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ROOT-TO-SHOOT COMMUNICATION IN *RICINUS COMMUNIS* L.

PLANTS SUBJECTED TO DRYING A PART OF THE ROOT SYSTEM

A thesis submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy in the Faculty of Science, Department of Agricultural Sciences, Long Ashton Research Station

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Root-to-Shoot Communication in *Ricinus Communis* L. Plants Subjected to Drying a Part of the Root System

Abstract

This thesis examines the role of root-sourced abscisic acid in the regulation of stomatal closure and leaf expansion in response to drying approximately half of the roots of *Ricinus communis* L. plants. Drying part of the root system of *Ricinus communis* promoted stomatal closure and slowed leaf expansion in the absence of any disturbances in shoot water relations, implying the involvement of chemical rather than hydraulic signalling. Initially root-sourced ABA was believed to be responsible for these responses. Delivery rates (concentration x flow rate) of ABA out of the drying roots were calculated which took into account the effect of dilution on solutes in the well-watered and droughted plants due to different transpiration rates in these plants. The delivery term was further modified to account for the differences in sizes of their roots and shoots. ABA delivery out of the roots of plants with drying upper roots increased within the first 12 h and was maintained over the next 3 days. However, significant decline in stomatal apertures and leaf elongation occurred only 2 - 3 days after root drying began. During the early stages of drying upper roots (2-3 d) xylem sap pH, and delivery rate of nitrate and 1-aminocyclopropane-1-carboxylic acid were little changed, while hydraulic conductivity of the root system as a whole was reduced approximately 25%, and ABA accumulation (synthesis?) in roots increased. Increased ABA levels in phloem sap was not found, suggesting no enhanced re-cycling of ABA between shoots and roots was taking place in the plants during this time. Antitranspirant activity in xylem sap of droughted plants that was not ABA was sought as a possible cause of stomatal closure. However, convincing evidence of such activity was not found. Examination of ABA output by roots into shoot compared to that entering lamina of the 5th leaf in the canopy showed the attenuation of the signal as transpiration fluid moved up the plant. These observations indicate that ABA from roots is unlikely to be a highly active signal eliciting shoot responses to mild drought in *Ricinus communis*.

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Declaration

The work presented in this thesis is based upon the author's independent study under the supervision of Dr. MB Jackson. The author is responsible for all experimental work, interpretation of results, discussions and conclusions herein. All assistance and advice has been acknowledged. The views expressed in this dissertation are those of the author and not of the University.

AD Jokhan

April 1997

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Chapter 1 : LITERATURE REVIEW

In 1972, Jacob Levitt proposed a definition of biological stress derived from a concept taken from physical science that physical stress defines any force applied to an object. In biological terms, 'stress' has the connotation of causing injury (i.e., irreversible or plastic strain) to an object to which it is applied. A biological stress may, therefore, be defined as any environmental condition capable of inducing injurious strain in living organisms. Since a biological stress is not necessarily a force, the biological strain is not necessarily a change in dimension. An organism may suffer physical strain (e.g., cessation of cytoplasmic streaming) or a chemical strain (e.g., a shift in metabolism), therefore if either strain is sufficiently severe, the organism could suffer permanent injury or death (Levitt, 1972). In botanical terms, stress is any change in environmental conditions that reduces or adversely changes a plant's growth or development. Although such effects are of intrinsic botanical interest, understanding how they are brought about is of pressing concern since environmental stresses represent the major limiting factor to agricultural productivity. Furthermore, in addition to having an impact on crops which are presently being cultivated, environmental stresses also influence the possibility of introducing particular crop plants into areas which are not at this time being used for agriculture (Cherry, 1989). This is increasingly important for World food production since, as the human population increases and utilises more land for housing and industrial activities, agriculture is being forced into marginally productive areas (Hale and Orcutt, 1987).

The problem is exacerbated by human activities that degrade the environment and climate, thus increasing the stress under which plants must grow and survive.

Environmental stresses most often encountered by plants are drought, waterlogging, nutrient deficiencies and toxicity, salinity, temperature extremes, air pollution and chemical interference. Each stress can limit plant growth individually. However, their influences on growth and survival are frequently exerted through interaction and covariation (Jones *et. al.*, 1989). In a stressful situation there is an imposed depression of the rate of dry matter production of all or part of a vegetation to below its full "genetic potential" (Jones *et. al.*, 1989). In terms of yield, plant responses to stress will depend upon the severity of the stress, time over which it is imposed, stage of its life, and the extent to which a plant can adapt to and recover from its effects.

Drought

Although the earth carries a large amount of water, only a small fraction of this water is available to use as "fresh" water by land-dwelling organisms (Eagleson, 1970). Ninety seven percent of the earth's total water resource is ocean, 2% is frozen in icecaps, 0.31% is stored as groundwater and 0.37% of the remainder has a potential of being useful as "fresh" water. This fraction includes water in lakes, streams, shallow ground, soil moisture and atmospheric moisture. The demand for water varies greatly in different countries, depending on population and pattern of socio-economic development. Agriculture is the main drain on water supply (Tolba, 1992). Plants use

immense amounts of water during their lifetime. A single mature plant weighing 1 kg in dry weight, excluding roots, may contain 8 kg of water. The same plant may have transpired 50 kg of water during its growth (Tekinel, 1979). On an average, globally, 69% of water withdrawn is used in agriculture (Tolba, 1992). In the United States alone, 85% of the total water consumption is used for irrigation of crops for the production of food and fibre (Davidson *et. al.*, 1986). Adequate supply of water alone is not the only problem, good quality water is also necessary for both agricultural and human consumption. Although irrigation is heavily used in successful agricultural farming and no doubt increases yields (Briggs and Courtney, 1989; Tolba, 1992), it wastes large quantities of water (Tolba, 1992). This can lead to significant changes in soil conditions, some of which are long-lasting and potentially detrimental to the soil, such as soil erosion, especially when water is supplied by surface methods, and when salt accumulates due to poor quality water used for irrigation (Briggs and Courtney, 1989) or to rising water tables which raise salt from depth.

In higher plants, water is absorbed by roots from the soil and transported to shoots as a result of gradients in water potentials developed within the plant from evapotranspiration or root pressure. When rates of water loss by transpiration exceed rates of water absorbed by roots, water in the conducting vessels (xylem) is subject to an under pressure or negative pressure. Cells of the shoot system are hydraulically linked to the conducting vessels and thus will experience a degree of water shortage. In wet, well-aerated soils, water movement through the soil-plant-air interface is in a

dynamic state. Drought in plant tissues occurs when the availability of water in the soil is reduced while atmospheric conditions favour the continued loss of water by transpiration and evaporation. As soil dries, because of drainage, evaporation and absorption by roots, the continuity of liquid water is interrupted. Some water remains trapped in smaller pores of < 0.5 μm and some remains in the form of vapour in soil pore spaces. The drier the soil becomes the more tightly the remaining water is held on the soil particles. There comes a stage when forces holding the remaining water are so strong that roots are unable to extract any from the soil so that although transpiration will have almost stopped (due to stomatal closure), the shoot has dehydrated so much that it would be unable to recover if placed in a high humidity environment. At this stage the plant has permanently wilted and the soil is at its permanent wilting point (PWP). This occurs at soil water potentials between -1.0 MPa to -2.0 MPa (Hale and Orcutt, 1987). On a global basis, drought limits plant growth and crop productivity more than any other single environmental factor (Jones, *et. al.*, 1989).

As a result of global warming, more frequent droughts are predicted (Hsiao, 1993). Carbon dioxide emissions into the atmosphere are increasing at approximately 0.25% each year and at this rate by the year 2060 atmospheric carbon dioxide concentrations would have doubled its present level of 350 ppm (Tolba, 1992). Other greenhouse gasses besides carbon dioxide (e.g., methane, nitrous oxide, chloroflurocarbons) are also increasing and will contribute to global warming. The

impact of climate change on world food production is not certain (Parry and Rosenzweig, 1993). Generally, more atmospheric carbon dioxide would increase carbon assimilation by plants while reducing transpiration as a result of stomatal closure, thus increasing yields and water use efficiency by plants. However, plants will also be prone to more frequent droughts. It appears that the influence of global warming on plant growth and yield is very complex. Simulations of global warming scenarios using crop models predict different effects on different crops (Parry and Rosenzweig, 1993). A number other factors will influence plants such as the species, climate of the country in which they are grown, altitudes at which they are grown, and the farming techniques used (Tolba, 1992; Hsiao, 1993).

Effects of Drought on Plant Performance

The awareness of the importance of plant responses to water deficits is great, but the complex ways in which water deficits restrict plant growth are not understood, nor are mechanisms of drought tolerance and resistance which occur in certain species (Smith and Griffiths, 1993). Studies of effects of drought entail investigation not only at the whole-plant level but also at cellular and molecular levels. Water deficits can influence plants either by causing desiccation of cells and organs, or may cause regulation at whole-plant levels without necessarily causing cell dehydration (Tardieu, 1996).

Cell expansion is the most sensitive process to tissue hydration (Hale and Orcutt, 1987; Davies and Zhang, 1991). As the cell loses water, loss of cell turgor

slows cell expansion and decreases ultimate cell size, resulting in smaller stems, leaves and stomatal apertures (Hale and Orcutt, 1987). According to Hsiao (1973) the effects of water deficits on plant cells are reduced water potential (or activity of cellular water), reduced cell turgor, increased concentration of solutes and macromolecules as cell volume is reduced at lower turgor, altered spatial relations in plasma, tonoplast and other cell organelles due to volume changes and changes in structure and configuration of macromolecules by removal of water of hydration or through modification of the structure of adjacent water. Under mild to severe desiccation, the release of hydrolases (which would normally be isolated into compartments) destroys membranes in susceptible plants. The effect of mild water deficit on photosynthesis may be brought about by reduced carbon assimilation due to the reduced carbon dioxide uptake as stomata close (Hsiao, 1973; Schulze, 1986; Kaiser, 1987). Reduction in leaf water content during more severe stress alters photosynthetic competence in many plants. Leaf water deficits can have effects on chloroplast biochemistry that result in reduced photosynthetic performance. Reduced biochemical capacity for photosynthesis is due to decreased electron transport through photosystem II and this occurs under severe drought stress (Baker, 1993). Drought stress favours the synthesis of amino acids over organic acids and sugars (Hale and Orcutt, 1987). Legumes experiencing water deficits show reduced nitrogenase reductase activity in roots (Sundström and Huss-Danell, 1995). This is probably caused by a reduction in the continuous supply of photoassimilates to roots (Huss-Danell and Sellstedt, 1985) and levels of oxygen in root nodules (Silvester *et.*

al., 1990). Overall protein synthesis is also affected by drought stress and there is a shift from polyribosome to monoribosome populations that are inactive in protein synthesis (Hasio, 1973).

Water is the main component of plant cells. At cellular level it dissolves and/or imbibes hydrophilic molecules, gives rise to membrane structure, orientates protoplasmic molecules in hydrophobic and hydrophillic poles. It is also the main medium for biochemical reactions and is involved as a reactant in several processes such as photosynthesis. At tissue level, water is the link between cells and it forms continuity between apoplasm and symplasm. At whole-plant level, water carries sap containing assimilates, minerals and hormones. Water moving through the plant is transpired through stomata which helps cool plants. Hence, water deficits would disrupt any or several of these activities at any level of organization, slowing down metabolism, growth and reducing rigidity (Monneveux and Belhassen, 1996).

Surprisingly perhaps, some effects of soil drying on shoot growth and functioning can be seen in the absence of shoot dehydration (Bates and Hall, 1981; Neales *et. al.*, 1989; Gowing *et. al.*, 1990; Bano *et. al.*, 1993; Schurr and Schulze, 1996). In such instances chemical messages from the drying roots are thought to be responsible for bringing about changes such as increased stomatal closure and inhibition of cell division and expansion (Passioura, 1988a; Davies and Zhang, 1991; Tardieu *et. al.*, 1992; Gowing *et. al.*, 1993).

Drought and Agriculture

In World agriculture, drought ranks among the most devastating causes of economic losses (Martin *et. al.*, 1992). In agricultural terms drought refers to periods in which rainfall fails to keep up with evapotranspiration over prolonged periods. In such circumstances, plants suffer reduced growth or yield because of insufficient water supply. It is also possible for too large a humidity deficit to cause plant dehydration, in spite of seemingly adequate water available in soil. For example, this can occur when a sudden increase in air temperature takes place and humidity decreases, especially at critical stages of plant growth such as pollen meiosis or anthesis (Passioura, 1996). Whereas, over time, drought can cause plant death in the wild, plant survival is usually not an issue in cultivated crops (except in perennial pastures) because farmers either irrigate or choose crops or sowing dates so that plants do not experience such extreme stress. In crops, what is sought is a maintenance of productivity under relatively mild stresses (Tardieu, 1996). The water that is finally available to the crop from its water supply is that water which is stored in the soil and is accessible to that plant plus rainfall during the life of the crop, minus any losses from drainage beyond the reach of the roots and direct evaporation. The most effective use of the available water involves: (a) capturing as much as possible; (b) using the captured water as effectively as possible during carbon dioxide assimilation; (c) converting as much of this assimilate as possible into harvestable form such as grain (Passioura, 1996).

In Thailand, almost all maize is grown under rainfed conditions (Kitbamroong and Chartachume, 1992). Crop yield was found to vary from year to year, depending on the amount and distribution of rainfall. Thus, drought stress during the growing season is the major factor in determining yield (Kitbamroong and Chartachume, 1992). Water shortage can be long-term (e.g., seasonal) or transient (e.g., at noon on hot days). When cereals reach the developmental stage prior to kernel filling, drought causes the formation of wizened, undeveloped seeds. Untimely drought can also cause premature drop of orchard fruits (Kitbamroong and Chartachume, 1992). In maize, silking and pollen shedding occur simultaneously and drought stress can delay silking. Therefore, selection against silk delay has been found to be the most effective way of breeding maize plants for drought tolerance. Transient water deficiency can also be damaging to plants at the reproductive stage (Briggs and Courtney, 1987; Passioura, 1996). For example, transient water deficit coupled with low atmospheric relative humidity can cause irreparable harm to the pollination of orchard trees, maize and vegetable crops. The germination of pollen on the stigma requires droplets of the stigma fluid, and the stigma is only receptive for a few hours. Desiccation of the stigma fluid will lead to pollen abortion during this crucial time.

Heavy crop yields require a large total production of dry matter. At a given time, total dry matter produced by a plant is the result of cumulative gains and losses by the plant. Between eighty to ninety percent of plant dry matter is produced by photosynthesis and the remainder by the absorption of mineral nutrients (Karamanos,

1979). The economic yield of a crop will ultimately be determined by the translocation of assimilates from other parts of the plant to the economically important part after gain by photosynthesis, subject to any losses by respiration, senescensing and abscission, and translocation to non-harvested organs, most notably the roots which can require up to 50% of the total photosynthate for growth and maintenance. Water shortage reduces photosynthesis, therefore reducing dry matter assimilation not only by reducing leaf area for assimilation, but also by interfering with the electron transport in photosystem II. The loss of leaves by senescence and death during water stress also reduces assimilation area. Overall, drought affects yield by reducing both sink (the harvestable part) and the source (dry matter assimilation part) of the plant, depending on the timing and severity of stress with respect to plant phenology (Blum, 1996).

Expansion Growth

As already stated, expansion growth is especially sensitive to water stress (Jones *et. al.* 1989; Salisbury and Ross 1991). Cell enlargement occurs when cell walls are deformed plastically under the influence of turgor pressure and wall loosening factors. Water enters the cell down a water potential gradient, expanding the cell by raising turgor. At the same time solutes must enter to provide metabolites for cell wall synthesis and to maintain osmotic potential inside the cell necessary to drive enlargement. The biophysical basis of cell expansion can be described by the steady state growth equation, formulated by Lockhart in 1965. One form of the Lockhart

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equation is: $1/V.\ dV.dt = \varphi(P - Y)$ where V is the increase in cell volume: φ is cell wall extensibility; Y is the yield threshold turgor - defined as the minimum turgor pressure required to initiate cell expansion, and P is cell turgor (Dale, 1988). The Lockhart equation applies to single cells and is strictly unsuitable for multicellular systems. Cell wall properties change as cells mature and so does cell turgor and in an organ such as a leaf, overall expansion is much more complex than that which can be explained reliably by the Lockhart equation. Another flaw in the Lockhart equation is that it represents that rate of cell expansion as being dependent on turgor while it has been shown by Zhu and Boyer (1992) that even for a single cell of *Chara* the rate of growth did not depend on turgor. They showed that over a threshhold of turgor growth was highly dependent on energy metabolism.

Growth in size is the result of an irreversible enlargement of cells. Each step in growth represents a minute irreversible increase in cell volume which will be limited by the cell wall. Ray (1962) showed cell elongation in oat coleoptile to occur without the synthesis of any new cell wall material. As a consequence of this the wall became thinner. This would not occur normally during sustained growth. Sustained growth would require differentiation, proliferation of membranes and organelles, and increases on protein and cell wall material (Hsiao, 1973). Turgor provides physical force for cell expansion and a reduction in cell water potential of approximately -0.2 MPa usually reduces leaf growth (Hsiao, 1973). Studies showing immediate growth response to water deficits indicate that water deficits directly and physically reduce

growth and that this response would be too rapid to be mediated by metabolism. For example, Acevedo *et. al.* (1971) found that rewatering the soil of mildly stressed maize resulted in the resumption of rapid elongation of young leaves within seconds. Where reduced leaf water potential is evident, growth inhibition can be explained in terms of cell turgor. However, leaf expansion is also inhibited during drought stress in the absence of any measurable changes in shoot hydration (Davies and Zhang, 1991). In such circumstances cell expansion is thought to be controlled by some substance arising from drying roots. In an experiment using clonal apple trees grown with twin root system showed that droughting approximately half of the roots reduced leaf area development by approximately 50%. Rewatering the roots restored rates of leaf expansion and initiation rates close to those of the well-watered plants. More intriguingly, removing the droughted roots from the plants also gave the same effect, suggesting that water supply could not have been limiting leaf expansion and initiation in these plants (Gowing *et. al.*, 1990). Instead a positive message that is chemical in nature rather than hydraulic appears to be generated by the drying roots that is responsible for slowing leaf expansion.

Slow drying of roots over 21 days can give rise to smaller, thicker, greener leaves which leads to a reduction in leaf fresh weight in unwatered plants, although no effect on shoot dryweight may be found (Zhang and Davies, 1989b). Drying part of the root system stimulates root extension in the hydrated parts (Zhang and Davies, 1989b; Jones *et. al.* 1989; Davies and Zhang, 1991; Sharp and Davies, 1979). As the

roots elongate they are capable of obtaining water from deeper into the soil, while inhibition of shoot growth together with stomatal closure restricts transpiration, thus conserving water.

The combined effects of reduced shoot growth accompanied by increased root growth, changes root to shoot ratios during droughting. Jackson (1993) suggested that roots and shoots occupy different environments and utilize their own respective environments so that each may control the size of the other by limiting the supply of one or more constituents to the dependent part, exerting a negative control. Root-shoot interactions may have important implications for crop improvement since the extent to which roots can modulate shoot behaviour is essential for identifying the most efficient level in the hierarchy of regulation at which to direct efforts toward selecting stress tolerant genes from the progeny of conventional breeding or direct transformation programmes (Meinzer *et. al.*, 1991).

Water Relations

Mechanism of stomatal action

Stomata play a regulatory role in shoot water relations and transpiration.While providing the means by which plants obtain carbon dioxide for photosynthesis they also control water loss from plants. The stomatal complex consists of two guard cells and a variable number of subsidiary cells (sometimes called neighbouring cells). The kidney-shaped or elliptical guard cells of dicots have a radial orientation of the cellulose microfibrils in the cell walls so that on inflation they change shape and

stretch in the direction of their longitudinal, curved axes. Since the micellae in the walls fan out radially from the pore to the periphery of the stomatal apparatus, the lengthening of the guard cells results in an increase in curvature. As the guard cells inflate, they push one another apart which results in the middle portions of the cells being displayed to form the pore (Raschke, 1975; Palevitz, 1981). Stomatal frequency (or density) of a stomata-bearing epidermis varies from 20 - 2000 pores per mm^2, majority being between 40 - 350. Pore diameter at the widest point varies from 6 - 20 μm (Weyers and Meidner, 1990).

The driving force for stomatal opening is increased turgor pressure of the guard cells (Willmer, 1983). Changes in guard cell turgor and that of neighbouring cells give rise to stomatal movements due to alterations in water potentials of either or both of these groups of cells, or their solute potential. Since neighbouring cell volume is ten times the volume of guard cells, stomatal aperture is more influenced by changes in guard cell solute level than those in the neighbouring cells (Weyers an Meidner 1990). Changes in guard cell solute content occur as a result of the metabolism of organic compounds within the guard cells and, import and export of osmotically active inorganic substances from the surrounding cells. Potassium ions are the major species of cations that move into the guard cell. Potassium accumulation in the guard cells mostly result in stomatal opening. Proton (H^+) efflux precedes stomatal opening in epidermal strips of *Commelina communis* (Edwards *et. al.*, 1988). Membrane potential difference inside guard cell plasma membrane is negative (as in most plant

cells). Membrane hyperpolirization in light and depolarization in the dark suggests the presence of light-dependent electrogenic proton efflux pumps in guard cells (Zeiger, 1983; Assmann *et. al.*, 1985). Zeiger (1983) proposed the chemiosmotic mechanism which links H^+ efflux/ K^+ influx pumps on guard cell. The chemiosmotic theory models the metabolic and electrical events associated with ion transport, depending on a primary solute gradient across a semi-permeable membrane (Poole, 1978). According to Zeiger, guard cell properties point to a chemiosmotic mechanism that is dependent on a primary proton gradient as a driving force for all the ion fluxes associated with stomatal movement. An active H^+ pump, moving H^+ out of the guard cells would generate electrical and concentration (pH) gradients across the plasmalemma. As a result K^+ would move down the electrochemical gradient into the guard cells passively. However, objections to the possibility that total uptake of K^+ is passive were raised by Clint and Blatt (1989) who claimed that uptake may be against a potassium electrochemical equilibrium which would imply active movement across the plasmalemma. The stoichiometry of K^+ and H^+ is not 1:1, and so electro-neutrality is maintained in the guard cells by the production of organic ions, especially malic acid, and to a small extent the import and export of chloride. All species utilize Cl^- as a counterion for K^+ but species having starch in guard cell chloroplast synthesise organic ions, mainly malate as a counterion for K^+. The relative proportions of malate and Cl^- vary widely with species and experimental conditions. Besides malate, citrate, possibly glutamate and aspartate can also serve as counterions for K^+ (Allayway, 1981). Protons inside guard cells arise from water. Reduction of

oxaloacetate to malate in the guard cells generates $2H^+$ which could also be a proton source, although there is no direct evidence for this (Zeiger, 1983).

MacRobbbie (1983) suggested that stomatal closure in the dark was due to a stimulation of ion efflux instead of inhibition of influx. The work of Edwards *et. al.* (1988) also supports a similar idea when they claim that the processes involved in stomatal closure are different from those involved in bringing about stomatal opening. Calcuim ions are though to be involved in inhibiting stomatal opening (Raschke, 1979; Mansfield *et. al.*, 1990), and their possible role is discussed later in this Chapter.

Stomatal closure

Plants usually respond to soil drying by closing their stomata, thus slowing transpiration. Stomatal closure provides the principal resistance to water loss from plants and the only site of resistance that is under short-term regulation. The ability of stomata to close in response to water deficit can play a significant role in the survival of plants under environmental conditions characterized by low water uptake and high water loss (Schutle and Hinckley, 1987). Stomata play a key role in the regulation of gas exchange and water economy in higher plants (Hsiao, 1973; Cornish and Zeevart, 1986; Spence *et. al.*, 1986). According to Tardieu and Davies (1993), the major function of stomata is to prevent leaf or shoot water potential falling below the threshold at which xylem cavitation occurs. In the absence of stomatal regulation, cavitation would lead to embolism and the loss of functional xylem vessels, thereby breaking the hydraulic connection between root and shoot. Under such circumstances

leaf water potential would be lowered even further leading to final deleterious tissue dehydration. The fact that partial leaf dehydration increases ABA concentrations in the leaves in association with stomatal closure has been well documented. These linked effects are seen as essential components in the regulatory system that guards the leaves against rapid and severe desiccation. ABA may be synthesised in the leaves as leaf water deficits develop (Hiron and Wright, 1973; Hornberg and Weiler, 1984; Behl and Hartung, 1986; Cornish and Zeevart, 1986), redistributed in the leaf from symplast to apoplast as the pH changes during water stress (Dörffling *et. al.*, 1980; Hartung, *et. al.*, 1981; Daeter and Hartung, 1995), or originate in the roots experiencing drought and move up to the shoot dissolved in the transpiration stream (Zhang and Davies, 1987; Davies, *et. al.*, 1990; Gowing, *et. al.*, 1990).

Osmotic adjustment

Cellular water deficit can enhance the concentration of solutes, change cell volume and membrane shape, disrupt water potential gradients, decrease turgor, disrupt membrane integrity, and denature proteins (Bray, 1997). To minimise cellular water deficits, plants can carry out osmotic adjustments whereby the osmotic potential of the cell is lowered in order to maintain a gradient in water potential which will favour water uptake and retention of turgor. Turner and Jones (1980) distinguished between the terms osmoregulation and osmotic adjustment. While osmoregulation (or turgor regulation) involves the regulation of osmotic potential within the cell by addition or removal of solutes from the cell until intracellular osmotic potential is approximately equal to the potential of the medium surrounding the cell, osmotic adjustment refers

to the lowering of cell osmotic potential by active accumulation of solutes in higher plants in response to water deficits. Osmotic adjustment can occur either through uptake of solutes, breakdown of larger molecules into osmotically active solutes or the synthesis of new molecules. Within a plant, factors such as reduced osmotic potential, the capacity to actively accumulate solutes, increased elasticity of cell walls and small cell sizes help maintain turgor (Hale and Orcutt, 1987).

Osmotic adjustment in plants is influenced by the rate at which stress develops. A water potential decrease in cells of -0.1 to -0.5 MPa allows for osmotic adjustment while reductions in water potentials of -1.0 to -1.2 MPa per day is too rapid for osmotic adjustment to compensate (Hale and Orcutt, 1987). Also, early in the stress cycle full turgor may be maintained, but continuation of stress lowers the capacity of cells to adjust fully. Plant age, differences in cultivars, organs and tissues, and environmental conditions also contribute towards a plant's ability to osmotically adjust to water deficit stress (Hale and Orcutt, 1987). Osmotic adjustment takes place in leaves, hypocotyls, roots and reproductive tissues of many species resulting in full or partial turgor maintenance under conditions of water deficit. Plants produce a few species of low-molecular weight solutes as osmolytes for reducing osmotic potential in the cells. These are known as compatible solutes because even in high concentrations they are not deleterious to cellular functioning. Quaternary ammonium and tertiary sulfonium compounds can act as compatible osmolytes or osmoprotectors in cytoplasm of higher plants (Rhodes and Hanson, 1993). These include compounds

such as glycine betaine, choline-O-sulfate, β-alanine betaine, proline betaine, *trans*-4-hydroxyproline betaine and dimethylsulfoniopropionate (Hanson, 1993). It appears that of the osmoprotectants, glycine betaine appears to be the most common compound accumulated in plants which are adapted to dry or saline soils (Hanson and Hitz, 1982). Other osmolytes, such as sucrose, and lyoles such as mannitol, sorbitol, and pinitol are also synthesized in plants exposed to water deficit stress (Jones *et. al.*, 1989). Carbon compounds for osmotic adjustments in leaves can be derived from photosynthesis, stored carbon (starch) or amino acid (storage protein) reserves (Mullet and Whitsitt, 1996). Accumulation of osmolites within the cell occurs in the cytoplasm although in higher plants vacoules can occupy up to eighty percent of the cell volume. This is because the water-requiring macromolecules and metabolic processes of the cells are located in the cytoplasm. Water in the cytoplasm would keep the macromelecules hydrated and the metabolic process functioning, thus keeping the cells alive (Borowitzka, 1981).

Osmotic adjustment in growing cells has been found to be essential for continued growth under conditions of restricted water availability. Creelman *et. al.* (1990) showed osmotic adjustment to occur in the growing zones of the stem of soybean seedlings. Sugar accumulation in response to mild drought in these cells was accompanied by turgor maintenance and sustained growth, while in the non-elongating zone there was no accumulation of sugars in the cells which also had lower turgor relative to those in the growing zone. Osmotic adjustment in the growth zone

of primary roots of maize has also been found to occur at low water potentials (Sharp *et. al.*, 1993). Accumulation of proline in the cells of these roots at low water potentials accompanies their growth. These workers claim that this finding suggests a role for increased rates of solute deposition in osmotic adjustment of growing regions in higher plants at low water potentials.

The accumulation of non-toxic (compatible) compounds in cells as a means of maintaining turgor for cellular growth and function is an important tool for maintaining tolerance against water deficit stress. Several water deficit inducible genes are known to encode enzymes in pathways that lead to compatible solutes, for example, genes encoding enzymes for betaine biosynthesis are activated by water deficit (Mullet and Whitsitt, 1996). The biosynthetic pathways of such compounds are the potential targets for genetic engineering for osmotic stress tolerance in crop plants.

Cell water potential

Early work on water relations included measurements of transpiration rates (Knight, 1917; 1922, cited in Vaadia *et. al.*, 1961) and rates of water uptake by roots (Kramer, 1938, Prat, 1948, cited in Vaadia *et. al.*, 1961). More recent studies in water relations have addressed such problems as the measurement of changes in fresh weight relative to tissue water content, and the concomitant changes in stomatal aperture using more advanced techniques (Brown *et. al.*, 1976). Water relations is now studied at cellular, tissue, whole plant, and whole canopy levels of organisation. Studies of water

relations now involves the determination of water potential (ψ_w), solute potential (ψ_s), turgor potential (ψ_ρ), hydraulic conductivity (Lp), and bulk volumetric elastic modulus (ε). Techniques such as the Shardakov dye method (Knipling, 1967; Knipling and Kramer, 1967), thermocouple psychrometry (Boyer, 1969) and the pressure volume analysis (Kim and Lee-Stadleman, 1984), and more recently the use of pressure probes of various designs (Steudle, 1993), have been used to determine the water relations of individual cells and whole root or shoot systems.

Definition of water potential

Water potential is a measure of the free energy status of water (Kramer, 1983). The chemical potential is the free energy per mole of any substance in a chemical system (Devlin and Witham, 1983). In plant water relations, the chemical potential is referred to as the water potential. Water potential, (ψ_w), of pure water is taken as zero. Under isothermal conditions, the factors involved in cell water relations at equilibrium can be summarised as: $\psi_w = \psi_s + \psi_\rho + \psi_m$. Solute potential (ψ_s) expresses the effect of solutes in the cell cytoplasm and vacuole. As solute concentrations increase in the cell cytoplasm, the free energy of water decreases (becomes more negative). Matric potential (ψ_m) expresses the effect of water binding to colloids and surfaces, and the capillary effects of cells and cell walls. Generally, matric potential is insignificant inside a fully hydrated cell, and therefore usually ignored in the water potential equation. However, as cells become dehydrated matric potential becomes more apparent. Turgor potential (ψ_ρ) arises from hydrostatic forces exerted by the cell wall against water driven into the cell osmotically. It is usually positive, except in the

xylem of a rapidly transpiring plant. There is evidence of negative turgor in severely droughted cells which can often lead to plasmolysis and the collapse of cell wall (cytorrhysis) (Oertli, 1990).

Leaf water potential is the most commonly used indicator of shoot water status. As plants experience water loss, leaf water potential becomes more negative. However, in moderately droughted plants leaf water potential can remain unchanged (Bates and Hall, 1981; Blackman and Davies, 1985; Zhang and Davies, 1989 a, b) or even be increased (Davies and Zhang, 1991). In such cases, leaf water potential by itself does not provide a good indication of imposed stress or of the absence of a response to the stress. Nevertheless, this does show that plant responses occurring due to mild water stress can mitigate the normal dehydrating influence soil water shortage effects (Blackman and Davies, 1985; Passioura, 1988a; Jackson, 1993). This is brought about by a variety of means, principally osmotic adjustment, slower leaf expansion, stomatal closure and earlier abscission.

Root hydraulic conductivity (Lp)

Roots perform many functions for higher plants including anchorage, absorbing water and nutrients, and producing signals that integrate the plant's response to the soil environment. Roots offer large radial and axial resistance to water flow. The permeability of roots per unit surface area varies between species, between the different parts of the same plant, and with age (Newman, 1976). The permeability of a root cell to water is responsive to a wide range of environmental factors including

temperature, water, oxygen, and salinity (Newman, 1976). Plant growth regulators and inorganic ions also regulate root Lp. Anderson *et. al.* (1989) found that phosphorus deficiency reduced root Lp in *Fraxinus pennsylvanics.* In a detailed study of the effect of phosphorus deprivation on root Lp, Wahab (1995) showed that within 1 day of withdrawing phosphorous from the external solution bathing the root of *Macroptilium atropurpureum,* root Lp was reduced in the absence of any changes in leaf water potential. Recovery of Lp was detected within 2 hours of restoring phosphorous supply. Reduced Lp was also shown by Chapin *et. al.* (1988) and Chapin (1990) when the roots of tomato and barley plants were deprived of nitrogen.

Roots experiencing drying synthesize increased amounts of ABA. The influence of ABA on ion uptake into, and water and ion flow through roots, has been studied but results have been contradictory. An increase in root Lp in response to exogenous ABA was reported by Glinka (1973, 1977), Glinka and Reinhold (1972), Davies *et. al.* (1982), Eamus (1984) in a range of monocotyledonous and dicotyledonous species. However, Markhart *et. al.* (1979) and Fiscus (1981) showed that ABA decreased hydraulic conductivity in soybean or *Phaseolus vulgaris* roots, while Eamus and Narayan (1990) reported a similar effect in *Abelmoschus esculentus* L. roots. Erlandsson *et. al.* (1978) found exogenous ABA to have no effect on sunflower root hydraulic conductivity.

Although a considerable amount of work has been done on looking at the effect of exogenous ABA on root Lp, in contrast, only a few studies of the influence of

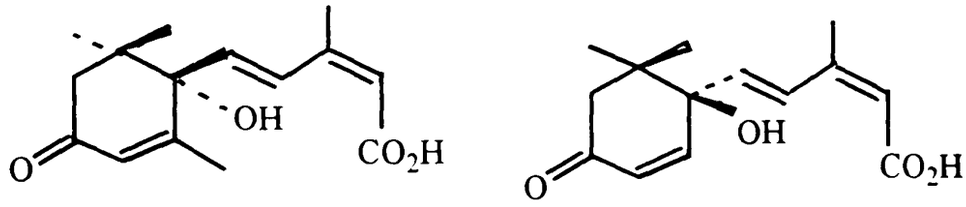
drought on root Lp have been made (Gregory and Brown, 1989). Inferences concerning soil and root conductance and the relative importance of each as a function of drying soils have been made in the past, but rarely have direct measurements of root Lp been made (Boyer, 1985). In one study by Blizzard and Boyer (1980) where rates of water flux and water potentials were measured directly, root conductance decreased as soil conductance decreased. In another study by Eamus and Narayan (1990), droughting the roots of *Abelmoschus esculentus* also reduced Lp, a response that appears to exacerbate the damaging effect of soil water shortage on shoot hydration levels.

Plant Growth Substances

Abscisic acid

During the 1950's, in several laboratories around the world, research was being carried out on growth-inhibiting substances in relation to abscission, dormancy and germination but identification was difficult because of the minute amounts of this substance present in plants. Carns and Addicott, in collaboration with Ohkuma, were the first to isolate and publish the structure of what it was then called abscisin II in 1965 (Addicott and Lyon, 1969). ABA is the trivial name for 3-methyl-5-(1'-hydroxy-4'oxo-2',6',6'-trimethyl-2-cyclohexen-1'-yl)-*cis*,*trans*-2-4-pentadienoic acid. The naturally occurring enantiomer of ABA, (S) *cis*-ABA, is the potent regulator of stomatal apertures amongst the known hormones. On exposure to sunlight the -S form is converted to the less active -R form and in equilibrium the mixture would have equal amounts of the two enantiomers. ABA has a molecular weight of 264, is soluble

in many organic solvents and has a melting point at 160 to 161 °C. The structure of ABA is shown below. Only (S) *cis*-ABA occurs naturally in plants.



