as vertical slabs between the two glass plates. The separating gel contained 10.0% (w/v) acrylamide and 0.08% (w/v) bisacrylamide (Bis), 0.1% (w/v) SDS, and 0.375 M Tris made to pH 8.8 with HCl and the stacking gel contained 3% (w/v) acrylamide,. 0.08% Bis (w/v), 0.1% (w/v) SDS and 0.125 M Tris made to pH 6.8 with HCl. Both gels were polymerised with N,N,N',N"-tetramethyl-ethylenediamine (TEMED) and ammonium persulphate. The electrode buffer, for both upper and lower tanks contained 0.1% (w/v) SDS and 0.025 M Tris made to pH 8.3 with glycine. Before electrophoresis, samples were centrifuged at 10,000 rpm for 2 min and 10 µl of clear supernatant was loaded into each slot. The electrophoresis was carried out at a constant current of 50 mA/gel until the marker dye front reached the bottom of the gel (for about 2 h).

Gels were stained overnight in a staining solution consisting of one part of 1% (w/v) Coomassie Brilliant Blue R mixed with 40 parts of 6% (w/v) trichloroacetic acid in water:methanol:glacial acetic acid (80:20:7) as described by Lawrence and Shepherd (1980), and destaining was carried out in water for 48 h.

4.4.3 *Results*

The 1-D SDS-PAGE between AN*, and CN and AN showed considerable differences in the banding pattern (Fig. 27) which may be a result of selection pressure in CN. In the absence of such selection AN and CN showed many similar bands. However, the band pointed by an open arrow showed a minor difference in electrophoretic mobility with that in the other sample in both AN and CN.

4.5 Discussion

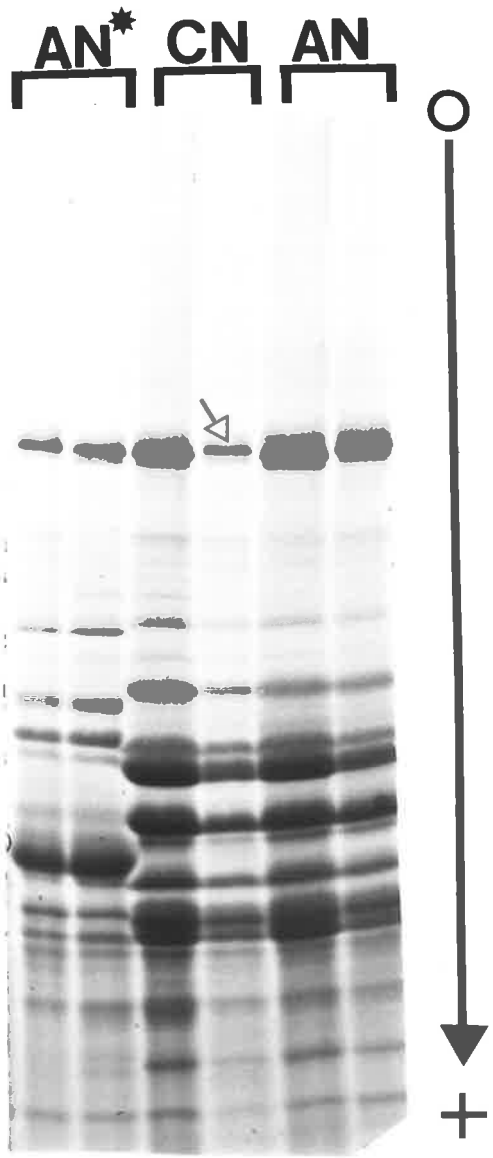
It was not possible to obtain seed of Excelsior and Proctor grown in different environments to study the influence of seed sources on possible variability in solute accumulation so that a direct comparision could be made with the results of Hanson *et al.* (1977) and Singh *et al.* (1972). However, the work reported with barley cultivar Norbert grown from different seed sources gives an indication of possible variation in solute accumulation within a single cultivar.

When Norbert seed grown in Canada and in Australia was used in seedling water stress experiments, the proline accumulating capacity of CN was greater than that of AN* (Fig. 26a), while there was no difference in Ψ or glycinebetaine accumulating capacity between AN* and CN (Table 18 and Fig. 26b). Variability in the proline content of individuals within a barley cultivar grown in controlled conditions has been observed previously (Aspinall and Choi, unpublished; see Aspinall and Paleg, 1981 for details), this variability being independent of leaf water status since excised leaves from individual plants were water stressed by floating on PEG. These authors were able to develop two genetically distinct progenies with low or high proline accumulating capabilities by selection within a cultivar. These populations also differed in seed size and when this was taken out, selection for proline levels within a variety was not possible. In the present investigation, since there was limited selection of AN* for shattering resistance, it is possible that there could have been a genetic differences between AN* and CN populations. Such a genetic difference was confirmed by the SDS-PAGE test of the seed proteins (Fig. 27). Further, the minor difference in the banding pattern between the

Fig. 27 One-dimensional SDS-PAGE patterns of reduced extracts of seed protein from AN*, CN, and AN. The open arrow points at a hordein band showing relative difference in electrophoretic mobility between samples of CN and AN.

O, origin and negative terminal

+, positive terminal


AN*
CN
AN
O
+

samples of both CN and AN shows that original seed CN must have been heterogenous and contained genetic heterogeneity for the proline accumulation response so that a genetically different type AN* could be selected from within it. However, the difference in banding pattern similar to that in AN* was not found in either CN or AN. This failure could be due to the limited number of grains used for SDS-PAGE. The existence of considerable intra-cultivar variation in barley for proline accumulating capacity, as shown by Aspinall and Choi (unpublished) and the present work (Fig. 26a), may explain some of the disagreement between the results of Hanson *et al.* (1977) and Singh *et al.* (1972), since Hanson *et al.* (1977) used Excelsior seed which was a selection from the original parent. However, no mention was made of the pedigree of the seed used by Singh *et al.* (1972) except the seed source (Singh, 1970).

In the absence of any considerable genetic difference, evident from the SDS-PAGE banding patterns of the seed proteins (Fig. 27), the proline accumulating capacity did not differ between seedlings grown from CN and AN (Table 19). This lack of difference in proline content between seedlings grown from seeds from different environments differs from reported variations in growth, yield and disease incidence of crops grown from different seed sources (Lyon and Keyser, 1905; Sunesen and Peltier, 1936; Holton and Heald, 1936; Tervet, 1944; Quinby, 1962). This variation in yield has been thought to be due mainly to seed size, however (Lafond and Baker, 1986). In the present case lack of difference in solute content between AN and CN may be due to the similar seed weights (AN: 4.51g/100 seeds; CN: 4.43g/100 seeds). AN was grown in Australia for only one generation (in 1983) and this may not have induced sufficient variation in seed weight. One other possible reason for the presence of variation in yield due to seed sources in the results of the previous workers may be the presence of undetected genetic differences, similar to those in the experiment 4.2, although the observed differences in yield were assumed to originate solely from seed related factors such as seed weight. Gebre *et al.* (1986) have shown that two seed sources of the same barley cultivar, Betzes, obtained from the USDA small grains collection and Montana State University (USA), showed differences in the SDS-PAGE banding pattern of seed

proteins, suggesting the presence of minor genetic differences.

The lack of differences in glycinebetaine content between two seed sources, despite selection induced variation in the proline content and the genetic composition, suggests that the triggering mechanisms in the metabolism of these two solutes may be controlled by different genes. This will be discussed further in the final chapter.

