SENSITIVITY OF GRAMINEACOUS LEAF GROWTH TO ABSCISIC ACID

ITS POTENTIAL IMPORTANCE TO LEAF GROWTH OF PLANTS IN DRYING SOIL

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A thesis submitted to the University of Lancaster for the degree of Doctor of Philosophy

May, 1996

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To Mum, Dad and Helen,

for offshore support

and

To Sarah,

for infinite patience

ABSTRACT

Recent reports have indicated that leaf growth of plants in drying soil can be regulated by chemical signals originating from the roots. A potential signal is the plant hormone abscisic acid, ABA. This thesis aimed to identify sources of variation in the sensitivity of cereal leaf growth to ABA using a detached shoot leaf elongation assay. Preliminary experiments showed that altering the nutrient composition or pH of the feeding solution had no effect on sensitivity. Assay variability resulted in considerable uncertainty over whether observed genotypic differences in sensitivity were real, or reproducible. Increasing the air temperature around the detached shoots greatly increased the sensitivity of leaf growth to ABA. Increased concentrations of ABA in the elongation zone ([EZ-ABA]) of detached shoots seemed to account for this increased sensitivity. When detached shoots were fed sap from droughted plants, sap ABA concentration could not explain the growth inhibitory activity. Measurement of [EZ-ABA] accounted for this "unexplained" growth inhibition. When intact plants were subjected to a slowly developing drought, growth was reduced by 35 % without any increases in [EZ-ABA]. Measurement of leaf water relations and xylem sap pH similarly failed to account for the growth inhibition. The diurnal growth pattern of droughted plants suggested an interaction of water relations with chemical signals. Use of a coleoptile growth assay showed that individual application of mild osmotic stresses or ABA did not inhibit growth. However, osmotic stress and ABA applied together significantly reduced growth. This interaction may be an important mechanism in explaining leaf growth inhibition of droughted plants. It also accounts for the relative (compared to droughted plants) insensitivity of leaf elongation to ABA in the leaf elongation assay.

ACKNOWLEDGEMENTS

I would like to thank Professor Bill Davies for his advice, encouragement and friendship throughout the course of this project.

On a professional level, I would like to thank

- Mr. Steve Palmer for assistance in the experiment described in Section 7.2 and for invaluable criticism.
- Ms. Jane Martin for assistance with the innumerable ABA assays.
- Dr. Damon Berridge for statistical advice.
- Mr. Phil Smith for construction of the growth cabinet used in this thesis.
- Dr. Andrew Sier for the superb drawings in Figures 2.1 and 3.1.
- Messrs H. Latham, J. Wilkinson, P.W. Williams, and W.A. Wood for workshop support.
- my colleagues in Lab B14 (too numerous to mention individually) for their advice, constructive criticism and the friendly lab. atmosphere.

On a personal level, I would like to thank

- my parents and sister Helen for their unfailing encouragement and communication.
- my mates back home, the Bowerham and Lancaster University tennis teams, and my new world of friends in Lancaster for preservation of my sanity.
- Mr Adam Holloway, tennis partner extraordinaire, for services beyond the call of duty in proof reading this thesis.
- Miss Sarah Honour for always being there when needed.

I am especially grateful to my sponsors, the Commonwealth Scholarship Commission, for their financial support and the opportunity to experience life in another country. Thanks also to the staff at The British Council for administering my award.

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LIST OF ABBREVIATIONS

Some non-standard abbreviations used in this thesis

ABA	abscisic acid
ANOVA	analysis of variance
AX	artificial xylem solution
[compound]	concentration of a given compound
cv.	cultivar
cv%	coefficient of variation
DI	distilled water
DW	dry weight
EZ	elongation zone
[EZ-ABA]	concentration of ABA in the bulk (the portion 10 to 40 mm from the crown node) elongation zone
FW	fresh weight
θ	gravimetric soil water content
GA	gibberellic acid
g_s	stomatal conductance
IA	immunoaffinity
IAA	indole acetic acid
leaf X	leaf on the main stem of a cereal shoot, numbered from the base
LER	leaf elongation rate
mM	millimolar
M	molar
MAN	mannitol
MES	2- [N-Morpholino] ethane sulphonic acid
MPa	megapascals
MTW 10.2	Minitab for Windows 10.2
PEG	polyethylene glycol
ψ_g	gravitational potential
ψ_L	leaf water potential
ψ_m	matric potential
ψ_p	turgor
ψ_w	water potential
ψ_π	osmotic potential
RIA	radioimmunoassay
RSER	relative segmental elongation rate
S.E.	standard error
S.D.	standard deviation
SPW 1.0	SigmaPlot for Windows 1.0
T	temperature
TRANS	transpiration
VPD	vapour pressure deficit

CHAPTER 1.

INTRODUCTION

1.1 WHY STUDY DROUGHT EFFECTS ?

In surveying agricultural productivity in the United States, Boyer (1982) concluded that unfavourable physicochemical environments depressed crop yields by 71 %. Of the various stresses faced by plants, drought was shown to be responsible for 41 % of insurance payments to farmers for crop losses in the period 1939 to 1978 (Boyer, 1982). It is likely that similar statistics apply globally. Such statistics lead Kramer (1974) to conclude that "in fertile soils plant growth and yields are reduced more often by water deficits than by any other cause."

1.2 WHY EMPHASISE LEAF GROWTH ?

As noted by Hsiao (1973), drought can affect almost any aspect of plant performance and biochemistry; including cell growth, cell wall and protein synthesis, cell division, enzyme activities, hormone levels, stomatal behaviour, photosynthesis, respiration, phloem translocation, ion uptake, proline and sugar accumulation, xylem cavitation, organ senescence and abscission. Of all these processes, a restriction of leaf growth is among the most sensitive to soil drying (Hsiao, 1973).

Leaf growth greatly affects plant productivity. Monteith (1977) demonstrated a single unifying relationship for a variety of crops where dry matter production increased linearly with the amount of radiation intercepted, this interception being dependent on leaf growth. While the relationship between leaf growth and crop yield is a direct one for forage species, grain crops which are source-limited (i.e. the limitation is dependent on the availability of assimilate to fill the grain) show a relationship between crop Leaf Area Index and grain yield (e.g. Aggarwal and Sinha, 1987).

Therefore the understanding of the regulation of canopy expansion is of great economic importance.

Leaf growth depends on both the supply of new cells and the enlargement of those cells, determined by the rates of cell division and cell expansion respectively. There is evidence that both processes are co-ordinated (Ben-Haj-Salah and Tardieu, 1995). In this thesis, only leaf elongation is considered.

1.3 WHY STUDY THE *GRAMINEAE* ?

The family *Gramineae* (commonly known as the grasses) contains most of the staple food crops of the world such as rice, wheat, corn, oats, barley, and sorghum. Grasses are also the basis of most pastures used for grazing. The economic importance of these crops, and the regulation of their leaf growth under drought, would therefore be impossible to overstate.

The grasses are especially suitable to the study of leaf growth as changes are chiefly in extension growth. This is in contrast to dicotyledons, where growth involves changes in length, width and thickness. Although grass leaves also exhibit changes in width and thickness, such changes are mostly noticeable between leaves of different insertion levels on the main stem. Over the development of a single leaf, the most noticeable single change is in length.

Grasses are also especially amenable to the study of the spatial distribution of growth. While dicotyledons have complex spatial patterns of expansion across their leaf surface (Maksymowych, 1962), the visible portion of the grass leaf (lamina) has actually ceased elongation. Cell expansion in grasses is restricted to a small zone of cells at the base of the leaves. Files of cells are produced by cell division, and expand through the elongation zone. Although the elongation zone is enclosed by the sheaths

of subtending leaves, there are ways of elucidating the spatial patterns of growth in the elongation zone (Section 5.2).

Scientists have long been interested in the effects of plant hormones and ionic solutions on leaf growth. Previous investigations have been hampered by the unavailability of a technique to manipulate chemical concentrations around the expanding cells in a non-destructive manner. The recent development of a leaf elongation assay with detached cereal shoots (Munns, 1992) appeared to offer a means of reliably assaying growth in response to a hormone application, where the concentration of hormone reaching the elongation zone via the transpiration stream was accurately known. For these reasons, the study of gramineacous growth was of practical advantage.

1.4 WHY CONSIDER ABA IN A STUDY OF LEAF GROWTH UNDER DROUGHT ?

1.4.1 The occurrence and importance of chemical signalling in droughted plants

For a plant cell to grow, water in the cell must exert a hydrostatic pressure on a cell wall that is capable of deforming. This hydrostatic pressure (measured in MPa) is known as the cell's turgor pressure or turgor potential or simply turgor (ψ_p). Turgor may be simply expressed as the difference of its component potentials, the water potential (ψ_w) and the osmotic potential (ψ_π), by the equation: $\psi_p = \psi_w - \psi_\pi$ (In actual fact, there are other terms such as ψ_m, the matric potential due to surface tension and ψ_g, the gravitational potential; both of which are important only in certain cases. For these reasons, the simplified version of the equation is usually given.). Water potential is a fundamental measure of the free energy status of water. By convention, ψ_w of a tissue (any organ such as leaf, stem, or root) or cell or subcellular compartment is usually given a negative sign to indicate that water will flow from a

higher ψ_W (closer to 0 MPa) down a chemical potential gradient towards that leaf. The more negative the ψ_W, the greater the chemical potential gradient existing. The osmotic potential of a tissue is dependent on the presence of dissolved substances, which lower the free energy status of that water. As solutes accumulate in a tissue, the value of ψ_π becomes more negative. The measurement of these potentials is central to the field of plant water relations.

The development of the thermocouple psychrometer (Richards and Ogata, 1958; Boyer and Knipling, 1965), and the pressure bomb (Scholander *et al.*, 1965) gave plant physiologists the ability to measure ψ_W and ψ_π, allowing the calculation of ψ_p. The development of the cell pressure probe (Green *et al.*, 1971), and its subsequent use in intact plants (Husken *et al.*, 1978) allowed the direct measurement of ψ_p. The theory and operation of these pieces of equipment have been reviewed extensively (e.g. Boyer, 1969; Boyer and Nonami, 1990) and will not be dealt with here. When carefully applied, estimates of ψ_p using different techniques can yield essentially the same results (e.g. Boyer and Potter, 1973; Nonami *et al.*, 1987).

Use of these devices allowed investigators to describe relationships between leaf elongation rate of grasses and the leaf water potential (ψ_L), when the water relations of plants were altered by changing the water potential around the roots by addition of osmotica (e.g. Acevedo *et al.*, 1971) or allowing the soil to dry out (e.g. Boyer, 1970). These studies confirmed that growth was most rapid at higher values of ψ_L or ψ_p.

The values of ψ_L at which leaf growth ceased was found to vary greatly between studies according to species (Boyer, 1970), the time of day (Chu and McPherson, 1977), the rate of soil drying .(Cutler *et al.*, 1980) and the growth environment of the plants (Watts, 1974). These differences were accounted for by

differences in solute accumulation (and hence ψ_π) during stress. Hence turgor was considered to be the principal driving force for growth.

However, the measurements of ψ_L in grasses had been made on mature, non-expanding tissue. Although the presence of water potential gradients existing between mature and expanding parts of cereal leaves had been suggested by Watts (1974), Michelena and Boyer (1982) showed that perturbed water relations in the lamina of droughted maize plants was not reflected in decreased turgor in the elongation zone. At about the same time, observations were made that stomata, whose movements were also assumed to be dependent on leaf water potential and turgor in the same way as leaf growth was (e.g. Turner, 1974), closed in response to low soil water potential without there being any change in shoot water potential (Bates and Hall, 1981; 1982). Such responses of stomata or leaf growth required that the plants had some mechanism other than leaf turgor for sensing the availability of the water in the soil. Jones (1980) and Cowan (1982) suggested that such a mechanism(s) would involve the transfer of chemical information from the roots to the shoots via the xylem. Such control has been termed non-hydraulic or chemical signalling. This distinguishes it from hydraulic signalling, as discussed above, which represents transmission of reduced soil water availability via changes in the xylem sap tension, which are reflected in lower ψ_Ls.

Since these early reports there has been a proliferation of reports in the literature where limitation of leaf growth or stomatal conductance (g_s) has been attributed to non-hydraulic signalling. This type of information has been collected in response to different plant stresses such as salinity (e.g. Termaat *et al.*, 1985), soil compaction (e.g. Masle and Passioura, 1987) and soil drying (see Appendix 1 for a non-exhaustive list). Researchers have devised a number of means to apparently unequivocally demonstrate the operation of chemical signals, by breaking the link between soil drying and perturbed water relations. The whole plant (or "Passioura-type") pressure chamber grows the plant in a pot to which a balancing pressure can be

applied to the roots to maintain shoots at full turgor, such that xylem sap exudes from a cut surface (Passioura and Munns, 1984). Split root techniques divide the plant root system between 2 or more containers so that some roots are exposed to drying soil (and hence generate a chemical signal) while others remain under well watered conditions to supply the shoot's water requirements. This system is considered to be similar to many field conditions, where drying of the surface soil exposes only a small proportion of the plant's root system to drying soil. This situation has been mimicked by growing plants in large soil columns.

Analysis of the reports in Appendix 1 shows some interesting trends in the pursuit of research on chemical signalling of drought, with respect to the system of soil drying and species used and the physiological processes and putative chemical signals measured (see Table 1.1). It is important to note that a number of very comprehensive field studies have validated the existence of chemical signalling in the field, supporting the more artificial laboratory studies which are characterised by low light intensities and VPDs. Researchers have concentrated on crop species, which is not surprising considering the economic argument. Interestingly, all reports for herbaceous dicotyledons concerned one species (*Helianthus annuus*). A disproportionate number of studies have measured only stomatal conductance; which probably reflects the relative ease of porometric measurements of g_S, as opposed to the relative sensitivities of gas exchange and leaf growth to drought. This bias is reflected in the discussion of the following sections. Of the studies which have sought to identify a potential chemical signal, a majority have measured the plant hormone abscisic acid (ABA) to the exclusion of other signals. This emphasis may simply reflect the relative ease of measurement of ABA using modern immunological techniques, or may be taken as indirect evidence of an important role for ABA in the control of leaf growth and g_S. However, it is necessary to critically examine the physiological evidence in favour of a role for ABA in controlling shoot physiology.

Table 1.1: Analysis of experimental trends in studies of soil drying-induced root to shoot communication. The references and data base used in compiling this Table may be found in Appendix 1.

Experimental Details	Reports
Drying Protocol:	
Whole Plant Pressure Chamber	4
Vertically Split Root System (Two or more Pots)	9
Horizontally Split Root System (Soil Columns or Large Pots)	12
Field Studies	5
Species Used:	
Herbaceous Monocotyledons	16
Leguminous Dicotyledons	5
Herbaceous Dicotyledons	5
Woody Species	6
Physiology Measured:	
Stomatal Conductance / Transpiration only	20
Leaf Elongation Rate only	3
Both	6
Putative Chemical Signals Measured:	
No	11
ABA only	13
ABA and other signals	4
Other only	1

1.4.2 The evidence for ABA as a root signal molecule

The early observations of stomatal closure in response to external ABA treatment (Mittelheuser and Van Steveninck, 1969; Jones and Mansfield, 1970) and the observation that bulk leaf ABA content could increase in detached leaves in response to dehydration (Wright, 1969), specifically in response to changes in leaf water potential (Zabadal, 1974; Wright, 1977) and turgor (Pierce and Raschke, 1980; 1981), meant that ABA has always been implicated in stomatal closure. The supposed mechanism was that drought-induced changes in leaf water potential would liberate ABA from the mesophyll chloroplasts where it is normally sequestered in unstressed

leaves (Heilmann *et al.*, 1980), and that this ABA would move to the guard cells to initiate stomatal closure (see e.g. Mansfield and Davies, 1981).

To fulfil the requirements of a root signal molecule, it was necessary to show that roots were capable of ABA synthesis, that the ABA could move from the roots to the shoots, and be capable of quantitatively affecting shoot physiology.

1.4.2.1 ABA Synthesis in the roots

A number of experimental approaches have been applied to establish that roots can accumulate ABA without import of ABA from the shoot. Experiments with detached roots have shown increased ABA concentration in response to dehydration in air (Milborrow and Robinson, 1973; Walton *et al.*, 1976; Cornish and Zeevart, 1985; Zhang and Davies, 1987b) and in response to osmotic stress (Cornish and Zeevart, 1985; Lachno and Baker, 1986). Experiments with whole plants which have been phloem-girdled, to prevent movement of ABA in the phloem to roots, have also shown increases in root ABA concentration when the root system was subjected to an osmotic stress (Walton *et al.*, 1976) or partially dehydrated in air (Cornish and Zeevart, 1985). However, detailed investigations are necessary to show that enhanced root ABA concentration in intact, droughted plants is a result of synthesis and not redistribution of ABA as a result of perturbed leaf water relations in older leaves. Growth cabinet studies have shown that this is probably the case, with enhanced root ABA concentration in the upper part of the soil profile preceding decreases in leaf water relations and stomatal conductance of droughted plants (Zhang and Davies, 1989a; Trejo and Davies, 1991). A tight relationship between root ABA concentration and soil water content can exist over a drying cycle and for roots from different parts of the soil profile (Zhang and Davies, 1989a). However, this may occur only under growth cabinet conditions when VPDs and water fluxes are low. In contrast to growth cabinet studies, where dehydration of only part of the soil profile increased root and xylem

ABA concentrations to initiate stomatal closure, field studies showed appreciable increases in root ABA concentration only when the whole soil profile was close to depletion (Tardieu *et al.*, 1992a).

1.4.2.2 Transport of ABA in the xylem

Although ABA was detected in xylem sap as early as 1968 (Lenton *et al.*, 1968), indicating its mobility in the plant, the origin of such ABA was uncertain. The experiments of Hoad (1975) suggested that placing the roots under osmotic stress did result in increased xylem sap ABA concentration, but that this ABA originated in the leaves. Recently, computer modelling suggested that a root stress treatment would result in the liberation of ABA normally sequestered in the leaf mesophyll chloroplasts, and that this ABA would move into the phloem to travel to the root and only from there, via the xylem sap to sites of action in the leaf (Slovik *et al.*, 1995).

To test whether ABA could move from a droughted root to the leaf to initiate stomatal closure, Zhang and Davies (1987b) loaded the root system of *Commelina* plants with ABA by overnight immersion of roots in an ABA solution. Some of the leaves were covered in aluminium foil to prevent stomatal opening when the lights came on. Immediately prior to the light coming on, the ABA was removed and the roots allowed to stand in moist air. When the light came on the following morning, transpiring leaves showed stomatal closure; closure being proportional to the accumulation of ABA in the epidermis. This provided clear evidence that ABA was capable of moving from roots, which were not contributing to the transpiration stream, (analogous to roots in dry soil) to the leaves and promoting stomatal closure.

1.4.2.3 Effects of xylem sap ABA on shoot physiology

Relationships between ABA concentration and shoot physiology were initially difficult to demonstrate since stomata could close without an increase in the ABA content of the bulk leaf (e.g. Beardsell and Cohen, 1975; Burschka *et al.*, 1983). It was shown that g_S in split-root maize plants could decline by 30-40 % before changes in leaf ABA content were detected (Blackman and Davies, 1985; Zhang and Davies, 1990a). In other studies, leaf ABA content only increased once the stomata had closed (Trejo and Davies, 1991). However, it was recently shown that xylem sap ABA concentration increased much earlier and to a greater extent than bulk leaf ABA content (Zhang and Davies, 1989b; 1990a). Such studies have also demonstrated correlations between xylem sap ABA concentration and stomatal conductance or leaf growth rate of droughted plants in growth cabinet studies (Zhang and Davies, 1989b; 1990a; 1990b; Khalil and Grace, 1993) and in the field (Tardieu *et al.*, 1992b).

1.4.2.4 Testing the physiological significance of increased xylem ABA

However, as the soil dries, the concentration of all xylem sap constituents will necessarily rise as transpirational fluxes decrease, as noted by Tardieu and Davies (1993). Thus any solute, irrespective of its effect on stomata, should give the negative exponential relationship demonstrated between xylem sap ABA concentration and stomatal conductance. Consequently, more rigorous tests of the physiological significance of ABA are required, such as those formulated by Jacobs (1959), and subsequently modified by Jackson (1987). **Correlation** and **duplication** can indicate a potential regulatory role of a particular hormone in a particular process. In these types of experiments, the link between xylem ABA concentration and stomatal conductance generated by soil drying can be compared with a relationship generated by external application. External application of ABA by root-feeding plants with different concentrations of synthetic ABA (Zhang and Davies, 1990a) or stem injection of ABA

(Tardieu *et al.*, 1993), has demonstrated relationships between stomatal conductance, leaf growth and xylem ABA concentration that were closely comparable to those caused by roots in drying soil. **Deletion and re-instatement** are more certain criteria to test the specificity of hormone action, achieved by manipulating endogenous hormone levels. One type of deletion experiment has employed the split-root system (Gowing *et al.*, 1990) to allow excision of the putative source for extra ABA (i.e. roots in drying soil). The result that partially dried, split-root apple seedlings showed growth recovery when the roots in drying soil were excised provided clear evidence of an inhibitory chemical signal (presumably ABA). Another type of deletion and re-instatement experiment in assessing the role of ABA in controlling stomatal conductance and leaf growth is the use of the immunoaffinity (IA) column to remove ABA from xylem sap, as discussed below.

In this type of experiment, a portion of the xylem sap of interest is passed through an IA column (composed of ABA antibodies) to remove ABA. The antitranspirant activity of the xylem sap can then be tested in the presence (unprocessed sap) and absence (after passage through the IA column) of ABA by feeding the sap to detached leaves and gravimetrically monitoring transpiration (a transpiration bioassay), or incubating epidermal strips in the sap and measuring stomatal aperture (an epidermal strip bioassay). In a similar manner, the growth inhibitory activity of the sap can be tested using a leaf elongation assay (Munns, 1992).

Using a transpiration bioassay system, Munns and King (1988) and Zhang and Davies (1991) obtained contradictory results. Munns and King (1988) demonstrated that ABA added to distilled water, at a concentration comparable to that found in the xylem sap of unwatered wheat plants, failed to reduce transpiration rate in detached leaves by as much as xylem sap from unwatered plants. They concluded that 100 times more ABA than was apparently present in sap of unwatered plants was needed to promote the same effect. They also showed significant antitranspirant activity in xylem

sap after removing ABA. On the basis of these results they proposed that xylem sap of wheat plants contained an unidentified compound with antitranspirant activity. Zhang and Davies (1991), however, demonstrated that removal of ABA, by means of an immunoaffinity column, eliminated the antitranspirant activity of maize xylem sap. Like Munns and King (1988), Trejo (1994) showed that ABA was unable to account for all the antitranspirant activity in *Phaseolus vulgaris* xylem sap.

Recent results of Munns *et al.* (1993) apparently confirmed the existence of another compound with antitranspirant activity, although there is some doubt that their compound occurs *in vivo*, since significant antitranspirant activity only developed with sap storage at -20°C. The antitranspirant activity of freshly collected sap is apparently explicable by ABA concentration, although the variability of the transpiration bioassay sensitivity (up to a 20 % change in antitranspirant activity on particular days) makes it difficult to be certain.

Although 2 of 3 of the immunoaffinity column experiments apparently demonstrated the existence of other antitranspirant activity, it must be noted that such experiments employ fully turgid tissues and thus do not address the important concept of tissue sensitivity (see Section 1.5) which may be crucial in arguing a regulatory role for ABA. Another criticism of such immunoaffinity column experiments is that they may collect sap from relatively stressed plants. It is noted that the effectiveness of ABA diminishes with increasing severity of stress (Correia and Pereira, 1995) and thus xylem sap may contain a compound which is important in maintaining long term stomatal closure and does not regulate stomatal behaviour in the early stages of a drying cycle.

Since Munns' group has so far failed to unequivocally demonstrate the *in vivo* occurrence of the proposed antitranspirant compound, and since other growth inhibitory compounds have not yet been identified (but see Campbell *et al.*, 1995 for a report of a novel growth inhibitory compound in grape vine which seems to be

involved in the regulation of bud dormancy and not extension growth *per se*); there remains a compelling reason to examine the effects of ABA on leaf growth.

1.4.2.5 What "measure" of hormone do cells respond to ?

In testing the effects of a xylem sap component on shoot physiology, it is necessary to determine whether the cells are responding to a concentration or a flux of that hormone. Concentration is the number of molecules in a given volume while flux is the concentration multiplied by the flow rate (of the transpiration stream). The calculation of hormone flux to growing leaf cells is uncertain since it is not known how the vasculature ensures a supply of water to the elongation zone. The elongation zone of leaves may also be a zone where phloem sieve tubes unload, which would also bring hormones to growing cells. The discussion which follows is therefore of most relevance to stomata.

Useful information has chiefly been obtained from laboratory studies, where ABA is fed to detached leaves and shoots. In analysing stomatal responses to a pulse of ABA supplied via the petiole to detached cherry leaves, Gowing *et al.* (1993) found that concentration alone could account for 30 % of the variance, while flux accounted for 74 %. Use of a simple model to calculate apoplastic concentration showed that this variable had the greatest explanatory power, but information from the model cannot be validated in the absence of data on the rate of ABA metabolism. It seems likely that this rate will be highly variable depending on environmental variation such as the water status of the plant.

Trejo *et al.* (1995) were able to change ABA flux at a given ABA concentration fed by altering the temperature and VPD surrounding detached shoots. At a given concentration, a 3-fold variation in ABA flux had no effect on the restriction of conductance; while the same flux achieved by increasing the ABA concentration was

able to further decrease conductance. The same authors also used a microscopic system to vary the ABA flux to detached epidermes. Significantly, stomata were only capable of responding to an increase in flux of an order of magnitude. Field data also indicate that rather limited (less than 2-fold) changes in ABA flux could not account for afternoon stomatal closure (Correia *et al.*, 1995).

However, the importance of hormone concentration alone has been challenged by Trewavas (1981), who showed that in a number of systems, sensitivity of cells to the hormone was at least as important as changes in concentration in determining tissue response. This tenet has been adopted by proponents of chemical signalling of soil drying, with a perceived need for more information about the sensitivity of various systems to the ABA signal (e.g. Davies *et al.*, 1994). It would therefore seem important to investigate the phenomenon of hormone sensitivity.

1.5 WHAT IS SENSITIVITY ?

Since Trewavas' (1981) paper, there has been controversy concerning the nature of plant sensitivity to hormones. While original controversy centred on the importance of sensitivity versus changes in endogenous concentration (Cleland, 1983 vs. Trewavas, 1983), in more recent times the definition of sensitivity has been controversial (Firn, 1986; Weyers *et al.*, 1987).

In Trewavas' (1981) definition, sensitivity was referred to as the competence of the tissue to respond to the hormone. It could be measured by tissue response to a single, usually saturating, hormone concentration. The examples of sensitivity given by Trewavas (1981) measured a given plant parameter (e.g. growth rate) after a pre-determined time. Determination of the dose-response curve was necessary to ensure that a saturating hormone concentration was applied. However, it has been found that different dose-response curves could be obtained with the same system applied with

same species (e.g. the response of coleoptile growth to exogenous IAA) according to whether the initial rate of response or the response after a pre-determined time was measured (Cleland, 1972).

This dichotomy of measurement approach has resulted in researchers on the stomatal response to ABA offering different definitions of sensitivity according to whether the equilibrium response (e.g. Snaith and Mansfield, 1982b) or the initial rate of response (e.g. Weyers *et al.*, 1987; Paterson *et al.*, 1988; Peng and Weyers, 1994) were measured. The latter response has been considered by its proponents to be a more realistic indicator of sensitivity due to the highly variable nature of the physical environment (Weyers *et al.*, 1995). However, there is evidence that ABA can set the magnitude of the response around which perturbations in the physical environment, or other chemical signals, can alter conductance (Wartinger *et al.*, 1990). In such cases, estimates of the equilibrium response may provide a more realistic measure of sensitivity.

Unfortunately, there have been very few attempts to compare data from the two measurements of response. Trejo *et al.* (1995) provide an example where detached shoots were fed the same concentration of ABA at different temperatures. Although lower temperature resulted in a slower (less sensitive) response, transpiration was inhibited to the same percentage of the control shoots (equally sensitive). This example shows that the two measurements of sensitivity may not be directly comparable, and some judgement must be made as to the physiological relevance of each. In this thesis, only equilibrium sensitivity has been studied. This decision was based partially on the perceived physiological relevance of the response; and partially on the fact that ruler measurement of detached leaves in a leaf elongation assay would provide sufficient replication to allow the elucidation of dose-response curves for leaf growth response to ABA under different environmental conditions.

It is necessary to identify the environmental and endogenous variables which have been shown to affect stomatal or leaf growth response to ABA. Table 1.2 presents data from experiments performed with a number of experimentally convenient assay systems and in whole plants. While assay systems may be of use in defining potential interactions, only experiments with whole plants will determine whether the interaction is of any physiological significance. It becomes more difficult to satisfy Jacobs' Rules (Section 1.4.2.4) when a certain hormone concentration produces vastly different effects according to the plant's circumstances. From an ecophysiologists perspective, the presence of such interactions could provide mechanisms to dynamically link the plant and its environment.

It is important to note that such interactions are not ubiquitous and different species may show contrasting behaviour. Tardieu *et al.* (1996) showed that the leaf water potential x ABA interaction that is critical for the control of stomatal behaviour in field-grown maize (Tardieu and Davies, 1992; Tardieu *et al.*, 1993) does not exist in sunflower. Identification of an interaction may depend on the range over which an environmental variable is altered. Rodriguez and Davies (1982) showed that ABA became more effective in an epidermal strip bioassay as the temperature increased from 18 to 22^{o}C. It is therefore not surprising that Tardieu *et al.* (1993) did not observe any difference in stomatal sensitivity to ABA in the field grown plants subjected to a temperature range of 27 to 40^{o}C. It is also important to note that the pretreatment history of the plants can be important (Allan *et al.*, 1994), which may explain apparently conflicting results.

Table 1.2: Factors which may modify the stomatal or leaf growth response to ABA. Experimental systems are as follows: ES - Epidermal strip bioassay, ES(P) - leaf fragments incubated on solutions, then epidermal strips removed to allow stomatal measurement, TB - Transpiration bioassay, LG - Leaf growth assay, WP - Whole plant. NI indicates no interaction between ABA and the variable.

FACTOR		Species	System	Reference
TEMPERATURE				
Assay		*Zea mays*	ES (P)	Rodriguez and Davies, 1982
		Phaseolus vulgaris	TB	Eamus and Wilson, 1983
		Triticum aestivum	TB	Ward and Lawlor, 1990
		Phaseolus vulgaris	TB	Cornic and Ghashghaie, 1991
	NI	*Zea mays*	WP	Tardieu *et al.*, 1993
		Phaseolus vulgaris	TB	Trejo and Davies, 1994
		Bellis perennis	ES	Honour *et al.*, 1995
		Cardamine pratensis	ES	Honour *et al.*, 1995
		Commelina communis	ES	Honour *et al.*, 1995
Pretreatment		*Commelina communis*	ES	Allan *et al.*, 1994
WATER STATUS				
		Commelina communis	ES	Tardieu and Davies, 1992
		Zea mays	WP	Tardieu and Davies, 1992
		Phaseolus vulgaris	TB	Trejo and Davies, 1994
	NI	*Lupinus cosentinii*	WP, TB	Correia and Pereira, 1994; 1995
	NI	*Helianthus annuus*	WP, TB	Tardieu *et al.*, 1996
	NI	*Acacia confusa*	WP, TB	Liang *et al.*, 1996
		Lutsea glutinosa		
WATER STRESS HISTORY				
	NI	*Vicia faba*	ES	Davies, 1978
		Vicia faba	WP	Davies, 1978
		Gossypium hirsutum	TB	Ackerson, 1980
		Commelina communis	ES	Wilson, 1981
		Solanum melongena	TB	Eamus and Narayan, 1989
		Commelina communis	ES	Peng and Weyers, 1994
CO_2		*Xanthium strumarium*	TB	Raschke, 1975
	NI	*Xanthium strumarium*	TB	Mansfield, 1976
		Xanthium strumarium	TB	Dubbe *et al.*, 1978
		Commelina communis	ES	Wilson, 1981
		Solanum melongena	TB	Eamus and Narayan, 1989
ASSAY TYPE				
		Commelina communis	ES ES(P)	Blackman and Davies, 1983
		Commelina communis	ES ES(P) TB	Trejo *et al.*, 1993
LEAF AGE				
		Triticum aestivum	TB	Mittelheuser and van Steveninck, 1969
		Zea mays	ES	Blackman and Davies, 1984b
		Commelina communis	ES	Willmer *et al.*, 1988
		Triticum aestivum	TB	Atkinson *et al.*, 1989

Table 1.2 cont:

FACTOR		Species	System	Reference
GENOTYPE				
		many spp.	TB	Kriedemann *et al.*, 1972
		wheat cultivars	WP, TB	Quarrie, 1983
		maize cultivars	ES	Rodriguez and Davies, 1982
XYLEM SAP COMPOSITION				
		Helianthus annuus	WP	Schurr *et al.*, 1992
NUTRIENT STATUS OF PLANTS				
Nitrogen		*Gossypium hirsutum*	TB	Radin *et al.*, 1982
Phosphorous		*Gossypium hirsutum*	TB	Radin, 1984
INCUBATION SOLUTION COMPOSITION				
Potassium		*Commelina communis*	ES	Wilson *et al.*, 1978
		Commelina communis	ES	Willmer *et al.*, 1978
		Commelina communis	ES	Snaith and Mansfield, 1982b
		Phaseolus vulgaris	LG	Van Volkenburgh and Davies, 1983
		Pisum sativum	ES	Zhang and Davies, 1987a
Calcium		*Commelina communis*	ES	De Silva *et al.*, 1985
Sodium		*Commelina communis*	ES	Jarvis and Mansfield, 1980
pH		*Commelina communis*	ES	Ogunkanmi *et al.*, 1973
	NI	*Valerianella locusta*	ES	Hartung, 1983
		Commelina communis	ES	Paterson *et al.*, 1988
Cytokinin		*Triticum aestivum*	TB	Mittelheuser and van Steveninck, 1969
	NI	*Commelina communis*	ES	Tucker and Mansfield, 1971
		Hordeum vulgare	TB	Cooper *et al.*, 1972
	NI	*Commelina communis*	ES	Ogunkanmi *et al.*, 1973
	NI	*Commelina communis*	ES ES(P)	Blackman and Davies, 1983
		Zea mays	ES	Blackman and Davies, 1983; 1984a; b
IAA	NI	*Commelina communis*	ES	Tucker and Mansfield, 1971
	NI	*Commelina communis*	ES	Ogunkanmi *et al.*, 1973
		Commelina communis	ES	Snaith and Mansfield, 1982a, b
		Vicia faba	ES	Dunleavy and Ladley, 1995
NAA		*Commelina communis*	ES	Snaith and Mansfield, 1984
GA_3	NI	*Commelina communis*	ES	Ogunkanmi *et al.*, 1973
Phenolics		*Commelina communis*	ES	Rai *et al.*, 1986
Phytoalexins		*Commelina communis*	ES	Plumbe and Willmer, 1986

Questions which should be addressed in discerning the relevance of a particular study include "How closely does the assay situation mimic the intact plant ?" and "What concentrations of compound, or what environmental conditions, were used to detect the interaction ?" Some of the interactions described in Table 1.2 have been criticised on the basis that the assay conditions may be non-physiological. For example, Cooper *et al.* (1972) were able to demonstrate that 10^{-4}M kinetin fed to detached

barley leaves in the transpiration stream was able to reverse ABA-induced stomatal closure. It is doubtful whether such an interaction is of relevance to the whole plant as xylem sap cytokinin concentrations in grasses rarely reach 10^{-7}M (Bano *et al.*, 1993). The relevance of some of the interactions described in Table 1.2 is therefore open to debate. Trewavas (1991) has suggested a specific measure of sensitivity, the "control strength", which measures the sensitivity of response to a hormone at the endogenous concentration, thus avoiding problems of non-physiological hormone concentrations. This approach has not seen much use due to potential difficulties of obtaining a dose-response curve at concentrations below the endogenous level, which is necessary to accurately assess the control strength.

Part of the reason for the conflicting nature of the reports in Table 1.2 is our lack of understanding of the mechanisms of sensitivity variation and our failure to measure ABA concentrations at the active site. The differences shown in Table 1.2 may, in some cases, simply be a reflection of different ABA concentrations at the active site under different treatments, and not any actual difference in *absolute sensitivity*. For stomata, the active site is thought to be the ABA concentration at the guard cell apoplast (Gowing *et al.*, 1993). For growing tissues, the epidermis is often considered to be growth limiting (Kutschera, 1992) and thus measurements of ABA in the leaf epidermis may be of relevance. In the absence of measurements of ABA at the active site, the differences in Table 1.2 are best thought of as differences in *apparent sensitivity*.

A good example of the difference between *apparent* and *absolute* sensitivity is provided by Trejo *et al.* (1993). Different assay techniques provided different dose-response curves for ABA-induced stomatal closure in the same plants. When isolated epidermal strips were incubated on 10^{-6}M ABA, stomata closed by 76 % while incubation of leaf pieces on the same concentration closed stomata by only 13 %, indicating a difference in *apparent* sensitivity. The difference in the degree of closure

was accounted for by measurement of ABA in the epidermis; indicating no change in the *absolute* sensitivity of stomatal response to ABA. Given this result, it is surprising that the plethora of ABA interactions identified in epidermal strips of *Commelina communis* have not been pursued in transpiration bioassays of the same species where the epidermis can be removed at the end of an assay for analysis of epidermal ABA concentration. Such studies would add validity to the epidermal strip work, by reproducing the interaction in the more realistic situation where the mesophyll can exert a controlling influence over epidermal ABA accumulation. Assays using isolated portions of tissue, while valuable in assessing sensitivity variation in the absence of the external factors (such as cell to cell communication), may be of limited relevance in assessing responses of intact plants.

Harris *et al.* (1988) have demonstrated the ability to measure ABA in individual guard cells pairs using a very sensitive enzyme-linked immunosorbent assay, allied to single cell methodology. Combination of this measurement technology with a microscopic system of measuring the sensitivity to ABA of individual stomata (McAinsh *et al.*, 1990; Trejo *et al.*, 1995) may go some way toward explaining the large variability in responses of individual stomata to external stimuli (Spence, 1987) if a relationship exists between guard cell ABA concentration and stomatal response. However, it is still likely that cells with identical ABA concentrations may show altered sensitivity to ABA due to other endogenous factors (such as those listed in Table 1.2).

Disregarding potential problems of accurately measuring ABA concentration at the active site, it should be recognised that signal transduction into a physiological response involves the interaction of a hormone with a receptor (Weyers *et al.*, 1987). The initial observation of ABA-binding proteins on the guard cell plasmalemma (Hornberg and Weiler, 1984) has not yet been repeated. Positive identification of the ABA receptor(s) and their quantification on a cellular or tissue basis may allow the calculation of absolute sensitivity on a "response per [ABA] at the active site per

number of hormone receptors" basis. Such a definition, although purely hypothetical at present, may further contribute to the distinction between apparent and absolute sensitivity.

Despite the cautions involved in the interpretation of results which purport to show differences in the sensitivity of plant response to ABA and the difficulties in distinguishing absolute changes in sensitivity from apparent ones, it is clear that changes in sensitivity do occur. In some cases, such changes may be of more relevance in controlling plant response than actual changes in ABA concentration.

1.6 ABA AND LEAF GROWTH

Recent research has shown that the dose-response curve for leaf growth of maize plants fed ABA hydroponically in a nutrient solution (Zhang and Davies, 1990a) was practically identical to that generated by withholding water (Zhang and Davies, 1990b), indicating a possible regulatory role for ABA in controlling leaf growth of droughted plants. However, Munns (1992) obtained contradictory results with a leaf elongation assay. Xylem sap collected from droughted barley plants contained $2\text{-}8 \times 10^{-8}$M ABA, and inhibited growth by 60 %. For exogenous ABA to inhibit leaf growth to the same extent, 10^{-5}M ABA was required, indicating that xylem ABA was not regulating leaf growth in barley. This discrepancy may be rationalised on the basis of sensitivity differences between the two experimental systems employed in the two studies. In view of this contradictory evidence, it is not surprising that a recent review (Munns and Sharp, 1993) called for a greater research effort in this area.

An examination of Table 1.1 indicates that the role of chemical signals in controlling leaf growth has historically been less-studied than in the control of stomatal behaviour. The paucity of leaf growth sensitivity data in Table 1.2 identifies an area of research that is in clear need of addressing. A possible reason for this paucity has

probably been the lack of a suitable assay technique, since the response of leaf growth to ABA in disc bioassays may be complicated by wounding effects. The recent development of a detached cereal shoot leaf elongation assay (Munns, 1992), which isolates the area of excision from the leaf elongation zone to avoid wounding responses, provides a tool to allow the systematic identification of variables affecting the response of leaf growth to ABA.

The aim of this thesis was to identify variation in the sensitivity of the leaf growth response to ABA, and assess its relevance in the control of leaf growth in droughted plants. Initial experiments considered the possible variation in leaf growth response to ABA induced by different nutrient ions and pH (Chapter 2), genotypes (Chapter 3) and temperature (Chapter 4). The possibility that temperature-induced variation in ABA response was a result of differences in ABA concentration at the active site was assessed by measurement of ABA in the elongation zone (Chapter 5). The response of leaf growth to actual xylem sap was considered in Chapter 6. The measurement of ABA in the elongation zone allowed a comparison of ABA response in the detached shoots with that of intact plants of a similar age which were subjected to drought (Chapter 7). The small size of plants used in the soil drying experiments precluded comparison on the basis of xylem sap ABA concentrations. The results of Chapter 7 showed differences in the sensitivity of detached shoots and intact plants to ABA. In an attempt to reconcile these differences, a coleoptile growth assay was used to examine any interaction of water potential and ABA (Chapter 8). The relevance of these changes in sensitivity to the leaf elongation response of whole plants to drought are discussed.

CHAPTER 2.

RESPONSE OF LEAF GROWTH AND TRANSPIRATION TO ABA IN A LEAF ELONGATION ASSAY

2.1 INTRODUCTION

Traditional methods of chemical application of ABA, such as spraying onto leaves (Jones and Mansfield, 1970) or injection of ABA into the stem (Quarrie and Jones, 1977) are limited in that hormone concentrations at the active site(s) are not known. Although the active site(s) for leaf growth would appear to be the cell walls (since ABA can decrease extensibility - Van Volkenburgh and Davies, 1983), correlations have been demonstrated between xylem sap [ABA] and leaf growth in maize (Zhang and Davies, 1990a; b). Presumably a proportion of the xylem sap must reach the elongating cells for such a correlation to be demonstrated. Van Volkenburgh and Davies (1983) incubated discs of *Phaseolus* in ABA solutions or fed ABA into the transpiration stream of detached shoots. The former method was better able to determine the response, presumably since the ABA could penetrate the wound sites and bath the elongating cells. However, interpretation of the growth response in leaf discs may be complicated by wounding responses and uncertainty of penetration of the hormone into the tissue (Pratt and Matthews, 1971). Bunce (1990) was able to manipulate ABA concentrations in the transpiration stream of intact *Glycine* plants by syringe injection into the petiole. In young cereal shoots, this approach would not be feasible since the elongation zone is at the base of the stem. Injection in this zone may result in wounding, thus syringe injection of ABA would need to be into the roots.

Recently, Munns (1992) reported a novel method of assaying detached cereal shoots for leaf growth response to plant growth regulators. By feeding solutions through the cut sub-crown internode, the concentration of ABA in the transpiration stream is precisely known. Since plants are assayed at the same stage of development,

the assay is particularly suited to comparing species differences, as it is possible to avoid the age-related changes in ABA sensitivity that can occur in stomata from leaves of different ages (Blackman and Davies, 1984b; Atkinson *et al.*, 1989). Another advantage is that both growth and transpiration can be measured in the same plants, allowing comparisons to be made of the sensitivity of different physiological processes to the same hormone.

To the author's knowledge, the leaf elongation assay (Munns, 1990; 1992) has not seen use in other laboratories. This is in contrast to other bioassay systems used to evaluate stomatal responses to ABA, such as the use of epidermal peels (Willmer and Mansfield, 1969; Tucker and Mansfield, 1971; Ogunkanmi *et al.*, 1973) and detached leaves (Mittelheuser and van Steveninck, 1969; Cummins *et al.*, 1971; Kriedemann *et al.*, 1972), which have seen extensive use in the *circa* 25 years since their development.

Munns (1992) gives several criteria to support the use of the assay:

1. detached shoots fed a control solution are able to maintain a constant leaf elongation rate (as do intact plants) over the 24 hour period of the assay.
2. leaf growth in the detached shoot responds in the expected way to the plant hormones GA (growth promoter) and ABA (growth inhibitor).
3. there is a negative log-linear relationship between ABA concentration and leaf growth inhibition, as found in other bioassay systems.

These findings were re-examined here. The response of leaf growth to temperature and of transpiration to ABA treatment were also assessed to further validate the leaf elongation assay technique. Additionally, the effect of altering the chemical composition of the feeding solution on the sensitivity of leaf growth to ABA was examined.

2.2 MATERIALS AND METHODS

Seeds were sown in pots containing a commercial potting compost (Sinclair Horticultural Products, Lincoln, England). Seeds were sown 6 cm below the surface of the compost to allow the development of a sub-crown internode. Plants were well watered every day and raised in a growth cabinet, with temperature maxima varying between 22-29^{o}C and minima between 9-16^{o}C and a light intensity at plant height of 200-300 µmol m^{-2} s^{-1}. Assays were routinely performed with various commercially available barley (*Hordeum vulgare* L.) cultivars (Klaxon, Firefly and Hanna). The GA sensitivity tests employed spring wheat (*Triticum aestivum* L. cv. RL 4137 and cv. Highbury). The latter contains the *Rht 1* allele for partial GA insensitivity.

The plants were excavated when the third leaf (all leaves specifically referred to in this thesis are on the main stem) was in its exponential phase of growth. The assay was performed at this growth stage since the sub-crown internode is still expanding when leaf 1 is expanding (which may result in wounding effects); and when leaf 4 was expanding, nodal roots were budding from the crown, which may have contributed growth regulators to the transpiration stream (Munns, 1992). Shoots can be assayed when leaf 2 is expanding (Munns, 1992), but the growth rate was reduced (compared to leaf 3) at a given temperature (data not shown).

Prior to excavation, the plants were placed in the dark for 15 minutes to reduce transpiration, reducing the likelihood of gas emboli entering the xylem vessels when cut. Plants were excavated *circa* 2 hours into the photoperiod. The roots were briefly washed to remove adhering compost then the coleoptile was stripped away. Removing the coleoptile had no significant effect on the subsequent growth of control shoots (data not shown) in 3 separate experiments.

The stems were cut in distilled water approximately 2 cm below the crown (node). Development of the sub-crown internode was essential. Shoots excised from plants without the internode showed a rapid reduction of growth, presumably due to wounding in the leaf elongation zone, or submergence of the crown in the feeding solution resulting in oxygen deficiency (Munns, 1992). The detached shoots were then transferred to vials containing either distilled water (DI) or an artificial xylem (AX) solution (ionic composition in mM: K^+, 4; NO_3^-, 4.5; Ca^{++}, 0.5; Mg^{++}, 0.5; SO_4^{++}, 0.5; Na^+, 0.2; Cl^-, 0.7) as a control or a treatment of ABA (Lancaster Synthesis, Morecambe, England) made up in the appropriate solution. Concentrations of ABA were made up for the active (+) enantiomer. Gibberellic acid (GA_3) (Sigma, Poole, Dorset, England) solutions were made up in artificial xylem solution. The pH of the solutions was not adjusted except in experiments specifically designed to examine the effect of pH on the ABA response (Section 2.3.5). This employed the same artificial xylem solution, except for the addition of 1 mM KH_2PO_4 and 1 mM K_2HPO_4. The pH of the solution was then adjusted by the dropwise addition of either 1 M HCl or 1 M KOH. The detached shoots were supported in the vials by 5 mL disposable pipette tubes as shown in Figure 2.1. A minimum of 5 replicate shoots per treatment were used.

The vials were transferred to a growth cabinet with a light intensity at plant height of 200-300 $\mu mol\ m^{-2}\ s^{-1}$. During the course of the assays, air temperature and relative humidity were monitored using a Solexpress SE-100 sensor (Solexpress Ltd., Astley, England). Soil temperature (at crown-node depth for intact plants not used in leaf elongation assays) was monitored using a mercury immersion thermometer. Temperatures and relative humidities were recorded at the times of leaf measurement and averaged over the course of the assay. Temperatures did not vary by more than 3^oC during each assay.

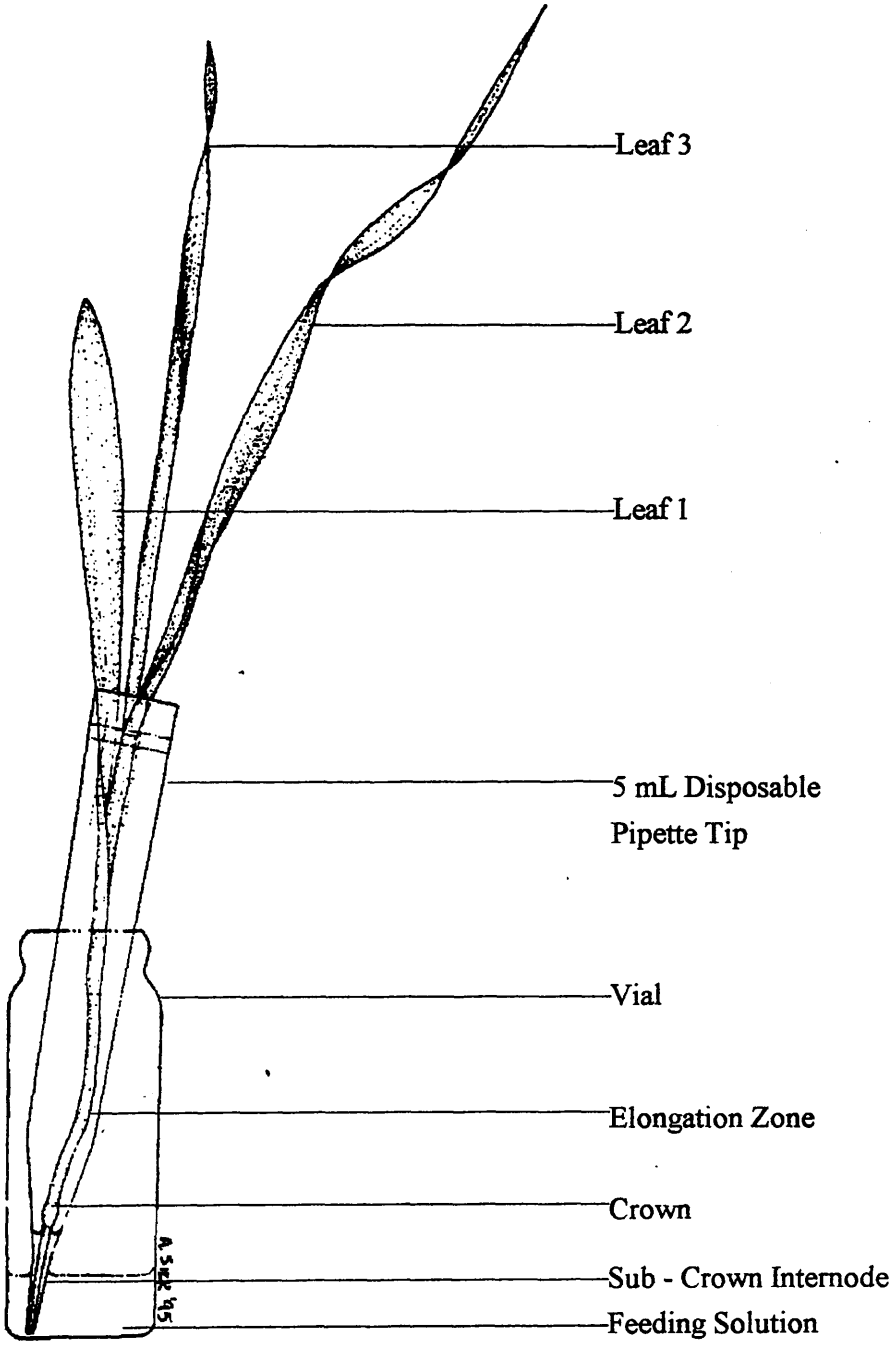

Figure 2.1: Detached barley (*Hordeum vulgare* L cv. Klaxon) shoot used for leaf elongation assays (0.9 x actual size). Drawing by Andrew Sier.

The length of leaf 3 was measured, with a piece of graph paper photocopied onto acetate, at intervals over a 10 hour period. Average leaf elongation rates (LER) in mm h^{-1} were calculated. The effectiveness of ABA in inhibiting leaf growth can be expressed as a percentage of control shoots (= LER of ABA-fed shoot / mean LER of control shoots x 100) at each measurement period (e.g. Figure 2.2b), or combined for all measurements taken during the steady-state phase of leaf growth (or transpiration) inhibition to generate a dose-response curve (e.g. Figures 2.4b, 2.5b).

Water loss was measured by placing 6 vials per treatment on an electronic balance (Precisa 125A, PAG Oerlikon, Zurich, Switzerland) at 1 hour intervals. At the end of the assay, leaf area was determined using a planimeter (LI 3100, Li-Cor Inc., Lincoln, NE, USA). Water loss per unit leaf area (transpiration) was calculated, after subtracting losses from blank vials (without a detached shoot), in mmol m^{-2} s^{-1}.

Routinely, less than 5 % of detached shoots wilted over the course of an assay, although certain batches showed higher percentages. Given this generally low percentage, the water potential of the shoots was not measured. It was assumed that cutting the detached shoots under water had released xylem tension and that the shoots would be at full turgor.

Means were discriminated using unpaired t-tests in SigmaPlot for Windows Version 1.0 - SPW 1.0 (Jandel Scientific, Erkrath, Germany). The significance of the linear regression of barley LER on temperature was tested in MINITAB for Windows 10 - MTW 10.2 (Minitab Inc., PA, USA). Some data were subjected to analysis of variance (see Appendix 2) using GENSTAT 5 (Rothamsted Experimental Station, Harpenden, England) for all measurement periods.

2.3 RESULTS AND DISCUSSION

2.3.1 Response of leaf growth to ABA in different ionic solutions

The effect of different feeding solutions on the growth of the detached shoots (distilled water- DI or artificial xylem solution - AX) was tested (Figure 2.2a). Control (not supplied with ABA) shoots showed a 25-30 % increase in LER over the first 4 hours of the assay. This was a common feature in many leaf elongation assays, although the magnitude of the increase was highly variable (compare Figures 2.2a, 2.3a). This response may be a recovery from any stresses imposed by excavation of the seedlings. A stable maximum LER was attained by control shoots between 4 and 8 hours. Although statistical comparisons (Student's unpaired t-test, $P > 0.05$) revealed no difference in LER between AX-fed and DI-fed controls at any time (as shown by Munns, 1992); the LER of AX-fed shoots was consistently 10 % higher than the LER of DI-fed shoots over the first 8 hours of the assay. Stimulation of growth by a dilute ion solution (10 mM KCl) has been previously reported in a leaf disc bioassay system (Van Volkenburgh and Davies, 1983). By 10 hours, a 35 % (DI) or 20 % (AX) decline in LER, relative to the stable maximum LER, had occurred. This decline was greater in DI-fed shoots, as observed in 2 subsequent replications of the same experiment (data not shown). For this reason, an artificial xylem solution was routinely used in all subsequent assays.

There was no change in LER of shoots fed 10^{-6}M ABA (compared to control shoots) after 2 hours. By 4 hours, a 50-55 % reduction in LER had occurred, which was maintained for the duration of the assay. The timing of the steady-state phase of leaf growth inhibition for 10^{-6}M ABA was consistent with Munns (1992).