(S) *cis*-ABA (R)- *cis*-ABA

Naturally occurring S- ABA and its R- enantiomer

ABA Biosynthesis

ABA is a sesquiterpene. Some details of the pathway of its biosynthesis remain obscure but it is thought that ABA is derived from a xanthophyll 9'-*cis*-neoxanthin which is itself derived from all-*trans*-violaxanthin. 9'-cis-neoxanthin is cleaved to form one C-15 compound (xanthoxin) which is then converted to ABA *via* ABA aldehyde. Localization of enzymes in the pathway are unknown although it appears that the conversion of xanthoxin to ABA occurs in the cytoplasm (Walton and Li, 1995).

Work using ABA-deficient mutants (*Flacca* and *Sitiens*) have shown that lesion occurs in the enzyme which oxidises ABA aldehyde to ABA so that in these mutants this conversion does not occur. Instead, ABA aldehyde is converted to ABA alcohol which accumulates. Sindhu *et. al.*, (1990) found that enzyme catalysing synthesis of ABA aldehyde from xanthoxin (xanthoxin oxidase) was present in both the wild type and mutant plants while the mutant plants did not have the enzyme ABA aldehyde

oxidase, which catalyses the conversion of ABA aldehyde to ABA. There is indirect evidence to suggest that ABA synthesis in leaves is controlled by the activity of 9'-*cis*-neoxanthin cleavage enzyme but whether this step is rate-limiting has not been demonstrated (Walton and Li, 1995). Using etiolated bean seedlings Li and Walton (1990) showed that the loss of xanthophylls violaxanthin, 9'*cis*-neoxanthin and 9-*cis*-violaxanthin, and increase in ABA and its metabolites, were related with a 1:1 stoicheometry, suggesting these xanthophylls to be the source of ABA. A similar pathway of ABA biosynthesis has also been shown to occur in roots (Parry and Horgan, 1992).

ABA metabolism

The first compounds described as ABA metabolites were ABA-glucose ester (ABA-GE), phaseic acid (PA), and 6'-hydroxymethyl ABA (HM-ABA) (Milborrow 1984). HM-ABA easily rearranges to PA. PA is reduced to dihydroxyphaseic acid (DPA) and both PA and DPA are found to occur in large concentrations in plants (Walton and Li, 1995). Activity of ABA depends on the presence of a free carboxyl group, a 2-*cis*, 4-*trans* pentadienoic side chain, a 4'-ketone and a double bond in the cyclohexane ring. The metabolites lack one or more of these functional groups and therefore lack the activity. ABA-GE lacks the free carboxyl group, PA lacks the ring double bond, and DPA lacks both the ring double bond and the 4'-ketone group. While PA has greatly reduced activity, DPA has no activity. ABA metabolism occurs in roots, stems, leaves, seeds and fruits, and metabolites are found in xylem and phloem sap. Cornish and Zeevaart (1984) showed that both ABA synthesis and

metabolism occurred more in mature leaves than in young leaves. It is possible that in leaves, one way of removing ABA from the active ABA pool is to compartmentalize the metabolites. For example, it has been shown that in mesophyll cells, ABA-GE is sequestered in vacoules while the active ABA remains in the cytoplasm (Bray and Zeevaart, 1985). Since little is known about ABA metabolic enzymes, knowledge of the regulation of metabolism is also limited (Walton and Li, 1995).

Functions of ABA

While ABA is generally thought of as a growth inhibitor, it can act as a promoter in storage protein synthesis in seeds and root extension at low concentrations (Davies, 1995). More ABA is present in young, growing tissues such as leaves than in older organs although its role in such tissues is unclear (Milborrow, 1984). The functions of ABA include the promotion of stomatal closure and inhibition of shoot elongation. ABA also has a role in maintaining seed dormancy; it counteracts the effects of gibberellic acid on amylase synthesis in germinating cereal grains. It also increases the response to wounding, inducing gene transcription for proteinase inhibitors, and may be involved in defence against insect attack (Davies, 1995).

Water deficiency and ABA

Elevated levels of ABA in dehydrated plant tissue were first detected by Wright and Hiron (1969). Increases in ABA are commonly seen in plants subjected to environmental stress. ABA synthesis has been shown to occur in dehydrating tissues

such as leaves when the bulk leaf turgor reaches zero (Pierce and Raschke, 1980). Stomatal closure occurs as leaves start to become dehydrated while ABA synthesis in detached leaves occurs after leaf turgor reaches zero. Dörffling *et. al.* (1980) showed that there was a 30-60 minute delay between stomatal closure and the synthesis of ABA in dehydrating leaves while stomata began closing as early as 5-15 minutes after drying. This shows that in detached leaves stomatal closure precedes ABA increase and could be attributed to stomatal guard cells being hydroactive, thus responding to turgor loss. In intact leaves these workers found a good relationship between increase in ABA in leaves and stomatal closure. ABA is believed to act initially on the outer surface of the guard cell plasmalemma (Hartung, 1983) which indicates the prime importance of apoplastic ABA in stomatal regulation. However, in a recent study by Allan *et. al.* (1994), when photoactive, caged ABA was photo-released inside the cytoplasm of guard cells of *Commelina*, stomatal closure was brought about just as efficiently suggesting that ABA can act on the inner surface of guard cell membranes as well as the outer surface of the plasma membrane.

It has been found by many authors that drying roots close stomata and reduce leaf expansion in the absence of any changes in leaf hydration implying that ABA from the drying roots is responsible for the responses (Blackman and Davies, 1985; Gowing *et. al.*, 1990; Khalil and Grace, 1993). The fact that drying roots synthesize ABA is well established (Walton *et. al.*, 1976; Lachno 1983; Zhang and Davies, 1987; Zhang and Davies, 1989b). It is possible that ABA from drying roots may be

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responsible for many of the physiological responses brought about by water deficiency. In addition, ABA induces regulation of the expression of genes under drought stress (Bartels *et. al.*, 1996). The importance of this in stress adaptation is unclear and is discussed in a later section.

ABA action on stomatal guard cells

Stomatal closure and leaf growth are most commonly experienced by plants subjected to environmental stress, in particular drought stress. There is extensive literature showing that as soil dries ABA levels in plants increase. ABA at stomatal guard cells influences the permeability of the plasma membrane to, and active transport of, K^+ and possibly hydraulic conductivity in complex ways (Mansfield, 1987). ABA action on the stomatal guard cell appears to be either on plasmalemma or a location easily accessible from outside (Hartung, 1983). Although the recent work of Allan *et. al.* (1994) showed the possibility that ABA action on the guard cells could also be intracellular.

Some research shows that guard cells have the ability to synthesize ABA but there is strong evidence that much ABA is synthesised in the cytoplasm of mesophyll cells. The protonated form (ABAH) easily penetrates the chloroplast membrane and moves into the stroma. Since ABA is a weak acid (pKa = 4.5) and the pH of the stroma is 7.5 it dissociates easily in this compartment; the anion being trapped here. When leaves lose water, and water potential of the mesophyll membrane falls sufficiently to impair the function of the chloroplast, ABA is released into the cytoplasm as the

stroma acidifies. This ABA is then free to move out into the cytoplasm and down concentration gradients to the transpiration stream, and thus on to the apoplast of stomatal guard cells.

The role of Ca^{2+} and IP_3 as second messengers in ABA signal transduction

The mechanisms by which plant cells detect environmental changes and transduce them into a physiological response is known as signal transduction. An external stimulus (primary signal or messenger) is perceived by a cell at the plasma membrane which causes the synthesis or mobilization of an intracellular 'second messenger'. The interaction of second messengers with components of the transduction pathway (e.g., enzymes, proteins, etc.) initiates a physiological response (McAinsh *et. al.*, 1991). Signal transduction has been studied extensively in animal cells. However, it is now becoming apparent that similar transduction mechanisms occur in plant cells. Calcium ions are important second messengers involved in signal transduction pathway in both animal and plant cells. In animal cells perception of some hormonal signals at the plasma membrane have been found to activate a phospholipase C that releases at least two second messenger molecules, inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) by the hydrolysis of a membrane-bound inositol lipid, phosphatidylinositol-4,5-bisphosphate (PIP_2). IP_3 can diffuse through the cytoplasm and cause Ca^{2+} efflux from endoplasmic reticulum or calcisome (Berridge and Irvine, 1989). In plants, the photoactivation of caged IP_3 introduced in guard cell cytosol causes Ca^{2+} release from internal stores (Gilroy *et. al.*, 1992). Stimulation of Ca^{2+} from microsomal and vacuolar preparations by IP_3 has also been reported

(Schumaker and Sze, 1987).

There have been several reports that stomatal closure induced by ABA is associated with increased cytoplasmic calcium (De Silva *et. al.*, 1985; Irving *et. al.*, 1992; McAinsh *et al.*, 1992). According to the Ca^{2+}-ABA second messenger model described by McAinsh *et. al.* (1990) ABA interacts with the plasma membrane of guard cells to cause elevation of Ca^{2+} in the cytoplasm. ABA interaction with the plasma membrane requires some from of external receptor on the plasma membrane. One possibility is the presence of a high-affinity proteinaceous ABA-binding site located on the plasma membrane (Hornberg and Weiler, 1984). Alternatively, ABA may interact with patches of membrane phosphlipids (Stillwell *et. al.*, 1989). An increase in cytosolic Ca^{2+} may result from either activation of inwardly directed Ca^{2+} channels located on the plasma membrane allowing influx, and/or the release of Ca^{2+} from intracellular stores, possibly through interaction with the phosphoinositide mechanism. However, there is no evidence of ABA-induced increase in IP_3 levels in guard cell cytoplasm.

Ca^{2+} must not only inhibit stomatal opening, but also induce closure. Inhibition of the inwardly directed K^+ channels present on the plasma membrane would inhibit stomatal opening (Schroeder and Hagiwara, 1989) while promotion of stomatal closure is brought about by the activation of plasma membrane anion efflux channels. McAinsh *et. al.* (1991) suggest a pre-depolarization of the plasma membrane occurs that is mediated by Ca^{2+}, which in turn activates the anion efflux channels leading to

the depolarization of the plasma membrane which activates the outwardly directed K^+ channels.

In an unstimulated cell, cytosolic Ca^{2+} concentrations range from 20 - 300 nM while ABA-stimulated Ca^{2+} concentration in the cytosol of guard cells can be in the micromolar range (Schroeder and Hagiwara, 1989; McAinsh *et. al.*, 1990). It is unclear whether ABA causes an influx of Ca^{2+} or releases it from intracellular stores, or both. Extracellular Ca^{2+} has been shown to be essential for ABA-induced stomatal closure in *Commelina communis* by DeSilva *et. al.* (1985). Since there are no plasmodesmatal links between guard cells and neighbouring cells, apoplastic Ca^{2+} would be the source for extracellular Ca^{2+}. Using EGTA, a chelator of Ca^{2+}, to remove apoplastic Ca^{2+} the effect of ABA on guard cells was eliminated. There is the possibility that EGTA can bind to the cell-wall associated Ca^{2+} and alter the mechanical properties of guard cell walls (De Silva *et. al.*, 1985). However, in a study by Schwartz *et. al.* (1988) stomatal opening was actually stimulated by EGTA, indicating that Ca^{2+} chelation with EGTA did not adversely affect membrane integrity or uncontrolled ion leakage. These studies underline the requirement of extracellular Ca^{2+} for elevated levels of cytosolic Ca^{2+} induced by ABA, thus indicating increased influx of Ca^{2+} as a contributing factor in the elevation of cytosolic Ca^{2+} levels.

MacRobbie (1989, 1992) used direct measurement of $^{45}Ca^{2+}$ fluxes into guard cells to show that ABA had variable effects on Ca^{2+} fluxes ranging from stimulation

to inhibition. Micro-injection of caged Ca^{2+} into guard cell cytoplasm and the subsequent photo-release of this Ca^{2+} into the cytosol induces stomatal closure (Gilroy *et. al.*, 1990). Removing extracellular Ca^{2+} by chelation with EGTA, thereby preventing Ca^{2+} influx into the cytosol, still allowed ABA to raise Ca^{2+} levels in the cytosol and cause subsequent stomatal closure (Gilroy, *et. al.*, 1991). Therefore, it appears that increases in cytosolic Ca^{2+} levels in response to ABA can originate from both extracellular and intracellular sources.

ABA-induced stomatal closure does not always accompany increased cytosolic Ca^{2+} concentrations. Some guard cells fail to show any evidence of increased cytosolic Ca^{2+} levels when treated with ABA, although stomatal closure is always observed (Gilroy *et. al.*, 1991; McAinsh *et. al.*, 1992). Photo-release of caged ABA in guard cells of *Commelina communis* caused elevated levels of cytosolic Ca^{2+} in plants that were grown at temperatures of 25°C and above, but failed to show any elevation in cytosolic Ca^{2+} levels in guard cells from plants grown at 10 to 17°C (Allan, *et. al.*, 1994) although stomata still closed. They propose that a change in cytosolic Ca^{2+} concentrations is not the only route whereby ABA induces stomatal closure. Thus, two transduction routes for ABA action appear to exist in *Commelina communis*, depending on previous growth temperature; one is a Ca^{2+}-dependent route while the other is Ca^{2+}-independent. Similar suggestions have been made by Gilroy *et. al.* (1991). This raises the question of the involvement of Ca^{2+} in ABA action on guard cells in these instances. If there is no detectable increase in cytosolic Ca^{2+}

concentration, the chain of events induced by Ca^{2+} is unlikely to be occurring here to bring about stomatal closure (Allan *et. al.*, 1994).

ABA and Growth

In many species when plants experience water deficits shoot growth is more inhibited than root growth (Sharp and Davies, 1989). Leaf tissue treated with ABA shows reduced growth resulting from decreased cell wall extendibility (Van Volkenburg and Davies, 1987). However, in unstressed plants, the younger, growing leaves contain higher concentrations of ABA than the mature leaves (Osborne *et. al.*, 1972; Zeevaart, 1977). Tal *et. al.* (1979) showed that leaves of ABA mutants of tomato (*flacca*) developed normally, suggesting that ABA appears not to be involved in the growth regulation of these plants. However, it has been shown on many occasions that ABA arising from drying roots could be responsible for the inhibition of shoot expansion in plants where root drying did not reduce shoot hydration (Gowing *et. al.*, 1990; Passioura and Gardner, 1990; Blum *et. al.*, 1991). Munns (1990), on the other hand, failed to explain the inhibition of leaf expansion in droughted wheat and barley as an ABA response and implicated an alternative growth inhibitor in the xylem sap of intact plants growing in drying soil. Exogenously applied ABA has been shown to inhibit root elongation (Pilet and Chanson, 1981; Watts *et. al.*, 1981;Pilet and Saugy, 1987), promote it (Watts *et. al.*, 1981; Pilet and Saugy, 1987) or to have little effect (Creelman *et. al.*, 1990). The different effects of applied ABA on root growth may depend on the initial rate of growth (Pilet and Saugy, 1987) or the concentrations of ABA used in the different experiments (Pilet and Chanson, 1981). ABA concentration

of 10^{-6} M caused inhibition of root elongation while at a concentration of 10^{-8} M there was no significant effect of ABA on root elongation (Pilet and Chanson, 1981). It is quite possible that the mechanism of action and compartmentation of exogenous and endogenous ABA may be different.

The work of Sharp and co-workers has shown that increased concentrations of endogenous ABA promote root extension; promotion at low water potentials being inhibited by fluridone, which prevents ABA accumulation. This led Sharp and co-workers to suggest that ABA was involved in the regulation of growth in the growth zone of maize roots (Sharp *et, al.*, 1990a; 1990b; 1994). Two possible roles of ABA were suggested. Firstly, the accumulation of proline in these cells. Proline contributes to osmotic adjustment in cells in the growth zone. Inhibition of proline accumulation was observed in the presence of fluridone and this effect was reversed by externally applied ABA, suggesting a potential role of ABA here. Secondly, the enzyme, xyloglucan endotransglycosylase (XET), which is believed to be important in cell wall yielding, markedly increases in the growth zone at low water potentials. This increase was inhibited by the application of fluridone, suggesting that ABA may be influencing the accumulation of this enzyme (Sharp *et. al.*, 1993). Thus, endogenous ABA appears to be involved in sustaining rates of root elongation during water deficiency that is slower than well-watered roots but faster than would be the case in the absence of the influence of endogenous ABA.

Regulation of gene expression by ABA

Plant survival in most environments requires an ability to withstand extremes of osmotic stress caused by drought, salinity and temperature. Two major evolutionary mechanisms operate in plants to help their survival in such conditions. The first is stress avoidance which depends upon specialised adaptations of root and shoot architecture and phenology (Aspinal and Paleg, 1981). Some plants also exhibit long-term changes in photosynthetic chemistry to reduce water loss through evapotranspiration. The second adaptive mechanism is a more direct stress tolerance involving changes in cellular chemistry, which appears to accumulate compatible solutes and specific proteins (Skriver and Mundy, 1990). Water deficit is accompanied by the accumulation of ABA which in turn influences the pattern of gene expression (Bray, 1991). Genes that are naturally expressed at specific times of vegetative and reproductive growth can also be activated at other times in response to ABA or desiccation. Young seeds are desiccation intolerant and as they mature many structural, metabolic and genetic changes occur. Similarly, in other parts of the plant desiccation leads to a number of physiological, metabolic changes as well as gene expression (Bray, 1991). To understand the molecular mechanisms of gene expression underlying water deficits, desiccation-induced genes have to be isolated to study their functions and pathways that lead to the induction of such genes. Genes expressed during drought stress are believed to promote cellular tolerance to desiccation through protective functions in the nucleus, cytoplasm and membranes, although direct proof is lacking. They may also cause osmotic adjustment to induce

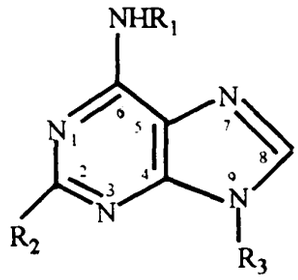
water uptake, control ion accumulation and further regulate gene expression (Jensen *et. al.*, 1996).

Many examples of ABA-regulated genes have been identified in droughted plants. These include the *rab* genes in maize (Pagè *et. al.*, 1993). *Rab* (responsive to ABA) genes have been isolated by differential screening of cDNA libraries synthesized from mRNAs of ABA-treated tissues (Skriver and Mundy, 1990). A set of new mRNAs have been isolated from detached pea leaves (Guerrero and Mullet, 1988). The expression of *Lea* (late embryogenesis abundant) genes is commonly seen in plants under osmotic stress (Dure, 1993). These *Lea* genes are thought to function in the protection of other proteins and membranes, renaturation of unfolded membrane, and sequestration of ions (Bray, 1991).

Little is known about the nature of signal transduction and putative second messages in ABA-induced gene expression. It is not known for certain if ABA regulated gene products promote drought resistance in plants. Nevertheless, genetic research has opened up the possibility of transferring suitable genes to economically important species in order to test their involvement in tolerance and possibly improve it (Jones *et. al.*, 1989). Up to now (Ceccarelli and Garando 1996) there is still a lack of success in breeding for stress conditions in general, and for drought stress in particular.

Cytokinins

The first naturally occurring cytokinin was purified in 1963 by Letham from immature kernels of *Zea mays* and identified as 6- (4-hydoxy-3-methylbut-*trans*-2-enylamino) purine, commonly known as zeatin (M^{c}Gaw, 1987). Most naturally occurring cytokinins are substituted purines with a 5-carbon branch at the N^6 position in the purine nucleus (R_1 in the skeletal structure).



Structural skeleton of most naturally occurring cytokinins

Most cytokinins are classified into three groups, zeatin, dihydozeatin, and isopentenyl adenine. It is widely believed that cytokinins are synthesized mostly in roots and move in the xylem to the shoot. However, they can also be produced in the shoot (Wang and Wareing, 1979) and there is little reason to believe that roots exert a monopolistic control over whole plant cytokinin levels. Functions of cytokinins include: cell division, morhogenesis in tissue culture, growth of lateral buds, leaf expansion resulting solely from cell enlargement, delay of leaf senescence, enhancement of stomatal opening in some species, and chloroplast development (Davies, 1995).

Certain *t*RNA species contain cytokinins and their turnover is a potential route to free cytokinin biosynthesis although it appears unlikely that this would be a major source of free cytokinins. It is also likely that certain enzymes exist in plants which are capable of direct synthesis of cytokinins (Horgan, 1984). The proposed pathway of cytokinin biosynthesis involves the reaction between 5'-AMP and i^2-isopentenylpyrophosphate (i^2-iPP) resulting in the synthesis of i^6A-5' monophosphate, which must be stereospecifically hydoxylated to form *trans*-zeatin derivatives (M^{c}Gaw, 1987).

Cytokinin metabolism involves two types of reactions. The first metabolic reaction is one in which the side chain of ^{6}N is retained but modified, and some biological activity is maintained. The second metabolism results in the irreversible loss of biological activity and cleavage of the side chain produces inactive products such as adenine (Horgan, 1984).

Water deficit and cytokinins

Early work of Itai and Vaadia (1965) showed that when leaf senescence is promoted by drought stress it is associated with a marked reduction in cytokinin activity in sap from detopped sunflower plants. Since roots are believed to be major sources of cytokinins, decreased cytokinin synthesis in roots is assumed to influence leaf functions. Itai and Vaadia (1971) induced water deficit in intact leaves by using a fan for 30 minutes and found decreased cytokinin concentrations in these leaves while the roots had not experienced any water deficit. In similar studies by Aharoni *et. al.*

(1977) and Ong (1978) changes in cytokinin levels in detached leaves were observed with water loss. These observations imply that changes in cytokinin levels in leaves can occur in the absence of roots.

There is little consistency in the reported effects of exogenous cytokinins on the stomatal complex. In many studies using excised leaves or epidermal strips exogenous cytokinin promotes stomatal opening (Livne and Vaadia, 1965; Blackman and Davies, 1983; Incoll and Jewer, 1986; Incoll and Jewer, 1987). However, in other studies externally applied cytokinins failed to influence stomatal aperture (Aharoni *et. al.*, 1977; Incoll and Jewer, 1986). In the study by Blackman and Davies (1983) high concentrations of exogenous cytokinin inhibited stomatal opening. The lack of response of stomata to exogenous cytokinin is possibly attributable to the presence of an optimal concentration of cytokinin in the tissues in these instances (Incoll and Jewer, 1987), while the inhibiting effect could have been due to supraoptimal concentrations of cytokinin present in the vicinity of the guard cells (Blackman and Davies, 1983).

The role of cytokinins as a possible root message has been suggested (Davies *et. al.*, 1986; Mansfield, 1987). Blackman and Davies (1985), using a split-root system and subjecting part of the root system to drought found that partial stomatal closure occurred although leaf water relations and ABA content remained unchanged. They also found that the effect of soil drying on stomatal closure could be reversed if leaf pieces were floated on solutions of kinetin or zeatin, and suggested that cytokinins

from the roots may be necessary to sustain maximum stomatal opening and that soil drying could cause an interruption in the supply of this which may cause stomata to close partially during drying of part of the root system. Davies *et. al.* (1986) proposed that drying roots reduce cytokinin synthesis and transport which may influence shoot physiology in the absence of changes in shoot hydration. Contrary to this, when Incoll *et. al.* (1990) imposed slow drought stress on roots of *Phaseolus vulgaris* they found that ABA, and not cytokinins was the likely root signal originating from the dehydrating roots which might cause stomatal closure in the absence of any changes in shoot turgor. Furthermore, an increase in cytokinin concentration in roots and decreased concentration in shoots in response to water deficit found by Hubick *et. al.* (1986) was interpreted as the result of either an inhibition of synthesis by shoots or an inhibition of transport by roots. Using one drought susceptible and two drought resistant species of tomato plants, Pillay and Beyl (1990) showed that osmotic stress applied to roots caused the largest reduction in cytokinin concentration in roots of the most resistant species (*Solanum pennellii*) while roots of the susceptible species failed to show any changes in cytokinin concentration. This finding is conflicting to the idea that drying roots synthesize less cytokinin, and further questions the involvement of cytokinin as a root signal. Moreover, in studies where cytokinin in xylem sap was analysed, concentrations of this hormone were measured. Since transpiration is slower in plants experiencing drought stress compared to the well-watered controls, concentrations of cytokinins in the transpirational fluid are likely to be overestimates of the actual amounts of cytokinins arising from the roots due to lack of dilution.

Hence, deliveries of cytokinins out of the roots are possibly more reduced than those observed as concentrations.

Ethylene

Ethylene was first recognised as having growth-regulating properties in 1901 by a Russian botanist, Neljubow. However, it was not until 1934 that there was positive proof that ethylene was a natural plant product (Beyer *et. al.*, 1984). Ethylene is a gaseous plant hormone and is produced by most plants, although it was recently reported by Summers *et. al.* (1996) that *Potamegeton pectinatus*, an aquatic weed does not synthesize this hormone. Effects of ethylene are many including abscission of leaf, flower and fruit, fruit ripening, accelerated underwater extension by many aquatic and semi-aquatic plants, aerenchyma development in poorly aerated plants, root hair formation, lateral cell expansion and nodal root outgrowth (Jackson, 1993). Since ethylene is a gas, it moves within the plant by diffusion. However, its immediate precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is transported in the xylem and this probably accounts for some long distance effects of root stress.

Ethylene is synthesised from methionine in all higher plants, where it has been studied (Beyer *et. al.*, 1984). Methionine is converted to *S*-adenosylmethionine (SAM) in an ATP-requiring step. In the next step of ethylene biosynthesis SAM is converted to 5'-methylthioadenesine (MTA) and 1-aminocyclopropane-1-carboxylic acid (ACC). This is the principal rate-limiting step and the reaction is catalysed by ACC synthase. MTA formed here is then hydrolysed to methylthioribose (MTR) and

the CH_3S group of MTR is recycled back into methionine in what is known as the Methionine Cycle. ACC produced from SAM is oxidized (i.e., in an oxygen-requiring step) to ethylene gas, the reaction catalysed by ACC oxidase (or ethylene-forming enzyme) (Beyer *et. al.*, 1984), which can also limit the rate of ethylene biosynthesis in some circumstances (English *et. al.*, 1995; Summers *et. al.*, 1996). Metabolism of ethylene leads to the formation of carbon dioxide and ethylene oxide (Beyer et. al., 1984). Soluble metabolites such as glycols and its glucose conjugates are also formed (Bolmstrom and Beyer, 1980).

Water deficiency and ethylene

The effects of flooding on ethylene-mediated responses in plants was first suggested in 1944 by Turkova. However, Kawase (1972) was the first to measure increased concentration of ethylene extracted from shoot tissues above the water line. Extensive work on ethylene action during flooding has been carried out since then. In contrast to the work on flooding, seemingly less work has been done on investigating the effects of drought stress on ethylene production. Many of the physiological responses brought about by water deficit such as increased respiration, enhanced senescence and abscission of flowers, leaves and fruits and reduced growth rates, are responses usually brought about by ethylene (Wang *et. al.*, 1990; El-Betagy and Hall, 1974). Ethylene does not usually affect stomatal conductance (Pallaghy and Raschke, 1972; Aharoni, 1978).

Drought stress can increase ethylene accumulation or synthesis in plants (Apelbuam and Yang, 1981; Aharoni, 1978; McKeon *et. al.*, 1982; El-Betagy and Hall, 1974; Wang *et. al.*, 1990) although the report presented by Hubick *et. al.* (1986) is contrary to this. In excised leaves, water deficits increase ethylene production rapidly, within hours (Apelbuam and Yang, 1981; Aharoni, 1978; McKeon *et. al.*, 1982). However, the initial increase in ethylene at the beginning of water stress is followed by a fall soon after. There is a further increase in ethylene accumulation which is accompanied by senescence (El-Beltagy and Hall, 1974). Increased ethylene production in response to drought stress is a result of increased ACC synthesis and increased activity of ACC oxidase which catalyses the conversion of ACC to ethylene (Apelbuam and Yang, 1981). Ethylene production has been found to be influenced by treating leaves with other hormones. For example, indole-3-acetic acid (IAA) and benzyladenine (BA) increase ethylene production while ABA pre-treatment of the leaves can reduce it, possibly by modifying the levels of ACC, although the activity of ACC oxidase was not affected (McKeon *et. al.*, 1982). Aharoni (1978) however, reported that an increase in leaf ABA content, induced by water deficit stress, was not related to the internal concentration of ethylene.

Study by Morgan *et. al.* (1990) showed that intact plants subjected to water deficit stress did not promote ethylene production and suggested that excised leaves may respond differently to rapid drying compared to intact plants. There is, therefore, the need to regard the complications that injuring tissues can cause in ethylene synthesis.

Root-to-Shoot Communication

Introduction

The function of roots as an anchorage for plants, as well as for absorption of water and minerals for growth and development, is unquestionable (Salisbury and Ross, 1991). However, the role of roots as sensors of perturbation in the soil environment is also of interest to many plant physiologists who have attempted to demonstrate that roots perform this vital function and then influence shoot behaviour through some form of systemic signalling. The communication between roots and the aerial parts of the plant may help survival by allowing the shoot to sense adverse conditions in the soil early enough to induce physiological changes within the shoot which can minimize damage as the roots increasingly fail to provide the shoot with its requirements of water and minerals.

The main link between roots and shoots is through the conducting vessels (i.e., xylem and phloem). Messages borne in xylem and phloem vessels connect the different parts of the plant. There could be three types of messages generated by stressed roots that induce changes in shoot development. These are:

(i) **Positive Messages**: These would take the form of an increase in output of physiologically active substances from the stressed roots, which might influence shoots when transported there in xylem sap moving either in transpiration fluid during daytime or in pressure sap at night.

(ii) **Negative Messages**: Shoots may sense that roots are under stress because the output of water, minerals or organic compounds into the xylem sap is decreased.

(iii) **Accumulation messages**: These are conceived as substances made by leaves that accumulate at the sites of synthesis because stress has reduced their rate of export to sites of previously vigorous growth in root or shoot apices.

The notion of roots exerting a hormonal control over shoots was proposed by Went (1938) when he reported a factor arising from the roots of dark-grown pea plants which promoted stem elongation. In 1939, Chibnall suggested that some influence of roots, possibly hormonal, was responsible for the regulation of levels of proteins in leaves (Chibnall, 1954). Many experiments followed these initial findings which suggested control of shoot behaviour by hormones originating in the roots.

Effects of soil drying on plants

Extensive work on soil drying has been carried out in the past 3 to 4 decades, and its effects on the inhibition of leaf growth and the promotion of stomatal closure are well known. Subsequently, it was realised that the changes in plants with all roots in dry soil are severe and affect the whole plant. They are mostly due to the dehydration of plant parts, especially the shoots since they are exposed to the drier atmosphere above the ground. Nevertheless, during mild soil drying, plants can maintain shoot hydration while shoot characteristics alter.

Stomatal closure

Stomatal closure due to the loss of hydration of leaves as plants lose water, could be the consequence of at least three processes. Firstly, stomatal guard cells may be hydroactive and dehydration could cause a loss of cell turgidity leading to their closure (Mansfield, 1987). Secondly, stomatal closure could also be induced by increased amounts of ABA in or around the guard cells (Hartung and Baier, 1990). Thirdly, any slowing of the photosynthesis would increase internal CO_2 levels, leading to stomatal closure. Current thinking is that apoplastic ABA is more important than symplastic ABA in causing stomatal closure (Hartung, 1983). Leaf dehydration can inhibit plasmalemma ATPase activity, slowing proton efflux, thereby increasing the alkalinity of the apoplast. This would induce undissociated ABA which is membrane-permeable, to diffuse out of the storage compartment into the more alkaline apoplast, down a pH gradient. This would increase ABA levels in the apoplast thus increasing their action on the guard cells (Hartung, 1983). More ABA synthesis would follow due to a loss of leaf turgor (Wright and Hiron, 1967, Pierce and Raschke, 1980). There is some evidence that ABA may accumulate in discrete compartments within the cells. When leaf water potential or leaf turgor reaches a threshold, ABA is redistributed and this leads to a reduction in ABA concentration in the sites of synthesis, relieving product inhibition of the pathway (Beardsell and Cohen, 1975). However, evidence contrary to this was presented by Ackerson (1982) who claimed that stomatal closure in cotton leaves occurred before a rise in apoplastic ABA. When intact cotton plants were allowed to dry, stomata began closing as the

leaf water potential reached -1.2 MPa. These leaves began to accumulate ABA at -1.4 MPa but apoplastic ABA levels did not rise until leaf water potential reached -1.6 MPa. Since as little as 0.64 fmol ABA is enough to cause stomatal closure (Ackerson, 1982), the apoplast of well watered plants already contains enough ABA to induce stomatal closure. However, ABA may not act on guard cells unless it is released from the apoplast through the pH effect into the transpiration stream and travel to the evaporative surfaces where it affects stomatal apertures. Ackerson believes that this happens before ABA synthesis and/or release from the leaf cells occurs, which leads to an increase in apoplastic ABA. Using monosilicic acid as a tracer for water movement in the transpiration stream, Aston and Jones (1976) showed that the most intense evaporative sites were stomatal guard cells and subsidiary cells compared to the rest of the epidermal cells where accumulation of the tracer was found, making these possible sites for the accumulation of ABA moving in the transpiration stream. Another possible reason why apoplastic ABA may not be as important as currently believed is the fact that guard cells are able synthesise their own ABA, in which case it will be available to the guard cells considerably quicker than ABA from any other part of the leaf. Weiler *et. al.* (1982) argue that the suggested redistribution of ABA from the mesophyll cells and its transport to the guard cells, or release from chloroplasts into the cytoplasm of mesophyll cells then transport to the guard cells (Milborrow, 1980; Hartung *et. al.*, 1981) would need to occur within seconds to explain rapid stomatal closure. They found that under the same level of osmotic stress guard cell and mesophyll protoplasts synthesised almost the same amount of ABA

and suggested that guard cells respond to ABA from within the guard cells in the initial stages of osmotic stress. Furthermore, Allan *et. al.* (1994) have shown that symplastic ABA is just as efficient in causing stomatal closure as apoplastic ABA, confirming the ability of symplastic ABA in causing stomatal closure.

However, it has also been shown that during mild soil drying, or when a few roots are experiencing drying, plants can maintain shoot hydration while stomata close. In such circumstances increased ABA output from the drying roots is believed to be causing stomatal closure.

Leaf expansion

Enlargement of a leaf depends upon the expansion of cell walls in response to turgor pressure from within the cell. When, under turgor, a cell wall stretches irreversibly, cell volume increases (Dale, 1988). Where leaves experience water loss, growth is inhibited because of loss of turgor. Leaf expansion is regarded as the most sensitive to all physiological processes to drought, more so than stomatal closure (Mansfield, 1987; Jones *et. al.*, 1989; Salisbury and Ross, 1991). The response of leaf expansion to drought stress is probably too rapid to be metabolically controlled (Hsiao, 1973).

During drought stress, leaves accumulate ABA and so the role of ABA in the inhibition of leaf expansion has been sought. ABA does not appear to reduce turgor and therefore does not influence growth in this way. Stiffening of leaf tissue has been found with ABA treatment (Van Volkenburg and Davies, 1983). They suggested that,

although ABA had no effect on the ability of cells to acidify the leaf surface, response of cell walls to acidification was reduced by ABA, hence inhibiting cell wall extensibility although the cells might be turgid. The fact that higher levels of ABA are found in young, growing leaves, however, questions its role in inhibiting growth (Osborne *et. al.*, 1972; Zeevaart, 1977), although in the youngest leaves the inhibitory influences of high ABA may be more than counteracted by the presence of growth promoting hormones such as auxin.

Concentrations of ABA in the xylem sap of plants experiencing water deficits has been correlated with the inhibition of leaf expansion while leaf turgor remains unaltered (Saab *et. al.*, 1990; Zhang and Davies, 1990b). Munns (1990) showed, using leaf elongation bioassays, that there may well be as yet unidentified growth inhibitors in the xylem sap of wheat plants experiencing water deficits.

Root drying methods used in the study of root signals

To study chemical messages arising in roots during soil drying, laboratory experiments using specialized systems such as roots grown in long columns and split root systems have been used. In one of the earliest experiments using vertically divided roots of wheat grown in nutrient, Meyer and Gingrich (1964) showed that osmotic stress applied to half the root system influenced metabolism within the plant in the absence of any change in water content of the shoot. They used relative turgidities of the shoots as a measure of shoot hydration.