It can be concluded that grain of the same genetic background grown at different locations does not differ in response. However, small genetic changes may cause differences in the response. Such genetic changes could be a consequence of growing a population which is not completely homozygous, which Norbert is not, in different environments for a number of generations. It is unlikely that seed sources caused the differences in response recorded for Excelsior and Proctor, provided that both groups (Singh *et al.*, 1972; Hanson *et al.*, 1977) used the same genetic material.

Section 5: Variability in the accumulation of proline and glycinebetaine associated with vapour pressure deficit (VPD) during barley seedling growth and water stress

5.1 Introduction

It has been shown in the previous sections of this chapter that solute accumulating capacity of barley or wheat cultivars could vary significantly depending on seedling growth-temperature history (Section 2), seed related factors such as temperature during seed maturity or seed weight (Section 3) or the source of seed for the same cultivar which might contain genetic differences (Section 4). Some of these factors might be responsible for the discrepancies between the results of Singh *et al.* (1972) and those of Hanson *et al.* (1977). However, VPD is another environmental factor which is not controlled in most studies although other environmental factors may be closely controlled. The VPD regime in the field normally is different from that in the glasshouse or growth cabinet; glasshouse VPD being usually low because of inadequate ventilation. The general VPD pattern even in growth chambers would vary between geographical locations and within a geographical location with season, unless specifically controlled.

Hanson *et al.* (1977) grew barley seedlings in their experiments under low VPD (as low as 0.18 KPa; see Materials and Methods of Hanson *et al.*, 1977), however, VPD was neither controlled nor measured in the experiments of Singh *et al.* (1972). The experiments in this section are designed to test the effects of VPD during barley seedling growth and water stress on solute content.

5.2 Proline and glycinebetaine content of barley seedlings (cv. Clipper) at high (1.2 KPa) or low (0.12 KPa) VPD during growth and water stress

5.2.1 Introduction

Water stressed *Dactylis glomerata* L. and wheat plants had a lower Ψs when grown at a high VPD than plants grown at a low VPD (Gavande and Taylor, 1967; Morgan, 1984). These observations raise the possibility that growing plants at a high VPD leads to the accumulation of more osmotic solutes such as amino acids and sugars. The present experiment was designed to evaluate the effect of VPD during growth and water stress on the contents of proline and glycinebetaine in barley (cv. Clipper) seedlings.

5.2.2 Methods

Two days after sowing, the emerging barley (cv. Clipper) seedlings were transferred to cabinets at either a high (1.2 KPa) or a low (0.2 KPa) VPD. In all other respects these two cabinets provided similar growth condtions (20°C; 16 h photoperiod and 200 $\mu E\ m^{-2}\ s^{-1}$ photon flux density from fluorescent lights). Eleven days after sowing, half the pots of seedlings in each cabinet were transferred to the alternate environment. Plants in both the cabinets were either water stressed by flooding the rooting medium with -1.5 MPa PEG or maintained on nutrient solution (control). The cabinets were programmed to initiate the photoperiod 1 h before subjecting the plants to water stress, to minimise initial differences in leaf water status caused by differences in transpiration due to growth VPD. The treatments were replicated thrice. First leaves were sampled 1 day after water stress to measure water status, proline and glycinebetaine content.

5.2.3 Results

5.2.3.1 Water status

Growing plants at high or low VPD produced no differences in RWC, Ψ, Ψ_s or Ψ_p of the first leaves without the imposition of water stress (Table 20). Water stress reduced leaf RWC, Ψ, Ψ_s and Ψ_p (Tables 21a,b; 22a,b). Differences in VPD during growth (0.12 or 1.2 KPa) followed by stress at a common VPD (1.2 KPa) caused no significant effects on any of the water status parameters measured. However, differences in atmospheric VPD during stress resulted in such differences. High VPD during stress produced a lower RWC, Ψ, Ψ_s and Ψ_p than low VPD (Tables 21a,b; 22a,b). There were no significant interactions between the effects of water stress, VPD during growth and VPD during stress on any of the leaf water status parameters.

5.2.3.2 Proline content

Differences in atmospheric VPD had no significant effect on the leaf proline content of well-watered plants (Table 20), but when these plants were subsequently water stressed at the same VPD, seedlings grown in a high VPD environment accumulated more proline than plants grown at a low VPD (Fig. 28a). This difference occurred in the absence of any difference in leaf water status between plants grown at the two VPD levels. Subjecting plants to water stress in a high VPD environment resulted in the accumulation of more proline than stress at a low VPD (Fig. 28b). High VPD during stress resulted in greater water loss (Tables 21a,b; 22a,b), which presumably accounts for the increased proline accumulation.

5.2.3.3 Glycinebetaine content

VPD during growth had no effect on leaf glycinebetaine content in well-watered plants (Table 20) but differences in VPD during both growth and water stress significantly affected glycinebetaine accumulation of water stressed leaves (Figs. 29a,b). This is similar to the response observed with proline. As with proline, the response in glycinebetaine content to VPD during growth cannot be related to leaf water status, but

Table 20: Leaf water status, proline and glycinebetaine content of barley (cv. Clipper) grown at a low (0.12 KPa) or a high (1.2 KPa) VPD, before subjecting to water stress.

	Grown at low VPD	Grown at high VPD
RWC (%)	97.9	97.6
Ψ (-MPa)	0.44	0.49
Ψ_s (-MPa)	0.84	0.87
Ψ_p (MPa)	0.40	0.38
Proline (μmol g^{-1} dw)	0.98	1.09
Glycinebetaine (μmol g^{-1} dw)	2.27	2.82

Table 21: RWC and Ψ of barley seedlings (cv. Clipper) grown and water stressed at a low (0.12 KPa) or a high (1.2 KPa) VPD.

A: RWC (%)

Water stress	Control	Stress	LSD (P = 0.05)
	98.2	89.8	0.96
Growth VPD	Low	High	
	94.2	93.8	NS
Stress VPD	Low	High	
	94.9	93.1	0.96

B: Ψ (-MPa)

Water stress	Control	Stress	LSD (P = 0.05)
	0.56	1.38	0.11
Growth VPD	Low	High	
	0.94	0.99	NS
Stress VPD	Low	High	
	0.82	1.11	0.11

Table 22: Ψ_s and Ψ_p of barley seedlings (cv. Clipper) grown and water stressed at a low (0.12 KPa) or a high (1.2 KPa) VPD.

A: Ψ_s (-MPa)

Water stress	Control	Stress	LSD (P = 0.05)
	0.87	1.28	0.09
Growth VPD	Low	High	
	1.03	1.12	NS
Stress VPD	Low	High	
	0.98	1.17	0.09

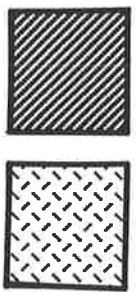
B: Ψ_p (MPa)

Water stress	Control	Stress	LSD (P = 0.05)
	0.32	-0.10	0.06
Growth VPD	Low	High	
	0.09	0.13	NS
Stress VPD	Low	High	
	0.16	0.13	0.06

Fig. 28 Effect of a low (0.12 KPa) or a high (1.2 KPa) VPD during barley seedling (cv. Clipper) growth and water stress on leaf proline content.