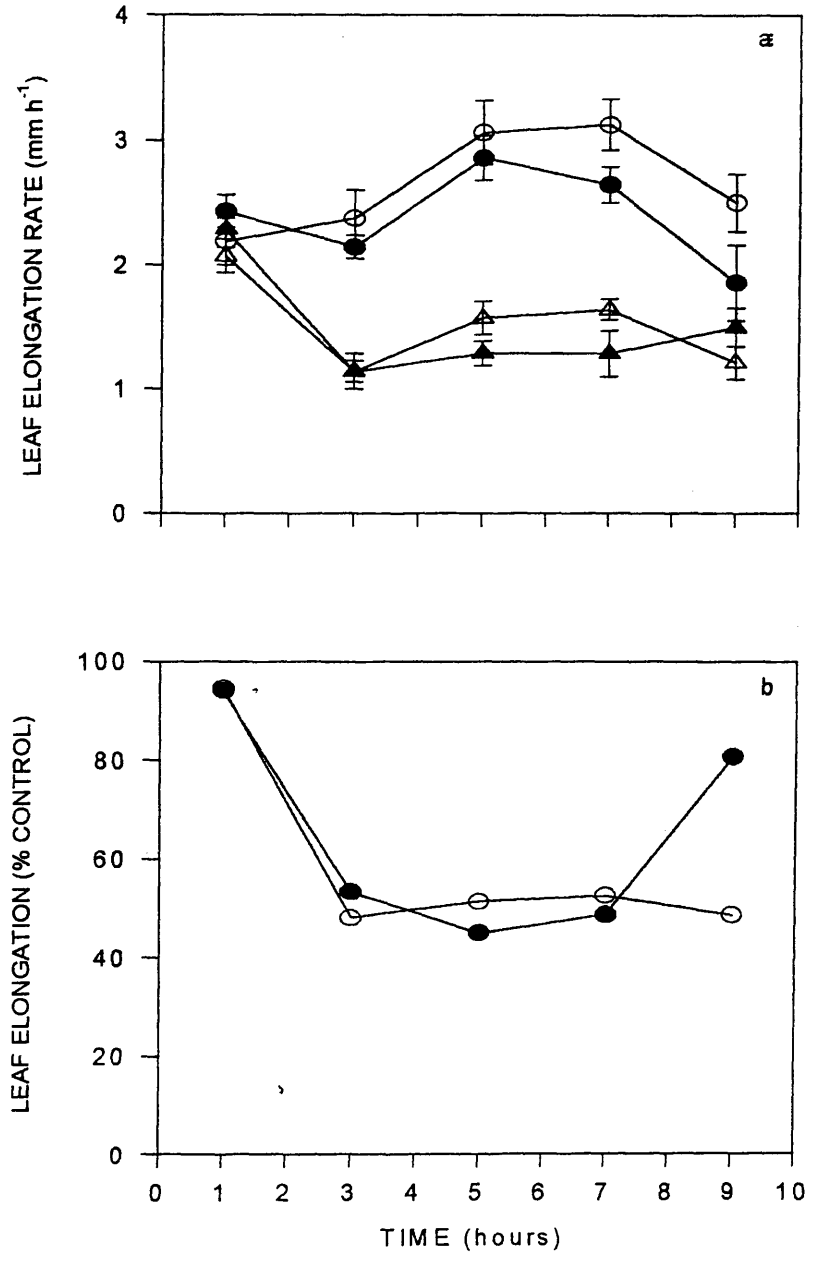

Figure 2.2: Effect of different feeding solutions on leaf elongation rate of detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots of maintained at 25°C (a). Treatments were 10^{-6}M ABA dissolved in distilled water (▲) or artificial xylem solution (Δ) while controls were distilled water (●) or artificial xylem solution (○). Points are means ± S.E. of at least 7 shoots. (b) Leaf elongation of ABA-treated shoots expressed as a percentage of the controls for distilled water (●) or artificial xylem solution (○). Error bars have been omitted for clarity.

Expressing the results as a percentage of the controls (Figure 2.2b) revealed the attainment of a steady-state level of leaf growth inhibition after 4 hours. The decline in LER of the DW-fed controls resulted in a spurious value for the inhibition caused by ABA at 9 hours. When this point was disregarded, it was apparent that adding a dilute solution of ions to the feeding solution did not affect the response to ABA. This was supported by analysis of variance, which showed no nutrient x ABA interaction (see Appendix 2). This finding is in contrast to other workers who have found that incubation of epidermal strips in ions such as K^+ (Wilson *et al.*, 1978; Snaith and Mansfield, 1982b), Na^+ (Jarvis and Mansfield, 1980) and Ca^{++} (De Silva *et al.*, 1985) can increase (Ca^{++}) or decrease (K^+, Na^+) stomatal sensitivity to ABA. Similarly, Van Volkenburgh and Davies (1983) found that the inhibitory effect of ABA on leaf disc growth was completely reversed when the discs were incubated in 50 mM KCl. These observations were given some support in the whole plant study by Schurr *et al.* (1992), who found significant correlations between stomatal sensitivity to ABA and concentrations of NO_3^- and Ca^{++} in the xylem sap of droughted sunflower plants. However, transpiration bioassays have shown no difference in stomatal response to ABA when leaves were incubated in either distilled water or 10 mM KNO_3 (Munns and King, 1988). Therefore the results of Figure 2.2b, showing no difference in leaf elongation response to ABA in distilled water and a composite nutrient solution, are not entirely surprising. These results were confirmed with solutions of 0, 5 and 10 mM KCl and KNO_3, which also showed no interaction between nutrient concentration and leaf elongation response to ABA (data not shown). When 50 mM KCl was substituted as a feeding solution in the leaf elongation assay, transpiration was inhibited by 30 % in comparison to shoots fed deionised water (data not shown), presumably due to osmotic effects. For this reason, the possibility of a KCl x ABA interaction affecting leaf growth was not pursued.

However, such assays have employed tissues detached from plants grown under optimum nutrient supply. The stomata of leaves detached from nutrient stressed plants (Radin *et al.*, 1982; Radin, 1984) are more sensitive to ABA; however the effect of different nutrient solutions on the leaf elongation response of such leaves to ABA has not been assessed. It is surmised that differences in stomatal response to ABA in epidermal peels in the presence of individual ions may be the result of an unrealistic assay situation; since feeding identical solutions via the transpiration stream, where the mesophyll has the possibility of controlling the flow of ions to the epidermis, elicits no difference in ABA response between distilled water and ion solutions (Munns and King, 1988). The correlations described in the study of Schurr *et al.* (1992) are believed to be merely correlations since changes in stomatal sensitivity will occur over the course of a drying cycle and could be related to an increase or decrease of any one of a number of xylem sap components (Gollan *et al.*, 1992). In the absence of bioassay evidence to confirm the conclusions of the whole plant study, the interactions between specific nutrient ions and stomatal sensitivity to ABA, as demonstrated by Schurr *et al.* (1992), are best treated as correlations, and not cause and effect.

The possibility that the initial increase in LER (25-30 % over the first 4 hours) of control detached shoots (Figure 2.2a) may have affected the response to ABA was tested by supplying ABA to treatment shoots at either 0 hours (usual assay procedure) or after 4 hours when leaves had attained their maximum LER. No differences in the response to ABA (expressed as a percentage of control values) were demonstrated (data not shown). In all further experiments, ABA was supplied at 0 hours.

In another test of whether the assay procedure affected the response of leaf growth to ABA, two assays were conducted using the same batch of plants, but excavation of 2 separate groups of plants was separated by 6 hours. No differences in the response to ABA (expressed as a percentage of control values) were demonstrated (data not shown), indicating that levels of carbohydrate in the growing zone (assumed

to be much higher 6 hours further into the photoperiod) did not affect the ABA response. In all further experiments, plants were excavated *circa* 2 hours into the photoperiod.

2.3.2 Response of leaf growth to temperature in intact and detached shoots

The increase (first 6 hours) and subsequent decrease (last 2 hours of the assay) of LER in control detached shoots seen in Figure 2.2a (but not apparent in Figure 2.3a) is very different from the response of intact plants over a similar measurement period which show a reduced, though stable, LER (Figure 2.3a). The reduced LER of soil-grown plants may be explained in terms of the differences in the meristem temperatures experienced, since the meristem is the site of temperature perception for leaf growth (Watts, 1972; 1974). Intact plants are expected to have a meristem temperature equivalent to soil temperature while the meristem temperature of detached shoots was assumed to be that of air temperature. A highly significant ($P<0.001$) linear regression ($LER = -0.65 + 0.132T$, $r^2 = 0.85$, d.f. = 27) described the relationship between the stable maximum LER of detached shoots and average air temperature during leaf elongation assays (Figure 2.3b), which suggested that there was close coupling between air and meristem temperature under the assay conditions. Although fitting a second order regression gave an r^2 of 0.91, a linear relationship has been favoured by most investigators (Gallagher and Biscoe, 1979; Gastal *et al.*, 1992; Ben-Haj-Salah and Tardieu, 1995). The non-linearity in the relationship for the detached shoots was noticed only for temperatures of *circa* 25°C, and perhaps indicates the development of stress in the shoots. However, this appears to be a normal response also in intact plants, which commonly show a reduction in leaf growth above a temperature optimum (e.g. Watts, 1972; Ben-Haj-Salah and Tardieu, 1995). It is difficult to know whether this is in response to temperature *per se*, or in response to the high VPDs which accompany high temperature (Squire *et al.*, 1983). Watts (1972) accounted for a high

temperature-induced growth reduction in terms of water loss exceeding water uptake, which induced low water potential in the elongating cells and restricted growth.

Intact plants showed the same LER response to (soil) temperature (Figure 2.3b) as did detached shoots. Although more sophisticated temperature measurement procedures (such as the use of hypodermic thermocouples to allow precise recording of meristem temperature) could have been employed, it is clear that detached shoots are capable of responding to temperature similarly to intact plants, further evidence of the validity of the leaf elongation assay procedure.

2.3.3 Response of leaf growth to GA_3

Table 2.1 shows the growth response of two different wheat cultivars (cv.s RL4137 and Highbury), and a barley cultivar (cv. Firefly) to 10^{-5}M GA_3. The results are very similar to those of Munns (1992), who found that detached wheat and barley shoots in a leaf elongation assay increased their growth by about 30 % when supplied with 10^{-5}M GA_3; with the exception of cultivars containing the reduced-height gene (*Rht 1*), which show partial insensitivity to GA_3 and are unresponsive to external GA_3 application. Table 2.1 shows that the response of cereal shoots that do not contain *Rht 1* appeared to be strongly cultivar specific, although growth of Firefly shoots at higher temperature may have produced the greater growth promotion than RL4137, since the effect of endogenous GA seems to be temperature dependent (Stoddart and Lloyd, 1986).

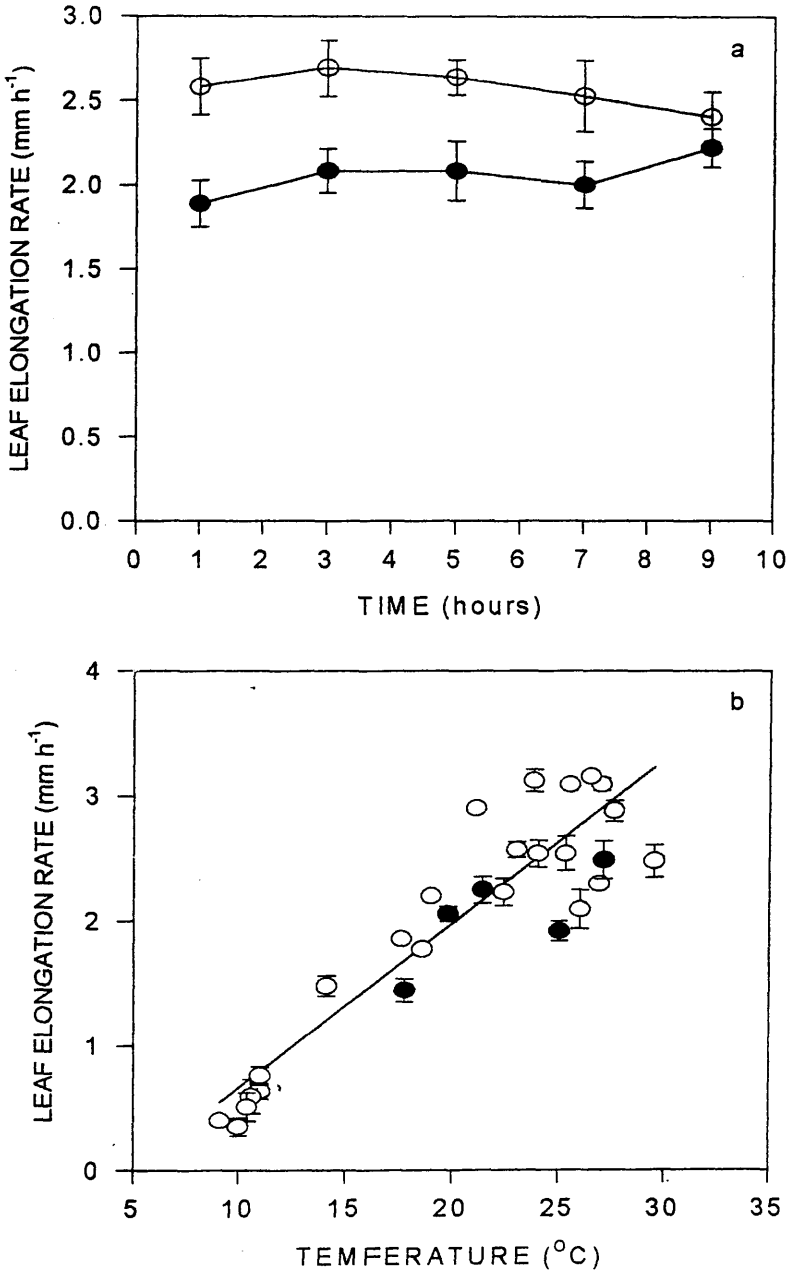

Figure 2.3: Leaf elongation rate of detached shoots (○) and intact plants (●) of barley (*Hordeum vulgare* L. cv. Klaxon) during a leaf elongation assay conducted at 23°C (a) and plotted as a function of air temperature (○) and soil temperature (●) (b). Linear regression in (b) fitted to all data points in SPW 1.0. Points in (a) are means ± S.E. of 9 shoots, while points in (b) are means ± S.E. of at least 24 measurements taken at least 4 times during an assay.

Table 2.1: Leaf elongation rate of different wheat and barley cultivars supplied with 10^{-5}M GA_3. Data are means ± S.E. of at least 16 measurements taken during steady-state phase of growth promotion (3-5 hours after supplying GA_3).

Cultivar	Temperature (°C)	Leaf elongation Rate (mm h^{-1}) Control	10^{-5}M GA_3	Percentage of Control
Highbury (*Rht 1*)	23	2.89 ± 0.19	2.91 ± 0.17	103
RL 4137	18	1.81 ± 0.11	2.09 ± 0.11	115
Firefly	27	2.47 ± 0.12	3.42 ± 0.11	139

2.3.4 Response of leaf growth and transpiration to a range of ABA concentrations

The response of LER and transpiration to a range of ABA concentrations was examined (Figures 2.4, 2.5). Feeding ABA significantly ($P<0.10$) reduced LER within 2 hours at all concentrations tested in Figure 2.4a. This result was unexpected considering the data in Figure 2.2a showed that 10^{-6}M ABA does not reduce LER after 2 hours. This inconsistency is an example of the day-to-day variability inherent in the assay. A relatively stable LER (unique according to concentration) was reached after 4 hours, with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}M ABA reducing LER by 11 %, 24 %, 58 % and 77 % respectively. All of these leaf growth reductions were significant with the exception of 10^{-8}M ABA. The sensitivity of the measurement technique in detecting the onset of the ABA response (within 2 hours) in the leaf elongation assay is similar to that in the leaf disc bioassay system of Van Volkenburgh and Davies (1983).

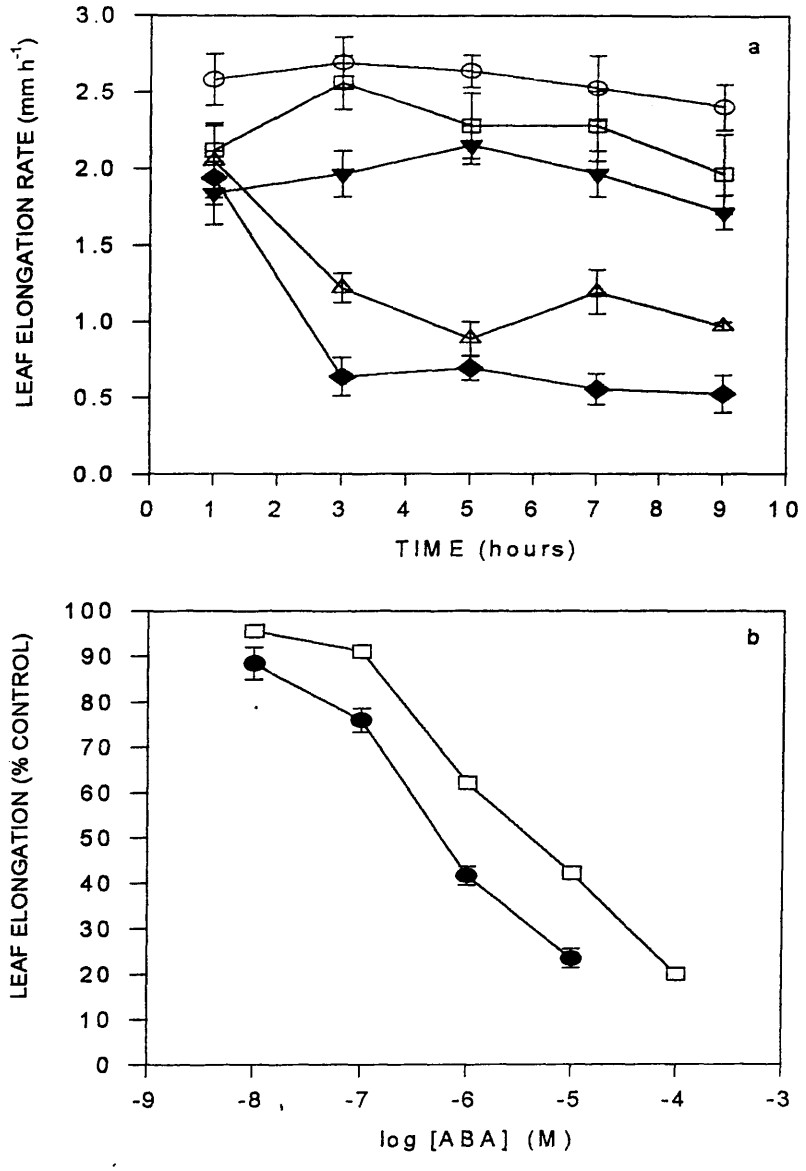

Figure 2.4a: Leaf elongation rate of detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots maintained at 23°C and supplied with artificial xylem solution (○), 10^{-8}M (□), 10^{-7}M (▼), 10^{-6}M (Δ) and 10^{-5}M (◆) ABA. Points are means ± S.E. of at least 8 shoots. (b) Dose-response curve generated from data in (a). Data (●) are expressed as a percentage of controls and are means ± S.E. of at least 32 measurements taken during the steady-state phase of leaf growth inhibition (3-9 hours after supplying ABA). The dose-response curve for detached shoots of *Triticum aestivum* L. cv. Egret maintained at 18°C (□) (from Munns, 1992) is included for comparison.

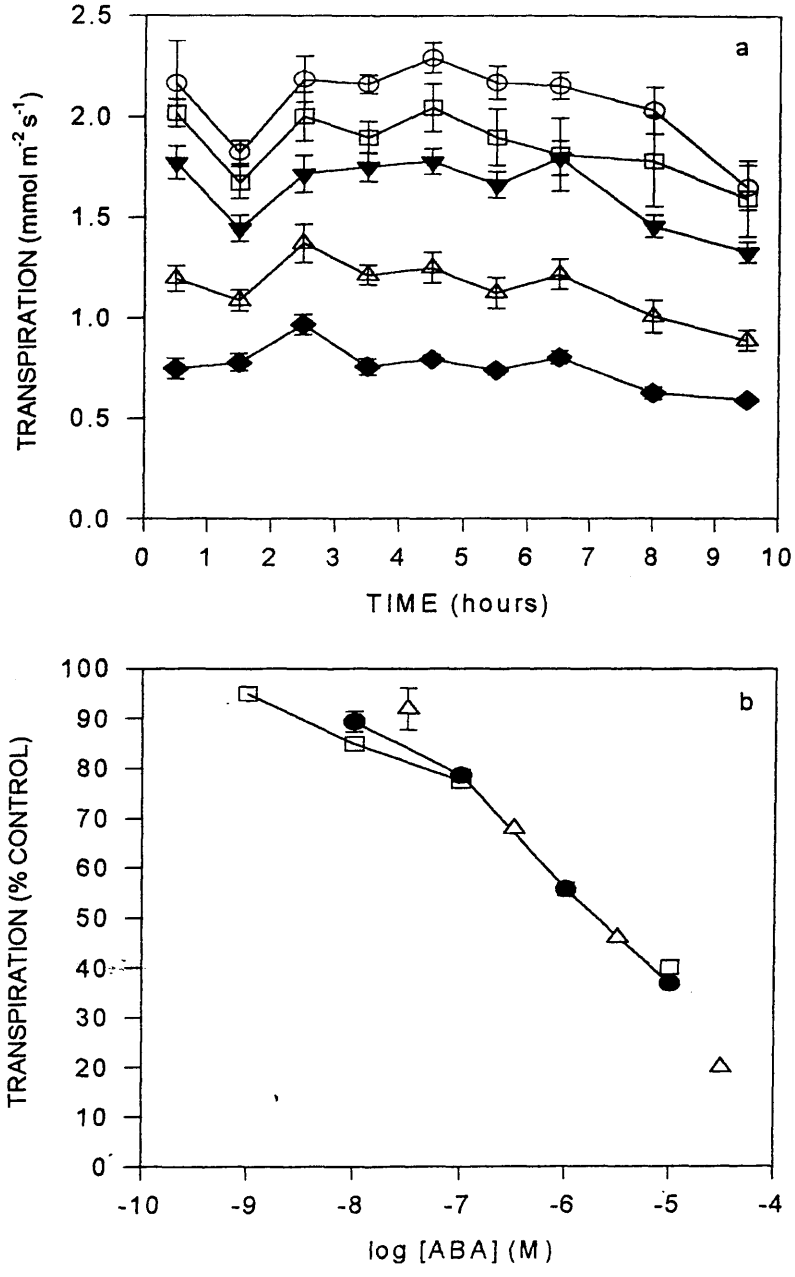

Figure 2.5a: Transpiration of detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots maintained at 23°C and supplied with artificial xylem solution (○), 10^{-8}M (□), 10^{-7}M (▼), 10^{-6}M (Δ) and 10^{-5}M (◆) ABA. Points are means ± S.E. of 6 shoots. (b) Dose-response curve generated from data in (a). Data (●) are expressed as a percentage of controls and are means ± S.E. of 42 measurements taken during the steady-state phase of transpiration inhibition (1.5-9.5 hours after supplying ABA). The dose-response curves for detached leaf 2 of *Triticum aestivum* L. cv. Egret maintained at 22°C (Δ) (from Munns and King, 1988) and detached primary leaves of Egret at 18°C (□) (from Munns *et al.*, 1993) are included for comparison.

Feeding with ABA reduced transpiration within an hour (Figure 2.5a). Stable transpiration rates were maintained in all treatments up to 6 hours. After 6 hours, transpiration rates in all treatments declined over the remainder of the assay period to reach 76 % of the stable reading obtained between 2 and 6 hours for control shoots. A similar decline has been noted before for detached leaf transpiration bioassays but previous reports indicate this occurs more rapidly (a linear decline with time, reducing transpiration by 23 % within 7 hours - Talha and Larsen, 1975) and with greater severity (50 % within 4 hours - Willmer *et al.*, 1978). Despite this decline, stable transpiration rates were obtained after 2 hours with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}M ABA reducing transpiration by 14 %, 21 %, 45 % and 64 % respectively. All these transpiration reductions were significant with the exception of 10^{-8}M ABA. It is interesting to note that the response to ABA remained reasonably stable over the 10 hour assay period, although at the end of the assay declines in the control transpiration rates resulted in 10^{-8}M ABA no longer having an effect. This confirmed the results of Talha and Larsen (1975), who showed that long measurement periods (up to 11 hours) reduced the effect of 10^{-8} and 10^{-7}M ABA.

Although ABA appeared to affect transpiration more rapidly than leaf elongation, these experiments were not designed to obtain data on the rates of response to ABA, merely the steady-state rates of leaf elongation and transpiration. Leaf elongation assays using ruler measurements in this thesis have a temporal resolution of 2 hours while simultaneous transpiration measurements were made hourly. Greater temporal resolution of these techniques could be attained by reducing replication. However, the limits of ruler detection (0.5 mm) would permit, at best, a measurement every 15 minutes at high temperatures (30°C) assuming an LER of 4 mm h^{-1}. Alternatively, studies on the rate of ABA-induced leaf growth inhibition could be undertaken using high resolution linearly variable displacement transducers (LVDTs).

ABA at concentrations from 10^{-8} to 10^{-5} M inhibited growth and transpiration according to the typical log-linear relationship between ABA concentration and the steady-state rates of the process described previously for leaf elongation (Munns, 1992), transpiration and stomatal opening (Trejo *et al.*, 1993). Expression of the data as a percentage of the control values allowed a comparison of the steady-state responses of the two processes in the same plants (Table 2.2). At low ABA concentrations (10^{-8} and 10^{-7}M), there was no difference in the magnitude of the response of transpiration and leaf elongation. At high ABA concentrations (10^{-6} and 10^{-5} M), leaf elongation appeared to be more inhibited. However, these results are from only one assay and Munns *et al.* (1993) stress the variability in ABA response that can occur between identical assays under similar conditions. An example of this variability is given in Figure 4.2. The relative responses of transpiration and leaf elongation to ABA at different temperatures are more fully considered in Figure 4.6.

Table 2.2: Sensitivities of growth and transpiration to ABA in the same leaf elongation assay. Data are means ± S.E. taken from Figures 2.4b, 2.5b, with the number of replicates indicated in parentheses. P value obtained by applying Student's unpaired t test (P<0.05 *), (P<0.01 **), (P<0.001 ***).

log [ABA] (M)	Leaf elongation (% Control)	Transpiration (% Control)	P Value
-8	88.5 ± 3.5 (32)	89.4 ± 2.0 (46)	0.805
-7	76.0 ± 2.6 (32)	78.6 ± 1.2 (48)	0.329
-6	41.7 ± 2.1 (36)	55.7 ± 1.2 (48)	0.000 ***
-5	23.5 ± 2.0 (36)	36.9 ± 0.8 (48)	0.000 ***

Expressing data as a percentage of control values allowed comparisons with results obtained by other workers. Figure 2.4b compares the dose-response curve of leaf elongation with that obtained by Munns (1992). Although 10^{-8}M ABA inhibited leaf elongation to a similar degree in both systems, leaf elongation appeared to be less responsive to ABA in Munns' system at all other concentrations. Two possible

explanations may account for this. Genotypic variation in ABA response may be involved since different species were used (*Triticum aestivum* cv. Egret in Munns, 1992 vs. *Hordeum vulgare* cv. Klaxon in Figure 2.4). Alternatively, the 5°C difference in temperature may explain the discrepancy. The roles of genotypic variation and temperature in altering ABA dose-response curves of leaf elongation are considered in Chapters 3 and 4, respectively.

Figure 2.5b compares the dose-response curve of transpiration with those obtained by Munns and King (1988) and Munns *et al.* (1993). Although there are differences of genotype and temperature, there is a close similarity of the dose-response curves over a range of 10^{-7} to $5x10^{-5}$M ABA. At concentrations lower than 10^{-7}M, there are some differences between the dose-response curves, and considerable variability within a single dose-response curve. Munns and King (1988) indicate that in five experiments, $5x10^{-8}$M ABA gave transpiration rates of 83, 85, 88, 101 and 103 % of leaves fed distilled water. This variability is important, for it is in this range that xylem sap ABA concentrations occur in droughted cereal shoots (Munns and King, 1988; Zhang and Davies, 1990a). It is important to note that the dose-response curve attained for transpiration using detached shoots (from Figure 2.5a) was very similar to that found by Munns' group using single detached leaves, which validates the use of detached shoots for assessing transpiration responses.

Table 2.3 compares the sensitivity of the leaf elongation assay described here with other commonly used growth and stomatal bioassays. Immediately obvious is the wide range of concentrations at which ABA has an effect in different stomatal systems. While a fuller discussion of factors affecting ABA sensitivity may be found in Section 1.5, this table does highlight important differences that occur due to species, different incubation solutions and method of assay. The ability of stomata to respond to very low ABA concentrations (10^{-11}, 10^{-10}M) in epidermal strip bioassays is very different from growth assays, which typically show significant growth reduction at 10^{-7}M ABA.

Only one "growth" assay, using the floating aquatic duckweed *Lemna*, showed ABA-induced growth inhibition at 10^{-11}M (Tillberg, 1975).

Table 2.3: What ABA concentrations are needed to demonstrate a significant effect in bioassay experiments ?

Assay technique	Species	log [ABA] (M)	Reference
Stomatal			
Epidermal	*Commelina communis*	-8	Tucker and Mansfield, 1971
Strip Bioassay	*Commelina communis*	-7	Ogunkanmi *et al.*, 1973
	Commelina communis	-10	Ogunkanmi *et al.*, 1973
	Commelina communis	-8	Trejo *et al.*, 1993
	Commelina communis	-10	Trejo *et al.*, 1995
Epidermal Strip Bioassay with whole leaf pieces	*Commelina communis*	-5	Trejo *et al.*, 1993
Detached Leaf	*Hordeum vulgare*	-7	Cummins *et al.*, 1971
Transpiration	*Zea mays*	-8	Talha and Larsen, 1975
Bioassay	*Commelina communis*	-5.5	Weyers and Hillman, 1979
	Commelina communis	-6	Trejo *et al.*, 1993
	Hordeum vulgare	-7	Figure 2.5
Growth:			
Coleoptile	*Avena sativa*	-7	Tillberg, 1975
Growth	*Avena sativa*	-8	Wright, 1969
Inhibition	*Avena sativa*	-9	McWha and Jackson, 1976
	Triticum aestivum	-8	McWha and Jackson, 1976
Lemna Growth	*Lemna gibba*	-11	Tillberg, 1975
Inhibition	*Lemna minor*	-7	McLaren and Smith, 1976
	Lemna gibba	-8	DeKock *et al.*, 1978
	Lemna minor	-6	Campbell *et al.*, 1995
Leaf Disc Inhibition	*Phaseolus vulgaris*	-7	Van Volkenburgh and Davies, 1983
Intact Leaf Bioassay	*Glycine max*	-6	Bunce, 1990
Detached Shoot	*Phaseolus vulgaris*	-4	Van Volkenburgh and Davies, 1983
Leaf Elongation	*Triticum aestivum*	-6	Munns, 1992
Assay	*Hordeum vulgare*	-7	Figure 2.4

Apparent differences in stomatal response to ABA caused by different assay techniques can be explained in terms of stomata responding to epidermal ABA concentration (Trejo *et al.*, 1993). More ABA accumulated in epidermal strips with their mesophyll removed, which emphasised the controlling influence of the mesophyll on epidermal ABA, casting doubt on the value of the epidermal strip assay in forecasting conditions in the intact plant. This example highlights an important consideration; that choice of a suitable bioassay to study potential variation in ABA sensitivity should mainly depend on its ability to estimate or reproduce whole plant response to ABA.

The leaf elongation assay described here was preferred over the other growth assays described in Table 2.3 due to its morphological similarity to intact plants (unlike coleoptile or leaf disc systems), its ease of application (unlike intact plant assays which rely on stem injection of ABA - Bunce, 1990) and the ability to alter the feeding solution. Although the leaf disc assay fulfils the latter criterion, it is unsuitable for monocotyledons since the lamina does not contain expanding cells. It is also possible that wounding effects and uncertainty of penetration of the feeding solution into the tissue (Pratt and Matthews, 1971) may be problems with the leaf disc assay.

2.3.5 Response of leaf growth and transpiration in the presence or absence of ABA supplied at different pHs

The response of leaf elongation and transpiration to ABA supplied at different pHs showed considerable variability between different batches of plants; a typical result is shown in Figure 2.6. It is apparent that both processes show stability in their response to solutions across a pH range of 5-8. At pH 9, both processes were reduced, however a large percentage (75 %) of the plants showed wilting symptoms. Only 15 % of the plants at other pHs were similarly afflicted. On occasion, solutions of pH 8 were shown to reduce leaf growth and transpiration, again with a disproportionate

percentage of these plants showing wilting. It is hypothesised that the wilting originates from the blockage of the xylem vessels; this could have been tested by re-cutting the shoots and transferring them back to solutions of a lower pH to see if growth recovery occurred. The wilting is not altogether surprising, as pH 9 is considered to be non-physiological, even in water stressed plants which show an alkalisation of the xylem sap (see Table 2.4).

It is unfortunate that there are no data available on the pH of barley xylem sap collected from water stressed plants to directly assess the physiological relevance of the imposed pH changes. Some comparative data are presented in Figure 7.8, which showed maize xylem sap to have a pH of 6.3-6.4 with no change occurring with a mild soil drying (which reduced LER by 35 %). Considering that drought-induced pH changes are much less than 1 pH unit for quite severe (indicated by the increase in ABA concentration) drying treatments (Table 2.4) and the considerable variability within a treatment, it is unlikely that pH has a direct effect on leaf growth.

Table 2.4: Changes in xylem sap pH and ABA concentration in well watered (WW) and water stressed (WS) plants. Values are means ± S.E. where possible. Data from the sunflower study were re-elaborated.

Stress	Reference	Species	Xylem Component	Water Stressed	Well Watered
WS <0.10 g g^{-1} θ WW 0.15-0.20 g g^{-1} θ	Gollan *et al.*, 1992 Schurr *et al.*, 1992	*Helianthus annuus*	pH ABA (nM)	6.30 ± 0.04 31 ± 4	6.64 ± 0.07 371 ± 76
WS - 3-4 days withholding water	Trejo, 1994	*Phaseolus vulgaris*	pH ABA (nM)	5.66 ± 0.12 72	6.07 ± 0.05 900

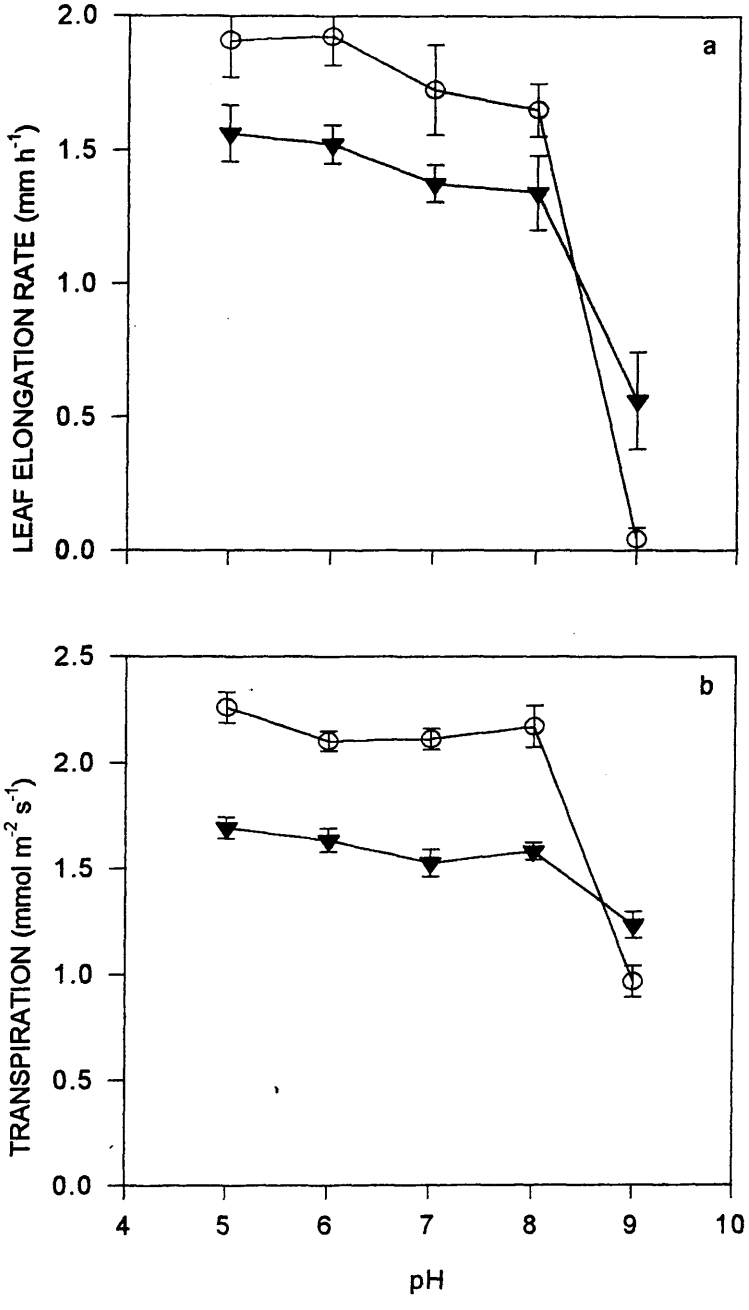

Figure 2.6: Effect of pH on leaf elongation rate (a) and transpiration (b) of detached barley (*Hordeum vulgare* L. cv. Hanna) shoots maintained at 24°C and supplied with artificial xylem solution (○) or 10^{-7}M ABA (▼). Points are means ± S.E. of at least 10 (a) and 15 (b) shoots. Data analysis excluded shoots which showed wilting symptoms (15 % of total) for pHs 5-8, but included shoots which had wilted (75 % of total) for pH 9.

This conclusion goes against a large body of literature showing effects of pH on the growth of coleoptiles (see e.g. Figure 8.9) and stem segments, although the controversy over the so-called "acid growth theory" continues (Kutschera and Schopfer, 1985; Schopfer, 1989, Cleland *et al.*, 1991; Rayle and Cleland, 1992). However, most of the supporting data for this theory comes from excised stems and coleoptiles incubated in solutions of pH < 5.5, and little evidence has been found to demonstrate effects of pH on intact leaves. In monocotyledons, a rather inconclusive correlation between leaf surface pH and maize leaf growth occurred in plants subjected to a soil drying treatment (Van Volkenburgh and Boyer, 1985), but there was no attempt to manipulate pH and examine the effect on leaf growth. The evidence for acid growth in gramineacous leaves is therefore rather weak.

Studies examining coleoptile growth have often employed stronger buffers than the 2 mM phosphate buffering system applied here in the detached shoot experiments, and found in wheat xylem sap (Munns and King, 1988). The failure to detect an effect of pH on growth in Figure 2.6 may therefore represent a failure to over-ride the plant's natural buffering system. It may have been worthwhile to have determined the osmotic effects of different phosphate concentrations on growth and transpiration of shoots, to determine the maximum buffering capacity that could have been used.

Failure to over-ride the plant's buffering system may also explain why ABA applied at a higher pHs did not further reduce either leaf growth or transpiration over the pH range 5-8. This contradicts data obtained from epidermal strip bioassays, which show greater stomatal closure at higher pHs in some (Ogunkanmi *et al.*, 1973; Paterson *et al.*, 1988) but not all (Hartung, 1983) cases. Although a whole plant study has shown a correlation between stomatal sensitivity to ABA and pH (Schurr *et al.*, 1992), correlations also existed for NO_3^- and Ca^{++}. As considered earlier, the multiplicity of inner correlations between different nutrient ions in the soil drying system make it unrealistic to pronounce conclusively on a specific interaction.

Epidermal strip assays may also be flawed since they do not consider the role of the mesophyll in regulating the concentrations of ions around the guard cell apoplast. Until some reconciliation between epidermal strip assays, transpiration bioassays and whole plant studies (in the case of stomata) or coleoptile, leaf elongation assays and whole plant studies (in the case of leaf growth) can be achieved, it is difficult to state with certainty the existence of a pH x ABA interaction affecting the leaf growth response of plants. Accordingly, the leaf elongation assays described in the remainder of this thesis have employed an artificial xylem solution in which the pH was not adjusted.

2.4 CONCLUSIONS

The leaf elongation assay appeared to be a reliable means of assaying for chemicals which can affect leaf growth. A potential problem existed when control shoots showed declines in LER over the latter stages of a 10 hour assay period. However, a stable response to ABA treatment occurred after 4 hours. Therefore control shoots maintained their growth for a sufficient period to allow detection of the ABA response. Further validity was added to the assay by the observations that LER was responsive to temperature as in intact plants, transpiration was affected to a similar extent as that found in detached leaf systems and the shoots responded to a growth promoter (GA_3) in the expected manner. Dose-response curves for leaf elongation and transpiration were similar to those reported by other workers.

CHAPTER 3.

DO GENOTYPIC DIFFERENCES EXIST IN LEAF ELONGATION RESPONSE TO ABA ?

3.1 INTRODUCTION

One interesting result from the previous section was that the dose-response curves for ABA-induced inhibition of leaf growth were very different between the present study and that of Munns (1992) (Figure 2.4b). One possibility for the discrepancy may be that different species were used. The possibility that different genotypes may vary in their response to ABA may be of considerable adaptive significance. Genotypes which show a sensitive response to ABA may be able to effectively restrict their leaf area development, allowing more soil water to become available later during grain filling. Alternatively, in environments where there is a positive correlation between early vegetative growth and grain yield, it may be desirable for genotypes to be relatively insensitive to ABA.

Such differences are of physiological relevance only if ABA is regulating growth. The role of ABA in controlling the leaf growth of droughted plants remains controversial (Section 1.6) and thus differences in leaf growth response to ABA may not be of adaptive significance. Regardless, it would be of considerable intrinsic interest to identify genotypes differing in their ABA response. If considerable differences were detected, it would be a logical development to see if leaf growth behaviour under drought was affected. The leaf elongation assay has an advantage in screening for genotypic differences since it is possible to avoid confounding effects of genotypic differences in water relations that may occur in a soil drying experiment. Although it can be argued that a system which examines the ABA response in essentially well-watered plants is artificial, it must be noted that considerable leaf growth inhibition can occur without the detection of any changes in the water relations of droughted plants

(Passioura, 1988a; Saab and Sharp, 1989; Gowing *et al.*, 1990). The leaf elongation assay approach also has the advantage of being less labour intensive than a soil drying experiment. Before embarking on a screening program, evidence of genotypic differences in ABA response should be located.

Most of the available evidence has come from studies of the stomatal response to ABA. Considerable variation between (Kriedemann *et al.*, 1972) and within (Rodriguez and Davies, 1982; Quarrie, 1983; Blum and Sinmena, 1995) species has been shown. It is therefore likely that the leaf elongation response to ABA may show intergenotypic variation. An exhaustive study of wheat genotypes (Blum and Sinmena, 1995) revealed significant variation in growth responses to ABA supplied in hydroponic solution. However, this study measured relative growth rate after 7 days and thus integrated effects of ABA on leaf area expansion and photosynthesis. Differences in coleoptile growth response to ABA have also been demonstrated (Filiti and Cristoferi, 1977; Lercari *et al.*, 1978 - both cited in Quarrie, 1983) in wheat cultivars. However, preliminary studies with the leaf elongation assay have not revealed consistent genotypic differences (Munns, pers. comm.). Therefore, comparisons of a number of selected genotypes were initiated.

The possibility of intercultivar variation was tested using wheat cultivars known to differ in their detached leaf and field ABA accumulation (Quarrie, pers. comm.) and their root growth response to soil drying (Rigby *et al.*, 1994). Two wheat cultivars from the study of Blum and Sinmena (1995), known to differ in transpiration and growth response to ABA, were also obtained. As a matter of practical necessity, the two barley cultivars on which most of the work in this thesis is based were also tested for differences. Species differences were assessed by comparing the responses of barley and maize. This comparison was considered important since contradictory data have been obtained using different techniques on the importance of xylem sap ABA in controlling leaf growth in these species (Zhang and Davies, 1990a, b; Munns, 1992),

despite the close similarity of the dose-response curves for leaf growth response to synthetic ABA obtained in different systems in the two studies. The work reported here on maize extends the use of the leaf elongation assay to an additional species.

3.2 MATERIALS AND METHODS

Seeds of the wheat (*Triticum aestivum* L.) cultivars SQ1 and Chinese Spring were kindly provided by Dr. S.A. Quarrie, while seed of the cultivars Sunstar and Sundor were kindly supplied by Dr. A. Blum. Seeds of the barley (*Hordeum vulgare* L.) cultivars Klaxon and Firefly and the maize (*Zea mays* L.) cultivar Earliking were obtained through commercial suppliers.

Preliminary experiments with maize (*Zea mays* cv. Earliking) showed it to be a less suitable candidate for the assay than wheat or barley. Lateral roots could form on the base of the sub-crown internode and nodal roots could appear at the crown at the time leaf 3 was ready to assay. These roots were removed to avoid introducing potting compost to the feeding solution. After preparation, the detached shoots appeared as in Figure 3.1. The developmental window in which plants were suitable for assay was reduced (compared to barley) since plants whose third leaf was greater than 10 cm at the time of assay would show a reduced LER 6 hours after the commencement of an assay (data not shown). Despite these difficulties, care with selection and preparation of plants ensured enough material.

The use of maize shoots with their larger leaves permitted the use of porometry to determine if detached shoots maintained similar gas exchange characteristics to intact plants. A Delta-T AP4 porometer (Delta - T Devices, Burwell, Cambridge, England), which was calibrated before each measurement period, was used.

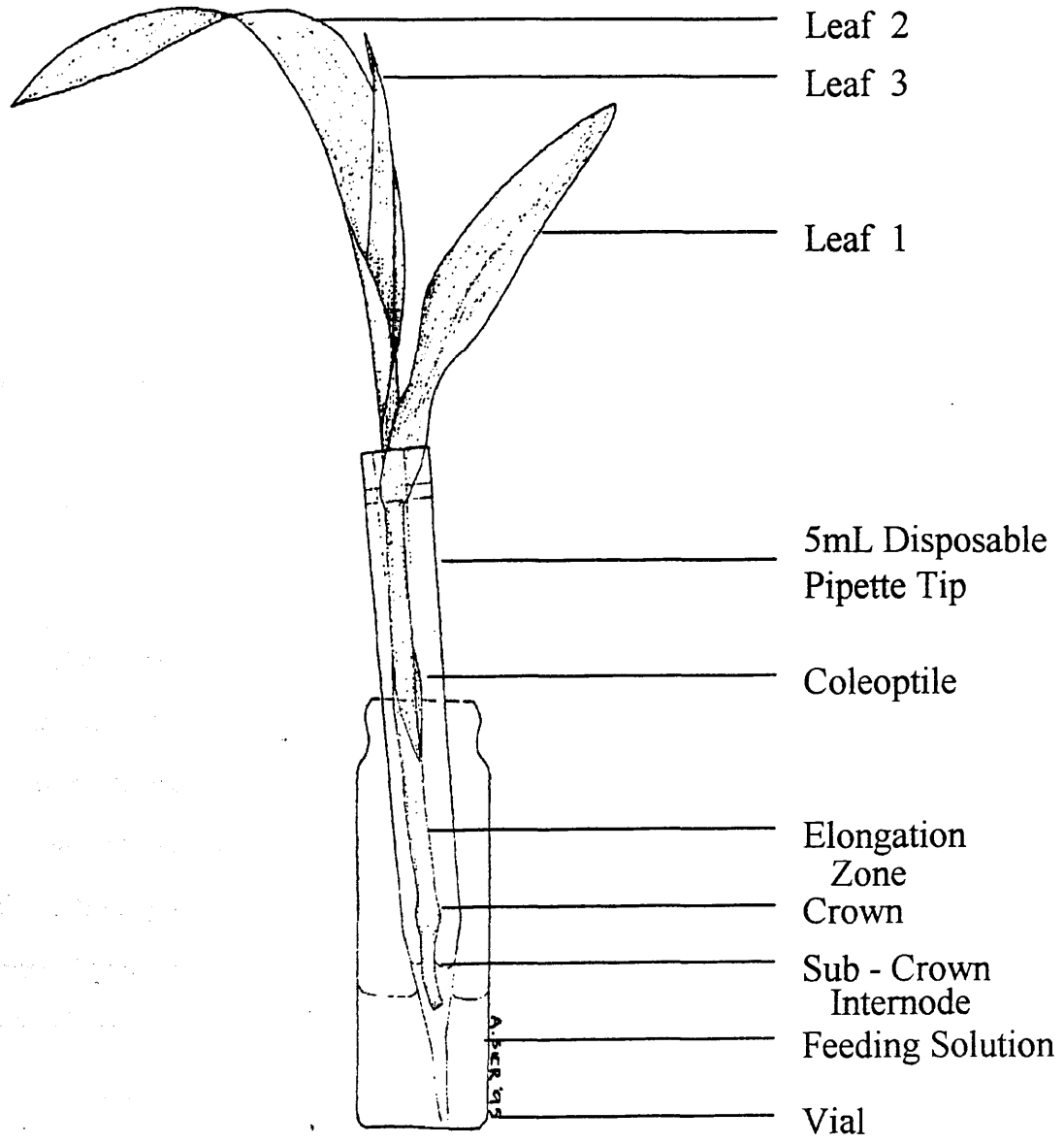

Figure 3.1: Detached maize (*Zea mays* L. cv. Earliking) shoot used for leaf elongation assays (0.85 x actual size). Drawing by Andrew Sier.

All plants were grown at 30/20°C to avoid any possibility of chilling injury in maize. Seed of both cultivars in each pair was planted at the same time (except in the maize vs. barley comparison, where maize seed was sown 3 days later) in the hope of obtaining plants at the same stage of development when the shoot was ready to assay. This was not achieved in all cases, and care was taken in selecting shoots for assay that they were of the same developmental stage. This reduced the replication from what was thought desirable (9 shoots per cultivar per treatment) in some comparisons. Leaf elongation assays, as described in Section 2.2, were performed at temperatures of 25-30°C using 5 ABA concentrations. Experiments for each pair of cultivars were repeated on 2 (Sunstar vs. Sundor) or 3 (all other pairs) occasions.

Data were analysed in two ways. Using the LER data obtained when leaf growth inhibition was maximal, LER as a percentage of the control values was calculated for each ABA concentration (as in Figures 2.2b, 2.4b). Leaf growth inhibition was maximal at 3, 5 and 7 hours after feeding ABA, however in one case (Chinese Spring vs. SQ1) only data from 3 hours was used since the LER of control Chinese Spring plants had declined by 5 hours (Figure 3.2). This problem was previously noted (see the data for 9 hours for Figure 2.2a) and seems to be a cultivar-specific difference in the maintenance of LER in detached shoots at high temperature. The data (LER % Control) for the different genotypes were compared by applying Student's unpaired t-test in SPW 1.0 at each ABA concentration using the data combined from 3 measurement occasions (t= 3, 5, 7 hours). Data from each experiment were tested (see Appendix 3) and the combined data from all experiments also assessed. Data from each experiment are presented graphically in one case (maize vs. barley - Figure 3.5a-c) and for the combined data in all other cases (Figures 3.5d, 3.6). As a comparison, the two data sets for Klaxon were also compared (Figure 3.6d).

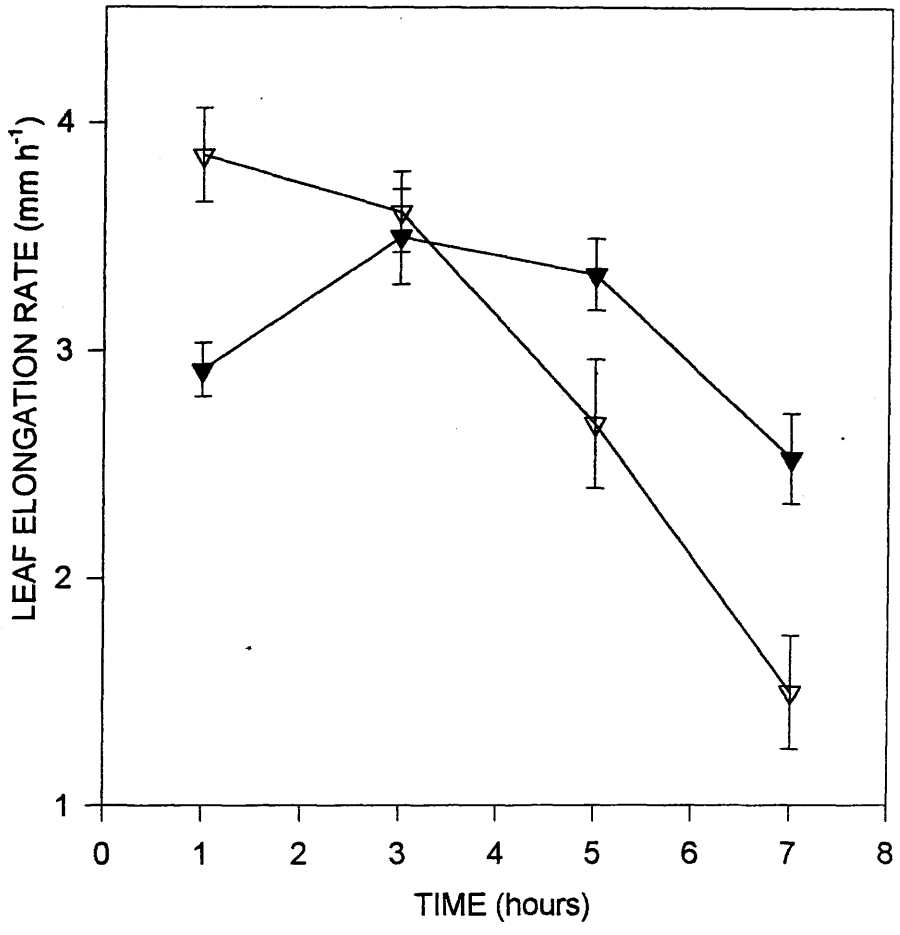

Figure 3.2: Leaf elongation rate of detached shoots of wheat (*Triticum aestivum*) cultivars Chinese Spring (▽) and SQ1 (▼) supplied with artificial xylem solution. Points are means ± S.E. of 7 (Chinese Spring) and 9 (SQ1) shoots.

Alternatively, primary data on LER for each experiment and a combined data set including all experiments were analysed in GENSTAT 5 (Rothamsted Experimental Station, Harpenden, England) for all measurement periods (except in the case of the Chinese Spring vs. SQ1 comparison as discussed above). Data were arcsin transformed to satisfy normality of variance. Sample ANOVA tables can be found in Appendix 2. The significance of the Genotype x ABA (G x ABA) interactions are presented in Table 3.1.

In addition to the leaf elongation assays, a detached leaf transpiration bioassay was employed to screen for variation in stomatal responses to ABA. Growth of the plants was as described above, and leaf 2 was detached at the same stage plants were ready for a leaf elongation assay. After leaving plants in the dark for 15 minutes, leaves were detached, re-cut under distilled water (DI), placed in Eppendorf tubes containing DI and allowed to recover in the dark for 30 minutes. They were then placed in a growth cabinet for 45 minutes to allow the stomata to open, then transferred to a solution of AX or various ABA concentrations. Transpiration was determined gravimetrically as in Section 2.2.

3.3 RESULTS AND DISCUSSION

Figure 3.3 shows that leaf growth and abaxial stomatal conductance in detached maize shoots were very similar to intact plants (no significant differences, $P>0.05$, at any time during the assay). Detached maize shoots showed reduced leaf growth and stomatal conductance when supplied ABA (in a similar manner to detached barley shoots - see Figures 2.4, 2.5), the percentage reduction being dependent on ABA concentration.

The response of LER to temperature (T) in detached maize shoots in a number of experiments was described by a highly significant ($P<0.001$) linear regression (LER = -2.60 + 0.161T, $r^2 = 0.73$, d.f. = 11) over the restricted temperature range of the experiments (24-30^{o}C). Comparison with a similar regression for barley over that temperature range was not possible as barley shoots showed a higher, but variable, LER (see Figure 2.3b), such that the regression was non significant ($P=0.92$). Comparison of the maize regression with the barley regression (from Figure 2.3b) showed that for a given temperature, maize LER was, on average, only 60 % that of barley.