Early experiments by Jackson and Campbell (1975) and Jackson (1980) used roots of tomato plants which were divided vertically so that the xylem of each half of the root system was linked to that side of the shoot (certainly the lower leaves). The approach enabled them to distinguish the effect of waterlogging one half the root system on changes such as epinastic curvature brought about on that half of the shoot. The side that had its roots in well drained soil did not show epinasty, suggesting that whatever was causing the response was coming from the oxygen-deficient, waterlogged roots since the effect could not be minimised by simply removing roots. Results from horizontally divided root system of tomato plants led Jackson and Campbell (1979) to propose the involvement of positive and negative messages arising from stressed roots which bring about certain physiological responses in the plants independently of changes in leaf hydration. The presence of a second root system (adventitious roots induced to grow on the stem of these plants) enabled the supply of the substance required for continued stem elongation while the lower roots were waterlogged. While waterlogging all the roots inhibited stem elongation in these plants, the inhibitory effect of this was overcome by the presence of the second unstressed root system. With this set-up they were able to show that other shoot responses to waterlogging such as epinastic curvature and ethylene production in the shoot were not influenced by the presence of this second root system. These observations suggested that healthy well-watered roots exported substances to the shoot that sustained stem elongation and that this export was inhibited by oxygen deficiency (negative message). Similarly, split root systems in drought root research

have made it possible to study the effects of soil drying on shoot physiology before the shoot begins to experience water deficit. Again, clear evidence of chemical messages passing from roots to shoots was obtained. Roots of plants have been divided in various ways to study the effects of soil drying. Splitting the roots vertically using a sharp razor blade when the plants are young then growing the two halves in different containers provides two root systems, originally divided equally. Assuming that root growth in the two halves occurs at the same rate, this provides a mechanism whereby half of the root system can be dried to examine root messages while the other half of the root system supplies the plants with water for transpiration and growth (Saab and Sharp, 1989; Blackman and Davies, 1985; Gowing *et al.*, 1990; Blum and Johnson, 1992; Khalil and Grace, 1993; Zhang and Kirkham, 1995). However, it is not the case that in nature one half of the root experiences drought while the other, vertical half remains well hydrated. Hence, although a system such as this does allow the study of chemical messages originating from drying roots, a system which simulates the natural drying of roots near the surface of the soil is preferred. In the field, when drought occurs soil around the surface becomes dry first and hard while the deeper soil layers can contain sufficient moisture for plant growth. In such cases although the roots are able to obtain water for growth, the signalling effects of soil and root drying near the surface can be experienced by the plant. Attempts to simulate such a drying situation led to a system where plants are grown in long soil columns (Blum *et al.*, 1991). In a system such as this there is no control over the amount of roots that are being dried at any one time which means that the drying roots cannot be

separated and analysed for water content and hormone levels. However, this does provide a system for studying more naturalistically the effects of mild root drying on shoot responses.

A split root system which allows: (a) drying of upper roots while the lower roots supply the plant with water, maintaining shoot hydration; (b) determination of the amount of root drying; (c) the intensity of drying by looking at water content of the drying roots; (d) analysis of hormones in the drying roots is the most desirable for studying root signals. Split root systems using horizontally divided roots have usually had the upper roots grown in soil while the lower roots were grown in nutrient solution. Attempts have been made to expose either the lower roots (Neales *et. al.*, 1989) or the upper roots (Bano *et. al.*, 1993; Masia *et. al.*, 1994) to drying but because the two roots are grown in two different media (i.e soil and nutrient) the roots may not be structurally identical nor respond to drought in the same way. Furthermore, roots grown hydroponically may not be suitable for using in pressure chambers since pressurizing these roots could lead to the intercellular spaces in the cortex becoming water-filled and thus interfere with aeration. Soil, on the other hand, provides more structural support and strength to the roots, while the gas-filled pores in the soil allow gas-filled air spaces in the cortex to withstand applied pressures (Passioura 1988b).

Methods of obtaining xylem sap for ABA analysis

Analysis of components of xylem sap gives a good indication of their output by the roots. There are several ways in which xylem sap can be collected, although some

methods are less satisfactory than others. It is common practice to collect exudation sap which is osmotically driven from detopped plants (Lachno, 1983; Zhang and Davies, 1990a, 1990b, 1991; Neales and McLeod, 1991; Correia and Pareira, 1995). The flow of osmotically driven sap is rarely more than 10% of daytime transpiration (Jackson, 1993). Studies using sap collected in this way have usually ignored the effect that this reduced flow would have on the concentration of solutes in the sap. The effect of flow rates on the concentration of ABA can be seen in Figure 28B (Chapter 5). Concentrations of solutes such as ABA in osmotically driven sap are likely to be gross overestimates of the amount of ABA in xylem sap of intact plants. Moreover, drought stress is likely to change root hydraulic conductivity which will influence sap flowing through the treated plants, thus misrepresenting concentrations of solutes in the osmotically driven sap. As shown in Figure 28B, while sap dilution of well-watered controls lies close to that of the predicted curve of dilution of water, dilution of sap from the droughted plants was less than proportional to flow. This further emphasises the complexity of interactions of inherent factors within the roots which influence osmotically driven flow.

Collecting xylem sap from detopped plants placed in pressure chambers and arbitrarily applying pressure to the roots also proffer the problem of dilution with increasing flow rates. In studies using this method of collecting xylem sap, pneumatic pressures of between 0.1 to 0.5 MPa are usually applied to induce sap flow out of the cut sump (Zhang and Davies, 1987; Neales *et. al.*, 1989, Zhang and Daives, 1990a;

Bano *et. al.*, 1993; Masia *et. al.*, 1994). The above argument applies in these instances as well since, depending upon the rate of sap flow through these detopped roots concentrations will vary. Shashidhar *et. al.* (1996) used leaf water potentials as a guide to the amount of pneumatic pressure applied to roots of detopped plants to collect sap from well-watered controls, moderately stressed, and severely stressed plants. This is a plausible method of obtaining xylem sap. Here the assumption is that leaf water potentials were similar in the well-watered and treated plants so the driving force for water uptake will be similar despite differences in stomatal conductances and transpiration of these plants (Newman *et. al.*, 1990; Smit *et. al.*, 1990). This assumption is not entirely true however. Sap flow rates in intact droughted plants are often slower because of slow transpiration although leaf water potential may be the same, while sap flows through root systems of droughted and well-watered plants pressured to the same extent will not reproduce the required differential flow. In addition, leaf water potentials over estimate. the driving force for the movement of water through roots. This is because of the existence of hydraulic resistance positioned in leaves between the xylem and the evaporating sites. This is seen very clearly in experiments showing leaf water potentials to retain a negative value (approximately -0.2 MPa) when xylem potentials are rendered zero by the application of a pneumatic balancing pressure to the roots (Table 8, Chapter 7).

In an experiment where they compared different methods of collecting xylem sap from plants in drying soil using maize and sunflower, Zhang and Davies (1990a)

showed that a few hundred microlitres of exudate collected after excising a shoot, or sap collected by pressurising whole root system of detopped plants were suitable for estimating ABA concentrations in the xylem sap of these plants. They did not encounter the problem of contamination of sap resulting from handling and injury which is often found to occur in the first few hundred microlitres of sap following excision as shown by Else *et. al.* (1994). Zhang and Davies (1990a) found that in sap obtained from centrifuging stem segments of sunflower plants ABA concentration was diluted, and suggested that this could be because of sap being drawn from the parenchyma tissue in the segments. ABA concentration in xylem sap was higher than that in the parenchyma tissue. The same method was used for collecting sap from two cultivars of *Phaseolus vulgaris* by Trejo and Davies (1991). There is a possibility that sap obtained in these ways may not represent authentic transpiration fluid as it leaves the root system. This is because of flow-dilution effects already discussed and because ABA concentrations may change as water moves up the plant from the roots to shoot tissues. A further problem is the unsuitability of concentration term as a measure of ABA output. Since it is subject to dilution effects it is preferable to replace it with delivery rates since this (concentrations x sap flow rate) gives a value that is less affected by flow. It takes into account the different flow rates in control and treated plants, and their effects on concentrations of solutes in sap flowing in these plants. Where delivery rates have been calculated by various authors, osmotically driven sap was usually sampled and flow rates used in these calculations were those of sap flow out of the detopped plants at the time of sap collection (Carlson *et. al.*,

1987; Meizner *et. al.*, 1991). This is a reasonable approach but assumes that hormones are diluted strictly in proportion to the rate of transpiration, with no effect on total delivery (Jackson, 1993). This may often hold for unstressed plants, as dilution is found to be proportional to flow, but this was often not the case with stressed plants (Else *et. al.*, 1995; Jackson, 1997).

With faster sap flows, delivery rates of solutes such as hormones or their precursors can increase and these increases may not be in proportion (Meizner *et. al.*, 1991; Jackson 1997). Thus, it is preferable to collect xylem sap at flow rates close to that expected at whole plant transpiration to allow the study of individual components of the xylem sap, such as ABA, as a signal from roots. Neuman *et. al.* (1990) used pressure driven flows to collect xylem sap and calculated delivery rates of ABA. However, the pressures used were determined by leaf water potential of these plants. As discussed earlier, these pressures are unlikely to induce similar flows to those in intact plants. Where no dilution of sap with increasing flow rates is assumed (see King, 1976; Smith and Dale, 1988) delivery rates can be grossly overestimated.

To avoid making misjudgements when calculating delivery rates, xylem sap needs to be sampled when it is flowing at rates at, or close to, whole plant transpiration. This can be attained either by inducing sap to flow at whole plant transpiration rate from detopped plants by applying pneumatic pressure to the roots in a pressure chamber or by collecting xylem sap directly from **intact**, transpiring plants. The latter is not easily accomplished because sap in xylem is usually under negative

tension and puncturing the xylem to obtain sap would release this tension as air would enter the vessels. This would stop all flow except for an osmotically driven component. However, sap from intact, transpiring plants can be collected by applying balancing pressures to the plants in pressure chambers to overcome this negative tension in the xylem so that severing xylem vessels will allow sap to be collected when a slight overpressure is applied (Passioura, 1980).

Yet another method used in attempting to obtain authentic xylem sap is to place an excised leaf or branch in a Scholander bomb and pressurising the tissue to express sap from the xylem (Tardieu *et. al.*, 1991; Tardieu and Davies, 1992; Khalil and Grace, 1993; Correia and Pereira, 1994, 1995; Jackson *et. al.*, 1995). Although some of the sap collected in this way will undoubtedly be transpiration fluid, there may also be sap expressed from within the cells. Overpressures of up to 1.0 MPa are used to collect sap from these tissues (Khalil and Grace, 1993). Many factors will influence the sample of sap collected by this method. Since only minute amounts of sap can normally be expressed from leaf tissues in this way, and because the initial sap expressed is most likely to be authentic transpiration fluid this first volume of sap expressed is normally used for analysis. However, concentration of solutes in this sample can be distorted because of contamination due to cutting and handling (Else *et. al.*, 1994). Also, in small leaves, collecting 50-100 μl of sap for analysis may exceed xylem volume (Hartung *et. al.*, 1988). Moreover, since sap in xylem is under negative tension, cutting a leaf would cause the sap to be withdrawn into the cells.

Applying pressure to the leaves would re-draw sap from the cells and in doing so it could become contaminated with solutes from within the cells, changing the concentration of solutes in the re-emerging sap (Jackson, 1993). However, Correira and Pereira (1994) used two sets of overpressures to express sap from leaves of lupin. In the first instance, overpressures of 0.2 to 0.25 MPa was applied and sap collected. Another 0.2 to 0.25 MPa overpressures were applied to collect more sap. A total of 10-15 mm^3 sap was collected from each leaf. They found that concentration of ABA in sap from well watered plants varied from 16 to 738 $\mu mol\ m^{-3}$ and no significant difference in concentrations of sap collected using the two overpressures was seen.

Sampling xylem sap from intact, transpiring plants

Split top chambers and the balancing pressure approach is the preferred method to sample xylem sap from intact, transpiring plants. In 1980 Passioura designed a pressure chamber which would (i) determine the drop of water potential, Ψ_w, between the soil and leaf of a plant; (ii) allow the sampling of xylem sap. Roots offer resistance to water flow through the plant. Water is continuously being evaporated from the cells in the sub-stomatal cavity in leaves. The flow of water through the plant is a function of Ψ_{xylem} and the hydraulic resistance (R) offered by the roots and they influence sap flow through the plants (Q) as follows: $Q = \delta\Psi//R$ where $\delta\Psi$ is the difference in water potentials between leaf and soil (Passioura, 1980). Xylem sap in a transpiring plant is under tension. Applied pneumatic pressure in the pressure chamber helps bring Ψ_{xylem} to zero (relative to atmospheric). The way in which applying such a pneumatic pressure influences the water relations of the plant, is explained by Passioura and

Munns (1984), and is paraphrased as follows:

The water relations of a plant is considered before and after applying pneumatic pressure. Water is evaporating from the leaves at a constant rate, Q, and there is negligible frictional losses of pressure during to flow through the xylem or cells (i.e., no hydraulic resistance in xylem conduits). A pressure drop of 0.5 MPa exists across the endodermal membrane of roots which has a constant hydraulic resistance, R. If, in a hypothetical situation, osmotic potential (solute potential) Ψ_s and turgor pressure in the medium surrounding the roots are zero, then hydrostatic pressure in the xylem is -0.5 MPa. Furthermore, supposing that Ψ_s of root cortex is -0.3 MPa, then turgor in these cells will be +0.3 MPa ($\Psi_w = \Psi_s + \Psi_p$). If solute potential in the leaf is -0.8 MPa then turgor in the leaf cells will be +0.3 MPa (i.e., (-)0.5 MPa = (-)0.8 MPa + (+)0.3 MPa). If a pneumatic pressure of 0.5 MPa is applied to the root system in a pressure chamber, and Q and R remain constant, (a) Ψ_{xylem} will be raised to zero; (b) in the leaves while the cells are still experiencing atmospheric pressure, they will be in contact with xylem with zero hydrostatic pressure. Before applying pneumatic pressure Ψ_{xylem} = -0.5 MPa; Ψ_s = -0.8 MPa; Ψ_p = + 0.3 MPa in the leaf cells. After applying a pneumatic pressure of 0.5 MPa Ψ_{xylem} = 0 MPa; Ψ_s = -0.8 MPa; Ψ_p = +0.8 MPa. Leaf turgor has increased by the same amount as the applied pneumatic pressure. No change in root cell water relations occurs since this increased pressure is transmitted uniformly throughout the system. Although the absolute pressure within the cells is higher the differential pressure across the cell wall (i.e., turgor) remains unaltered. This applies whether cells are inside or outside the membrane of roots that intercept the transpiration stream.

However, solute potential of soil is not zero. Suppose soil Ψ_s = -0.2 MPa. While Q and R remain constant, hydostatic pressure in the xylem is now

-0.7 MPa, and therefore 0.7 MPa pneumatic pressure will be needed to raise hydrostatic pressure in the xylem to zero. This is not to say that the driving force across the roots is 0.7 MPa because it is still 0.5 MPa. Leaf water relations remain the same as before. If hydrostatic pressure in xylem is unknown, pneumatic pressure needs to be applied in the pressure chamber to just raise Ψ_{xylem} to zero, and this is equal to and opposite to Ψ_{xylem}. This is known as the balancing pressure.

In an intact, transpiring plant balancing pressure is determined by first removing sufficient leaf lamina to expose a few millimetres of the central vein. A short piece of clear tubing is then fitted to this exposed vein. Pneumatic pressure in the pressure chamber is raised slowly to bring the xylem to the verge of bleeding. A little more pressure applied to the roots will induce xylem sap to drip out of the severed end. Alternatively, increasing the humidity of the air around the plant will also encourage xylem sap to bleed. The original experiment by Munns and Passioura (1984), where they sampled xylem sap from intact plants, was followed by a number of experiments by others with some modifications (Gollan *et. al.*, 1992; Schurr *et. al.*, 1992; Schurr and Schulze, 1995; 1996; Heckenberger *et. al.*, 1996). There are uncertainties, however, concerning the suitability of sampling sap high in the canopy and sap flow rates at which sap is sampled after a balancing pressure is set despite the sophistication with which these experiments have been carried out. These experiments are summarised below and the uncertainties reviewed.

Munns and Passioura (1984): Barley and lupin plants were detopped approximately 10 mm above the roots after setting balancing pressure, and sap collected from the cut stump was analysed. Sap flow rates out of the cut stump were not checked to see if they were indeed similar to those occurring in the intact plants and thus not subject to flow-related dilution errors.

Munns and King (1988): After balancing pressure was set, the second leaf of wheat seedling was removed from near the base and sap was collected from this

severed end. It was assumed that xylem sap bled out of this cut at a rate similar to that at which the leaf was transpiring, but this was not checked.

Munns (1990): Balancing pressure was set in wheat seedlings in the Passioura-type chamber and sap was collected from the remaining half of a leaf blade after the distal portion was removed. Sap flow rate out of the cut surface was stated to be that of the transpiration rate of the distal half prior to its removal. This assumption might hold true but no data on flow rates were presented to support this claim and calculations of ABA delivery rates used transpiration rates derived from whole plants rather than the actual flow rate of the sap analysed.

Gollan, Schurr and Schulze (1992): These authors set balancing pressure at the distal tip of the 6th oldest leaf of sunflower plants and then raised Ψ_{xylem} above zero by reducing transpiration of the plant by spraying the plants with water. They assumed that although transpiration was reduced, this did not alter the actual flow rate in the plant. Sap then issued from the leaf tip previously used to set a balancing pressure and was analysed to estimate solutes in the transpiration stream. A problem with this method is that whole plant transpiration could not be at the normal rates despite the puzzling claim by these authors that reducing transpiration did not alter total water flow from the root to the shoot. Clearly this is impossible since spraying with water slowed transpiration. A further problem is that the rate of sap flowing from the leaf tip was about 50% of the total transpiration rate of the plant while the leaf would only constitute about 14% of the transpirational surface (assuming a 7-leaf

plant). Since sap flow into this leaf was changed vastly the sap analysed would not be a good guide of what was entering the leaf in terms of water, solute concentration and delivery prior to changing transpiration rate of the plant. Another shortcoming is that sap was sampled after it had passed through the leaf. As sap moves through the leaf there may be exchange of solutes between the sap and the leaf cells so whatever appears in the sap after it has passed through the leaf may not be the same as what entered it.

Schurr, Gollan and Schulze (1992): The methods were as in Gollan *et. al.* 1992. Additionally, delivery rates were calculated for an individual but attached leaf enclosed by a gas exchange cuvette. These delivery rates were calculated by multiplying water flow through this leaf, as measured by gas exchange, with the concentration in sap from the tip of a different leaf (leaf six) where the balancing pressure was set prior to spraying with water. The accuracy of the resulting calculation depends upon how well concentrations in the flowing sap estimated what enters the in the gas exchange cuvette where sap flow would be much slower. Clearly it would be preferable to measure flow rate and solute concentration from the same leaf using sap flowing at near normal rates.

Schurr and Schulze (1995) and subsequent papers: Using *Ricinus communis* these authors changed their earlier method of inducing sap flow out of the plant in a way that avoided large changes in sap flow rate. Instead of spraying with water to slow transpiration, one cotyledon was excised. This presumably slowed transpiration

slightly thereby generating a positive Ψ_{xylem} that forced sap to flow from the petiole stump from where the cotyledon was removed. This slow flow close to the base of the plant was only 3% of total transpiration although still likely to be faster than the original rate of water loss from the cotyledon. It is likely that the solute content of the sap may approximate what is leaving the roots of an intact plant. However, the method does not help assess what is being delivered in sites higher in the canopy. This is unfortunate since the split top bomb approach offers the most promising means of testing changes in sap content as it rises up the plant. A further shortcoming of the Schurr and Schulze (1995) approach is that it involved constant adjustment of pneumatic pressure with changes in the movement of the meniscus. If removing the cotyledon influenced the movement of the meniscus, balancing pressures would adjust accordingly thus possibly changing overall water movement into the plant while the sap is being sampled. It would seem to be preferable NOT to change the pneumatic pressure at the roots to regain a balancing pressure once the cotyledon or other site of hydraulic resistance (e.g., another leaf) is removed.

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Chapter 2 : MATERIALS AND METHODS

Ricinus communis L.

Ricinus communis L., the castor oil (bean) plant belongs to the family Euphorbiaceae. It is a perennial herb, cultivated in the tropics, subtropical and warm temperate regions as an annual crop. Castor is a major oil seed crop and has been known since ancient times, originating possibly in India or Africa. It is a hardy, drought resistance plant. The seeds of castor oil comprise about 50% oil, 18% protein. The seed itself contains three toxic compounds: (i) Ricinine, which is a mildly toxic alkaloid which is not considered dangerous; (ii) Ricin, which is an extremely poisonous protein present in large amounts and can be detoxified by moist cooking; (iii) a powerful, heat-stable allergen which forms about 12% of the castor cake.

The best-known use of these plants is as a source of medicinal laxative. Other uses are: (a) dehydrated oil for paints, varnishes and other protective coatings. Derivatives of the oil are used in lubricants, greases, hydraulic fluids, soaps, printing inks, linoleum and oil cloth; (b) the cake (casto pomace) is used as a fertilizer; (c) pulp of the stem is suitable for the production of wrapping papers and strawboards.

Physiological work using *Ricinus* plants from the early 1970's exploited the ease with which phloem sap can be obtained in studies of the content of this exudate from sieve tubes

(Hall and Baker, 1972; Millburn 1970; Hoad, 1973). Phloem sap is easily extracted from *Ricinus* plants by making incisions lightly on the stem to puncture the phloem. Positive pressure in the sieve elements then result in a slow release of sap that can readily be collected for analysis of sugars, minerals and hormones.

Plant Material

Lycopersicon esculentum Mill.(Tomato)

A preliminary experiment used tomato (*Lycopersicon esculentum* Mill., cv Ailsa Craig). Seeds were sown in 0.4 l plastic pots in peat based compost (Fisons' Levington M3) and a slow release fertilizer (Osmocote, 2 kg m^{-3},Grace Sierra UK, Nottingham, UK). These were kept in a Fisons' 1550 controlled environment room providing a 16 h photoperiod (2000-1200 h) with photosynthetically active radiation (PAR) of 400 μmol m^{-2} s^{-1} at bench height, supplied by metal halide lamps. The average day/night temperatures were 25°C/20°C with a relative humidity of 50%. All pots were watered daily via capillary matting which was irrigated automatically. Side shoots were removed regularly.

Ricinus communis L. (Castor oil)

Commercially available seeds of castor oil (*Ricinus communis* L., cv, Carmencita supplied by Nickerson,s Seeds Ltd., Lincoln, UK) were germinated individually in small disposable containers (30 mm X 30 mm X 50 mm) in soil (70% loam, 30% grit, plus 3.3 g l^{-1} Miniosmocote, Grace Sierra UK, Nottingham, UK). On day 8, the containers were removed and the seedlings with the soil attached were transferred into

0.5 l plastic pots containing a 20 mm layer of polystyrene beads at the base of the pots while the rest of each pot was filled with the same soil mixture in which seeds were germinated. Plants were grown in a Fisons' 1550 controlled environment room providing a 16 h photoperiod (2100 h-1300 h) with a PAR of 540 μmol m^{-2} s^{-1} supplied by metal halide lamps. The average day/night temperature were 25°C/20°C with a relative humidity of 80%. These pots were watered twice daily by hand. When roots began to emerge from the base of the pots (15 d old), they were placed on top of second pots of the same size, containing the same growth medium. This encouraged the roots to enter the second pots. Both the pots were watered by hand at least once daily. Plate 1 shows a 4-week-old *Ricinus communis* plant grown in a Fisons' 1550 controlled environment room with twin root system, and Plate 2 shows a 3-month-old plant grown in a glasshouse. Figure 1 shows a cross-sectional diagram of a twin root system with distribution of roots in the two pots before droughting experiments. The development of this twin-root system is the subject of a separate chapter (Chapter 3).

Plate 1 : A 4-week-old *Ricinus communis* plant with a twin-root system, grown in a Fisions' 1550 controlled environment room.

Plate 2 : A 3-month-old *Ricinus communis* plant grown in single pot in a glasshouse.

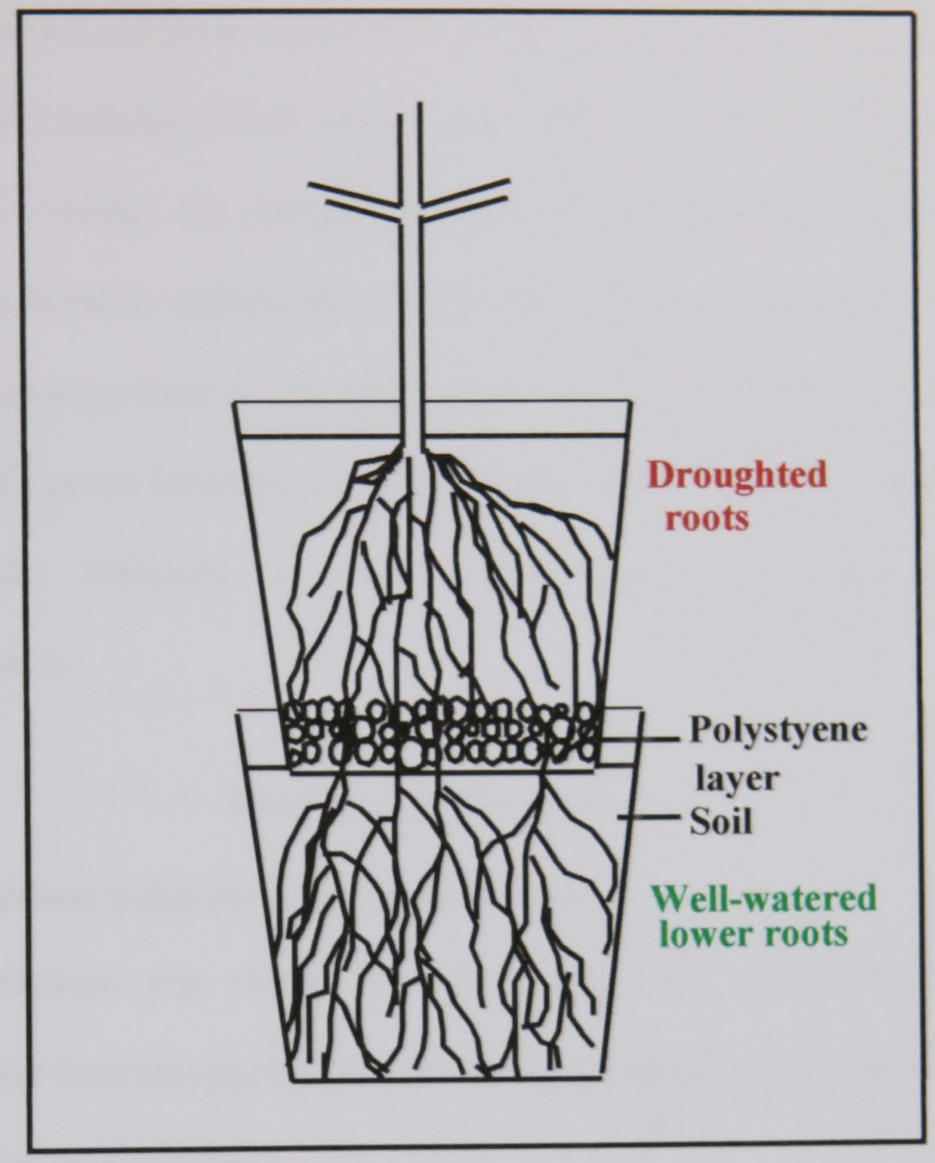

Figure 1 : Diagrammatic representation of the twin root system, showing the distribution of roots in the two pots. The polystyrene layer at the base of the upper pots acts as a barrier to water movement from the lower, well hydrated soil into the upper soil after water additions to the upper pot were halted.

Commelina communis L.

Commelina communis L. was grown to provide a source of epidermal strips used to test for the presence of anti-transpirants in xylem sap. Seeds were sown in John Innes No.2 compost in a growth chamber with a day/night temperature of 24°C/12°C and a photoperiod of 14 h with a PPFD of 320 μmol m^{-2} s^{-1}, conditions described by Trejo

et al., 1993. After emergence, the seedlings were transplanted into 0.4 l pots and left in the same growth room for a further week. These were transferred into a Fisions' 1550 controlled environment room with PAR of 400 μmol m^{-2} s^{-1} and automatic irrigation. After 3 weeks, the youngest, fully grown leaf was used as a source of epidermal strips. In earlier experiments it was found that although the size of stomatal pore in epidermal strips from the 3rd oldest leaves were bigger, those obtained from the youngest fully grown leaves were more stable and remained open for longer in the incubation buffer. Therefore, the youngest, fully grown leaves were used in antitranspirant tests.

Experimental Methods

Drought Treatment Using *Ricinus communis* Plants

Droughting treatment was carried out on 4-5-week-old plants. It involved withholding water from the upper pots which had approximately half of the total root system at the start of droughting.

Stomatal Conductance

Stomatal density varies between the upper and lower surfaces of most dicotyledenous plants. Typically the lower (abaxial) surface of such plants may have approximately 200 stomatal pores mm^{-2} of leaf with stomatal conductances of about 0.4 mol m^{-2} s^{-1} (Weyers and Meidner, 1990). Diffusive resistance of the abaxial surface of the youngest fully expanded leaves of 8 replicate plants were taken using a Delta-T Devices automatic porometer (MK3, Cambridge, UK). Calibration curves were obtained each day using a calibration plate and stomatal conductance calculated.

Leaf Elongation Rates

The youngest growing leaves of control and droughted plants were marked using type correction fluid to aid identification. The lengths of these leaves were measured to the nearest millimetre daily using callipers. Elongation rates per day were calculated by obtaining the growth of eight individual leaves each day.

Root and Shoot Fresh and Dry Weights

After the shoots were excised from the roots, they were weighed using a top pan balance sensitive to 3 decimal points. These shoots were then put into paper bags and dried in an oven at 80°C for 72 h prior to re-weighing. Roots in upper and lower pots were separated by cutting the roots at the point of contact between the base of the upper pots and the soil of the lower one. The growing medium was washed off with running tap water over a sieve. Segments of roots were collected, placed on tissue and blotted gently twice using new tissue each time. Fresh and dry weights of these roots were taken as described for the shoots.

Root hydraulic conductivity

Hydraulic conductivity of the whole root systems were determined using pressure chambers. Both root systems of well-watered and droughted plants were watered to saturation 60 minutes prior to detopping the plants and placing them into pressure chambers. The plants were sealed and pneumatic pressures increased slowly in steps of 0.05 MPa. At each stage 5 minutes were allowed for flow rates to stabilize before collecting xylem sap over a recorded time interval. The weight of the sap was

equivalent to its volume. Dividing volume of sap by the time in which it was collected determined the rate of sap flow out of the cut stump at each applied pressure. The slope of the linear portion of a graph of pneumatic pressure against sap flow rate was the Lp of that root system. At pneumatic pressures of less than 0.2 MPa the graph was non-linear. This occurs because at such low applied pressures sap flow is also influenced by osmotically-driven flow (Jackson *et. al.*, 1996).

Leaf Water Potential

The youngest fully grown leaf was excised and, at once, placed in a Scholander bomb (Scholander *et. al.*, 1965), the inside of which was lined with damp tissue paper to reduce evaporation from the leaves while they were enclosed. Pressure was increased slowly in steps of 0.05 MPa, while observing the petiole through an illuminated magnifying lens for xylem sap to appear at the cut surface. The pressure at which this occurred was taken to be the water potential of the leaf with the arithmetic sign reversed. In many instances, the pressure bomb was operated in the same growth room as the plants. This minimised the time from excision to measurement, thereby decreasing errors from water losses.

Leaf Solute Potential

The youngest fully grown leaf was excised, wrapped in aluminium foil while keeping it flat, and frozen immediately in liquid nitrogen. Leaves were then placed in a freezer at -20°C. When required, leaves were removed from the freezer and left in the cold room to thaw at 4°C for approximately 20 minutes. While the leaves were thawing,

thin strips (about 3 mm) were cut and placed in plastic chromatography columns (Bio-Rad, 731-1550) and centrifuged at 3600 rpm at 4°C. Approximately 100 µl of sap was collected from each column in Eppendorf tubes and stored on ice for a maximum of 30 minutes. Some extract was diluted in 1/4, 1/2, and 3/4 aliquots and 50 µl samples assayed for solute content using a Roebling milli-osmometer. The straight line of the dilution curve (Fig. 2) indicated that the concentration of the sap for the instrument did not contain compounds that interfered significantly with the readings or were subject to dissociation on dilution. It also demonstrated that the concentrations of the solutes in the sap were within the range of the instrument. The milli-osmometer utilized the depression of the freezing point principle for solute potential determination. The instrument was calibrated using standard solutions of potassium chloride. The principle of the instrument is based on the fact that in water, 1 mole of a substance in 1 litre (1.0 mol l^{-1}) depresses the freezing point by -1.858°C. In the present work, the osmolarity measurements obtained were converted to Megapascals (MPa) using the arithmetic relationship of 0.1 MPa = 41.53 mOsmol.

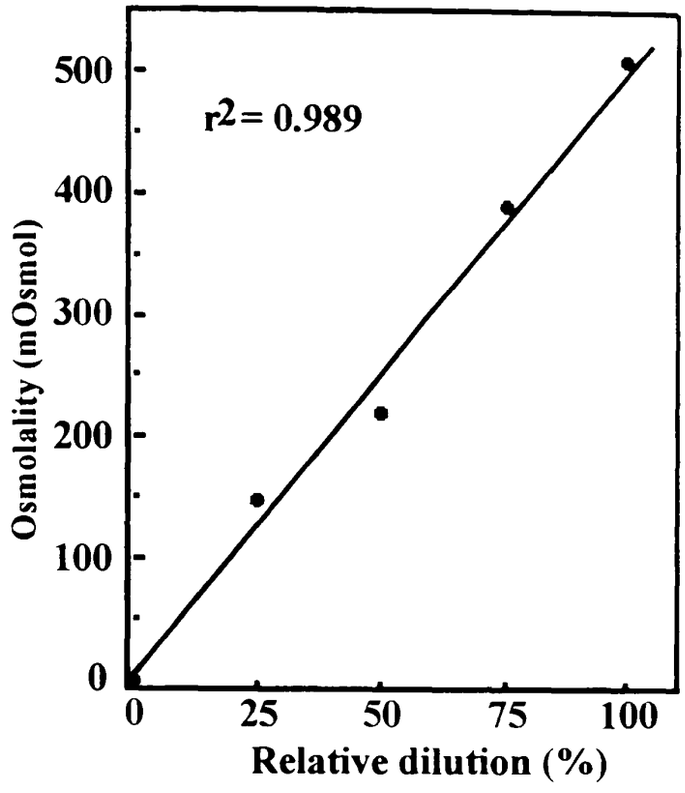

Figure 2 : A dilution curve using leaf sap extract assayed in the milli-osmometer. The straight line indicates that there was no interfering compounds or dissociations caused by dilutions.

Whole Plant Transpiration

To estimate whole plant transpiration, plants were grown in single pots. On day 0 these plants were watered and left on the bench to allow excess drainage of water from the pots. These were then weighed using a top pan balance to 3 decimal points at time (To), and left on the bench without irrigation for 1-2 hours prior to weighing them again. This time (T_1(+) was noted accurately. These measurements were repeated three time a day for both the control and droughted plants, for 5 days. Similarly, weights of pots with soil but without plants were also measured alongside to determine rates of evaporation from the soil surface. Whole plant transpiration and evapotranspiration was calculated by (Weight at T_0 - Weight at T_1)/(T_1 - T_0). This

was corrected for the evapotranspiration component by subtracting the rate of water loss measured from pots without plants.

Soil Water Content

To determine soil water content, plants were grown in single pots and on day 0 these were watered and left on the bench to allow for excess drainage of water from the pots while preventing surface evaporation by covering the soil surface with aluminium foil. At this stage the soil was at field capacity (strictly speaking 'pot' capacity). Soil from a depth of 20 mm at the centre of five pots was weighed and placed in an oven at 105°C for 72 h prior to taking dry weight measurements. Thereafter, soil samples were removed from 5 replicate pots twice daily, their fresh weights measured, and placed in the oven.