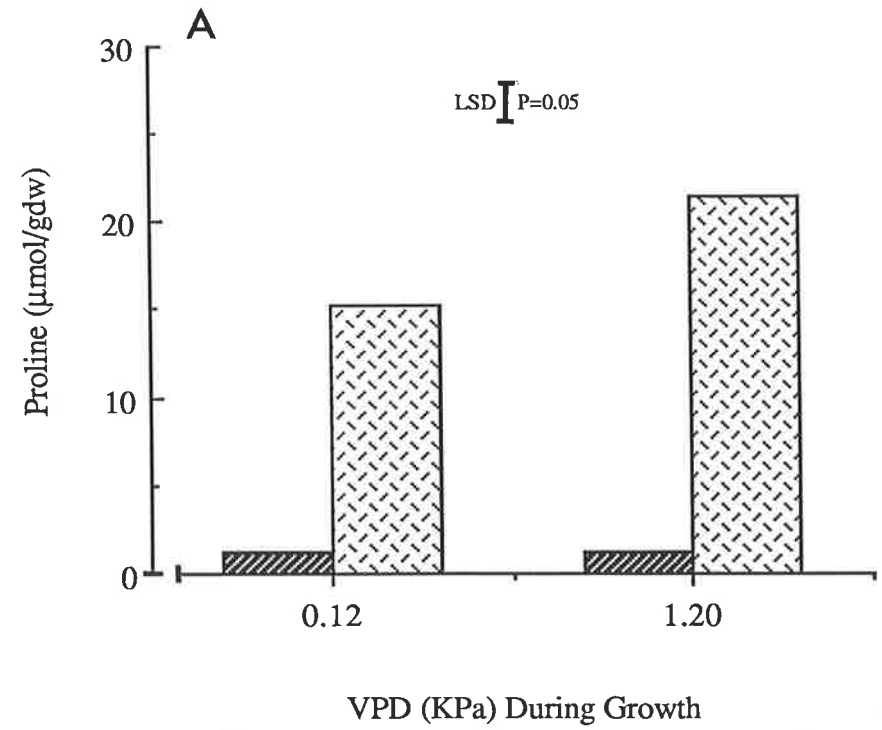
A. VPD during growth

B. VPD during stress



Control

Stress


A
30
20
10
0
LSD P=0.05
Proline (µmol/gdw)
0.12
1.20
VPD (KPa) During Growth

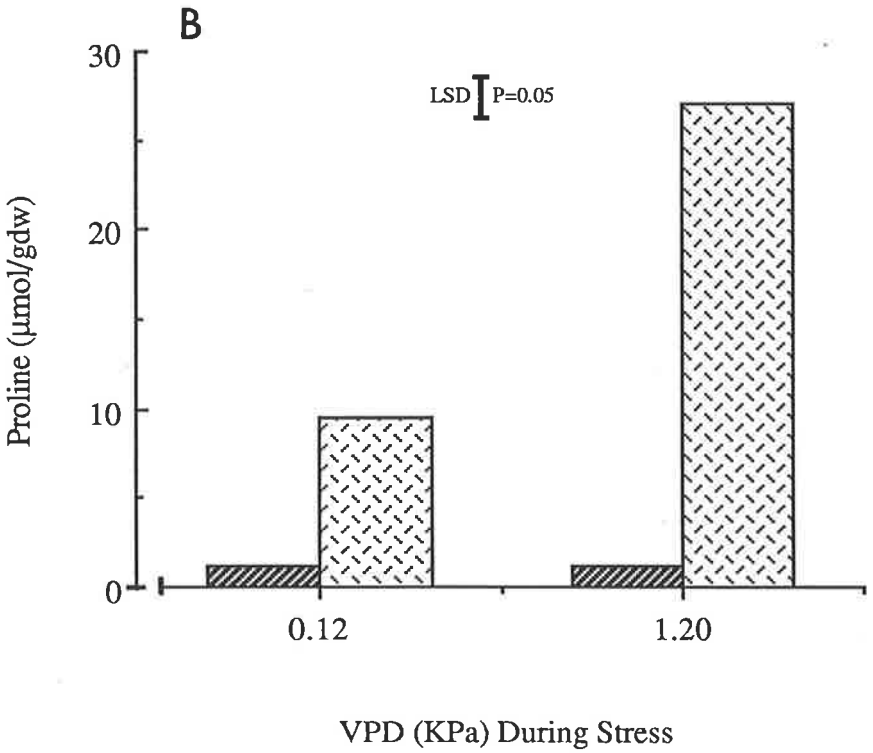
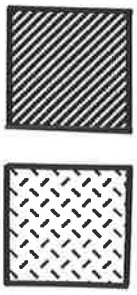

B
30
20
10
0
LSD P=0.05
Proline (µmol/gdw)
0.12
1.20
VPD (KPa) During Stress

Fig. 29 Effect of low (0.12 KPa) or high (1.2 KPa) VPD during barley seedling (cv. Clipper) growth and water stress on leaf glycinebetaine content.

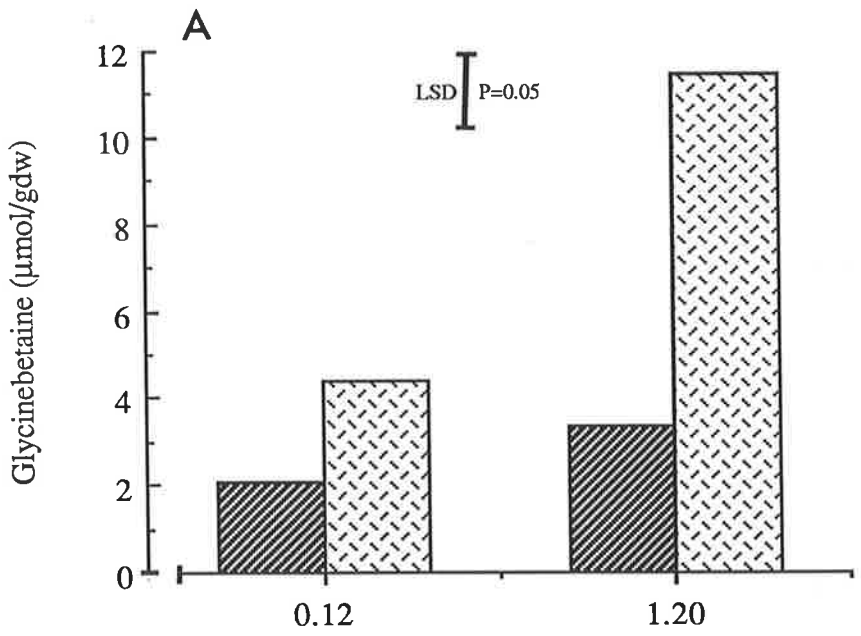
A. VPD during growth

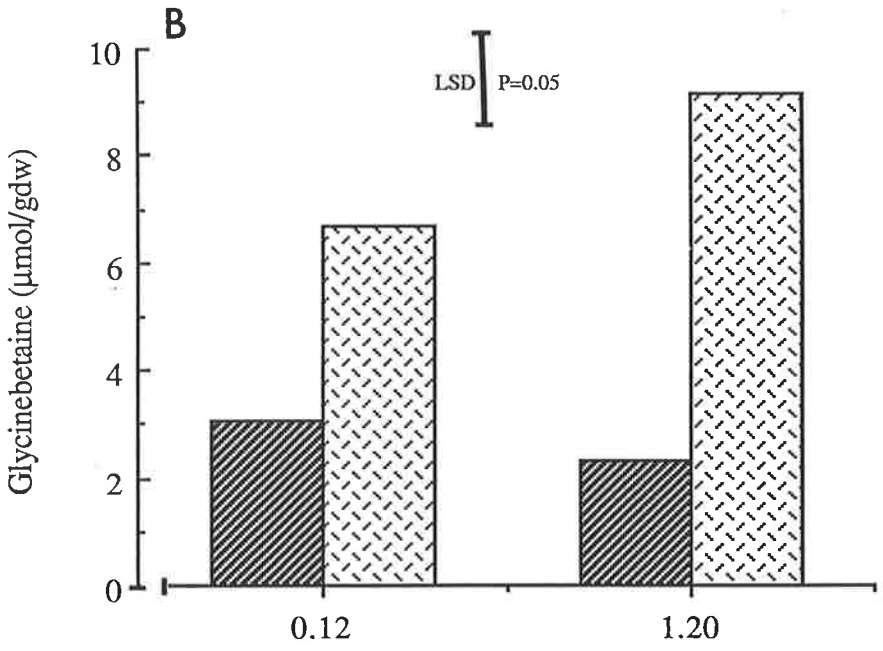
B. VPD during stress



Control

Stress


A
LSD P=0.05
Glycinebetaine (µmol/gdw)
12
10
8
6
4
2
0
0.12
1.20
VPD (KPa) During Growth


B
LSD P=0.05
Glycinebetaine (µmol/gdw)
10
8
6
4
2
0
0.12
1.20
VPD (KPa) During Stress

the response to VPD during water stress may be the result of a lowered leaf water status (see Tables 21a,b; 22a,b).