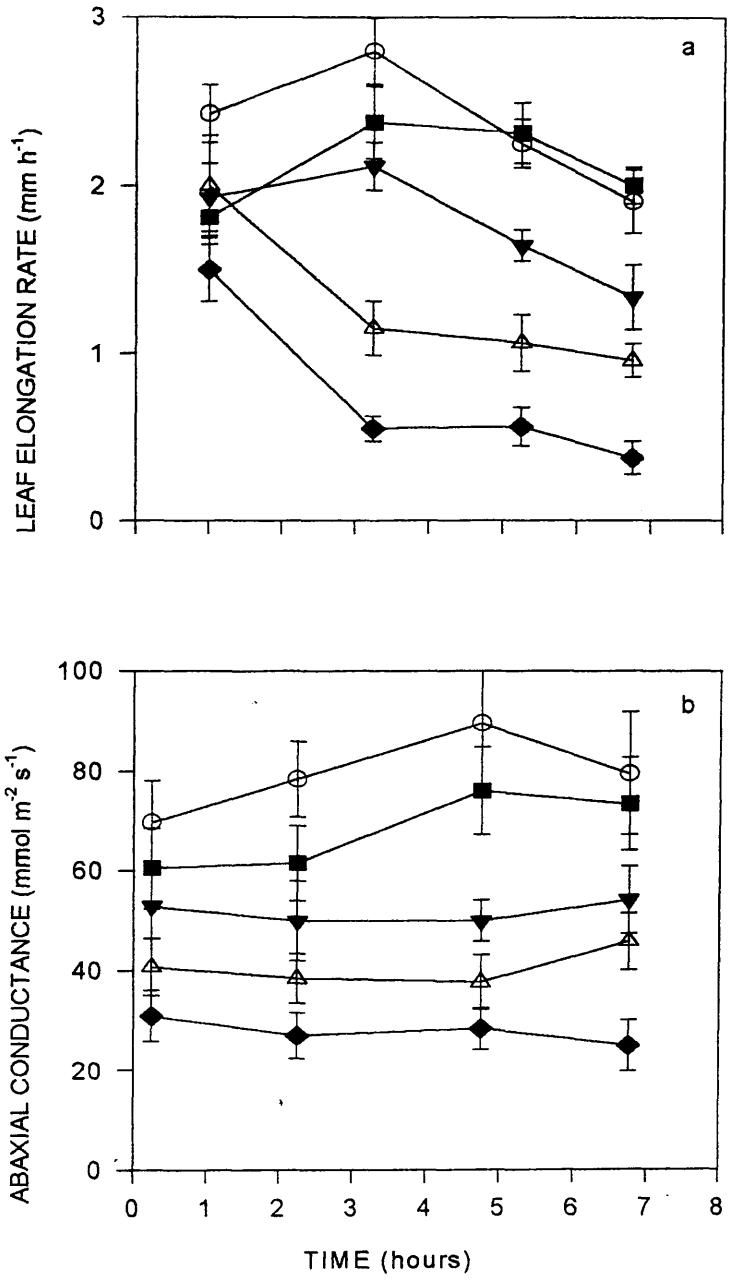

Figure 3.3: Leaf elongation rate (a) and abaxial conductance (b) of intact maize plants (■) or detached maize shoots maintained at 30°C and supplied with an artificial xylem solution (○), 10^{-7}M (▼), 10^{-6}M (Δ) and 10^{-5}M (◆) ABA. Points are means ± S.E. of 8 shoots.

To generate a dose-response curve for the inhibition of maize leaf growth by ABA, further experiments were performed. Figure 3.4 shows the characteristic log-linear response to ABA in the maize leaf elongation assay system, which is similar to the relationship between leaf growth and xylem ABA concentration determined by Zhang and Davies (1990a, b) for intact maize plants grown in drying soil or supplied with ABA via a hydroponics solution. The similarity of the responses provided further evidence that the bioassay technique may provide valuable information on the responses of whole plants.

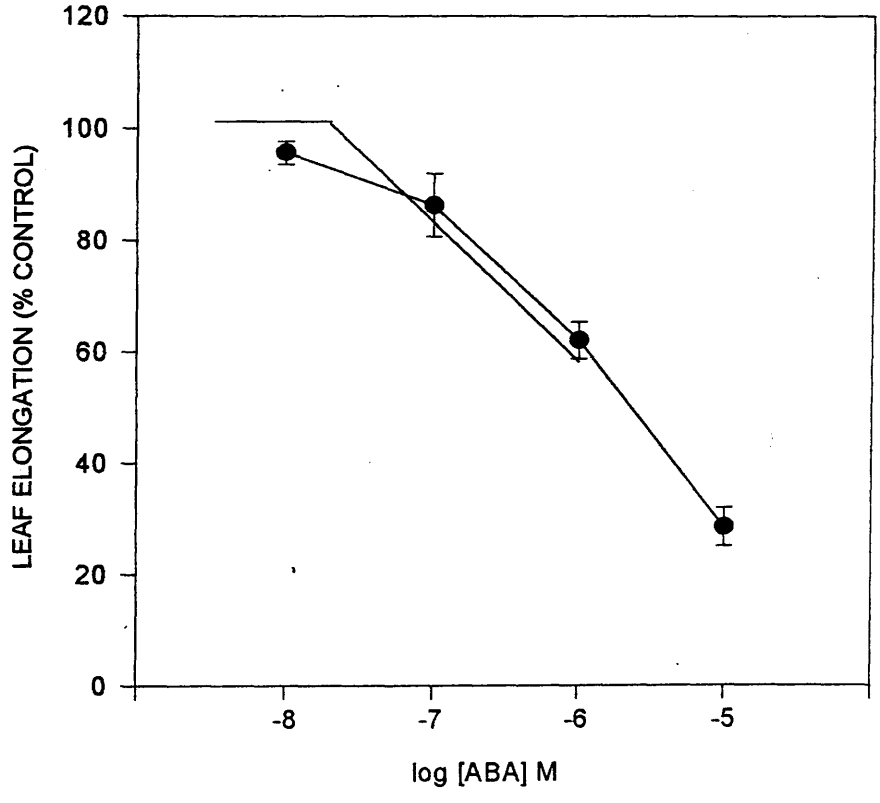

Figure 3.4: Response of leaf elongation to xylem sap ABA concentration in intact maize plants (solid line) and detached shoots (●). The solid line, redrawn from Zhang and Davies (1990b), plots the leaf elongation response versus xylem sap ABA concentration for whole plants subjected to a soil drying treatment or fed a nutrient solution containing ABA. The symbols refer to maize leaf elongation assays conducted at an average temperature of 27°C. Points are mean leaf elongation, expressed as a percentage of controls, ± S.E. of n experiments for 10^{-8}M (n=3), 10^{-7}M (n=9), 10^{-6}M (n=11) and 10^{-5}M (n=8) ABA.

Figure 3.5 compares dose-response curves of leaf growth versus ABA concentration generated in 3 separate experiments for maize and barley (cv. Klaxon) plants assayed under the same conditions. In one of 3 experiments (Figure 3.5b) there were highly significant ($P<0.001$) differences in ABA response between maize and barley. This suggested that it would be prudent to perform 5 assays per pair of cultivars; unfortunately lack of time limited replication. This one experiment resulted in there being a significant difference with the combined data (Figure 3.5d) at 10^{-7}M ABA, with no significant differences in ABA response at other concentrations. This occurred in spite of large differences in growth rate (as discussed above) between the two species. ANOVA confirmed the presence of a significant ($P<0.01$) genotype x ABA (G x ABA) interaction in Experiment 2, even when the data from 10^{-6}M and 10^{-5}M ABA were omitted from the analysis (Table 3.1). This one experiment resulted in a significant G x ABA interaction when the data from the 3 experiments were combined. The highly significant ($P<0.01$) genotype x ABA x experiment (G x ABA x E) interaction indicates that further experiments should be performed to discriminate whether maize and barley differ significantly in their response to ABA.

Comparison of the two barley cultivars (Figure 3.6a) showed that Firefly was significantly more sensitive to ABA than Klaxon at all concentrations tested. However, analysis of variance (Table 3.1), revealed no significant G x ABA interaction in any experiment. It is concluded that the two cultivars do not differ in their response to ABA, which highlights the inadequacy of comparing data strictly on the percentage inhibition of growth shown.

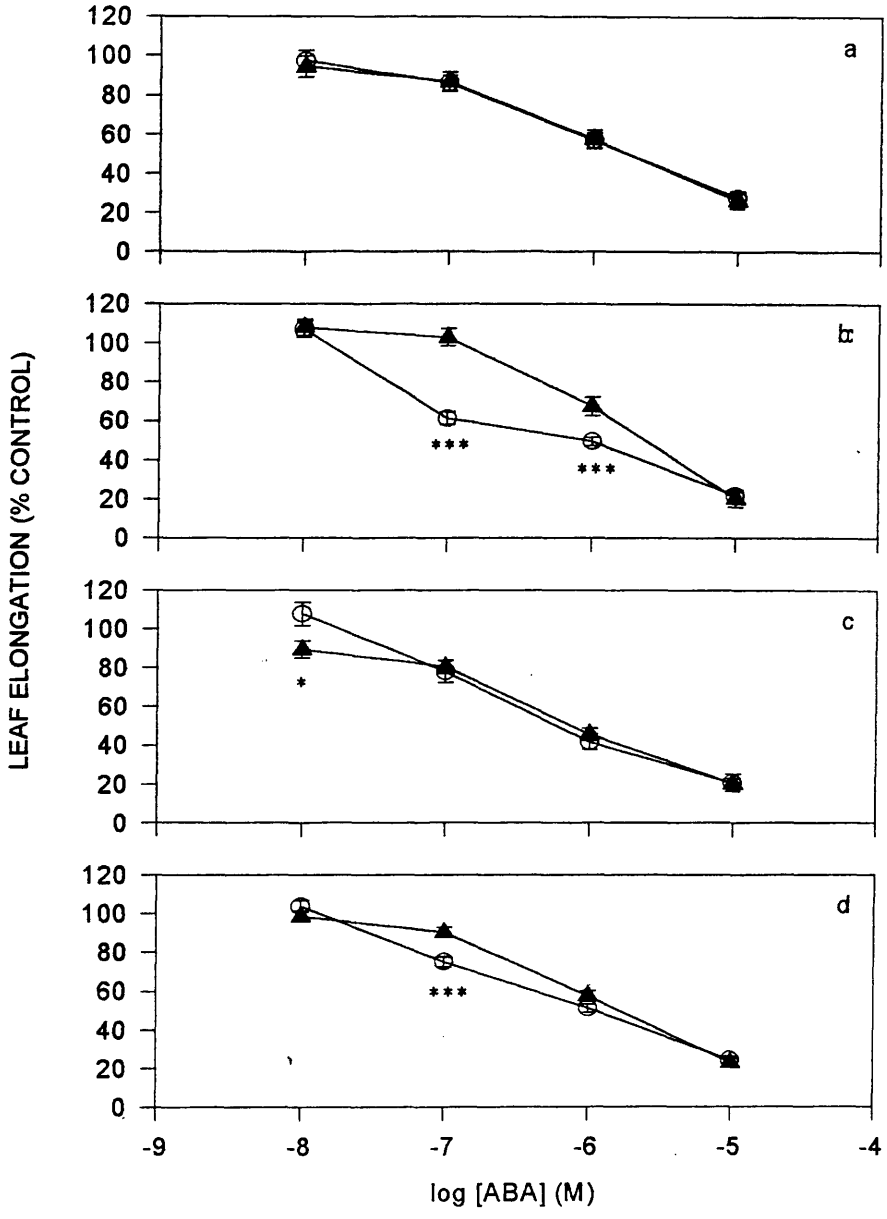

Figure 3.5: Dose response curves (as generated in Figure 2.4b) for leaf growth of *Hordeum vulgare* cv. Klaxon (○) and *Zea mays* cv. Earliking (▲) in response to ABA for 3 separate experiments: (a), (b) and (c) where the temperature was 26-28°C. Data expressed as a percentage of controls, and represent means ± S.E. of at least 18 measurements taken during the steady-state phase of leaf growth inhibition (3-7 hours after supplying ABA). Data from the 3 separate experiments were combined to generate the dose-response curve in (d). Significant differences ($P<0.05$ *), ($P<0.01$ **), ($P<0.001$ ***) between species at each ABA concentration, as determined by Student's unpaired t test, are indicated on graphs.

Table 3.1: Table of significance of Genotype x ABA interactions for various data sets. with (All [ABA]s) or without the data for 10^{-6} and 10^{-5}M ABA. (P<0.05 *), (P<0.01 **), (P<0.001 ***).

Genotypes	Data Set	Exp. 1	Exp. 2	Exp. 3	Overall
Klaxon	All [ABA]s	NS	**	NS	**
vs. Earliking	- $10^{-6}/10^{-5}$ M ABA	NS	**	NS	**
Klaxon	All [ABA]s	NS	NS	NS	NS
vs. Firefly	- $10^{-6}/10^{-5}$ M ABA	NS	NS	NS	NS
Sunstar	All [ABA]s	NS	NS		NS
vs. Sundor	- $10^{-6}/10^{-5}$ M ABA	NS	NS		NS
Chinese Spring	All [ABA]s	*	NS	*	**
vs. SQ1	- $10^{-6}/10^{-5}$ M ABA	*	NS	NS	NS
Klaxon No. 1	All [ABA]s	NS	*	NS	**
vs. Klaxon No. 2	- $10^{-6}/10^{-5}$ M ABA	NS	*	NS	NS

Comparison of the wheat cultivars Sunstar and Sundor was hampered by poor germination. Combined data from only two experiments showed a significant (P<0.05) difference in ABA response at 10^{-7}M (Figure 3.6b), the same concentration routinely used by Blum and Sinmena (1995) in detecting genotypic differences. This difference was not supported by ANOVA, with no significant G x ABA interactions detected in either experiment, even when the data from 10^{-6}M and 10^{-5}M ABA were excluded (Table 3.1). Considering this negative result, it was decided to repeat Blum and Sinmena's (1995) experiment using these two cultivars in a transpiration bioassay. When detached leaves were fed 10^{-7}M ABA, both cultivars exhibited transpiration that was 60 % of the controls (data not shown), a result at odds with the large differences (15 % of controls for Sundor and 58 % for Sunstar) noted by Blum and Sinmena (1995).

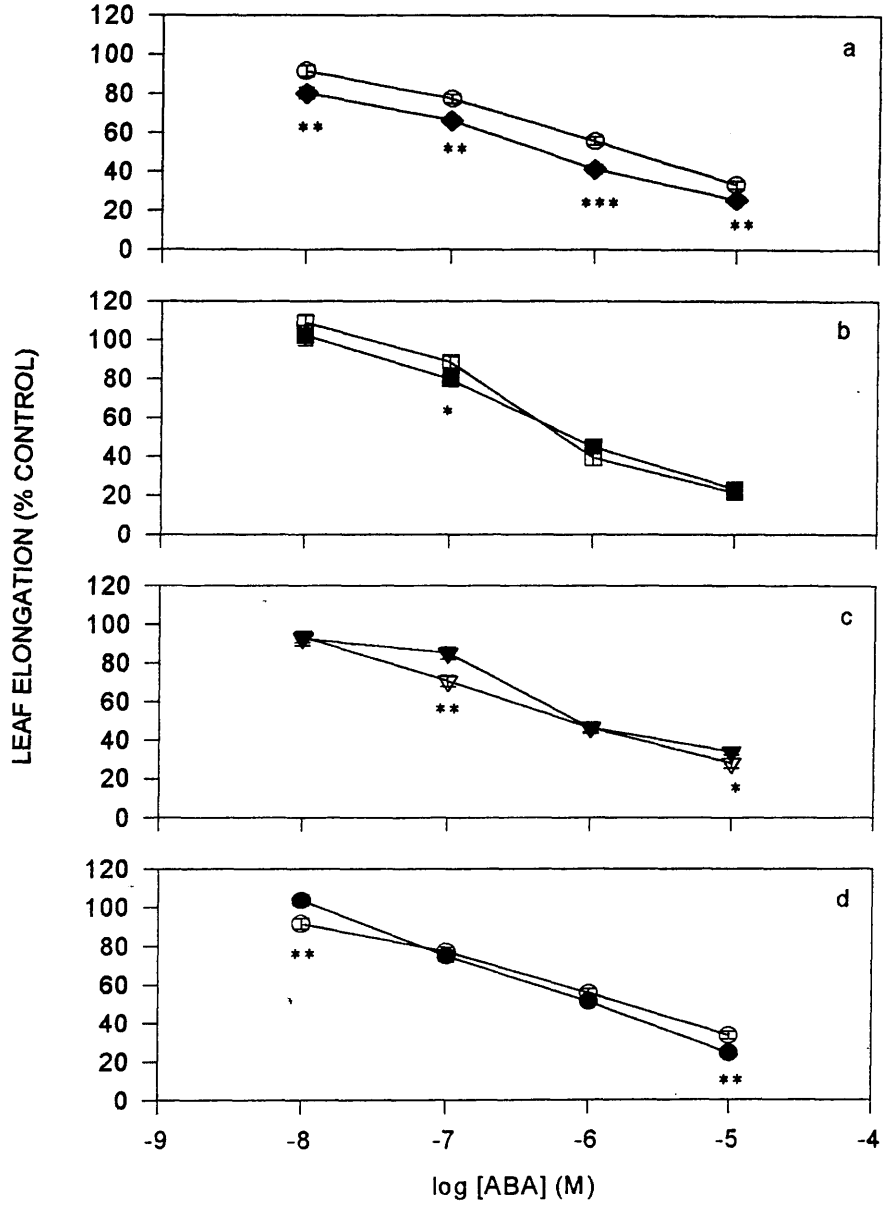

Figure 3.6: Dose-response curves (as generated in Figure 3.5d) for leaf growth in response to various ABA concentrations for *Hordeum vulgare* cv. Klaxon (○) and *Hordeum vulgare* cv. Firefly (◆) (a), *Triticum aestivum* cv. Sunstar (□) and *Triticum aestivum* cv. Sundor (■) (b), *Triticum aestivum* cv. Chinese Spring (▽) and *Triticum aestivum* cv. SQ1 (▼) (c), and two data sets for cv. Klaxon, from Figure 3.5d (●) and Figure 3.6a (○). Data expressed as a percentage of controls, and represent means ± S.E. of at least 12 (c), 21 (b) and 44 (a,d) measurements taken from 3 (a,c,d) or 2 (b) separate experiments during the steady-state phase of leaf growth inhibition when the temperature was 26-30°C. Significant differences between genotypes ($P<0.05$ *), ($P<0.01$ **), ($P<0.001$ ***) are indicated on graphs.

There was a significant ($P<0.01$) difference between Chinese Spring and SQ1 in response to ABA only at 10^{-7}M ABA (Figure 3.6c), with a weaker difference ($P<0.05$) noted at 10^{-5}M (a concentration too high to be regarded as physiologically relevant). Only ANOVA for the time period (t= 3 h) was considered relevant, since Chinese Spring showed reduced growth in control shoots over the later stages of the assay (Figure 3.2). This revealed a significant G x ABA interaction (Table 3.1) but only if the entire data set (all ABA concentrations) was used. The fact that the experiment x genotype x ABA interaction was not significant ($P>0.10$) for both ABA data sets indicates that the 3 experiments were consistent. It is concluded that these two wheat cultivars differ in their response to ABA.

The validity of these genotypic comparisons was examined by comparing the data from the two Klaxon data sets (Figure 3.5d vs. Figure 3.6a) in Figure 3.6d. Reassuringly, there was no difference in the percentage data for 10^{-7}M and 10^{-6}M ABA, but significant differences ($P<0.01$) at the two other concentrations (Figure 3.6d). Analysis of variance (Table 3.1) showed a significant G x ABA interaction for Experiment 2. This was confirmed by a highly significant ($P<0.01$) G x ABA interaction for the data set employing all ABA concentrations. These results are worrying, as the variability between different experiments using the one cultivar makes it difficult to be certain in comparisons of 2 cultivars, at least for comparisons using cv. Klaxon. The only solution would be to increase the replication of experiments. The variability in cv. Klaxon would also invalidate the comparisons with maize and cv. Firefly.

The only genotypic difference which can be stated with reasonable confidence is the comparison of Chinese Spring (CS) and SQ1, which shows that CS is more responsive to ABA, at least when expressed as a percentage of control leaves. However the detection of this interaction seemed to depend on the use of data from one measurement period and using 10^{-7}M ABA (Figure 3.6c). Rejection of data from

other time periods was considered valid as control shoots of CS were unable to maintain their growth for longer than 4 hours (Figure 3.2). This problem may not have been encountered at lower assay temperatures. Despite these cautions, it appears as though CS and SQ1 differ in their leaf elongation response to ABA.

It is difficult to account for this apparent change in sensitivity. One possibility is that there was greater accumulation of ABA in the elongation zone of CS shoots, perhaps due to greater transpiration rates. It was not possible to accurately assess transpiration in the CS detached shoots, as leaf 2 was too large and floppy and could not be contained within the balance. It was, however, possible to measure transpiration in detached single leaves. All 4 experiments revealed no difference in transpiration rates of leaves fed 10^{-6}M ABA, but there were consistently higher (12 % in 2 experiments, and 48 % in 2 experiments) transpiration rates in SQ1 leaves fed an artificial xylem solution (data not shown). This would tend to negate the idea of greater ABA accumulation in Chinese Spring shoots, if ABA accumulation is partially dependent on the initial transpiration rates of control shoots. When the percentage transpiration values were calculated in the experiments, SQ1 was shown to be 15 % more responsive to ABA than CS. Such differences may be meaningful if there is no relationship between the transpiration rates of the controls and the sensitivity, as demonstrated by Blum and Sinmena (1995) in a survey of the transpiration responses of 6 wheat cultivars. However, the data referred to here showed a significant correlation between sensitivity to ABA and the control transpiration rate (data not shown) of 5 wheat genotypes. This correlation revealed that the more rapidly transpiring cultivars (prior to ABA treatment) showed the greatest response to ABA. Could an analagous relationship hold for leaf growth ? Leaf elongation rates for CS and SQ1 were compared 3 hours into leaf elongation assays using the data from all experiments (Figure 3.7). There was no difference in the growth rates of solutions fed an artificial xylem solution, but CS shoots fed 10^{-7}M ABA showed significantly ($P<0.01$) less growth.

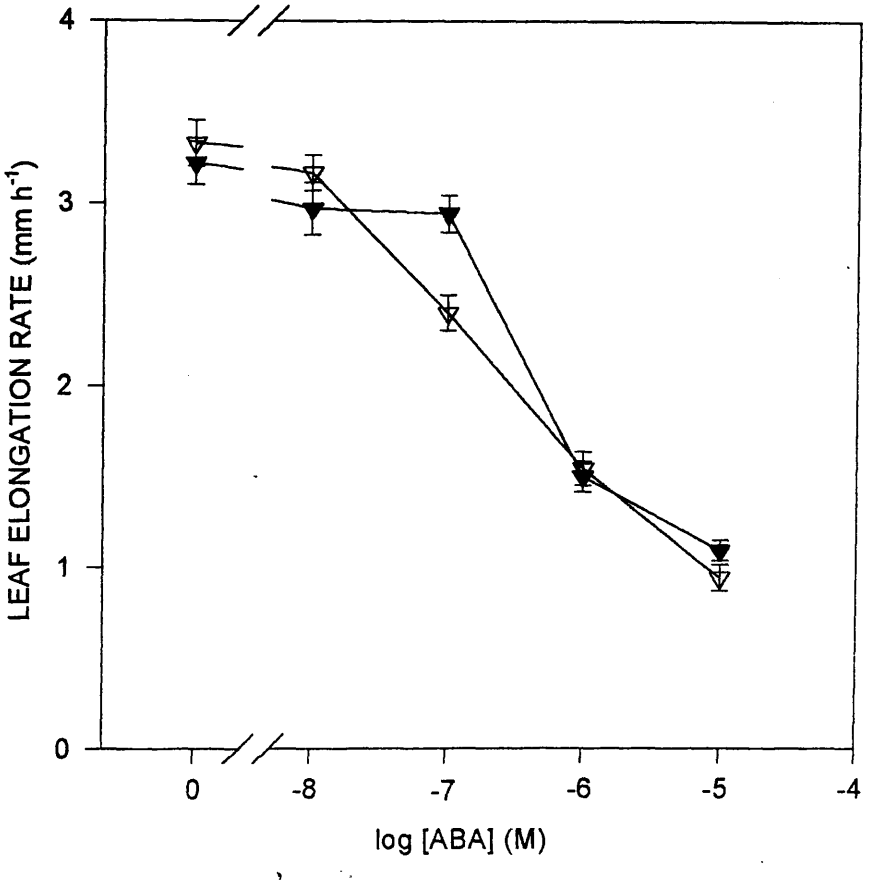

Figure 3.7: Dose-response curves for leaf elongation rate of *Triticum aestivum* cv. Chinese Spring (▽) and *Triticum aestivum* cv. SQ1 (▼). Points are means ± S.E. of at least 12 shoots from 3 experiments.

Future comparisons of genotypic differences in leaf growth response to ABA should employ a more narrow range of ABA concentrations (e.g. 0, 10^{-8}M, $5x10^{-8}$M, 10^{-7}M, $5x10^{-7}$M ABA). It is important to note that the LER data, when expressed as a percentage of controls and combined across experiments (Figures 3.5d, 3.6), showed a significant difference at 10^{-7}M in all 4 genotypic comparisons (excluding the data from the two Klaxon data sets). This confirmed the conclusions of Blum and Sinmena (1995) that this concentration best discriminated cultivar differences.

3.4 CONCLUSIONS

Identification of cultivars which differ in their sensitivity of leaf growth response to ABA seems to be fraught with difficulty, due to the apparent inconsistency of response between experiments in the one cultivar. Responses also seemed to be inconsistent between different laboratories, as the data here were unable to confirm previous differences shown by Blum and Sinmena (1995). Analysis of the results according to two different methods (ANOVA of LERs vs. plotting the percentage inhibition of growth) revealed some inconsistencies in the conclusions. Before cultivars can be indicated to differ in their ABA response, both types of analysis should give consistent results. Additionally, the value of employing a wide range of ABA concentrations in a dose-response curve is open to criticism as being not physiologically relevant.

Despite these cautions, CS seemed to be more sensitive to ABA than SQ1 when fed 10^{-7}M ABA. This difference did not seem to be related to faster growth or transpiration rates in CS. However, this conclusion is based from only 3 experiments and more rigorous experimentation is clearly needed to confirm this conclusion for plants grown and assayed under different conditions. The abundance of differences in stomatal sensitivity to ABA demonstrated by Blum and Sinmena (1995) may not hold for a more fundamental process such as growth. Despite the optimism of Munns (1992) that the leaf elongation assay would be a useful means of detecting differences in cultivar response, such differences (if indeed they exist) have proved difficult to resolve. It is therefore unlikely that genotypic variation in leaf growth response to externally supplied ABA can account for the different dose-response curves shown in Figure 2.4b, nor the difference in species response to xylem sap ABA concentration (cf. Zhang and Davies, 1990a; b vs. Munns, 1992).

CHAPTER 4.

DOES TEMPERATURE MODIFY THE EFFECTIVENESS OF ABA IN INHIBITING LEAF GROWTH ?

4.1 INTRODUCTION

Section 1.5 emphasised that the effect of ABA in bioassay and field situations can be modified by other environmental variables. It is thus unrealistic to expect a single dose-response curve for any process regulated by ABA. Two environmental factors which could influence the responsiveness of leaf growth to ABA are leaf water potential and temperature. However, a number of experiments have shown that the water relations of expanding tissues can be tightly controlled while leaf laminae show perturbed water relations (e.g. Michelena and Boyer, 1982).

Field and growth cabinet studies have revealed that temperature can exert a considerable effect on LER (Watts, 1974; Gallagher and Biscoe, 1979; Ong, 1983). Any interaction between ABA and temperature would be of considerable importance in determining plant growth under a mild water stress.

Previous demonstrations of temperature x ABA (T x ABA) interactions affecting stomatal behaviour have generally grown plants at a single temperature (pretreatment temperature) and imposed different temperatures during the assay. The significance of much of this work is questionable since data have been obtained with chilling sensitive species such as maize (Rodriguez and Davies, 1982) and bean (Eamus and Wilson, 1983; Cornic and Ghashghaie, 1991; Pardossi *et al.*, 1992; Trejo and Davies, 1994) and *Commelina communis* (Allan *et al.*, 1994; Honour *et al.*, 1995). The promotion of stomatal opening in such species when fed ABA at low temperature is thought to be an injury response due to a "locking open" of stomata. Growth of chilling-sensitive plants at a low pretreatment "hardening" temperature removed the T

x ABA interaction since stomata were able to close at low temperature in response to ABA (Eamus and Wilson, 1983).

Since a temperature x ABA interaction affecting stomata has been demonstrated in species such as *Triticum aestivum* (Ward and Lawlor, 1990), *Bellis perennis* and *Cardamine pratensis* (Honour *et al.*, 1995), which are not regarded as chilling sensitive, the phenomenon may be of general importance. The possibility that leaf growth may be similarly affected was tested in spring wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*).

4.2 MATERIALS AND METHODS

This work comprised 3 batches of plants raised under different conditions at different times of the year, as summarised in Table 4.1. Preliminary experiments, performed as described in Section 2.2, used a variety of wheat (*Triticum aestivum*) cultivars (cv.s Chinese Spring, Gaza, Highbury, Kharchia, RL 4137, Sicco, TW 161 - seed kindly provided by Dr. S.A. Quarrie) and an ABA concentration of 10^{-6}M. All other leaf elongation assays used 5 different ABA concentrations over a range of temperatures from 10°C to 30°C.

To evaluate the occurrence of any T x ABA interaction, a number of data sets were constructed, comprising leaf growth of *Triticum aestivum* cv. Tonic and *Hordeum vulgare* cv. Klaxon as well as transpiration of *H. vulgare* cv. Klaxon. Transpiration (determined gravimetrically as described in Section 2.2) was only determined for barley; wheat shoots being unsuitable as leaf 2 was too large and floppy to allow precise weight determinations. Primary data on leaf elongation or transpiration rates were log transformed to satisfy normality of variance then analysed by ANOVA in GENSTAT 5 (see Appendix 2 for table). Data are presented graphically as a percentage of the control (no ABA) values during the phase of steady state growth or

transpiration inhibition (period of maximal response) at each temperature (as in Figures 2.4b, 2.5b). Data in the graphs were subjected to linear regression analysis in MTW 10.2 to determine significance.

Table 4.1: Plant material used for leaf elongation assays. Growth environment refers to a naturally lit greenhouse (GH) or the growth cabinet (GC) referred to in Section 2.2. Maximum and minimum temperatures are means ± S.E. with the number of days in parentheses. The values in square brackets indicates the range of temperatures.

Species	Growth Environment	Temperature (°C) Maximum [Range]	Minimum [Range]	Time of Year
Triticum aestivum many cv.s	GH	12 ± 0.4 (28) [9-16]	25 ± 0.5 (28) [22-29]	Oct.- Nov.
	GC	20 ± 0.4 (13) [17-22]	29 ± 0.4 (13) [27-33]	Nov.-Dec.
Triticum aestivum cv. Tonic	GH	11 ± 0.4 (41) [6-16]	24 ± 0.3 (41) [20-27]	Jan. - Mar.
Hordeum vulgare cv. Klaxon	GH	15 ± 0.4 (32) [10-18]	26 ± 0.6 (32) [10-18]	Mar.- April
	GC	12 ± 0.4 (9) [10-14]	22 ± 0.8 (9) [18-26]	Oct. - Nov.

4.3 RESULTS

4.3.1 Preliminary experiments

Primary data on leaf growth of detached shoots of *Hordeum vulgare* cv. Klaxon which were fed a range of ABA concentrations at high (23°C) and low (10°C) temperature are given in Figures 2.4 and 4.1 respectively. Leaf growth at low temperature appeared to vary over the initial stages of the assay. This was thought to result from the frequency of measurement and the limits of accurate ruler measurement. When measurements were made less frequently toward the end of the assay, more stable growth rates were found. At low temperature, treatment differences due to ABA were more difficult to distinguish (than at higher temperature), with significant

differences in LER being noted for only 10^{-6} and 10^{-5}M ABA. Attainment of significant differences occurred later in the assay (5.5 hours at 11°C vs. 3 hours at 23°C). Expression of the steady-state values of LER as a percentage of control values revealed a different dose-response curve from that seen at 23°C (Figure 4.1b). Although there was no difference in the dose-response curve for 10^{-8} and 10^{-7}M ABA, the effectiveness of 10^{-6} and 10^{-5}M ABA was reduced at low temperature. Thus the response of leaf growth to ABA appeared to be highly temperature dependent.

This conclusion was evaluated using detached wheat shoots from a number of cultivars grown in either a growth cabinet or a greenhouse. Regardless of genotypic or pretreatment history, all data for leaf growth inhibition after 5 hours (when the effect of ABA was maximal and there had been no decline in the LER of control shoots) fitted a highly significant ($P<0.001$) relationship between leaf growth (% control) and temperature (Figure 4.2). Immediately obvious is the variability which occurred at the one temperature, which is consistent with data on transpiration bioassay variability obtained by Munns *et al.* (1993), where day-to-day variation in the sensitivity of the assay can be as much as 20 %. Despite this, a significant regression was obtained which shows that the effect of 10^{-6}M ABA increases with increasing temperature.

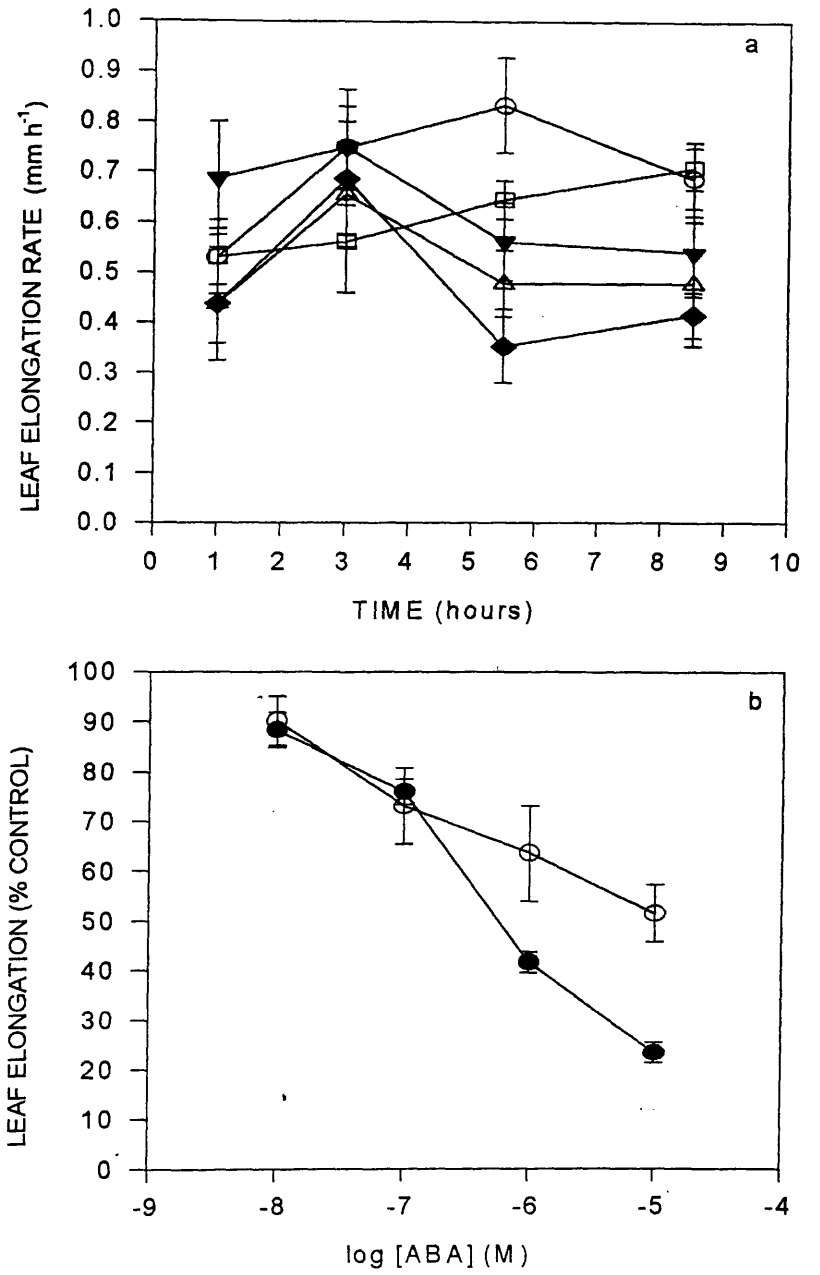

Figure 4.1a: Leaf elongation rate of detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots maintained at 11°C and supplied with artificial xylem solution (○), 10^{-8}M (□), 10^{-7}M (▼), 10^{-6}M (Δ) and 10^{-5}M (◆) ABA. Points are means ± S.E. of at least 8 shoots. (b) Dose-response curve generated from data in (a). Data (○) are expressed as a percentage of controls and are means ± S.E. of 16 measurements taken during the steady-state phase of leaf growth inhibition (5.5-8.5 hours after supplying ABA). The dose-response curve for detached shoots maintained at 23°C (●) (from Figure 2.4b) is included for comparison.

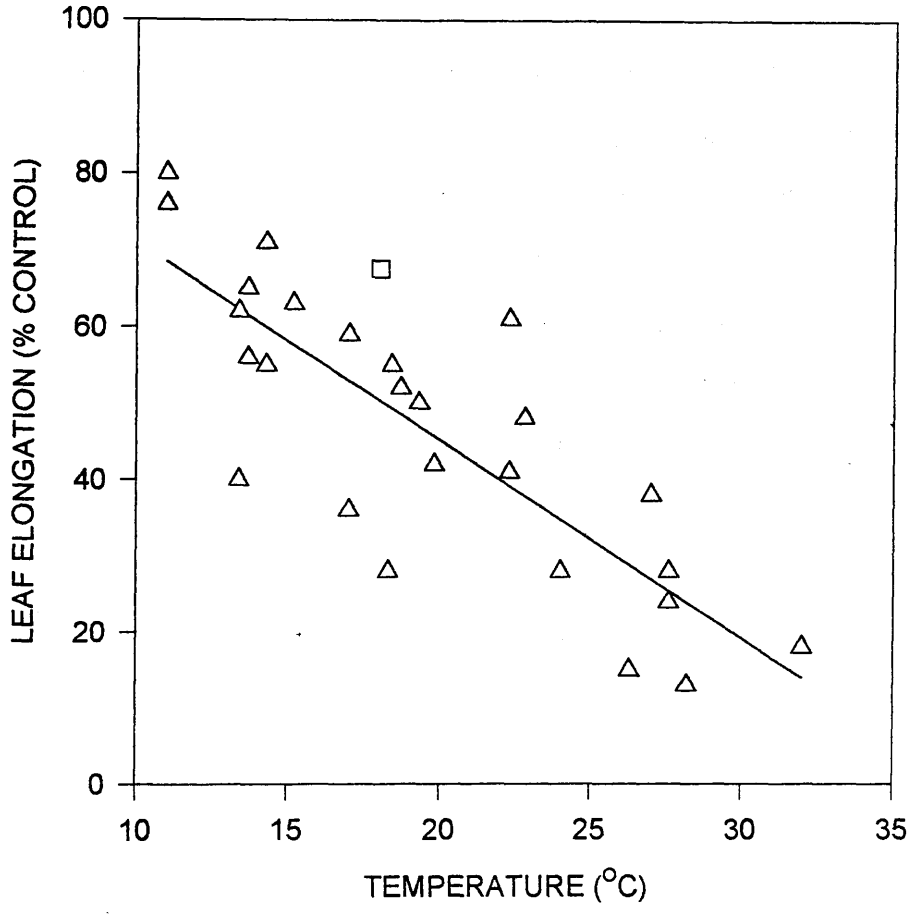

Figure 4.2: Effect of temperature on the response of leaf elongation to 10^{-6}M ABA supplied to detached wheat (*Triticum aestivum* L.) shoots of various cultivars (see text). No consistent genotypic variation in ABA response was detected. Each point is the mean value of % elongation taken 5 hours into an individual assay. Error bars omitted for clarity. Data point (□) of Munns (1992) also included (see also Figure 2.4b). Line fitted by linear regression in SPW 1.0 (LER % = 98.3-2.63T, r^2=0.66).

4.3.2 Leaf growth of *Hordeum vulgare* cv. Klaxon (Figure 4.3)

The ANOVA revealed highly significant effects ($P<0.001$) of temperature, ABA and their interaction (T x ABA) affecting leaf growth with the level of significance declining in this order. The model accounted for 64 % of the variance. The effect of time was non-significant ($P<0.05$), as expected since the primary data were rates. The interaction time x temperature was non-significant, indicating that temperature was held reasonably constant over the course each assay. The interaction time x ABA was highly significant ($P<0.001$), indicating that ABA was not immediate in its effects. However, the rapidity of ABA effects on leaf growth varied considerably between experiments (compare Figures 2.2a, 2.4a). The higher order interaction time x temperature x ABA was not significant.

The possibility that the T x ABA interaction resulted simply from the use of high ABA concentrations was examined by conducting a series of ANOVAs, sequentially removing higher ABA concentrations. The interaction remained highly significant ($P<0.001$) with only the data from 10^{-8} and 10^{-7}M ABA considered. When the data set consisted of only the control and 10^{-8}M ABA, no T x ABA interaction was detected, but there was also no significant ($P<0.05$) effect of ABA on LER.

4.3.3 Leaf Growth of *Triticum aestivum* cv. Tonic (Figure 4.4)

This data set was obtained to examine the generality of the results obtained with barley. As with barley, ANOVA revealed highly significant effects ($P<0.001$) of temperature, ABA and their interaction affecting LER. The fitted model accounted for 60 % of the variance. The interaction was not as significant as in the *H. vulgare* data (a variance ratio of 7.76 for Tonic vs. 25.38 for Klaxon). All model terms containing time (time, time x temperature, time x ABA, time x temperature x ABA) were significant ($P<0.05$).

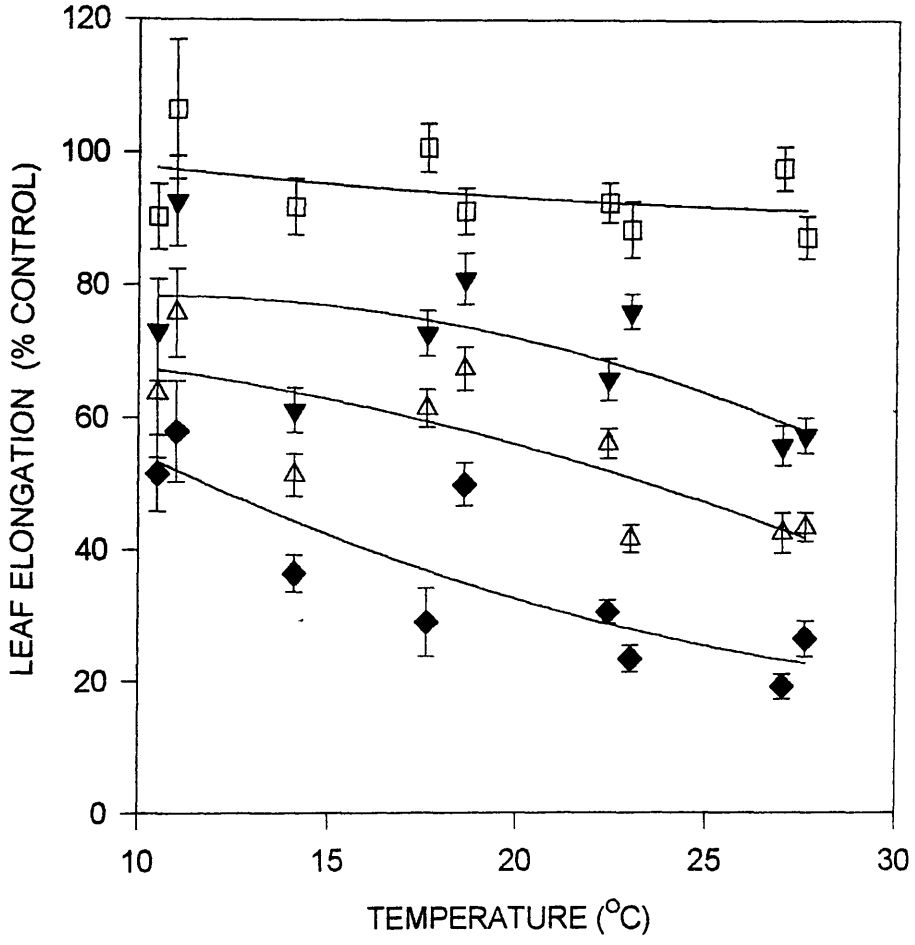

Figure 4.3: Effect of temperature on the response of leaf elongation to 10^{-8}M (□), 10^{-7}M (▼), 10^{-6}M (Δ) and 10^{-5}M (◆) ABA supplied to detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots. Point are means ± S.E. of at least 16 measurements taken during the steady-state phase of leaf growth inhibition (3-9 hours after supplying ABA) during an individual assay. Lines fitted by second order regression in SPW 1.0.

The physiological significance of the temperature x ABA interaction can also be assessed by obtaining the linear regression parameters for the data plotting leaf elongation (% control) against temperature (Table 4.2). Comparison of the slopes of the regressions indicates that the batch of Tonic plants were less sensitive to the interaction of temperature and ABA than in the other cases.

Table 4.2: Linear regression parameters for 3 data sets plotting leaf elongation (as a percentage of control) of detached shoots fed 10^{-6}M ABA at various temperatures. Intercept and slope, determined in MTW 10.2, are given with their S.D. in parentheses. Significant regressions as determined by MTW 10.2 are indicated ($P<0.05$ *), ($P<0.01$ **), ($P<0.001$ ***).

Data set	Intercept	Slope	P value	r^2
Figure 4.2	98.3 (7.6)	- 2.63 (0.37)	0.000 ***	0.66
Figure 4.3	84.2 (8.9)	- 1.48 (0.45)	0.013 **	0.61
Figure 4.4	76.2 (9.8)	- 0.84 (0.48)	0.128	0.34

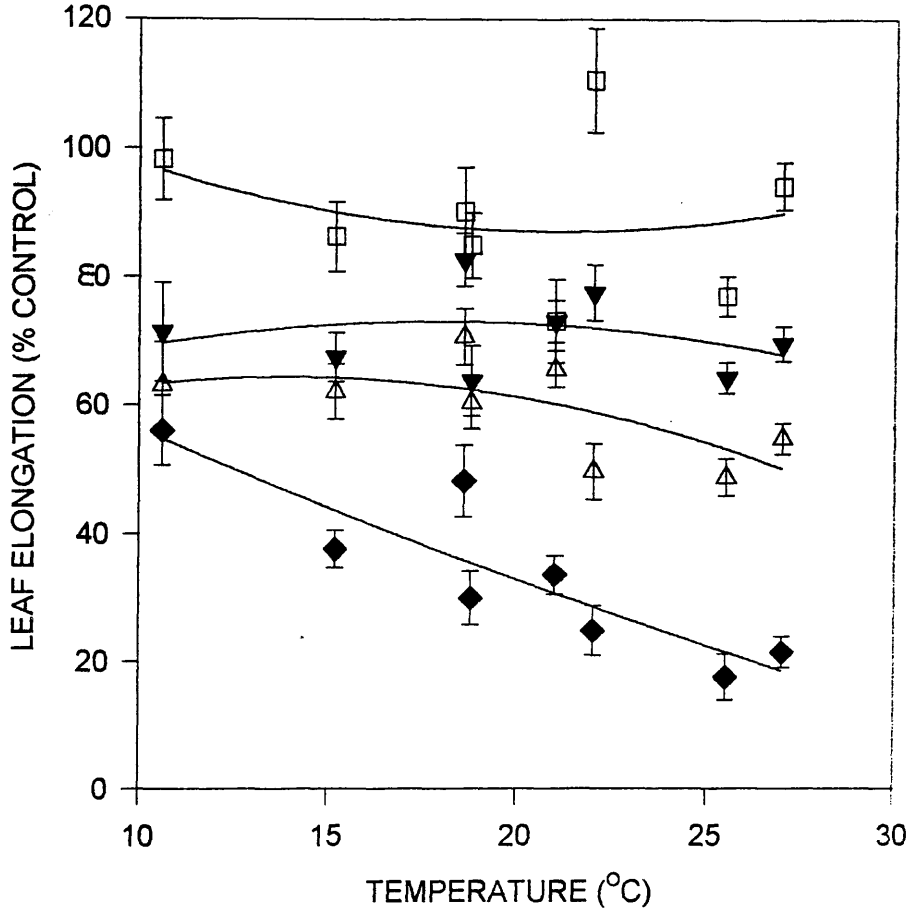

Figure 4.4: Effect of temperature on the response of leaf elongation to 10^{-8}M (□), 10^{-7}M (▼), 10^{-6}M (Δ) and 10^{-5}M (◆) ABA supplied to detached wheat (*Triticum aestivum* L. cv. Tonic) shoots. Points are means ± S.E. of at least 16 measurements taken during the steady-state phase of leaf growth inhibition (3-9 hours after supplying ABA) during an individual assay. Lines fitted by second order regression in SPW 1.0.

4.3.4 Transpiration of *Hordeum vulgare* cv. Klaxon (Figure 4.5)

Although the temperature and ABA main effects were highly significant ($P<0.001$), there was no detectable T x ABA interaction. The model accounted for 86 % of the variance. All model terms containing time were significant ($P<0.05$).

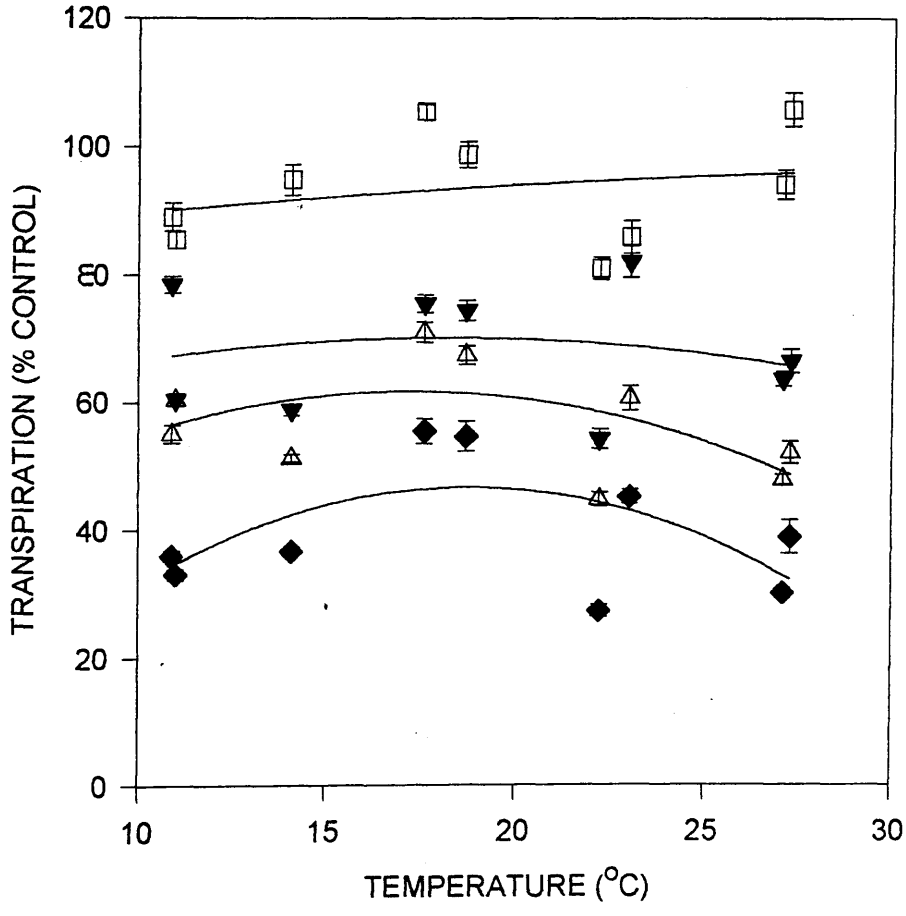

Figure 4.5: Effect of temperature on the response of transpiration to 10^{-8}M (□), 10^{-7}M (▼), 10^{-6}M (Δ) and 10^{-5}M (◆) ABA supplied to detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots. Points are means ± S.E. of at least 30 measurements taken during the steady-state phase of transpiration inhibition (1-9 hours after supplying ABA) during an individual assay. Lines fitted by second order regression in SPW 1.0.

Comparison of transpiration and leaf elongation data across different temperatures and ABA concentrations was attempted (as in Table 2.2) in the hope of determining which process was more affected by ABA treatment (Figure 4.6). The data on the steady-state rates of transpiration and LER inhibition (expressed as a percentage of control values) after ABA treatment were compared statistically by Student's unpaired t-test. If there was no difference, a value of 0 was recorded. In the case of a significant ($P<0.05$) difference, the mean value of transpiration inhibition was subtracted from the mean value of leaf elongation inhibition. Positive values indicated that transpiration was inhibited more than leaf elongation; negative values indicated that leaf elongation was inhibited more than transpiration. Linear regressions were fitted to the set of values obtained at a given ABA concentration.

Across all ABA concentrations, 44 % of experiments showed no difference in the relative response of transpiration and leaf elongation; with the percentages at 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}M ABA being 46 %, 63 %, 31 % and 44 % respectively. It should be noted that the data for 10^{-6}M ABA are biased due to the number of experiments performed at high and low temperature (where the chances of obtaining a difference in relative response are greater). At all ABA concentrations, there was a tendency for transpiration to be inhibited more at low temperatures; with leaf elongation being inhibited more at high temperatures. However, only the regressions for 10^{-7}M ($P=0.097$) and 10^{-6}M ($P<0.001$) ABA approached significance. As noted earlier, the data for 10^{-6}M ABA should be viewed with suspicion due to the uneven distribution of data points with respect to temperature. It is concluded that despite 56 % of experiments showing differences in the relative response of transpiration and leaf elongation, that there is probably no real difference in response. This contradicts the findings of Munns (pers. comm.) in the bioassay system, who found that transpiration was always inhibited more than leaf elongation in the same plants.

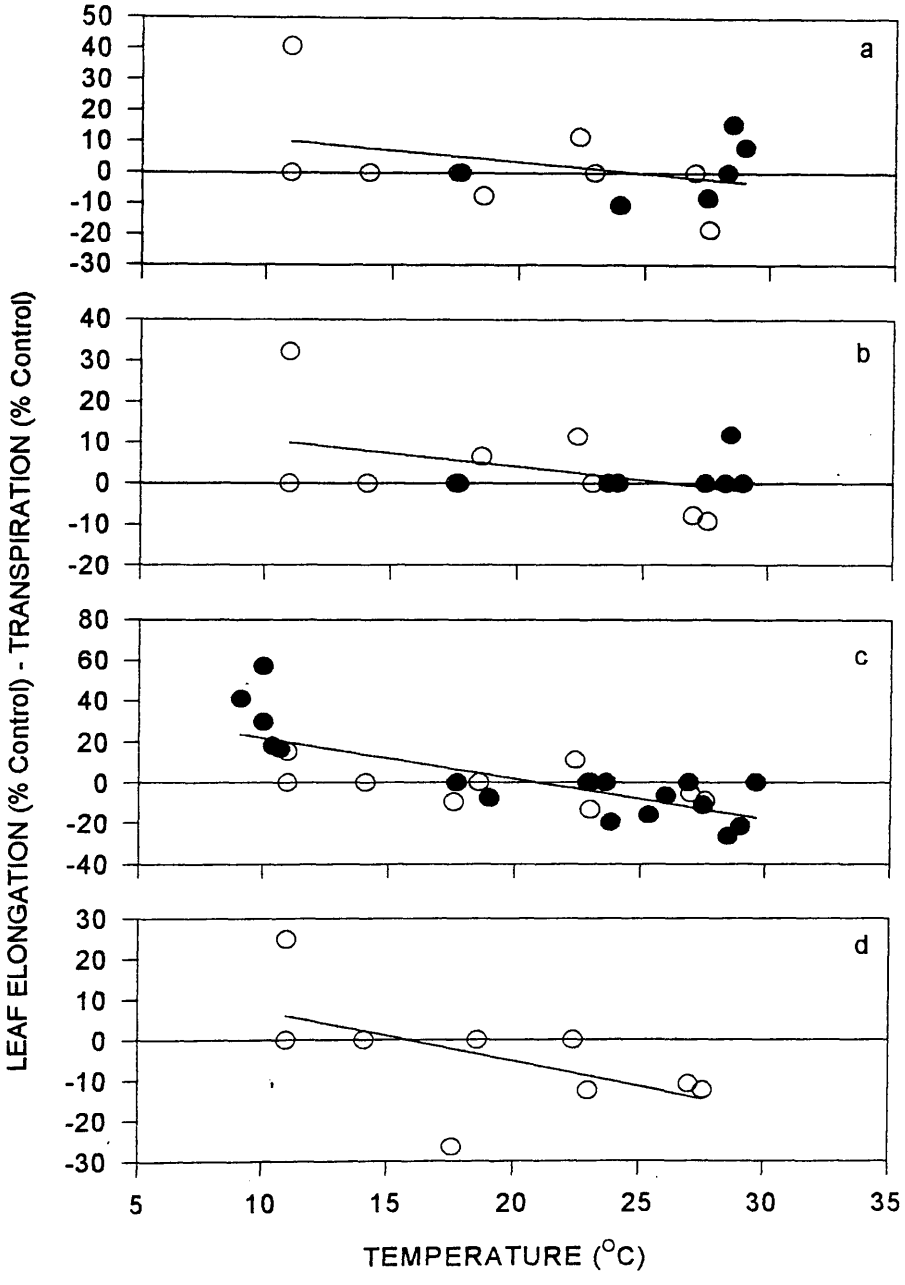

Figure 4.6: Effect of temperature on the relative sensitivity of transpiration and leaf elongation to 10^{-8}M (a), 10^{-7}M (b), 10^{-6}M (c) and 10^{-5}M (d) ABA supplied to detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots. Values of transpiration (% control) (see Figure 4.5) were subtracted from values of leaf elongation (% control) (see Figure 4.3). When there was no significant ($P<0.10$) difference between the two values, a value of 0 was recorded. Positive values indicate that transpiration was inhibited more by ABA treatment than leaf elongation; negative values indicate that leaf elongation was inhibited more than transpiration. Lines fitted by linear regression in SPW 1.0 between all points. Points were obtained from experiments summarised in Figures 4.3. and 4.5 (○) and experiments performed in Chapters 5 and 6 (●).