These soil samples were removed from the oven, cooled in airtight containers with silica gel to prevent the dry soil taking up moisture from the air, and dry weights were measured initially after 72 h of drying. These samples were then placed back into the oven for a further one day before measuring dry weights again. This was repeated until there was no change in the weights. This ensured that all free water had been lost from the soil sample. The difference between the fresh weights and the final dry weights was the amount of water that was present in the soil sample. Gravimetric soil water content was calculated by (Fresh weight - Dry weight)/Freshweight (x 100%)

Soil Water Potential

Water potential of the soil was determined using leaves as osmometers. Plants were grown in single pots, and on the day of the experiment all pots were saturated with water. Weights of 6 pots were taken immediately after water loss by drainage had stopped (To), then at regular intervals (e.g. after 4 h, 8 h, 12 h) for the first day then once daily for 3 d. Approximately 30 to 150 minutes prior to removing leaves for water potential measurement, 6 plants were covered with clear polythene bags to prevent water loss from the leaves, allowing their water potential to come to equilibrium with that of the soil. A smaller one covered the leaf which was to be excised for leaf water potential measurement (see Fig. 3). The plants were also covered with aluminium foil to reduce incident radiation and help prevent transpiration by the enclosed plants. The leaf in the smaller polythene bag was excised and transferred with its bag to a Scholander bomb (as described in the section for the determination of leaf water potential) for measuring leaf water potential. The assumption underlying this procedure is that, at equilibrium (i.e., no water flow through the plant), the leaf water potential of the enclosed plant is close to the water potential of the soil.

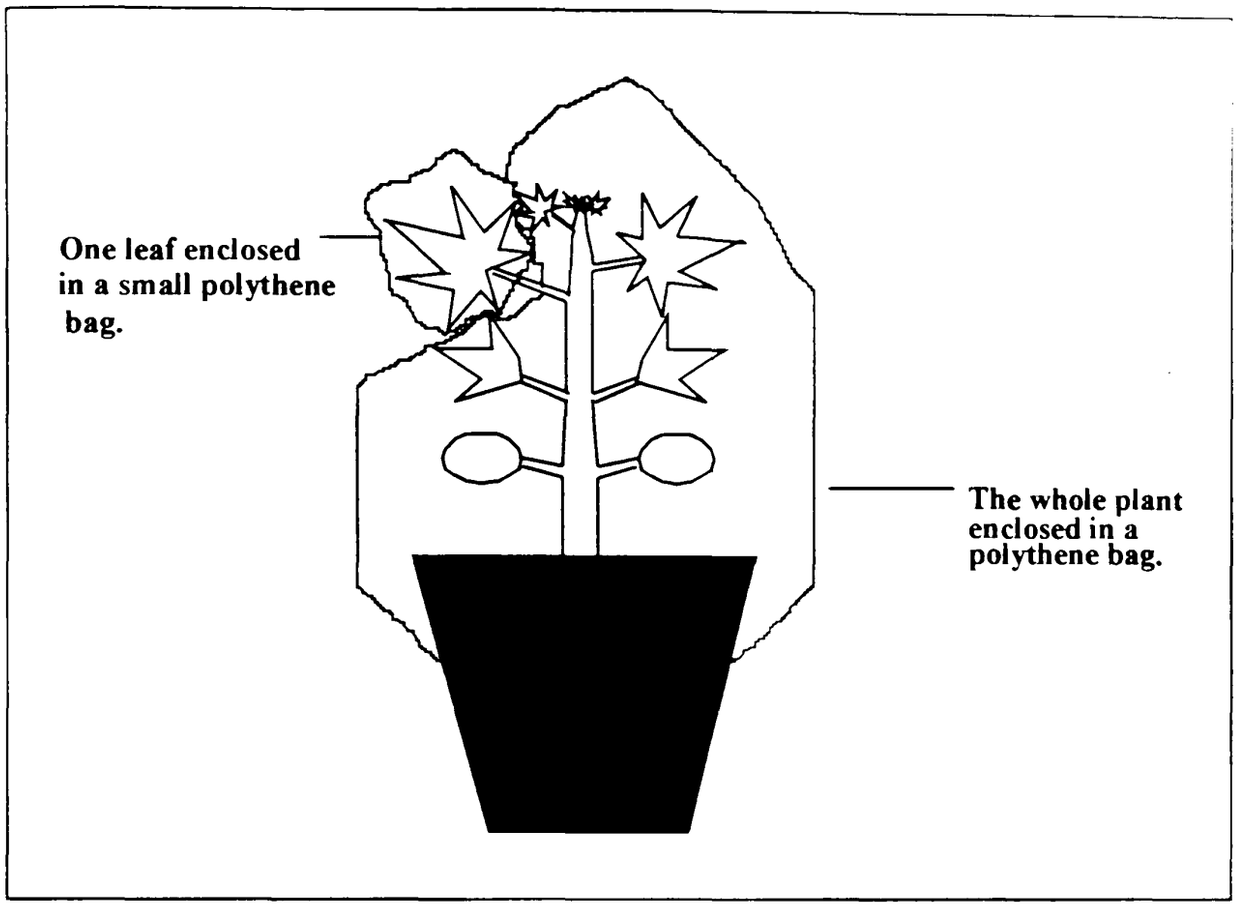

Figure 3 : Diagram showing the set-up to determine soil water potential using leaves as osmometers. The polythene bag around the plant increases the relative humidity inside it which almost totally halts transpiration by the plant. An outer aluminium foil cover ensured that stomata closed completely thus ensuring no water loss from the foliage. Once the leaf water potential equilibrated with the water potential of the soil, the single leaf in the smaller polythene bag was removed to measure leaf water potential.

To help decide the length of time required for the leaf water potential to equilibrate with the soil, plants were covered with polythene bags as described above and leaves were excised every 15 minutes for leaf water potential measurements. The time taken for the leaves equilibrate with soil water potential on the first day was 60 minutes, 90 minutes on the second and by day 3 it took 150 minutes. In the light of these results, 60 minutes was used to assess soil water potential on the first day, 90 minutes on the second day and 150 minutes on the third day. At the times soil water

potentials were measured drying soil drying, the water content of the soil was also measured and a relationship between soil water content and soil water potential was obtained (Fig. 4). A similar curve showing this relationship has been shown by Slayter (1967). Knowing this relationship allowed the intensity of droughting in terms of soil water potential could be estimated, in other experiments, simply from soil moisture contents alone.

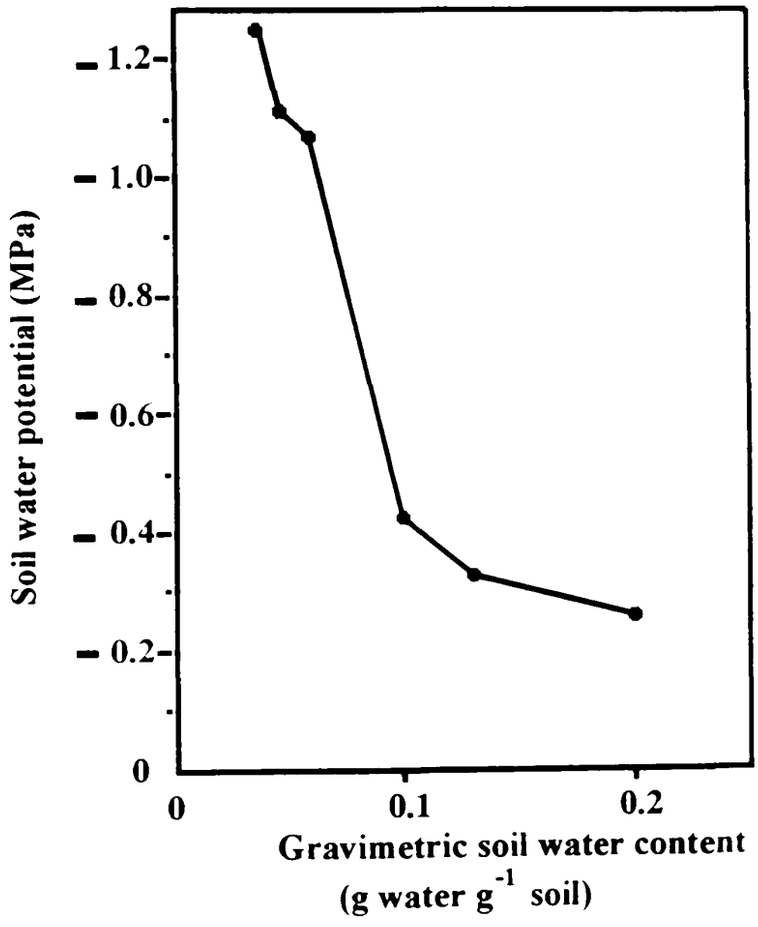

Figure 4 : Relationship between soil water content and estimates of soil water potentials obtained from using leaves of plants at zero transpiration. The soil consisted of 70% loam and 30% grit.

Xylem Sap Collection from Detopped Plants

Whole plant transpiration was determined gravimetrically prior to excising the shoot and placing the pot with its root system in a pressure chamber (Fig. 5) and sealing it. The cut stump was connected to piping which led to the outside of the pressure chamber where sap was passed through fine silicon rubber tubing. Pressure in the chamber was increased up to 0.4 MPa in 0.1 MPa intervals and up to 5 minutes was then allowed before collecting xylem sap in 1.5 ml Eppendorf tubes. This delay was needed to allow solutes that are discharged into the xylem sap as a result of handling procedures to be discarded (Else *et. al.*, 1994). A tared top pan balance was used to measure, by weight, the volume of sap collected. The time taken to collect xylem sap was recorded accurately with a stop watch.

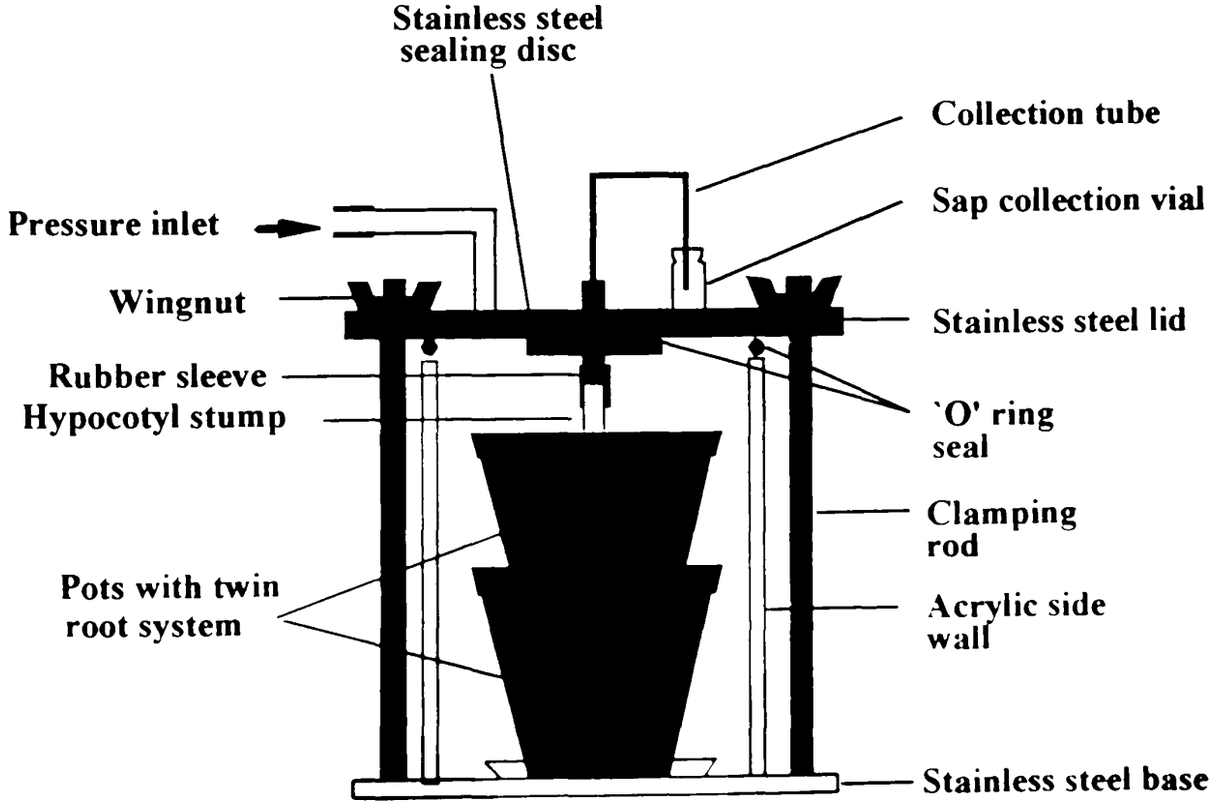

Figure 5 : Diagram showing the pressure chamber set-up with a detopped plant.

Xylem Sap from Intact Plants

Intact plants were placed in split-top chambers so that the pots containing the plants were inside the chamber while the shoot protruded above it (see Fig. 6). The basic design and concept of its use are those of Passioura (1980). The split-top of the chambers were sealed together by securing the metal rings at the top of the chamber and tightening horizontal screws. Sealing to the sides of the chamber was achieved by tightening wingnuts threaded onto vertical rods so that the stainless steel lid sections compressed a circular O-ring. The areas around the stem and the metal rings were then sealed using silicon rubber prepared by mixing silastic (Silicon rubber,Dow Corning, GMbH, Wiesbaden, Germany) 25 g of base (9161 RVT) with 1.5 ml of catalyst (N9162) and quickly pouring this around the stem using a 10 ml syringe. The proportion of the base and catalyst required to prepare a strong, quick-setting seal varied slightly with different batches of the product. Finally, brass compression pieces were secured tightly around the stem, pushing down on to flexible rubber inserts. While the plants were in the split-top chambers (approximately 60 minutes) about 30 ml of water was injected through a hole in the lid and onto a tray at the bottom of the pots ensure the lower roots remained well-hydrated. Prior to applying balancing pressure this hole was closed, with a threaded stud.

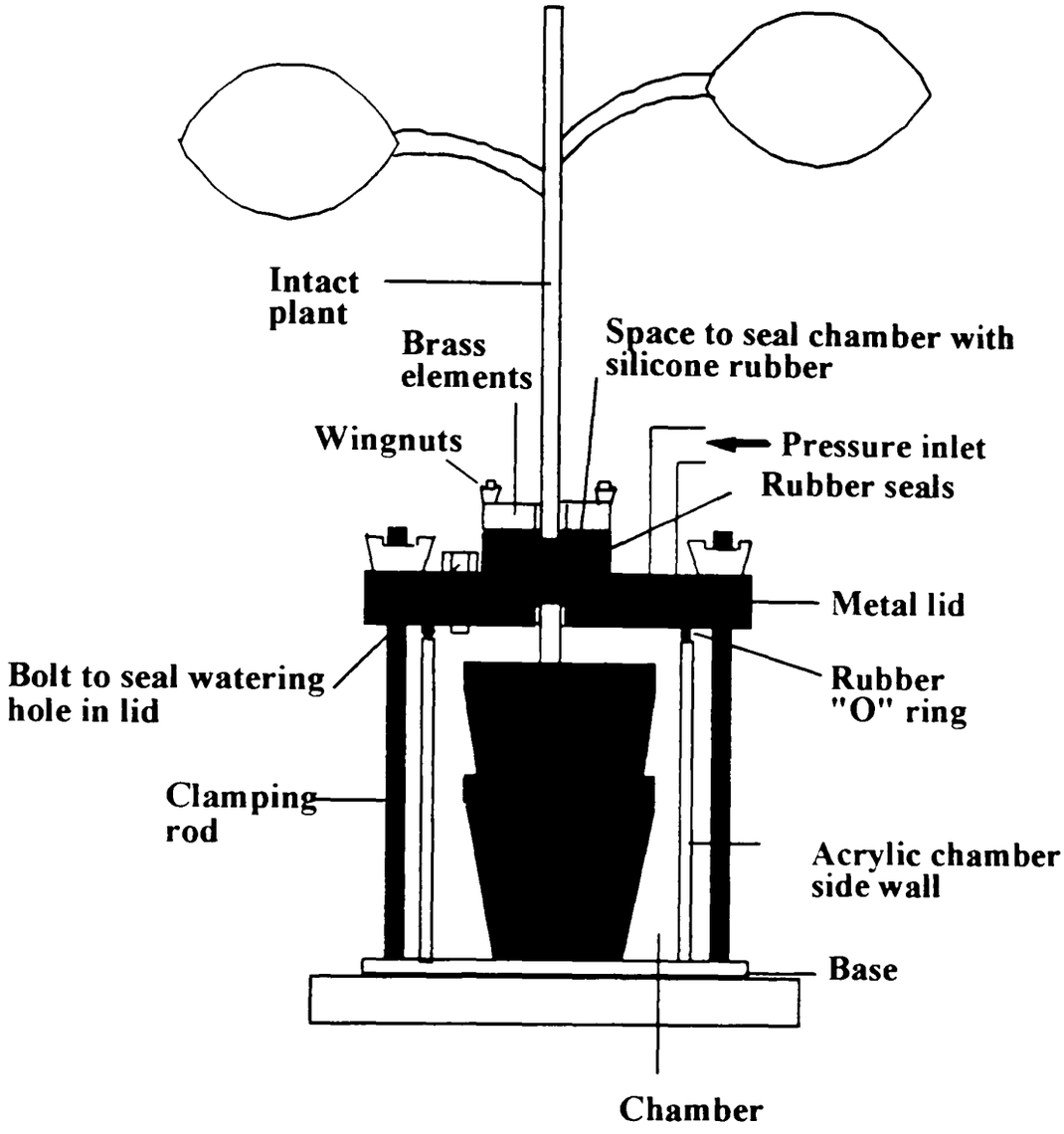

Figure 6 : Diagram of a plant inserted into a split-top pressure chamber.

Once the plant had sealed, about 1 cm^2 of the distil end of leaf 3 was removed to reveal a short stub of the central vein. A narrow silicon rubber tube (about 30 mm long) was carefully pushed onto the vein. Pneumatic pressure in the pressure chamber was then increased slowly in steps of 0.05 MPa and 5 to 10 minutes allowed at each step to check if xylem sap had emerged into the tube. Once sap was visible in the tube, pressure was adjusted so that movement was arrested. This was assumed to be balancing pressure. This balancing pressure is the pneumatic pressure applied to the

roots in the pressure chamber to just raise the combined hydrostatic and osmotic pressure in xylem sap to atmospheric (i.e., zero in relation to atmospheric pressure). The balancing pressure is equal and opposite to negative pressure in the xylem that would exist if the roots were outside the pressure chamber. It amounts to the hydrostatic force required to drive the transpiration flow of water radially across the roots from soil to xylem less outward osmotic potential created by different solute concentrations in soil and xylem sap.

In Situ Measurement of Sap Flow Rates

Estimates of sap flow rates at the base of the stem and through the petiole of a leaf higher in the canopy were made using electronic flow gauges that employed the heat balance principle. A commercially available system (Flow2 sap flow system) was used that was fitted with one small sensor (5.5 mm internal diameter) for petioles and a larger one (10 mm internal diameter) for the stem. Prior to using the Flow2 gauges they were tested for accuracy against a gravimetric method of determining sap flow rates at whole plant transpiration. Figure 7 below shows the graphs obtained which indicate that the two methods were in close agreement with both the gauges estimating flow within 10% of that obtained from weight loss. This is considered to be an acceptable figure (Sakuratani, 1981, 1984).

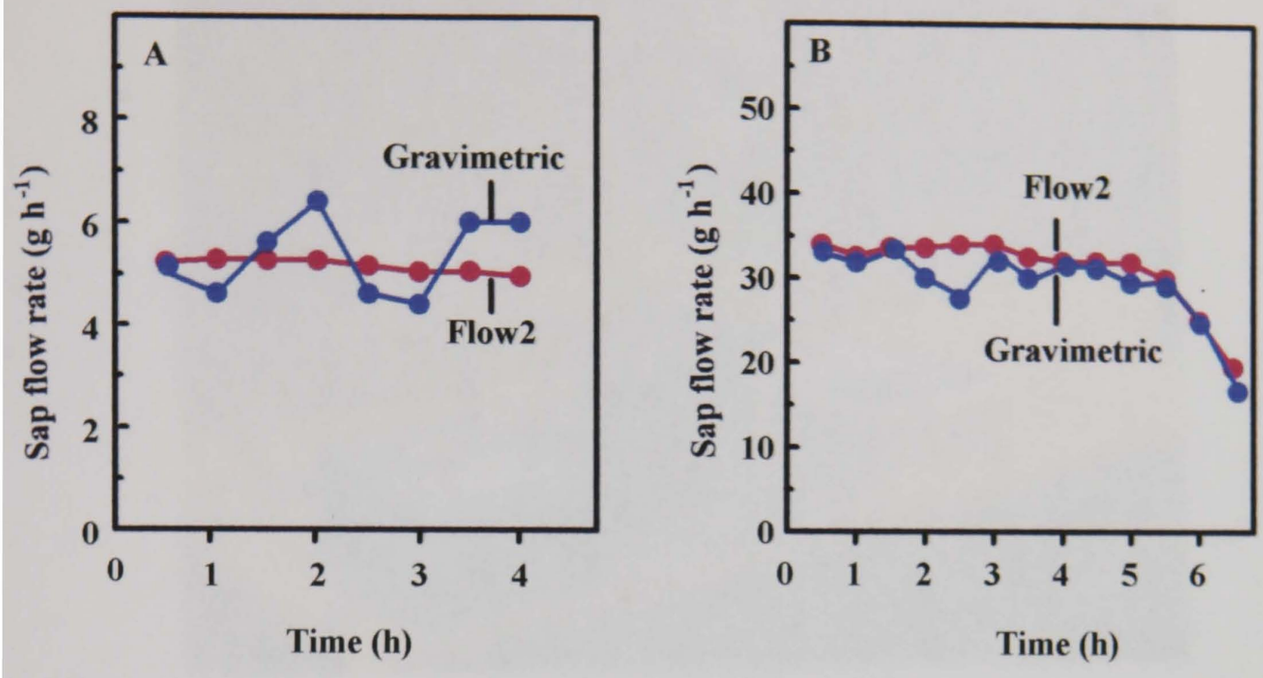

Figure 7 : A comparison between a Gravimetric method of measuring sap flow at the stem base and the two gauges of Flow2 heat balance method where a sensor is attached to the intact stem. (A) shows results obtained when the smaller gauge (SGA5) that was attached to the stem of a 3-week-old plant which had the correct diameter for this gauge. (B) shows results obtained when the larger gauge (SGA10) was attached to a 4-week-old plant. Sap flow was measured very 30 minutes in both the plants.

The Flow2 equipment operates on the stem heat balance theory where a constant heat is applied to the stem and sap moving up in the xylem carries this heat to heat sensors at the top of the gauge. Fast sap movement carries less heat in the sap. The larger gauge (SGA10) was clamped to the stem near to the plant while the smaller gauge (SGA5) was clamped to the petiole the leaf of which was later removed to collect sap after a balancing pressure had been established. These gauges were connected to a datalogger (Dynamax, Texas, USA) which collected the sap flow data. This data could be stored as a data file or a graph.

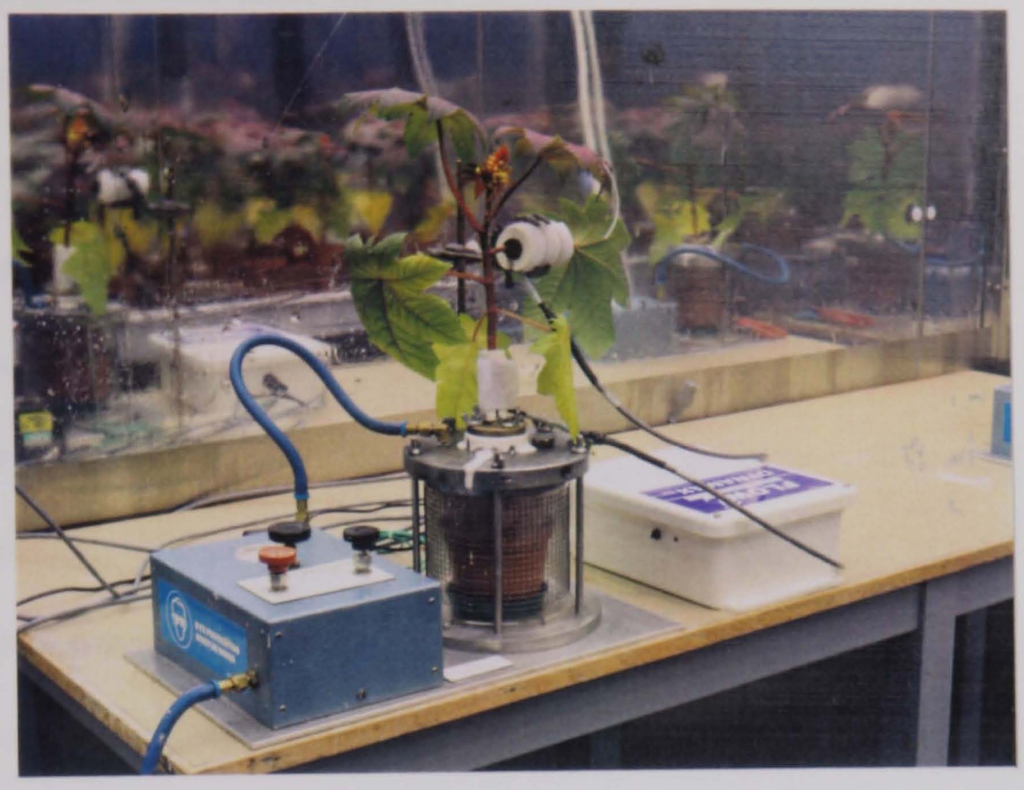

Plate 3 : Experimental set-up of the split-top chamber with the Flow2 meter attached to the plant to measure sap flow (a) into the petiole of the leaf; (b) into the stem of the plant.

When a balancing pressure was set and sap flow in the two gauges were stable, the lamina of the petiole to which the smaller flow gauge was attached was excised. Sap flow into the two gauges continued to be recorded and xylem sap collected and frozen in liquid nitrogen for ABA analysis or osmolality measurement.

Nitrate Measurement in Xylem Sap

Nitrate ions in xylem sap were measured using a high performance liquid chromatography (HPLC) technique. The ion chromatograph used to measure nitrate measures inorganic ions in sodium carbonate/sodium bicarbonate eluent. The

detection limit is 0.01 nmol ml^{-1}. In addition to the pump and column, the ion chromatograph also has a Dionex Anion Self-regenerating suppressor. This recycles the used conductivity cell effluent to neutralise the inflowing eluent giving a lower background conductivity which enables the detection of lower concentrations of anions. Ions are measured by a Dionex Conductivity Dector. The output was directed to Chromatography Data Handling System (Jones, Deeside, UK). 100 µl sap sample was diluted into 1000 µl (10-fold dilution). 100 µl of this diluted sample was injected in the chromatography columns (ASG4 and AS-SC), and The Jones chromatographic software was used to analyse this sample and determine the concentration of nitrate ions in the sample.

ACC Measurement in the Xylem Sap

1-aminocyclopropane-1-carboxylic acid (ACC) in xylem sap was converted to ethylene (conversion is equimolar) and gas chromatography (GC) was used to analyse ethylene released (Lizada and Yang, 1979). The GC (Pye Unicam Series 204) was fitted with a flame ionization detector and a 1-m long glass chromatography column containing activated alumina. The temperature of the column was maintained at 80°C. Each time the equipment was used it was calibrated by three replicate injections of 1 ml of 1 ppm (v/v) ethylene. One ml of sample was added to each of two 25 ml Erlnmeyer flasks, to which 100 µl of mercuric chloride was added with either 10µl of 78 mmol m^{-3} ACC as a conversion standard, or 10µl of water. The flasks were sealed with 'Suba-seal' rubber puncture caps. To this, 0.4 ml of a mixture comprising saturated sodium hydroxide, 12% (v/v) solution of household bleach (sodium

hypochlorite - 5% available chlorine) and water (ratios 4:8:3 respectively) was injected. The mixture was vortexed and left for 9-15 minutes at room temperature prior to removing 1 ml of the headspace gas to be analysed for ethylene by gas chromatography. The efficiency of conversion of xylem sap was approximately 68%.

Testing the Authenticity of the Radioimmunoassay

Prior to using the RIA there was a need to authenticate it to ensure that it was a suitable method for measuring ABA levels in the xylem sap and root tissues of *Ricinus communis*. RIA is an alternative method for determining ABA content in plant tissues for two reasons. Firstly, it requires small amounts of sample and secondly, it can be used with crude plant extracts. However, there are problems with their application such as interference in the antigen-antibody binding is prevalent with crude plant extracts. If no account is taken of possible interferences, the results obtained cannot be relied upon for accuracy. In immunoassays, quantification relies on competition between endogenous plant hormone and a synthetic radioactive equivalent for the active site on the antibody, and can therefore produce false positives due to non-specific interferences or cross-reaction from the plant extracts. To check for interference, increasing amounts of synthetic hormone are added to the experimental sample and the values of ABA concentrations obtained in the assay plotted against the amount of hormone added. If no interference is present then the line will be parallel to the standard line given by ABA in the absence of the plant extract, and its y-intercept will indicate the amount of endogenous compound in the extract (Wang *et. al.*, 1986). This is known as the spiked dilution test, and the results

of such a test is shown in Figure 8 for xylem sap from both well-watered and droughted *Ricinus communis* plants. In these samples the slopes were very close to 1 (not statistically different). If interference was present then the binding would have decreased, therefore the slope of the sample would be greater than unity. With root extracts some interference was found. The graphs obtained from spike tests using root extracts from both well-watered and droughted plants were non-linear (graphs not shown). Thus, the spiked dilution tests showed that there was no interfering compounds in the xylem sap of *Ricinus communis,* indicating that the immunoassay was suitable for quantifying ABA in xylem sap of *Ricinus*. However, due to the interference in the root extract, RIA was ruled out as a method for analysing root ABA.

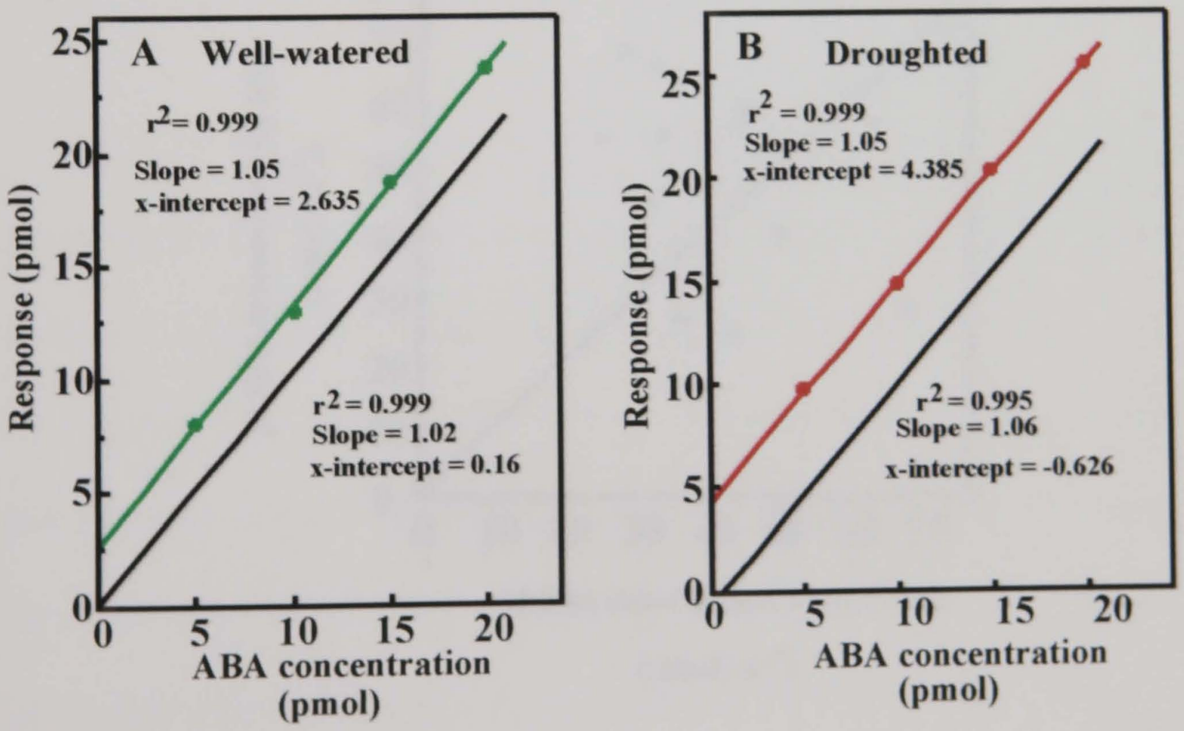

Figure 8 : Spiked dilution tests using xylem sap from (A) well-watered, (B) 48 h droughted *Ricinus communis*. Immunoreactive material in the sap samples was determined after the addition of different amounts of (S)-ABA.

Before RIA could be used to analyse ABA concentrations it was essential to validate that the quantity of ABA detected using the immunoassay were in agreement with those obtained using a physicochemical technique such combined gas chromatography mass spectroscopy (GC-MS). Once again using the sap samples of *Ricinus communis* it was found that there was reasonable agreement between the quantities of ABA determined by the immunoassay and the physicochemical methods (Fig 9). This was not the case with root extract and therefore the immunoassay technique was restricted to use with the sap samples only. ABA quantification using the root extract was made using GC-MS.

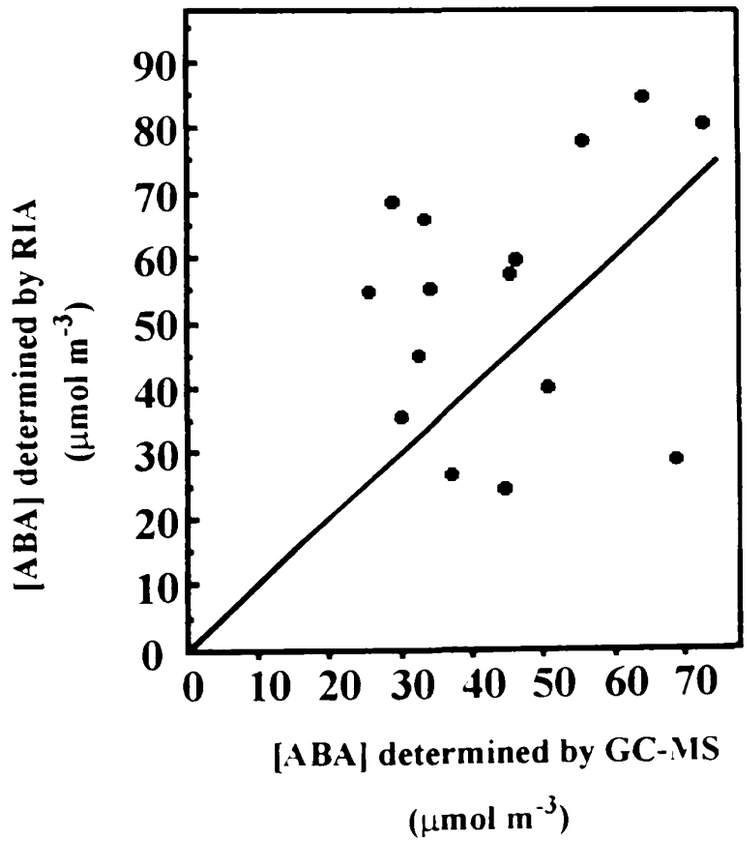

Figure 9 : The relationship between ABA concentrations in xylem sap of *Ricinus communis* obtained using GC-MS and RIA.

ABA Analysis using Scintillation Proximity Assay

The radioimmunoassay for ABA was a much modified version of Quarrie *et. al.*, 1988. The version used a scintillation proximity assay (SPA) which uses fluomicrospheres linked to protein-A (+PA-SPA). This protein link also binds to the FC region on MAC 252 which is a monoclonal antibody with high specificity and affinity for ABA. It is specific for (S)-ABA (therefore can discriminate against (R)-ABA). The use of fluomicrospheres removes the need to separate bound and unbound ABA prior to scintillation counting, permitting fast processing time (Whitford and Croker, 1991). MAC 252 is a rat gamma-globulin which recognises and binds specifically to for free acid of (S)-cis,trans-ABA or tritiated ABA([^{3}H] ABA). ABA binds to the antibody (Ab) in the following way:

$$ABA + [^3H]\ ABA + Ab \rightleftharpoons ABA\text{-}Ab + [^3H]\ ABA\text{-}Ab$$

Both ABA from the sample and the tritiated ABA compete for the less than equivalent amount of antibody attached to the beads. Once tritiated ABA binds to the antibody the beta particles emitted by [^{3}H] ABA are in close proximity with the fluomicrospheres. The energy emitted activates the fluor in the beads which results in emission of light which is measured by the liquid scintillation counter. Since the beta particles emitted from tritiated ABA have a range of 8μm in water, any tritiated ABA which is not bound to the beads will not be close enough to the fluomicrospheres to produce scintillations. More recently (Whitford *et al.*, 1997, in preparation) the methyl groups of the fluorophor-containing polyvinyltoluene beads were oxidised to

carboxyl groups using potassium permanganate as described by Udenfriend *et al.*, (1985). This oxidised beads are directly linked to the antibody (MAC 252) by amide bonds and do not require protein-A (-PA SPA). After some early work using the -PA SPA beads most of the RIA used was with -PA SPA beads. Comparison of ABA analysis using the two types of beads showed similar results.