5.3 VPD during the growth of barley cultivars and variability in the capacity to accumulate proline or glycinebetaine

5.3.1 Introduction

The results reported in the previous experiment (5.2) showed that differences in solute content accompanying differences in VPD during stress could be explained in terms of tissue water status, but that changes in solute content in response to growth VPD were unaccompanied by differences in leaf water status. In the present experiment only the effect of growth VPD was examined in barley cultivars, Excelsior, Proctor, Australian Norbert (AN*) and Canadian Norbert (CN). This is thought to be uncomplicated by concomitant changes in leaf water status of these cultivars on solute accumulating capacity when water stressed at a common VPD (1.2 KPa). The first two cultivars were included in this experiment as they were used by both Hanson *et al.* (1977) and Singh *et al.* (1972).

5.3.2 Methods

The four barley cultivars, Excelsior, Proctor, AN* and CN, were grown under high or low VPD under controlled environmental conditions (20°C; 16 h photoperiod, 200 $\mu E\ m^{-2}\ s^{-1}$ photon flux density from fluorescent lights) for 11 days in a similar way to the previous experiment. Then, plants grown under low VPD were transferred to the cabinet where the other set of plants were grown at high VPD (1.2 KPa). Both sets of plants were water stressed at an atmospheric VPD of 1.2 KPa (other environmental conditions being identical to the growth environment) by flooding the rooting medium with -1.5 MPa PEG. Well-watered control plants were maintained as before. Two days after water stress the first leaves were harvested for the measurement of water status, and proline and glycinebetaine content.

5.3.3 Results

5.3.3.1 Water status

There was no initial difference in leaf RWC or Ψ of Excelsior, Proctor, AN* and CN due to growing the seedlings at low or high VPD prior to subjecting plants to water stress (Table 23). When the plants were water stressed, RWC varied both between cultivars and with VPD during the seedling growth (Table 24a). Growing seedlings under a low VPD resulted in the lowest RWC in Proctor and the highest in Excelsior (Table 24a). However, when these cultivars were grown under high VPD, the lowest RWC was recorded in Excelsior and the highest in CN. Despite these differences in RWC, VPD during growth had no effect on leaf Ψ (Table 24b). There were cultivar differences in mean Ψ, however, CN having a higher Ψ than the remaining cultivars. The effects of VPD during growth, water stress and cultivar on Ψ did not interact.

5.3.3.2 Proline content

There were no cultivar differences in proline content in the absence of water stress, but the proline content of water stressed plants varied between cultivars and with VPD during the plant growth (Fig. 30a). All cultivars accumulated more proline when grown at a high VPD than at a low VPD. The relative proline accumulation by the various cultivars varied with VPD during growth, however, the lowest proline content found in water stressed seedlings was in cv. Excelsior grown at a low VPD. In this case, Excelsior accumulated less proline than did Proctor. However, when the plants were grown at a high VPD Excelsior accumulated more proline than Proctor. AN* and CN showed no differences in proline content when grown at a high VPD, but when these cultivars were grown at a low VPD, CN accumulated more proline than AN. See appendix 1b for the analysis of variance.

5.3.3.3 Glycinebetaine content

As with proline, the glycinebetaine content of the cultivars did not vary with VPD during growth when they were not subjected to stress (Table 23). When water stressed, plants grown at a high VPD accumulated more glycinebetaine than plants grown at a low

Table 23: Leaf water status, proline and glycinebetaine content of barley cultivars after growing for 11 days at low (0.12 KPa) or high (1.2 KPa) VPD before subjecting to water stress.

		RWC (%)	Ψ (-MPa)	Proline (μmol g^{-1} dw)	Glycinebetaine (μmol g^{-1} dw)
Excelsior	Low VPD	98.4	0.46	0.91	2.51
	High VPD	98.6	0.48	0.99	3.87
Proctor	Low VPD	97.8	0.47	0.98	2.16
	High VPD	98.0	0.49	0.97	2.58
AN*	Low VPD	98.7	0.53	0.96	2.09
	High VPD	98.3	0.51	1.01	3.11
CN	Low VPD	97.9	0.50	0.98	2.20
	High VPD	78.2	0.49	0.99	3.42

Table 24: RWC and Ψ of barley cultivars grown under low (0.12 KPa) or high (1.2 KPa) VPD and water stressed at a common VPD (1.2 KPa).

A: RWC (%)

	Excelsior	Proctor	AN*	CN	LSD (P = 0.05)
Control					
Low growth VPD	98.7	97.9	97.9	98.5	
High growth VPD	98.0	98.0	98.3	97.3	
Stressed					
Low growth VPD	76.8	66.2	70.4	76.0	
High growth VPD	67.6	73.6	78.0	81.4	3.3

B: Ψ (-MPa)

Low growth VPD	High growth VPD	LSD (P = 0.05)
1.28	1.31	NS

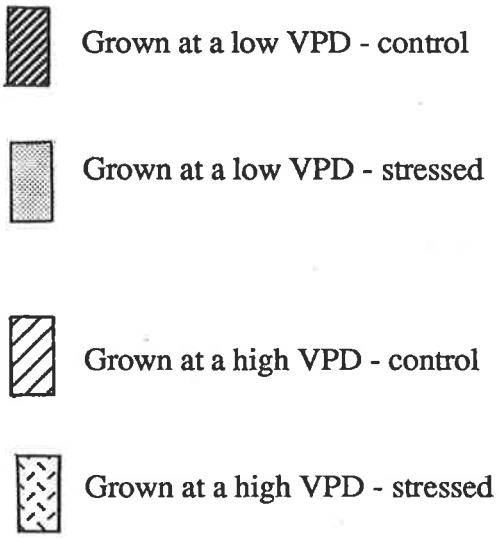
Control	Water stressed	
0.61	1.98	0.09

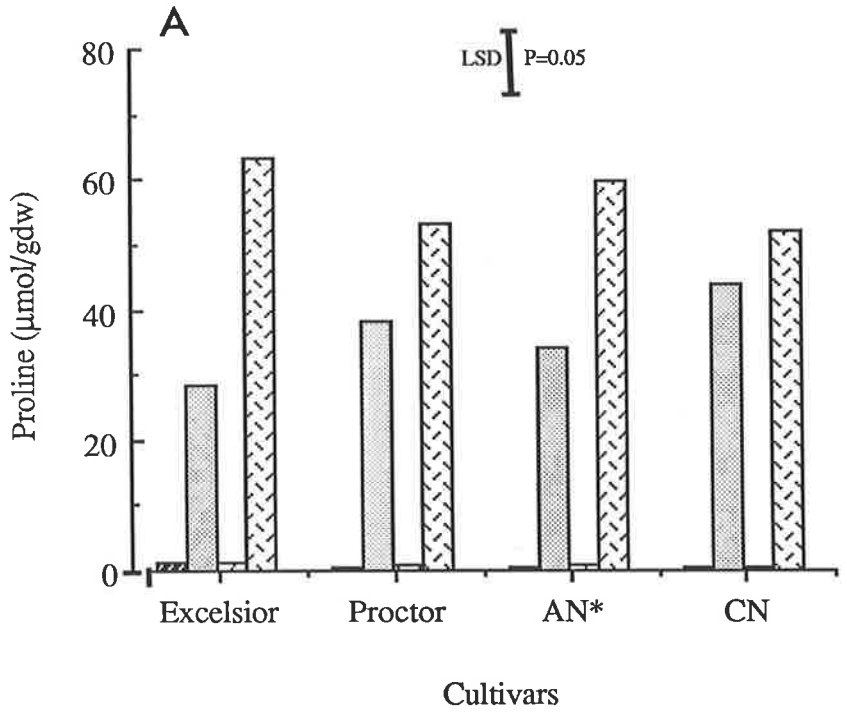
Excelsior	Proctor	AN*	CN	
1.37	1.34	1.33	1.14	0.13