4.4 DISCUSSION

This chapter shows a marked temperature dependence of leaf growth responses to ABA. High temperature and soil drying commonly co-occur and therefore any interaction between the effects of the two variables may have an important influence on leaf expansion and canopy development. Definition of the nature of this interaction is important even though the mechanisms involved are unknown. It may be that at higher transpiration rates at higher temperatures, more ABA accumulates in the elongation zone than in the assays performed at low temperatures, where the evaporative demand is lower. This possibility is examined in Section 5.3.3.2.

It is also possible that the interaction may be an artefact of employing unrealistically high ABA concentrations. This is a common criticism of studies purporting to show variation in tissue sensitivity when a range of hormone concentrations are employed to generate a dose-response curve. This deficiency lead Trewavas (1991) to suggest that sensitivity should only be measured at the concentration of hormone found endogenously. It is therefore important that ANOVA of the leaf growth data showed a significant T x ABA interaction at 10^{-7}M ABA. Although this is at the upper end of the range (2-8x10^{-8}M) detected in xylem sap of wheat and barley plants growing in drying soil (Munns and King, 1988), Mulholland *et al.* (1994) showed that barley plants growing in compacted (1.7 g cm^{-3}) soil had xylem sap ABA concentrations in excess of 10^{-7}M ABA. It therefore seems that the interaction described in this Chapter may be of physiological relevance.

Temperature modulation of plant response to hormones has been previously demonstrated when the amount of hormone does not vary. Stoddart and Lloyd (1986) showed that the stimulation of LER by an application of GA_3 to wheat plants increased with temperature. Walker-Simmons (1988) observed that ABA is 100 times more effective at 30°C than at 10°C in restoring dormancy to isolated wheat embryos.

Stomatal responses to ABA have also been shown to be temperature dependent in epidermal strip bioassays, where hormone flux cannot vary with temperature (Rodriguez and Davies, 1982; Honour *et al.*, 1995) and in transpiration bioassays where flux of ABA has been measured and shown not to vary with temperature in the range that influences ABA sensitivity (Trejo, 1994). Since temperature dependence of hormone responses appears to be a common mechanism of their action, it is worth considering the implications of the interaction reported here.

It is known that well-watered plants exhibit a tight relationship between leaf growth and temperature, which can be maintained over the diurnal temperature range (Gallagher and Biscoe, 1979; Ong, 1983). Plants in drying soil show an increasing divergence from this relationship as temperature increases during the day. Gallagher and Biscoe (1979) sought to relate this divergence to changes in bulk leaf water potential, which changed from -0.2 MPa pre-dawn to a minimum of -1.5 MPa in the middle of the day. Cabinet grown maize plants from which water was withheld reached a "pre-dawn" lamina water potential of -1.5 MPa, yet the turgor of the elongation zone was not affected (Michelena and Boyer, 1982) and we must therefore argue that some factor other than a decrease in turgor is responsible for the soil drying-induced limitation of leaf growth of grasses.

It is possible to re-interpret the results of Gallagher and Biscoe in the light of our knowledge of chemical signalling. As the temperature increases over the course of the day, the divergence from the expected LER-temperature relationship increases. This may be explained by an increased amount of ABA in the elongation zone (due to the chemical signal generated that day by roots in contact with drying soil) and temperature dependent increase in sensitivity of leaf growth to ABA. The relative contribution of ABA amount and sensitivity cannot be assessed at this time.

Temperature dependent variation in the response of leaf growth to ABA arriving in the transpiration stream is likely to be an important adaptive response which allows plants to control water loss when the soil dries. Drought is often associated with high temperatures and high evapotranspiration, which can rapidly deplete soil water if plant leaf area is large. Considering that leaf area production is usually temperature dependent, the increased effectiveness of ABA in inhibiting leaf growth at high temperature represents an important means of restricting leaf area development during periods when high rates of water loss can be a problem. Since leaf growth can be more sensitive than stomatal conductance to soil drought (e.g. Saab and Sharp, 1989), this mechanism represents an early response to chemical signals produced in the roots.

CHAPTER 5.

ABA ACCUMULATION IN THE LEAF ELONGATION ZONE OF DETACHED BARLEY SHOOTS

5.1 INTRODUCTION

A possible explanation for the apparent temperature-dependence of the leaf growth response to ABA (see Chapter 4) may be that different amounts of ABA accumulate in the growing cells under different environmental conditions. A given xylem sap ABA concentration may be more effective at high temperature because the transpirational flux is higher, delivering more ABA to the site(s) of action.

The experiments described in this Chapter aim to provide information on ABA concentrations in the elongation zone (EZ) in the leaf elongation assay system. Such data may permit a comparison of this system with intact plants subjected to soil drying (Chapter 7). Before measurements of ABA concentration in the EZ were made, it was first necessary to define the extent of the elongation zone in the detached shoot system.

5.2 SPATIAL DISTRIBUTION OF GROWTH AND ABA CONCENTRATION IN THE LEAF ELONGATION ZONE OF DETACHED SHOOTS

5.2.1 INTRODUCTION

The grass leaf meristem is located at the base of the stem and is enclosed by the sheaths of older leaves. Cell division occurs exclusively at the base of stem, then files of cells move through an elongation zone. The length of this elongation zone is highly dependent on genotypic, developmental and environmental factors. Cells in the exposed lamina have ceased elongating.

Definition of the elongation zone has usually been attempted by destructive techniques where cells in the elongation zone are marked in some way and the displacement of marks measured at a later time. In ink marking methods, the surrounding fully expanded leaves are peeled away and the leaf marked with indelible ink (Volenec and Nelson, 1981). A more commonly used technique involves piercing the base of the stem with a series of pins mounted at known distances (Davidson and Milthorpe, 1966; Kemp, 1980; Schnyder *et al.*, 1987). Results obtained from these two techniques show essentially the same spatial pattern of growth (Schnyder *et al.*, 1987). Since such methods injure the plant and reduce LER, an alternative technique is the use of anatomical markers as suggested by Silk *et al.* (1989). Markers that have been used include cell lengths (Volenec and Nelson, 1981; Schnyder *et al.*, 1990), interstomatal distances (Paolillo *et al.*, 1991), and patterns of separation of rings and gyres in the walls of protoxylem vessels (Paolillo and Sorrells, 1992). Comparison of destructive and anatomical techniques has been shown to give the same spatial distribution of growth in some studies (Schnyder *et al.*, 1990) but not in others (Paolillo and Sorrells, 1992).

Since the grass elongation zone contains a gradient of cells at different stages of maturity, gradients of major nutrients (Meiri *et al.*, 1992); cell wall loosening enzymes such as peroxidases (MacAdam *et al.*, 1992 a, b) and xyloglucan endotransglycosylase (Palmer and Davies, 1996); and carbohydrates (Spollen and Nelson, 1994) exist in this tissue. No data could be found in the literature on the spatial distribution of ABA in elongating grass leaves, although ABA showed a pronounced spatial gradient of accumulation in maize mesocotyls of seedlings grown at low water potential (Saab *et al.*, 1992). Accordingly, the spatial distribution of ABA in response to uptake via the sub-crown internode was studied.

5.2.2 MATERIALS AND METHODS

5.2.2.1 Spatial distribution of growth

The marking method of Schnyder *et al.* (1987) was employed. A mounted set of 10 entomological pins, each 2 mm apart, was used to pierce the elongation zone starting from the middle of the crown. Two sets of pin marks spanned the basal 36 mm of stem immediately after transfer of the shoots to their feeding solution. Eight shoots were marked per treatment. LERs of marked and unmarked shoots were measured during the assay. After a specified period of time (according to leaf growth rate), the first and second leaves of the shoot were stripped away to expose the third leaf. Using an eyepiece graticule (Ernst Leitz Wetzlar GMBH, D-6330 Wetzlar, Germany), the distance between successive pin marks on the third leaf was recorded. This distance was divided by the original distance between pin marks (2 mm) and the time elapsed to give the measured Relative Segmental Elongation Rate ($RSER_{meas}$) in h^{-1}. The growth inhibition caused by marking (which ranged from 27-50 % according to temperature and ABA treatment - see Table 5.2) was corrected for in the calculation of actual RSER according to the equation:

$$RSER = \frac{RSER_{meas}}{LER_{marked} / LER_{unmarked}}$$

Data on RSER were collected for AX-fed and 10^{-6}M ABA-fed barley shoots after 6 hour and 10 hour periods of growth at temperatures of 27°C and 11°C, respectively.

5.2.2.2 Spatial distribution of ABA concentration

Every 2 hours during leaf elongation assays performed under the same conditions as in Section 5.2.2.1, 8 detached shoots per treatment were removed for

analysis of ABA content. Shoots had their outer leaves removed to reveal leaf 3. This leaf was sectioned with a parallel series of razor blades, mounted 3 mm apart. Each sample also contained tissue from leaves of a higher serial number enclosed within the third leaf. Six mm segments were collected from the elongation zones of 8 shoots (necessary to bulk samples to provide enough tissue for one ABA determination) during the leaf elongation assays. Samples were collected over salted ice then stored at -20°C. Samples were then freeze-dried, powdered and extracted in deionised water (extraction ratio 1:30 for control plants; 1:50 for 10^{-6}M ABA-fed plants) at 5°C overnight. Samples were then centrifuged and the supernatant removed for analysis by the radioimmunoassay (RIA) protocol of Quarrie *et al.* (1988) as described below. Aqueous extracts of maize, barley and wheat leaves are reported to have immunoreactive contamination of between 5-15 % (Quarrie *et al.*, 1988; Quarrie pers. comm.). The assumption that barley elongation zones would show acceptable levels of contamination was tested by thin layer chromatography (TLC) of aqueous extracts (see Section 5.2.2.4), which revealed no significant contamination. Data on ABA concentrations have not been corrected for contamination.

5.2.2.3 Radioimmunoassay (RIA) for ABA

The ABA concentration of aqueous extracts of barley leaves and elongation zones was determined using a competitive RIA using the label, DL- *cis, trans* [^{3}H] ABA (Amersham plc, Little Chalfont, England) and the antibody AFRC MAC 252 (Dr. S. Quarrie, Cambridge Laboratory, Norwich, England), a re-cloned version of AFRC MAC 62, with which the immunoassay protocol (see below) was developed (Quarrie *et al.*, 1988; Quarrie, pers. comm.). AFRC MAC 62 is highly specific for the free acid (+)-ABA, as the cross reactivity table (Table 5.1) shows. AFRC MAC 252 is assumed to have the same cross-reactivity as MAC 62. Since the leaf elongation assays employed here have fed (±)-ABA to the detached shoots, it was considered important to determine that the antibody was only recognising (+)-ABA. This test was performed

by Quarrie (pers. comm.), who showed that a 125 pg (+)-ABA / 50 µL standard gave the same number of counts in the RIA using AFRC MAC 252 as did a 250 pg (±)-ABA / 50 µL standard. The reported ABA concentrations are for (+)-ABA, the physiologically active form.

The high specificity of AFRC MAC 252 allowed aqueous extracts to be used without prior purification. The immunoassay protocol is given below. Standards of synthetic (±)-ABA of known dilutions (125-2000pg (+)-ABA / 50 µL) were made up (as 250-4000 (±)-ABA / 50 µL) in deionised water. Other "standards" of water and a high ABA concentration (10^{-3}M) were used to determine the maximum binding (B_{max}) and non specific binding (B_{min}) respectively. All standards were assayed in duplicate in each batch of samples, while each sample was assayed once. A standard curve of counts recovered versus (+)-ABA added was produced for each batch of samples. The standard curve of counts per minute (B) against known ABA concentrations was linearised, after subtraction of B_{min} from all values (Figure 5.1), using the logit transformation to allow the calculation of a gradient, intercept and correlation coefficient. The logit transformation of variable B was given by:

$$\text{logit } B = \ln \frac{B / B_{max}}{1 - B / B_{max}}$$

Sample counts were converted to tissue ABA concentrations using the linearised plot. A successful assay was indicated by a correlation coefficient (r^2) greater than 0.99.

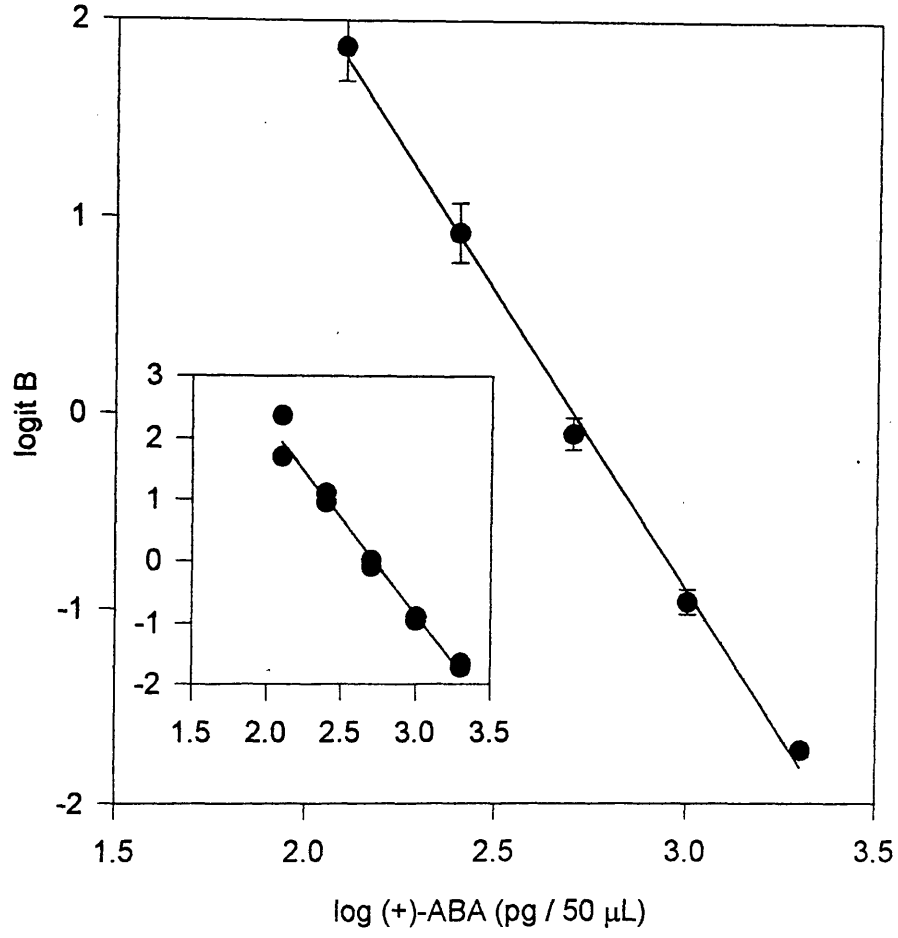

Figure 5.1: Standard curve for the RIA, plotting the number of counts (B) against known ABA concentrations. The main graph gives means ± S.D. of 4 replicate assays done on the same day; the inset gives the duplicate standards for one of these assays. Lines fitted by linear regression in SPW 1.0.

Table 5.1: Cross-reactivity of the monoclonal antibody MAC 62 with ABA and with some of its derivatives and metabolites (from Quarrie *et al.*, 1988).

Compound	Percentage cross-reactivity
(+)-2-*cis*-abscisic acid	100
(+)-2-*trans*-abscisic acid	<0.1
(±)-2-*cis*-abscisic acid	49
(+)-2-*cis*-abscisic acid methyl ester	0.4
(+)-2-*cis*-abscisic acid glucose ester	<0.1
phaseic acid	<0.1
dihydrophaseic acid	<0.1
xanthoxin	<0.1

Immunoassay Protocol:

1. Into a 2 mL Eppendorf tube (vial) add (in order)

- 200 μL of 50 % phosphate buffered saline (PBS) (50 mM NaH_2PO_4 and 100 mM NaCl, adjusted to pH 6.0)
- 50 μL of standard or sample
- 100 μL of tritiated ABA (*circa* 8000 cpm) dissolved in buffer solution (5 mg globulin / mL PBS)
- 100 μL of MAC 252 dissolved in buffer solution (5 mg bovine serum albumin and 4 mg polyvinylpyrrolidone / mL PBS).

Vials are contained in foam racks, 40 vials per rack.

2. Cap the vials, mix thoroughly and incubate in the dark at 4°C for 45 minutes.
3. Centrifuge at 8000g for 1 minute to remove any liquid on caps.
4. Remove caps, add 500 μL saturated NH_4SO_4 to each tube to precipitate antibody-antigen complex.
5. Mix thoroughly and incubate in the dark at room temperature for 30 minutes.
6. Centrifuge at 8000g for 4 minutes to precipitate pellet.
7. Remove caps and place on absorbent towelling to remove moisture.
8. Gently turn vials, in their foam rack, upside down to remove supernatant.
9. Place vials upside down on absorbent towelling for 2 minutes, tap gently to remove any remaining supernatant.
10. Add 1mL 50 % saturated NH_4SO_4, cap vials and resuspend pellet.
11. Centrifuge at 8000g for 5 minutes to precipitate pellet.
12. Remove caps and place on absorbent towelling to remove moisture.
13. Gently turn vials, in their foam rack, upside down to remove supernatant.
14. Place vials upside down on absorbent towelling for 2 minutes, tap gently to remove any remaining supernatant.
15. Add 100 μL deionised water to each vial and resuspend pellet.
16. Add 1.5 mL of scintillation cocktail, Ecoscint H (National Diagnostics, NJ, USA) and mix thoroughly.
17. Place vials into empty 20 mL scintillation vials and count once for 6 minutes on a scintillation counter (Tri-carbon 300, Canberra Packard, Pangbourne, England).
18. Data are converted from counts per minute (cpm) to ABA concentrations via the standard curve.

5.2.2.4 Thin Layer Chromatography (TLC) for detection of immunoreactivity in aqueous extracts

Barley plants were grown as in Section 2.2 until leaf 3 was ready to conduct a leaf elongation assay. The plants were left unwatered for 3 days prior to collection of tissue. The first and second leaves were removed and the portion 10-40 mm from the node of leaf 3 was removed from a number of plants. The sample was freeze-dried, powdered and extracted in deionised water (extraction ratio 1:25).

Pre-prepared silica gel TLC plates (Aldrich, Gillingham, Dorset, England) were cleaned by washing overnight in a 20:80 methanol:ethyl acetate mixture. After drying, they were marked with a soft pencil as in Figure 5.2. The silica from Zone B was carefully removed to isolate the ABA marker application zone from the aqueous extract (sample) application zone. Two plates, representing the extract and a water control, were run simultaneously. In the marker and sample application zones, respectively, 5 μL of 10^{-3}M ABA and 100 μL (repeated applications were necessary) of the extract (or control) were placed. The application zones were concentrated by placing the plate in a tank of ethyl acetate / water; and allowing the solvent to run to the edge of the application zone twice. The plates were then run twice until the solvent front reached Zone 10, with the plates allowed to dry between each run. The edge of the plate was briefly placed under a UV lamp to visualise the location of the ABA marker. The silica from Zone D was then removed (to avoid edge effects). Each zone (1-10) of silica was then carefully scraped off into an Eppendorf tube. To each tube, 200 μL of water was added to dissolve the silica prior to assaying each solution in duplicate in the RIA. The immunoreactivity (expressed in ABA equivalents / 50 μL) of each zone in the extract was calculated by subtracting the corresponding values for the water control. The distribution of immunoreactivity in barley elongation zones is given in Figure 5.3. The marker showed ABA to be in Zones 8 and 9. The percentage of immunocontamination was calculated as:

100- [(Immunoreactivity in Zones 8+9 / Total Immunoreactivity) x 100]

and found to be 16 %. This relatively insignificant amount of contamination meant that it was possible to assay barley elongation zones for ABA without prior purification.

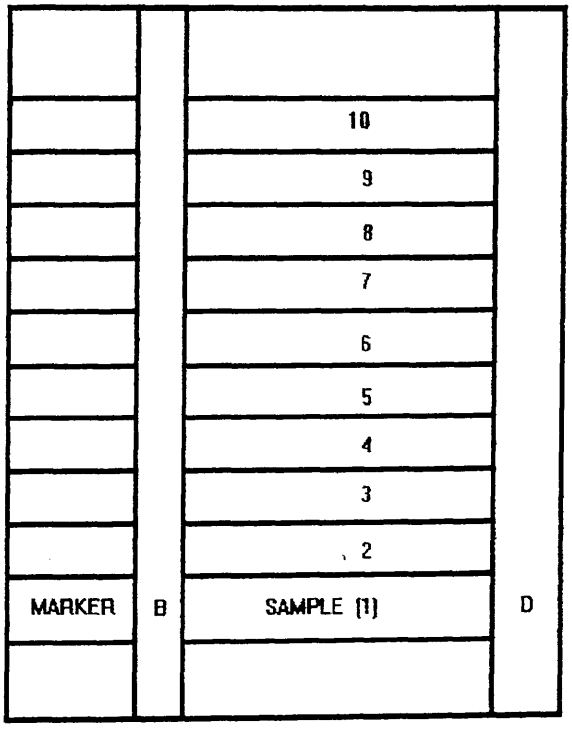

Figure 5.2: Marking on TLC plate to delineate marker and sample application zones, buffer strip (B) and silica removed at end (D) to minimise edge effect.

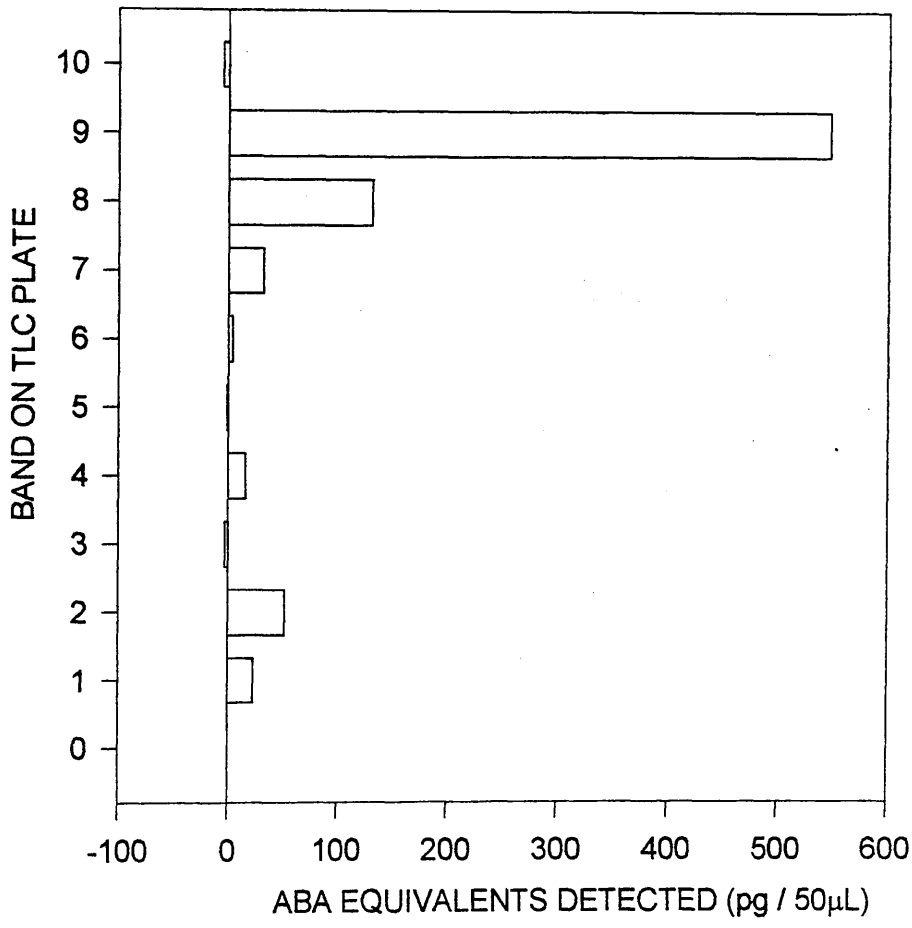

Figure 5.3: Immunohistogram of an aqueous extract of barley (*Hordeum vulgare* L. cv. Klaxon) leaf elongation zones. The bands on the y axis correspond to those marked on Figure 5.2. The ABA equivalents on the x axis are derived by subtracting the ABA eq.s in a water control from the ABA eq.s actually detected in the assay of the sample. Each band for both control and sample were assayed in duplicate. Each bar on the graph is the mean value.

5.2.3 RESULTS

In all treatment combinations, marking the detached shoots with needles was found to have a significant ($P<0.05$) effect on LER. At high temperature, marking reduced LER of control plants by 50 % from 3.2 mm h^{-1} to 1.6 mm h^{-1} (Table 5.2). In all other treatments, marking reduced growth by 23-31 %. These values compare with reported values of 21 to 41 % for similarly marked intact *Festuca arundinacea* plants (Schnyder *et al.*, 1987), and 40 % for marked *Zea mays* plants (Ben-Haj-Salah and Tardieu, 1995). Schnyder *et al.* (1987) showed that the effect of marking decreased with time elapsed, so that LER may be reduced by as much as 70 % after 1 hour and only 40 % by 6 hours. Hence the effect of marking in experiments with barley do not appear excessive given the time period of measurement. It is important to note that marking does not influence the spatial distribution of growth (Schnyder *et al.*, 1987).

The effects of temperature and ABA in unmarked plants were consistent with the responses seen in Chapters 2 and 4. ABA reduced LER in unmarked plants by 16 % (but not significantly) at 11°C and by 49 % at 27°C. At low temperature, there was no significant effect of marking on the ABA response (LER expressed as a percentage of control shoots). However, at 27°C, marking was found to reduce the inhibition of growth caused by ABA from 49 % to 23 % (Table 5.2).

Table 5.2: Effect of marking, temperature and feeding solution on leaf elongation rate (LER) and growth inhibition of detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots. Growth inhibition was calculated as 100 - [$LER_{Treatment}$ / $LER_{Control}$ x 100]. Feeding solutions were an artificial xylem solution (AX) and 10^{-6}M ABA made up in AX. LERs are means ± S.E. of the number of replicates in parentheses. Measurements taken 3-5 (27°C) and 3-8.5 (11°C) hours into each assay.

Temperature	Treatment	Leaf elongation rate (mm h^{-1}) Unmarked	Marked	*Marking-induced growth inhibition (%)*
27°C	AX	3.16 ± 0.11 (16)	1.59 ± 0.09 (16)	*50*
	10^{-6}M ABA	1.61 ± 0.16 (14)	1.21 ± 0.09 (14)	*24*
ABA-induced growth inhibition (%)		*49*	*23*	
11°C	AX	0.59 ± 0.06 (24)	0.40 ± 0.05 (27)	*23*
	10^{-6}M ABA	0.49 ± 0.05 (24)	0.34 ± 0.04 (24)	*31*
ABA-induced growth inhibition (%)		*16*	*19*	

Figure 5.4a shows the spatial distribution of growth in the elongation zone of detached barley shoots for 2 different temperatures and feeding solutions. The plotted growth trajectory was of the "local maximum" (*sensu* Paolillo and Sorrells, 1992) type; as found for other published studies of spatial distribution of growth quantified using needle marking techniques. The maximal RSER increased from 0.03 h^{-1} at 11°C to 0.20 h^{-1} at 27°C in AX-fed plants. While growth at 27°C resulted in a pronounced local maximum RSER between 10 and 20 mm; at 11°C the maximum RSER occurred over a greater length of the elongation zone. Thus low temperature flattened the growth profile. ABA feeding had a negligible effect on the growth profile at 11°C but inhibited growth at all positions in the elongation zone at 27°C. Neither temperature nor feeding ABA changed the length of the elongation zone.

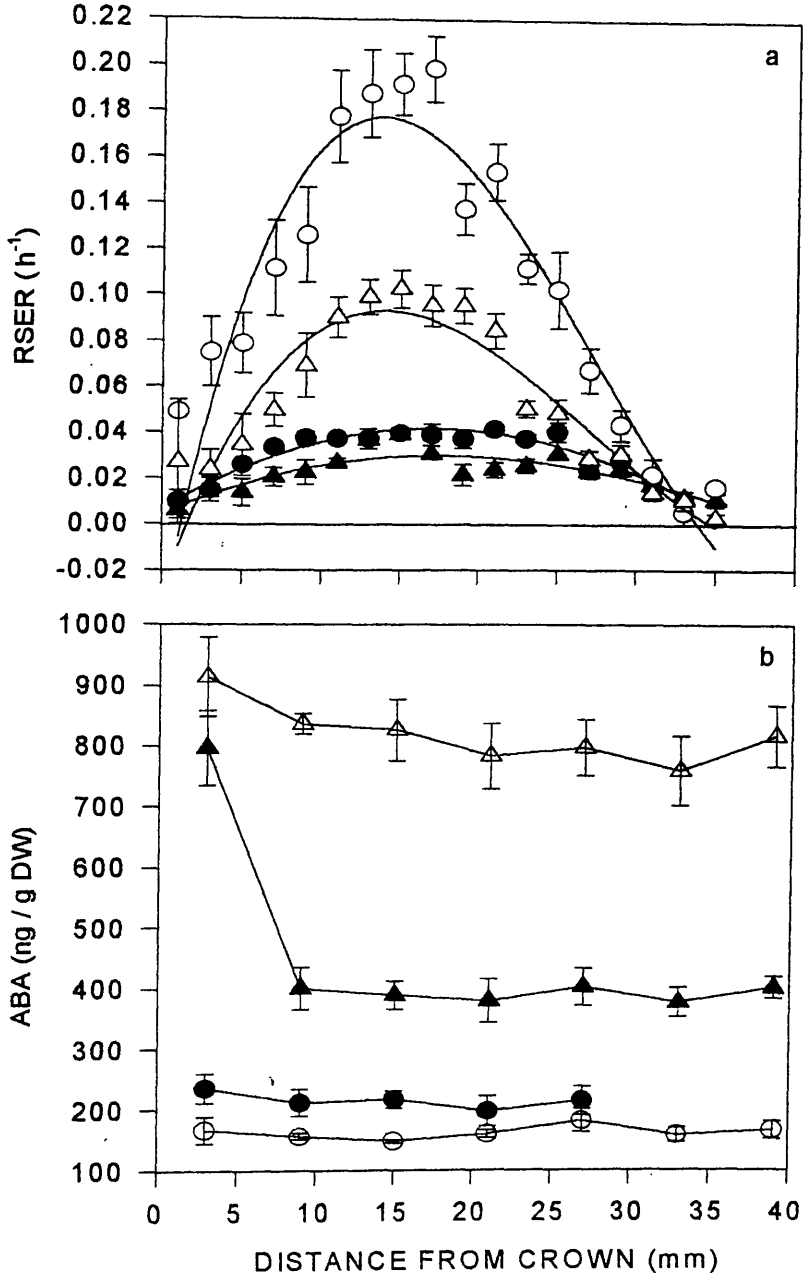

Figure 5.4: Spatial distribution of Relative Segmental Elongation Rate (RSER) (a) and ABA content (b) in detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots fed artificial xylem solution (○, ●) or 10^{-6}M ABA (Δ, ▲) at temperatures of 11°C (closed symbols) and 27°C (hollow symbols). Growth determinations were made after 6 (27°C) and 10 (11°C) hours. Lines in (a) are third order regressions fitted in SPW 1.0. Points are means ± S.E. of 8 shoots. ABA determinations are means ± S.E. of bulked samples (8 shoots required to make up sufficient sample) taken at 3 times during each leaf elongation assay when leaf growth inhibition was maximal.

For accurate analysis of growth, investigations should be elemental and instantaneous (Silk, 1984). In other words, the segments should be short in relation to the length of the EZ and elongate little between the time of marking and the time of measurement. Elongation of the marked control plants at 27°C over the measurement period was 9.6 mm, which is 27 % of the EZ length. At 11°C, 4 mm of growth occurred, representing 10 % of the EZ. These percentages are similar to those reported by other workers (e.g. 16 % -Schnyder *et al.*, 1987; 27 % - Meiri *et al.*, 1992; 14-32 % - Bernstein *et al.*, 1993) and thus may be taken as acceptable approximations of the instantaneous rates. The 2 mm segment length adopted in this study represents a compromise between high spatial resolution of growth and the increase in experimental error that would result from measuring distances between closer-spaced holes.

Figure 5.4b shows the spatial patterns of ABA accumulation (expressed on a dry weight basis) for barley plants either fed an AX solution or 10^{-6}M ABA at temperatures of 11°C and 27°C. For shoots which were fed 10^{-6}M ABA at low temperature there was a pronounced elevation of ABA concentration in the basal 10 mm of the leaf. The constancy of ABA content from 10-40 mm in all treatments pointed to this region being an appropriate one to sample in replicated measurements of bulk elongation zone ABA content, to allow greater temporal resolution than the 2 hourly determinations made here.

5.2.4 DISCUSSION

It was interesting that neither low temperature nor ABA treatment altered the length of the EZ in detached barley shoots although both treatments reduced leaf elongation rate. This is in contrast to the positive relationship between extension growth and the length of the EZ found for other treatments such as irradiance (Schnyder and Nelson, 1989), nitrogen nutrition (Volenec and Nelson, 1983), salinity

(Bernstein *et al.*, 1993), water deficit (Spollen and Nelson, 1994; Durand *et al.*, 1995) and genetic differences (Volenec and Nelson, 1981; Paolillo and Sorrells, 1992; Schunmann *et al.*, 1994) where reduced leaf extension growth has always been found to be associated with a shorter EZ. One possibility for the lack of this relationship in the current work may be the short time period over which the temperature and ABA treatments were applied, which may not have allowed time for the expression of altered EZ length. This was necessitated by the finite period of normal growth in the detached shoot system (as outlined in Section 2.3.1). Thus growth of cells in the first 2 hours of the assay (before the effect of ABA was maximal) may have resulted in the maintenance of EZ length under ABA treatment.

Evidence from similar spatial studies of growth in the maize primary root show that temperature does not affect EZ length (Pahlavanian and Silk, 1988) over a temperature range of 16 to 29°C. This contrasts with the effect of temperature on leaf growth of maize plants, where EZ length of leaf 6 declined from 75 mm at 25°C to 55 mm at 17°C (Ben-Haj-Salah and Tardieu, 1995). This reduction may contribute to the well-recognised (e.g. Capell and Dorffling, 1993) chilling sensitivity of maize. There is a lack of data on EZ length in response to temperature for chilling tolerant plants, so it cannot be said whether the response of detached barley shoots shown here is consistent with that of intact plants.

The effect of ABA accumulation on spatial patterns of mesocotyl growth varies according to soil water potential (Saab *et al.*, 1992). At high water potential, reduction of mesocotyl ABA concentration by application of the carotenoid biosynthesis inhibitor fluridone had little effect on EZ length. This result supports the leaf growth data presented here, which showed that varying the ABA concentration in the leaf elongation zone had no effect on EZ length. The maintenance of turgor by leaf elongation zones (Michelena and Boyer, 1982) suggests that the effects of ABA may be best studied at high water potential. At low water potential, fluridone treatment

restored mesocotyl EZ length (Saab *et al.*, 1992), indicating that ABA was reducing EZ length. However, the relevance of such results to leaf growth of intact, droughted plants may be questionable as the fluridone experiments were conducted with etiolated, non-transpiring seedlings.

Despite extensive validation, the needle-marking method used here continues to draw criticism since it lowers LER over the period that spatial growth analysis occurs (Paolillo and Sorrells, 1992). Thus it is necessary to adjust the measured RSER according to the marking-induced growth reduction. While anatomical markers may be a more suitable means of obtaining spatial growth distributions free of potential wounding artefacts, there is no suggestion that needle marking alters EZ length. Since neither ABA nor temperature altered EZ length, the area for analysis of ABA content has been defined.

5.3 MEASUREMENT OF ABA IN THE BULK ELONGATION ZONE OF DETACHED SHOOTS

5.3.1 INTRODUCTION

Although many studies of plants grown in drying soil have measured ABA concentrations in the roots (e.g. Zhang and Davies, 1989a; Tardieu *et al.*, 1992a), bulk leaves (e.g. Blackman and Davies, 1985; Zhang and Davies, 1989a), leaf epidermes (Zhang *et al.*, 1987), elongating stems (e.g. Creelman *et al.*, 1990) and xylem sap (e.g. Loveys, 1984; Zhang and Davies, 1989b), little data could be found in the literature on the ABA contents of the elongating cells of leaves. If the bioassay data from Chapter 4 are to be of use in interpreting the responses of whole plants, it becomes important to measure ABA accumulation in the leaf elongation zone.

Accounting for physiological effects in terms of tissue hormone concentrations has not proved universally successful in the field of ABA research. Early attempts to correlate stomatal closure with leaf ABA content showed that stomata could close in the absence of increased ABA (Beardsell and Cohen, 1975; Burschka *et al.*, 1983; Blackman and Davies, 1985) and stay closed after water stress when ABA concentrations had returned to basal levels (Beardsell and Cohen, 1975). However, Zhang *et al.* (1987) were able to account for stomatal closure in split-root *Commelina* plants in terms of epidermal ABA concentrations, when the ABA of the bulk leaf had not increased. This result was attributed to the epidermis' low buffering capacity for ABA. Recently, Trejo *et al.* (1993) were able to account for differences in apparent sensitivity of stomatal closure in different assay systems in terms of epidermal ABA concentrations, when the concentration of ABA fed to the stomata was the same. In this context, it seemed worthwhile to attempt to account for the variable leaf growth inhibition seen at one feeding ABA concentration in the leaf elongation assay system (Figure 4.2) in terms of ABA concentration in the EZ. Since the ABA concentration in the leaf elongation zone was relatively constant over a considerable length (Figure 5.4b), the experiments in this section sampled a portion of the elongation zone 10-40 mm from the node.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Dose-response experiments

Leaf elongation assays with barley plants (*Hordeum vulgare* cv. Klaxon) were performed as described in Section 2.2 at 25°C using a range of ABA concentrations. Periodically, 5 detached shoots were removed per treatment for sampling of the ABA content of leaf 2 (the principal transpiring organ of the shoot) and the region of the EZ 10 to 40 mm from the crown. Samples were frozen at -20°C before being freeze-dried.

Dried samples were powdered before being extracted in deionised water at a ratio dependent on the ABA concentration which they were fed (1:40 for control, 10^{-8}M and 10^{-7}M; 1:50 for 10^{-6}M). The average dry weight of the EZ was 0.02g, which provided enough extract (50 μL) for one ABA determination. To maintain consistency, one ABA determination of each leaf was performed. ABA analysis was performed according to the method of Quarrie *et al.* (1988) as described in Section 5.2.2.3.

5.3.2.2 Temperature experiments

Leaf elongation assays with barley plants (*Hordeum vulgare* cv. Klaxon) were performed as described in Section 2.2 over a range of temperatures and using 10^{-6}M ABA. This concentration was chosen as it was known to give a wide range of effects on leaf growth (e.g. Figure 4.2). ABA was measured in leaf 2 and the region of the EZ 10 to 40 mm from the crown. Processing of samples for ABA determination was as described above and the RIA procedure as described in Section 5.2.2.3.

5.3.2.3 ABA accumulation in detached, dehydrated tissues

Hordeum vulgare cv. Klaxon plants were grown as described in Section 2.2 in preparation for a leaf elongation assay. Plants were well-watered until leaf 5 was 5-10 cm in length. Shoots were then excavated, leaves 2 and 4 detached and a section 5 cm long cut from the middle of each leaf. The leaves surrounding the elongation zone of leaf 5 were removed, and a portion of the EZ 10-40 mm from the node was removed. The tissue portions of 6 shoots were then placed on a piece of aluminium foil on a balance (Precisa 125A, PAG Oerlikon, Zurich, Switzerland) in a growth cabinet and allowed to dehydrate until they had lost 10 % of their fresh weight. The pieces of aluminium foil containing the tissue portions were then placed on a saturated piece of

filter paper in a Petri dish. The lid of the Petri dish was replaced and the dishes placed in the dark for 18 hours to incubate at high relative humidity. The tissue portions from each Petri dish were then placed in Eppendorfs, stored at -20°C, freeze-dried, and subjected to ABA analysis as described in Section 5.2.2.3.

5.3.2.4 Experiment where ABA was withdrawn after two hours of feeding

A leaf elongation assay with barley plants (*Hordeum vulgare* cv. Klaxon) was performed as described in Section 2.2 at 24°C and using 10^{-6}M ABA. After 2 hours, a subset of plants fed 10^{-6}M ABA was transferred to artificial xylem solution. Every 2 hours, 5 detached shoots were removed per treatment for sampling of the ABA content of leaf 2 and the region of the EZ 10 to 40 mm from the crown. Collection of samples and ABA determination were as described above.

5.3.3 RESULTS

5.3.3.1 Dose-response experiments

Figure 5.5 shows the ABA accumulation over time in the elongation zones of detached shoots at the one temperature over a range of ABA concentrations. At all feeding concentrations, most accumulation occurred within the first 2 hours. Although uptake continued at the same rate after 2 hours (not shown here, but see Figure 2.5a), the ABA concentration stayed reasonably stable over the remainder of the assay, indicating that ABA metabolism and/or compartmentation had adjusted to the uptake rate. This finding allowed sampling of ABA concentration in subsequent experiments at only one point in time (6 hours), when LER was stable after ABA treatment.

The ABA contents detected in shoots fed 10^{-8} or 10^{-7}M ABA were indistinguishable statistically (Student's t test, P>0.05). This situation has been found previously in a transpiration bioassay of detached *Phaseolus acutifolius* leaves (Trejo, 1994), where the ABA contents of leaves fed either 10^{-7}M ABA or deionised water were indistinguishable after a 3 hour feeding period. At higher concentrations of ABA in the *Phaseolus* system, ABA accumulation was concentration dependent, as in the system described here.

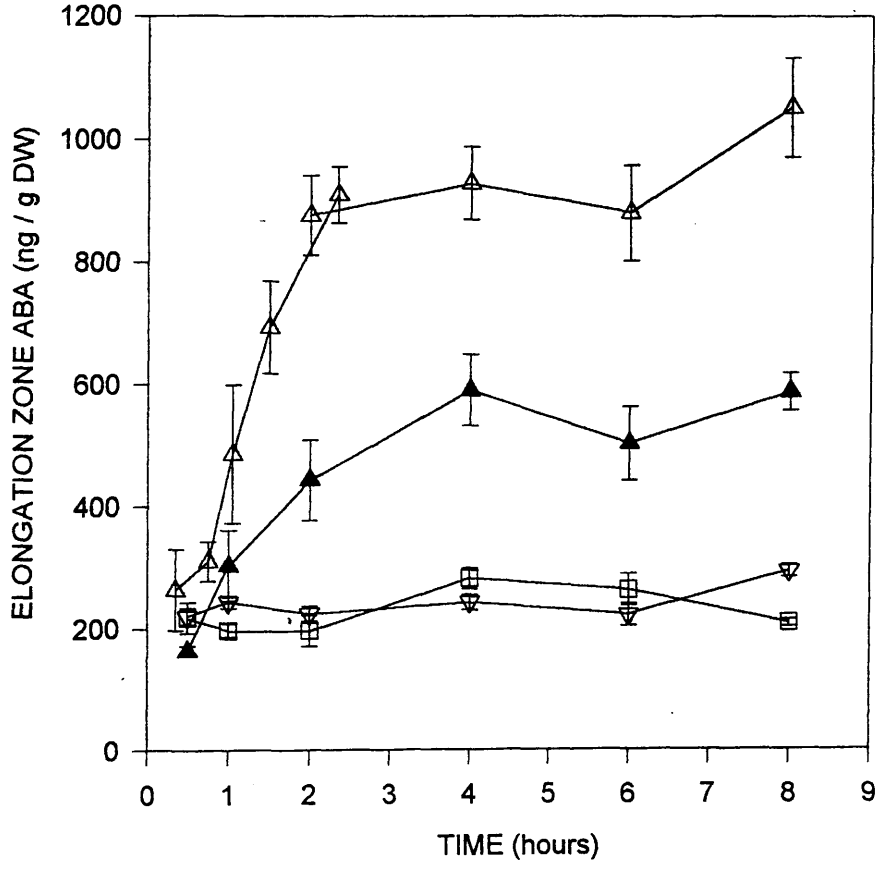

Figure 5.5: Accumulation of ABA with time in the elongation zone of detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots fed 10^{-8}M (□), 10^{-7}M (▽), $5x10^{-7}$M (▲) and 10^{-6}M (Δ) ABA at *circa* 25°C. Points are means ± S.E. of 5 shoots.

5.3.3.2 Temperature experiments

Figure 5.6 shows the effect of 2 different temperatures on the EZ and leaf 2 ABA contents and the LER. Growth (Figure 5.6a) and transpiration (data not shown) rates in this experiment were inhibited by 15 % and 38 % respectively at 10°C and 72 % and 56 % respectively at 24°C. Uptake of ABA into leaf 2 (Figure 5.6c) proceeded rapidly with most accumulation occurring over the first hour. At 10°C, ABA concentration remained stable over the course of the assay with a gradual increase with time at 24°C. Concentrations of ABA in EZ ([EZ-ABA]) increased rapidly at 24°C, with relatively stable concentrations occurring after 2 hours. At low temperature, ABA accumulation was slower in the EZ, eventually reaching half the concentration of plants assayed at 24°C. The magnitude of ABA accumulation in the EZ at the different temperatures may therefore account for the observed differences in leaf growth inhibition. This type of experiment was repeated 3 times. Statistical analysis revealed a significant ($P<0.05$) temperature x ABA interaction when LER was modelled according to feeding ABA concentration, but no interaction when the data were modelled according to [EZ-ABA]. The magnitude of ABA accumulation in the EZ at the different temperatures may therefore account for the observed differences in leaf growth inhibition.

This conclusion was confirmed by applying a wider range of temperatures. Figure 5.7 plots the leaf growth inhibition against both [EZ-ABA] and assay temperature for the same shoots. The similarity of the two relationships suggested that ABA in the elongation zone was responsible for the apparent effect of temperature on ABA-induced leaf growth inhibition.

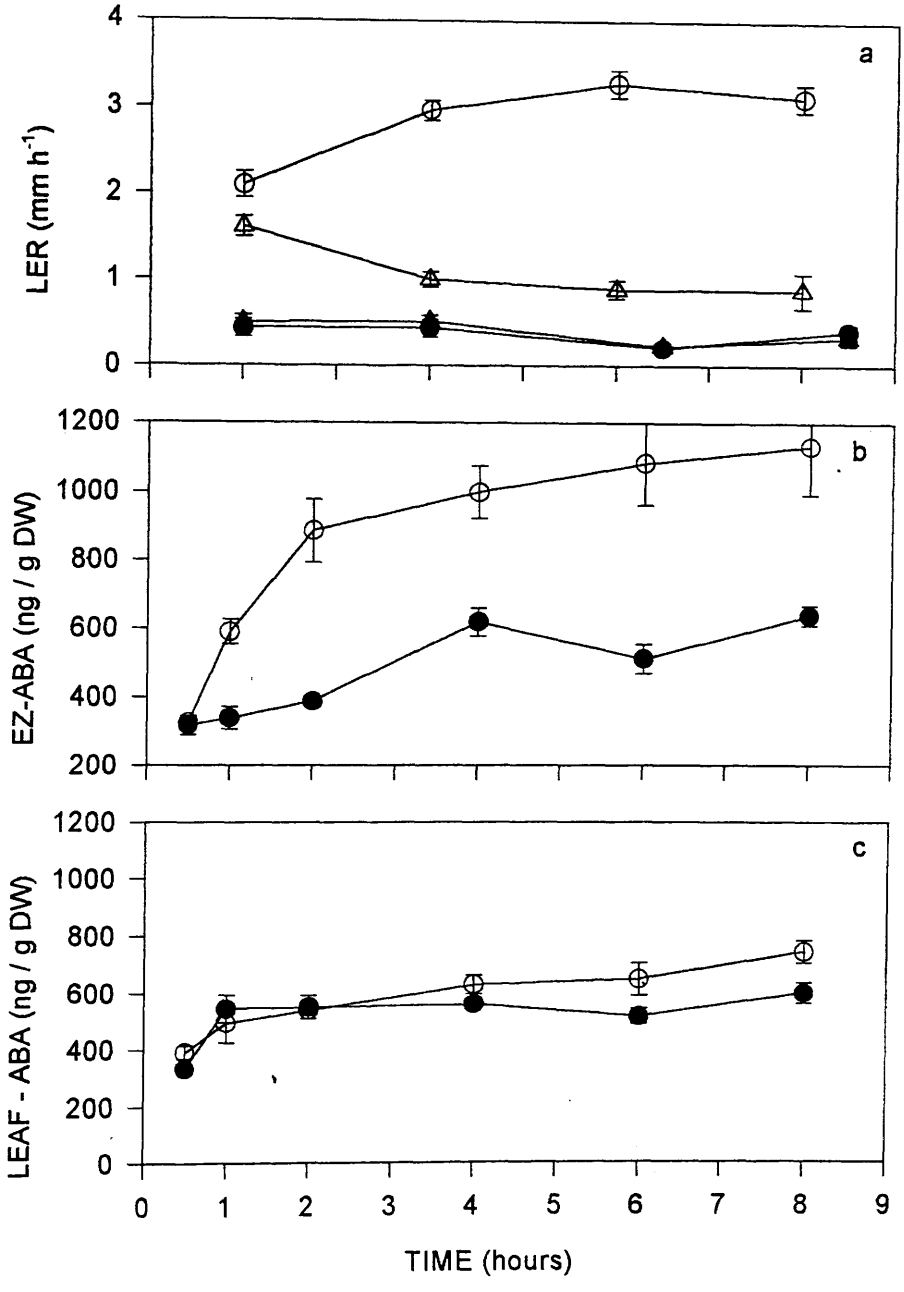

Figure 5.6: Leaf elongation rate (a) of detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots fed artificial xylem solution (○, ●) or 10^{-6}M ABA (Δ, ▲) at temperatures of 11°C (closed symbols) and 27°C (hollow symbols) and ABA content of the bulk leaf elongation zone (b) and the mature leaf (c) over time for detached barley shoots fed 10^{-6}M ABA at temperatures of 11°C (●) and 27°C (○). Points are means ± S.E. of 8 leaves and 5 ABA determinations.

To extend the idea that leaf elongation in the detached shoot system could be dynamically controlled by the ABA concentration of the elongation zone, and not the feeding ABA concentration, an experiment was performed where leaf elongation was monitored after a pulse of ABA had been supplied via the transpiration stream, then removed (Section 5.3.3.4).

A consistent feature of feeding ABA to detached shoots was a greater accumulation in the elongation zone compared to leaf 2. This was particularly noticeable when 10^{-6}M ABA was fed to detached shoots at high temperature (Figure 5.8), but also occurred with 10^{-7}M ABA (Figure 5.8 inset). However, shoots fed 10^{-6}M at low temperature seem to fall on the 1:1 relationship between the two tissues. Enhanced ABA concentrations in the elongation zone were also seen when the data were expressed on a weight of water basis (data not shown). The significance of this result is difficult to comment on since greater EZ ABA accumulation may reflect greater cell density and vacuolation in this tissue. Other possible explanations include greater uptake of ABA from the transpiration stream by cells of the EZ, increased synthesis of ABA in response to transient water deficits in the bioassay system, and a reduced rate of ABA catabolism by cells in the EZ. The option of increased ABA synthesis was examined in Section 5.3.3.3.

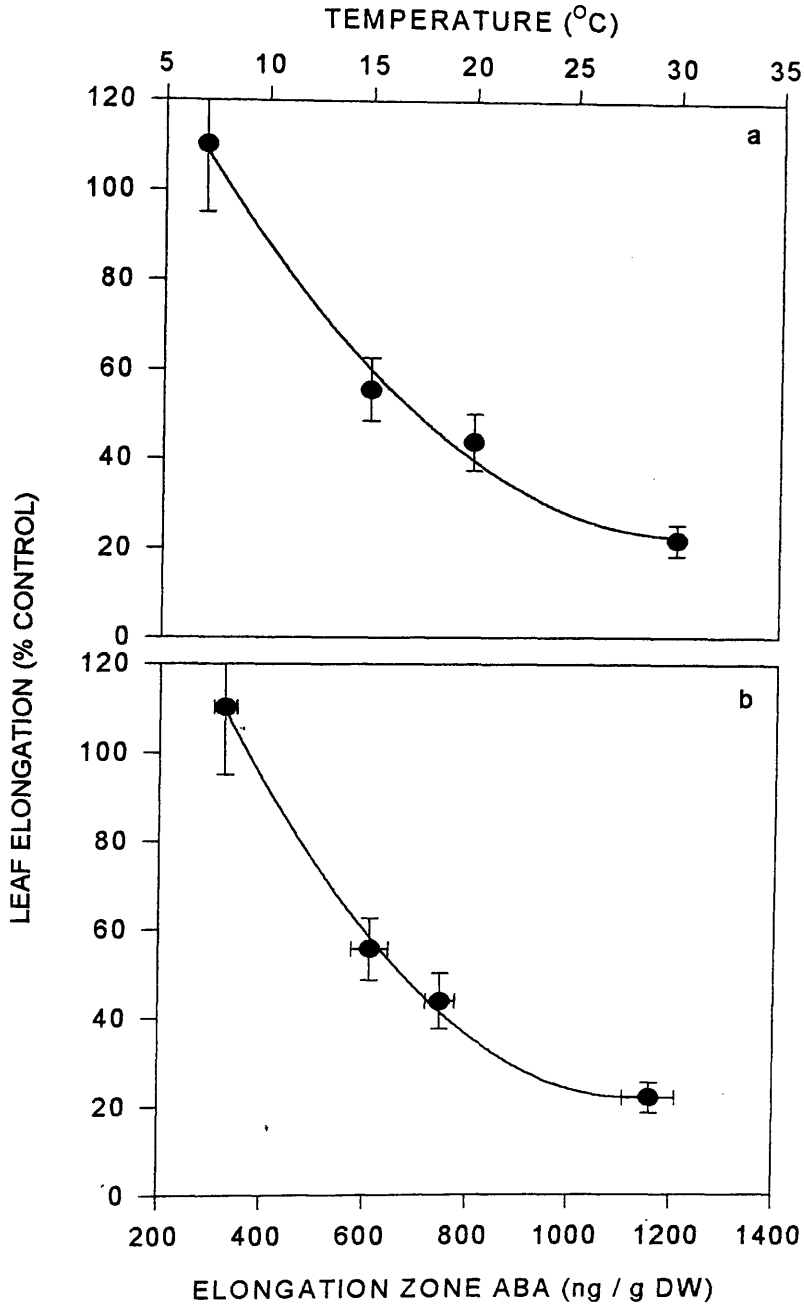

Figure 5.7: Leaf growth response of detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots supplied with 10^{-6}M ABA, plotted against the air temperature at which the leaf elongation assay was conducted (a) and the bulk elongation zone ABA content (b) after 6 hours of ABA feeding at the temperatures in (a), when leaf growth inhibition was maximal. Points are means ± S.E. of 8 leaves and 5 ABA determinations. Lines are second order regressions fitted in SPW 1.0.