To measure ABA concentration a calibration curve is required which covers the working ranges of 0.625 pmol to 50 pmol. The buffer used in RIA was prepared by dissolving 340 mg potassium dihydrogen orthophosphate in 50 ml deionised water, 435 mg of dipotassium hydrogen orthophophate in 50 ml deionised water with 292 mg of sodium chloride. To obtained the buffer at pH 6.0, 44 ml of potassium dihydrogen orthophosphate and 6 ml of dipotassium hydrogen orthophosphate was added, checking the pH with a pH meter. Prior to using the buffer 1% PVP (polyvinyl-pyrrolidone, BDH, England) was added. 200µl of buffer was required for each sample (plus 20 vials for standard calibration curve). Some of the buffer was used to prepare 100 µl ml^{-1} tritiated ABA (S,R-cis, trans [-^{3}H] abscisic acid, Amersham International). Buffer was used to make 7.5 µl of the monoclonal antibody (Mac 252) per ml, similar to that used by Quarrie, *et al.*, (1988). From a stock solution of (S)-ABA (synthetic mixed isomers, Sigma Chemical Co. USA) a 50 pmol per 100 ml solution was prepared. A series of standards were prepared by diluting the stock solution to give 50, 30, 20, 10, 5, 2.5, 1.25, and 0.625 pmol solutions. To determine ABA concentrations in the xylem sap 100 µl of each sample was transferred into

scintillation vials. 75 µl of tritiated ABA (30 000 DPM) was added to each vial, followed by 25 µl of the -PA ABA beads. Oxidised beads (not linked to MAC 252) were used to measure non-specific binding, and maximum binding (B_{max}) of tritiated ABA was also measured (75 µl of tritiated ABA plus 25 µl of the -PA SPA beads). Finally 75 µl of buffer was added to the mixture in each vial to make up the volume to 275 µl. All vials were capped, labelled, vortexed and placed in the dark at room temperature for only 6 hours for the antibody-antigen to develop prior to placing then in a LKB 1217 liquid scintillation counter. After counting, the samples were re-incubated and re-counted for approximately 24 hours after the start. However, it was found that most binding occurred after only 6 hours incubation, and binding activity declined if counting was delayed for more than 24 hours.

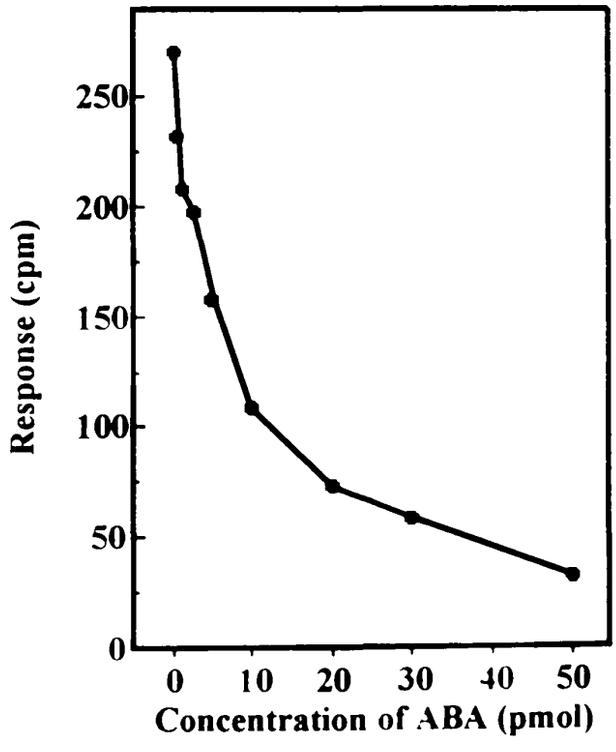

Figure 10 : A typical standard curve for (+) ABA obtained using the MAC 252 radioimmunoassay described above. Incubation time for this was 6 h.

Bulk Tissue ABA Content

Root samples were weighed, and 0.5 g sub-samples frozen immediately in liquid nitrogen and kept at -20°C for ABA analysis. To extract ABA from the tissue, 5 ml of 80% methanol (MeOH) and butylated hydroxy toluene (BTH) (20 mg l^{-1}) was added to the frozen tissue and homogenised with a mixer (Ultra-Turrax T25, IKA Labortechnik, Staufen, Germany). This mixture was clarified in a cooled centrifuge at 3500 rpm for 5 minutes and the supernatent decanted into vials. This removed much of the ABA from the tissue. To extract more, the residue was taken up in a further 5 ml of 80% MeOH + BTH, centrifuged and decanted as before. The final 10 ml of MeOH was evaporated in a Speedvac SC110 leaving 2 ml of aqueous solution. This solution was passed through a Nylon-66 membrane filter (Alltech, Carnforth, Lancashire, UK, pore size 0.2 μm) followed by a 1 ml water wash, and made up to 4 ml with deionised water. To check the extraction recovery, tritiated ABA (10 000 DPM) was added to the sample at the start of extraction and at the end of extraction the percentage recovery was determined by doing a tritiated ABA count. On an average, 91% recovery of the tracer was achieved. This mixture was used for ABA analysis either using proximity scintillation assay or combined gas chromatography mass spectroscopy (GC-MS).

Combined Gas Chromatography-Mass Spectroscopy

A C_{18} Sep-Pak reverse-phase cartridge (Waters) was attached to a 5 ml syringe which formed a column for purifying samples prior to the analysis of ABA by GC-MS. The column was washed twice with 4 ml of absolute alcohol each time, then 4 ml of

distilled water before loading in the following sequence: two ml of 20% methanol; 1 ml of the sample (or as much as possible), 10 µl [$^2H_3$] ABA internal standard (the amount of internal standard should be approximately equal to the amount of ABA in the sample). The volume was made up to 5 ml using 20% methanol. This was passed slowly through a Sep-Pak column, washed with 4 ml of distilled water, and the ABA eluted with 3 ml of 60% methanol. This was evaporated to dryness under vacuum, re-dissolved in 100 µl of 100% methanol, and methylated with ethereal diazomethane. The methylated samples were evaporated to dryness and re-dissolved in 20 µl of ethyl acetate. Three microlitre samples of this were injected into a fused silica WCOT capillary column (25 mm X 0.22 mm X 0.15 µm) with a coating of BPX5 (SGE, Milton Keynes, UK). Ions at *m/z* 190 and 162 (methyl abscisate) and *m z* 193 and 165 ([methyl-$^2H_3$] abscisate) were monitored and the 190:193 in ratio were used to calculate the ABA concentration.

***Commelina* assay for stomatal closing activity in xylem sap.**

Epidermal strips are detached leaf epidermal tissue used in the study of stomatal function in well-controlled environments. Assuming the strips are removed without any contamination by other leaf cells, the most common advantages of using epidermal strips are that responses are independent of influences from mesophyll cells, most of the environmental factors can be controlled and a large number of replicate measurements that are possible, allowing statistical analysis of the results. The drawbacks of such work is that it can only be carried out using a limited number of species. Further, epidermal strips are not used with the intention of measuring

responses of stomata that resemble precisely those of intact leaves but used merely as a model system where environmental parameters can be controlled (Spence, 1987).

Commonly used plants for epidermal strips are *Allium cepa* (because its guard cells do not utilize starch for carbohydrate storage), *Commelina communis* (it is remarkably easy to peel, yields strips with high viability and low mesophyll contamination, has large stomatal pores, and is easily grown), *Tradescantia virginiana* (has exceptionally large stomata and is used successfully in research involving direct pressure measurements and measurements of ion activities with microelectrodes), and *Vicia faba* (easily grown in a short time, strips easily although strips are highly contaminated with mesophyll). In *Commelina*, the frequency of stomata in epidermal peel is generally 50 stomata mm^{-2}. The guard cells are approximately 50 μm long and 27 μm wide in size with 6 subsidiary cells. With 90° peeling, mesophyll contamination is only 4 cells mm^{-2}, the strips comprise 100% living guard cells, 98% living subsidiary cells and 45% living epidermal cells. Stomata of *Commelina* open maximally at pH 5.5 and close at pH 7.5, require 30-100 mol m^{-3} monovalent cations supplied in the form of NaCl, KCl, RbCl, prefer Cl^- to Br^- as balancing monovalent anions and open without external Ca^{2+} (Weyers and Meidner, 1990).

Xylem sap samples were diluted in MeS buffer that was prepared fresh on the day of used by dissolving 1.952 g l^{-1} of MeS (2-[N-morpholino] ethanesulphonic acid (Sigma Chemical Co, St. Louis, USA) to make a 0.01 M concentration and

7.455 g l^{-1} (0.1 M) of KCl (BDH Chemicals, Poole, UK) in distilled water. This was placed on an magnetic stirrer to allow the buffer to dissolve properly. The pH of this solution was adjusted to 6.1 by adding a 0.1 M KOH (potassium hydroxide) solution drop by drop.

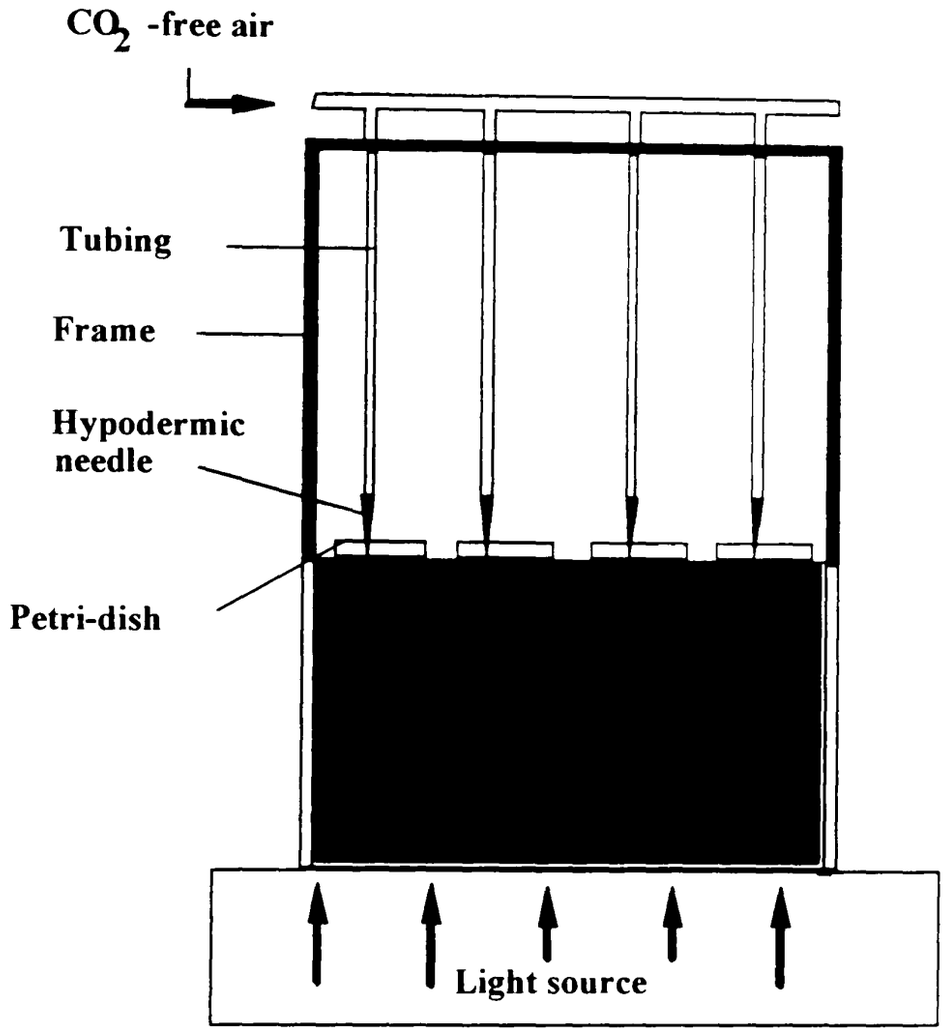

Figure 11 : Diagram showing the water bath over which the epidermal strips of Commelina were placed during the experiment. Temperature in the water bath was 25°C, with a PPFD of 280 μmol m^{-2} s^{-1}.

The youngest fully grown leaf from *Commelina communis* plants were floated on the buffer in the controlled environment room where the plants were grown and

epidermal strips were obtained using the method described by Weyers and Meidner, 1990. These leaves were placed on a wet board, sprayed with buffer to avoid drying, and their mid-veins removed. The two halves of each leaf were then floated in the MeS buffer, abaxial side down. The 80 mm diameter Petri dishes containing the leaf halves were brought back to the lab. Two further Petri dishes (80 mm diameter) were filled with the MeS buffer at 25°C ready to receive epidermal strips. The leaves were stripped of their abaxial epidermis after placing them, adaxial side down. The leaf was partly cut through at about 5 mm from the end by gently rocking a bluntish razor blade. This cut through the mesophyll layer but left the abaxial epidermis intact. The leaf was then turned over, held down with a blunt seeker resting on the rest of the leaf while the abaxial epidermis was stripped at an angle of 90° with forceps. These epidermal strips were placed in the Petri dishes with clean MeS buffer, their inner surface facing down. All the leaves were stripped in this way and a collection of epidermal strips made in Petri dishes in the MeS buffer. These strips were then cut into 5 mm long pieces in the buffer with sharp scissors and disrtibuted among smaller 50 mm diameter Petri dishes with 10 ml of the MeS buffer in each one; 15 sections in each dish. The Petri dishes were then placed on the illuminated water bath at 25°C (Fig. 9) for 90 to 120 minutes to allow stomata to open before transferring some of them to other Petri dishes containing the treatment solutions. CO_2-free air (ambient air passed through a column of three- to nine- mesh Carbosorb sodalime [BDH]) was bubbled through hypodermic needles into each Perti dish to help ensure that respiratory CO_2 did not accumulate and close the stomata. The treatments were either

different concentrations of (±) -ABA (Sigma), or different dilutions of xylem sap from the well drained or droughted plants.

At pre-determined time intervals, five strips were removed from Petri dishes at random and placed on a slide with a drop of incubation medium, covered with a cover slip. Stomatal apertures were measured using a light microscope on which was mounted a video camera which was linked to an Apple II computer. The image on the screen was focused using the X20 objective and stomatal apertures measured directly off the screen with a small flexible scale. One millimetre on the screen was equal to 1.32 μm. Ten stomata were measured at random from each of the strips. Five strips were used from each treatment. A mean and standard errors of the five means were estimates of stomatal apertures (strictly the maximum width of the apertures between the guard cells). Stomata at the edge of the strips were avoided because they may have been damaged when the strips were cut (Weyers and Meidner, 1990).

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Chapter 3 : DEVELOPING THE TWIN-ROOT SYSTEM

Introduction

An important requirement for the experimental protocol is a method that will allow hydraulic and non-hydraulic effects of drying roots on shoots to be distinguished. Simply withholding water from the entire root system is unsatisfactory because any impact of chemical signals from the roots will be masked by dehydration effects as the soil overall becomes exhausted of water. A more satisfactory approach is to expose only part of the root system to drought while the rest of the root system continues to supply the plant with adequate water to retain normal shoot hydration. The latter method requires the roots to be divided into two parts one of which is dried while the other remains well-watered. Various methods for doing this are discussed in Chapter 1. It is possible to grow a second root system below the first one (Zhang and Davies, 1987; Neales, *et al.*, 1989; Bano *et. al.*, 1993; Masia, *et al.*, 1994). In the experiments of Zhang and Davies, and Neales, *et al.* the second root system was grown hydroponically below the upper soil-grown roots. These lower roots were used for partial drying. In both these experiments roots were air-dried to allow them to lose some water (between 10% to 40%, Zhang and Davies); (around 10%, Neales, *et al.*). These roots were then placed in moist, dark containers to allow them to remain at similar water content during experimentation. Roots were kept in these containers for up to 49h (Neales, *et al.*). The positions of well-watered and droughted roots were

reversed in the experiments of Masia, *et al.* They grew the second root system hydroponically below the first soil-grown one but the upper (internal) roots were dried, while the lower (protruding) roots were kept in aerated nutrient solution. During droughting, the upper roots lost about 16% of their water while the lower roots also decreased by about 20%. Overall, approximately two-thirds of the root system was dried. However, the set-up of Masia *et al.* was not satisfactory for separating hydraulic and non-hydraulic signals. Although only part of the root system was droughted, and in a more natural way than the systems of Zhang and Davies (1987) and Neales *et al.* (1989) (i.e. the upper roots rather than the lower ones), the lower, supposedly well hydrated roots actually lost more water during this time than the drying roots. Furthermore, leaf water content declined about 14%. As a result, increases in ABA in the leaves could not be attributed solely to any increase in supply from the drying roots. Using roots of the same plant that were grown in two different media, namely compost or soil, and nutrient solution, may also be undesirable since the two kinds of roots may not necessarily behave in the same way. Notwithstanding these potential flaws, droughting only a part of the root system allows the study of possible chemical messages originating in the drying roots which may bring about physiological changes in the shoot in the absence of shoot water deficits.

This chapter presents the stages that led to the development of a double root system to allow the growth of plants successfully to achieve the following aims:

- Establish soil drying to allow for adaptive responses to occur in the shoots in the

absence of increased leaf water deficits.

- Establish a suitable method for inducing a second root system below the first.
- Determine the most suitable growing medium that: (i) supports vigorous growth; (ii) allows partial root drying to be spread over a few days; (iii) allows roots to be removed from the soil for accurate root fresh and dry weight determinations.

Simple Pot Tests Using *Lycopersicon esculentum* L.

As described in Chapter 2, droughting was imposed on 5-week-old tomato (*Lycpersicon esculentum* Mill.) plants growing in 0.4 l pots in peat compost. This simplest approach tried to vary the intensity of the droughting stress by watering once or twice daily, or halt watering completely at the start of the experiment.

Complete withholding of water, or watering once or twice a day were found to be unsatisfactory. The two most severe treatments resulted in severe wilting within two to three days, preceded and accompanied by large losses in leaf water potential (Fig. 12B) and stomatal closure (Fig. 12A). Watering twice daily imposed a less intensive stress but the severity differed at various times of the day since watering was discontinuous. Clearly, droughting the whole pot to different extents was not a suitable method for studying chemical signalling from roots of the droughted plants. Some alternative approach to droughting the roots was required. Accordingly, an attempt was made to grow plants with two separate root systems and to stress only one of these by withholding water. In this way it might prove possible to observe the effect of the drying roots on shoot behaviour while the second, well-watered roots

supplied the shoot with the water needed for growth and transpiration. In these circumstances any effects of drying roots could be ascribed to chemical rather than hydraulic messages passing to the shoots.

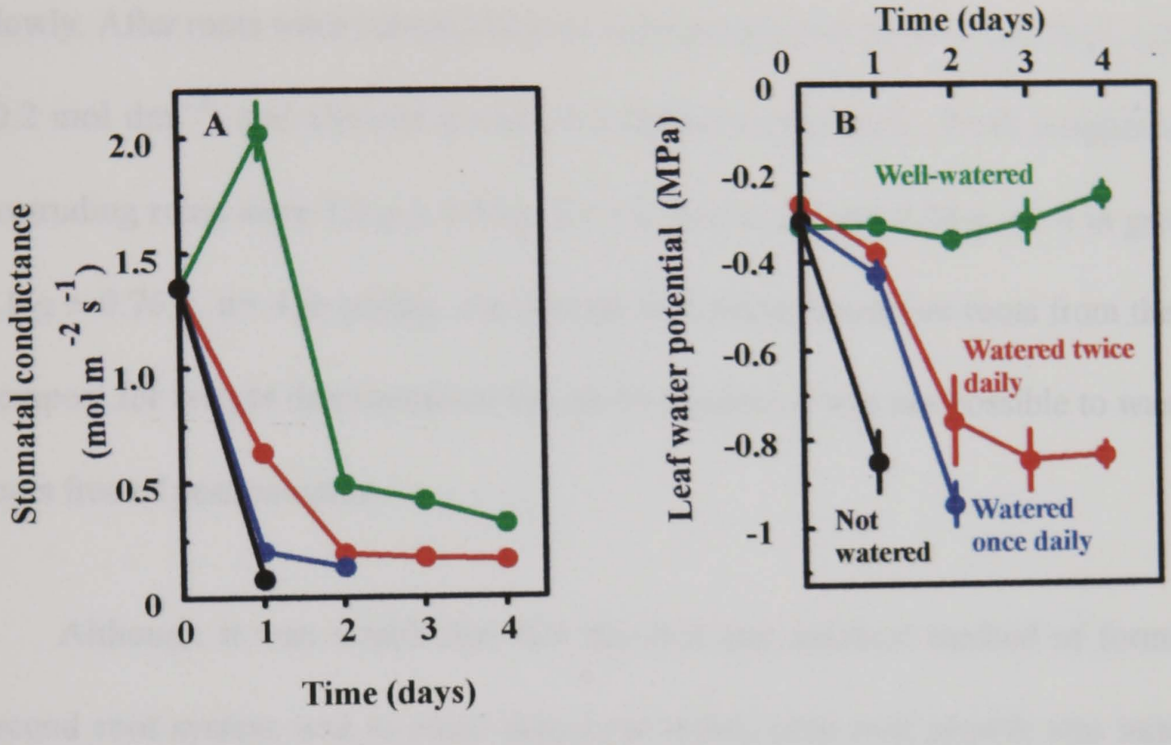

Figure 12 : The effects of carrying out the following treatments on 5-week-old plants of *Lycopersicon esculentum* Mill. growing in 0.4 l pots in Levington compost: well-watered (green); watered twice daily (red); watered once daily (blue); not watered (black). (A) Stomatal conductance of the abaxial sides of the youngest, fully expanded leaves of 8 plants were measured twice daily, once at approximately 4 hours into the photoperiod and again at 12 hours into the 16 hour photoperiod for up to 4 days. (B) Water potential of the youngest fully expanded leaves of 8 plants not used for stomatal conductance determinations. Vertical lines are standard errors of 8 replicates.

Inducing a Second Root System Using *Lycopersicon esculentum* Mill.

Plants were grown as described in Chapter 2. To induce a second root system at the base of the upper pots, 5-week-old plants were placed over a second lower pot containing moist sand, grit and perlag. A second set of 8 pots was placed approximately 5 mm above water in glass jars.

It took 5 days for roots to grow approximately 5 to 15 mm out of the base of the first pot into the medium below. Formation of a second root system below the first occurred faster from pots that were placed above water in the jars. Those placed on moist grit, perlag, and sand also produced roots from the bottom of the pots, but more slowly. After roots were initiated all pots were placed over aerated $Ca(NO_3)_2$ solution (0.2 mol dm $^{-3}$) and allowed a week for further root growth. Fresh weights of the protruding roots were 1.9 g ± 0.59 g, n= 5 in water; 1.3 g ± 0.48 g, n= 4 in grit; and 1.8 g ± 0.76 g, n= 4 in perlag. An attempt was made to remove roots from the peat compost for weight determination but failed because it was not possible to wash the roots free of peat compost

Although it was established that the best and quickest method of forming a second root system was to place pots over water, once root growth was initiated, continued growth was satisfactory in all the media tested. However, some drawbacks were recognised in these approaches. For example, roots grown hydroponically would be unsuitable for use in pressure chambers to be used in future experiments to obtain xylem sap. The problem here being that, under pressure, water can move into intercellular spaces more readily and asphyxiate the cells. As a result, it seemed preferable to adopt a system of growing plants with two root systems in a solid medium that would enable xylem sap collection using pressure chambers without the risk of damaging the roots. The work of Passioura (1988) showed that the presence of soil particles around the roots protects them from damage by water injection into

spaces at the pressures needed to obtain xylem sap from plants. In subsequent experiments with a solid rooting medium a switch was made from tomato to castor oil (*Ricinus communis* L.). This change was made because *Ricinus communis* readily releases phloem sap by making incisions into stems or petioles. In future work it was hoped to compare the effects of soil drying on hormones and other solutes moving between roots and shoots in both xylem and phloem transport systems. A second reason for preferring *Ricinus communis* was because it is known to be drought resistant. Studies on adaptation to drought stress are more likely to be critical using a species with a marked resistance to stress since mechanisms of drought resistance must occur more strongly in such plants.

The experiment with tomato demonstrated that a second set of roots can be induced to grow in a solid medium. The next step was to determine the most suitable growing medium for *Ricinus* that:

- Supported vigorous growth.
- Generated a second root system to from below the first when it was placed on a new medium.
- Allowed partial root drying to be spread over several days.
- Allowed roots to be removed cleanly and completely from the medium for accurate root fresh and dry weight determination.

As stated earlier, root fresh and dry weights could not be measured in tomato that was grown in the peat-based compost because the peat was difficult to remove from the roots, thus preventing accurate root weight measurements. To determine the

proportion of roots in the two 'half' root systems, a growing medium was needed that came away from roots easily without losing fragile secondary roots. This is important since it is essential to know the mass of both the root system before and after droughting begins. Only with such values can the intensity of a signal generation by roots be calculated.

Medium Comparison

Germinated seeds of *Ricinus* were transferred into 0.5 l plastic pots containing sand, perlag and soil (70% loam, 30% grit, plus 3.3 g l^{-1} of the slow release fertilizer miniosmocote). Since sand and perlag did not contain mineral nutrients, these pots were watered with a full strength nutrient solution (Long Ashton formula) everyday. When roots began to emerge from the base of the pots (13 d old) they were placed on top of second pots of the same size, containing the same growth medium. Measurements of leaf growth, shoot and root fresh and dry weights were taken 5 d and 1 d prior to droughting the plants.

Droughting experiment began when the 4th leaf had emerged (23 d old). By this time approximately 40% of the total fresh root was in the lower pots of the soil-grown plants but only 13% in the sand-grown plants, and 12% in the perlag-grown plants. Roots in the upper pots were exposed to drought while the lower pots were watered to 'pot' capacity twice daily. The control and droughted plants in each of the media tested were placed randomly in the growth room using random numbers.

Growth prior to droughting

A similar pattern of growth in total plant height was seen in plants grown in each of the three media (Fig. 13 A). Growth of the youngest leaf decreased in all plants during the 8 day measurement prior to droughting (Fig. 13 B) and was always slowest in perlag-grown plants up to the last set of measurements.

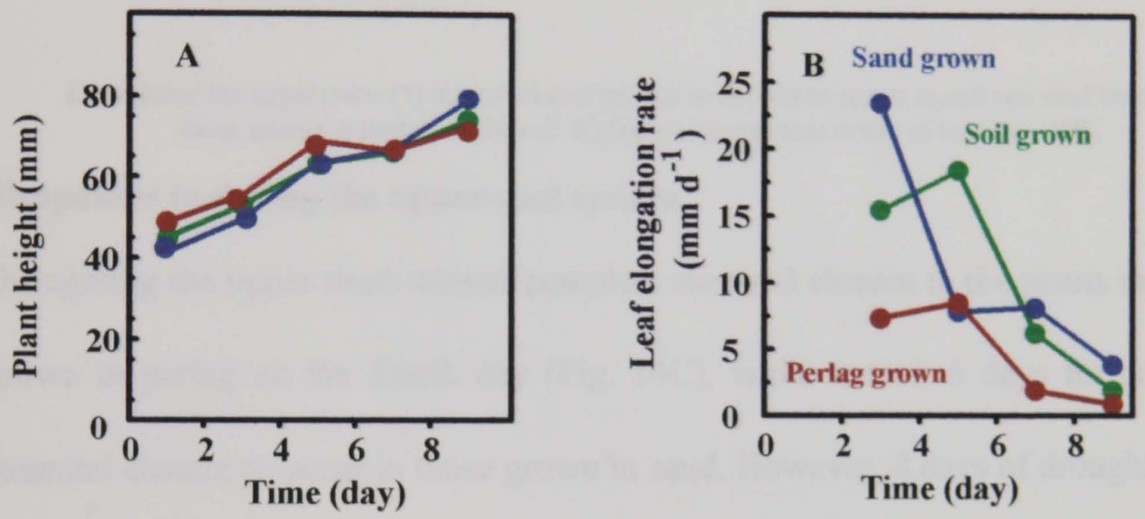

Figure 13 : *Ricinus communis* L. plants were grown with twin root systems in soil, sand or perlag and were subjected to drought treatment in the upper pots when 23-day-old. (A) shoot height during 8 days prior to droughting. (B) shows the rates of elongation of the second youngest leaf in plants in the three media prior to drought treatment. Means of 8 replicates with standard errors.

However, shoots in plants grown in sand were much heavier (freshweight 20.9 g ± 3.12 g, n= 8) than those grown in soil (freshweight 14.6 g ± 1.30 g, n= 8) or perlag (9.1 g ± 0.47 g, n= 8). After 11 d growth on the second pots, total root fresh weight was heavier in the plants in soil (18.2 g ± 2.07 g, n=8) than those grown in sand (16.1 g ± 1.29 g, n=8) or perlag (9.6 g ± 0.79 g, n=8). In plants grown in sand and perlag only a small fraction of these roots were in the lower pots (sand 2.4%, perlag 4.0%), while those grown in soil already had 21.6% of their roots in the lower pots (Table 1).

Table 1: Percentage of the total root system (freshweight basis) in the lower of 23-d-old *Ricinus communis* L. plants grown in soil, sand, or perlag.

Days	Soil	Sand	Perlag
0	21.6	2.4	4.0
5	41.6	12.8	11.5
13	51.0	33.0	58.0
*13	56.0	44.0	43.0

Droughting the upper roots (*) increased root growth in the plants grown in soil and sand but not in those grown in perlag. Means of 8 plants with standard errors of less than 10%.

Responses to drying the upper root system

Droughting the upper roots caused complete stomatal closure in the plants that were grown in perlag on the fourth day (Fig. 14C), while it took 6 days for complete stomatal closure to occur in those grown in sand. However, 8 days of droughting did not bring about complete stomatal closure in the plants that were grown in soil, although conductances were lower in these plants compared to well-watered ones on most days (Fig. 14 A,B,C). Conductances in perlag-grown plants were always small, suggesting even well-watered plants were stressed.

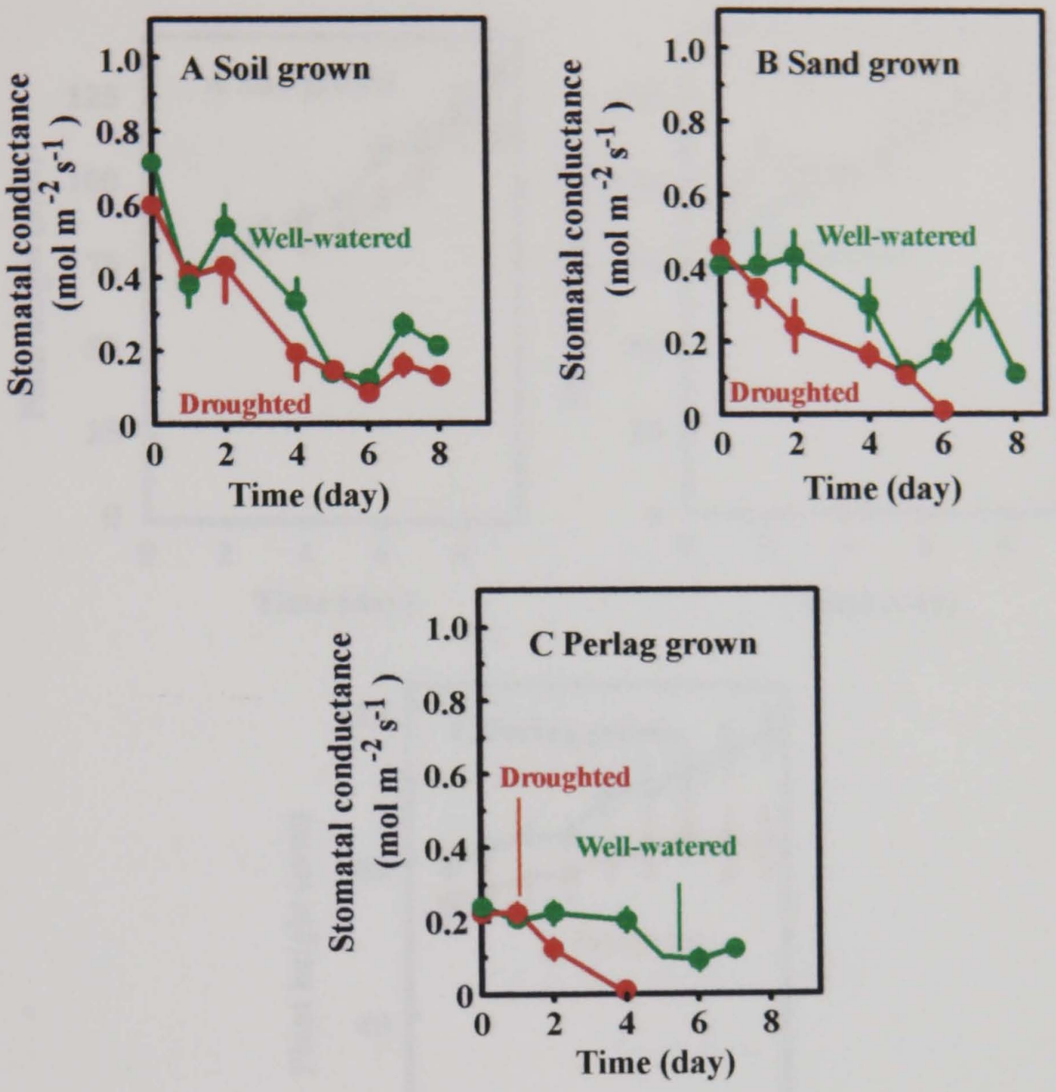

Figure 14 : The effect of drying the upper root system on stomatal conductance of the youngest fully grown leaves of 23-day-old *Ricinus communis.* Plants grown in (A) soil, (B) sand or (C) perlag. Means of 8 replicates with standard errors.

All plants increased height steadily over the experiment. The slope was less for perlag-grown plants, indicating stress, even in the well-watered ones. Except for the plants grown in soil, which showed a small reduction in shoot height, droughting the upper roots failed to slow shoot extension convincingly (Fig. 15A,B,C). The rate of expansion by the second youngest leaf declined over the 8 days of the experiment in plants in all three growth media. However, the decline was more marked in plants with droughted upper roots (Fig. 16 A, B, C)

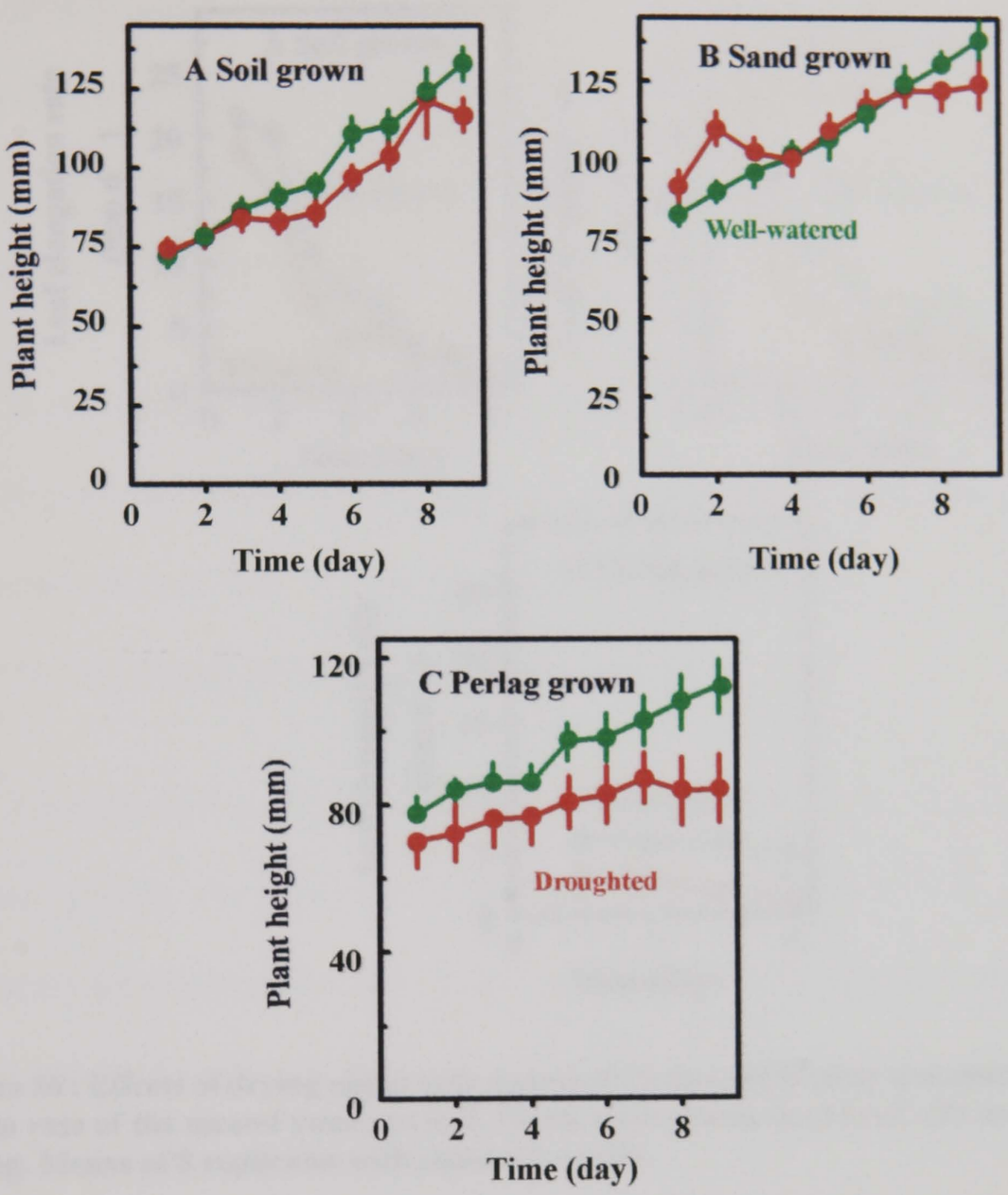

Figure 15 : Effects of drying the upper root system of 23-day-old *Ricinus communis* on total plant height. Plants were grown in (A) soil, (B) sand or (C) perlag. Means of 8 replicates with standard errors.