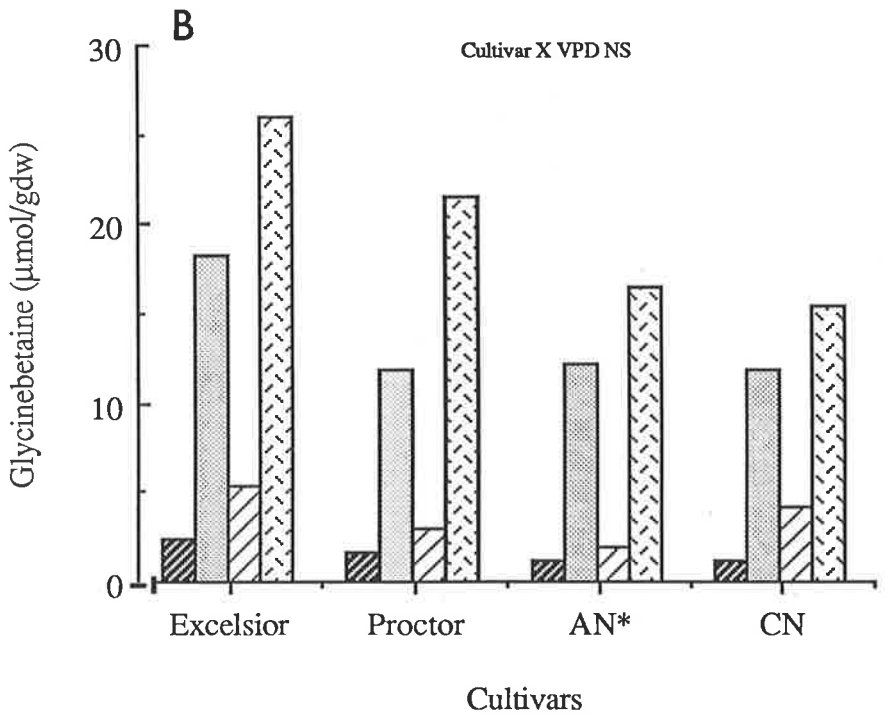
Fig. 30 Leaf proline and glycinebetaine content (μmol g^{-1} dw) of barley (cvs. Excelsior, Proctor, AN* and CN) seedlings grown at a low (0.12 KPa) or a high (1.2 KPa) VPD and water stressed (-1.5 MPa PEG) at a common VPD regime (1.2 KPa) for 2 days.

A. Proline content

B. Glycinebetaine content




A
LSD P=0.05
80
60
40
20
0
Proline (µmol/gdw)
Excelsior
Proctor
AN*
CN
Cultivars


B
Cultivar X VPD NS
30
20
10
0
Glycinebetaine (µmol/gdw)
Excelsior
Proctor
AN*
CN
Cultivars

VPD (Table 25). Excelsior contained more glycinebetaine than the rest of the cultivars, but there were no interactions in the effects of cultivar, growth VPD and water stress on glycinebetaine content (Fig. 30b).

5.4 Discussion

The solute content of water stressed barley seedlings has been shown to vary with changes in environmental factors (Aspinall and Paleg, 1981). The work reported in this section of the thesis demonstrates that the VPD regime during plant growth or water stress could also induce such a variability in the accumulation of proline and glycinebetaine. Differences in VPD during growth, induced variation in the contents of proline and glycinebetaine (Figs. 28a; 29a) independent of changes in leaf water status (Tables 21a,b). However, VPD differences during water stress caused variation in solute content mainly due to the control of evapotranspiration, thus plants undergoing water stress under high VPD lose more water, as evident from low Ψ and RWC; (Tables 21a,b) and this leads to the accumulation of a greater amount of proline and glycinebetaine (Figs. 28b; 29b). VPD during stress, in addition to this effect mediated through leaf water status, may have produced an effect on solute content identical to that caused by the growth VPD. However, it is difficult to disentangle such possible effects of VPD during stress from the direct effect on transpiration. Such an influence of VPD during growth or water stress on solute accumulation has not been reported earlier although D. Aspinall (pers. comm.) found that detached and water stressed barley leaves (floated on PEG) derived from plants grown at a high VPD accumulated more proline than those from plants grown at a low VPD.

This variation in the solute content of water stressed barley seedlings in response to VPD, could potentially be applied to understand the differences in response between plants grown under laboratory and field conditions (McCree, 1974; Morgan, 1983). Lesser osmotic adjustment (which arises from the accumulation of proline and glycinebetaine, as these are the principal solutes in wheat; Wyn Jones, 1984) of glasshouse grown than of field grown wheat plants of the same cultivar (Morgan, 1983)

may be related, at least in part, to the humid conditions in the glasshouse. Munns and Weir (1981) observed that plants grown in a phytotron during the winter season showed less osmotic adjustment than plants which were grown during spring. The difference in irradiance between these two seasons has been thought to be responsible for this seasonal variation since temperature was controlled (Morgan, 1984). However, based on the results of the present experiment, it is reasonable to argue that uncontrolled VPD differences in the phytotron, due to seasonal variation in ambient VPD, could also have been responsible.

The greater proline accumulating capacity of plants grown at a high VPD (1.2 KPa) may have been due to a process of 'hardening'. Hardening by various mild stresses results in the capacity to accumulate a greater amount of solute (Singh *et al.*, 1973c; Ladyman *et al.*, 1980; Meza-Basso *et al.*, 1986) in response to subsequent stress. For example, Singh *et al.* (1973c) reported that barley plants, previously exposed to water deficit on one or more occasions, had a considerably increased capacity to accumulate proline on subsequent exposure to water deficit. This increase in proline accumulation was not due to any change in leaf water status in the final exposure to water deficit, nor to any persistance of accumulated proline from previous stress episodes. An increased glycinebetaine accumulation was also found in barley plants previously exposed to drying cycles (Ladyman *et al.*, 1980). This increased accumulation was thought to be due to the persistance of accumulated glycinebetaine from previous stress episodes (Ladyman *et al.*, 1980). In the present experiment, however, the response when plants were grown at high VPD was not due to the same cause, as there was no difference in the initial glycinebetaine level between plants grown under high or low VPD (Table 20).