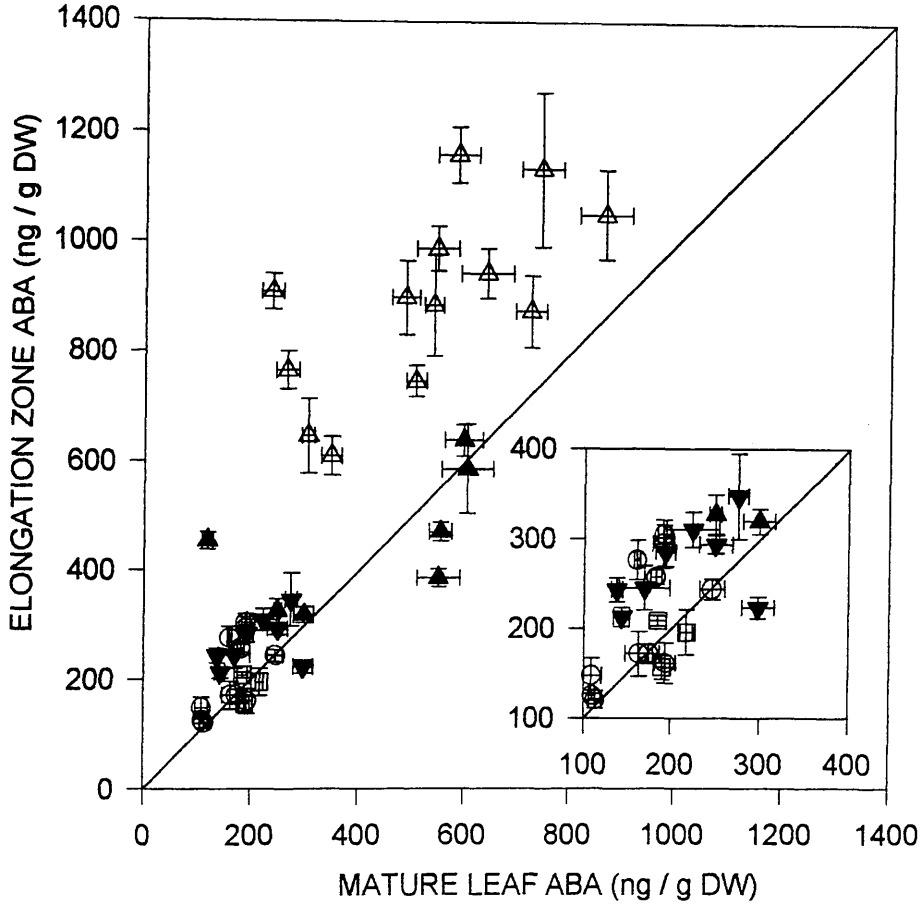

Figure 5.8: Relationship between mature leaf (leaf 2) ABA concentration and elongation zone ABA concentration for detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots fed artificial xylem solution (○), 10^{-8}M (□), 10^{-7}M (▼), 10^{-6}M ABA at *circa* 25°C (Δ) and 10^{-6}M ABA at 10°C (▲) for at least 2 hours. The line indicates the 1:1 relationship. The inset indicates data between 100 and 400 ng / g DW. Points are means ± S.E. of 5 shoots.

5.3.3.3 ABA accumulation in detached, dehydrated tissue

Table 5.3 shows that in the detached tissue test there was no statistically significant difference (Student's unpaired t test, $P>0.05$) between the laminae of the two leaf ages (leaves 2 and 4). Strictly, this is an inappropriate comparison to make as the 2 leaves differ both in their age and their point of insertion on the main stem. However, Quarrie and Henson (1981) showed that there was no statistical difference in ABA accumulation (expressed on a dry weight (DW) basis) in response to dehydration of wheat leaves of the same developmental age sampled at different insertion levels. Samples expressed on a fresh weight (FW) basis showed differences, but only between the flag leaf and other leaves (Quarrie and Henson, 1981). Although 3 different species showed effects of leaf age on ABA accumulation (Quarrie and Henson, 1981), the differences in leaf age between the leaf 2 and leaf 4 laminae sampled here must not have been sufficiently great to result in differences in ABA accumulation. The ABA concentrations in the barley leaf laminae here (*circa* 2000 ng g DW^{-1}after 18 hours incubation at 90 % FW) correspond well with those reports of Trejo (1994) using maize (1800 ng g DW^{-1}) and Henson and Quarrie (1981) using wheat (*circa* 3100 - 5400 ng g DW^{-1} depending on cultivar) and rice (*circa* 1400 - 5400 ng g DW^{-1} depending on cultivar). The values of Henson and Quarrie (1981) are approximate only since the original data were expressed in ng g FW^{-1}; a 10:1 FW:DW ratio was assumed. Comparison of the capacity for different genotypes to accumulate ABA after a pre-determined incubation period may be an inappropriate strategy, as different genotypes can differ considerably in their kinetics of ABA accumulation in the detached leaf test (Henson and Quarrie, 1981). An analogous criticism can be applied to determination of ABA accumulation in different tissues (expanding vs. non-expanding zones of leaves).

Table 5.3: ABA contents of the EZ of leaf 5 and the laminae of an expanding (leaf 4) leaf and a mature (leaf 2) leaf after 18 hours incubation at 90 % of original tissue fresh weight. ABA data are presented as means ± S.E. with the number of replicates given in parentheses.

Tissue Type	ABA (ng / g DW)
Leaf 2 Lamina	2139 ± 261 (4)
Leaf 4 Lamina	2074 ± 90 (5)
Leaf 5 Elongation Zone	1296 ± 45 (5)

Despite this potential criticism, Table 5.3 shows that the EZ accumulated less ABA than the mature and expanding leaves in the detached tissue test. This contradicts previous reports that younger leaves have a greater capacity for ABA synthesis (Quarrie and Henson, 1981), but such data were obtained only using leaf laminae. Therefore the greater ABA accumulation in the EZ seen in the bioassay system (Figure 5.8) is unlikely to be accounted for by an increased synthesis of ABA by cells in the EZ.

5.3.3.4 Experiment where ABA was withdrawn after two hours of feeding

Figure 5.9 shows the response of growth, transpiration and ABA concentrations in detached shoots when the ABA solution was replaced by the artificial xylem (AX) solution after 2 hours of feeding, and when a supply of ABA was maintained. After 2 hours, there was substantial accumulation of ABA in leaf 2 and the EZ in response to ABA feeding. These concentrations were maintained in shoots subjected to a continuous supply of ABA (data not shown but see Figure 5.5), as were the steady-state levels of leaf growth inhibition and transpiration (see Figure 5.9a). Removal of ABA from the feeding solution resulted in the partial recovery of transpiration and leaf growth. Although leaf growth increased over the remainder of the experiment for plants which were subjected to a 2 hour pulse of ABA followed by a chase of AX solution, a 15 % increase in transpiration occurred 1-2 hours after removal of ABA but no further increases in transpiration were noted. The reversibility of ABA-induced reductions in transpiration are consistent with previous reports

(Cummins *et al.*, 1971; Cummins, 1973) but this would appear to be the first report of the reversibility of ABA-induced reductions in leaf elongation.

The differential after-effects of drought stress on leaf elongation rate (immediate recovery following re-watering - Acevedo *et al.*, 1971) and transpiration (may be inhibited for several days following re-watering - Correia and Pereira, 1994) would seem to be consistent with the results described in Figure 5.9a. This provides additional circumstantial evidence for a role of ABA in the physiology of droughted plants.

Removal of ABA from the feeding solution also resulted in the decline of ABA concentrations in both mature leaves and elongation zones to new steady state values (which were higher than existed in the tissues before the experiment started - Figure 5.9b). The higher ABA concentration is likely to result from some ABA being made unavailable to degrading enzymes, possibly by sequestration in the chloroplasts (Heilmann *et al.*, 1980). Although estimation of rates of ABA breakdown was not the chief aim of this experiment, it is obvious that ABA catabolism in this system is rapid. Half-lives for ABA in the bioassay system are certainly within 2 hours, in agreement with the data of Gowing *et al.* (1993) who experimentally determined a half-life for externally supplied ABA in detached cherry leaves of 36 minutes. Comparison of other half-life data for ABA in the literature may not be meaningful, as many such experiments have measured half-lives for endogenously produced ABA (e.g. Zeevart, 1983). The data presented in Figure 5.9 do not allow comparison of catabolic rates of leaf and EZ tissue. The larger standard error bars on the EZ values may represent a slower rate of catabolism or may simply be a result of the greater variability associated with measurements of ABA concentration in the EZ. The possibility that cells in the EZ degrade ABA at a reduced rate would be consistent with data which suggest that young leaves are less able to break down ABA (Zeevart and Creelman, 1988). The possibility that cells in the elongation zone may metabolise ABA at a reduced rate is worthy of further investigation.

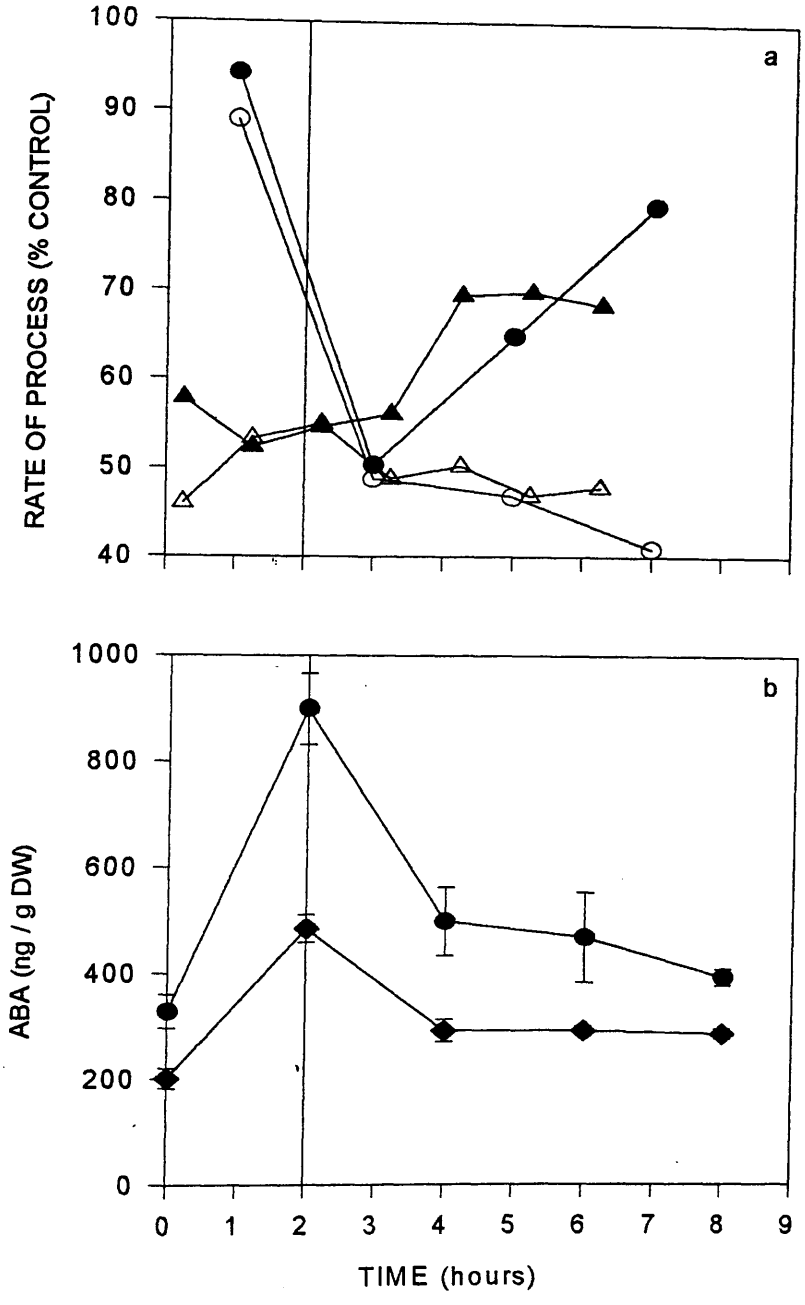

Figure 5.9: Response of transpiration (▲, Δ) and leaf elongation (●, ○) (expressed as a percentage of control shoots) in detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots to continuous feeding with 10^{-6}M ABA (hollow symbols) or 2 hours of feeding 10^{-6}M ABA, followed by transfer of shoots to artificial xylem solution (filled symbols) (a) and the accompanying changes in ABA content (b) of the elongation zone (●) and mature leaf (◆) in the transfer experiment. Points are means of 5 shoots, with error bars in (a) omitted for clarity and S.E.s given in (b).

5.3.4 DISCUSSION

The data in this Section indicate the usefulness of measuring EZ-ABA. Measuring [EZ-ABA] seemed to account for the putative temperature x ABA interaction. However, this cannot be stated unequivocally until experiments are performed which feed the same ABA concentration to plants at the same flux rate, but with the EZ held at different temperatures. This type of experiment may resolve the different effects of [EZ-ABA] and any putative T x ABA interaction affecting leaf growth. While both ABA concentration and amount are important (and intrinsically related), the evidence presented here from detached shoot experiments where the ABA flux is varied suggests that leaves are responding more to an accumulated amount of ABA than a xylem sap concentration. This situation is very different from bioassay studies analysing stomatal responses, which indicate that enhanced ABA accumulation under high temperatures and VPDs does not generally produce additional stomatal closure (Trejo *et al.*, 1995).

It is also difficult to completely discard the concept of a T x ABA interaction affecting LER given the numerous examples in the literature which report this interaction influencing stomata, both in chilling- (Rodriguez and Davies, 1982; Eamus and Wilson, 1983; Cornic and Ghasghaie, 1991) and non-chilling sensitive (Honour *et al.*, 1995) species. However, this type of interaction appears to be highly variable even within a well-studied species such as *Commelina communis* (cf. Rodriguez-Ontiverous 1982 vs. Honour *et al.*, 1995) and factors such as pre-treatment temperatures may be important (Allan *et al.*, 1994). The temperature range of the assay may also be important. Rodriguez and Davies (1982) report an increase in stomatal sensitivity to ABA for maize across the temperature range 18 to 22^{o}C. There was no increase in sensitivity for a range of 25 to 40^{o}C (Rodriguez and Davies, 1982; Tardieu *et al.*, 1993).

Although [EZ-ABA] may be a useful variable to measure in accounting for the LER of detached shoots fed synthetic ABA, it is uncertain whether ABA accumulation can explain the growth inhibition of detached shoots which are fed xylem sap from droughted plants, or the growth inhibition of droughted plants. These possibilities are addressed in Chapters 6 and 7 respectively.

CHAPTER 6.

RESPONSE OF LEAF GROWTH TO XYLEM SAP IN A LEAF ELONGATION ASSAY

6.1 INTRODUCTION

Although the transpiration bioassay has been used on at least 3 occasions to examine the antitranspirant activity of xylem sap (Munns and King, 1988; Zhang and Davies, 1991; Trejo, 1994), only one report (Munns, 1992) has fed xylem sap in the leaf elongation assay. In that system, sap ABA concentration was unable to account for the observed leaf growth inhibition. Xylem sap with an ABA concentration of $4x10^{-8}M$ inhibited leaf growth by 60 %, while synthetic ABA at $10^{-5}M$ ABA was required to reduce leaf growth by the same amount. It was concluded that there was an additional growth inhibitory substance in barley or wheat xylem sap.

However, more recent work by that group (Munns *et al.*, 1993) casts doubt on the presence of other antitranspirant (and by analogy, growth inhibitory) compounds in wheat and barley xylem sap. Freshly collected sap inhibited transpiration to a level commensurate with its ABA concentration, and only sap collected from droughted plants and stored at -20°C showed major antitranspirant activity, which was ascribed to a large polymeric compound which developed under the solute-concentrating conditions of storage (Munns *et al.*, 1993). In a similar experiment, Sinclair *et al.* (1995) showed that the antitranspirant activity of maize xylem sap increased with storage at -20°C, but that the majority of this activity could be accounted for by physical blockage of the xylem vessels.

In view of these recent reports which cast doubt on the existence of antitranspirant or growth inhibitory compounds other than ABA, and the differences in the dose-response of leaf elongation to ABA generated by different workers (Figure

2.4b), it was decided to investigate the potential growth inhibitory and antitranspirant effects of maize xylem sap.

6.2 MATERIALS AND METHODS

Maize (*Zea mays* L. cv. Earliking) plants were grown in 1.5 litre pots in a 50: 50 mixture of John Innes No.2 compost and gravel. Maize was chosen as it is relatively easy to collect large volumes of sap. When 8 leaves had appeared, 75 % of the plants were left unwatered for 3 days. A greater proportion of the plants was left unwatered since droughted plants exuded less sap when detopped (as found by Zhang and Davies, 1991). Plants were detopped, a tube placed over the cut stump and the sap allowed to exude overnight before collection the following morning, a procedure similar to that described by Zhang and Davies (1991) and Sinclair *et al.* (1995). The sap was centrifuged at 8000g for 5 minutes in Eppendorf tubes to spin down cell debris which occur in maize sap collected by this method (Zhang and Davies, 1991). The 100 μL remaining in the bottom of each Eppendorf tube was discarded. Centrifugation of sap from maize plants appeared to be a suitably quick method of preparation for leaf elongation assays, as sap from well-watered plants did not show any growth inhibitory activity when supplied to detached barley shoots (see Figure 6.1c). The sap was fed to detached barley shoots on the same day as sap collection, to avoid the development of antitranspirant activity in the sap with storage at -20°C (Munns *et al.*, 1993; Sinclair *et al.*, 1995).

Initial experiments fed xylem sap or ABA made up in artificial xylem solution to detached shoots at average temperatures and relative humidities of 18°C and 40 % respectively. Leaf elongation was recorded every 2 hours, and transpiration hourly. At the end of the assay, samples of the xylem sap and the ABA solutions were collected, stored at -20°C, and ABA concentrations quantified in a radioimmunoassay (RIA) protocol (Quarrie *et al.*, 1988), as described in Section 5.2.2.3.

Later experiments were conducted at average temperatures and relative humidities of 28°C and 40 % since leaf growth was more sensitive to ABA at this temperature (Figure 4.2). After 6 hours, samples were taken for determination of ABA concentration in the bulk elongation zone (10-40 mm from the crown as explained in Section 5.3) as described above, and any remaining maize sap collected.

6.3 RESULTS

Figure 6.1 shows that for both assays, and for both leaf elongation (Figures 6.1a, c) and transpiration (Figures 6.1b, d), that the points for xylem sap collected from droughted plants did not lie on the dose-response curve generated from ABA in artificial xylem solution. This contradicts the finding that maize xylem sap contains no additional antitranspirant activity (Zhang and Davies, 1991) but supports the finding that xylem sap contains growth inhibitory activity other than ABA (Munns, 1992). Sap from well-watered maize plants was found to inhibit transpiration to the same extent as sap from droughted plants (Figure 6.1d), yet leaf elongation was unaffected (Figure 6.1c). An antitranspirant effect of xylem sap from well-watered plants has previously been shown (Munns and King, 1988), although the effect was highly variable between different batches of sap. Maintaining well-watered plants at full turgor in a root pressure chamber was shown to eliminate most of the antitranspirant activity, suggesting that well-watered plants developed transient leaf water deficits during the day, which promoted antitranspirant activity in the sap (Munns and King, 1988).

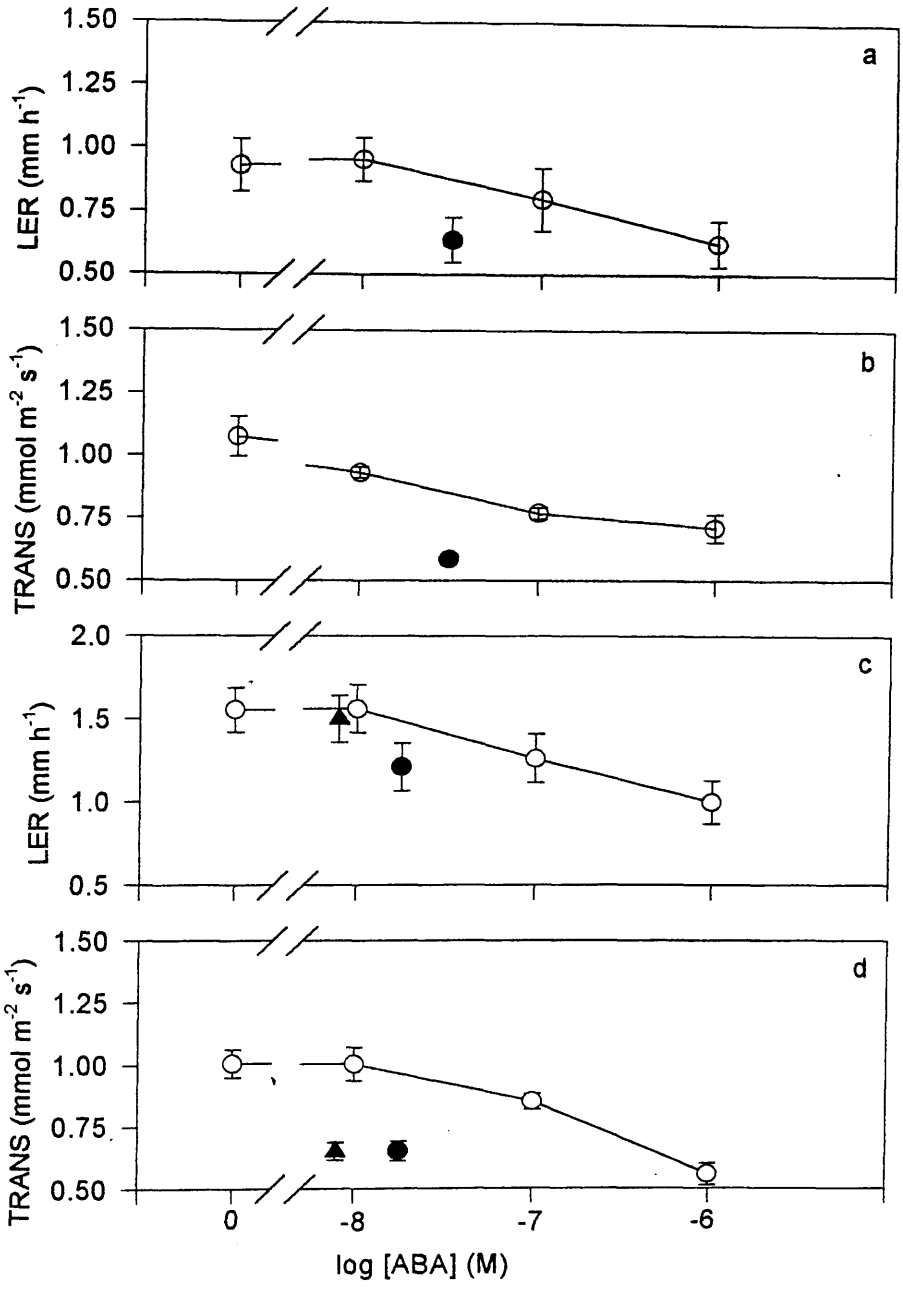

Figure 6.1: Response of leaf elongation (LER) (a, c) and transpiration (TRANS) (b, d) in detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots fed synthetic ABA dissolved in artificial xylem solution (○) or xylem sap collected from detopped, droughted (●) and well-watered (▲) maize plants in two separate experiments (a, b and c, d). Points are means ± S.E. of at least 12 measurements taken during the steady-state phases of leaf growth and transpiration inhibition (1-5 hours after supplying ABA). Shoots were maintained at 15-20°C.

Table 6.1 shows the effects of the different saps, and of ABA on the sensitivities of leaf elongation and transpiration (expressed as a percentage of the controls). In the first experiment (Figure 6.1a, b), there were no differences in the sensitivities of leaf elongation and transpiration to either ABA or droughted sap. This was confirmed in the second experiment, except for 10^{-6}M ABA and sap from water stressed plants, which inhibited transpiration more than leaf elongation.

In later experiments at the higher assay temperature, sap from droughted plants reduced growth by 45 % in a manner inconsistent with the ABA concentration of the xylem sap (Figure 6.2a), as seen before (Figures 6.1a, c). However, when leaf elongation rate was plotted against the ABA content of the EZ of leaves used in the same assay, the point for the droughted sap lay on the relationship (Figure 6.2b) generated with synthetic ABA.

Table 6.1: Sensitivities of leaf elongation and transpiration to synthetic ABA solution and sap collected from droughted (WS) or well-watered (WW) maize plants. Data are means ± S.E. with the number of replicates indicated in parentheses. P value obtained by applying Student's unpaired t test. ($P<0.05$ *), ($P<0.01$ **), ($P<0.001$ ***)

Feeding Solution	Leaf Elongation (% control)	Transpiration (% control)	P Value
Experiment 1:			
10^{-8}M ABA	103.0 ± 11.4 (12)	86.8 ± 2.5 (16)	0.124
10^{-7}M ABA	85.0 ± 14.6 (15)	71.7 ± 2.3 (20)	0.275
10^{-6}M ABA	67.2 ± 9.8 (18)	66.1 ± 5.3 (24)	0.920
WS SAP	68.7 ± 9.9 (18)	54.6 ± 1.6 (24)	0.109
Experiment 2:			
10^{-8}M ABA	100.5 ± 9.2 (16)	99.7 ± 6.7 (18)	0.944
10^{-7}M ABA	81.4 ± 9.4 (16)	84.5 ± 3.1 (18)	0.747
10^{-6}M ABA	64.3 ± 8.3 (12)	55.4 ± 4.4 (18)	0.000 ***
WS SAP	78.0 ± 9.3 (20)	64.6 ± 5.1 (12)	0.011 **
WW SAP	96.5 ±16.6 (12)	64.8± 3.9 (18)	0.359

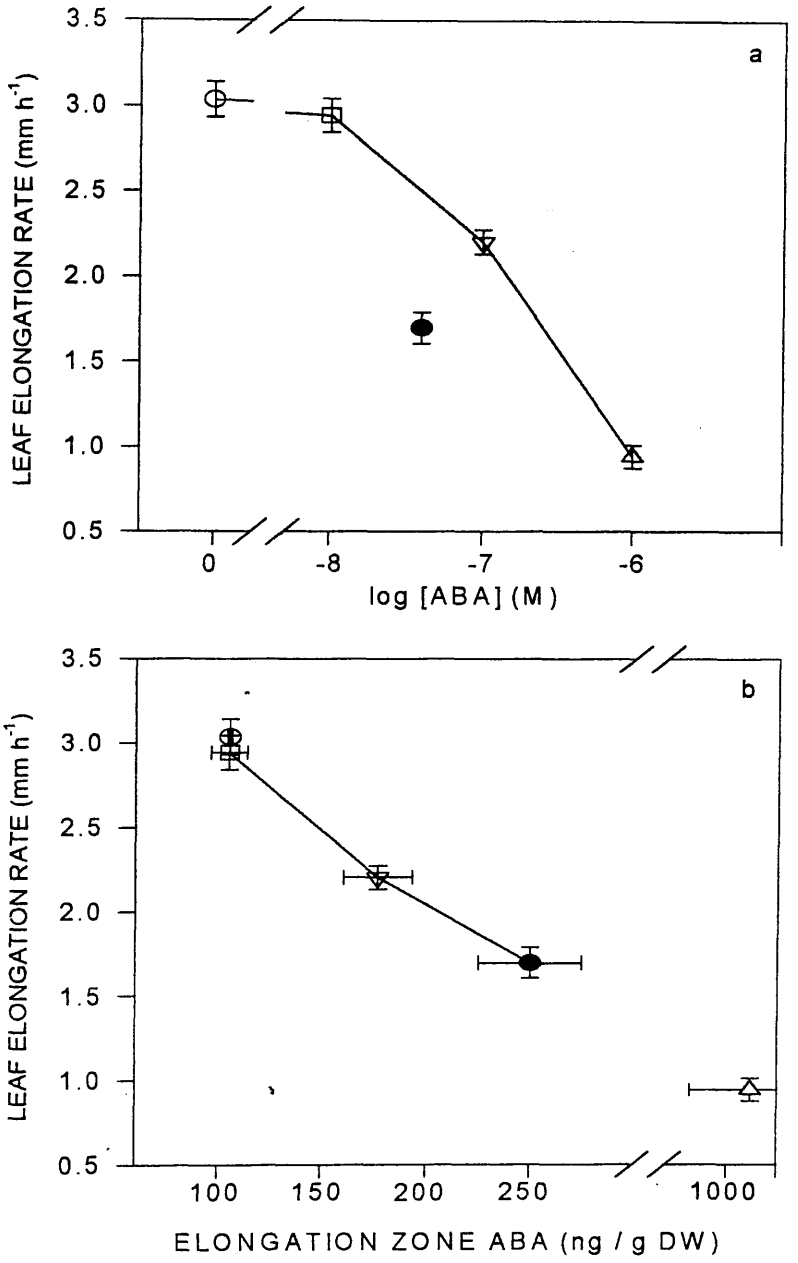

Figure 6.2: Leaf elongation rate plotted as a function of xylem ABA concentration (a) and elongation zone ABA concentration after 6 hours (b) for detached barley (*Hordeum vulgare* L. cv. Klaxon) shoots fed artificial xylem solution (○), 10^{-8}M (□), 10^{-7}M (▽), 10^{-6}M (△) ABA and xylem sap from droughted maize plants (●). Points are means ± S.E. of 8 leaves and 5 ABA determinations.

6.4 DISCUSSION

The literature on the effects of actual sap on plant growth and transpiration in bioassays is filled with contradictions and methodological problems, as Table 6.2 attempts to summarise. The results presented here seemingly add to an already confusing picture.

The fact that xylem sap from well-watered plants inhibited transpiration is worrying, although not without precedent. Meinzer *et al.* (1991) showed that root exudate from well-watered sugarcane plants inhibited stomatal conductance (g_s) in detached leaves by 40 to 80 % relative to distilled water fed plants; with increased inhibition as the leaf area of the plants from which the exudate was collected increased. The cause of the inhibition in the detached leaves was not determined, but was unlikely to have been ABA as the delivery rate from cut stumps declined with increasing leaf area (Meinzer *et al.*, 1991). This would suggest the existence of another antitranspirant factor in the exudate, perhaps an osmotically inhibitory or inappropriate combination of ions; or a physical blockage of the transpiration stream. Regardless of possible causes, the fact that sap collected from well-watered plants inhibited transpiration in a manner inconsistent with its ABA concentration (Figure 6.1d) would invalidate any judgements on a possible regulatory role for ABA in affecting transpiration in water-stressed plants.

Table 6.2: Summary of experiments which have fed actual xylem sap to detached tissues.

Reference:	Munns and King, 1988 Munns, 1992 Munns *et al.*, 1993	Zhang and Davies, 1991	Trejo, 1994	Sinclair *et al.*, 1995	This study
Species from which sap collected	*Triticum aestivum* *Hordeum vulgare*	*Zea mays*	*Phaseolus vulgaris*	*Zea mays*	*Zea mays*
Sap Collection methodology	root pressure chamber from plants maintained with or without balancing pressure	detopped shoots (0.5 mL per shoot)	leaf pressure chamber from detached shoots (0.1 mL per shoot)	detopped shoots overnight collection (up to 25 mL per shoot)	detopped shoots overnight collection (up to 3 mL per shoot)
Assay technique	- detached wheat leaf transpiration bioassay (TRANS) - detached wheat leaf elongation assay (LER)	- detached wheat leaf transpiration bioassay (TRANS) - *Commelina* epidermal strip bioassay (STOM)	- detached bean leaf transpiration bioassay (TRANS) - *Commelina* epidermal strip bioassay (STOM)	- detached maize leaf transpiration bioassay (TRANS)	- detached barley leaf elongation assay (TRANS) - detached barley leaf elongation assay (LER)
Does unaltered sap from well-watered plants (WW) inhibit response ?	YES (0-70 %) - without balancing pressure NO (15 %) - with balancing pressure	YES - TRANS - wilted YES - STOM - overcome by diluting sap by half	NO - TRANS YES - STOM	NO (15 %) after 1 day with storage at 4°C or - 80°C	YES (40 %) - TRANS NO - LER
Is inhibition caused by a physical blockage of the xylem vessels ?	NO - no filtration needed - no particulate matter in in sap	YES - large particles in sap	Not tested sap filtration routinely performed	YES - but small signal component - variable filter effect according to brand	Not tested -centrifugation employed

Table 6.2 cont: Summary of experiments which have fed actual xylem sap to detached tissues.

Reference:	Munns and King, 1988 Munns, 1992 Munns *et al.*, 1993	Zhang and Davies, 1991	Trejo, 1994	Sinclair *et al.*, 1995	This study
Does wilting occur when leaves fed sap from WW plants ?	NO	YES - wilting entirely removed by sap filtration	NO	YES - but wilting not adequately assessed	NO
Does inhibition increase with storage of sap ? at 4°C : at - 20°C : at - 80°C : at - 196°C :	1993 study YES - only WS plants YES - 50 % after 4 days NO	Not assessed Storage at - 20°C	Not assessed Storage at - 80°C	 YES - WW plants YES - 70 % after 10 days YES - 100 % after 1 day YES - 30 % after 10 days	Not assessed fresh sap used
Does ABA in droughted sap explain response ?	NO - TRANS (1988) NO - LER (1992) YES - TRANS fresh sap only (1993)	YES - TRANS YES - STOM	YES - TRANS YES - STOM - but invalid comparison as WW sap inhibitory	ABA not measured	NO - TRANS - but invalid comparison as WW sap inhibitory YES - LER - when [EZ-ABA] measured
Does inhibition remain after removal of ABA via an immunoaffinity column ?	YES - TRANS (1988)	NO - TRANS NO - STOM	YES - TRANS	Not assessed	Not assessed
Does ABA in actual sap have a synergistic effect on inhibition?	NO - TRANS	Not assessed	YES - 1 experiment NO - 1 experiment both TRANS	Not assessed	Not assessed

Maize xylem sap collected by the same method used in these experiments is known to contain large particles (>0.2 μm diameter) which may block xylem transport and result in wilting of large (16 cm long) detached leaves (Zhang and Davies, 1991). Although the method of sap preparation used here (centrifugation) was not as rigorous as Zhang and Davies (1991) (filtering sap), physical blockage seems unlikely since the leaf elongation rate of control shoots was essentially the same as shoots fed xylem sap (Figure 6.1c) and no wilting of the barley detached shoots was observed. It is assumed that growth would be more sensitive than transpiration to an interruption of the water supply. Although Zhang and Davies (1991) suggested that wilting may go undetected in small (8 cm long) cereal leaves, the average leaf area of detached shoots was 16 cm^2 (compared to 9 cm^2 for 16 cm long barley leaves) and thus it is difficult to sustain the physical blockage hypothesis for the experiments described here.

The sap collection technique used here has been frequently criticised for giving misleading data on hormonal and ionic contents of xylem sap (Munns, 1990; Jackson 1993), since the low sap flow rates (much less than those in the intact transpiring plant) result in the concentration of solutes. The osmotic potential (ψ_π) of xylem sap samples was determined by psychrometry to be -0.14 MPa. KCl of a similar osmotic potential (25 mM) inhibited transpiration by 10 % and had no effect on leaf elongation in the leaf elongation assay (data not shown) and thus the inhibition of transpiration by sap from well-watered plants does not appear to be a purely osmotic effect, but perhaps an effect of an unfavourable combination of ions.

Feeding xylem sap collected from well-watered plants, but of a different ionic composition to that which the bioassay material normally is in contact with (e.g. due to species differences), has been noted to prevent stomatal opening in epidermal strip bioassays (Zhang and Davies, 1991, Trejo, 1994) but feeding well-watered maize sap to detached wheat leaves did not inhibit transpiration to an extent greater than that expected on the basis of ABA concentration (Zhang and Davies, 1991). Why has sap

collected from well-watered maize plants by essentially the same method as Zhang and Davies (1991) and fed to barley leaves (assumed to behave similarly to wheat leaves) inhibited transpiration ? One difference in the sap collection technique is that Zhang and Davies (1991) only collected the first 0.5 mL collected from the cut maize stump, while the study here allowed exudation for 12 hours overnight. Perhaps the ionic composition of the exudate changes considerably from that found in the initial droplets (collected by Zhang and Davies and assumed to represent the actual transpiration stream existing in the vessels when the top was removed). Sequential analysis of K^+ in sap exuding from detopped maize plants showed a drop in K^+ concentration in the first 0.5 mL, but little change in later samples (Zhang and Davies, 1990b). This contrasts with data obtained using the same species and sap collection technique, which showed that NO_3^- concentration steadily increased over a 3 hour period, comprising 0.8-2.0 mL of sap (Canny and McCully, 1988). This shows that concentrations of ions in exudate may be affected by long collection times. Such concentration changes may have reduced transpiration in detached shoots fed sap from well-watered plants. However, this does not explain the observation that leaf elongation in these shoots was unaffected.

It is possible, but unlikely, that the leaf elongation assay was not sufficiently sensitive to detect reductions in the LER of detached shoots fed sap from well-watered maize plants. Certainly, the dose-response curve for Figure 6.1c shows a much reduced slope compared to that portrayed in Figure 2.4b, which probably results from the lower assay temperature. However, an experiment performed at 28°C where LER of shoots was much higher gave the same result for sap collected from well-watered plants: an inhibition of transpiration of about 40 % yet no effect on leaf growth (data not shown).

It seems that xylem exudate from well-watered maize plants contains a compound(s) which did not affect leaf elongation but had antitranspirant activity. Such activity is considered to be an artefact of the long sap collection times employed in this

study. Inhibition of transpiration by maize xylem sap was not further considered in later experiments, as different sap collection techniques would be required such as the use of a whole plant pressure chamber, which would allow sampling of xylem sap at flow rates comparable to those existing in the intact plant (Munns and King, 1988; Schurr and Schulze, 1995).

Although it appeared that there was another growth inhibitory compound in maize xylem sap from droughted plants (Figures 6.1 a, c, 6.2a), measurement of the ABA concentration of cells in the leaf elongation zone negated this idea (Figure 6.2b). This result would seem to confirm the suggestion of Munns and Sharp (1993) that the antitranspirant compound in wheat and barley sap caused ABA to accumulate in leaves to which it is fed. It would be interesting to see if confirmation of this suggestion was forthcoming in the form of some data.

One possible explanation for this result is that there is some other compound in the xylem sap (perhaps a conjugated version of ABA) which is converted to free ABA in the elongation zone. ABA conjugates such as ABA glucose ester and ABA methyl ester routinely occur in xylem sap but usually remain below 10 % of the free ABA (Hartung, pers. comm.). In some stress environments with some species, the levels of conjugates can increase by up to 4 fold (Bano *et al.*, 1993). The level of conjugates in the maize xylem sap, nor the potential identity of other possible compounds, has not been pursued since there was a highly significant relationship between [EZ-ABA] and LER, which justified the measurement of [EZ-ABA] in droughted plants (Chapter 7).

6.5 CONCLUSIONS

Long collection times for maize sap exuding from cut stumps resulted in sap from well watered plants showing antitranspirant but not growth inhibitory effects. Further judgements on the role of ABA in the antitranspirant activity of maize xylem sap from water-stressed plants were thus precluded. This artefact may have been removed by collecting sap using a whole plant pressure chamber, which is likely to be a more dependable method of collecting the large volumes of sap required for assays at high temperature. Leaf elongation was inhibited by sap from droughted plants in a manner inconsistent with the ABA concentration of the sap. Measuring [EZ-ABA] seemed to account for this growth inhibition, thus indicating the validity of measuring [EZ-ABA] in droughted plants.

CHAPTER 7.

ACCUMULATION OF ABA IN THE LEAF ELONGATION ZONE OF INTACT PLANTS DURING A SOIL DRYING EPISODE

7.1 INTRODUCTION

If the data collected on ABA accumulation in the bioassay system are to be compared with plants grown in drying soil, ABA concentrations ([ABA]s) and effects in the two systems should be comparable. As noted in Section 5.3.1, ABA has been measured in many plant parts, but never expressly in the leaf elongation zone of droughted plants. This is somewhat surprising considering that the mature and elongating parts of grass leaves differ in their capacity for osmotic adjustment (Munns *et al.*, 1979) and turgor maintenance (Michelena and Boyer, 1982) in response to drought. It might, therefore, be possible that the elongation zone accumulates more ABA in response to soil drying than do the mature leaves, as demonstrated for detached cereal shoots fed ABA in the leaf elongation assay (Figure 5.8). Any enhanced ABA accumulation may be physiologically important in explaining leaf growth inhibition under drought.

ABA accumulation in the EZ may be of considerable practical importance in the accurate measurement of plant ABA relations. Although xylem sap [ABA] is known to be more sensitive than bulk leaf [ABA] to soil drying (Zhang and Davies, 1989b; 1990a), there are considerable methodological difficulties in obtaining a valid sap sample. This is especially the case in monocotyledons, which generally contain less ABA than dicotyledons (Munns, 1990); thus it may be difficult to collect the volumes of sap needed by immunological methods. In many small-leaved grasses, it is often impossible to express sufficient xylem sap for ABA analysis using the Scholander pressure bomb. Investigators may then be forced to collect xylem sap from detopped shoots, a method of sap collection that has been criticised as providing unrealistically

high ABA concentrations (Munns, 1990; Jackson, 1993) since the sap is collected at low flow rates atypical of transpiring plants. The problem is exacerbated in small plants, as exudation would be the sum of the transpiration stream (the small amount of xylem sap existing in the vessels at the time of detopping) and later sap flow through the root system. The ABA concentration of exudates can only be regarded as a valid measurement of xylem [ABA] when only the transpiration stream is collected, as validated by anatomical measurements of xylem volume to allow calculation of the the volume of xylem sap existing in the cut stump at the time of detopping (Zhang and Davies, 1990b). Given the problems involved with sampling xylem sap from small plants, it would seem useful to pursue another measurement of ABA concentration which may be related to LER. Although correlations between lamina ABA concentration and LER have been shown (Puliga *et al.*, 1996), the enhanced [EZ-ABA] of detached shoots fed ABA (Figure 5.8) warrants further investigation of the ABA relations of elongating cells under drought conditions. The studies described in this chapter seek to relate leaf elongation rate to ABA accumulation in the leaf elongation zone of droughted plants.

7.2 INFLUENCE OF SPATIAL GROWTH DISTRIBUTION ON ABA CONCENTRATION DETECTED IN THE GROWING ZONE

7.2.1 INTRODUCTION

Before samples of the bulk elongation zone (as applied in the bioassay system - Section 5.3) could be relied upon as valid measures of ABA concentration in the elongation zone, it was necessary to consider any effects that drought-induced changes in the spatial distribution of growth (Spollen and Nelson, 1994; Durand *et al.*, 1995) may have had on the ABA content of the samples collected.

7.2.2 MATERIALS AND METHODS

Maize (*Zea mays* cv Earliking seeds) were deep-sown (to induce a sub-crown internode) in 9 litre pots (20 seeds per pot) in a 50:50 mixture of John Innes No. 2 compost and gravel. A 10 cm thick layer of vermiculite was placed over the seeds to permit ease of access for the marking experiments to determine the spatial distribution of growth. All plants were well watered until leaf 3 had emerged, when half the pots remained unwatered while the other half continued to be watered daily, at the beginning of the night period. Average maximum and minimum temperatures and relative humidities in the growth cabinet during the experiment were 20°C and 29°C, and 29 % and 44 % respectively.

Two days after withholding water, leaf length of six plants per treatment was measured. Length measurements occurred every 12 hours to investigate the daily patterns of elongation. Each day, eight plants per treatment were marked with a series of parallel entomological pins 2 hours into the light period, to determine the spatial pattern of growth (as in Section 5.2.2.1). The negative effect of marking on LER was not determined in soil-grown plants, and assumed to be *circa* 25-35 %, as shown for detached shoots (Table 5.2). Data on RSER have not been corrected for the effect of marking injury. Another set of 8 plants per treatment were sectioned with a parallel series of razor blades to determine the spatial pattern of ABA accumulation (as in Section 5.2.2.2). ABA concentration of tissues was measured according to the RIA protocol described in Section 5.2.2.3 (Quarrie *et al.*, 1988).

Statistical differences between droughted and well-watered plants were determined by Student's unpaired t-test in SPW 1.0

7.2.3 RESULTS AND DISCUSSION

Figure 7.1 shows the time course of leaf growth of the 2 emergent leaves and EZ ABA content (averaged over all positions in the EZ) over the experimental period. In well-watered plants, there is increased growth during the light period, which can probably be attributed to the higher average temperature in the growth cabinet during that period. However, even in growth cabinets which maintain a constant temperature day and night, leaves of well-watered grasses may show enhanced growth during the day (Watts, 1974; Christ; 1978), although higher night growth at constant temperature has been noted in tall fescue (Parrish and Wolf, 1983; Schnyder and Nelson, 1988; Durand *et al.*, 1995). None of these studies have expressly measured meristem temperature, which would seem to be a prerequisite for ensuring that any day/night growth differences at constant cabinet temperature are not due to different meristem temperatures, especially in the case of experiments showing reduced LER at night. Experiments showing enhanced LER at night are suggestive of plants developing water stress during the day despite high levels of soil water, which is indicative of LER being sensitive to VPD (Squire *et al.*, 1983). Watts (1972) explained this response to VPD in terms of transpirational water loss exceeding water uptake, decreasing water potential in the elongating cells. No attempt has been made to validate this suggestion by measuring ψ_W in elongating cells, although it is often favoured as an explanation for higher night-time LERs (Parrish and Wolf, 1983) in plants which are particularly sensitive to VPD.

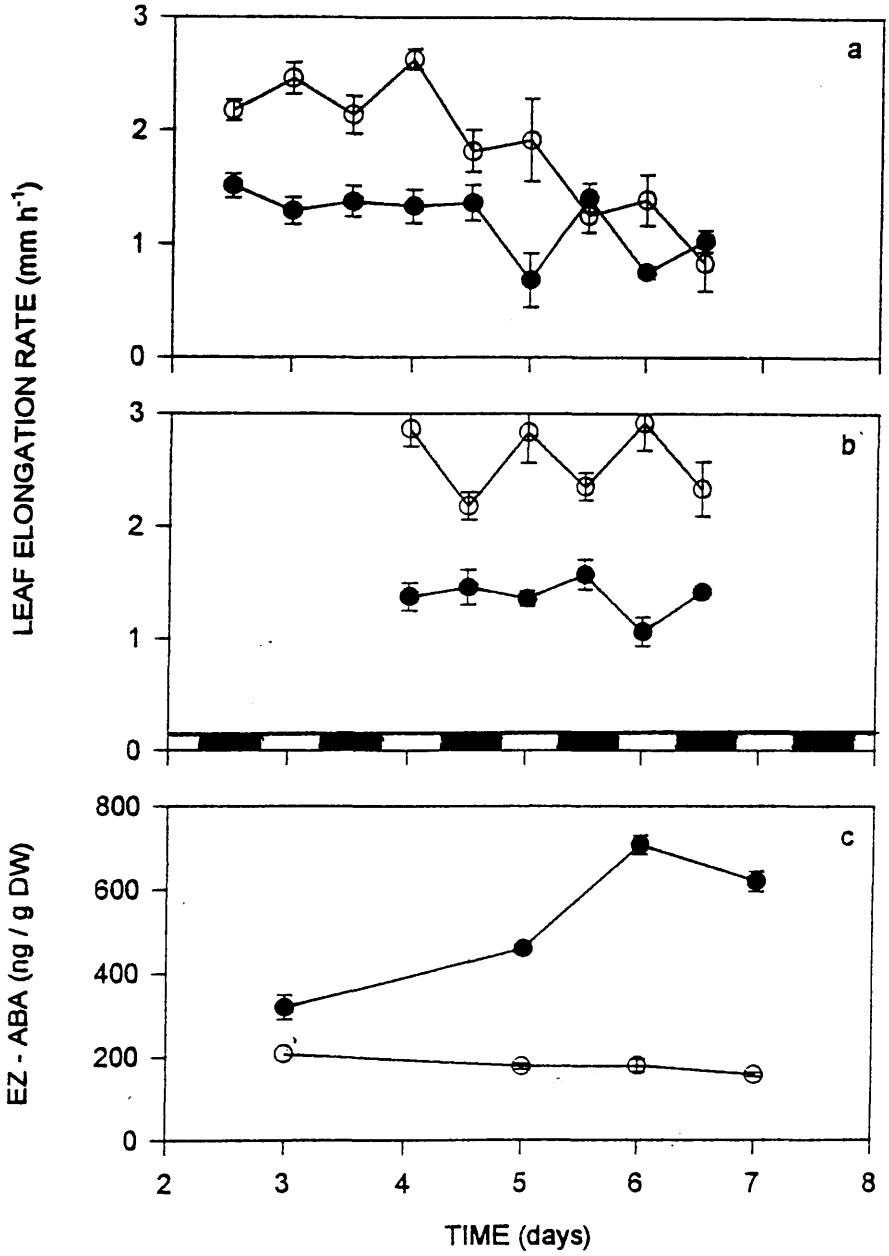

Figure 7.1: Time course of leaf elongation rate (LER) of leaf 3 (a) and leaf 4 (b) and average elongation zone (EZ) ABA concentration (c) of well watered (○) and droughted (●) maize plants over a soil drying cycle. Solid bars on the time axis indicate the night period. LERs are means ± S.E. of 6 (a) and 4 (b) leaves. ABA concentrations are means ± S.E. of 18 samples taken at different positions in the EZ (see Figure 7.3 for details of sampling regime). Samples from each position in the EZ were bulked from 6-8 plants.

Since there was a time lag between withholding water and the beginning of leaf length measurements, growth of leaf 3 of droughted plants had already been inhibited by 48 % during the light period of Day 3 (Figure 7.1a) and by 30 % in the previous nyctoperiod. Further inhibition of LER continued over the course of the drying cycle, although this was partially obscured in leaf 3 since the leaves of well-watered plants showed reduced LER as they approached maturity. At the end of the drying cycle (Day 6), day-time LER of leaf 4 of droughted plants was inhibited by 64 %.

Droughted plants did not show any pronounced diel variation of LER until late in the drying cycle (Days 5 and 6), when a reversal of the pattern exhibited in well-watered plants occurred such that nocturnal LERs of droughted plants were significantly ($P<0.05$) greater than day-time LERs for leaf 3, but not significantly ($P>0.10$) greater for leaf 4. Such enhancement of nocturnal LERs of droughted plants has previously been observed (e.g. Ephrath and Hesketh, 1991; Durand *et al.*, 1995) and probably reflects improved water relations of plants during the night. However, the signal which restricted leaf growth was not abolished by any improvement in plant water relations which occurred overnight. This may be interpreted as circumstantial evidence in favour of a chemical signal, although no definitive comments can be made in the absence of plant water relations measurements in this experiment. Since the restriction of leaf growth varied from day to night, it is likely that any putative chemical signal is interacting with plant water relations. This interaction has been demonstrated in the control of stomatal behaviour of field-grown droughted maize plants (Tardieu and Davies, 1992; Tardieu *et al.*, 1993) and is further investigated in Chapter 8.

By Day 3, ABA concentration in the bulk elongation zone had increased significantly ($P< 0.05$) by 1.8 times from *circa* 180 ng / g DW in well-watered plants to 320 ng / g DW (Figure 7.1c). ABA concentration increased with time up to Day 6, reaching a maximum of 710 ng / g DW, an increase of 3.9-fold. These ABA concentrations appear to be consistent with those previously reported in the leaves of

both well-watered and droughted maize plants (Zhang and Davies, 1990a; Jovanovic and Quarrie, 1990; Pekic *et al.*, 1995).

It was important to show that ABA could accumulate in the EZ for two reasons. Firstly, no data could be found in the literature which expressly measured the EZ-ABA concentration of droughted plants. Although Jovanovic and Quarrie (1990) studied the whole-plant distribution of ABA in droughted maize plants, their data refer to "young leaves and apical tissue", and no determinations of the extent of the leaf elongation zone were performed. Secondly, there is no reason to automatically suspect increased ABA concentration in the EZ under drought, since the stimulus for ABA synthesis (i.e. turgor loss) is usually absent in the EZ of droughted plants (e.g. Michelena and Boyer, 1982). Although the enhancement of [EZ-ABA] shown here is good evidence of a chemical signal, the origin (Is ABA synthesised in the leaves and transported in the phloem to the EZ, or synthesised in the roots and transported in the xylem to the EZ, or synthesised in the EZ ?) and physiological significance (What are the primary events regulating leaf expansion in the early stages of drought ?) of the signal are unknown due to the inadequate time course of the leaf length measurements and the failure to document plant water relations.

The reduced sensitivity of changes in [EZ-ABA] (as opposed to changes in xylem sap [ABA]) in indicating drought-induced changes in hormonal relations is illustrated by the fact that [EZ-ABA] was only increased 1.8-fold in this experiment by a drought which reduced LER by 50 %; whereas a similar drought-induced reduction in LER elicited a 30-fold increase in xylem [ABA] (Zhang and Davies, 1990b). Thus it would seem difficult to discriminate drought-induced changes in plant ABA relations by measuring [EZ-ABA]; in a similar manner to which changes in bulk leaf [ABA] are not detected despite large increases in xylem ABA (Zhang and Davies, 1989b; 1990a). Although this would appear to negate the rationale behind measuring [EZ-ABA], it must be noted that it was impossible to obtain a large enough sap sample for ABA

analysis (using the RIA described in Section 5.2.2.3) from the plants used in this experiment, either by expressing xylem sap from detached leaves using the Scholander pressure bomb or collecting xylem sap from a single detopped shoot. This is quite apart from the technical uncertainties of whether the sap actually represents a captured portion of the transpiration stream, as discussed above in Section 7.1.

Figure 7.2 shows the spatial patterns of growth, or relative segmental elongation rate (RSER) at Days 3 and 5. The initial stages of water deficit (Day 3) reduced maximum RSER by 22 % but did not affect the length of the EZ of leaf 3 (Figure 7.2a), which remained at 36 mm. Although a similar EZ length (36 mm) was found in soil-grown maize and detached barley shoots (Figure 5.4a), the RSER data cannot be directly compared since the RSER of barley shoots was corrected to allow for marking injury. Data from Day 5 also show that water deficit had no effect on the EZ length of leaf 3 (Figure 7.2b). As LER of leaf 3 declined (Figure 7.1a), the EZ length of well-watered plants was reduced from 36 mm (Days 3 and 5) to 28 mm on Day 6 (data not shown). This reduction in EZ length as leaves approach maturity has been previously described (Schnyder *et al.*, 1990; Palmer and Davies, 1996). Despite this reduction, there was no indication that drought reduced the EZ length of leaf 3. However, it is difficult to be certain that drought *per se* had no effect on the EZ length of leaf 3, as the soil drying is likely to delay leaf development, thus any comparisons are confounded by the treatments differing in both the intensity of drought stress and the developmental stage of the leaf.

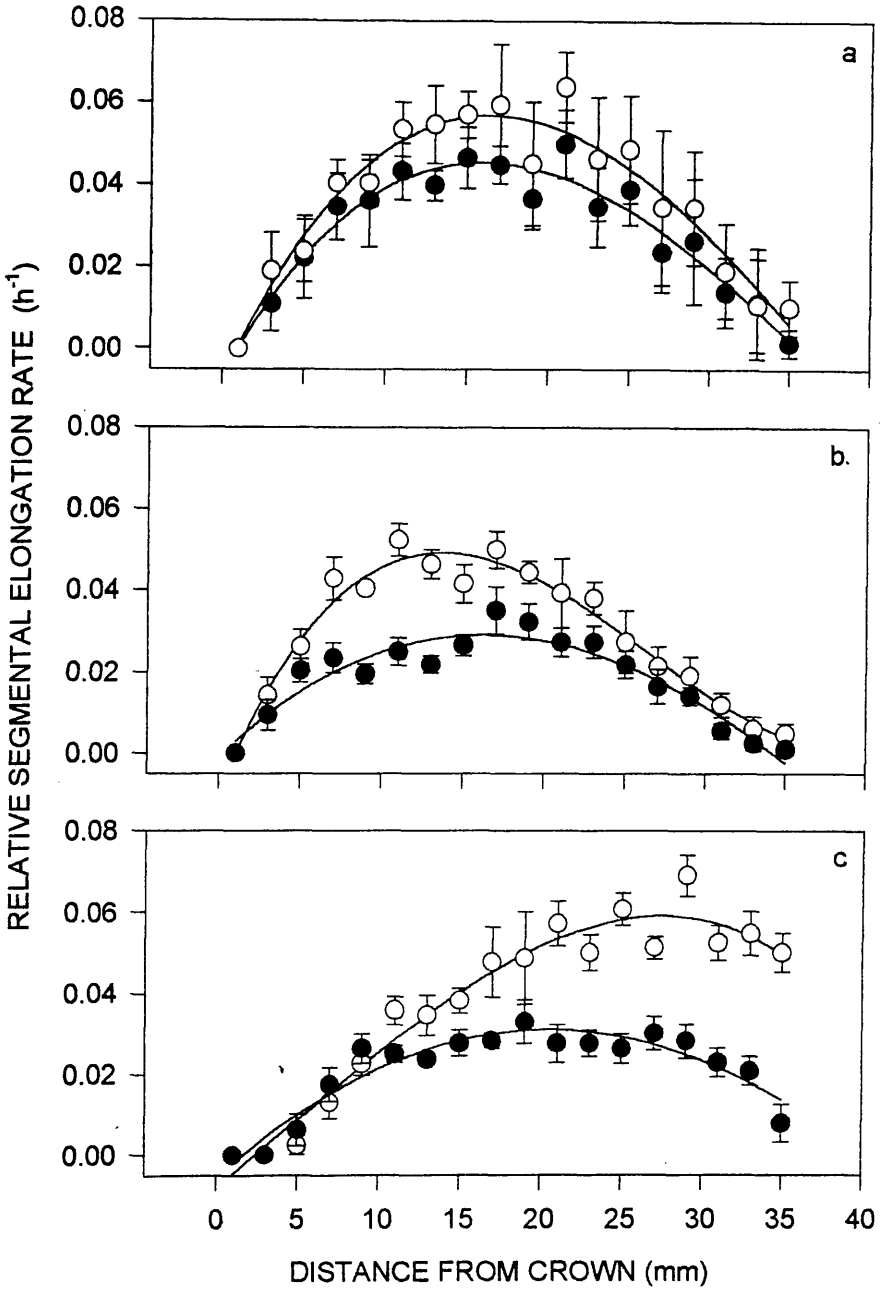

Figure 7.2: Spatial distribution of Relative Segmental Elongation Rate in well-watered (○) and droughted (●) maize plants for leaf 3 on Day 3 (a), leaf 3 on Day 5 (b) and leaf 4 on Day 5 (c). Points are means ± S.E. of 8 replicates. Lines are third order regressions fitted in SPW 1.0.