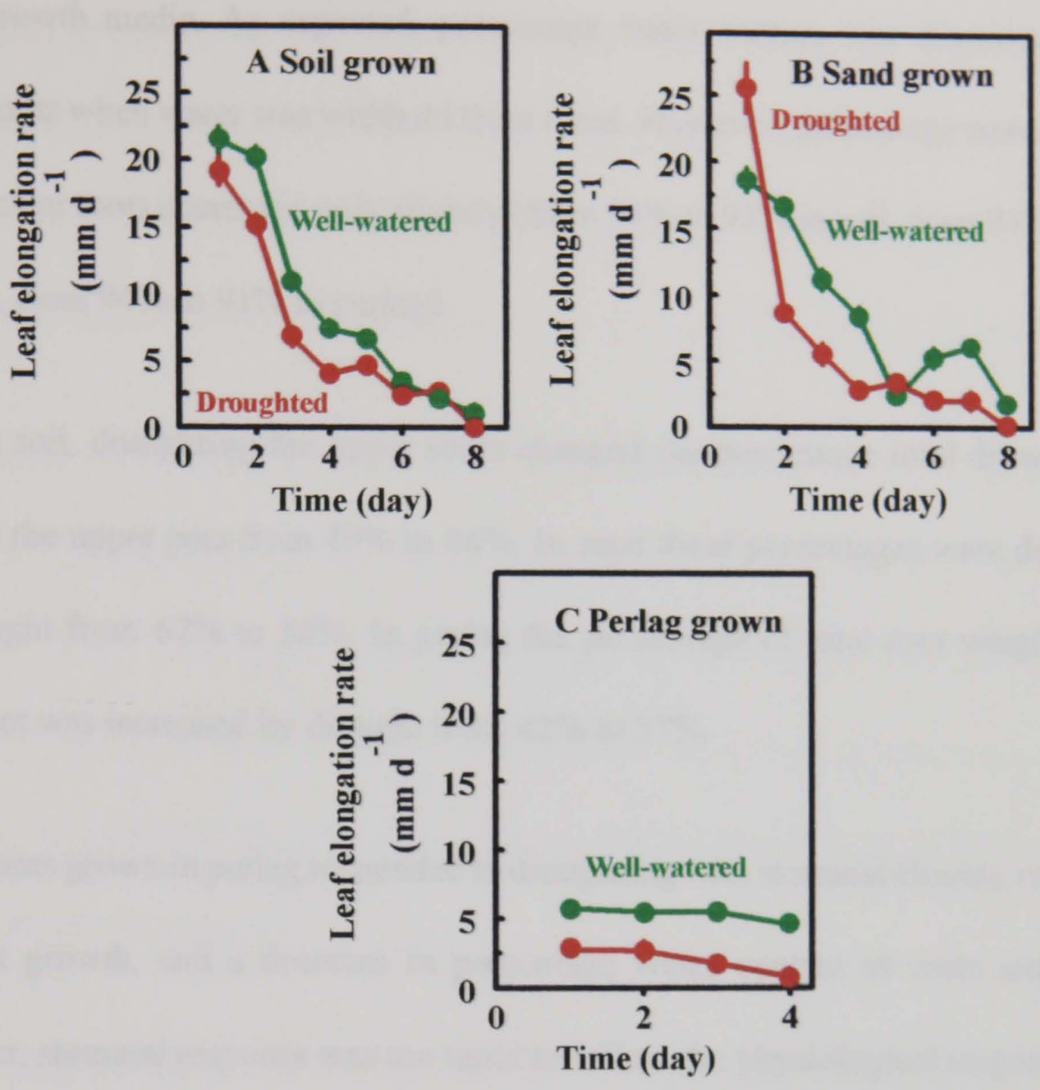

Figure 16 : Effects of drying upper root system of 23-day-old *Ricinus communis* on elongation rate of the second youngest leaf. Plants were grown in (A) soil, (B) sand, or (C) perlag. Means of 8 replicates with standard errors.

When plants were harvested at the end of the experiment, shoot freshweights were decreased by the drought treatment (soil: well-watered 33.77 g ± 1.178 g, n= 8; droughted 26.95 g ± 1.93 g, n= 8, sand: well-watered 46.75 g ± 1.76 g, n= 8; droughted 27.29 g ± 1.16 g, n= 8, perlag: well-watered 19.33 g ± 0.78 g, n= 8; droughted 8.07 g ± 0.82 g, n= 8). There was also a small reduction in the percentage water content of the shoot of plants with droughted upper roots (2% in soil, 3% in sand and 5% in perlag), although these changes were not stastistically different.

Droughting the upper roots depressed the growth of the upper roots of plants in all three growth media. As expected, percentage water content was decreased in the upper roots when water was withheld from them. However, percentage water content of the lower roots decreased only slightly (from 94% to 93% in soil, from 94% to 92% in sand, from 94% to 91% in perlag).

In soil, droughting the upper roots changed the percentage total dryweight of roots in the upper pots from 49% to 44%. In sand these percentages were decreased by drought from 67% to 56%. In perlag the percentage of total root weight in the upper pot was increased by drought from 42% to 57%.

Plants grown in perlag responded to droughting with stomatal closure, reduction in shoot growth, and a decrease in percentage water content of roots and shoot. However, stomatal response was too rapid to follow the physiological responses that accompany it or cause it. Root growth in both the pots was very poor. Furthermore, all perlag-grown plants showed some symptoms of stress even when well-watered, making them unsuitable to use in further experiments. Similarly, plants grown in sand showed very slow growth of roots into the lower pot unless the upper roots were droughted. However, droughting decreased water content of the roots, and complete stomatal closure occurred after 6 days, which was slower than for plants grown in perlag. This advantage for future work is offset by poor root growth into the lower pots prior to droughting. Root growth into the lower pot occurred most readily in plants that were grown in soil. Although complete stomatal closure did not occur in

these plants during the 8 day experimental period, there was some reduction in conductance during droughting. Clearly, there was decreased percentage water content of the upper roots (showing some water deficit) while the lower pots and the shoot showed only a small decrease in percentage water content, which is probably a developmental effect and not an indication of any water shortage in the lower roots.

Based on the findings it was clear that soil (70% loam, 30% grit) would be the preferred growing medium for *Ricinus communis* L. plants, and that the twin-pot approach where only upper roots were droughted held promise for changing shoot behaviour in the absence of foliar water deficits, but further development was still required. In particular some method of increasing the intensity of drought stress on the upper roots was needed.

Preventing Water Transfer From Lower To Upper Pots During Droughting

The principal modification to the set-up was to place a barrier to upward water movement in the base of the upper pots. Thus, prior to the transfer of germinated seeds to pots of soil, perlag was placed at the base to form an approximately 20 mm layer, before soil was added. This was expected to act as a barrier to capillary water movement from the lower, well-hydrated soil to the upper drying soil. In this way withholding water from the upper pots would impose a more intense drought stress on the upper roots.

At the beginning of drought, approximately 43% of the roots were in the lower pots, but by the end of the experiment this increased to 52% in the controls and 65% in droughted plants. During the drought treatment, expansion of the second youngest leaf decreased in both well-watered and droughted plants (Fig. 17B). However, the slower growth rate of the droughted plants is a clear indication that a marked effect on the shoot was achieved by withholding water from the upper pots. In contrast, plant height was similar in both sets of plants (Fig. 17C). Leaf expansion was slowed considerably. The overall pattern of stomatal conductance over the 7 day treatment was similar in droughted and control plants (Fig.17A). However, after day 2, conductances of the droughted leaves were lower than those of the leaves of well-watered plants (except on day 6).

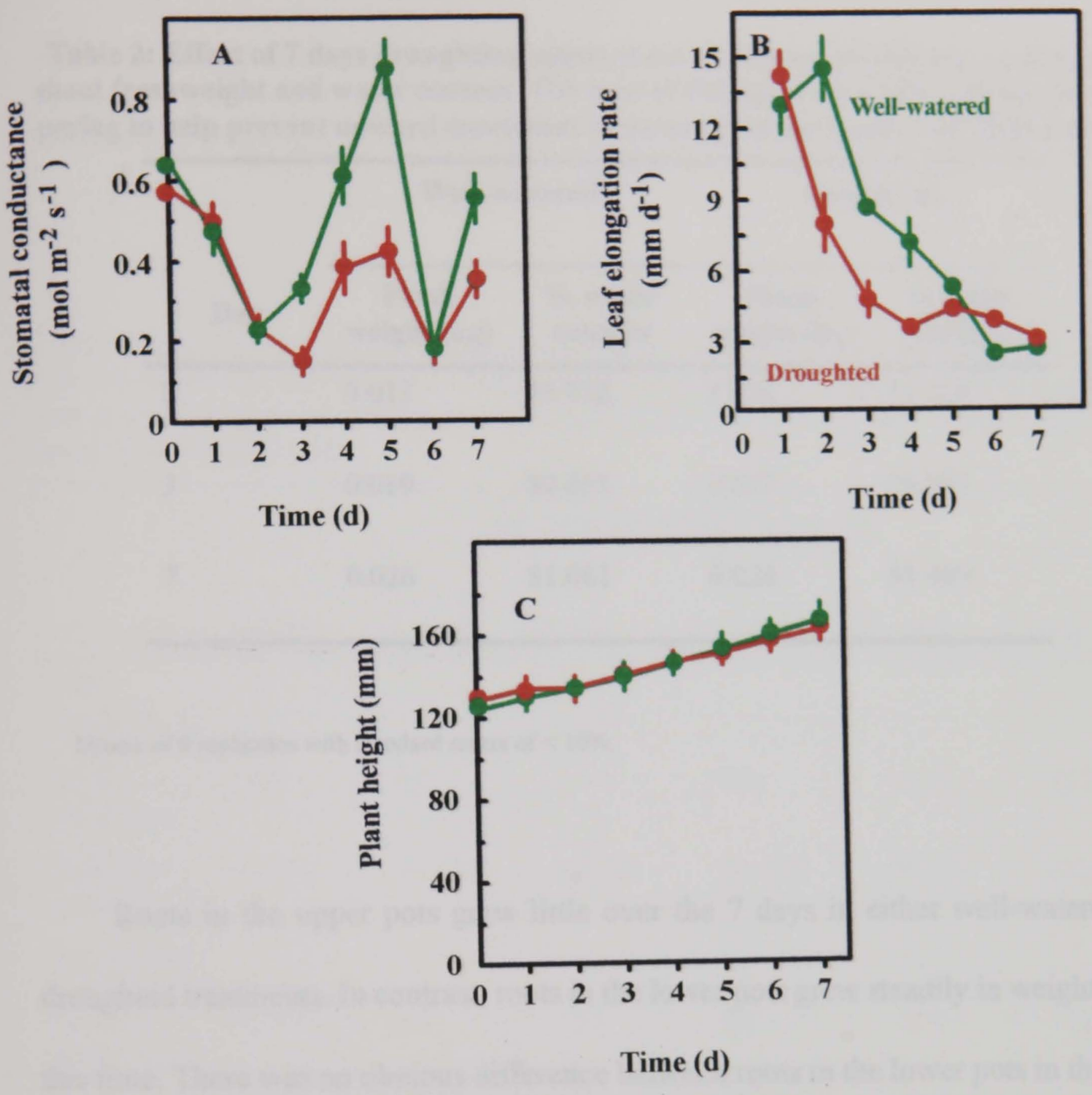

Figure 17 : The effects of 7 days drying upper roots of *Ricinus communis* grown in soil with roots divided into upper and lower pots. The basal 20 mm of the upper pots was perlag while the lower pots had soil. The perlag layer at the base of the upper pots was a barrier to upward water movement from the lower, well- watered soil into the upper, drier soil. (A) Stomatal conductance of the youngest, fully grown leaves. (B) Elongation rate of the second youngest growing leaf. (C) Shoot height at daily intervals. Means of 8 replicates with standard errors.

Throughout droughting, shoot fresh weight and shoot water content were very similar irrespective of treatment (Table 2). Thus there was little evidence of water deficit or overall loss of shoot growth.

Table 2: Effect of 7 days droughting upper roots of 23-day-old *Ricinus communis* on shoot freshweight and water content. The base of the upper pots had a 20 mm layer of perlag to help prevent upward movement of water from the lower, well-hydrated soil.

Day	Well-watered		Droughted	
	Fresh weight (kg)	% water content	Fresh weight (kg)	% water content
0	0.013	78.922	0.014	79.414
3	0.019	80.655	0.017	79.991
7	0.026	81.081	0.028	81.409

Means of 8 replicates with standard errors of < 10%.

Roots in the upper pots grew little over the 7 days in either well-watered or droughted treatments. In contrast, roots in the lower pots grew steadily in weight over this time. There was no obvious difference between roots in the lower pots in the two treatment (Table 3).

The results of this experiment showed that withholding water from the upper pots in which a water barrier was present in the base gave clear effects on stomata and leaf expansion but without any marked effect on overall shoot growth or water content. There was no clear influence on root growth or water content, this was even true for the upper root, unwatered roots. This indicates considerable water transport internally from lower to upper roots to maintain hydration. This would have been

expected to occur at night. The root weights shown in Table 3 were taken in the morning. It may well be that as the photoperiod progressed, the upper roots lost water content and set up root to shoot signals that closed stomata and slowed leaf growth.

Table 3: Effect of 7 days of drying upper roots of 23-day-old *Ricinus communis* with twin root systems on root freshweight and water content. The base of the upper pots had a 20 mm layer of perlag to help prevent upward movement of water from the lower well-watered pots.

	Duration of droughting (days)					
	0		3		7	
	Upper	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**
Well-watered						
Fresh-weight (g)	10.0	5.0	13.0	9.0	13.0	15.0
% water content	92.370	94.752	91.432	93.928	88.316	93.891
Droughted						
Fresh-weight (g)	11.0	4.0	10.0	9.0	13.0	15.0
% water content	92.205	94.51	89.349	94.196	87.848	93.891

Means of 8 replicates with standard errors of < 10%.

It is also possible that the roots actually present in the 20mm perlag layer were less drought stressed than those above, since they were in such close proximity to the wet soil of the lower pot.

The results so far led to two further questions:

- Is there a difference in the amount of water in roots around the perlag, and the rest of the roots in the upper pots as the soil in these pots dried?
- Based on the observation that stomata were only partially closed, were stomata capable of closing more fully when the stress was made more severe e.g. by drying all the roots?

The following experiment tried to answer these questions. Plants were grown as described in Chapter 2 but with the following modifications. Plants were divided in three batches: (a) well-watered plants where both the upper and lower pots were watered daily; (b) upper pot droughted, lower pot watered twice daily [d]; (c) water withheld from both [sd].

Stomata of the plants where both root systems were dried closed completely and promptly by the second day of the experiment (Fig. 18A) showing that severe stress was capable of stopping almost all transpirational loss of water from leaves. Stomatal conductances of the plants that received water in the lower pots only were not much lower than those of the control plants for the first 3 days but after the 4th day the conductance of these gently droughted plants were smaller than in well-watered plants (Fig. 18A).

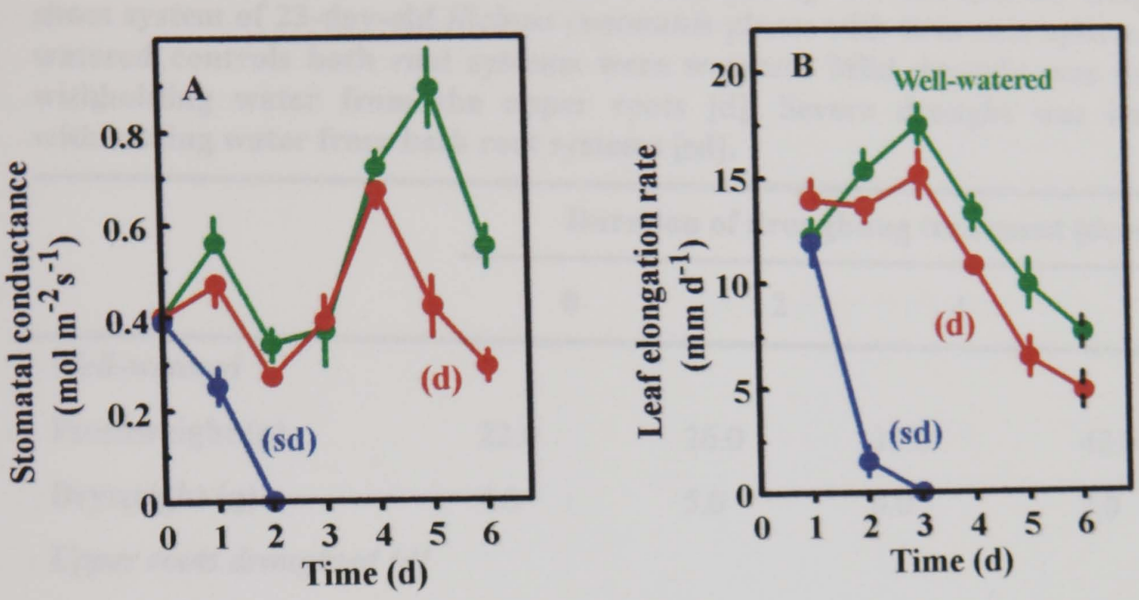

Figure 18 : The effects of droughting the upper root system [d], or both root systems [sd] on stomatal conductance of the youngest fully grown leaves (A), and elongation of the second youngest leaves (B) of 23-day-old *Ricinus communis* plants.

Similarly, leaf elongation was arrested by the severe droughting on the third and subsequent days. Drying only the upper roots slowed leaf expansion on day 2 and all later days (Fig. 18B) compared to the controls.

The water content of shoots of the severely droughted plant reduced by about 4% within two days of droughting. The plants were visibly and severely wilted by the third day and no further measurements were made. Water content of the well-watered and mildly droughted plants remained unchanged during the experiment (Table 4). The effect of mild drought treatment on root distribution and hydration were examined. The proportion of roots in the lower pots of the well-watered and the droughted plants remained similar throughout the experiment. When the water content of roots in the upper, droughted, pots and the lower, watered pots were determined upper roots were slightly less hydrated then lower ones (Table 3)

Table 4: Effect of different intensities of droughting on fresh and dry weights of the shoot system of 23-day-old *Ricinus communis* plants with twin root systems. In well-watered controls both root systems were watered. Mild drought was imposed by withholding water from the upper roots [d]. Severe drought was imposed by withholding water from both root systems [sd].

	Duration of droughting treatment (days)			
	0	2	4	6
Well-watered				
Freshweight (g)	22.0	26.0	35.0	42.0
Dryweight (g)	4.0	5.0	6.0	7.0
Upper roots droughted [d]				
Freshweight (g)	22.0	27.0	33.0	39.0
Dryweight (g)	4.0	5.0	6.0	7.0
Both roots drought [sd]				
Freshweight (g)	22.0	27.0		
Dryweight (g)	4.0	6.0		

Means of 8 replicates with standard errors of < 10%.

Table 5: Effect of withholding water from the upper root system of 23-day-old Ricinus communis plants for up to 6 days on the percentage water content of roots in the upper and lower pots, in roots in the basal perlag layer of the upper roots and in the roots in soil above the perlag layer.

	Duration of drought treatment (days)			
	0	2	4	6
	Percentage water content			
Roots of lower pots				
Well-watered	94.12	94.44	93.68	94.35
Droughted	94.12	94.44	95.00	93.63
Roots of upper pots				
Well-watered	91.17	92.32	91.11	91.74
Droughted	91.17	90.84	89.58	88.42
Roots of upper pots				
Perlag layer		91.67	91.89	92.22
Above perlag layer		90.00	87.27	84.62

Means of 8 replicates with overall standard error of < 10%.

However, when roots in the perlag base were separated out from the roots above, it was clear that they were as fully hydrated as roots in the lower, watered pot (about 92%). However, roots in the soil above were much drier, with a mean water content of 84.6% after 6 days. Clearly, the absence of direct watering the upper pot did result in considerable water deficits developing in the bulk of the upper root system. These data are reworked for greater clarity in Fig. 19 where root dryweight is expressed as a percentage of freshweight.

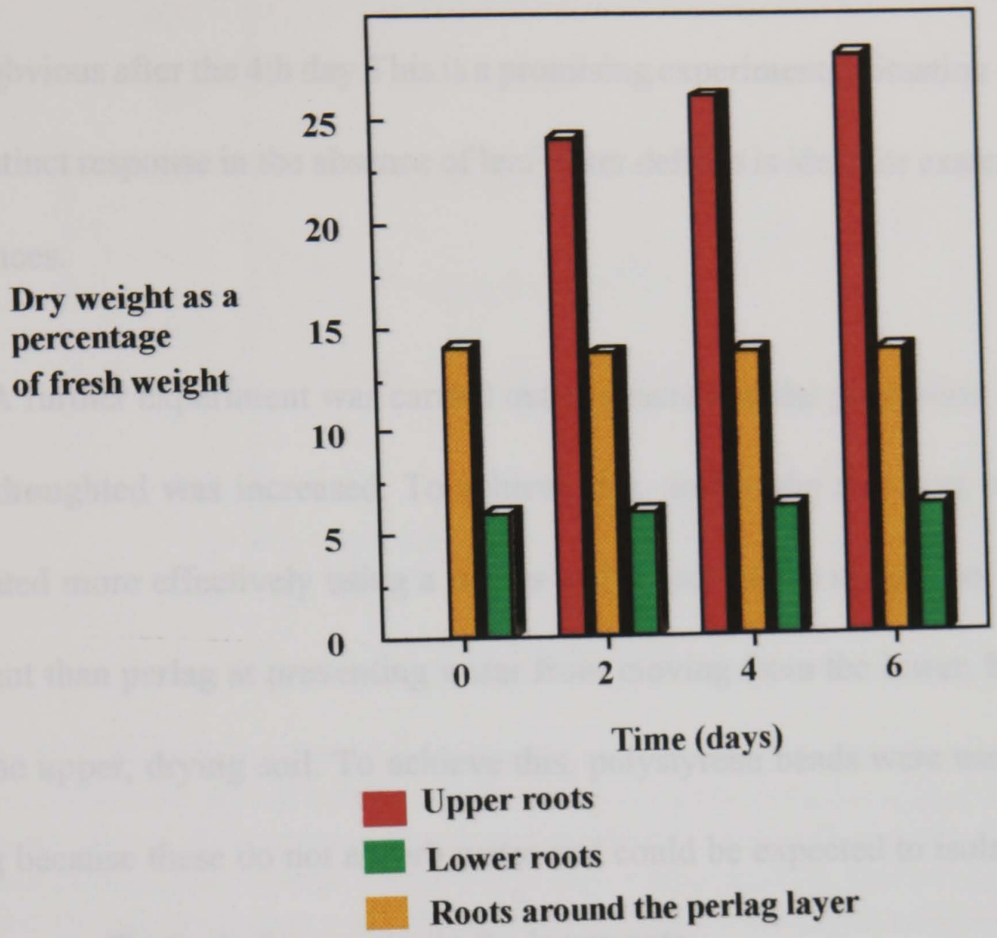

Figure 19 : The effect of withholding water from the upper root system for up to 6 days on the level of hydration of upper roots in 23-day-old *Ricinus communis*.

The first conclusion that can be drawn from the above experiment is that stomata of *Ricinus communis* are capable of complete stomatal closure if the drought is sufficiently severe. Leaf elongation is also sensitive to severe droughting, but less so, because on the second day when stomata were already closed and the leaves were wilted, the second youngest leaf still showed some growth, though much reduced. The slower response of the mildly droughted plants was perhaps because of the smaller number of roots that were droughted. The remaining well-hydrated roots of these plants were supplying shoots with sufficient water to maintain stomatal opening and

leaf elongation, although at reduced rates. In this experiment approximately half of the overall root system was droughted. The response developed slowly, becoming more obvious after the 4th day.This is a promising experimental situation since a slow but distinct response in the absence of leaf water deficits is ideal for examining causal influences.

A further experiment was carried out to ensure that the proportion of roots that were droughted was increased. To achieve this, soil in the two pots needed to be separated more effectively using a barrier at the base of the upper pots that is more efficient than perlag at preventing water from moving from the lower, hydrated soil into the upper, drying soil. To achieve this, polystyrene beads were used instead of perlag because these do not absorb water and could be expected to isolate the upper roots more effectively from water in the lower pots.

Two sets of leaves were used for the measurement of elongation rates. These were the youngest leaf (leaf number 5 just emerging) and the second youngest leaf (leaf number 4). Roots in the upper pots of the control and the droughted plants were divided into (a) portion of roots around the polystyrene layer; (b) portion of roots above the polystyrene layer.

Stomatal conductances of plants with droughted upper pots were smaller than well-watered plants throughout the experiment which lasted 10 days. Differences in conductances between the well-watered and the droughted leaves varied from 13% to 58%, reaching the largest differences on days 9 and 10 (Fig. 20).

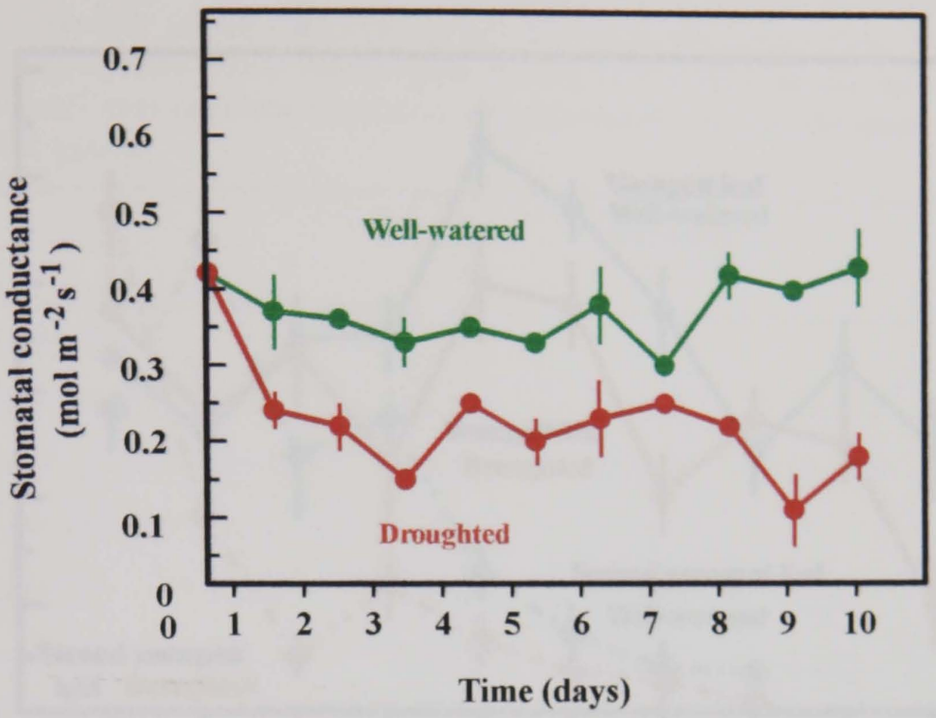

Figure 20 : Stomatal conductance of the youngest fully grown leaves of 25-day-old *Ricinus communis* plants when roots in the upper pots were droughted for 10 days. In the upper pots a 20 mm layer of polystyrene beads, rather than perlag, was placed at the base of the upper pots to help prevent water moving from the lower, well hydrated soil into the upper, drying soil. Means of 8 replicates with a standard errors.

As seen in Fig. 21, elongation rates of the youngest and second youngest leaves were different during the experiment. The youngest leaves gave a sigmoidal growth curve with elongation rates reaching a maximum on day 5 before decreasing during the rest of the experiment. Elongation rates of the leaves of plants with droughted upper roots showed a similar pattern of growth, although at much slower rates. Expansion by the second youngest leaves (4th leaf) slowed throughout the experiment in both the well-watered and the droughted plants but the decline was much more extensive in the latter. Similar trends were obtained in earlier experiments (Fig. 12b, 14, 16, 18a).

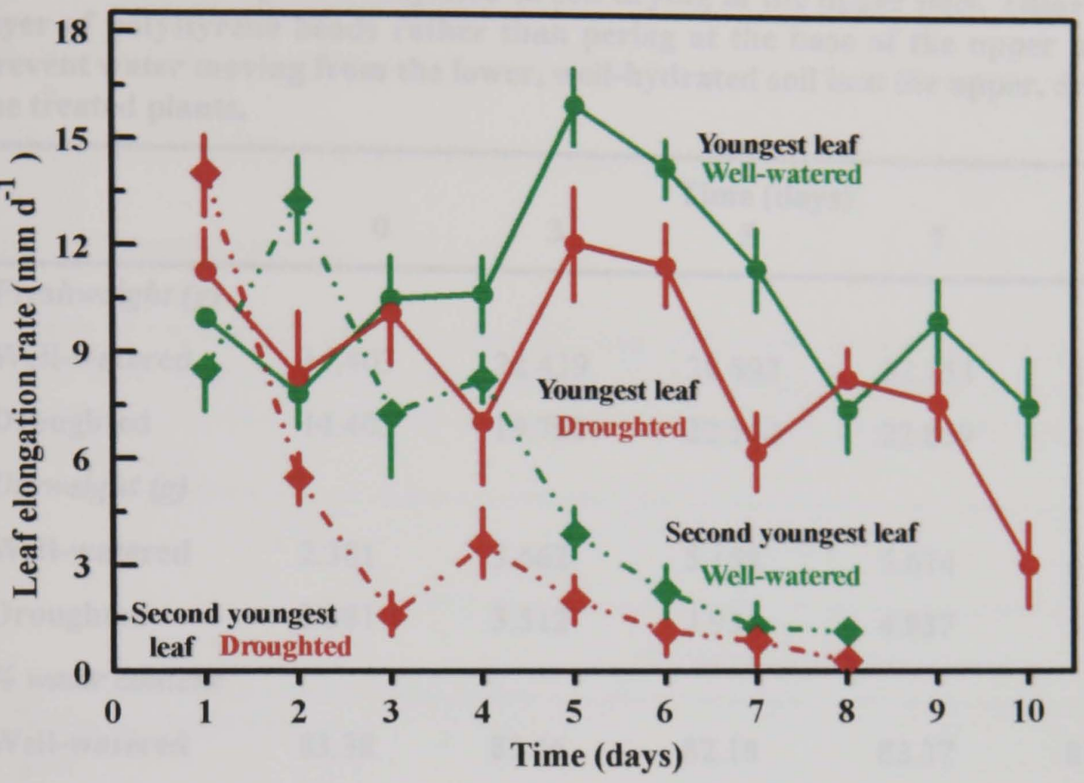

Figure 21 : Elongation rates of the youngest and the second youngest leaves of 25-day-old *Ricinus communis* plants the upper roots of which were subjected to drying over 10 days. In the upper pots a 20 mm layer of polystyrene beads, rather than perlag, was placed at the base of the upper pots to help prevent water moving from the lower, well hydrated soil into the upper, drying soil. Means of 8 replicates with standard errors.

While the percentage water content of the shoot of both well-watered and droughted plants remained unaltered, there was a significant reduction in the fresh and dry weight of the plants with their upper roots in drying soil ($P<0.05$) unlike the observation made in an earlier experiment (compare Tables 4 and 6). The only difference between the two set-ups was that the basal perlag layer in the earlier one was replaced by polystyrene layer to help prevent water moving from the lower, well-watered pots into the upper drying soil.

Table 6: Fresh and dry weights and percentage water content of shoots of 25-day-old *Ricinus communis* plants subjected to soil drying in the upper pots. These pots had a layer of polystyrene beads rather than perlag at the base of the upper pots to help prevent water moving from the lower, well-hydrated soil into the upper, drying soil in the treated plants.

	Time (days)				
	0	3	5	7	10
Freshweight (g)					
Well-watered	14.403	22.439	28.892	32.153	37.086
Droughted	14.403	19.750	22.564	22.859	31.587
Dryweight (g)					
Well-watered	2.381	3.662	5.158	5.674	6.694
Droughted	2.381	3.512	3.934	4.837	5.947
% water content					
Well-watered	83.38	83.66	82.18	82.37	81.95
Droughted	83.38	82.19	82.30	81.21	81.17

Means of 8 replicates with <10% standard error.

Percentage water content of the lower roots in both the well-watered and the droughted plants remained constant throughout the experiment at 92-95% (Table 7). As expected, there was a gradual reduction in the percentage water content of roots in soil above the polystyrene layer in droughted pots from 92% to 82%. However, the water content of roots in the isolating layer of polystyrene beads was not reduced by droughting the pot and remained at values close to those of roots in the lower, watered pot. Because of this, the extent of drying the soil above the polystyrene layer was underestimated if all roots in the upper pots were grouped together. There is a problem

in interpreting all the percentage root water content data in this and previous experiments. There is also a risk that all the roots re-hydrated to some extent during the brief washing required to separate them from the soil, but there is no alternative method that would avoid this water uptake problem which is likely to uplift values most for droughted roots.

Table 7: Percentage water content of roots of *Ricinus communis* (25-day-old) with twin root systems. Both root systems of the plants were either watered (well-watered) or watering of the upper root system was stopped for up to 10 days (droughted).

	Time (days)				
	0	3	5	7	10
	% water content				
Roots in the lower pots					
Well-watered	92.95	94.56	93.92	93.95	95.18
Droughted	92.95	93.68	93.63	94.27	92.99
Roots in the upper pots					
Well-watered:					
polystyrene layer	92.92	91.99	91.68	91.68	89.13
above polystyrene layer	92.59	92.23	91.08	90.88	94.55
overall	92.76	92.11	91.28	91.28	91.84
Droughted:					
polystyrene layer	92.92	90.18	91.44	90.87	90.70
above polystyrene layer	92.59	89.37	88.19	87.25	82.45
overall	92.76	89.78	90.32	89.06	86.58

Means of 8 replicates with <10% standard error.

The lower stomatal conductances of leaves of the droughted plants indicated some stomatal closure during the droughting period. This was accompanied by a reduction in leaf expansion rates of the youngest growing leaves of the droughted plants. The difference in the two sets of leaves was most likely due to the stage at which leaf expansion was measured. For the youngest growing leaves elongation was measured following emergence, hence a stage of rapid growth was seen reaching a maximum on day 5 before growth rates gradually decreased. Thus a sigmoid curve, typical of growth, was obtained. In the second youngest leaves, on the other hand, the initial stage of rapid growth was missed before taking measurements of leaf expansion. As in the previous experiments the slowing-down phase of expansion was maintained. However, in both the cases the retarding effect of drying roots in the upper pots on elongation of the leaves was still visible.

There was no apparent change in the shoot water content of the droughted plants throughout the experiment; the well-watered lower roots supplying adequate water to maintain shoot hydration.