The mechanism of these various hardening effects on solute accumulation is unknown, however changes at the metabolic level are indicated. The rate limiting step in proline biosynthesis is P5C formation (Boggess *et al.*, 1976b) and efficiency of this step may be enhanced by hardening. Drought and possibly hardening treatments in general result in changes in the integrity and composition of mitochondrial membranes (Xiao-Nan

et al., 1986) which could lead to a loss of mitochondrial compartmentation of proline oxidisation products (Stewart *et al.*, 1977; Stewart and Boggess, 1978). This damage to mitochondrial membranes may persist, if only partially, when the hardened plants are well-watered before subjecting to a further water stress. Stewart (1972a) and Joyce *et al.* (1984) have shown that leaf carbohydrate status plays an essential role in determining proline level during water stress. It is possible that hardened plants may have had a greater level of carbohydrate. Although the accumulation of ABA is not an essential pre-requisite for proline accumulation (Stewart and Voetburg, 1987), its application does increase the proline level in most plants (Aspinall *et al.*, 1973; Aspinall, 1980; Parameshwara, 1984; Stewart and Voetburg, 1985; Goring and Plescher, 1986) and hardened plants may have a greater capacity to accumulate the hormone when water stressed. If these responses occur, hardened plants will have a greater capacity to synthesise proline and inhibit proline oxidation, both of which will contribute to the greater capacity to accumulate proline. Similarly, the rate limiting step in glycinebetaine biosynthesis, oxidation of glycinebetaine aldehyde to glycinebetaine (Hanson and Scott, 1980; Ladyman *et al.*, 1983), may also be stimulated in hardened plants. Alternatively, since the glycinebetaine biosynthetic pathway involves a membrane-bound intermediate, phosphatidylcholine (PC) (Hanson and Rhodes, 1983), the possible persistance of stress-induced modification to membrane lipid composition (Christiansen, 1984) in the hardened plants may also account for the increased capacity to accumulate this solute.

The response to atmospheric VPD during growth is dependent upon genetic factors. Four barley cultivars grown in two contrasting VPD regimes showed enhanced capacity to accumulate proline and glycinebetaine after growth at high VPD when water stressed subsequently at a common VPD . This confirms the VPD response observed with Clipper. However the level of response to growth VPD varied between the cultivars such that the proline accumulating capacity of Excelsior and Proctor were in reverse order in the two VPD regimes (Fig. 30a). A similar reversal in response pattern, in this case with growth rate, was observed by Lauter and Munns (1987) with two Chickpea cultivars (L-550 and E-100) when they were grown and salinised in two different VPD

regimes. At low VPD cv. L-550 was more salt resistant and produced greater shoot dry matter than cv. E-100, but, at high VPD cv. E-100 was more salt resistant and produced greater shoot dry matter than L-550.

The alteration in the relative proline accumulation potential (Fig. 30a) of different barley cultivars with growth VPD is a potential explanation of the observed discrepancy between the results of Singh *et al.* (1972) and of Hanson *et al.* (1977). When barley cultivars were grown under low VPD, Excelsior (a cultivar with greater stability in yield under dry areas; Finlay and Wilkinson, 1963) accumulated less proline than Proctor (a cultivar with lesser stability). A similar relationship in proline accumulation between these two cultivars was reported by Hanson *et al.* (1977) with barley seedlings grown under a low VPD (as low as 0.18 KPa). This led Hanson and co-workers to the conclusion that greater proline accumulating capacity is associated with drought susceptibility. However, when the same cultivars were grown at high VPD, water stressed Excelsior accumulated more proline than Proctor (Fig. 30a) which is similar to the results reported by Singh *et al.* (1972). Although Singh *et al.* (1972) made no mention of the VPD during their experiments, it is possible that VPD during their experiments was high, since in South Australia VPD is usually high (as high as 1.9 KPa at 20°C). Differences in VPD during seedling growth and water stress seems to be one of the most likely causes for the discrepancies between these two groups. However, the levels of proline accumulated by the seedlings of the present experiment were much lower than those in the investigations of Singh *et al.* (1972) and Hanson *et al.* (1977). This could have been due to the lower light intensity, shorter duration of stress and PEG with lower osmotic strength used in the present investigation.

Glycinebetaine content also varied with seedling growth VPD, but there was no reversal in response by different cultivars under two VPD regimes. Lack of this reversal in the response of these cultivars in glycinebetaine accumulation may simply result from differences in its biosynthesis or metabolism. For example, inhibition of oxidation of glycinebetaine is not a factor controlling stress induced level of glycinebetaine accumulation as it is for proline (Wyn Jones and Storey, 1981; Stewart, 1981);

similarly, ABA had no effect on glycinebetaine metabolism (McDonell *et al.*, 1983), and there is no evidence that carbohydrate plays a role in determining the level of glycinebetaine. Excelsior contained a greater glycinebetaine content than Proctor in both VPD environments. This shows that the correlation between drought resistance of these cultivars and glycinebetaine content does not change with a change in VPD, as did for proline.

CHAPTER V
General Discussion

Water is the major constraint on productivity of temperate cereals in many climates. As the annual rainfall varies, extreme fluctuations in crop yield can occur in dryland farming areas (Aspinall, 1984). There has been considerable work done in the past two decades to identify suitable physiological parameters which confer drought resistance (Turner, 1986). For this purpose, cultivar differences in the accumulation of proline (Singh *et al.*, 1972) and glycinebetaine (Ladyman *et al.*, 1983) have been thought to be of a great value because of the ease in cultivar selection by traditional breeding or genetic manipulation with genetic engineering involving these metabolic traits (Le Rudulier *et al.*, 1984; Hanson *et al.*, 1986; Wyn Jones and Gorham, 1986). However, the accumulation of these two solutes has been postulated to be merely a metabolic breakdown process associated with drought susceptibility (Hanson *et al.*, 1977; Hanson and Nelson, 1978; Hanson, 1980). Although the extent of accumulation of these two solutes in response to stress is acknowledged to be genetically controlled (Hanson *et al.*, 1979; Richard and Thurling, 1979; Grumet *et al.*, 1985; Grumet and Hanson, 1986), there exists a difference in opinion about the significance of solute accumulation, particularly with regard to proline accumulation (Aspinall and Paleg, 1981; Hanson and Hitz, 1982). This follows from the apparent failure of proline to satisfy some of the metabolic criteria for consideration as an adaptive solute and from the conflicting responses of two contrasting barley cultivars in the accumulation of proline (Hanson *et al.*, 1977, 1979) and glycinebetaine (Hanson and Nelson, 1978). These differences in observed response suggest that accumulation of the solutes may vary with environmental and experimental factors including the nature of stress treatment. The objective of the work reported in this thesis was to elucidate this possibility.

The water relations (Kanemasu and Tanner, 1969; Jordan and Ritchie, 1971; Turner and Begg, 1973; McCree, 1974; Morgan, 1984) and solute accumulating ability (Naidu *et al.*, 1987) of plants grown in controlled environment have been found to differ from those of field-grown plants. This has been found to be due to differences in rapidity of stress imposition or other environmental conditions (Gavande and Taylor, 1967; McCree, 1974; Thomas *et al.*, 1976; Jones and Rawson, 1979). The laboratory

experiments reported in this thesis demonstrate that rapidity of stress imposition is a factor which will strongly influence solute accumulation and therefore may account for reported differences between experiments carried out in the field or laboratory. In the present investigation the progressive water stress imposed by withholding water (Experiments 1.3 and 1.4) resulted in a reduction in ψ of 0.4-0.5 MPa per day. Even this rate of stress development is relatively faster than in the field,where a reduction in ψ of 0.1 MPa per day is more normal (Morgan, 1984). In this situation, proline and glycinebetaine dominate the accumulated nitrogen-containing solutes (Tables 4 and 6). The amino acid accumulation pattern found here is similar to that of wheat cultivars water stressed in the field (Drossopoulos *et al.*, 1985) and also the laboratory experiments of Barnett and Naylor (1965; 1966) with *Cynodon dactylon* and of Singh et al. (1973a) with barley seedlings, where water stress was imposed progressively by withholding water. However, the imposition of water stress in laboratory experiments is often by an abrupt osmotic shock, which here resulted in a reduction of ψ of about 1.5 MPa per day (Fig. 6b and Table 5) as in the experiments of Hanson and Tulley (1979). This leads to amide domination of the amino acid pool (Tables 3 and 5) which is reflected even in the phloem sap composition (Hanson and Tulley, 1979). The difference in response of amino acids to rapidity of stress imposition in this study suggests that the abrupt osmotic shock imposed by Hanson and co-workers was the basis of their assertion that proline is not a major amino acid in nitrogen (ammonia) turnover during water stress. The dominant metabolic responses to stress in field situations still remains to be established, but the present results suggest that the involvement of proline and glycinebetaine is more likely.