Although the EZ of leaf 4 was not fully determined at Day 5, it is clear that maximum RSER has been reduced and extrapolation of the regressions shows that EZ length has been reduced (Figure 7.2c). Data from Day 6 confirmed this conclusion, with EZ length of leaf 4 being reduced by drought from 50 mm to 36 mm (data not shown). Although the EZ of leaf 4 was reduced, there was maintenance of growth at the base of the leaf (0-10 mm from the crown in Figure 7.2b). A similar basal maintenance has been shown for growing leaves (Spollen and Nelson, 1994; Durand *et al.*, 1995) and roots (Sharp *et al.*, 1988) subjected to drought.

It is interesting to note that the spatial pattern of growth for leaf 4 on Day 5 (Figure 7.2c) is very different from that in leaf 3 (Figure 7.2b) on the same day and from that seen when detached shoots are fed ABA (Figure 5.4a), in that EZ length is not maintained under drought. Although this would appear to be evidence against the role of ABA in controlling leaf growth in droughted plants, by Day 5 it is likely that water relations in droughted plants were affected, so the growth profile in Figure 7.2c may represent an interaction of perturbed water relations and increased ABA accumulation. When the water stress is less severe (Day 3), there is agreement in the general profile of spatial distribution of growth under drought stress (Figures 7.2a, b) and ABA treatment (Figure 5.4a).

Figure 7.3 compares the spatial pattern of ABA in the elongation zone at Day 5 The spatial distribution of ABA in detached maize shoots fed ABA via the sub-crown internode is included for comparison. ABA content is expressed on a on a weight of water basis, which emphasises (compared to expression on a dry weight basis) the spatial variation in ABA content. The spatial pattern of ABA accumulation is essentially similar regardless of whether the ABA is produced endogenously in response to drought, or supplied externally via the sub-crown internode in a leaf elongation assay. Thus sampling of a bulk elongation zone (as in Section 5.3) appears to be a valid measurement to make since ABA concentration is reasonably

homogenous in the expanding leaf, 10 to 40 mm from the node, under drought stress treatment.

It is interesting that the spatial pattern of ABA in the EZ is similar irrespective of whether the source of ABA is endogenous (drought stress treatment) or exogenous (feeding via the sub-crown internode). This would suggest that ABA in the EZ of droughted plants accumulates as a result of transport from other parts of the plant, and not as a result of synthesis. A possible lack of ABA synthesis in the elongation zone is consistent with results showing turgor maintenance in the EZ (e.g. Michelena and Boyer, 1982), since turgor loss is considered to be the signal for ABA synthesis (Pierce and Raschke, 1980; 1981). However, potential spatial patterns of ABA synthesis using detached, dehydrated elongation zones (as in Section 5.3.3.3) were not examined.

7.2.4 CONCLUSIONS

This experiment shows the plant response to a rapid and moderately severe soil soil drying, where the initial changes in LER and ABA were not observed due to the measurement regime. Although it is impossible to speculate on the possible causes of the leaf growth inhibition, ABA may play a role since it has been shown to inhibit maize leaf growth (Figure 3.3a); and since it has been shown to accumulate in the EZ (Figure 7.1c), which previously has not been conclusively demonstrated. Importantly, spatial analysis of growth and ABA accumulation have allowed the definition of an essentially homogenous (with respect to ABA) part of the elongation zone to sample in more detailed studies of plant water and ABA relations in a milder soil drying treatment.

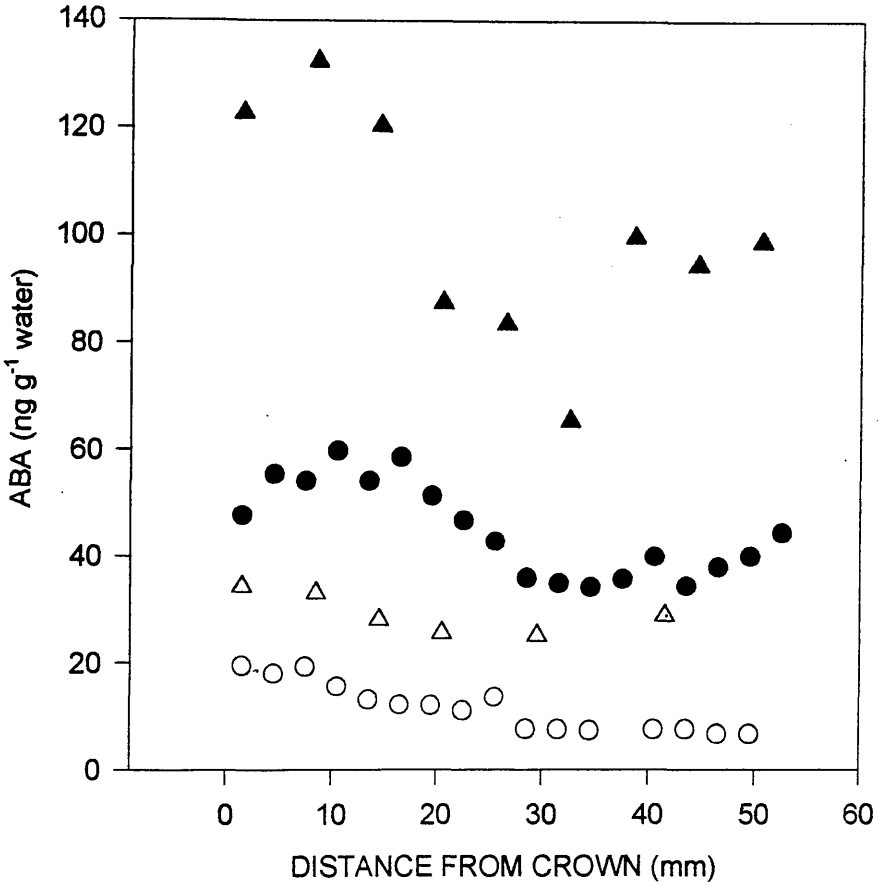

Figure 7.3: Spatial distribution of ABA in the leaf elongation zone of well-watered (○) and droughted (●) maize plants on Day 5; and in detached maize shoots fed an artificial xylem solution (Δ) or 10^{-6}M ABA (▲) in a leaf elongation assay for 8 hours. Samples are from leaf 3 and are bulked from 6-8 plants.

7.3 DAILY VARIATION IN LEAF ELONGATION AND ABA CONTENT OVER A SOIL DRYING CYCLE

7.3.1 INTRODUCTION

The experiment in the previous Section was not performed with sufficient temporal resolution to allow determination of the primary events associated with leaf growth reductions. While daily measurements provide useful temporal information on changes in LER, it is possible to investigate the time course of drought-induced growth inhibition over the course of a day using ruler measurements (e.g. Chu and McPherson, 1977). Such a procedure was adopted as it allowed the determination of whether the reduction in afternoon leaf elongation previously described for droughted plants (Chu and McPherson, 1977; Van Volkenburgh and Boyer, 1985) could be explained in terms of increased ABA accumulation in the leaf elongation zone. Previous studies have shown that diurnal changes in g_s of droughted plants could not be explained in terms of diurnal variation of xylem sap ABA concentration (Loveys *et al.*, 1987; Wartinger *et al.*, 1990; Tardieu *et al.*, 1992a; Correia *et al.*, 1995). Although such examples negate the possibility of changes in chemical signalling *per se* (but not potential changes in sensitivity) accounting for growth changes, the leaf growth system could well be different from stomatal systems since the magnitude of growth inhibition in the bioassay system increased with ABA accumulation under high temperatures and VPDs (Figure 5.7). It is therefore possible that the higher temperatures and VPDs seen in the afternoon (the time at which leaf growth inhibition is first detected in a drying cycle - Chu and McPherson, 1977; Van Volkenburgh and Boyer, 1985) may allow an accumulation of ABA in the elongation zone. As far as the author is aware, this is the first attempt to ascribe diurnal responses of LER in droughted plants to a specific chemical signal.

7.3.2 MATERIALS AND METHODS

Two experiments were conducted at different times of the year using both barley (*Hordeum vulgare* cv. Firefly) and maize (*Zea mays* cv. Earliking). In both experiments, plants were grown in a 50:50 mixture of John Innes No. 2 compost and gravel in rectangular drainpipes (6 cm x 6 cm x 30 cm). Two pre-germinated barley seeds or one seed of maize were deep-sown (to induce a sub-crown internode) in each drainpipe. For barley, seedlings were thinned to 1 plant per drainpipe when leaf 2 appeared. All plants were well watered until leaf 3 had emerged, when half the drainpipes remained unwatered for the rest of the experiment while the other half continued to be watered daily at the beginning of the night period. Average maximum and minimum temperatures and relative humidities in the growth cabinet during the experiment were 24°C and 14°C, and 80 % and 52 % respectively for barley; and 36°C and 22.5°C, and 69 % and 28 % respectively for maize. A 12 hour photoperiod was maintained.

Three (barley) or two (maize) days after withholding water, twelve plants per treatment were randomly selected for measurement of leaf length of all expanding leaves. Measurement of labelled plants occurred 4 times per day so that LER could be calculated for 3 periods during the light period and overnight. LER was also calculated for 12 hour periods to compare daily leaf elongation rates with the literature. On certain occasions, three (barley) or five (maize) randomly selected plants per treatment were chosen for sampling ABA and lamina water relations.

Water relations data was obtained by removing a 7 mm diameter disc of lamina from leaf 2 and immediately sealing it in a psychrometer cup. The cup was loaded into a C-52 chamber (Wescor Inc., Logan, UT, USA) and incubated at 25°C for 3 hours before ψ_L was read with a dew point microvoltmeter (HR-33T, Wescor Inc., Logan, UT, USA). The disc was then removed, wrapped in aluminium foil, and plunged into

liquid nitrogen to disrupt the cell membranes. The frozen disc was allowed to thaw for 5 min before being re-sealed in the chamber to incubate for 30 minutes prior to the determination of osmotic potential ψ_π. Water relations data were used to calculate lamina turgor (ψ_p) according to the equation: $\psi_p = \psi_L - \psi_\pi$.

Barley samples for ABA determination were taken from leaf 2 and a 30mm section of the EZ (10-40 mm from the node, containing tissue from the youngest emergent leaf and any enclosed leaves). In maize, it was possible to separate the expanding leaves, therefore sampling comprised leaf 2 and a 30 mm section (10-40 mm from the node) of each expanding leaf. All samples were analysed by the RIA (see Section 5.2.2.3) of Quarrie *et al.* (1988).

Every second day, 3 drainpipes per treatment were opened up, the soil column divided into 7 cm sections, and a sample of soil collected from each section. The soil was transferred to a pre-weighed glass vial (W_v = weight of vial), weighed within an hour ($W_{v+wet\ soil}$), and allowed to dry for 1 week at 70°C before re-weighing ($W_{v+dry\ soil}$), allowing the determination of gravimetric moisture content (θ) according to the equation:

$$\theta\ (\%) = (W_{water} / W_{dry\ soil}) \times 100$$
$$= (W_{v+wet\ soil} - W_{v+dry\ soil}) / (W_{v+dry\ soil} - W_v) \times 100$$

From Day 6 in the maize experiment, it was possible to extract a sample of xylem sap from leaf 4. Sap was collected at 0.5 MPa above the balancing pressure for a period of 5 minutes, which generally produced < 30 µL of sap. The sap was immediately frozen at -20°C after collection. Although the volume of sap did not permit quantification of ABA concentration using the ABA assay described in Section 5.2.2.3, it was possible to measure xylem pH using a microelectrode (Model 9802 BN,

Orion Instruments, Boston, MA, USA) coupled to a pH meter (DR 359 Tx, EDT Instruments, Dover, England).

Statistical differences between droughted and well-watered plants were determined by Student's unpaired t-test in SPW 1.0. The significance of correlations between variables measured in the soil drying experiments was tested by linear regression in MTW 10.2.

7.3.3 RESULTS

The leaf growth data for maize and barley were very similar; therefore Figure 7.4 provides an example of changes in LER of all emergent maize leaves over a drying cycle. The leaves which achieved full expansion during the experiment (leaves 3 and 4) maintained rapid growth from emergence for a period of 3-4 days before their growth rate fell, as described by Palmer and Davies (1996). There was a pronounced diurnal rhythm to their growth, which probably reflected changes in temperature in the cabinet, since temperature has a major effect on LER (Watts, 1974). Interestingly, for the well watered treatment, leaf growth fell in the late afternoon, despite no change (or a slight increase) in temperature, as noted previously for well-watered plants in a growth cabinet (e.g Parrish and Wolf, 1983) and in the field (e.g. Squire *et al.*, 1983).

Figure 7.5 shows the diel effects of drought on leaf elongation expressed as a percentage of control plants in both experiments. In constructing this figure, the percentage inhibition values have been taken only from those leaves which were growing rapidly. Leaves at different stages of development were inhibited by drought to different degrees at any one measurement period (Figure 7.4). Younger leaves will

show differences between treatments while older leaves may not (as was shown for differences in spatial distribution of growth - Figures 7.2b, c). When leaves 3 and 4 approached the end of their period of expansion, the LER of the droughted plants matched or sometimes exceeded the LER of the well-watered plants. This occurred despite the LER of droughted plants being depressed during the period of rapid growth and may be a result of delayed development of the droughted leaves.

In the barley experiment (Figure 7.5a), as the drought progressed, differences in LER were noticed earlier in the light period, such that significant ($P<0.05$) differences were detected on Day 5 for the period 1500-1900, Day 6 for 1100-1500, Day 7 for 0700-1100 and not until Days 8, 10 and 11 for the dark period. A similar pattern was observed for maize (Figure 7.5b). Withholding water first had a significant ($P<0.05$) effect on LER of maize on Day 4 for the period 1500-1900, Day 5 for the period 0700-1100 and Day 6 for the period 1100-1500. By the end of the drying cycle, growth was progressively inhibited over the course of the day (Figure 7.5b). Growth was inhibited at night on Day 9, but only for leaf 5. This pattern of growth (inhibition of LER only during the day in the initial stages of soil drying with night-time reductions in LER occurring only later in the drying cycle) has been noted previously for field (Tardieu and Ben-Haj-Salah, 1995) and slowly-droughted cabinet-grown (Chu and McPherson, 1977) plants. It is important to note the similarity between the diurnal patterns of leaf growth inhibition in the two experiments (Figure 7.5), which would seem to indicate that the same mechanisms of growth inhibition are involved.

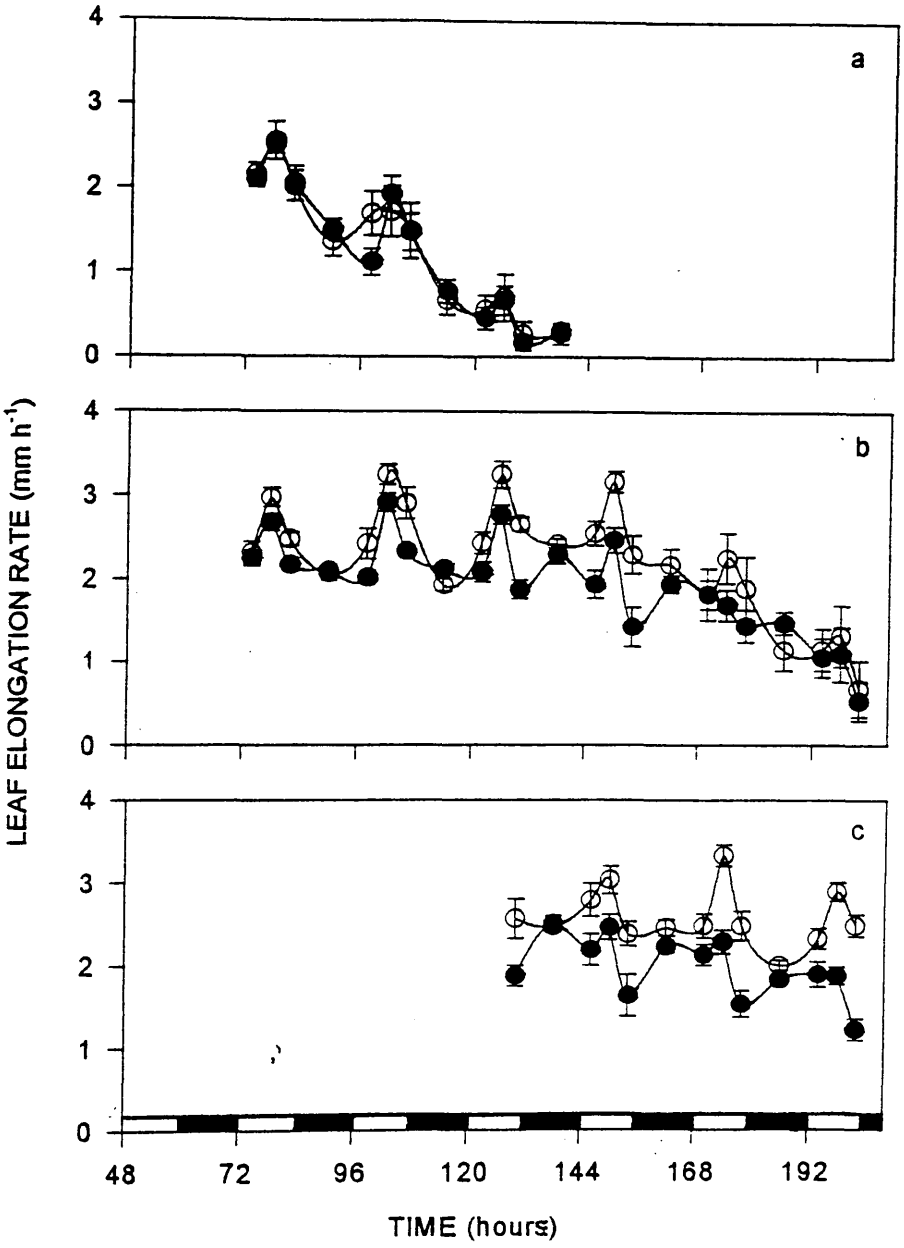

Figure 7.4: Time course of leaf elongation rate of leaf 3 (a), leaf 4 (b) and leaf 5 (c) in well-watered (○) and droughted (●) maize plants over a soil drying cycle. Solid bars on the time axis indicate the night periods. Points are means ± S.E. of 12 leaves.

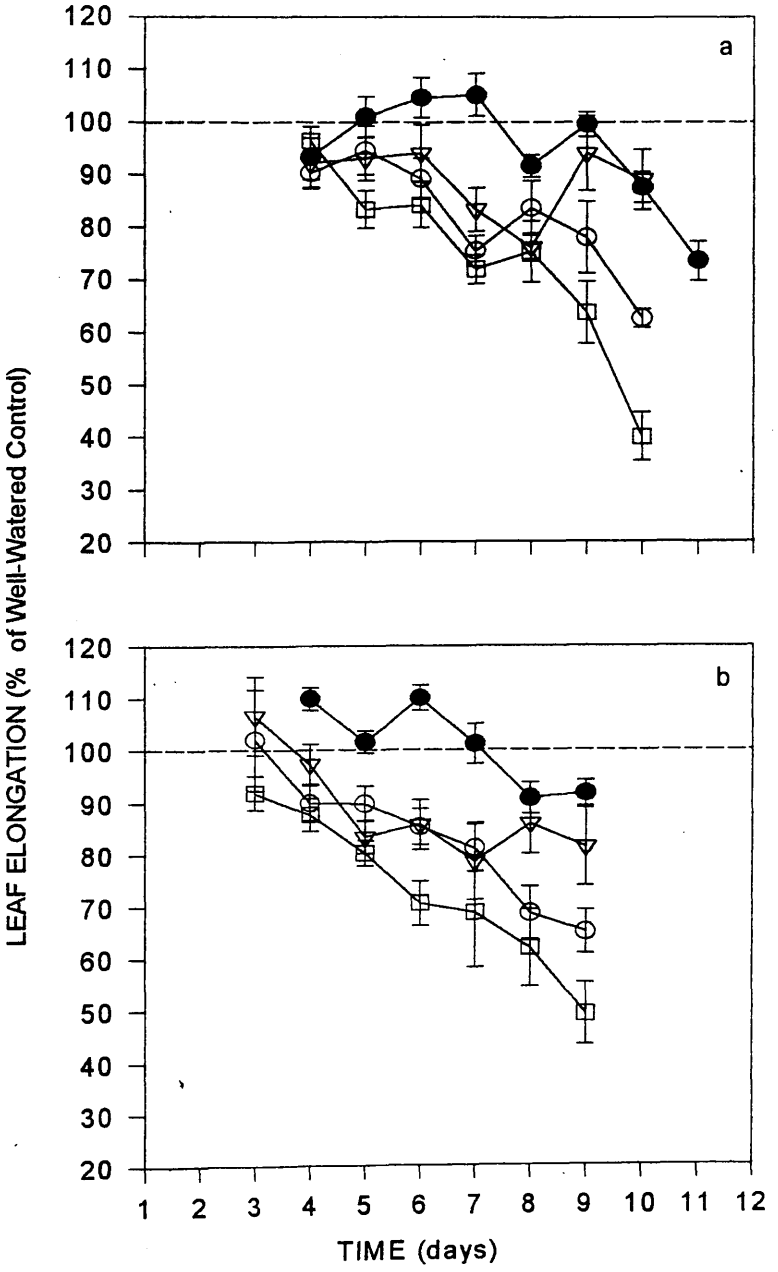

Figure 7.5: Time course of diel response of leaf growth in barley (a) and maize (b) over soil drying cycles. Points are means ± S.E. of 12 leaves at 3 times during the day: 0700-1100 (▽), 1100-1500 (○), 1500-1900 (□) and overnight (●).

Figure 7.6 shows the daily (calculated for the 12 hour period 0700-1900) leaf growth inhibition, lamina water relations and ABA relations for the barley experiment. Daily leaf elongation declined by 37 % by the end of the experiment (Figure 7.6a). Although ψ_L of droughted plants appeared to be more negative from Day 5, these differences were significant ($P<0.10$) only on Day 8 (Figure 7.6b). Water relations data are absent for Day 9. The ψ_π of droughted plants was significantly ($P<0.10$) reduced on Days 6, 8 and 10 (Figure 7.6c). Due to compensatory changes in ψ_L and ψ_π by droughted plants, the turgor of droughted plants was not different ($P>0.30$) at any stage in the drying cycle, except on Day 10, when droughted plants showed higher turgor (Figure 7.6d). Presentation of such data as daily means obscures the diurnal changes in lamina water relations which are noted in the field (Acevedo *et al.*, 1979) and which may (e.g. Chu and McPherson, 1977) or may not (e.g. Saab and Sharp, 1989) be detected in growth cabinet studies. In the early stages of the drying cycle (Days 5, 6), ψ_L and ψ_π of droughted plants were not different from well-watered plants in samples taken at 1300 but were significantly ($P<0.05$) reduced in samples taken at 1700 ($n=3$). However, these differences were not observed at any other stage in the experiment. Leaf ABA of droughted plants was significantly increased only on Day 10 (Figure 7.6e). An increase was also noted in the elongation zone on Day 10 at this time (Figure 7.6f), but this was not significant as there was a simultaneous increase in the [EZ-ABA] of well watered plants. There were no diurnal changes in leaf 2 [ABA] (data not shown) or [EZ-ABA] (Table 7.1) in either watering regime. Consequently, the number of replicates for ABA analysis at each time of day was increased from 3 (barley) to 5 (maize).

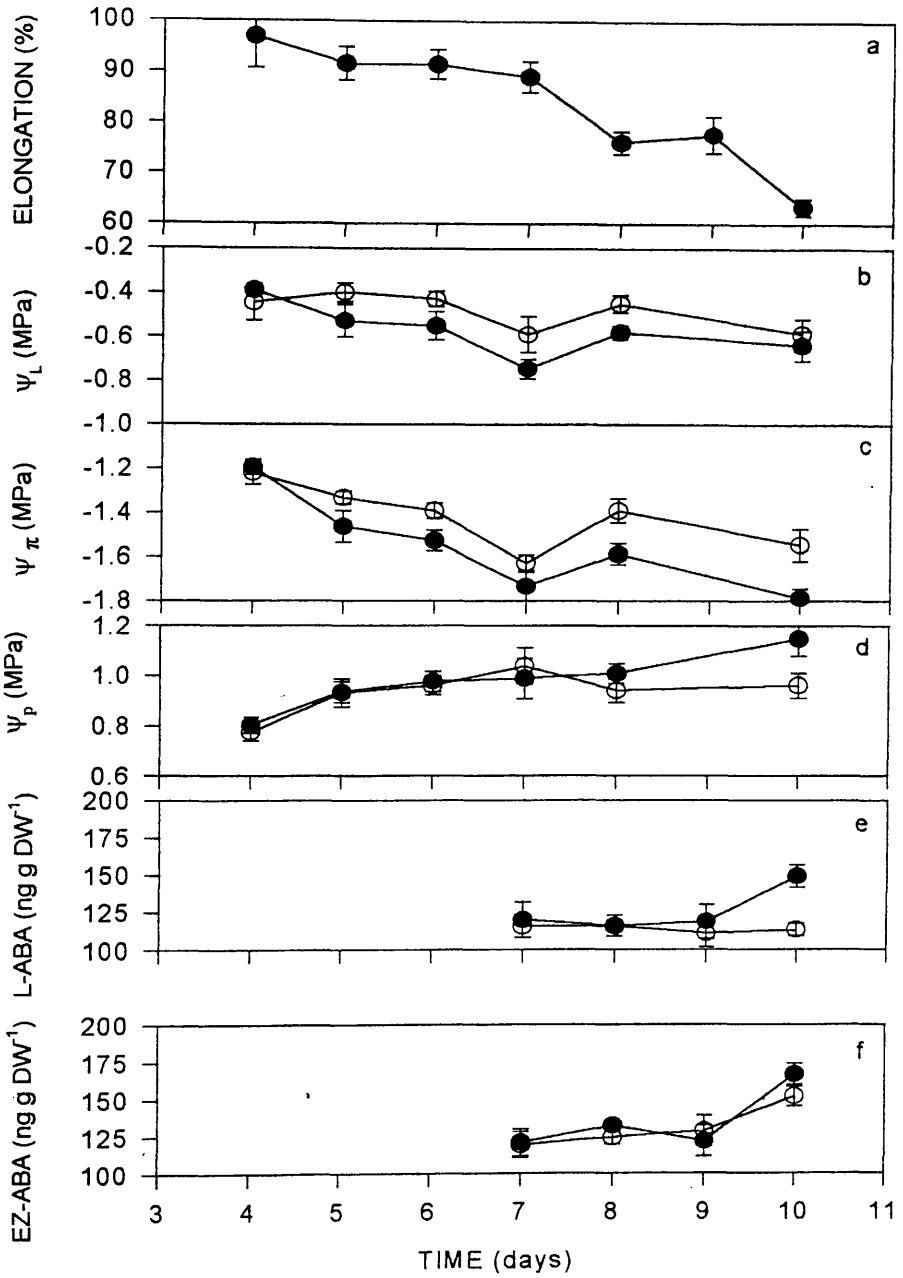

Figure 7.6: Time course of daily (calculated for the period 0700-1900) leaf elongation of droughted plants expressed as a percentage of well-watered controls (a); and of lamina water potential (ψ_L) (b), lamina osmotic potential (ψ_π) (c), lamina turgor (ψ_p) (d), mature leaf ABA concentration (L-ABA) (e) and bulk elongation zone ABA concentration (EZ-ABA) (f) of well-watered (○) and droughted (●) barley plants over a soil drying cycle. Points are means ± S.E. of 12 (a) or 6 (b-f) measurements. In b-f, 3 measurements per treatment were taken during each period 1100-1500 and 1500 - 1900.

Table 7.1: ABA concentration of the bulk elongation zone [EZ-ABA] of well-watered and droughted barley (*Hordeum vulgare* L. cv. Firefly) plants for two periods of the day: 1100-1500 and 1500-1900. Values are means ± S.E. of 3 replicate plants. Differences between periods were discriminated by Student's t-test in SPW 1.0. NS = not significant at $P < 0.10$.

	Well-watered plants			Droughted plants		
Day	[EZ-ABA] (ng / g DW)		P value	[EZ-ABA] (ng / g DW)		P value
	1100 - 1500	1500 - 1900		1100 - 1500	1500 - 1900	
8	115.7 ± 4.1	120.3 ± 6.7	NS	136.0 ± 2.9	133.7 ± 3.5	NS
9	113.3 ± 11.3	144.3 ± 11.8	NS	106.0 ± 6.4	132.0 ± 14.7	NS
10	151.3 ± 11.5	160.0 ± 5.0	NS	161.3 ± 11.4	176.3 ± 13.2	NS

Figure 7.7 shows the daily leaf growth inhibition, lamina water relations and ABA relations for the maize experiment. The growth data are similar to the barley experiment, with growth being inhibited by 35 % by the end of the drying cycle (Figure 7.7a). Due to poor germination in the maize experiment, there were insufficient well-watered plants to allow collection of psychrometric and ABA data each day. To allow statistical comparisons between treatments, the data from well-watered maize plants, (sampled on 3 occasions) was combined (n=15) since ANOVA indicated no significant differences between measurement periods in either water or ABA relations. For maize, the only significant ($P<0.10$) differences in water relations were detected for ψ_L on the afternoon (1500-1900) of Day 7 (Figure 7.7b), and ψ_π on the same day for both measurement periods (1100-1500 and 1500-1900) (Figure 7.7c). The only significant ($P<0.10$) difference in lamina turgor was seen on Day 9, when water-stressed plants showed higher turgors (Figure 7.7d). Figure 7.7e shows that there was no consistent increase in the ABA content of the maize mature leaf. In the elongation zone (Figure 7.7f), ABA had apparently increased by the end of Day 6, but the changes were not significant ($P<0.05$) until Day 8.

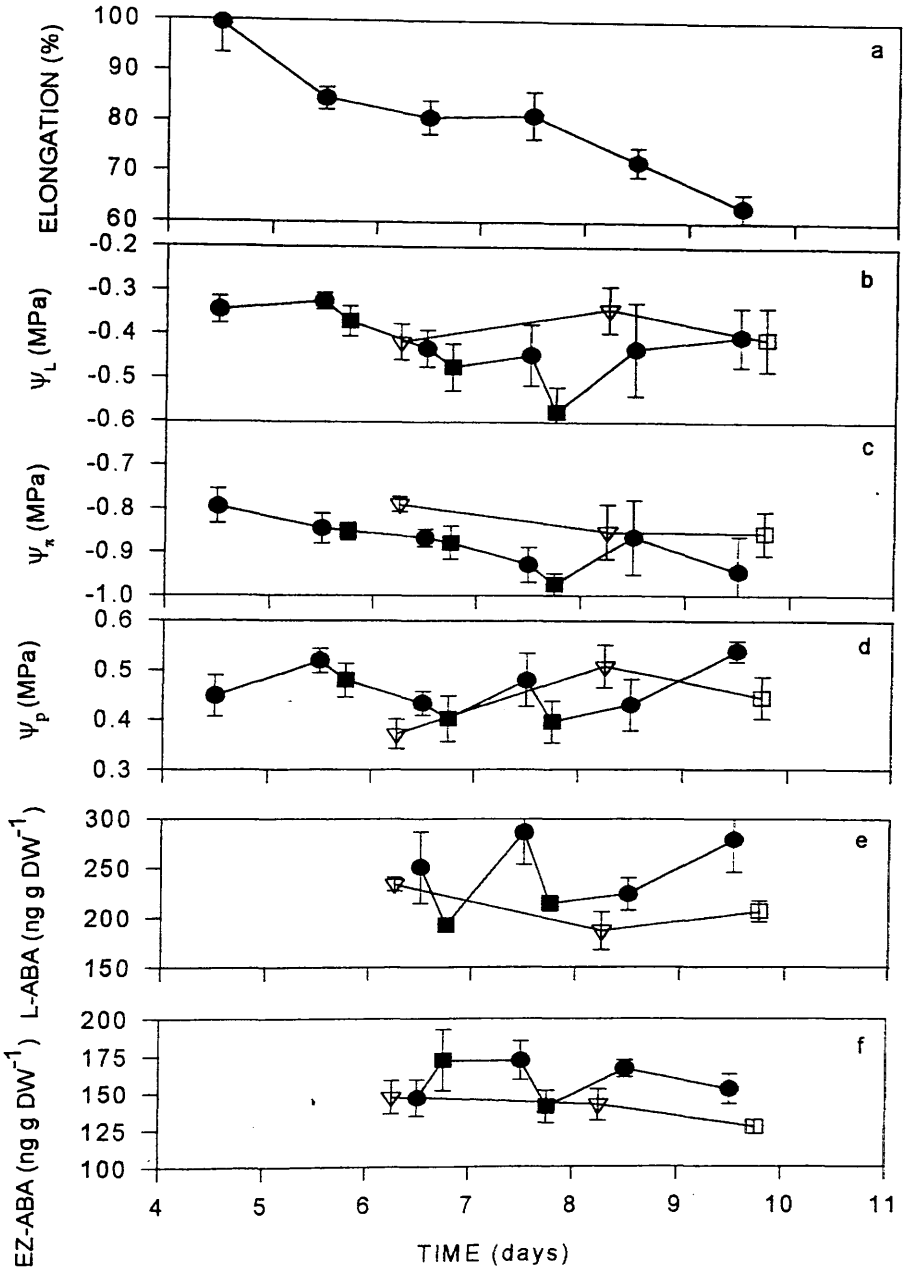

Figure 7.7: Time course of daily (calculated for the period 0700-1900) leaf elongation of droughted plants expressed as a percentage of well-watered controls (a); and of lamina water potential (ψ_L) (b), lamina osmotic potential (ψ_π) (c), lamina turgor (ψ_p) (d), mature leaf ABA concentration (L-ABA) (e) and bulk elongation zone ABA concentration (EZ-ABA) (f) of well-watered (hollow symbols) and droughted (filled symbols) maize plants over a soil drying cycle. Measurements were taken during the periods 0700-1100 (∇, ▼), 1100-1500 (○, ●) and 1500-1900 (□, ■). Points are means ± S.E. of 12 leaf elongation measurements (a), 5 water relations measurements (b-d) and 5-15 ABA determinations (e, f).

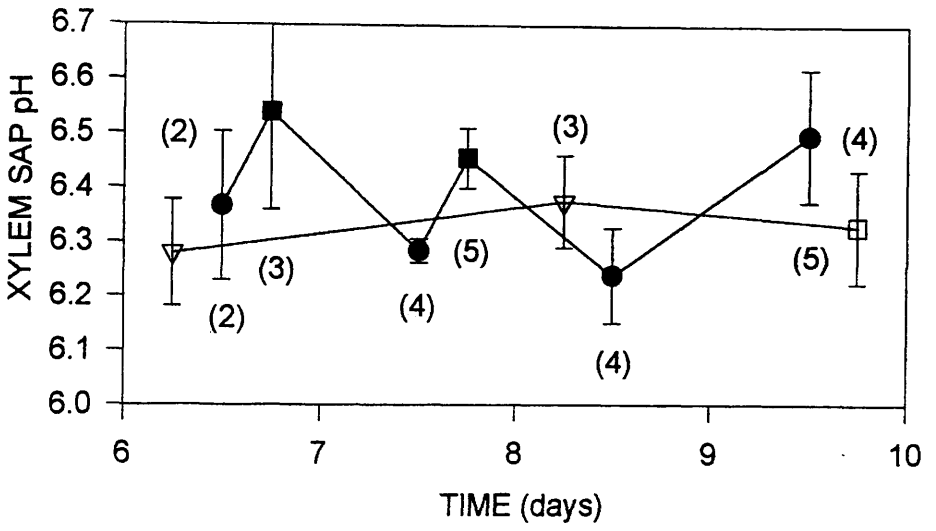

Figure 7.8: Time course of xylem sap pH of well-watered (hollow symbols) and droughted (filled symbols) maize plants over a soil drying cycle. Measurements were taken during the periods 0700-1100 (▽, ▼), 1100-1500 (○, ●) and 1500-1900 (□, ■). Points are means ± S.E. of the number of replicates given in parentheses above (well-watered) or below (droughted) each symbol.

Figure 7.8 shows the response of xylem sap pH in the maize experiment over the last 4 days of the drying cycle. There were no differences in pH between droughted and well-watered plants. In only one instance (Day 7) did sap pH in droughted plants increase (0.2 units) over the course of the day, which contradicts the characteristic diurnal variation regularly seen by Schurr and Schulze (1995) in intact well-watered and droughted plants of *Ricinus communis*.

Plotting leaf elongation rate against [EZ-ABA] for the 2 experiments (Figure 7.9) failed to produce any relationship between the two variables (cf. the data from the detached shoot system in Figure 6.2b). The greater inhibition of LER seen in the afternoon (1500-1900) measurements was not accompanied by increased [EZ-ABA] (half-filled symbols in Figure 7.9b). The fact that leaf elongation declined in the droughted plants without enhanced ABA accumulation indicated that the leaves were either responding to some other compound (although the data in Figure 6.2b negate this idea), or that the measurement techniques for determining [EZ-ABA] were not sufficiently precise to discriminate any differences, or that the ABA in the elongation zone of the droughted plants was more effective in inhibiting growth.

Figure 7.10 shows the relationship between elongation zone ABA and mature leaf ABA for the 2 experiments reported in this Section. In contrast to experiments where ABA was fed to detached shoots (Figure 5.8), there was no preferential accumulation of ABA in the elongation zone under the mild water stress treatments applied here.

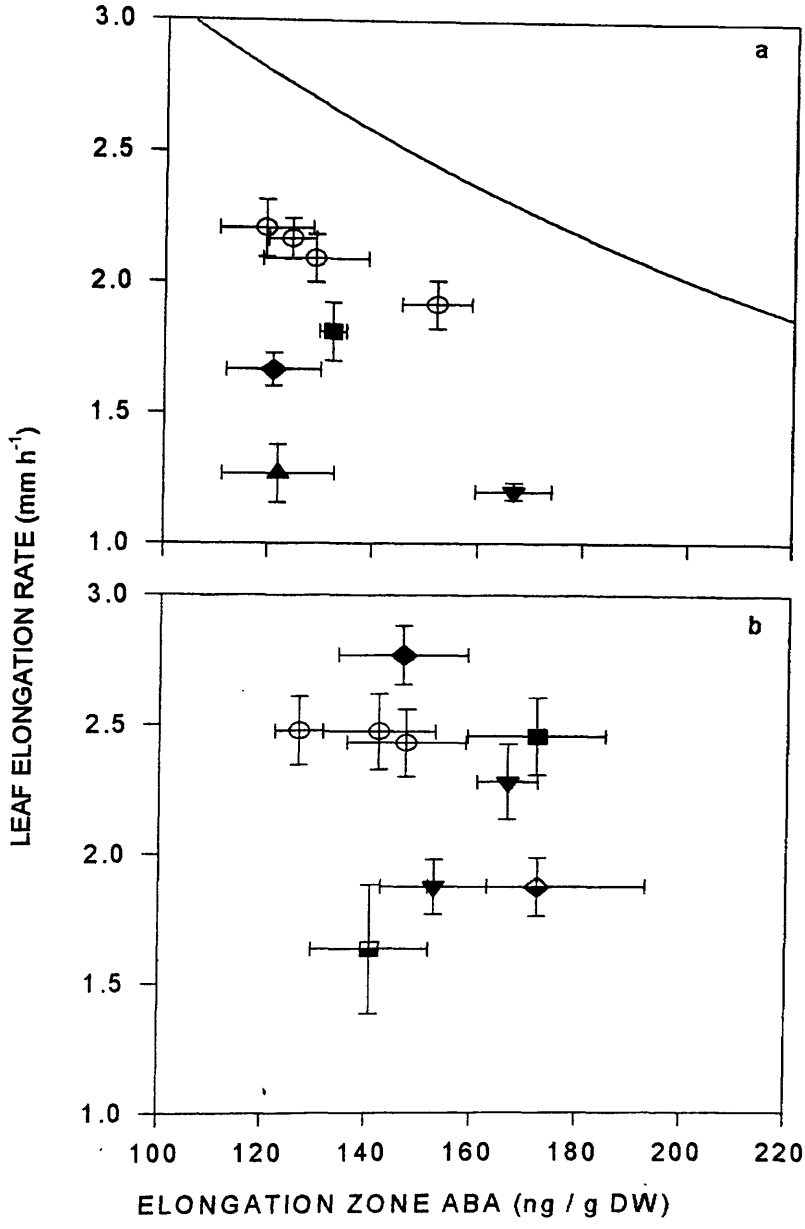

Figure 7.9: Relationship between leaf elongation rate and the ABA content of the bulk elongation zone over 4 sequential days of a soil drying cycle during the barley (a) and maize (b) experiments. Well watered plants are hollow symbols, while filled symbols refer to droughted plants on Days 1 (◆), 2 (■), 3 (▲) and 4 (▼) (corresponding to Days 7-10 in Figs. 7.5a, 7.6 for (a) and Days 6-9 in Figs. 7.5b, 7.7 for (b)). Half-filled symbols in (b) are for plants sampled between 1500-1900 while the filled symbols are for plants sampled between 1100-1500. Points are means ± S.E. of 12 leaves and 5-15 ABA determinations. The line in (a) is from Figure 6.2b. ABA values in (a) are from plants sampled 2 or 3 times during the day for which no diurnal variation in ABA content was detected. Values in (b) are from up to 3 leaves in the elongation zone for which no inter-leaf variation in ABA content was detected.

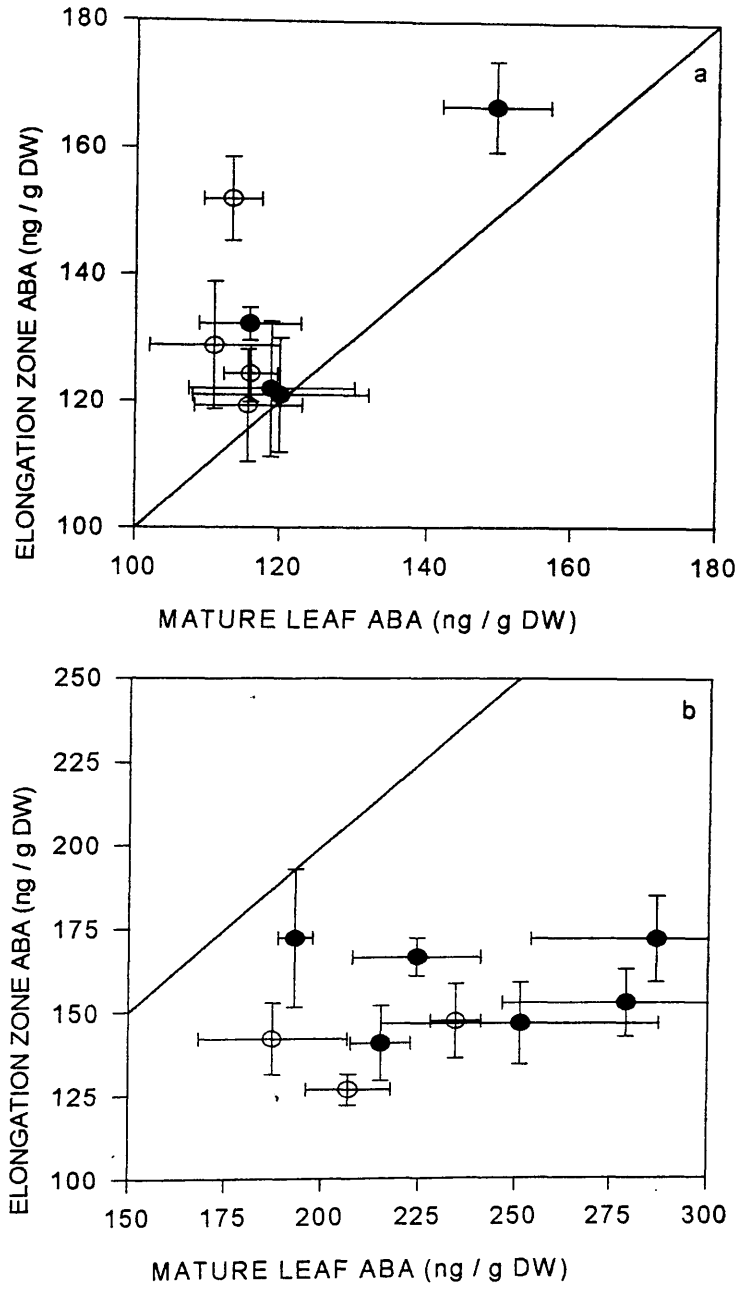

Figure 7.10: Relationship between mature leaf (leaf 2) ABA content and elongation zone ABA content for well-watered (○) and droughted (●) barley (a) and maize (b) plants during the soil drying experiments. The line in each indicates the 1:1 relationship. Points are means ± S.E. of 6 (a) or 5 (b) plants.

In contrast to the lack of relationship between LER and EZ-ABA, there were significant (P=0.075 for barley; P=0.021 for maize) linear relationships between LER and ψ_L (Figure 7.11) in the two experiments, with LER decreasing in conjunction with ψ_L. Data on LER were expressed as a percentage of well-watered plants to allow for changes in LER due to daily changes in temperature and developmental age of the plants in both experiments, and time of day in the maize experiment. It should be noted that the relationship in Figure 7.11a was only significant when the data point from Day 7, which appeared to be an anomalous point in Figure 7.6b, was excluded. It should be emphasised that the ψ_L data were collected from a different set of leaves (leaf 2) from those that were measured for LER (leaves 3, 4 or 5), and from different plants. It is assumed that the ψ_L measurements would be representative of changes occurring in the plants on which LER was measured. It is likely that collection of data for paired determinations of ψ_L and LER would have increased the scatter of points and weakened the significance of the regression.

In the maize experiment, data on LER and ψ_L collected at two different times of the day (1100-1500 and 1500-1900) seemed to fit the one relationship (Figure 7.11b). This normalisation of the LER data has previously been shown to allow a number of unique relationships between LER (in mm h^{-1}) and ψ_L, differing in the time of day at which measurements were taken, to fit the one relationship (Chu and McPherson, 1977). It is, however, unfortunate that only 3 comparisons between the two time periods are available for Figure 7.11b, precluding the possibility of testing whether the normalisation procedure for LER actually allowed the data to fit the one relationship.

It was interesting that the correlation between ψ_L and LER seen in Figure 7.11 was linear, as noted by Acevedo *et al.* (1971) for similar data. This contrasts with the more commonly found negative exponential relationship between ψ_L and LER (Boyer,

1970; Ludlow and Ng, 1976; Chu and McPherson, 1977; Squire *et al.*, 1983) seen in droughted grasses.

There was no significant (P=0.59) relationship between LER and lamina turgor for the maize experiment (Figure 7.12b). However, in the barley experiment, a significant ($P<0.05$) relationship between LER and ψ_p (Figure 7.12a) was found, with turgor of droughted plants increasing as LER declined. It should be noted, however, that there are only a limited number of sample points, and the significance of this regression hinges on the points for Days 4 and 10; it is unlikely there would have been any significant relationship if these two points had been different. The direction of the relationship was at variance with similar relationships in the literature, which have shown LER to decrease as turgor decreases. It should be noted that most of these data have imposed a "drought" by altering the osmotic solution around the roots or rapidly droughting pot-grown plants (Hsiao *et al.*, 1985), although some data have been obtained from the field (Squire *et al.*, 1983). An example from the literature, which paralleled the form of the relationship shown here, was the data of Jones (1985), who showed that over a 10 week period, unirrigated apple trees had higher water potentials than irrigated plants.

A similar pattern of soil water depletion was found in both experiments. Both experiments showed that gravimetric soil water content (θ) did not vary significantly with time for any soil layer in the well-watered treatment. Figure 7.13 shows the changes in θ over the course of the maize experiment. Initial θ reductions occurred in the uppermost soil layer at Day 4, which is characteristic of drying experiments conducted in soil columns (Zhang and Davies, 1989a), and may reflect water use by a proliferation of surface roots. As the experiment progressed, θ fell in all layers of the soil.

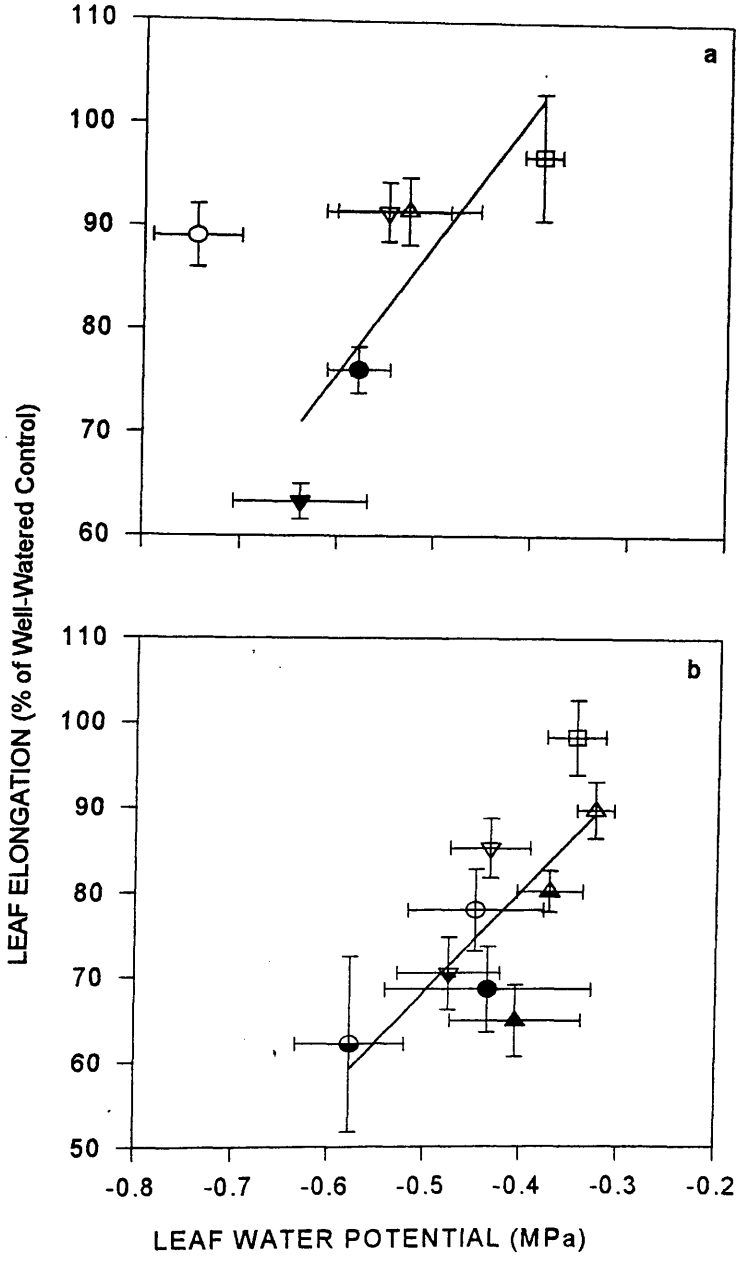

Figure 7.11: Relationship between leaf elongation rate of droughted plants (expressed as a percentage of the well-watered controls) and the leaf water potential during the barley (a) and maize (b) soil drying experiments. Symbols refer to Days 4 (□), 5 (Δ), 6 (▽), 7 (○), 8 (●), 9 (▲) and 10 (▼) of a drying cycle. Half-filled symbols in (b) are for plants sampled between 1500-1900 while the filled or open symbols are for plants sampled between 1100-1500. Points are means ± S.E. of 12 elongation rates and 6 (a) or 5 (b) water potential determinations. Linear regression in (a) excludes data from Day 7, while linear regression in (b) includes data from measurements taken from both 1100 to 1500 and 1500 to 1900. Linear regressions are LER % = $152.4 + 127.5\psi_L$; r^2=0.71 for (a) and LER % = $128.1 + 119.3\psi_L$; r^2=0.56 for (b).

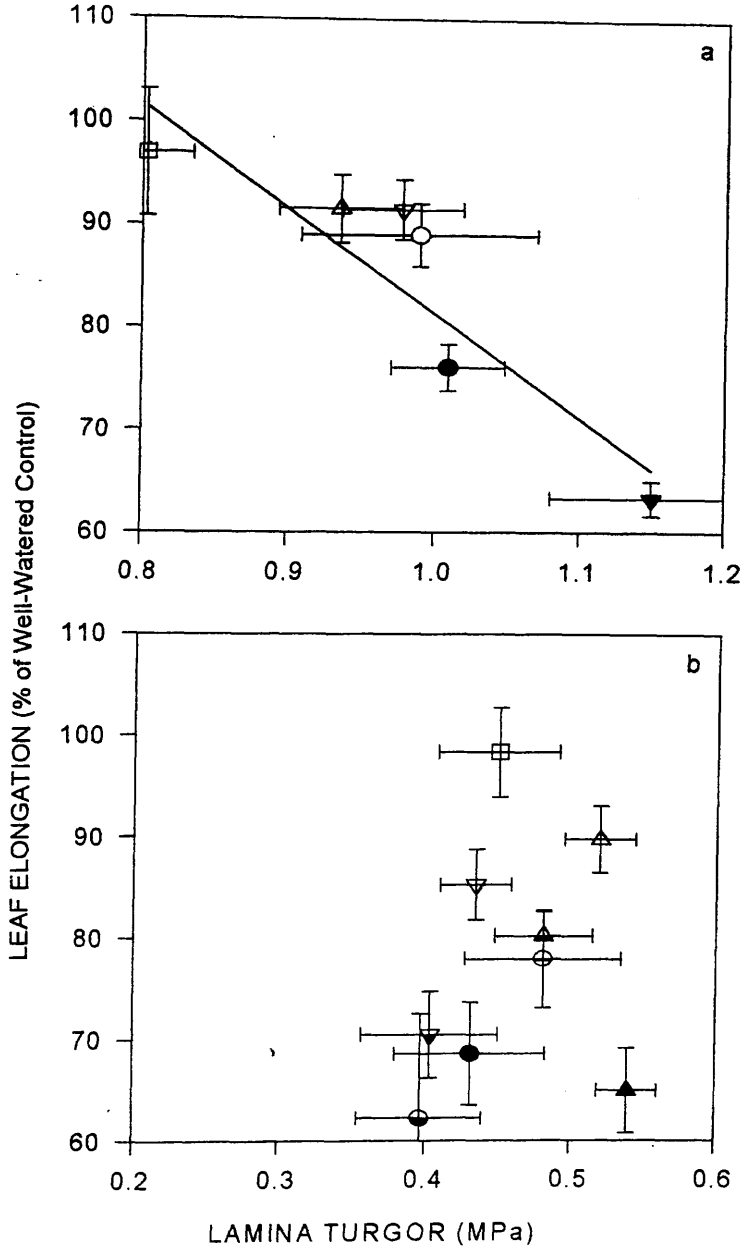

Figure 7.12: Relationship between leaf elongation rate (LER) of droughted plants (expressed as a percentage of the well-watered controls) and the lamina turgor (ψ_p) during the barley (a) and maize (b) soil drying experiments. Symbols refer to Days 4 (□), 5 (Δ), 6 (∇), 7 (○), 8 (●), 9 (▲) and 10 (▼) of a drying cycle. Half-filled symbols in (b) are for plants sampled between 1500 to 1900 while the filled and open symbols are for plants sampled between 1100 to 1500. Values are means ± S.E. of 12 elongation rates and 6 (a) or 5 (b) turgor determinations. Linear regression in (a) excludes data from Day 7 (to maintain consistency with Figure 7.11a) and is described by LER % = 167 - 83ψ_p; r^2=0.84. There was no significant relationship between LER and ψ_p in (b).