Not watering the upper roots reduced root water content from 92% to 82% in roots above the polystyrene layer but, as in the case of the perlag layer, roots in the polystyrene layer remained fully hydrated. These results indicate that roots around the polystyrene layer should be considered as unstressed lower roots. It seems that although the polystyrene layer inhibits movement of water from the lower well hydrated pots into the upper drying pots, this is not sufficient to prevent roots within

the layer from obtaining water by this means. At the end of the droughting experiment there was twice as much roots in the upper pots (i.e. roots above the polystyrene layer) of the well-watered plants (18.215 g) as in the droughted plants (9.486 g), indicating a definite inhibition of root growth in this region from where water was withheld.

Ten days of droughting of roots in the upper pots has the following influences on the pattern of growth. Total plant dry matter accumulation was inhibited by 3-4%. However, within this figure root mass was stimulated by about 11%, while shoot mass decreased by about 14%. The promoting effect of root growth was largest in the lower, well watered pots. There was an overall increase in root:shoot ratio from 0.45 to 0.57. These effects were brought about by a modest decrease in the hydration of upper roots.

As in the previous experiment, approximately 50% of the roots were exposed to drying at the beginning of the drought treatment. However, drying the upper roots promoted root growth in the lower pots of the droughted plants so that at the end of the experiment 61% of the roots of the well-watered plants were found in the lower pots while 70% of the roots was found in the lower pots of the droughted plants.

From the previous two experiments it can be seen that both perlag and polystyrene beads at the base of the upper pots were sufficient as a barrier to upward movement of water to cause water deficits in the majority of roots in droughted pots. Drying approximately 50% of the roots of *Ricinus communis* was sufficient to bring

about a reduction in stomatal conductance and leaf expansion, in the absence of gross changes in the shoot water content. Thus, the twin root system with polystyrene as the isolating layer was adopted in all subsequent experiments to study signalling by drying roots.

General Discussion

Split root systems where one root system is always fully watered, allows the study of putative 'messages' arising from a second stressed root system and transported to the shoot. Using such a system, hydraulic messages can almost be entirely eliminated to allow the study of chemical messages alone. Such approaches have a long history.

In the twin-root system set up designed in this chapter, the idea of a split root system has been refined so that both the upper and the lower roots have been grown in the same medium and drying is imposed naturally around the upper roots of known mass, while the rest of the root system supplies water to retain shoot hydration. Since both the root systems are present in the same kind of growing medium it eliminates any possible difference between the structure or water conductance pathways in the these roots. Passioura (1988) argues that when roots grown hydroponically are placed in pressure chambers and pressurized the intercellular spaces in the cortex of the roots become infiltered and therefore become abnormal conduits for the longitudinal transport of water. This artefactual longitudinal transport is not a problem in roots growing in soil with a large air-filled porosity because most of the gas-filled intercellular spaces survive the application of pressure.

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Chapter 4 : WATER RELATIONS OF SHOOTS OF DROUGHTED PLANTS

Introduction

In Chapter 3, the effect of drying the upper root system of *Ricinus communis* grown in twin pots was shown to promote stomatal closure and inhibit leaf expansion. These effects were seen in the absence of any loss in the water content of the shoot, strongly suggesting that they may be brought about by non-hydraulic signals from drying upper roots. Water content is a crude measure of shoot hydration, and taken only once a day, is inadequate in separating the hydraulic and non-hydraulic effects as drought stress develops. To investigate the possibility of non-hydraulic signalling in these plants, water relations was studied in more detail.

In this Chapter, a detailed study of the effect of drying soil around upper roots of *Ricinus communis* over 48 to 96 h was carried out. It involved measuring stomatal conductance, leaf elongation rate and leaf water potential [leaf water potential (Ψ_L) is almost always negative and is the algebraic sum of the component potentials arising from the effects of turgor (ψ_p), solutes (ψ_s), and matrix (ψ_m)]. Plant tissues, such as leaves, comprise 80-90% water and in such tissues matrix potential is negligible and therefore ignored as a variable of significant influence on water potential (Kramer, 1983). So, leaf water potential is influenced mainly by leaf solute potential and leaf turgor. Leaf water potential is acceptable as a fundamental measure of plant water status that is related to the water content of the tissue and other components which

describe the free energy attributable to water (Meidner and Sheriff, 1976). Moreover, water potential is a more sensitive measure of the change in water relations than is tissue water content. In a fully turgid tissue, the initial decrease in tissue water content causes large reductions in water potential. Changes in Ψ, ψ_p, and ψ_s as tissue water declines are described in a classical Höfler diagram (Kramer, 1983). Leaf and shoot water potentials have been measured in previous studies involving drying part of the root system (Saab and Sharp, 1989; Gowing *et al.*, 1990; Ebel *et al.*, 1994), but in these studies only a few measurements have been made over the duration of the experiment, which can be many days. Such scattered measurements may overlook hydraulic changes occurring within the root and shoot and that may influence shoot physiology.

Hydraulic conductivity of the whole root system was determined during drying of the upper roots to observe its effect on root permeability to water as soil in the upper pot became depleted of water. A detailed study of shoot water relations together with root Lp should give a good indication of the processes occurring in the whole plant as drought stress sets in and help assess the relative contributions of hydraulic and non-hydraulic signalling during stomatal closure and slowing of leaf expansion as plants experience drought.

Method

Plants were grown in twin pots as described in Chapter 2. Drought was imposed on six, 35-day-old *Ricinus communis* plants for 48 to 96 hours. Stomatal conductance

was measured every 3 hours for the first 12 hours. Using a second set of six plants, stomatal conductances of leaves of two different ages (youngest and second youngest fully expanded leaves) were measured twice daily over 3.5 days of drying the upper roots. Leaf elongation rate was measured every 4 hours for the first 12 h, then finally after 24 h of drought treatment. Using a separate set of plants, leaf elongation rate was measured twice daily for 2.5 days of drought treatment. Leaf water potential was measured twice daily, once between 4-5 hours and again between 9-10 hours into the 16 h photoperiod over 2.5 days. In another experiment, leaf water potential was measured once daily for 4 days. Leaf solute potential was also measured at the same time using leaves from different plants. Root hydraulic conductivity was measured once daily over 3 days of droughting the upper roots.

Results

Stomatal conductances of the abaxial surfaces of leaves of two different ages (the youngest and the second youngest fully expanded leaves of the same plant) were found to respond similarly to drying upper roots. Fig. 22A shows that 3.5 days of drying upper roots (when soil water potential in the upper pots would have been less than -1.1 MPa) reduced stomatal conductance on most days, particularly in the morning, in the youngest leaves, although this reduction was not significantly different ($P>0.05$) except on the morning of day 3. Similarly, no significant difference was found in the stomatal conductance of the abaxial surface of the second youngest leaves of the droughted plants ($P>0.05$) (Fig.22B). However, towards the end of the

experiment a greater difference between conductances of droughted and control plants was seen in the youngest leaves while the stomata of the second youngest appeared to be less responsive to root drying even 3.5 days into the drought stress treatment. The youngest fully expanded leaves were used to measure stomatal conductance over the first 12 h of drying in more detail. Soil water potential would have reached approximately -0.6 MPa.

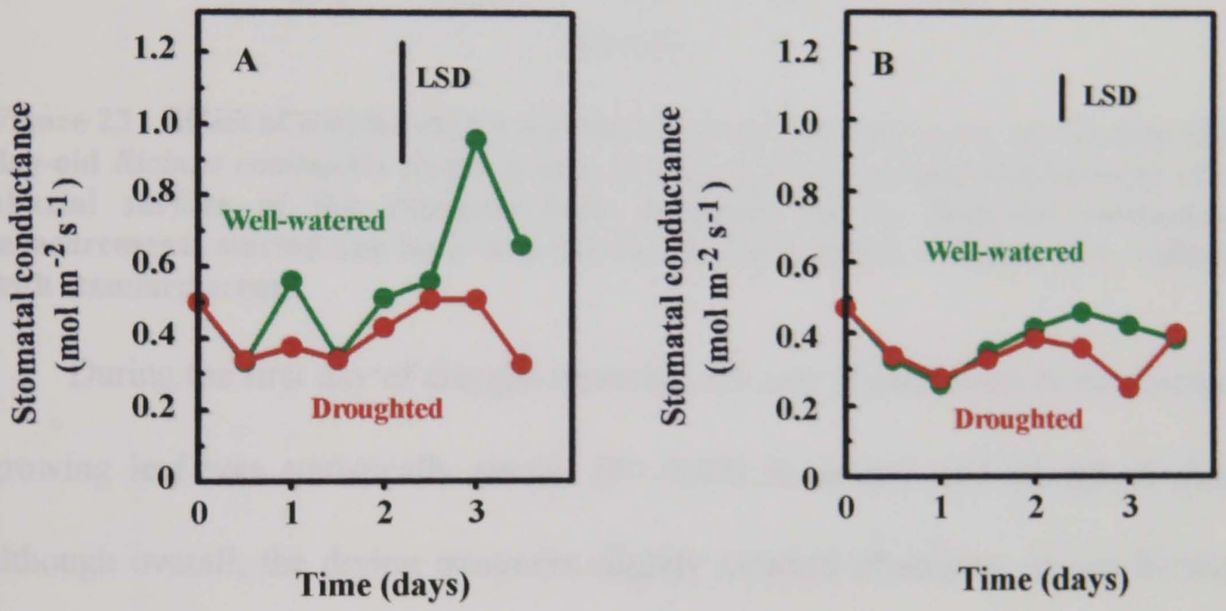

Figure 22 : Effect of drying the upper roots of 35-day-old *Ricinus communis* plants grown in twin pots on stomatal conductance of the abaxial surface of (A) the youngest leaf, (B) the second youngest leaf. Means of 6 replicates with LSD.

Stomata of the well-watered plants showed slightly increased conductance 4 hours into the photoperiod which then declined over the remaining 9 hours. Stomatal conductance of the youngest fully expanded leaf of six plants was measured every 3 hours for the first 12 hours as drought developed in the upper pots. However, there was no statistically significant difference between stomatal conductances of the two sets of plants ($P>0.05$) over this time (Fig. 23).

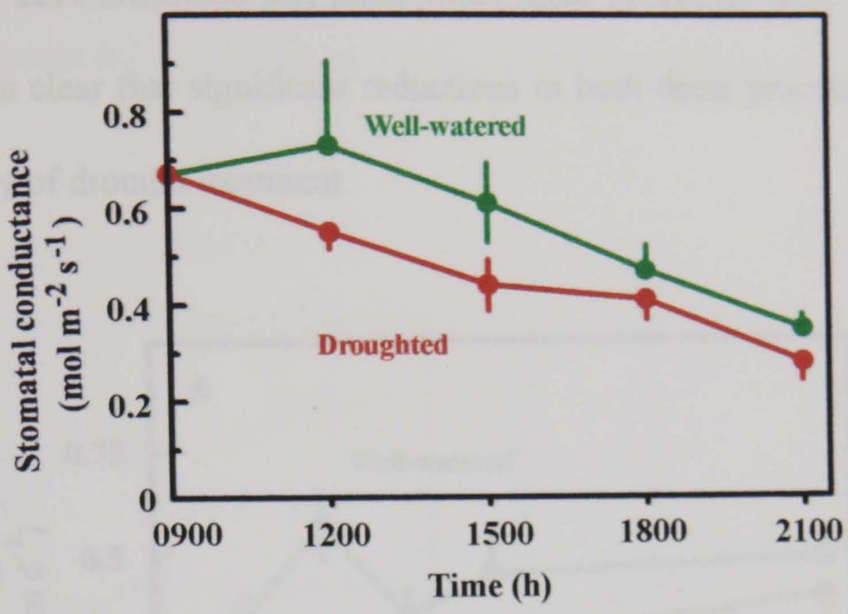

Figure 23 : Effect of withholding water for 12 hours from the upper root system of 35-day-old *Ricinus communis* plants grown in twin pots on stomatal conductance of the abaxial surface of the youngest fully expanded leaves. Stomatal conductance measurements started one hour into the 16 hour photoperiod. Means of 6 replicates with standard errors.

During the first day of drought treatment the rate of expansion of the youngest growing leaf was statistically similar ($P > 0.05$) in control and droughted plants although overall, the drying treatment slightly reduced expansion, except between 1200 h and 1500 h when these differences were larger (Fig. 24 A). Twenty four hours later, at soil water potential of almost -1.0 MPa, (0900 h the next day) rate of expansion of the leaves had increased somewhat in both sets of plants and still there was no significant difference between the treatments. In a separate set of six plants, leaf elongation rates of the well-watered plants were found to be slightly higher than in the droughted plants during the 2.5 days of droughting but this difference was statistically insignificant, except in the afternoon of the second day (Fig. 24B). As mentioned at the beginning of this Chapter, shoot processes such as stomatal

conductance and leaf expansion respond to drying upper roots after a delay of 2-3 d. Both, Fig. 22A illustrated this point when these processes were looked at in more detail. It is clear that significant reductions in both these processes begin after the second day of drought treatment.

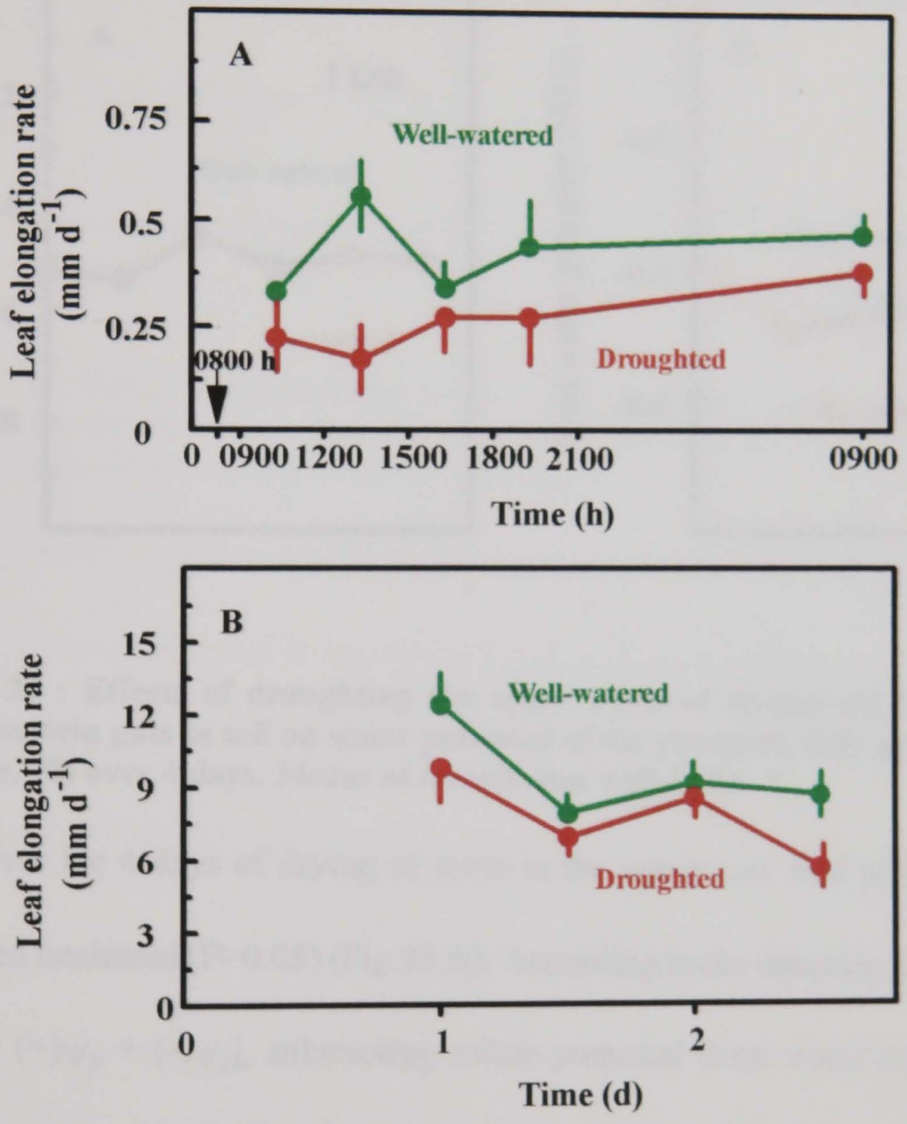

Figure 24 : Effect of droughting the upper roots of 35-day-old *Ricinus communis* grown in twin pots on elongation rate of the youngest, growing leaf (A) during the first 24 hours of droughting, (B) during the first 2.5 days of droughting.

In one experiment leaf water potential was measured six times during the first two photoperiods after starting drought treatment to the upper roots. Readings in

control plants were stable at approximately -0.5 MPa. The results from treated plants were indistinguishable from those of control plants (Fig 25A). A similar picture was obtained when daily readings were taken over 4 d of drying treatment (Fig. 25B).

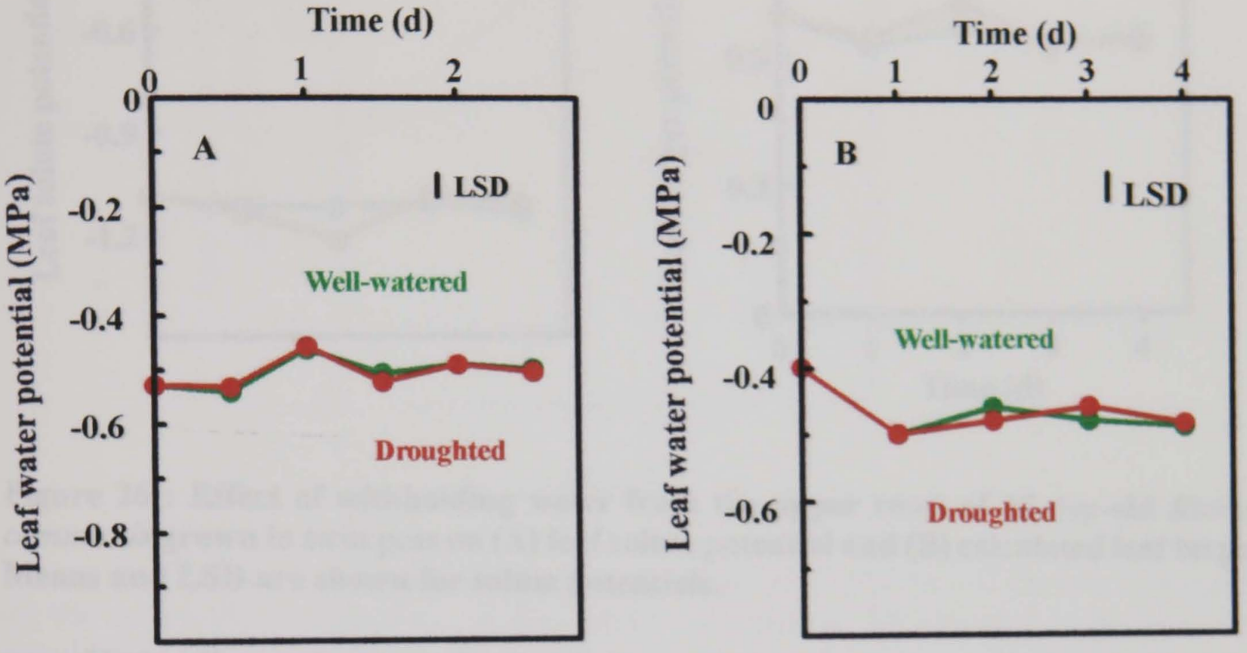

Figure 25 : Effects of droughting the upper roots of 35-day-old *Ricinus communis* grown in twin pots in soil on water potential of the youngest, fully grown leaf (A) over 2.5 days, (B) over 4 days. Means of 6 replicates with LSD.

Over the 4 days of drying of roots in the upper soil, leaf solute potential also remained unaltered ($P>0.05$) (Fig 26 A). According to the equation for water potential [$(-)\Psi = (+)\psi_p + (-)\psi_s$], subtracting solute potential from water potential gives the turgor component of the tissue. From the water potential and solute potential measurements obtained here leaf turgor was calculated for the 4 days of droughting (Fig 26 B) and was found to be unaltered during this time.

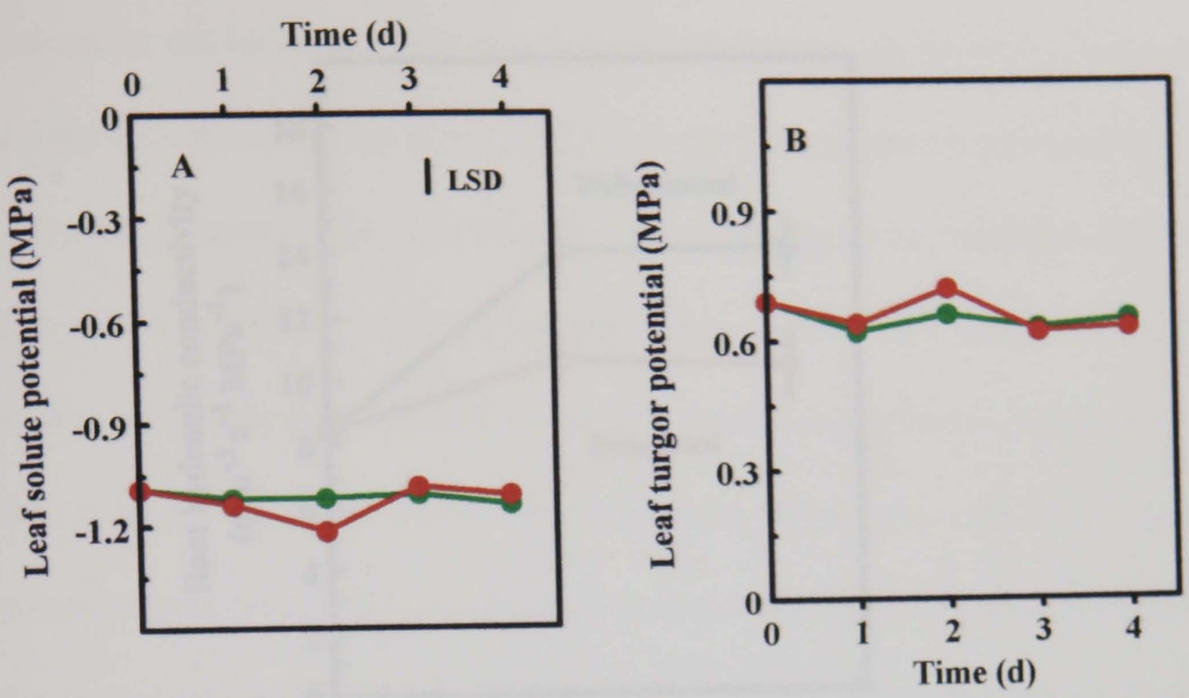

Figure 26 : Effect of withholding water from the upper roots of 35-day-old *Ricinus communis* grown in twin pots on (A) leaf solute potential and (B) calculated leaf turgor. Means and LSD are shown for solute potentials.

Hydraulic conductivity (Lp) of the root system as a whole was determined over 2 days of droughting and it was found that Lp of the whole root system of the plants with upper roots drying was reduced by about 30%, a statistically significant reduction ($P<0.05$) (Fig. 27).

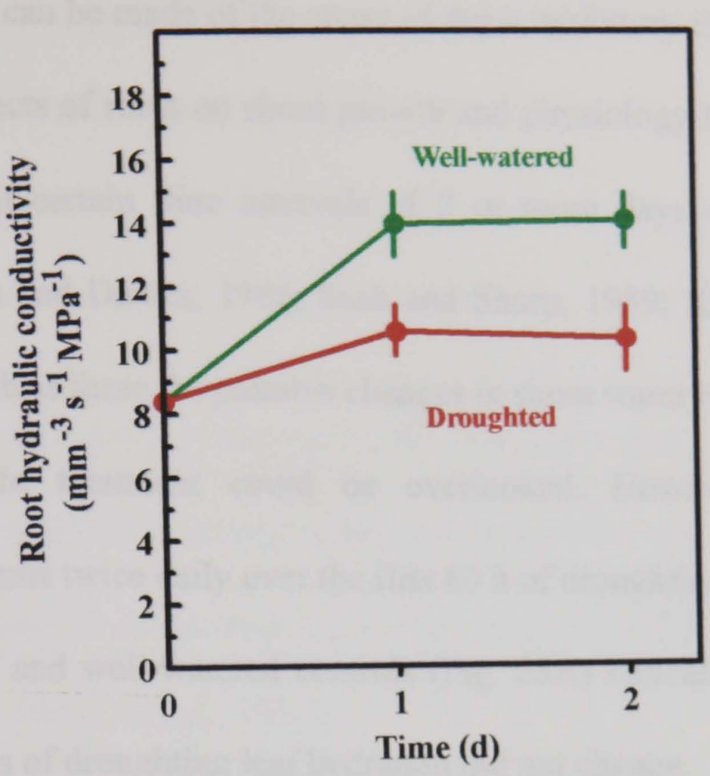

Figure 27 : Effect of 2 days of drying the upper roots of 35-day-old *Ricinus communis* on the hydraulic conductivity of the whole root system. Means of 6 replicates with standard errors.

Discussion

Drying upper roots of *Ricinus communis* clearly shows reduced stomatal conductance and inhibition of leaf elongation within 2 - 3 d (Fig. 20 & 21,Chapter 3). This has been found to occur in the absence of any significant loss in shoot water content. But shoot water content is not a sensitive measure of shoot water relations, therefore, a more detailed look at water relations of the shoot was necessary. One possibility for the delayed shoot responses could be that as roots in the upper pots start to dry, the shoot could be experiencing some dehydration. Reduced leaf water potentials would induce ABA synthesis and release within the leaves which could then influence these

processes. A careful documentation of shoot water status is therefore crucial before inferences can be made of the cause of these inhibitory effects. Many studies on the drying effects of roots on shoot growth and physiology have determined leaf water potential at certain time intervals of 2 or more days after drought stress began (Blackman and Davies, 1985; Saab and Sharp, 1989; Khalil and Grace, 1993). In studies such as these the possible changes in shoot water relations at the beginning of the drought treatment could be overlooked. However, leaf water potential measurements twice daily over the first 60 h of droughting showed no differences in the treated and well-watered controls (Fig. 25A) indicating that indeed during the early stages of droughting leaf hydration did not change.

In this study, both shoot water content (Chapter 3) and leaf water potential (Fig. 25) of plants with their upper roots in drying soil were found to remain at control levels, suggesting no perturbations to water relations of the shoots. However, water potential can remain unaltered although the other parameters of shoot water relations may change which may in turn affect shoot growth and physiology. In other words, leaf solute potential and turgor may change in concert without there necessarily being any change in leaf water potential. The importance of determining these components of water potential was seen in the work of Gallardo *et. al.* (1994) where they found no difference between Ψ_L of lupin plants with partially dried and fully dried roots during the first 15 days of drought treatment. Seemingly it was found that Ψ_L remained unchanged in the leaves of severely dried plants because as soon as drying began, leaf

ψ_s began to decrease and leaf ψ_p increased. However, drying upper roots of *Ricinus communis* did not alter leaf water potential (Fig. 25) or solute potential (Fig. 26A), and therefore leaf turgor (Fig. 26B), calculated by subtracting ψ_s from Ψ_L, remained unaltered. Thus, no change in the gross tissue water relations of the leaves was brought about by drying the upper root system.

Hydraulic conductivity (Lp) of the whole root system of *Ricinus communis* was reduced by about 30% during 2 days of drying of the upper roots. Drying whole roots has been found to reduce root Lp in other species (Drake and Carr, 1978; Nobel and Sanderson, 1984; Jokhan, 1990).

The results indicate overall that the twin root system set-up using, *Ricinus communis*, enables part of the roots to be dried to prompt physiological changes within the shoot that develop in the absence of marked changes in the water relations of the shoot. It seems unlikely, therefore, that the slowing of leaf expansion and partial closing of stomata that drying about 50% of the roots brings about are simply the result of water shortage being sensed directly by leaves. Instead, the results point to some other means of communicating water deficiency at the roots and of ultimately changes in leaf growth and stomatal behaviour. Similar conclusions from twin-root experiments have ben drawn by Blackman and Davies, Neales *at al.*, 1989; Bano *et. al.*, 1993; Khalil and Grace, 1993. Experiments where whole root systems have been dried slowly from the top soil profile also point to this conclusion (Zhang and Davies, 1989; 1990a, 1990b; Blum *et. al.*, 1991).

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Chapter 5 : QUANTIFYING CHEMICAL SIGNALS FROM PLANTS WITH DRYING UPPER ROOTS

Introduction

Drying upper roots of *Ricinus communis* increases stomatal closure and inhibits leaf expansion. In this study it has been found that although these physiological processes are affected slightly soon after drying of the soil in the upper pots begins, significant reductions in these processes occur only 2-3 days after the onset of drought stress (Chapter 4); these changes being brought about in the absence of any change in shoot hydration. In recent years, much research has focused on physiological changes such as stomatal closure and reduced leaf expansion brought about by mild drought stress since it has been shown by others that, as soil starts to dry, regulatory effects can be exerted on the shoots prior to any detectable changes in shoot water status (Bates and Hall, 1981; Zhang and Davies, 1987; Saab and Sharp, 1989; Schurr *et. al.*, 1992). Seeing that regulatory effects, such as slower cell division and growth and reduced stomatal conductance, can take place in the absence of detectable losses in shoot hydration, a chemical rather than an hydraulic control of these physiological processes is indicated. This suggests that a chemical message or signal arises in the drying roots and moves in the transpiration stream to the shoot as a positive message. It is believed that abscisic acid (ABA) moving in the transpiration stream from roots to shoots is one such message that slows leaf expansion and closes stomata in plants experiencing root drying. Concentrations of ABA in the xylem sap of droughted

plants have been measured on a number of occasions (Lachno 1983; Cornish and Zeevaart, 1985; Zhang and Davies, 1987; Neales *et. al.*, 1989; Tardieu *et. al.*, 1992; Bano *et. al.*, 1993; Masia *et. al.*, 1994). Increases in xylem sap concentrations associated with soil drying have been interpreted as a measure of increased ABA passing to the shoots from drying roots. However, this approach can be misleading. The sap sampled may not always be the same as actual transpiration fluid and the concentrations therein can vary, not only in response to changes in the amount of the hormone entering the transpiration stream, but also in response to changes in sap flow rate through dilution (Jackson, 1993; Else *et. al.*, 1994). The latter is problematic since methods for obtaining xylem sap so commonly involve collection when flow is slower than transpiration. Furthermore, droughting reduces transpiration rate, thus reducing ABA dilution. In these circumstances, increases in concentration of solutes such as ABA in sap samples will be, in some measure, a result of less dilution rather than an increase in amount transported. Clearly, if droughting roots really do generate an ABA message that initiates and sustains stomatal closure and suppresses transpiration, the message must take the form of increased export of ABA rather than simply a higher concentration in the transpiring solvent.

The first part of this chapter describes an approach to replacing the concentration term with various expressions of delivery rate as a means of quantifying reliably the influence of drought on the putative ABA message transmitted from drying roots into the base of shoots (Jokhan *et. al.*, 1996). Droughting is known to

enhance root growth relative to shoots (Passioura, 1988; Sharp and Davies, 1989). As a result, absolute sizes of root and shoot systems and the root:shoot ratio may change (Chapter 3). However, little or no account is normally taken of the amounts of root and shoot tissue when comparing ABA transport from roots to shoots of droughted and well-watered plants. In this chapter the influence of the size of root and shoot are taken into account by expressing ABA delivery in several alternative ways. Each gives a different perspective on hormonal intercommunication between below- and above- ground parts.

Ethylene is a hormone accumulated by plants under stress especially water logging (Turkova, 1944; Kawase, 1972, Jackson, 1993). Its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) levels have been studied in response to flooding and the results show that there is an increase of ACC transport in the transpiration stream during waterlogging (Bradford and Yang, 1980; Else *et. al.*, 1995). Such an increase in droughted plants would be of great interest since ethylene derived from this ACC might also explain the slower leaf expansion of droughted *Ricinus communis*. A number of publications suggest that drying roots cause ethylene synthesis in leaves, probably increasing the synthesis of ACC (El-Betagy and Hall, 1974; McKeon *et. al.*, 1982; Wang *et. al.*, 1990). These claims have usually used some method whereby detached leaves were used to measure ethylene. The study of Morgan *et.al.* (1990) however, showed that when intact plants were used, soil drying failed to stimulate ethylene synthesis, and suggested that studies should be carried out

using intact plants rather than excised parts to avoid ethylene production due to injury. In the present work the possibility was tested that drying roots export increased amounts of ACC to the shoots.

Nitrogen is an essential mineral required by plants for growth and much of it is obtained from soil in the form of nitrate. From studies with sunflower plants it has been shown that along with abscisic acid, pH, phosphate and nitrate could potentialy contribute to root-to-shoot communication during soil drying (Schurr and Schulze, 1996). As the soil dries, water flow through the soil slows and this in turn changes the water status of the growing region of the root (Passioura, 1984). A change in root water status will affect active ion uptake by the roots (Boyer, 1985) which can alter ion concentrations in the xylem sap (Schulze and Bloom, 1984). However, a change in ion concentration in the xylem sap can also result from a lack of dilution as stomata of these stressed plants close. Although Gollan *et. al.* (1992) showed reduced nitrate concentrations in xylem sap obtained from intact sunflower plants in drying soil, the work of Schurr and Schulze (1996) failed to find any change in nitrate concentration in the xylem sap from intact plants of *Ricinus communis*. Delivery rates of nitrate rather than concentrations may thus be a preferable expression of changes in nitrate output by the roots. Accordingly, the delivery rates of nitrate in *Ricinus communis* plants with upper drying roots were measured.

ABA in phloem was also determined. ABA is a weak acid with a dissociation constant of 4.8. It dissociates in the following way:

$$HABA + H_2O \equiv\equiv\equiv ABA^- + H_3O^+$$ (Slovik *et.al.*, 1992)

In its protonated form (HABA) ABA is very permeable and can move easily across membranes. The anion (ABA^-) is impermeable (Hartung, 1983; Hartung and Slovik, 1991). Phloem is more alkaline compared to the other components of the leaf. Since HABA is very permeable it can preferentially move along a pH gradient into the phloem, dissociate here and the ABA^-, being impermeable, become trapped in this alkaline compartment. If this actually happens *in planta* then any increased ABA output from the roots may eventually become trapped in the phloem and this should become detectable as the plants are droughted. In more general terms, phloem ABA may be a sensitive indicator of ABA enrichment of shoot tissues.

Methods

Basic methods are as described in Chapter 2 but with some modifications. Thirty-day-old *Ricinus communis* plants grown in twin root systems were subjected to soil drying in the upper pots for up to 4 days. Soil water potential in the upper, drying pots reached -1.2 to -1.3 MPa by this time. Xylem sap was collected from six detopped plants every 3 hours on the first day for ABA analysis, and then once daily over the next 3 days. Whole plant transpiration rate was determined for each plant before the shoot was excised and the detopped plant placed in a pressure chamber. Xylem sap

was made to flow at rates similar to those occurring during whole plant transpiration by adjusting the pressure. Sap exuding for the first 5 minutes was discarded and approximately 1.5 ml sap was then collected for analysis of ABA, ACC and NO_3^-. A different set of plants were droughted for up to 3 days and used to determine ABA delivery to illustrate the various expressions of delivery rate. Root freshweight and leaf areas of these plants were also taken. Phloem sap was collected from a separate set of plants by making a light incision on the stem approximately 50 mm above the soil surface, as described by Hoad (1973). The bleeding sap was collected initially at different time intervals from the time of incision, using microcapillary tubes, to check if there was a critical time after the incision when sap had to be collected. It was found that sap collected over the first 10 minutes after incision was made had similar levels of ABA. After 10 minutes the wound either stopped bleeding or ABA levels in the sap collected became low (data not shown). Thus, phloem sap was collected from the plants within the first 10 minutes of making incisions.

Results

Delivery rates as a means of quantifying ABA output from roots

Increasing hydrostatic pressure around both root systems of freshly decapitated *Ricinus communis* plants increased sap flow rate (Fig. 28A). Droughting the upper root systems for 24 h slowed the flows at each applied pressure. The slope of the response, which represents the hydraulic conductance of the double root system, was reduced by 24% 24 h after the start of droughting. As sap flow rate was increased with pressure, the concentration of ABA in droughted and well-watered plants declined

(Fig. 28B). The diluting effects of faster flow rates is seen in Fig. 28B. While dilution was proportional to sap flow rate in control plants it was less than proportional in droughted plants. A similar effect of stress on dilution characteristics has been found previously in waterlogged tomato plants (Else *et. al.*, 1995).