It has been postulated that plant species with the ability to accumulate a re-utilizable and energy yielding compound such as proline during stress (Table 4) have an advantage (Stewart *et al.*, 1966; Stewart, 1972b). This is particularly so in the rainfed environment due to the possibility of stress relief with intermittent rains. This re-utilizable character of proline is also evident in plants undergoing diurnal variations in leaf water status. Rajagopal *et al.* (1977) showed that in unirrigated field-grown wheat the proline content reaches a maximum at mid day, corresponding closely with the time of minimum

RWC in those leaves. The diurnal range was 2.5 fold and low levels of proline at other times of the day suggested that the proline accumulated at mid day has been re-utilized. It would appear, therefore, that severe water stress imposition by the application of an osmoticum such as PEG which leads to the partial (Hanson *et al.*, 1977) or no reduction (Lewin, 1980) in accumulated proline following stress relief may represent an extreme situation of little relevance to that of plants in the natural habitat. PEG itself does not seem to prevent the re-utilization of proline. Singh *et al.* (1973a, 1973c) and Riazi *et al.* (1985) imposed water stress with PEG and proline levels declined rapidly upon stress relief presumably because the plants in those experiments and in this study (experiment 1.3) were not severely water stressed. The severe treatments in the experiments of Hanson *et al.* (1977) and Lewin (1980) may have damaged cell membranes and the vascular system and so prevented the further metabolism and/or transport of proline.

Leaf proline and glycinebetaine concentrations varied in water stressed barley and wheat seedlings with a range of factors including temperature during seedling growth prior to water stress (Chapter IV, section 2), temperature during parent seed maturation (Chapter IV, section 3), genetic differences arising from the use of seed from different sources (Chapter IV, section 4) and VPD during seedling growth or water stress (Chapter IV, section 5). Although the content of both proline and glycinebetaine varied with these factors, the responses of the two solutes were distinctly different.

Water stressed barley seedlings which experienced low or high temperature during growth (Chapter IV, section 2) showed different abilities to accumulate proline and glycinebetaine; proline responded to low temperature whereas glycinebetaine levels increased in response to both high and low temperature, the response to high temperature being the greater. Accumulation of the two solutes may also be affected differentially by salinity since Chu *et al.* (1976) reported that elevated internal Na level inhibited the rise in proline caused by water stress, whereas no such inhibition of the accumulation of glycinebetaine was found by Wyn Jones and Storey (1981).

Differences in the sensitivities of the biosynthetic pathways of thse two (and other) compatible solutes strongly suggest that there is an evolutionary advantage conferred on

plant species which have the ability to accumulate more than one solute. In the case of high temperatures, for example, it is rare for plants to be subjected to high temperature stress in the absence of concomitant water stress, but it is possible, and, thus, the ability to accumulate both proline and glycinebetaine, rather than just proline, would be useful. Similar factors pertain to a consideration of the interaction of salinity and water stress.

Both solutes (and the other compatible solutes accumulated) have the capacity to protect enzymes maintaining their activity under less favorable conditions, and sustain membrane integrity and function against the effect of environmental stress (Pollard and Wyn Jones, 1979; Paleg *et al.*, 1981, 1984, 1985; Nash *et al.*, 1982; Jolivet *et al.*, 1982, 1983). Protection is afforded against heat (Paleg *et al.*, 1981; Jolivet *et al.*, 1982, 1983), cold (Bornman and Jansson, 1980), salt (Pollard and Wyn Jones, 1979; Manetas *et al.*, 1986), drought (Itai and Paleg, 1982), pH (Paleg *et al.*, 1984), and possibly other stresses, increasing the range of adaptability of plant species to a wide variety of environmental conditions.

Although the biosynthetic pathway of glycinebetaine is less affected than that of proline by external influences such as heat and salt, the susceptibility of proline to very rapid and complete reutilization following the release from stress, confers yet another advantage on the plant able to accumulate both solutes. Not all of the synthetic and energetic reserves funnelled into the accumulation of the solutes are beyond recall, but with the accumulation of a glycinebetaine as well as proline, the plants which form both are better able to approach the onset of a subsequent stress.

In the absence of seed size differences between the seed matured at different temperatures, proline content did not differ whereas glycinebetaine responded to an additional carryover effect of parent temperature (Table 15). This response of glycinebetaine is comparable to the results of Ketellaper (1963) who found that growing plants at an unfavourable temperature resulted in growth reductions due to imbalances in some essential metabolites such as vitamins and amino acids. These imbalances have been thought to be responsible (Ketellapper, 1963) for temperature-induced carryover effects of reduced growth in subsequent generations (Highkin, 1958). In the present

investigation, the carryover effects of parent temperature may be due to the differences in precursor content for the synthesis of glycinebetaine.

A minor genetic difference resulting from the use of two seed sources of barley (cv. Norbet) induced differences in proline content but not in glycinebetaine content.

These distinct differences in the response of proline and glycinebetaine to similar stimuli indicate that the accumulation of these two solutes is activated by different control factors. Although water stress activates these control factors, the environment before or during water stress may have varying effects in different cultivars leading to the same cultivars accumulating different quantities of these two solutes. This possibility was examined with barley cultivars (Chapter IV, sections 3.4 and 5.3). When seedlings were grown from small seed or average-sized seed at a high VPD (1.2KPa), Excelsior accumulated more proline than Proctor as reported by Singh *et al.* (1972). This relationship between these cultivars was reversed (as reported by Hanson *et al.*, 1977) either by the use of large seeds or by growing seedlings at a lower VPD (0.12KPa). The glycinebetaine content of these cultivars did not show such significant reversal in response to seed size or VPD differences. Hanson *et al.* (1977) grew their seedlings at a low VPD (reaching a low value of 0.18 KPa) compared to that likely to be found in the field (about 1.2 KPa) and in the South Australian conditions of Singh *et al.* (1972). Although both groups conducted their experiments under controlled conditions, neither group controlled VPD. The ambient VPD between these two geographical locations show two fold difference (Table 25) and this difference increases even more in some seasons (Grentilli, 1971; Bryson and Hare, 1974). For most of the year VPD in Adelaide is higher than in Lansing. This, then, is the likely cause of the apparent reversal in the capability to accumulate proline of these cultivars in the two studies. Lack of this reversal in the response of these cultivars to accumulate glycinebetaine at different VPD regimes may simply result from differences in its biosynthesis or metabolism.

Table 25: Climatic data* for Adelaide (South Australia) and Lansing (Michigan, USA) during the period of barley grain development to maturity. The values are averages over at least 10 years.

		Temperature (°C)		Precipitation (mm)	Mean vapour pressure deficit (KPa)
		Daily Mean	Extreme (max.)		
Adelaide					
	Sep.	13.4	35.1	49	0.56
	Oct.	16.0	39.4	47	0.84
	Nov.	18.5	45.2	36	1.14
Lansing					
	Jul.	22.1	39.0	66	0.86
	Aug.	21.2	39.0	77	0.72
	Sep.	16.7	36.0	66	0.50

*Adapted from Gentilli (1971) for Adelaide and Bryson and Hare (1974) for Lansing.