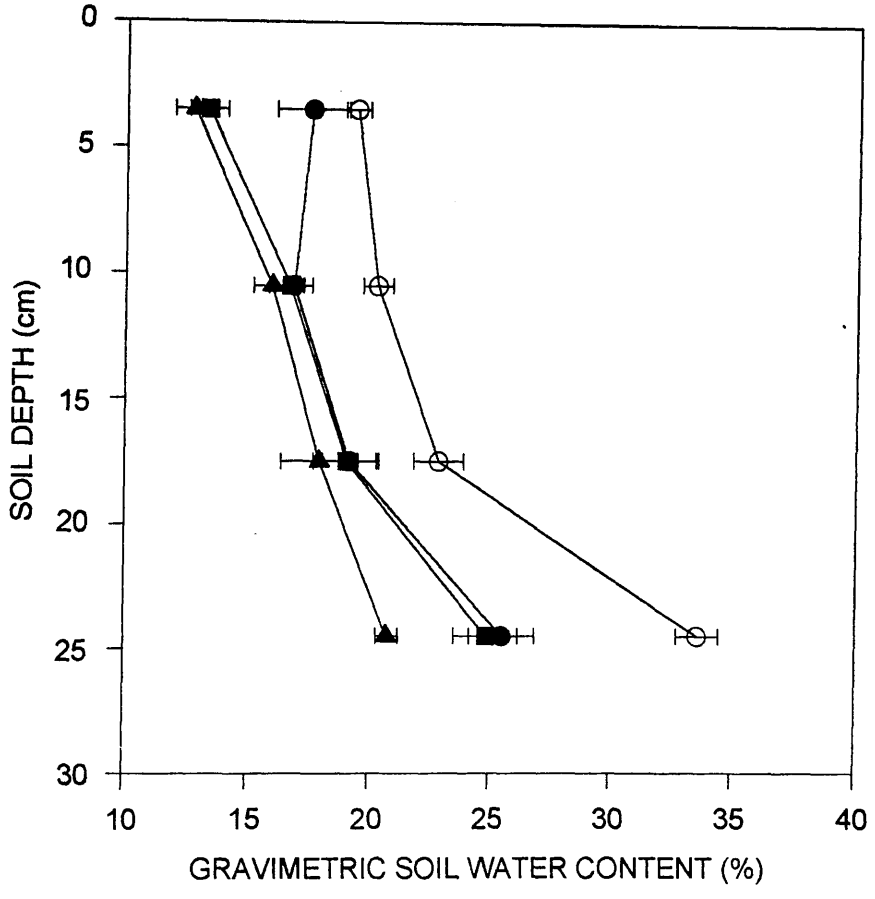

Figure 7.13: Changes in gravimetric soil water content at different soil depths during the maize experiment. Well-watered soil columns (○) showed no variation with time over the course of the experiment, so data from all days were combined. Points are means ± S.E. of 8 replicates. Data for droughted soil columns were collected on Days 4 (●), 6 (■) and 8 (▲). Points are means ± S.E. of 4 replicates.

Table 7.2 compares the different parameters measured in these drying experiments to show when significant treatment differences were detected. In determining the importance of any changes, it is necessary to compare the average coefficient of variation (cv%) for the different measurements (Table 7.3) to gauge some likelihood of treatment differences being detected for different parameters.

Table 7.2: Time series of changes in soil and plant parameters measured in soil drying experiments with barley and maize.

Parameter	Day on which significant ($P<0.05$) treatment differences seen	
	Barley	Maize
Leaf Growth - Day	5 (1500-1900)	4 (1500-1900)
Leaf Growth - Night	8	9
Leaf Water Potential (ψ_l)	5 (1500-1900), 8 (all data)	7 (not sustained)
Leaf Solute Potential (ψ_π)	5 (1500-1900), 6 (all data)	6 (not sustained)
Lamina Turgor	No Change	No Change
Soil Water Content (θ)	6 (possibly 5 -unmeasured)	4
Elongation Zone [ABA]	No Change	8
Mature Leaf [ABA]	10	7 (not sustained)

In both experiments, leaf growth was amongst the first parameters to differ, which was accompanied by changes in the water content of the upper layers of the soil profile in the maize experiment (the soil sampling regime for the barley experiment precluded identification of synchrony of changes). The timing of changes in water relations varied between experiments. In both cases, ψ_π appeared to be a more sensitive indicator of soil drying than leaf water potential. Changes in ψ_π apparently compensated for any reductions in ψ_L such that lamina turgor did not change in either experiment. Changes of ABA concentration in the elongation zone and mature leaves also appeared to be poor indicators of soil drying, as these occurred 3-5 days after the onset of leaf growth inhibition.

In the barley experiment, it was impossible to evaluate the relative importance of soil water content and changes in leaf water relations in influencing leaf growth; since the transient reductions in ψ_L and ψ_π on the afternoons that leaf growth was first inhibited were unaccompanied by measurements of soil water content. It is surprising that reductions in ψ_L and ψ_π were detected then, as much greater reductions in leaf growth were unaccompanied by perturbed water relations. The uncertainty over the detection of water relations in the barley experiment was

compounded by the need to combine data from two measurement periods to increase replication for statistical comparisons, and the greater cv% associated with water relations measurements (Table 7.3). In an attempt to remedy this situation, water relations variables in the maize experiment were sampled using a greater number of replicates at each observation period, precluding the need for pooled data.

In the maize experiment, leaf growth reductions preceded changes in lamina water relations by 2 days. Leaf growth was inhibited at the same time that changes in soil water content were detected, apparently indicating the regulation of growth by chemical signals. As noted previously, the similarity between the diel patterns of leaf growth inhibition in the two experiments (Figure 7.5) suggests that the same mechanisms of growth inhibition are involved.

Table 7.3: Average coefficient of variation for soil and plant parameters of well-watered and droughted soil columns during the maize soil drying experiment. Values are means ± S.E. of the number of observation periods shown in parentheses. The n value refers to the number of replicates taken at one observation period.

Parameter	Coefficient of variation (cv%)		Observation periods
	Well-watered	Droughted	
Leaf Elongation Rate	12.2 ± 1.4 (8) n=12	16.2 ± 1.8 (8) n=12	leaf 4, Days 4, 5
Leaf Water Potential	31.0 ± 5.1 (3) n=5	28.0 ± 4.8 (8) n=5	All periods, Fig 7.7b
Leaf Osmotic Potential	11.5 ± 3.5 (3) n=5	10.9 ± 2.3 (8) n=5	All periods, Fig 7.7c
Lamina Turgor	19.4 ± 1.0 (3) n=5	18.1 ± 2.5 (8) n=5	All periods, Fig 7.7d
Soil Water Content	9.1 ± 1.1 (8) n=4	11.2 ± 1.2 (12) n=4	All depths, Fig 7.13
Elongation Zone [ABA]	22.5 ± 6.6 (3) n=5	21.3 ± 5.5 (6) n=5	All periods, Fig 7.7f
Mature Leaf [ABA]	10.7 ± 4.5 (3) n=5	22.6 ± 4.5 (6) n=5	All periods, Fig 7.7e

It is important to note that the cv% of different parameters will vary over the course of a drying cycle, and that the data presented in Table 7.3 are averages. This is especially the case for leaf growth, where plant-to-plant differences in the timing of the cessation of leaf growth resulted in a doubling of the measured cv% (data not shown). However, measurement of all expanding leaves (Figure 7.4) ensured that one leaf was always in the period of rapid expansion, thus reducing the cv%. This technique ensured that there was roughly equal likelihood of detecting changes in leaf growth, soil water content and ψ_π (Table 7.3). Changes in ψ_L, lamina turgor and ABA concentrations were more difficult to detect due to inherent variability in their measurement. The difference in cv% between ψ_L and ψ_π (Table 7.3) is probably responsible for ψ_π being considered a more sensitive indicator of soil drying than ψ_L or turgor (Gallardo *et al.*, 1994; Auge *et al.*, 1995). Therefore, when determining on a time course basis (disregarding any underlying physiological reasons) whether soil or plant variables controlled growth, the most valid comparison would be between soil water content and ψ_π. Comparisons using lamina turgor and ψ_L are prejudiced by the higher cv% of ψ_L. Comparing the time course of ψ_π and θ (Table 7.2), it is apparent that θ changed 2 days before ψ_π in the maize experiment.

It is unfortunate that measurements of stomatal conductance (g_S) were not attempted in these experiments for comparison of the time course of changes. Leaf growth is usually considered to be more sensitive than g_S to soil drying (Hsiao, 1973, Passioura, 1988a; Saab and Sharp, 1989; Auge *et al.*, 1994; Ebel *et al.*, 1994), although contradictions can occur (Auge *et al.*, 1995). Table 7.4 compares data from two other soil drying experiments with maize where g_S was measured. The cv% data from these two maize experiments seem consistent with those described earlier for leaf growth (Table 7.3) and in the literature for g_S (25-35 % - Zhang and Davies, 1990a) Comparison of the cv% data shows that it is more likely for differences in leaf growth to be detected before changes in g_S. In cases where chemical signalling is suspected, this statistical anomaly may explain why leaf growth is inhibited prior to g_S, when there

appears to be no difference in transpiration and leaf growth responses to ABA over a wide temperature range (Figure 4.6).

Table 7.4: Average coefficient of variation for plant parameters of well-watered and droughted plants during two other maize soil drying experiments. Values are means ± S.E. of the number of observation periods shown in parentheses. Only the initial stages of soil drying were considered in each experiment. The n value refers to the number of replicates taken at one observation period.

Experiment	Treatment	Coefficient of Variation (%)	
		g_s	LER
Greenhouse	Well-watered	16.4 ± 3.9 (3), n=8	10.3 ± 2.5 (3), n=12
	Droughted	31.2 ± 4.9 (3), n=8	12.8 ± 6.1 (3), n=12
Growth Cabinet	Well-watered	33.7 ± 4.3 (5), n=8	16.7 ± 2.0 (5), n=12
	Droughted	41.7 ± 6.8 (5), n=8	24.3 ± 4.1 (5), n=12

7.3.4 DISCUSSION

Table 7.5 attempts to compare the experiments described here with others reported in the literature. Comparisons between drying experiments are difficult to make due to differences in the lag period, rate, duration and intensity of soil drying. The lag period refers to the number of days after the initiation of soil drying that the first significant effects on plant performance (in this case leaf growth) are detected. Although the rate of soil drying can be measured by the decay of pre-dawn water potential expressed in MPa day^{-1}(e.g. Wilson and Ludlow, 1983; Toft *et al.*, 1987), not all investigators have measured this parameter. Comparison of water potential measurements between experiments can be difficult due to differences in the timing of water relations measurements. For this reason, the rates of soil drying in the experiments were expressed as the decay of leaf elongation expressed on a

LER% day^{-1} basis. Duration refers to the number of days since the previous watering. Unless the investigator is interested in the recovery of plants from drought or the survival of tillers in a prolonged drought, the imposition of drought will only proceed until leaf growth stops. In the experiments described in this Section, the aim was to impose a mild (*sensu* Hsiao, 1973 where a mild stress is defined as one which decreases Ψ_L by up to 0.3 MPa) soil drying treatment, as it is under these conditions that chemical signalling is likely to be of most importance. Intensity refers to the magnitude of stress imposed, usually measured by the minimum leaf water potential obtained. Examination of Table 7.5 shows that the maize and barley experiments (unlike many reports) have imposed a slowly developing drought of mild intensity which is likely to simulate field conditions in the initial stages of drought. It is only by imposing a realistic drying regime that it is possible to elucidate mechanisms which may operate in the field.

Many experiments fail to simulate a realistic drought by employing an unsuitable combination of plant size (actually transpiring leaf area, since this determines water loss), pot size (determining available water) and environmental conditions (high VPDs can promote rapid soil drying). Only experience can help in the design of an appropriate system in which to impose a drought; although the provision of a drainpipe of adequate length to allow normal root development would seem to be essential. Pots mimic the field situation of certain duplex soils, where a shallow layer of arable soil overlays an impenetrable clay barrier. In such situations, there is a proliferation of roots in the upper profile (or pot), and the root system simply uses water from the limited depth of soil until the water is exhausted. Under such conditions, there is likely to be a long lag period of normal growth followed by rapid growth reductions. In such cases, hydraulic signalling is likely to be important in controlling plant response. Adaptive responses such as the control of water loss by chemical signals would have less time to operate effectively in such rapidly drying soils (pots).

Table 7.5: Characteristics of soil drying experiments. ? indicates information unavailable, NM indicates parameter not measured. +P indicates held at balancing pressure, -P indicates allowed to dry normally.

Reference	Species Cultivar	Soil Volume (litres)	Leaf Area at Start (initial leaf) (cm^2)	Duration (days)	Lag Period (days)	LER at End (% Control)	Rate of Drying (% Control / day)	Ψ_l at (Start) End (MPa)
Chu and McPherson, 1977	*Bromus catharticus* cv. Grasslands Matua	2.3	? (leaf 6)	10	3	18	-9.9	(-0.85) -2.50
Michelena and Boyer, 1982	*Zea mays* cv. Crow 226	?	? (leaf 5)	4	0	4	-31.2	(-0.10) -1.00
Van Volkenburgh and Boyer, 1985	*Zea mays* cv. Mol17 x B73	?	? (leaf 5)	5	0	20.5	-19.9	(-0.40) -0.80
Passioura, 1988a	*Triticum aestivum* cv. Egret	0.18	13-14 (leaf 3)	5	1	49 -P 64 +P	-17.3 -P -12.5 +P	(-0.35) -1.20
Saab and Sharp, 1989	*Zea mays* cv.FR27 x FRMol7	2 x 2.5	? 5520 at end	14	4	75	-2.7	(-0.40) -0.40
Spollen and Nelson, 1994	*Festuca arundinaceae* cv. V2-29 (Exp. 2)	1.0	?	5	2	14	-30.3	(-0.60) -2.00
Durand *et al.*, 1995	*Festuca arundinaceae*	1.0	?	5	0	6	-21.4	NM
Figure 7.6	*Hordeum vulgare* cv. Klaxon	1.08	16 (leaf 3)	10	5	63	-4.3	(-0.40) -0.60
Figure 7.7	*Zea mays* cv. Earliking	1.08	18 (leaf 3)	9	4	65	-5.4	(-0.35) -0.55

It was fortunate that a slow rate of drying was imposed in the maize and barley experiments. Detection of a chemical signal is often dependent on the rate of soil drying as determined by pot size (cf. Henson *et al.*, 1989 vs. Blum *et al.*, 1991) and the intensity of stress (Ebel *et al.*, 1994). An inappropriate stress intensity can result in a hydraulic signal occurring, or failure to detect any changes in plant parameters (see certain experiments in Saab and Sharp, 1989; Ebel *et al.*, 1994). If an experiment progresses long enough, changes in hydraulic parameters may interact with a chemical signal.

Therefore in attempting to explain the leaf growth inhibition seen in these experiments, the role of chemical and hydraulic messages should be taken into account. Initial growth reductions of droughted plants were observed solely during the day, as observed in field crops of maize (Tardieu and Ben-Haj-Salah, 1995) and slowly droughted pot-grown prairie grass (Chu and McPherson, 1977). It is thus tempting to speculate that the message was purely hydraulic, with enhanced turgor of the growing cells at night being responsible for growth recovery. Despite apparently convincing relationships between leaf elongation rate and leaf water potential (ψ_L) in droughted plants (Figure 7.11) over a small ψ_L range (0.2 MPa), there was either no relationship (Figure 7.12b), or a positive,one (Figure 7.12a) between LER and lamina turgor. Since turgor is usually considered to be the driving force for cell growth, it is difficult to argue for a purely hydraulic control of LER.

The unlikelihood of ψ_L regulating LER is reinforced by the fact that well-watered plants can show a much greater variation in ψ_L (than the 0.2 MPa shown in Figure 7.11) with no change in LER (McCree and Davis, 1974; Chu and McPherson, 1977). It is thus difficult to argue that ψ_L *per se* has a controlling role in leaf growth, especially in the case of the data presented here, when LER and ψ_L were not measured on the same leaves or even on the same plants ! It is also difficult to maintain a case for ψ_L as this was measured on cells that are not actually elongating. It has been

frequently demonstrated that the elongating regions of the leaf can maintain turgor while the lamina shows reduced ψ_L and turgor (Michelena and Boyer, 1982; Westgate and Boyer, 1984).

However, it is known that growth is dependent on the transfer of water from the supplying tissue (xylem) to elongating cells and the maintenance of a (growth-induced) water potential gradient between the two tissues (recently elegantly demonstrated by Nonami and Boyer, 1993). If the water potential of the xylem were to decrease during the afternoon, the supply of water to the elongating cells would be disrupted and growth would decline. Such a mechanism may account for the daily variation in leaf growth inhibition. It would be difficult to test this possibility experimentally as transpiration-induced water potentials during the day would mask any growth-induced water potentials, which can only be detected at night (Westgate and Boyer, 1984).

Despite the attraction of this hypothesis (the disruption of the growth-induced water potential), droughted plants show a reduction in growth even when the water potential of the xylem is maintained at atmospheric pressure by pressurising the roots (Passioura, 1988a). Pressurised and unpressurised droughted plants show a very similar pattern of daily growth reduction over a drying cycle in which growth was reduced by up to 50 % in unpressurised plants (Passioura, 1988a), which seems to indicate both the existence and potency of root-supplied chemical signals.

The diurnal variation of leaf growth inhibition (Figure 7.5) may be accounted for by a chemical message being generated by the surface roots in drying soil (Figure 7.13) during the day and being transmitted to the shoots. At night, these surface roots may be re-hydrated by transfer of water from deeper, well-hydrated roots, so-called "hydraulic lift" (Richards and Caldwell, 1987). However, it was not possible to detect an ABA message (as accumulation in the leaf elongation zone) despite leaf growth

reductions of 37% (barley) and 35% (maize) at the end of the experiment. This may be a consequence of sampling leaf (or EZ) tissue, since tissue measurements of ABA are much less sensitive than measurement of xylem sap (Zhang and Davies, 1989b; 1990a). Unfortunately, it was not possible to extract xylem sap from the barley plants in this study using the pressure bomb. The sap samples obtained from the fourth leaf of maize plants (only after Day 6 when LER had already dropped by 18 %) were not of sufficient volume to allow quantification by the RIA used in Section 5.2.2.3. Collection of xylem sap samples by root exudation wasn't feasible since the crown was destroyed in sampling the bulk EZ. The existence of a chemical message in droughted maize plants was suggested by an increase (relative to control plants) in the ABA concentration of the guttation fluid on Day 9 (data not shown).

Another possibility for the inability of bulk [EZ-ABA] to explain leaf growth inhibition is that epidermal ABA concentration of expanding leaves may be the controlling variable. It has been established that the epidermis is frequently the limiting factor for growing tissues (Kutschera, 1992) and epidermal ABA concentration may mediate this inhibition. Until it is possible to sample the epidermis of monocotyledenous leaves, this question cannot be resolved.

From these experiments, it is difficult to argue that either a hydraulic message or an ABA message is controlling the growth rate of the droughted plants. It seems that the measurement techniques employed in this study were not sufficiently sensitive to enable the detection of possible controlling variables such as growth-induced water potential and xylem (or epidermal) ABA concentrations.

Other possible explanations which may explain the growth inhibition seen in these experiments include:

1. that drying-induced changes in xylem pH can have a direct effect on growth (Van Volkenburgh and Boyer, 1985) or redistribute ABA to the sites of action (Hartung *et al.*, 1988). These two possibilities would be difficult to distinguish experimentally.
2. that ionic changes in the xylem sap can alter the sensitivity to ABA (Schurr *et al.*, 1992)
3. that other compounds with growth inhibitory activity are involved (Munns, 1992).

With reference to pH, it must be noted that the xylem can be an extremely well-buffered system (Gollan *et al.*, 1992) and pH is unlikely to be greatly affected by the early stages of soil drying seen in these experiments. Although a line was fitted by eye to describe the relationship between sunflower xylem pH and θ , the large scatter of points in the relationship between pH and θ makes it difficult to accept that xylem pH can directly control stomata (Gollan *et al.*, 1992). This data is supported by the observation that a 35 % reduction in daily maize leaf growth rate was not accompanied by any increase in xylem sap pH (Figure 7.8). Such data also support the findings of studies using the leaf elongation assay (Figure 2.6).

Greatly different sensitivity of g_s to soil drying (and xylem [ABA]) in sunflower seemed to be correlated to differences in the ionic composition of the sap (Schurr *et al.*, 1992). It is unlikely that any potential nutrient x ABA interaction was inhibiting leaf growth in this study, since no corroborating evidence could be found with bioassay studies (Section 2.3.1). The interaction found by Schurr *et al.* (1992) was characterised by large differences in g_s between plants, which would have produced a large cv% (characteristic of many studies investigating the effect of soil drying on stomatal behaviour). The relative uniformity of the leaf growth inhibitions (cv% < 20 %) detected in the drying experiments suggests that the plants were either uniformly sensitive to any potential nutrient x ABA interaction, or that it did not exist.

The results of Figure 6.2b show no compelling evidence for the existence of other growth inhibitory compounds in maize sap.

A further possibility is that leaf growth inhibition may be caused by an interaction of chemical and hydraulic factors, as suggested by Sharp and Davies (1989). Such an interaction can influence stomatal behaviour in the field (Tardieu and Davies, 1992). The possibility that such an interaction affects growth is considered in Chapter 8.

CHAPTER 8.

USE OF A COLEOPTILE ASSAY TO INVESTIGATE THE INTERACTION OF ABA AND WATER POTENTIAL ON GROWTH

8.1 INTRODUCTION

The previous chapter demonstrated the apparent failure of changes in elongation zone ABA concentration to account for drought-induced changes in leaf elongation rate. Although there was a clear relationship between [EZ-ABA] and LER in the detached shoot system, droughted plants showed a substantial inhibition of leaf elongation in the absence of increased ABA concentration. Measurement of plant water relations similarly failed to account for changes in the growth of droughted plants. One explanation for the declines in leaf growth may be an interaction between ABA and water potential occurring across ranges which were not detectable using the measurement techniques used in Chapter 7.

Stomatal research has indicated the presence of a ψ_L x ABA interaction in 3 systems. Tardieu and Davies (1992) applied the *Commelina* epidermal strip bioassay using a range of ABA concentrations and osmotica (PEG solutions). The sensitivity of stomatal aperture to ABA was found to increase as the osmotic potential of the incubation medium fell from -0.3 MPa to -1.5 MPa. These experiments corroborated field data, which showed that the response of maize stomatal conductance to ABA was dependent on the water potential of the leaf (Tardieu and Davies, 1992; Tardieu *et al.*, 1993). Inclusion of such an interaction term was essential in modelling stomatal conductance in the field, as purely chemical or purely hydraulic control of stomata did not provide sensible (as obtained in the field) information (Tardieu and Davies, 1993). Further evidence of a ψ_L x ABA interaction has been provided by Trejo and Davies (1994), who generated a tension in the xylem of detached *Phaseolus* shoots in a transpiration bioassay by attaching a 30 cm long capillary tube to the base of the

shoots. Shoots which drew water through this tube showed a more rapid initial response to ABA. However, the existence of an ψ_L x ABA interaction is not ubiquitous. Data from field and growth cabinet studies and from transpiration bioassays on sunflower failed to show the interaction found in maize (Tardieu *et al.*, 1996).

Choice of a model system in which to investigate a potential ψ_L x ABA interaction affecting growth was between the capillary tubing experiment, or incubating coleoptiles in ABA and osmotic solutions. The coleoptile system was chosen as it is easily reproducible, less technically demanding, and it allowed comparison with the wealth of information attained with this system in the study of coleoptile growth responses to indoleacetic acid (IAA). It is worth reviewing the growth of excised coleoptiles, to assist in interpretation of the results presented in this chapter.

Coleoptile growth is assumed to be under control of endogenous IAA levels, as indicated by Went's classic experiment that showed exogenous IAA could substitute for the tip, the assumed site of IAA synthesis, (Went and Thimann, 1937) in allowing coleoptile growth. However, an alternative explanation is provided by Trewavas (1981), who considers that a concentration of IAA in the tip is due to diffusion from the endosperm. In processing coleoptiles for experiments, the IAA-producing tip is routinely decapitated then the isolated coleoptiles are usually floated on distilled water until sufficient are accumulated for experimentation. In the 30 minutes following tip decapitation, there is a transient burst of elongation. This may not be seen by workers who float coleoptiles on distilled water for an hour before experimental use. Following this burst, growth slows for 75 minutes, the "latent period", and is resumed with another transient burst of elongation lasting 30-120 minutes, after which a steady rate of growth is maintained for hours (at least 6 in maize coleoptiles - Evans and Schmitt, 1975; Kutschera and Schopfer, 1985) the "spontaneous growth response". Although some studies have shown that the "spontaneous growth response" can be strongly gravity-dependent (e.g. Evans and Schmitt, 1975), comparable growth shown by

coleoptiles floated on solutions in Petri dishes (e.g. Pope, 1993) suggests that the same mechanisms are involved. In the absence of absorbable solutes in the incubation solution, the spontaneous growth response eventually declines due to insufficient solute uptake to maintain turgor (Oertli, 1975). In maize coleoptiles maintained in distilled water, this decline occurs 6 hours after excision (Kutschera and Schopfer, 1985). Although the length of time of each phase varies between species (Macdowall and Sirois, 1977), the time scale above is given for wheat (Macdowall and Sirois, 1976), the principal species used in this Chapter.

The initial burst of elongation is thought to represent the response of the coleoptile to residual IAA in the section (Evans and Schmitt, 1975). The second burst of elongation is thought to represent resumed IAA synthesis in the section (Went and Thimann, 1937; Evans and Schmitt, 1975; Weiler *et al.*, 1981). The growth rate of excised coleoptiles in the steady phase of elongation is reduced by 37 % compared to intact ones (Kutschera and Schopfer, 1985).

A treatment which affects growth may either delay the resumption of normal growth (increasing the latent period) or change the steady growth rate. The latent period varies from 15 to 45 minutes across an IAA concentration range of 10^{-7} to 10^{-4}M (Macdowall and Sirois, 1977). Calculation of coleoptile length after 6 hours (using the growth rates determined by Macdowall and Sirois (1977) for each phase of coleoptile growth) shows a growth reduction of only 8 % attributed to a longer latent period. Given this relatively small variation in coleoptile length due to latent period effects for such a large difference in hormone concentration, it is assumed that the coleoptile length measured at a single point in time in this study reflects differences in the spontaneous growth rate.

In applying a treatment to coleoptiles, there is the concern that the tissue will only take up the hormone of interest slowly since the cuticle forms a barrier.

Macdowall and Sirois (1977) present indirect evidence that IAA penetration into coleoptiles is a statistical function of diffusive entry through cut segments. Thus some investigators have peeled epidermal strips off coleoptiles (Rayle, 1973) or abraded their surface (Evans and Vesper, 1980) to improve hormone penetration. Abrasion to remove the cuticle usually only exposes a minority of cells to the outside solution, which complicates interpretation of the physiological response, since the response integrates the growth of cells protected by the cuticle and those open to the external solution (Rayle and Cleland, 1992). Abrasion also physically damages the coleoptiles, which may reduce growth rates by about 30 % compared to non-abraded controls (Kutschera and Schopfer, 1985). Peeling coleoptiles is technically demanding, and there is a risk of damaging the tissue. For these reasons, neither peeling nor abrasion were applied to coleoptiles in this study.

8.2 MATERIALS AND METHODS

Wheat (*Triticum aestivum* cv. Tonic) or maize (*Zea mays* cv. Earliking) seeds were soaked in water for 3 hours, then planted in a tray filled with wet vermiculite. The tray was covered with aluminium foil to exclude light, placed in a plastic bag and placed in a dark room at 25°C. After 3 days, when the coleoptiles were 10-35 mm long, sections were prepared with a 3 bladed cutting device with razors mounted 3 mm and 6 mm apart. The apical 3 mm segments were discarded, while the 6 mm segments were floated on deionised water until (*circa* 1 hour later) sufficient coleoptiles were available to allow 10 treatments with at least 10 coleoptiles each. No abrasive or peeling treatments were performed since both treatments can cause potential problems as outlined in Section 8.1 (Rayle and Cleland, 1992). The primary leaf was not removed. All preparation was performed under a green safe light.

Randomly selected coleoptiles (between 10 and 18 per treatment) were transferred to Petri dishes containing 25 mL of treatment solution. Air was bubbled

through the solutions to maintain aeration. After 6 hours growth in the dark, the coleoptiles were measured with an eyepiece graticule to the nearest 0.1 mm. Six hours was chosen as the incubation time since previous studies have shown a steady elongation rate at this time in wheat (Macdowall and Sirois, 1976) and maize (Kutschera and Schopfer, 1985) coleoptiles, which may (Kutschera and Schopfer, 1985) or may not (Macdowall and Sirois, 1976) be followed by a deceleration of growth rate in the next hour. A short incubation time also prevents the occurrence of secondary growth responses to the applied treatments (Pope, 1993). Measurement of all treatments took an hour.

Following measurement, all coleoptiles in each Petri dish were washed in deionised water for 5 seconds to remove any incubation solution adhering to the surface, blotted dry and placed in Eppendorfs over ice. The Eppendorfs were then frozen at -20^{o}C and stored for subsequent ABA determination using the radioimmunoassay in Section 5.2.2.3 (Quarrie *et al.*, 1988).

Solutions of PEG 10 000, mannitol and KCl were made up in deionised water. The pH of these solutions was not adjusted. Experiments investigating the effect of pH on growth used the buffer MES (2-[N-morpholino] ethanesulphonic acid) adjusted to the appropriate pH by the dropwise addition of 1M KOH. A stock solution of 10^{-2}M IAA was made by initially dissolving the IAA in ethanol. Once soluble, the solution was made up in deionised water.

The osmotic potential (ψ_π) of these solutions was determined psychrometrically with Wescor C-52 chambers. Data on coleoptile growth are presented graphically as a function of solute concentration since there was considerable uncertainty in the determination of ψ_π at high ψ_π. Figure 8.1 shows the measured values of ψ_π plotted against various known solute concentrations.

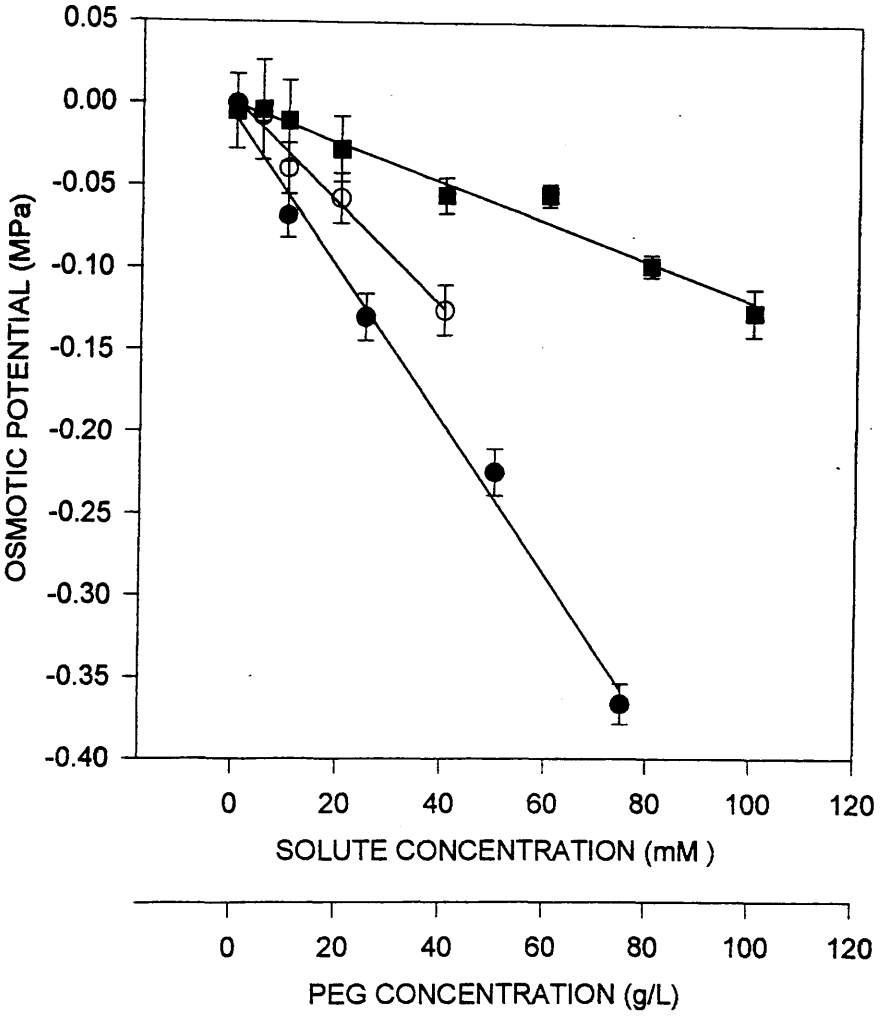

Figure 8.1: Osmotic potential determined psychrometrically plotted against solute concentration in mM for mannitol (○) and KCl (●), and PEG (■) concentration in g/L. Points are means ± S.E. of 2-4 psychrometric determinations. Lines are linear regressions fitted in SPW 1.0.

Each experiment was repeated on at least 2 occasions. The presented Figures combine data from the two or more experiments. Analyses of variance were performed using the general linear model function (to allow for unbalanced replication) in MINITAB 10.2 for Windows (MINITAB Inc, PA, USA). Sample ANOVA tables for

each section can be found in Appendix 2. Means were discriminated using unpaired t-tests in SPW 1.0.

8.3 RESULTS

8.3.1 Responses of maize and wheat coleoptiles to ABA and PEG applied separately

These experiments were necessary to establish the influence of a range of ABA and PEG concentrations on coleoptile growth. Wheat coleoptiles placed on deionised water grew to an average length of 7.17 mm after 6 hours (Figure 8.2a), or 20 % of their original length; while maize coleoptiles grew to an average length of 6.55 mm, or 9 % of their starting length. This compares with other published reports of elongation of unpeeled or unabraded coleoptiles in the absence of externally supplied IAA at 25°C (19 % for maize, Kutschera and Schopfer, 1985; 10-15 % for wheat, Macdowall and Sirois, 1977; 10 % for *Avena*; Hasegawa *et al.*, 1992; 5 % for *Avena*, Cleland, 1992). While the elongation of wheat coleoptiles compared favourably with other species, the maize growth was much less than expected.

Only PEG at concentrations greater than 60g/L (ψ_π = -0.08 MPa) significantly ($P<0.10$) inhibited maize and wheat coleoptile growth (see Figure 8.2a), with growth reductions of 12 %, 13 % and 39 % at PEG concentrations of 60, 80 and 100 g/L respectively in wheat and reductions of 23 %, 42 % and 56 % in maize. It is difficult to compare these data directly with other studies, since these have favoured PEGs of a lower molecular weight (e.g. Hohl and Schopfer, 1991). There was no difference between wheat and maize in their response to PEG, as indicated by a non-significant ($P>0.10$) species x PEG interaction.

Figure 8.2b shows that 10^{-7}M ABA had no significant ($P>0.10$) effect on either wheat or maize coleoptile growth. All higher concentrations were found to depress growth, with growth inhibitions of 40 %, 50 % and 78 % for ABA concentrations of $5x10^{-7}$, 10^{-6} and 10^{-5}M respectively in wheat, and inhibitions of 15 %, 28 % and 44 % in maize. There was a significant ($P<0.001$) species x ABA interaction.

The finding that 10^{-7}ABA had no effect on wheat or maize coleoptile growth contradicts previous reports that 10^{-7}M ABA reduced coleoptile growth by 30 % in wheat (Wright, 1969) and 32 % and 67 % in wheat and oat respectively (McWha and Jackson, 1976). However, the response of coleoptile growth to ABA is variable between days, as indicated by the highly significant ($P<0.01$) Experiment x ABA interaction for wheat in the two experiments performed. Certainly, later experiments (e.g. Figure 8.9) show a significant effect of 10^{-7}M ABA on wheat coleoptile growth.

8.3.2 Responses of wheat coleoptiles to KCl and mannitol applied separately

Since wheat showed greater growth under the assay conditions, it was chosen for further studies. It was decided to expose wheat coleoptiles to other osmotica. Mannitol was chosen as it was thought necessary to confirm any PEG responses with a similar non-ionic osmoticum; even though mannitol is taken up by coleoptiles and thus coleoptiles do no behave as ideal osmometers in the presence of mannitol (Hohl and Schopfer, 1991). KCl was chosen as an ionic osmoticum as it was hoped to further investigate, using the coleoptile growth assay, the observation that high (50 mM) concentrations of KCl can overcome the inhibitory effect of ABA on (leaf disc) growth (Van Volkenburgh and Davies, 1983).

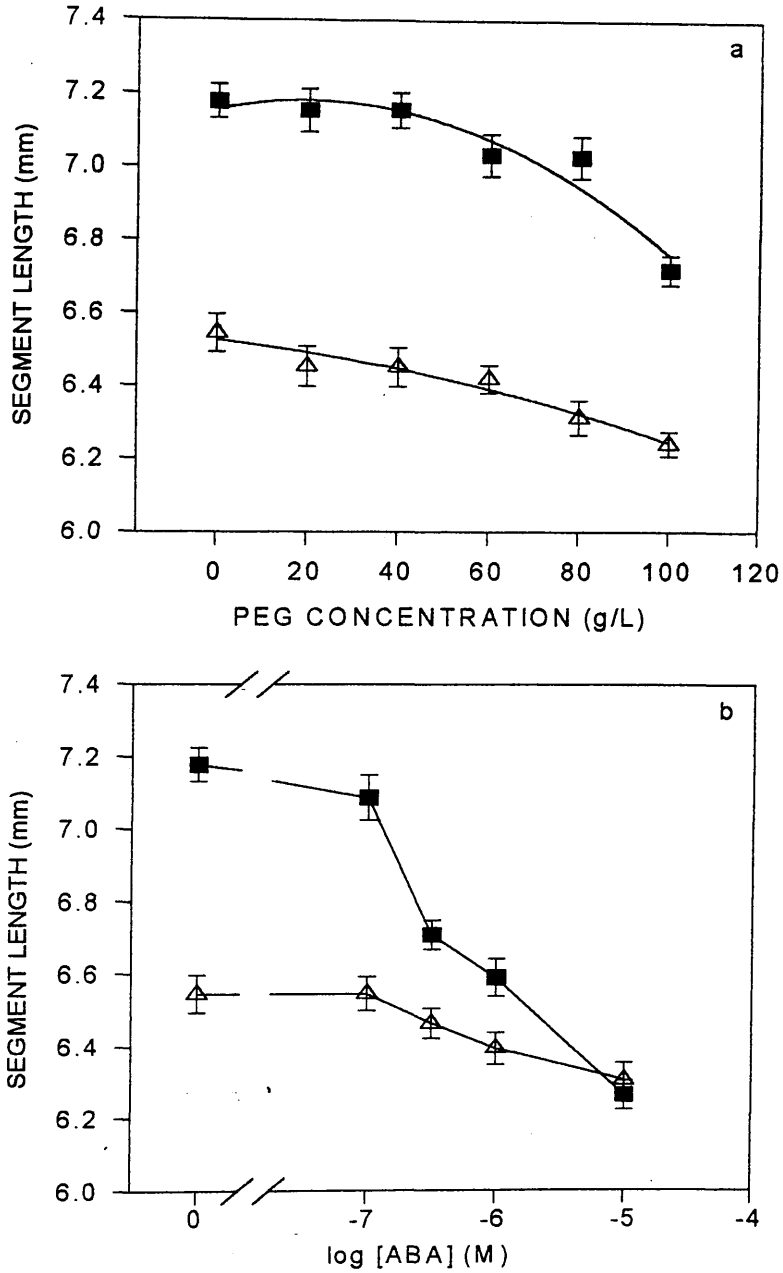

Figure 8.2: Segment length of wheat (■) and maize (Δ) coleoptiles after 6 hours growth in solutions of PEG (a) and ABA (b) at various concentrations made up in deionised water. Points are means ± S.E. of 17-25 coleoptiles from 2 separate experiments. Lines are in (a) are second order regressions fitted in SPW 1.0.

Both mannitol and KCl had highly significant ($P<0.001$) effects on coleoptile growth in the range 0-100 mM. Figure 8.3 shows inhibitory effects of mannitol and KCl at concentrations of 60 mM (ψ_π = -0.020 MPa) and 10 mM (ψ_π = -0.068 MPa) respectively. Comparison of the 2 solutes at the same osmotic potential (e.g. 10 mM KCl vs. 40 mM mannitol at ψ_π = -0.13 MPa) shows that growth was much more sensitive to the ionic osmoticum, which precluded any further investigation of the interaction between KCl and ABA. The extreme sensitivity of coleoptiles to KCl was surprising. Although transient reductions in coleoptile growth in response to 30 mM KCl have been observed previously (Oertli, 1975; Stevenson and Cleland, 1981), presumably due to a reduction in turgor pressure, longer term (22 hours) studies showed that 30 to 50 mM KCl was optimal for growth (Stevenson and Cleland, 1981), with growth being promoted relative to 0 mM KCl controls. This growth promotion may be explained in terms of continued solute uptake (of KCl) being necessary for long-term growth. Thus the dose-response curve for an ionic osmoticum such as KCl will depend on the duration of growth, with short-term studies showing growth reductions, and longer-term studies showing growth promotion. Although Oertli's (1975) study of *Avena* coleoptiles showed that after 6 hours, 30 mM KCl promoted growth (and inhibited growth relative to distilled water controls up to 6 hours), one can only conclude from the KCl data in Figure 8.3 that the wheat coleoptiles hadn't adapted to their new osmotic environment.

Mannitol at concentrations of 60, 80 and 100 mM inhibited growth by 21 %, 32 % and 30 % respectively. This is slightly more sensitive than described by Cleland (1959), who found that incubation of *Avena* coleoptiles in 50 and 100 mM mannitol (in the absence of IAA) reduced growth after 4 hours by 9 % and 20 % respectively.

Comparison of coleoptile growth in the 2 non-ionic solutes at the same osmotic potential revealed inconsistencies. Growth was more inhibited by 20 mM mannitol than 40 g/L PEG (ψ_π = -0.055 MPa) yet growth was more inhibited by 100 g/L PEG than

40 mM mannitol (ψ_π = -0.125 MPa). However, the results are not strictly comparable since the sets of experiments were performed on different sets of coleoptiles. Studies on cucumber hypocotyl elongation showed a greater inhibition of growth by PEG 6000 than mannitol at the same ψ_π (Michel, 1970).

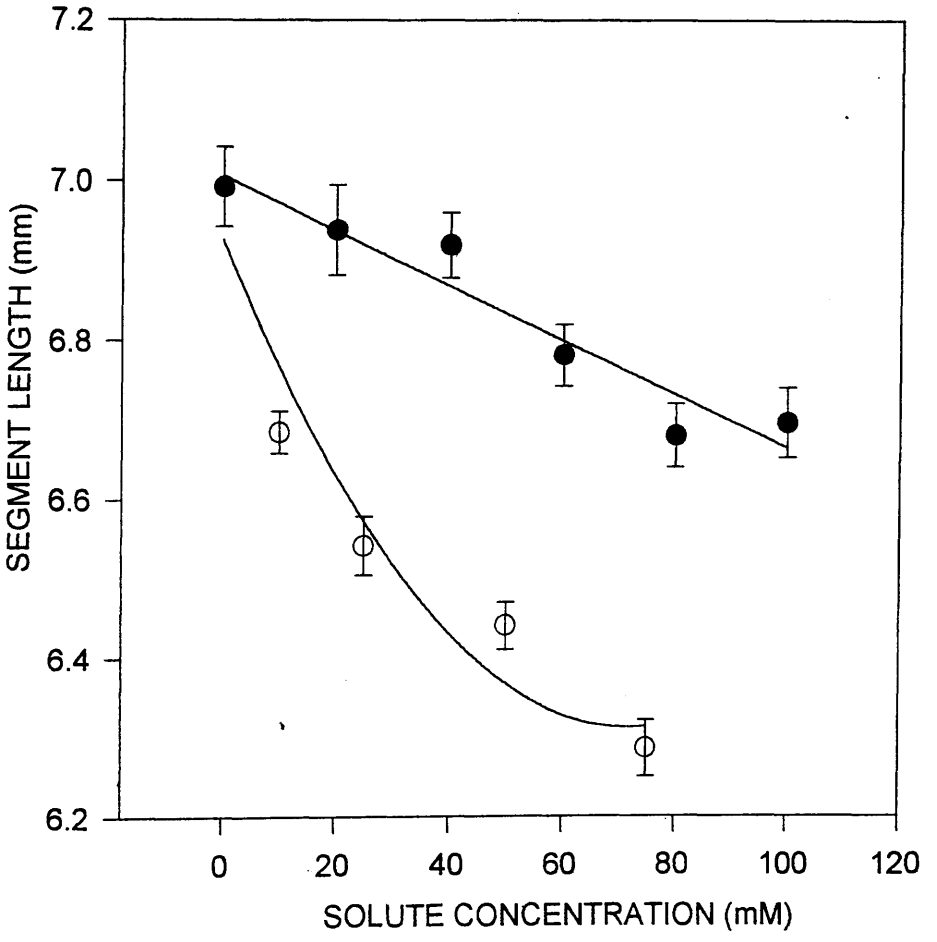

Figure 8.3: Segment length of wheat coleoptiles after 6 hours growth in solutions of mannitol (●) or KCl (○) at various concentrations made up in deionised water. Points are means ± S.E. of 20-29 coleoptiles from 2 separate experiments.

8.3.3 Responses of wheat coleoptiles to ABA and osmoticum applied together

It was decided to apply the two agents (ABA and PEG or ABA and mannitol) together in a concentration range where the individual agents had no effect on growth. Figure 8.4a confirms that PEG concentrations up to 40 g/L in deionised water had no effect on coleoptile growth, as indicated by the non-significant ($P>0.10$) main effect of PEG in the ANOVA. Although 10^{-7}M ABA in deionised water did not reduce growth, ABA inhibited growth by 16 %, 14 %, 26 % and 32 % at PEG concentrations of 5, 10, 20 and 40 g/L respectively. The bulk of this response took place between 0 and 10 g/L (ψ_π = -0.011 MPa), which indicates the extreme sensitivity of growth to the highly significant ($P<0.001$) PEG x ABA interaction. The interactions of Experiment x ABA, Experiment x PEG and Experiment x ABA x PEG were all non significant ($P>0.10$).

This interaction was confirmed applying a broader range of ψ_πs using mannitol (Figure 8.4b). In contrast to PEG, there was a highly significant ($P<0.001$) main effect of mannitol, with growth being reduced by a relatively uniform 13 % at concentrations of 5 mM or higher. This differed from the results of Figure 8.3, where mannitol had no inhibitory effects up to 60 mM. Although 10^{-7}M ABA did not significantly ($P>0.10$) inhibit growth at mannitol concentrations up to 10 mM, it reduced growth by 20.5 % and 17.3 % at 20 and 40 mM mannitol respectively. Consequently, there was a highly significant ($P<0.01$) mannitol x ABA interaction.

A possible explanation for the increased effectiveness of ABA in inhibiting growth at higher osmoticum concentrations may be that more ABA accumulated in the tissue. This was not found, as all ABA-treated coleoptiles had the same bulk ABA content (Table 8.1), as indicated by the non-significant ($P>0.10$) osmoticum x ABA interaction for both PEG and mannitol when the data on tissue ABA content were subjected to ANOVA.

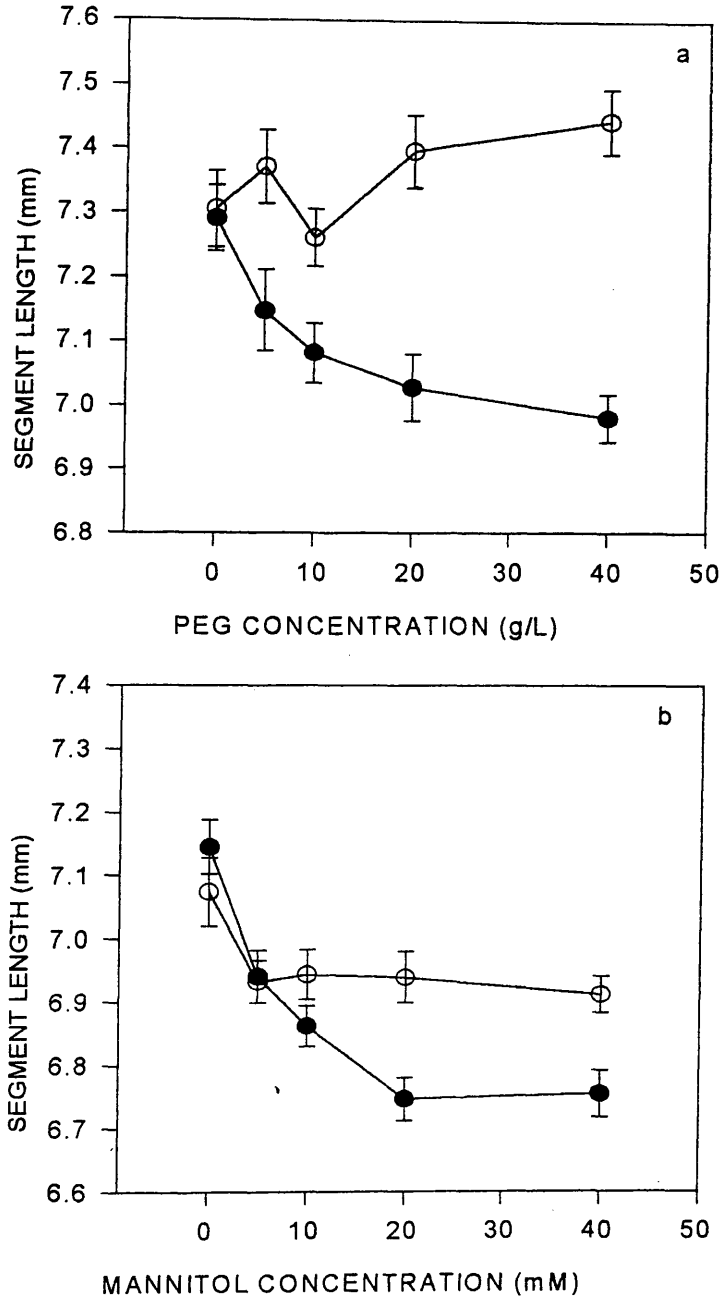

Figure 8.4: Segment length of wheat coleoptiles after 6 hours growth in solutions of PEG (a) and mannitol (b) at various concentrations with (●) or without (○) 10^{-7}M ABA made up in deionised water. Points are means ± S.E. of 25-37 coleoptiles from 3 separate experiments.

Table 8.1: ABA contents of coleoptiles incubated in various concentrations of PEG or mannitol (MAN) for 6 hours in the presence or absence of 10^{-7}M ABA. Values are means ± S.E. of the number of experiments indicated in parentheses. The results from ANOVA of the data are also given.

[PEG] (g/L)	ABA ng / g DW - 10^{-7}M ABA	ABA ng / g DW + 10^{-7}M ABA	[MAN] (mM)	ABA ng / g DW - 10^{-7}M ABA	ABA ng / g DW + 10^{-7}M ABA
0	154.0±18.2 (4)	188.5±16.8 (4)	0	187.0± 0.0 (1)	154.5± 5.5 (2)
5	141.3± 9.2 (4)	169.8±12.1 (4)	5	200.5±16.5 (2)	213.0±12.0 (2)
10	148.5± 6.1 (4)	176.5±17.3 (4)	10	181.5± 2.5 (2)	191.0±18.0 (2)
20	155.8±13.8 (4)	191.3±32.2 (4)	20	158.0± 9.0 (2)	212.0±10.0 (2)
40	138.5±14.5 (2)	166.3± 7.4 (4)	40	214.5±32.5 (2)	224.0± 5.0 (2)

P Values:

PEG	ABA	PEG x ABA	MAN	ABA	MAN x ABA
0.683	0.008	0.999	0.088	0.316	0.233

Although PEG had no significant (P>0.10) effect on ABA content, there was a weakly significant (P=0.088) mannitol effect. Thus mannitol at the concentrations applied may stimulate ABA production by wheat coleoptiles, as shown for much higher concentrations of mannitol (0.6 M) in *Avena* coleoptiles (Weiler *et al.*, 1981).

It was considered important to demonstrate the occurrence of this interaction using two different solutes as studies using PEG have been criticised due to the presence of toxic impurities in PEG (Jackson, 1962). It should be noted that the interaction occurred over a different range of ψ_π for each solute, which may result from uptake of mannitol by the coleoptile (Hohl and Schopfer, 1991).

8.3.4 Responses of wheat coleoptiles to ABA, IAA and osmoticum applied together

Since the growth of isolated coleoptiles is strongly dependent on external IAA concentration, it was decided to investigate whether an external source of IAA affected

the response of coleoptiles to ABA, and the Osmoticum x ABA interactions found above.

The response of coleoptile growth to IAA was highly variable from day to day, as shown previously (Shinkle and Briggs, 1984), indicated by a highly significant ($P<0.001$) Experiment x IAA interaction for the data shown in Figure 8.5. Wheat coleoptiles showed a significant promotion of growth (relative to deionised water controls) with 10^{-7}M IAA and continued to show increased elongation with $5x10^{-6}$M IAA. It is unfortunate that a larger range of [IAA]s was not spanned to determine the concentration at which the effect of IAA became saturating. Other reports indicate that coleoptiles respond to IAA over a concentration range of 10^{-7}M to 10^{-4}M at pH 5 in wheat (Macdowall and Sirois, 1977); and 10^{-7}M to 10^{-4}M (Nissl and Zenk, 1969) and 10^{-8}M to 10^{-6}M (Cleland, 1972) in oat. The range of IAA concentrations over which coleoptiles of a particular species are responsive is dependent on such factors as coleoptile age (Nitsch and Nitsch, 1956), the photoenvironment during coleoptile growth (Shinkle and Briggs, 1984), time of incubation (Nissl and Zenk, 1969; Cleland, 1972; Pope, 1993), incubation medium pH (Macdowall and Sirois, 1977; Shinkle and Briggs, 1984) and sucrose concentration (Cleland, 1972), and pre-incubation of coleoptiles in distilled water (Macdowall and Sirois, 1977; Vesper and Evans, 1978). The time-dependent effect of pre-incubation is assumed to be an effect of depletion of endogenous IAA concentration, which sensitises the tissue to subsequent external IAA application (Vesper and Evans, 1978). Particularly striking are the different sensitivity ranges obtained by Nissl and Zenk (1969) with the same material according to whether the initial growth rate was measured in individual coleoptiles maintained under a flowing solution which continually brought fresh IAA to the tissue (responsive from 10^{-10}M, saturating at 10^{-8}M IAA); or whether growth rate was measured after 24 hours incubation of many coleoptiles in Petri dishes (10^{-7} to 10^{-4}M IAA). The difference in sensitivity was attributed to the breakdown of IAA by epiphytic bacteria

during long incubation times. Thus the range over which coleoptiles respond to IAA is likely to be unique to each study.

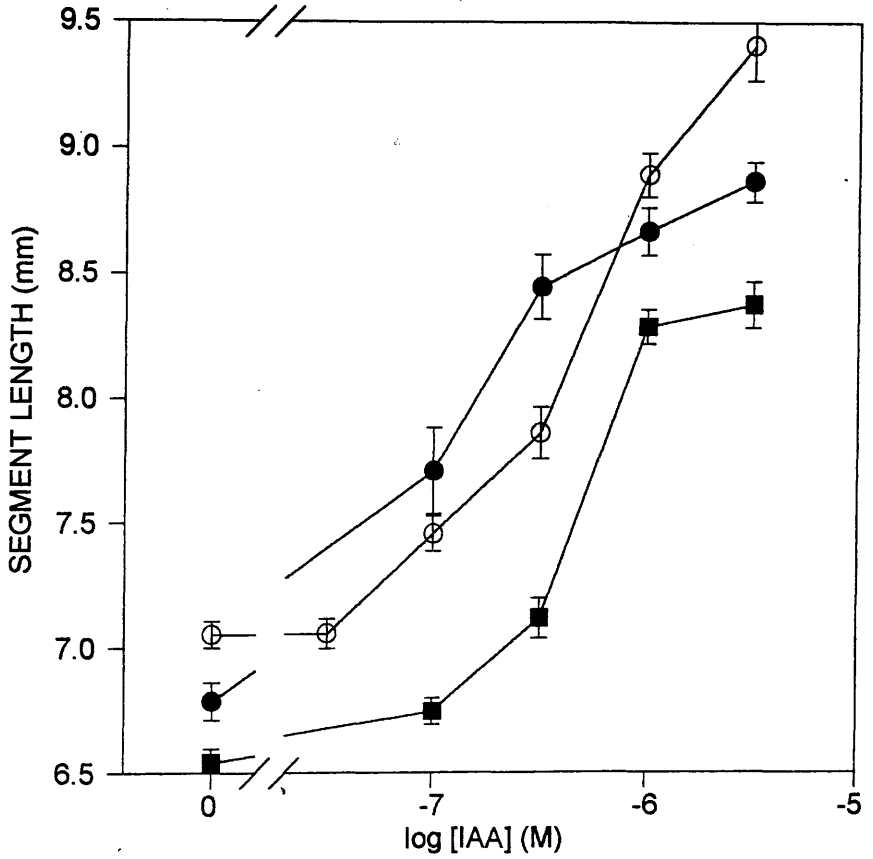

Figure 8.5: Segment length of wheat coleoptiles after 6 hours growth in solutions of IAA at various concentrations made up in deionised water for 3 separate experiments (●, ○, ■). Points are means ± S.E. of 9-15 coleoptiles.

Despite the highly significant effect of IAA on coleoptile growth, there was no significant ($P>0.10$) interaction of IAA x ABA across the IAA concentration range tested (Figure 8.6). ABA at 10^{-7}M reduced IAA-induced coleoptile growth by 9-25 %, confirming previous observations with higher ABA concentrations (10^{-4}-10^{-6}M) (Rehm and Cline, 1973) that external ABA application reduces IAA-induced elongation.

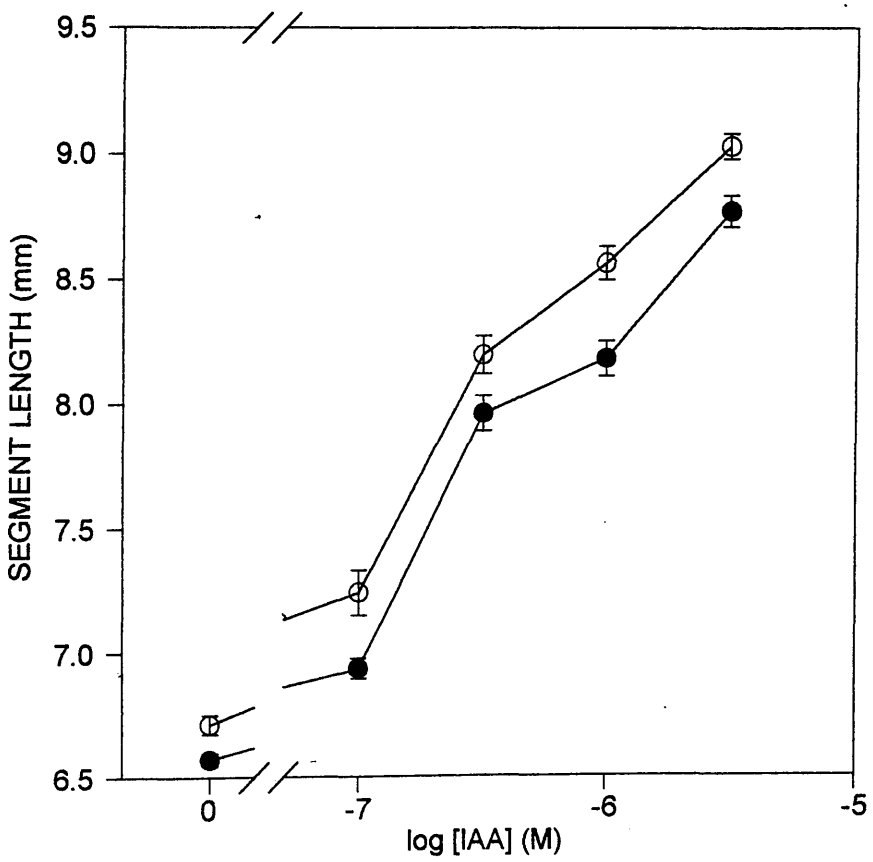

Figure 8.6: Segment length of wheat coleoptiles after 6 hours growth in solutions of various concentrations of IAA in the presence (●) or absence (○) of 10^{-7}M ABA made up in deionised water. Points are means ± S.E. of 26-37 coleoptiles from 3 separate experiments.

Figures 8.7 and 8.8 show the response of coleoptile growth to ABA and an osmoticum applied at 3 IAA concentrations. Table 8.2 summarises the statistical analyses showing the significance of the osmoticum x ABA interaction in all experiments. IAA had a considerable effect on the significance of the osmoticum x ABA interaction; with $5x10^{-7}$M IAA rendering the interaction non-significant ($P>0.10$) for both mannitol and PEG, and 10^{-7}M IAA rendering the interaction non-significant ($P>0.10$) for only mannitol.

Analysis of the entire PEG data set (Figure 8.7) shows highly significant ($P<0.01$) main effects of ABA, PEG and IAA. There was also a highly significant ($P<0.001$) IAA x PEG interaction, and confirmation of the PEG x ABA interaction seen in the absence of external IAA. The interaction IAA x ABA was non significant ($P>0.10$), as seen before in Figure 8.6. The tripartite interaction (ABA x IAA x PEG) was also highly significant ($P<0.01$).

Table 8.2: Table of significance (P values given) for the osmoticum x ABA interaction in all experiments. NS = not significant, $P<0.05$ *, $P<0.01$ **, $P<0.001$ ***

Osmoticum	[IAA]	Exp. 1	Exp. 2	Exp. 3	All Exp.s	All [IAA]s
PEG	0 M	0.000 ***	0.211	0.179	0.000 ***	
	10^{-7}M	0.001 **	0.004 **		0.001 **	0.000 ***
	$5x10^{-7}$M	0.655	0.610		0.698	
Mannitol	0 M	0.880	0.009 **	0.061	0.003 **	
	10^{-7}M	0.863	0.221		0.427	0.082
	$5x10^{-7}$M	0.587	0.142		0.455	

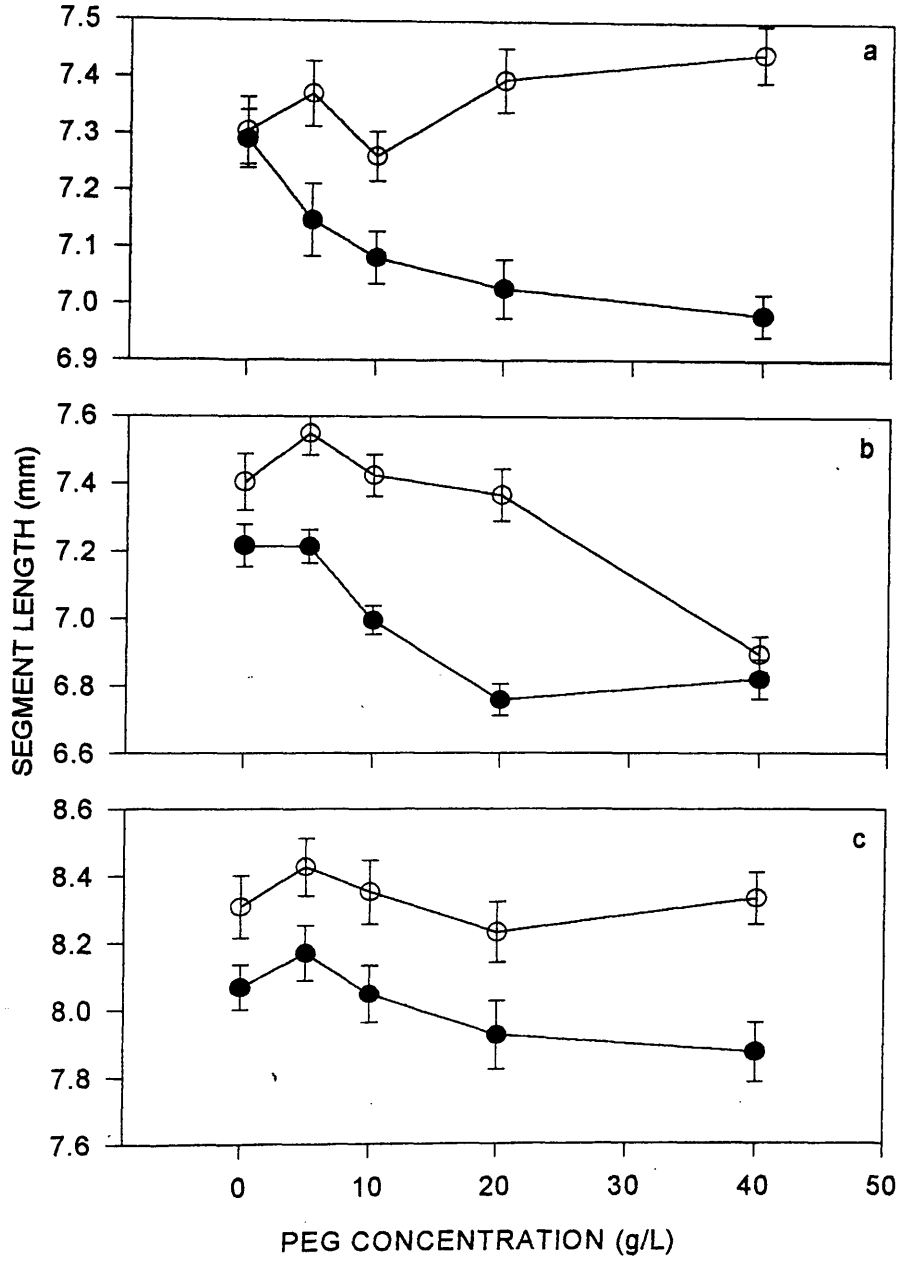

Figure 8.7: Segment length of wheat coleoptiles after 6 hours growth in solutions of PEG at various concentrations in the presence (●) or absence (○) of 10^{-7}M ABA and with no IAA (a), 10^{-7}M IAA (b) and $5x10^{-7}$M IAA (c) in the incubation medium. Points are means ± S.E. of 19-29 (b,c) or 25-33 (a) coleoptiles from 2 (b,c) or 3 (a) separate experiments.

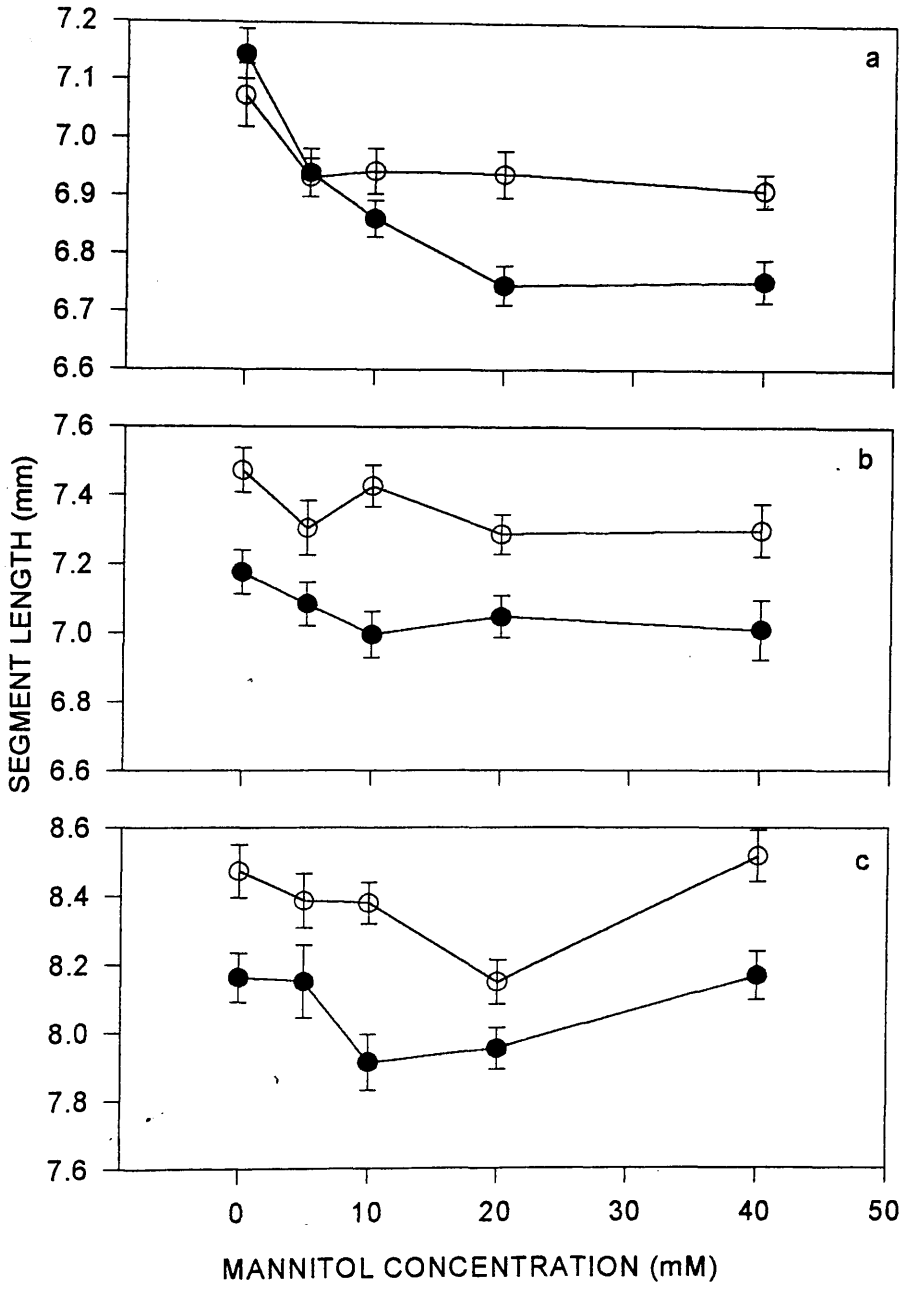

Figure 8.8: Segment length of wheat coleoptiles after 6 hours growth in solutions of mannitol at various concentrations in the presence (●) or absence (○) of 10^{-7}M ABA and with no IAA (a), 10^{-7}M IAA (b) and $5x10^{-7}$M IAA (c) in the incubation medium. Points are means ± S.E. of 23-33 (b,c) or 31-37 (a) coleoptiles from 2 (b,c) or 3 (a) separate experiments.

Analysis of the entire mannitol data set (Figure 8.8) similarly showed highly significant ($P<0.001$) main effects of IAA, ABA and mannitol. However, the interaction terms differed in their significance from those seen with PEG, with significant ($P<0.01$) IAA x ABA and IAA x mannitol interactions, and a weakly significant ($P=0.082$) ABA x mannitol interaction. The tripartite interaction (ABA x IAA x mannitol) was not significant ($P>0.10$).

Thus the osmoticum x ABA interaction seen in Figure 8.4 would seem to be abolished at high external IAA concentrations. Such data do not indicate whether the lack of interaction is due to IAA concentration or the higher growth rate *per se*. In an attempt to resolve this, it was decided to investigate the effect of ABA in concert with another treatment (pH) known to affect coleoptile growth.

8.3.5 Responses of wheat coleoptiles to ABA applied at various pHs

In a study of pH effects on growth, Cleland (1992) used a buffer concentration of 20 mM MES (2-[N-morpholino] ethanesulphonic acid) as a compromise between minimal osmotic inhibition of growth and maximum buffer effectiveness However, preliminary experiments with wheat coleoptiles revealed a non-significant ($P=0.103$) 12 % growth reduction with 20 mM MES, so 10 mM MES was used in further studies.

Figure 8.9 shows the response of coleoptile growth to 10^{-7}M ABA across a range of pHs. Coleoptile length in the absence of ABA declined from 6.88 mm at pH 5.5 by 37 % to 6.55 mm at pH 6.7. This alkali-induced growth reduction is broadly comparable to studies described in the literature with *Avena* and *Zea* coleoptiles (Kutschera and Schopfer, 1985; Schopfer, 1989; Cleland *et al.*, 1991) however differences in the buffers used and preincubation times given in these studies precludes specific comparisons with the results here. A similar pH-induced growth reduction (34 %) was noted in the presence of 10^{-7}M ABA. ABA reduced growth by 24 %, 18 %

and 19 % at pHs of 5.5, 6.1 and 6.7 respectively. Both ABA and pH main effects were highly significant ($P<0.001$) but there was no significant ($P>0.10$) interaction between the two agents.

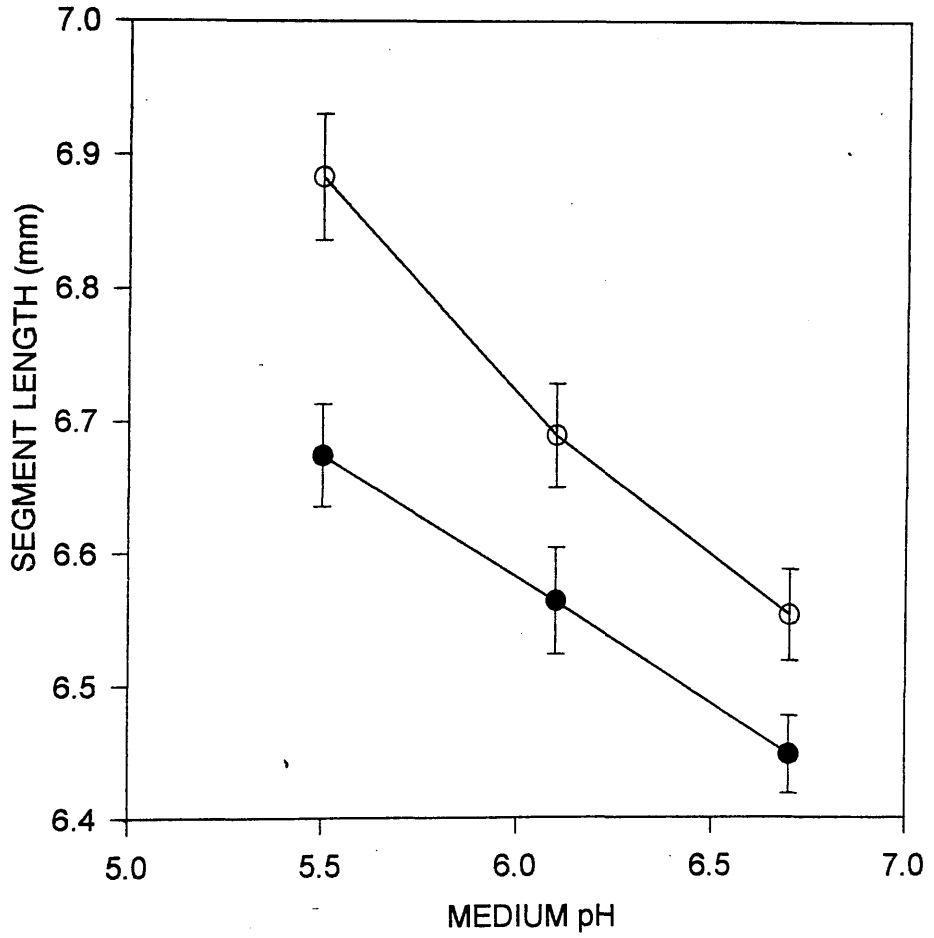

Figure 8.9: Segment length of wheat coleoptiles after 6 hours growth in solutions of 10 mM MES made up in deionised water and adjusted to various pHs, in the presence (●) or absence (○) of 10^{-7}M ABA. Points are means ± S.E. of 25-31 coleoptiles from 2 separate experiments.

8.4 DISCUSSION

The rationale behind the coleoptile experiments described in this chapter was to investigate a possible interaction between water potential and ABA, which was clearly observed (Figure 8.4) in the absence of external IAA. This interaction did not appear to be dependent on the ABA concentration of the expanding tissue (Table 8.1), in contrast to the apparent temperature x ABA interaction described for the leaf elongation assay system in Section 5.3.3.2 (see Figure 5.7). Again, a bulk tissue measurement was made, even though the epidermis is considered to be the limiting factor for coleoptile growth (Kutschera, 1992). Although it is possible to remove the epidermis of coleoptiles (Rayle, 1973), the number of coleoptiles that would be required for analysis of ABA using the RIA employed in this study (assuming an arbitrary doubling of ABA concentration in the epidermis compared to bulk coleoptile tissue, and an epidermal weight of 10 % of the entire coleoptile) was calculated to be prohibitive in terms of time.

It would be desirable to confirm the existence of this interaction using continuous recording of growth in a transducer (e.g. Kutschera and Schopfer, 1985) or by observing coleoptiles using a time lapse camera allied to image analysis equipment. This approach is necessary to show that the effects reported here are not just an artefact of a single measurement time being biased by treatment-induced differences in lag times for the resumption of growth following coleoptile excision. However such an explanation for the effects observed here is unlikely as an analysis of lag times for IAA-induced growth showed that a 1000 fold decrease in IAA concentration did not alter calculated growth after 6 hours by more than 10 %. This compares with the 30 % growth reduction seen when osmotica and ABA are applied together (Figure 8.4).

It was of concern that the osmoticum x ABA interaction disappeared with the application of external IAA. This occurred despite there being no IAA x ABA

interactions in 2 of 3 data sets (Figures 8.6, 8.7). It might be argued that the interaction described here is somewhat artificial in that the coleoptiles (in Figure 8.4) are elongating, presumably as a result of their endogenous IAA production, at a reduced rate compared to intact coleoptiles. Application of external IAA of the appropriate concentration can induce isolated coleoptiles to grow at the same rate as intact coleoptiles (Kutschera and Schopfer, 1985). Shouldn't, therefore, the interaction be of most relevance when the isolated coleoptiles are growing at the same rate as intact coleoptiles ? It is likely, however, that the appropriate concentration of IAA required for "intact-like" elongation in isolated coleoptiles results in tissue IAA concentrations that are many times those that occur endogenously, perhaps due to a wounding-induced change in coleoptile sensitivity to IAA. Therefore it is possible to argue that IAA treatment is similarly artificial and more credence should be given to the data obtained when the coleoptiles are elongating under their endogenously produced IAA. Whichever argument applies, it would seem imperative to confirm the existence of a water potential x ABA interaction in another growing system, such as the leaf elongation assay system using capillary tubing to impose an osmotic stress (Trejo and Davies, 1994) or growing plants in vermiculite of low water potential and manipulating ABA concentrations by external application (Sharp *et al.*, 1994). If we assume that the water potential x ABA interaction (in Figure 8.4) shown by coleoptiles is indicative of leaf growth behaviour in intact plants, we have a possible explanation for the leaf growth responses to drought seen in Section 7.3.

The magnitude of the interaction accounted for a coleoptile growth inhibition of 32 % (in the PEG data set) over the range of osmotic stresses applied, which is quantitatively sufficient to explain the leaf growth reductions (35-37 % - see Figures 7.6a, 7.7a) seen in the soil drying experiments. However, the ψ_π x ABA interaction described in Figure 8.4 occurred over a very narrow range of ψ_π (0 to -0.06 MPa). It seems unlikely that intact, droughted plants would show a growth response to ABA over the same ψ_π range. However, the coleoptile may be a model system for the whole

plant, although any ψ_π x ABA interaction in intact plants may occur in a different range. This may still be a restricted range, which would be difficult to quantify using psychrometric techniques due to the inherent variability of measurements (Figures 7.6b, 7.7b). If growth of droughted plants is affected by the ψ_π x ABA interaction over such a restricted range, it would explain the high sensitivity of leaf growth to drought observed in many studies (Hsiao, 1973; Saab and Sharp, 1989).

The existence of such an interaction in droughted plants would also explain the diel pattern of leaf growth inhibition (Figure 7.5). Reductions in LER were first detected in the afternoon as in previous studies (Van Volkenburgh and Boyer, 1985), when xylem tension would be maximal. At night, there is no growth reduction since xylem tension is reduced and the ABA signal is diminished as surface roots re-hydrate due to the transfer of water from deeper roots which are well-supplied with water.

An interaction of water potential and ABA in controlling growth may also explain the frequently observed correlation of lamina water potential and LER in droughted grasses (Boyer, 1970; Chu and McPherson, 1977). The significance of such correlations has usually been dismissed since well-watered plants may show the same range of ψ_Ls, and the fact that cells in the elongation zone can maintain turgor despite reductions in ψ_L (Michelena and Boyer, 1982). We also observed a correlation of ψ_L and LER in the drying experiments (Figure 7.11), which is similar to the correlation between medium osmotic potential and the growth of "droughted" (ABA-supplied) coleoptiles.

In addition to the explanatory value of this interaction in accounting for the growth of droughted plants, it may have important implications for the assessment of inhibitory activities of xylem sap. Although changes in growth inhibitory and antitranspirant compounds can regulate shoot physiology in the absence of changes in plant water relations (e.g. Saab and Sharp, 1989; Zhang and Davies, 1990a), more

recent reports have emphasised that ABA and water relations interact in their effects (Tardieu and Davies, 1992; Tardieu *et al.*, 1993). Evidence of such an interaction is provided by the requirement of an unrealistically high [EZ-ABA] in the detached shoot in order to inhibit LER to a comparable amount as intact plants subjected to drought (Figure 7.9a). Thus the failure of the leaf elongation assay system to take account of any interaction, by using shoots detached from well-watered plants, may limit its usefulness in assessing the growth inhibitory properties of xylem sap.

CHAPTER 9.

DISCUSSION

WHAT CONTROLS LEAF GROWTH OF GRASSES IN DRYING SOIL ?

IS THERE A ROLE FOR SENSITIVITY MODULATION OF ABA IN THIS CONTROL ?

The traditional answer to the first question is that soil drying results in a decline in leaf water potential (ψ_L) and turgor, which reduces growth. This mechanism is supported by relationships between growth and ψ_L (e.g. Boyer, 1970). It should be noted that the bulk of the growth reduction (50-80 %) occurs over a very narrow range of water potentials (e.g. 0.3 MPa) and that well-watered plants can show a similar variation in ψ_L over the course of the day, yet show no growth reduction. The observation that turgor can be maintained in the elongation zone (Michelena and Boyer, 1982) despite reductions in ψ_L also argues against simple hydraulic control of leaf elongation rate. Specialised experimental systems, which allow maintenance of high ψ_L in plants which have roots in contact with drying soil, have convincingly demonstrated that leaf growth can be controlled by chemical signals originating from the roots (Passioura, 1988a; Saab and Sharp, 1989; Gowing *et al.*, 1990). While the relevance of these results to the control of leaf growth in the field has generated controversy (cf. Kramer, 1988 vs. Passioura, 1988b), Ludlow *et al.* (1990) provide many examples of the apparent control of leaf growth and stomatal conductance by root-supplied signals in the field.

It is unfortunate that two of the most compelling examples of the operation of chemical signalling in the control of leaf growth (Saab and Sharp, 1989; Gowing *et al.*, 1990) have not quantified any potential signal. By virtue of its ability to inhibit leaf growth (Van Volkenburgh and Davies, 1983), its almost universal ability to mimic the responses of drought (Trewavas and Jones, 1991) and its well-recognised role in the control of stomatal conductance under drought (Davies and Zhang, 1991; Munns and

Sharp, 1993), ABA clearly must be a contender for a role in the inhibition of leaf growth of droughted plants. However, relatively few studies have examined the role of ABA in controlling leaf growth of droughted plants (Munns and Sharp, 1993). While whole plant studies have indicated a correlation between xylem ABA concentration and leaf growth of droughted plants, which is identical to the relationship found by feeding ABA solutions to well-watered plants (Zhang and Davies, 1990b); the evidence from leaf elongation assay studies is contradictory (Munns, 1992). When xylem sap from droughted plants was fed to detached shoots in the assay, growth was inhibited in a manner inconsistent with the sap ABA concentration.

However, the results from the bioassay study must be treated with caution for two reasons. Recent evidence from that group has indicated that storage of xylem sap at -20°C (as indicated in the original report) produced a large molecular weight compound which seemed to be responsible for the antitranspirant activity of stored sap (Munns *et al.*, 1993). Freshly collected sap had an antitranspirant activity apparently explicable in terms of its ABA concentration (although variability of assay sensitivity in the physiological range makes it difficult to be sure). If these results can be extrapolated to leaf growth, it may reconcile the difference between bioassay and whole plant studies. Secondly, the concentration of ABA in the elongating cells was not measured in Munns' study.

Although a recent study has indicated the presence of another growth inhibitory compound in xylem sap (Campbell *et al.*, 1995), this compound seems to be involved in the phenological development of the plant and not involved in the drought stress response. Although growth promoting compounds such as cytokinins may be important in chemical signalling (Meinzer *et al.*, 1991), the experiment of Gowing *et al.* (1990) appears to negate their role under drought stress. In a split-root experiment, excision of the dried root system resulted in growth recovery. Such a treatment cannot increase the supply of growth promoters to the shoot but would eliminate a supply of growth

inhibitors (such as ABA). Therefore assessment of a possible chemical control of leaf growth must surely focus on ABA, in the absence of knowledge of other inhibitors.

One of the dogmas of hormone physiology is that variation in response may be as much a function of changes in tissue sensitivity to the hormone as changes in endogenous concentration (Trewavas, 1981). Previous studies examining the role of ABA in controlling leaf growth have usually measured endogenous ABA concentrations; there has been no systematic attempt to identify sources of sensitivity variation, as there has been for research into stomatal responses to ABA (see Table 1.2). One report indicated that the effect of ABA on leaf growth could be reversed by high KCl concentrations (Van Volkenburgh and Davies, 1983), indicating that sensitivity modulation of leaf growth could occur. The recent development of a detached cereal shoot leaf elongation assay (Munns, 1992) provided a means of identifying sensitivity modulation of the leaf growth response to ABA.

Initial studies attempted to validate the leaf elongation assay as a reliable means of assaying growth, and attempted to verify the previously described report of a nutrient ion x ABA interaction (Chapter 2). The reversal of ABA-induced leaf growth inhibition by high KCl concentrations (Van Volkenburgh and Davies, 1983) could not be directly tested in detached cereal shoots as the KCl concentrations required were osmotically inhibitory to growth. There was no difference in the short-term leaf growth response of shoots fed ABA made up in distilled water or a dilute solution of nutrient ions, as shown previously for transpiration by Munns and King (1988). This result contradicts the report of Schurr *et al.* (1992), who demonstrated correlations between xylem sap nutrient concentrations and stomatal sensitivity to ABA in individual droughted sunflower plants with greatly different sensitivity to ABA. The surprising thing about this set of data is that all the plants were well fertilised, yet individual plants had different nutrient xylem nutrient concentrations. The multiplicity of inner correlations between different nutrient ions in the soil drying system make it unrealistic

to test for specific mechanisms of nutrient x ABA interactions. Alternatively, the leaf elongation assay system can be criticised as being too short term to allow expression of any interactions. Until the two types of study are able to provide reconcilable evidence, the idea that limited nutrient uptake by droughted plants can modify the leaf growth response to ABA is best treated as untested.

It is important to note that the above discussion of possible nutrient ion x ABA interactions refers to well-fertilised plants, or in the case of detached tissue assays, tissues detached from well-fertilised plants. Tissues detached from nutrient-stressed plants commonly show a greater stomatal response to applied ABA (Radin *et al.*, 1982; Radin, 1984). In a similar manner, it would be interesting to test whether detached cereal shoots from nutrient stressed plants showed a greater response to ABA. Whole plant studies which have imposed different fertilisation regimes on droughted plants have failed to demonstrate a nutrient stress effect on the sensitivity of the leaf growth response to soil drying (Saab and Sharp, 1989; Passioura and Gardner, 1990; Auge *et al.*, 1995). This may be taken as circumstantial evidence of a lack of nutrient stress x ABA interaction if it is assumed that ABA was involved in the drying response. However, Auge *et al.* (1995) found that the magnitude of the leaf elongation restriction in individual plants depended on the measured leaf phosphate concentration, and not the applied phosphate concentration. It is clear that future studies of the soil drying response would benefit from more rigorous measurement of plant nutrient concentrations (in both leaves, xylem saps and ideally the leaf elongation zone) to distinguish individual plant response; although the soil drying studies reported in Chapter 7 showed little between-plant variation (an average coefficient of variation for LER of 12-16 % - Table 7.3) in the sensitivity of LER to soil drying.

One of the potential causes of the apparent nutrient-induced sensitivity variation in the study of Schurr *et al.* (1992) may have been xylem pH. Varying the pH of the feeding solution from 5 to 8 had no effect on the response of leaf growth of

ABA-supplied shoots. In shoots fed an artificial xylem solution, there appeared to be a variable reduction in leaf growth at high pHs, but such reduction usually appeared to be associated with wilting of the detached shoots. It was concluded that pH does not affect leaf growth of either control or ABA-fed shoots, but it is possible that the artificial xylem solutions used may not have had sufficient buffering capacity to over-ride the plant's endogenous capacity.

The initial work with the leaf elongation assay also provided further evidence of its validity. Leaf growth responded to temperature in the detached shoots in a similar manner to intact plants. The dose-response of transpiration was very similar to that previously described for detached leaves (Munns and King, 1988; Munns *et al.*, 1993). However, leaf growth was found to be roughly an order of magnitude more sensitive than that reported by Munns (1992), indicating that sensitivity modulation of the leaf growth response to ABA in the detached shoot system could occur. Possible sources of this variation were considered to be genotype and temperature.

Genotypic variation in stomatal response to ABA has been previously described (Rodriguez and Davies, 1982; Quarrie, 1983; Blum and Sinmena, 1995). Bioassay approaches are particularly amenable to identifying this variation as they avoid possible complications in field experiments such as genotypic differences in plant water relations. Despite selection of genotypes which were thought likely to give different responses (and indeed had been demonstrated to differ in their transpiration response to ABA), there was only one case (comparison of 2 cultivars) where a consistent difference in leaf growth response was noted (Chapter 3). This was not due to greater uptake of ABA, or a greater LER of control plants, by the more responsive genotype. Further experimentation is clearly necessary to test whether this interaction would occur if the plants were grown or assayed under different conditions to those under which this study was conducted. Given the relatively small differences in leaf growth response (< 20 %) and potential variability between assays (demonstrated by a

significant G x ABA interaction comparing two batches of plants of the same cultivar), future work on variation in these genotypes seems difficult to justify from a logistics perspective.

Despite this pessimistic appraisal, genotypic differences in stomatal response to a soil drying-induced chemical signal have been previously demonstrated (Blum and Johnson, 1993). In this experiment, differences in cultivar response were related to differences in root growth; such that genotypes which showed a reduced stomatal response to soil drying had fewer roots exposed to dry soil (and hence fewer sites for the generation of a chemical signal). Although further work by this group has shown one of the cultivars which was less responsive to soil drying was relatively insensitive to application of ABA in a transpiration bioassay, comparative tests were not conducted with cultivars which were more responsive to the soil drying treatment (Blum and Sinmena, 1995).

A related program has been conducted by the Maize Research Institute in Belgrade in collaboration with Dr. S. Quarrie, with the aim of characterising genotypic differences in ABA accumulation (e.g. Pekic *et al.*, 1995). Despite successful efforts in identifying genotypes with different ABA accumulation in detached leaf and field studies, there has been a notable lack of success in characterising such genotypes for differences in stomatal or leaf growth sensitivity to ABA. Again, possible relationships between ABA sensitivity and field response have not been elucidated.

In addition to questioning the logistics of further searches for genotypic variation in the leaf growth response to ABA, the rationale behind such a search for needs to be questioned. The relevance of genotypic differences in the leaf growth response to ABA in field situations is difficult to predict. It is obvious that the physiological significance of genotypic variation in ABA response will depend on the mechanisms of growth control in the field. If signalling is hydraulic, then differences in

ABA response are irrelevant. Under conditions where chemical signalling is important, the agronomic significance of genotypic differences in the ABA response of leaf growth to forage yield will depend mainly on the water stress regime (Ludlow *et al.*, 1989). If the crop is intermittently irrigated, a sensitive genotype will be at a yield disadvantage. In the unirrigated situation, a sensitive genotype may be able to conserve water by restricting canopy expansion, allowing survival until the next rain event. It may therefore be possible to optimise the crop for its leaf growth response to ABA in a given environment. Although the significance of genotypic differences in the ABA response of cereal leaf growth to grain yield may seem a tenuous link dependent on whether the crop is source limited, chemical signalling has been shown to increase tiller mortality and reduce biomass and grain yield (Blum *et al.*, 1991; Blum and Johnson, 1993). Currently, the doubts over the reality of genotypic differences in leaf growth sensitivity to ABA ensure that the objective is best pursued for its intrinsic interest. The application of sensitivity differences is more likely to be as a physiological tool for investigating the role of ABA in leaf growth, rather than in the agronomic situation.

Like genotypic variation, temperature is a factor which has repeatedly been shown to affect stomatal response to ABA (e.g. Rodriguez and Davies, 1982; Eamus and Wilson, 1983; Honour *et al.*, 1995). Analysis of LER data collected for detached shoots at different temperatures and ABA concentrations showed a highly significant interaction between temperature and ABA (Chapter 4). Low temperature reduced the effectiveness of ABA. This interaction is potentially of enormous importance in the control of leaf growth in the field, since LER of well-watered plants is strongly temperature dependent (Gallagher and Biscoe, 1979; Ong, 1983). Greater inhibition of leaf growth by ABA at high temperatures would be important in conserving water by restriction of canopy expansion, since soil drying and high temperature commonly co-occur .

In an attempt to ensure that the greater inhibition of leaf growth at high temperature was not an artefact of more ABA being present at the site(s) of action, ABA was measured in the leaf elongation zone at different temperatures (Chapter 5). This method of sampling was selected to determine whether the ABA content of the elongation zone was a more appropriate explanatory variable than the ABA concentration fed to the shoots. Either variable may still be regarded as an apology for lack of information of the ABA concentration at the active site(s), which is assumed to be the apoplastic ABA concentration of the tissue in the leaf elongation zone which is limiting growth. Analogies from other growing tissues (coleoptiles and stems) suggest that the epidermis is often growth limiting (Kutschera, 1992).

The similarity of the relationship between leaf growth inhibition and temperature, and leaf growth inhibition and elongation zone ABA concentration [EZ-ABA], in the same plants (Figure 5.7), suggested that the interaction was caused by ABA accumulation in the elongation zone. To resolve whether the interaction was a function of [EZ-ABA], or a genuine difference in sensitivity, it would be necessary to feed ABA to detached shoots at the same transpiration rate, while maintaining the elongation zone at different temperatures. Despite the correlation suggested above, it would be surprising if the interaction between ABA and temperature found in stomatal studies did not hold for leaf growth.

The temperature studies demonstrated a fundamental difference in the control of leaf growth and stomata by ABA in bioassay systems. Enhanced ABA accumulation in the elongation zone at high temperatures was able to reduce leaf growth (Chapter 5), yet such accumulation in the leaves at high temperatures does not generally produce additional stomatal closure (Trejo *et al.*, 1995). It would therefore appear that the detached shoot is able to regulate the amount of ABA reaching the stomata, but not the amount reaching the leaf elongation zone. Metabolic differences between the two tissues could be involved. If such a situation existed for intact plants, it would provide

a possible explanation for the increased sensitivity of leaf growth (relative to stomatal conductance) to drought (e.g. Saab and Sharp, 1989). An alternative explanation for the enhanced ABA accumulation in the elongation zone is that phloem transport of ABA (Wolf *et al.*, 1990) to the roots is disrupted in the detached shoot, resulting in a build-up of ABA.

The fact that differences in elongation zone ABA content seemed to account for differences in ABA response under different environmental (temperature) conditions prompted a consideration of experiments in which detached shoots were fed xylem sap (Chapter 6). In agreement with Munns (1992), the ABA concentration of xylem sap from droughted plants was unable to account for the leaf growth inhibition. However, when leaf growth inhibition was plotted against the concentration of ABA in the elongation zone, the point for xylem sap lay on the ABA dose-response curve. This suggested that the xylem sap contained a compound which could be converted to ABA in the elongation zone. Although high concentrations of ABA conjugates can occur in xylem sap from grasses (Bano *et al.*, 1993), they have traditionally been regarded as unimportant in the plant due to their stability. The result reported here seems to suggest a possible role for ABA conjugates in leaf growth inhibition additional to that of free ABA. The identity of any additional compounds was not pursued due to the difficulties of obtaining adequate quantities of sap and concerns over the validity of the sap sampling strategy. It would be desirable to repeat these experiments using sap collected from an intact transpiring plant using a whole plant pressure chamber to see whether the inhibition of leaf growth could still be attributed to the ABA content of the leaf elongation zone. Despite these methodological concerns, the clear relationship between elongation zone ABA content and leaf growth in the detached shoot system pointed to the necessity of measuring elongation zone ABA concentration in droughted plants.

The results of the temperature and xylem sap experiments indicated that xylem sap ABA concentration may not be the best indicator of leaf growth response. This seemed fortunate, as it was impossible to sample the xylem sap of soil-grown plants at a similar developmental stage to the plants used in the assay. Although xylem sap is regarded as the most sensitive indicator of changes in chemical signalling (Zhang and Davies, 1989b; 1990a), it must be noted that the means of sampling xylem sap may provide samples which are not indicative of concentrations in the transpiration stream (Munns, 1990; Jackson, 1993).

Another complication with analysis of xylem sap constituents from droughted plants is that reductions in transpirational water flow will result in less dilution of all solutes loaded into the transpiration stream. It is therefore difficult to discern a physiological role for a particular xylem sap constituent. However, observations which show reductions in leaf growth well before reductions in transpiration (e.g. Passioura 1988a; Saab and Sharp, 1989) may allow the identification of increases in xylem sap ABA concentration prior to reductions in transpiration. A detailed time course of leaf growth, transpiration and xylem ABA concentration may allow the detection of a relationship between leaf growth and xylem ABA concentration prior to decreases in transpiration, which would be extremely valuable in providing evidence of a role for ABA in controlling leaf growth. This was an original aim of the soil drying experiments, but problems of accurate gravimetric measurement of transpiration, and problems with obtaining a sufficient sample of xylem sap precluded such a temporal analysis. Instead, detailed sampling of leaf growth and elongation zone ABA content was achieved.

Although the soil drying studies imposed a slowly developing, mild water stress, ideal conditions for the generation of a chemical signal, there was only limited ABA accumulation in the elongation zone, despite daily growth reductions of up to 35 %. Changes in xylem sap pH (when it became technically possible to sample) also did

not account for the leaf growth reduction, in support of the bioassay evidence presented in Chapter 2. There was also no clear evidence of hydraulic signalling controlling growth (Chapter 7).

However, ψ_L and ψ_p have low intrinsic capacity for explaining changes in growth reductions due to the large random error associated with their measurement. There is also physiological uncertainty since current water relations measurements are principally directed at measurement of cells in the lamina, which may have very different water relations from elongating cells (Michelena and Boyer, 1982; Barlow, 1986). However, the lamina is likely to be the first tissue to show perturbed water relations (Michelena and Boyer, 1982), which provides some justification for the measurement of water relations of cells that are not actually growing.

Importantly, the relationship between LER and elongation zone ABA concentration was very different in the soil drying and in the detached shoot experiments. One possibility that may explain the greater ABA accumulation in the detached shoot system is the lack of roots. It is well known that the phloem is an alkaline trap for ABA (Hoad, 1978); any ABA transported from the root that is not metabolised by leaf tissue may find its way into the phloem for return to the roots. This recycling process may contribute to the drought-induced increase in xylem ABA concentration (additional to increased root ABA synthesis in response to soil drying) in intact plants. In detached shoots, the ABA is likely to accumulate at the base of the shoot. This would suggest that the leaf elongation assay system does not provide a realistic assessment of ABA accumulation in the elongating cells of intact plants.

Another reason for the difference in LER response to ABA in the two systems may be small differences in water potential in the elongating cells below the detection limits of psychrometric measurement. Such a hypothesis would support evidence of

Sharp and co-workers, who have shown that the effect of ABA on root growth is highly dependent on soil water status (e.g. Sharp *et al.*, 1994).

The possibility of a water potential x ABA interaction affecting growth was investigated using a coleoptile growth assay (Chapter 8). Although growth was not inhibited in the presence of ABA or osmoticum alone, application of both of these agents together resulted in a growth reduction of up to 35 %, using either PEG or mannitol as an osmoticum. Importantly, in contrast to the temperature experiments with the leaf elongation assay, the interaction could not be explained on the basis of ABA concentration in the coleoptile elongation zone.

The existence of such an interaction in intact plants may explain both the magnitude of the growth reduction, and the diurnal course of leaf growth inhibition, seen in the soil drying experiments. The extreme sensitivity of this interaction was demonstrated by the fact that the growth response occurred over a very narrow range of osmotic potentials. It seems unlikely that intact, droughted plants would show a growth response to ABA over the same ψ_L range. However, the coleoptile may serve as a model system for the whole plant, although any ψ_L x ABA interaction in intact plants may occur in a different range. This may still be a restricted range, which would be difficult to quantify using psychrometric techniques due to the inherent variability of measurements. If growth of droughted plants is affected by the ψ_L x ABA interaction over such a restricted range, it would explain the high sensitivity of leaf growth to drought observed in many studies (Hsiao 1973; Saab and Sharp 1989).

Although the coleoptile data alone do not provide a particularly strong case for the existence of a water potential x ABA interaction affecting growth, recent results from another system (Thompson and Davies, unpublished observations) appear to support the effect. The relationship between leaf growth rate and turgor pressure of the leaf elongation zone of the first maize leaf was investigated using plants grown in

hydroponic solutions. Addition of osmoticum to the solution failed to reduce growth or cell turgor, as did addition of ABA to the plants in the absence of an osmotic stress around the roots. However, the addition of osmoticum to the roots of ABA-supplied plants reduced growth, again without a measurable decrease in the turgor pressure of cells in the elongation zone.

The results presented in this thesis show two clear examples where the effect of ABA on leaf growth is highly dependent on the environmental conditions. Unfortunately, the existence of both interactions can still be regarded as potentially artefactual due to the nature of the assay systems used in their identification (leaf elongation assay in the case of temperature, and coleoptile assay in the case of water potential). Future investigations should attempt to manipulate these environmental variables in whole plants responding to chemical signals of soil drying.

It should also be noted that this study has chiefly addressed the equilibrium leaf growth response to ABA, and not the dynamics of the response. It would be instructive to compare the dynamics of leaf growth response to ABA when conditions such as temperature, and leaf water potential, are varied. This would assist in verifying the existence of the described interactions. While other interactions may also be important in certain contexts, it is likely that temperature and water potential are the two variables which are of most importance to droughted plants.

While there may still be some doubt as to the role of ABA in controlling leaf growth of plants grown in drying soil (Munns and Sharp, 1993), part of this doubt must surely result from a lack of understanding of the factors which can alter the sensitivity of leaf growth to ABA. It is hoped that the study described goes some way towards identifying such sensitivity variation. The challenges that lie ahead include the verification of the existence of these interactions in intact plants, and the elucidation of potential mechanisms.

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APPENDIX 1.

INSTANCES OF NON-HYDRAULIC SIGNALLING OF SOIL DRYING

The references (entire references to be found on preceding pages) below were considered in the compilation of Table 1.1. The data base (used to calculate percentages in Table 1.1) is given on p 235. When two experiments employed the same species/drying treatment combination, they are included as the one study.

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25 Zhang *et al.* (1987) *Journal of Experimental Botany* 38, 1174-1181.

26 Zhang and Davies (1989a) *Plant, Cell and Environment* 12, 73-81.

27 Zhang and Davies (1989b) *The New Phytologist* 113, 167-174.

28 Zhang and Davies (1990a) *Plant, Cell and Environment* 13, 277-285.

29 Zhang and Davies (1990b) *Journal of Experimental Botany* 41, 1125-1132.

Experimental details of soil drying studies - DATA BASE																														
Reference:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	No.
Whole Plant Pressure chamber												*	*			*			*											4
Vertically Split Root System (Two Pots)	*	*	*			*				*				*	*					*					*					9
Horizontally Split Root System (Columns)				*			*	*			*						*	*				*	*			*	*	*	*	12
Field Studies					*				*												*	*		*						5
Herbaceous Monocotyledons		*	*	*		*	*			*		*				*		*	*	*	*				*	*		*	*	16
Leguminous Dicotyledons					*			*			*					*							*							5
Herbaceous Dicotyledons												*	*									*					*		*	5
Woody Species	*								*					*	*		*							*						6
Stomatal Conductance / Transpiration only	*			*	*	*	*	*	*		*	*	*		*		*				*	*	*	*	*	*	*	*		20
Leaf Elongation Rate only																*		*											*	3
Both		*	*							*				*					*	*										6
No putative chemical signals measured	*	*	*		*		*			*		*		*		*			*	*										11
ABA only measured						*		*			*				*		*				*	*	*		*	*	*	*	*	13
ABA and other signals measured				*					*				*											*						4
Other signals only measured																		*												1

APPENDIX 2.

EXAMPLES OF STATISTICS

Sample ANOVA table from Chapter 2 (Figure 2.2a)

Effects of nutrient solution, ABA and their interaction on leaf elongation rate of detached shoots of *Hordeum vulgare* cv. Klaxon
NS = not significant, P<0.05 *, P<0.01 **, P<0.001 ***

Source of Variation	d.f.	s.s.	m.s.	v.r.	Significance
Variation between leaves					
Nutrient Solution	1	0.0562	0.0562	1.4839	NS
ABA	1	1.7983	1.7983	47.4987	***
Nutrient x ABA	1	0.0081	0.0081	0.2139	NS
Residual	25	0.9466	0.0379		
Total	28	2.8092			
Variation within leaves					
Time	1	0.0697	0.0697	4.44	*
Time x Nutrient	1	0.0438	0.0438	2.79	NS
Time x ABA	1	0.0860	0.0860	5.48	*
Time x Nutrient x ABA	1	0.0426	0.0426	2.71	NS
Residual	112	1.7587	0.0157		
Total	144	4.8100			

Sample ANOVA tables from Chapter 3 (Figure 3.5b)

Effects of genotype, ABA and their interaction on leaf elongation rate of detached shoots of *Hordeum vulgare* cv. Klaxon and *Zea mays* cv. Earliking in an individual leaf elongation assay.
NS = not significant, P<0.05 *, P<0.01 **, P<0.001 ***

Source of Variation	d.f.	s.s.	m.s.	v.r.	Significance
Variation between leaves					
Genotype	1	0.463	0.463	5.946	*
ABA	4	38.182	9.5456	122.52	***
Genotype x ABA	4	2.121	0.5302	6.805	***
Residual	75	5.843	0.0778		
Total	84	46.61			
Variation within leaves					
Time	1	0.9009	0.9009	19.03	***
Time x Genotype	1	0.9424	0.9424	19.9	***
Time x ABA	4	4.3749	1.0937	23.1	***
Time x Genotype x ABA	4	0.3815	0.0954	2.01	NS
Residual	245	11.600	0.0474		
Total	339	236.833			

Sample ANOVA table from Chapter 3 (Figure 3.5d)

Effects of experiment, genotype, ABA and their interactions on leaf elongation rate of detached shoots of *Hordeum vulgare* cv. Klaxon and *Zea mays* cv. Earliking.
NS = not significant, P<0.05 *, P<0.01 **, P<0.001 ***

Source of Variation	d.f.	s.s.	m.s.	v.r.	Significance
Variation between experiments					
Experiment	2	16.124	8.062	92.485	***
Variation between leaves					
Genotype	1	9.696	9.696	111.232	***
ABA	4	85.771	21.443	245.984	***
Genotype x ABA	4	1.281	0.320	3.675	**
Genotype x Experiment	2	2.536	1.268	14.548	**
ABA x Experiment	8	2.318	0.290	3.324	**
Genotype x ABA x Experiment	8	1.547	0.193	2.219	**
Residual	205	16.750	0.087		
Total	234	136.02			
Variation within leaves					
Time	1	5.800	5.800	100.48	***
Time x Experiment	2	0.794	0.397	6.88	**
Time x Genotype	1	3.364	3.364	58.28	***
Time x ABA	4	9.628	2.407	41.7	***
Time x Genotype x Experiment	2	0.050	0.025	0.43	NS
Time x Genotype x ABA	4	1.014	0.253	4.39	**
Time x ABA x Experiment	8	0.999	0.125	2.16	*
Residual	683	39.428	0.190		
Total	939	105.96			

Sample ANOVA table from Chapter 4 (Figure 4.3)

Effects of temperature, ABA and their interaction on leaf elongation rate of detached shoots of *Hordeum vulgare* cv. Klaxon
NS = not significant, P<0.05 *, P<0.01 **, P<0.001 ***

Source of Variation	d.f.	s.s.	m.s.	v.r.	Significance
Variation between leaves					
Temperature	1	242.6	242.6	933.08	***
ABA	4	180.02	45	173.08	***
Temperature x ABA	4	26.32	6.58	25.38	***
Residual	397	102.49	0.26		
Total	406	551.43			
Variation within leaves					
Time	1	0.17	0.17	1.42	NS
Time x Temperature	1	0.11	0.11	0.94	NS
Time x ABA	4	25.37	6.34	54.45	***
Time x Temperature x ABA	4	0.83	0.21	1.77	NS
Residual	1428	166.35	0.12		
Total	1844	744.25			

Sample ANOVA table from Chapter 8 (Figure 8.4a)

Effects of experiment, PEG, ABA and their interactions on segment length of coleoptiles of *Triticum aestivum* cv. Tonic
NS = not significant, P<0.05 *, P<0.01 **, P<0.001 ***

Source of Variation	d.f.	seq s.s	adj. s.s	m.s	v.r.	P
Experiment	2	0.39	0.45	0.23	2.78	0.064
ABA	1	4.59	4.57	4.57	55.94	0.000 ***
PEG	4	0.56	0.62	0.15	1.89	0.113
Experiment x ABA	2	0.04	0.06	0.03	0.39	0.675
Experiment x PEG	8	0.75	0.84	0.1	1.28	0.252
ABA x PEG	4	1.85	1.92	0.48	5.89	0.000 ***
Experiment x ABA x PEG	8	0.86	0.86	0.11	1.31	0.239
Residual	272	22.22	22.22	0.08	.	
Total	301	31.24				

APPENDIX 3.

GENOTYPIC COMPARISONS OF LEAF GROWTH RESPONSE TO ABA

Table of significance for experiments comparing leaf growth response to ABA of different genotypes. Values for LER were expressed as a percentage of controls and means discriminated by Student's unpaired t test. P<0.05 *, P<0.01 **, P<0.001 ***

Genotypic Comparison	Data Set	P values for unpaired t test on LER values expressed as a percentage of controls; values when leaf growth inhibition maximal			
		10^{-8}M ABA	10^{-7}M ABA	10^{-6}M ABA	10^{-5}M ABA
Klaxon vs. Earliking	Exp 1	0.709	0.931	0.915	0.740
	Exp 2	0.880	0.00 ***	0.00 ***	0.728
	Exp 3	0.020 *	0.675	0.465	0.926
	Combined	0.205	0.00 ***	0.070	0.608
Klaxon vs. Firefly	Exp 1	0.041 *	0.045 *	0.001 **	0.004 **
	Exp 2	0.283	0.352	0.004 **	0.108
	Exp 3	0.267	0.00 ***	0.122	0.945
	Combined	0.008 **	0.005 **	0.00 ***	0.009 **
Sunstar vs. Sundor	Exp 1	0.464	0.096	0.065	0.561
	Exp 2	0.005 **	0.349	0.515	0.778
	Combined	0.323	0.038 *	0.246	0.69
Chinese Spring vs. SQ1	Exp 1	0.935	0.00 ***	0.082	0.015 *
	Exp 2	0.631	0.009 **	0.399	0.450
	Exp 3	0.901	0.46	0.024 *	0.499
	Combined	0.881	0.002	0.976	0.038 *
Klaxon No 1 vs. Klaxon No 2	Exp 1	0.817	0.794	0.421	0.005 **
	Exp 2	0.001 **	0.046 *	0.139	0.007 **
	Exp 3	0.078	0.688	0.161	0.337
	Combined	0.004 **	0.503	0.131	0.001 **

APPENDIX 4.

PUBLICATIONS

Publications arising from this work:

Dodd IC, Davies WJ 1994. Leaf growth responses to ABA are temperature dependent. *Journal of Experimental Botany* **46,** 903-7.

Dodd IC, Stikic R, Davies WJ 1996. Chemical regulation of gas exchange and growth of plants in drying soil in the field. *Journal of Experimental Botany* **in press**.

Dodd IC, Davies WJ 1996. The relationship between leaf growth and ABA accumulation in the grass leaf elongation zone. *Plant, Cell and Environment* **in press**.