In the present investigation, when seedlings were grown from average sized seed the glycinebetaine content of Excelsior, a cultivar well-adapted to the limited soil moisture regimes of South Australia (Finlay and Wilkinson, 1963), was found to be greater than that of Proctor (Fig. 30b), the lesser adapted cultivar. This response of glycinebetaine agrees with the recent suggestion of Hanson and co-workers that this compound is involved in the resistance of a cultivar to environmental stress (Ladyman et al., 1983; Hanson and Grumet, 1985; Grumet *et al.*, 1985; Grumet and Hanson, 1986; Hanson *et al.*, 1986). These recent results of Hanson and co-workers contradict their earlier view that a greater potential of a cultivar to accumulate glycinebetaine is associated with drought susceptibility, as Proctor accumulated more glycinebetaine with a lower leaf ψ than did Excelsior (Hanson and Nelson, 1978). This was similar to the response of proline (Hanson *et al.*, 1977) in their cultivars, and based on these results, they suggested that

cultivar differences in solute accumulation rate depended on the rate at which ψ declines during water stress and, hence, there were no net cultivar differences in proline accumulation potential in barley. Hanson and co-workers compared the proline content at an identical ψ in different cultivars, but the time factor was not considered as Excelsior and Proctor took different times to reach the set ψ. Time is important as the proline content depends not only on ψ but also on the length of exposure to stress (Aspinall and Paleg, 1981) and, hence, the conclusion of Hanson and co-workers is questionable.

The work reported in this thesis also suggests that although proline accumulates in response to a reduction in ψ, this alone is not controlling the level of proline or glycinebetaine in plants. For an identical reduction in ψ (about 1.3 MPa, Table 24b) by both Proctor and Excelsior, the growth environment (VPD) modifies the level of solutes. The earlier work of Singh *et al.* (1972, 1973c) and of Lewin (1980) showed the lack of differences in ψ between these two cultivars, in spite of differences in proline content. Hanson *et al.* (1977) were of opinion that the reason for the lack of differences in ψ between the cultivars in the work of Singh *et al.* (1972) was the use of the whole leaf, which may have had dried portions which obscured the equilibration in the psychrometer chamber. However, this explanation does not seem to hold good as there was no leaf death in the present investigation, and Lewin (1980) measured ψ by pressure chamber thus eliminating the criticism of Hanson *et al.* (1977). Further evidence of cultivar differences in proline accumulating ability at an identical ψ is presented by Lewin and Sparrow (1975), and Aspinall and Paleg (1981). In this latter case, detached leaf segments were floated on PEG to obtain identical leaf water deficit in the various cultivars.

In addition, the use of large seeds to raise seedlings may have contributed to the differences in response of the two barley cultivars in the two studies. Hanson *et al.* (1977) multiplied the seed for their study in a glasshouse and such seed has been reported to be larger than field-grown material (Waldron, 1941). Although glasshouse-grown seed was multiplied in field plots (Hanson *et al.*, 1977), high rainfall (Table 25) during the grain filling stage in this region would have been conducive to the production of large

seed. This would be particularly so in comparision with grain grown with limited rainfall during grain filling as was that used by Singh *et al.* (1972) which was grown in the field in South Australian conditions (Table 25). Although the mean daily temperatures were lower in Adelaide than in Lansing (Table 25), the occurance of extreme temperatures later during ripening, coupled with generally drier conditions, would lead to the production of smaller seed in Adelaide than in Lansing.

Large seed has been favoured for crop production due to the advantage in plant growth and yield over plants from small seed (Brenchley, 1923; Kiesselbach, 1924; Evans and Bhatt, 1977; Lafond and Baker, 1986). If young barley seedlings derive N from seed reserves for proline accumulation, then seedlings grown from large seed must show a greater capacity to accumulate proline than those grown from small seed in all cultivars. However, this was not so in Excelsior which suggests that the N content of the seed interacts with seed size in different cultivars (Phillips and Schlesinger, 1974). This variation in N content may be responsible for cultivars showing different abilities to accumulate proline when seed size was a variable (Fig. 24a).

Another cause for the differences in proline content of the two barley cultivars in the two studies may be the use of genetically different seed of Excelsior by Singh *et al.* (1972) and Hanson *et al.* (1977). The latter group of workers used a selection from the original parent Excelsior used by Singh *et al.* (1972). Such seed, subjected to selection pressure, does show a different potential for proline accumulation (Chapter IV, section 4.2). However it was not possible to ascertain the contribution of this factor to the discrepency, as samples of the seed used by the two groups could not be obtained. If there were no genetic differences between the seed material used by these groups, then individual or combined effects of seed size or VPD differences may account for the differences in the proline accumulating capacity of Excelsior and Proctor (Singh *et al.*, 1972; Hanson *et al.*, 1977). Although the work reported in this thesis examined only a few obvious and possible differences in the environmental conditions between the experiments of these two groups, it by no means guarantees that other environmental factors were not involved.

The future use of these metabolic characters in plant breeding for drought resistance is not certain, although there is considerable *in vivo* and *in vitro* evidence from various sources to support the idea that the accumulation of solutes is positively correlated, due to many physiological advantages, with resistance to various environmental stresses (Paleg *et al.*, 1981, 1984, 1985; Jolivet *et al.*, 1982; Ahmad *et al.*, 1987; Lone *et al.*, 1987). However, this has yet to be proved unequivocally with a whole plant system. The evaluation of the suitability of metabolic characters is limited by the lack of a clear objective measurement of drought resistance. The possible yield advantage of a drought resistant cultivar is a product of many physiological characters. The greater solute accumulating potential of a drought resistant cultivar, for a given reduction in ψ, may represent only a part of the resistance mechanism. However, the possible physiological advantage of this metabolic character of cultivars has been compared with leaf survival in the experiments conducted at the seedling stage under laboratory conditions (Hanson *et al.*, 1977). This analysis has not been extended to study the implication of solute accumulating ability of cultivars to increase crop yield under dry farming situations, as with osmoregulation (Morgan *et al.*, 1986). If this character is proven to have a link with the yield advantage, then it is very well suitable for the selection of parents for breeding at the seedling stage as the method requires only a leaf sample and the rest of the seedling can be retained for breeding purposes. Further, as the work reported in this thesis shows that solute accumulation apart from differing between the cultivars, is greatly affected by environmental factors, the methods and environmental conditions should be standardised to minimise such differences. At this juncture, it is undoubtedly premature to select cultivars based on their high proline accumulating ability in laboratory experiments. It is reasonable to expect, however, that proline accumulating potential, together with glycinebetaine accumulating potential and other physiological parameters (Blum, 1982, Blum and Sullivan, 1986; Turner, 1986), will compliment field selection of cultivars for future cereal breeding programmes.

APPENDICES

APPENDIX 1a

F-values for results in Fig. 24a

Source of variation	Variance Ratio (F-value)
Seed size	0.596
Stress	2450.972**
Cultivar	0.778
Seed size x stress	1.559
Seed size x cultivar	3.596*
Stress x cultivar	0.878
Seed size x stress x cultivar	3.874*

APPENDIX 1b

F-values for results in Fig. 30a

Source of variation	Variance Ratio (F-value)
Growth VPD	21.968*
Stress	765.440**
Cultivar	0.065
Growth VPD x stress	39.249*
Growth VPD x cultivar	3.165*
Stress x cultivar	0.163
Growth VPD x stress x cultivar	3.144*

Significant level * $P \leq 0.05$
** $P \leq 0.01$

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