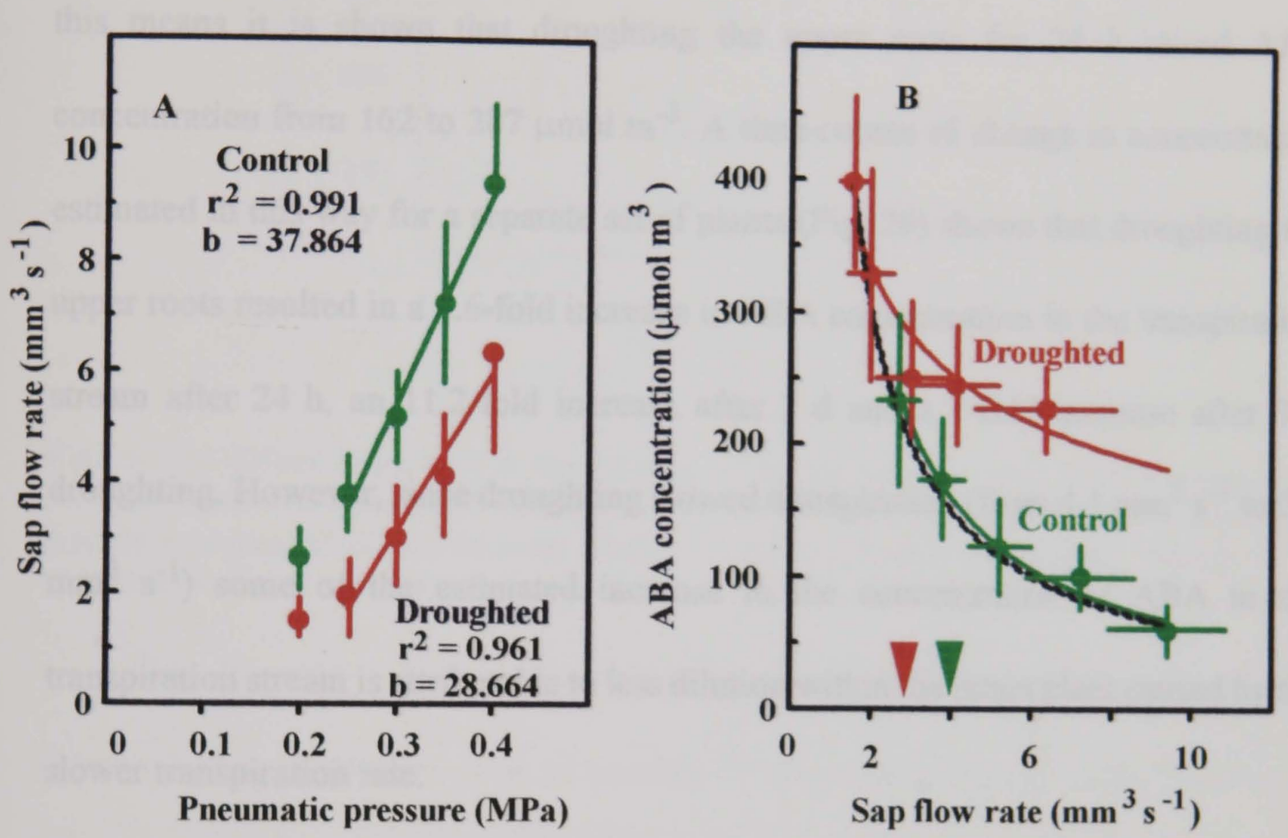

Figure 28 : Effect of pneumatic pressure applied to the roots of 1-month-old decapitated *Ricinus communis* plants with two horizontally separated root systems. Both root systems were well-watered (Control) or the upper root system was left unwatered for 24 h (Droughted). (A) Effect of increasing pressure on rate of sap flowing from the cut stump. Vertical lines about the means are standard errors (n=5). (B) Influence of increasing sap flow rates on concentration of ABA in xylem sap. Horizontal and vertical lines about each mean are standard errors (n=5). Dashed line shows the theoretical curve of dilution if this was fully proportional to flow. Points are fitted as a power curve with the regression formula $y = ax^b$ where $a > 0$. Arrows in (B) show the rates of transpiration on adjacent whole plants. The slopes in (A) represent root hydraulic conductance (Jackson *et. al.*, 1996). They are not significantly different ($P > 0.05$)

Since concentration varied strongly with sap flow rate, reliable estimates of the concentration likely to be present in the transpiration stream could only be made using values in sap flowing at rates of whole plant transpiration. These rates are shown by arrows in Fig. 28B. Reading up from the arrows to the appropriate dilution curves gives the predicted ABA concentration in the transpiration stream of intact plants. By this means it is shown that droughting the upper roots for 24 h raised ABA concentration from 162 to 287 μmol m^{-3}. A time-course of change in concentration estimated in this way for a separate set of plants (Fig. 29) shows that droughting the upper roots resulted in a 2.6-fold increase in ABA concentration in the transpiration stream after 24 h, an 11.2-fold increase after 2 d and a 7-fold increase after 3 d droughting. However, since droughting slowed transpiration (from 4.1 mm^3 s^{-1} to 2.7 mm^3 s^{-1}) some of the estimated increase in the concentration of ABA in the transpiration stream is attributable to less dilution within the intact plant caused by the slower transpiration rate.

Because ABA concentrations were shown to be vulnerable to dilution effects, a more reliable basis for estimating the impact of the drought treatment on ABA export was sought. Thus, the concentration terms were replaced with delivery rates obtained by multiplying concentration with sap flow rate. Since concentration does not always vary in strict proportion to flow rate (Fig. 28B), only sap flow rates that would give sound estimates of ABA deliveries were those close to whole plant transpiration rates.

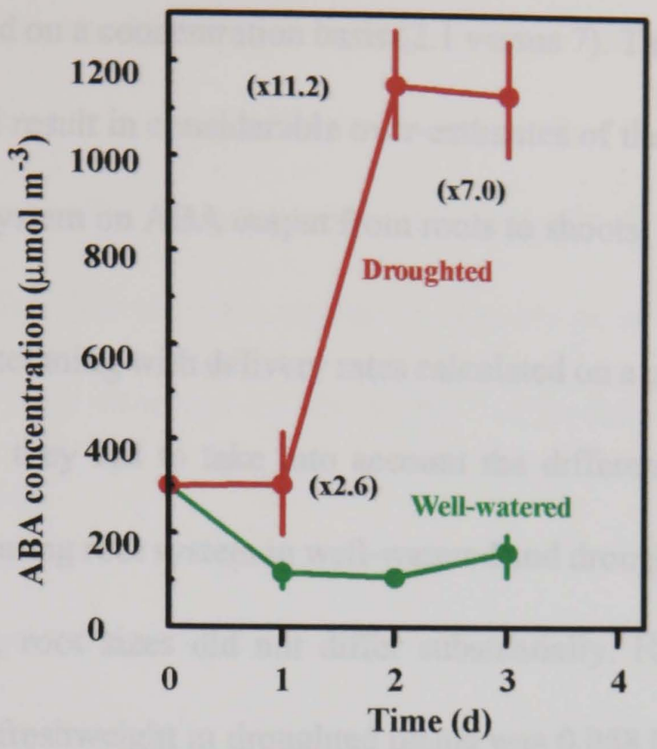

Figure 29 : Effect of up to 3 d droughting of the upper root system of 1-month-old *Ricinus communis* plants on the concentration of ABA in xylem sap flowing from freshly decapitated plants at rates similar to whole plant transpiration. Means of 6 replicates with standard error.

Thus, the approach was to use ABA concentrations in sap made to flow from detopped plants at rates as close as possible to whole plant transpiration. Therefore, routinely, ABA measurements were analysed in the sap of detopped *Ricinus communis* plants flowing at the whole plant transpiration rate or close to this. The values obtained estimate how much of the hormone is delivered into the base of the shoot by each double root system. These deliveries (Fig. 30A) showed an increase of 2.4-fold after 1 d of drought treatment which was similar to the proportional change in concentration at this time. However, after 2 d droughting, the increase in delivery was only 5.5 times that of well-watered plants, a much smaller effect than that recorded in terms of concentration. A similar picture was seen after 3 d droughting

when the impact of droughting on delivery rates was, again, substantially smaller than that observed on a concentration basis (2.1 versus 7). Thus, relying on concentrations alone would result in considerable over-estimates of the influence of droughting the upper root system on ABA output from roots to shoots.

A shortcoming with delivery rates calculated on a per plant basis, as used in Fig. 30A, is that they fail to take into account the differences between the size of the signal-generating root system in well-watered and droughted plants. In the short-term experiments, root sizes did not differ substantially. However, even after 3 d the overall root freshweight in droughted plants was 0.058 kg (± se, 0.004, n = 6) while that in controls was 0.047 kg (± se, 0.004, n = 6). Thus, even here delivery rates can be refined to advantage by dividing root weight to generate a specific delivery rate. In this way the extent to which, overall, each kilogram of root delivered ABA to the shoot can be determined (Fig. 30B). ABA delivery rates expressed in this way showed an increase in the amount of ABA transported from each kilogram of root into the base of the shoot of up to 6-fold after 2d droughting the upper roots. By the third day this had decreased substantially.

It is also of value to account for any differences in the amount of shoot tissue into which a given amount of ABA from the roots will ultimately be distributed. Clearly, the intensity of the message from a given delivery of ABA from a root system into the whole shoot will be much greater if the size of the shoot into which it becomes dispersed is smaller as a result of root stress. In the plants used here, 3 d of droughting

reduced total shoot area in relation to the controls from 0.077 m^2 (± se, 0.003, n = 6) to 0.071 m^2 (± se, 0.004, n = 6). Although this was only a loss of 7.8%, in other circumstances it could well be much greater. Any disparity in leaf area between treatments can easily be accounted for arithmetically. Such an adjustment is shown in Fig. 30C. ABA delivery rates expressed in terms of nmol delivered in to a unit area of leaf (m^2) were increased 2.1 times after 1 d droughting, 5 times after 2 d and 2.2 times after 3 d.

The most integrating expression of all is when delivery rate is adjusted for both the size of the source root system and the size of the shoot system (Fig. 30D). This indicates how much ABA is delivered into each unit area of leaf by each unit mass of root tissue (**nmol m^{-2} $kg^{-1}s^{-1}$**).This specific delivery rates amount to an efficiency statement and are useful for comparing results from different experiments, different species, different plant sizes and different treatments, where both the relative sizes of roots and shoots and their absolute sizes are different.

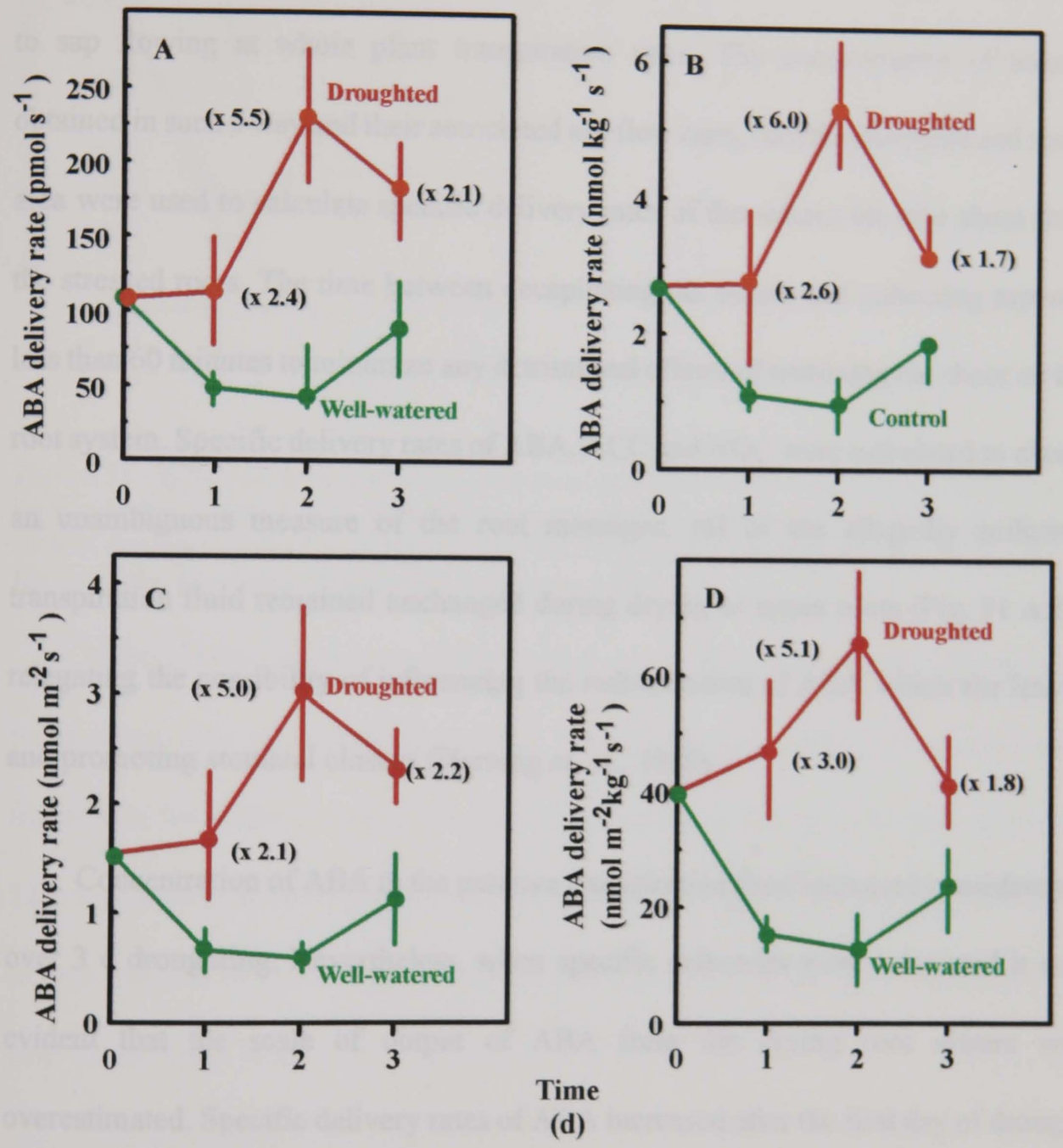

Figure 30 : Effect of up to 3 d droughting of the upper root system of 1-month-old *Ricinus communis* plants on delivery rates of ABA in xylem sap induced to flow from freshly detopped plants at rates similar to whole plant transpiration. (A) ABA delivery rate on a whole plant basis. (B) ABA delivery rate into the shoot per unit fresh weight of root system. (C) ABA delivery rate from whole root systems into per unit area of leaf. (D) ABA delivery from each kilogram of root into each square metre of leaf. Means of 6 replicates with standard errors. Figures in parenthesis show the effect of droughting compared to non-droughted controls at the same time.

When quantifying root messages the problems of dilution resulting from slower or faster flow rates were overcome by pressurising detopped plants in pressure chambers to generate sap flow rates in the root systems which were same as or close to sap flowing at whole plant transpiration rates. The concentration of solutes obtained in such a way and their associated sap flow rates, root freshweights and shoot area were used to calculate specific delivery rates of the solutes into the shoot from the stressed roots. The time between decapitating the shoots and collecting sap was less than 60 minutes to minimize any detrimental effects of removing the shoot on the root system. Specific delivery rates of ABA, ACC and NO_3^- were calculated to obtain an unambiguous measure of the root messages. pH of the allegedly authentic transpiration fluid remained unchanged during drying of upper roots (Fig. 31 A,B), relegating the possibility of influencing the redistribution of ABA within the leaves and promoting stomatal closure (Hartung *et. al.*, 1988).

Concentration of ABA in the putative transpiration fluid increased considerably over 3 d droughting. Nevertheless, when specific deliveries were calculated it was evident that the scale of output of ABA from the drying root system was overestimated. Specific delivery rates of ABA increased after the first day of drought treatment. A more detailed look at the time-course over which drying upper roots caused increased ABA output from the roots is seen in Fig. 31A. After only 9 h of drying the upper roots there was an increase in ABA delivery out of the roots.

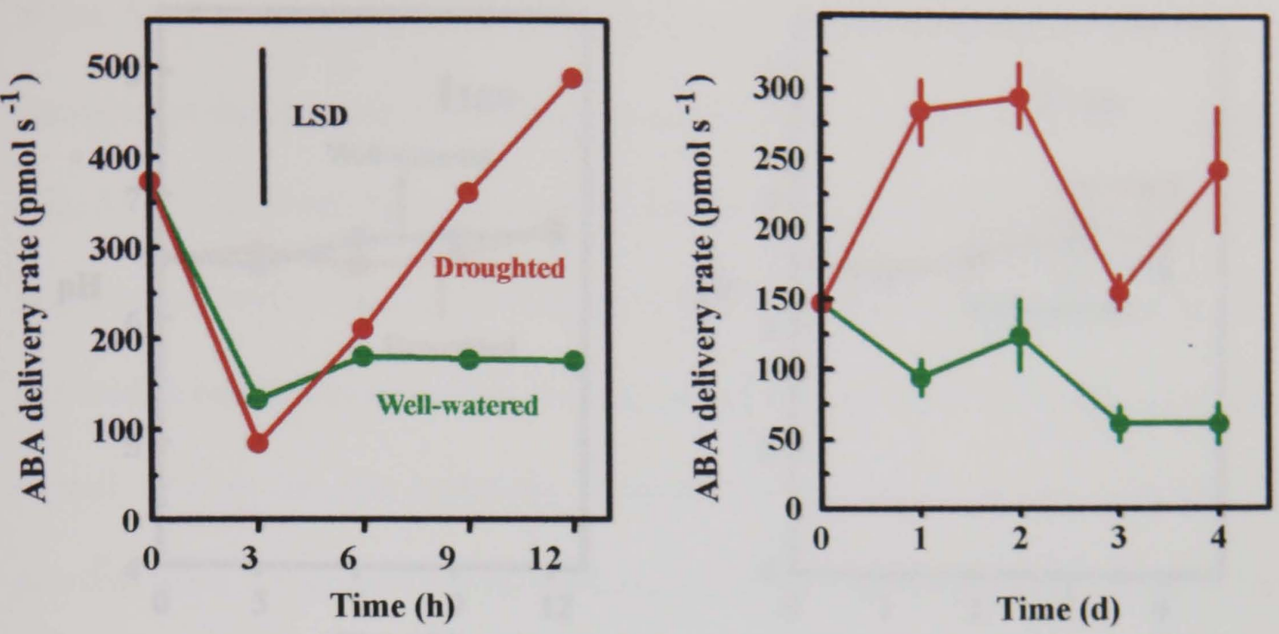

Figure 31 : Effect of drying of the upper roots of 1-month-old *Ricinus communis* plants on the delivery rate of ABA. (A) Delivery rate of ABA measured every 3 h over the first 12 h of droughting the upper roots. (B) ABA measured once daily for the next 4 days of the drought treatment. Means of 6 replicates with LSD.

Although this increase may look marked, a two-tailed t-test showed that this was not a statistically significant increase ($P > 0.05$). However, there was a significant increase in the delivery rate of ABA out of the roots of the droughted plants by the 12th h of drying the upper roots. This effect was not temporary because delivery rates of between 2.3- to 3.9-fold was sustained out of the roots of droughted plants for the next 4 days (Fig. 31B).

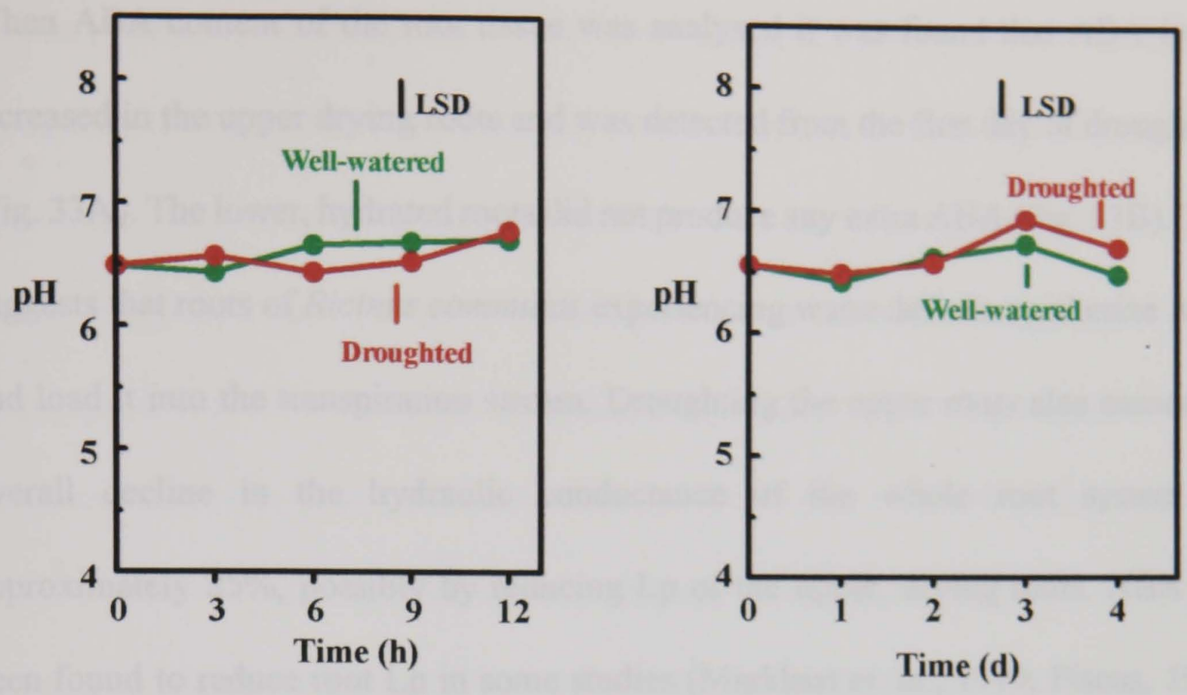

Figure 32 : Effect of 4 d droughting of the upper root system of 1-month-old *Ricinus communis* plant on pH of xylem sap induced to flow from freshly decapitated plants at rates similar to whole plant transpiration. (A) pH of sap sampled every 3 h over the first 12 of drying upper roots, (B) pH of sap measured once daily over 4 d droughting. No significant effect of root drying on pH was found (P>0.05). Means of 6 replicates with LSD.

pH of xylem sap

Figure 32 shows the effect of drying upper roots of *Ricinus communis* on the pH of xylem sap. pH remained unchanged during drying of upper roots. Even a closer look at the xylem sap pH during the first 12 h of droughting failed to show any differences (Fig. 31A). Thus there is no alkalinity based release ABA from the storage compartment and/or reduce uptake into the mesophyll cells of the ABA arriving in the transpiration stream, that might increase ABA in the apoplastand close stomata closure (Schurr and Gollan, 1990).

ABA content in drying roots

When ABA content of the root tissue was analysed it was found that ABA levels increased in the upper drying roots and was detected from the first day of droughting (Fig. 33A). The lower, hydrated roots did not produce any extra ABA (Fig. 33B). This suggests that roots of *Ricinus communis* experiencing water deficits synthesize ABA and load it into the transpiration stream. Droughting the upper roots also caused an overall decline in the hydraulic conductance of the whole root system by approximately 25%, possibly by reducing Lp of the upper, drying roots. ABA has been found to reduce root Lp in some studies (Markhart *et. al.*, 1979, Fiscus, 1981, Eamus and Narayan, 1990).

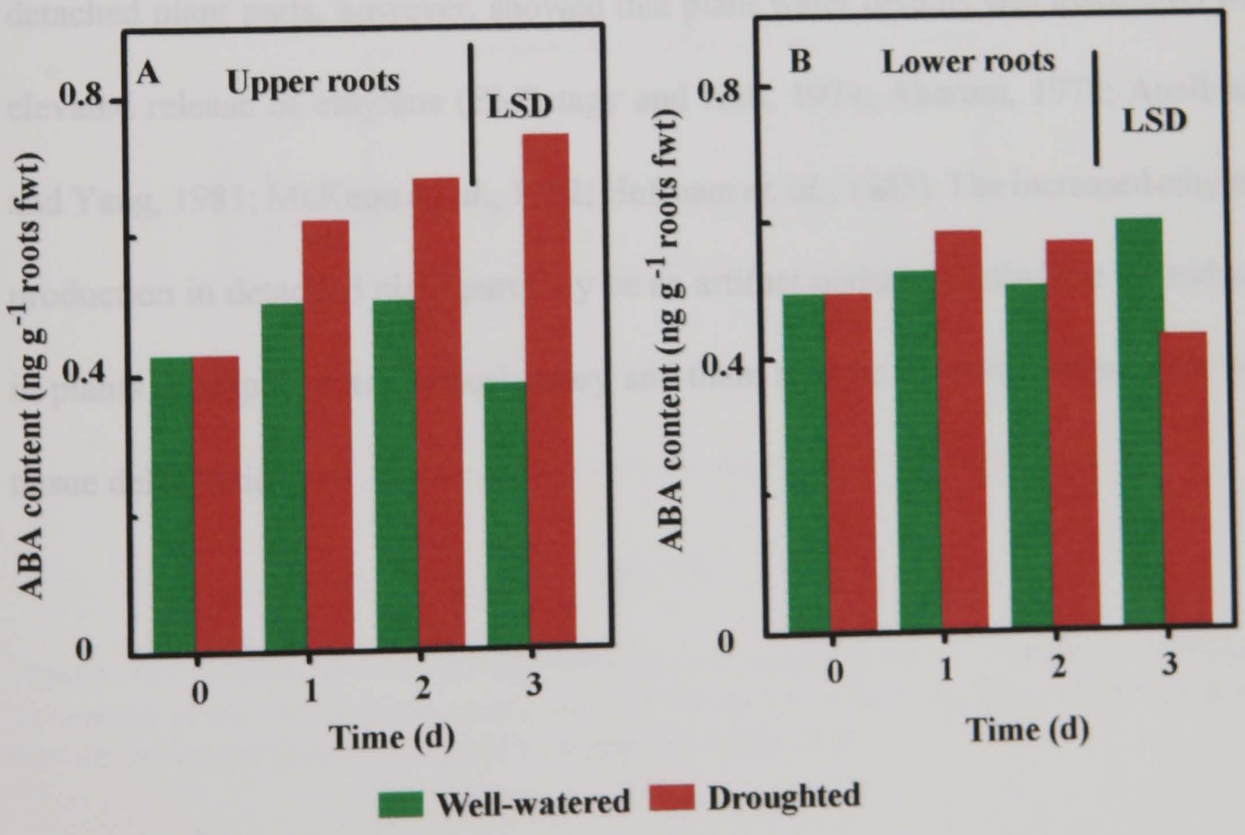

Figure 33 : Effect of 4 d drying of roots in the upper pots of 1-month-old *Ricinus communis* on ABA content of (A) upper, drying roots; (B) lower, well-watered roots. Means of 6 replicates with LSD.

Delivery of nitrates and ACC out of the drying roots

Delivery rates of nitrate ions from roots of the droughted plants were unchanged by droughting the upper roots of *Ricinus communis* (Fig. 34). This indicates that nitrate uptake and transport by the root system as a whole remained unaffected. Schurr *et. al.* (1992) suggested that apparent sensitivity of stomata to ABA increased with increasing nitrate concentrations. There was also no stress-induced synthesis of ACC, the precursor of ethylene, by these roots (Fig. 35). In fact, on the third day, delivery of ACC out of the drying roots was significantly less than that by the well-watered controls as shown by a t-test ($P<0.05$). Morgan *et. al.*, 1990 presented data which showed that when plants were subjected to water deficits there was no evidence of stress-induced ethylene production in intact plants. Numerous other studies using detached plant parts, however, showed that plant water deficits was associated with elevated release of ethylene (El-Betagy and Hall, 1974; Aharoni, 1978; Apelbaum and Yang, 1981; McKeon *et. al.*, 1982; Hoffnam *et. al.*, 1983). The increased ethylene production in detached plant part may be an artifact perhaps as ethylene is produced in plants in response to physical injury and there may be a positive interaction with tissue dehydration.

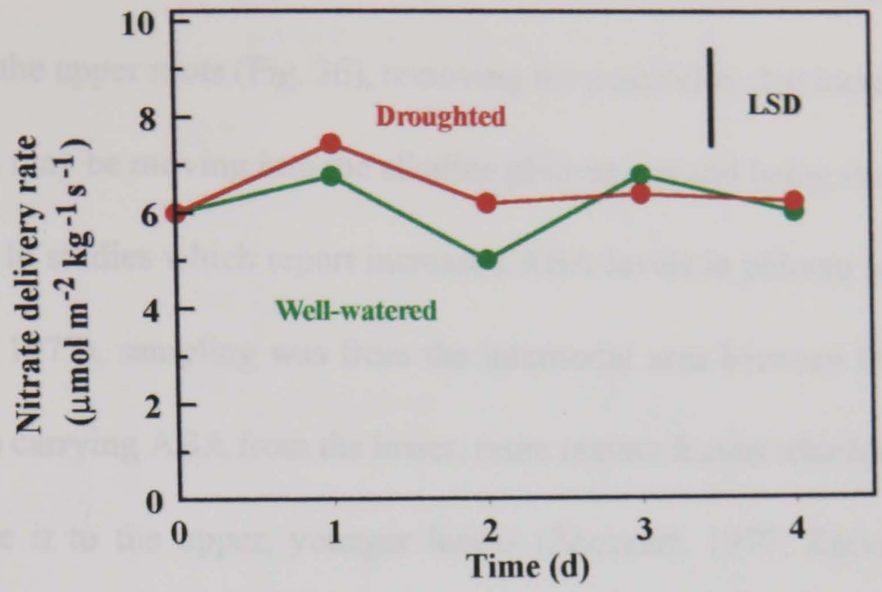

Figure 34 : Effect of 4 d droughting of the upper roots of 1-month-old *Ricinus communis* plants on delivery rate of nitrates from the roots into the base of the shoot. No significant change in delivery rate of nitrates from the treated roots was found (P >0.05). Means of 6 replicates with LSD.

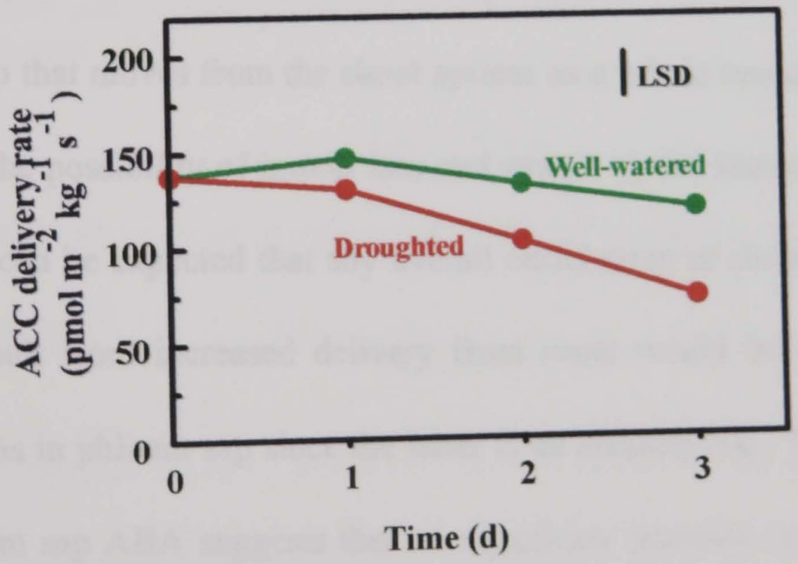

Figure 35 : Effect of 4 d drying roots in the upper pots of 1-month-old *Ricinus communis* plants on delivery rates of ACC from roots into the base of the shoots of freshly detopped plants. Means of 6 replicates with LSD.

Levels of ABA in the phloem sap

The levels of ABA in the phloem sap of *Ricinus communis* did not change during drying of the upper roots (Fig. 36), removing the possibility that increased ABA from the xylem may be moving into the alkaline phloem sap and being recirculated within the plant. In studies which report increased ABA levels in phloem sap (Hoad, 1973; Zeevaart, 1977), sampling was from the internodal area between leaves and would have been carrying ABA from the lower, more mature leaves which synthesize it and translocate it to the upper, younger leaves (Zeevaart, 1977; Zeevaart and Boyer, 1984). Drought stress increases ABA synthesis in the mature leaves as they lose turgor. This increases output from them into the phloem and movement upward, toward the sink (shoot apex). Phloem sap in the work presented here was taken from approximately 50 mm above the soil surface below the cotyledonus leaves, thus obtaining sap that moves from the shoot system as a whole toward the root system, eliminating the possibility of import into and export by the leaves of different ages. However, it can be expected that any overall enrichment of shoot tissue with ABA that may result from increased delivery from roots would be reflected in larger concentrations in phloem sap since the latter is an alkaline trap. The absence of any rise in phloem sap ABA suggests that no significant increase in shoot tissue-ABA resulted from increased ABA delivery from the drying upper roots, or no increase in circulation (Jia and Zhang, 1997).

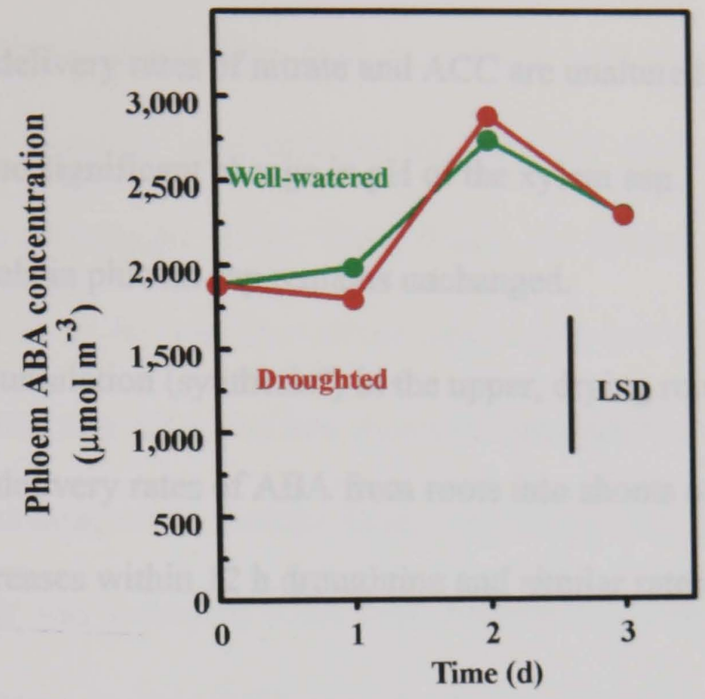

Figure 36 : Effect of 4 d droughting of roots in the upper pots of 1-month-old *Ricinus communis* plants ABA levels in phloem sap obtained by making light incisions on the stem approximately 50 mm above soil surface. ABA levels in phloem sap from the droughted plants and the well-watered were not significantly different (P>0.05). Means of 6 replicates with LSD.

Discussion

So far this study shows that drying upper roots of *Ricinus communis* results in the following changes:

- Increases stomatal closure. However, this response is delayed and significant reductions in stomatal conductance occur only 2-3 days after drought treatment begins (Chapter 3).
- Inhibits leaf expansion. Significant reductions are found after 2-3 days of drought-ting (Chapter 3).
- Shoot water status remains unaltered (Chapter 4).

- Total root hydraulic conductivity is decreased by 25% by the second day of root drying (Chapter 4).
- Specific delivery rates of nitrate and ACC are unaltered.
- There is no significant change in pH of the xylem sap.
- ABA levels in phloem sap remains unchanged.
- ABA accumulation (synthesis?) in the upper, drying roots increases.
- Specific delivery rates of ABA from roots into shoots of plants with drying upper roots increases within 12 h droughting and similar rates are sustained for the next 4 days.

It is, therefore, clear that roots of *Ricinus communis* plants synthesize ABA within 12 h of experiencing water deficit which is loaded into the transpiration stream. Delivery of nitrate or ACC, which are also likely to influence shoot response if changed, remains unaffected. The plants receive adequate water to maintain shoot hydration similar to those of the well-watered controls. Perhaps the slight reductions in stomatal conductance seen during the first day of drought assist in maintaining shoot hydration in spite of reduced root hydraulic conductivity. Significant stomatal and leaf responses occur after a delay of 2 days in spite of an increased output of ABA from the roots. This raises the question: '*Is root ABA acting as a signal to bring about stomatal closure and inhibited leaf expansion?*' Various studies have implied that ABA is the main chemical signal originating from drying roots responsible for closing stomata and, in many cases, inhibiting leaf expansion (Davies *et. al.*, 1990; Hartung

and Davies, 1991; Khalil and Grace, 1991; Gollan *et. al.*, 1992; Bano *et. al.*, 1993). Much of the work has involved measuring ABA concentrations in xylem sap of droughted plants and finding them to increase with the stress, suggesting a tight relationship between concentration of ABA in the xylem sap and stomatal conductance (Wartinger, *et. al.*, 1990;, Zhang and Davies, 1990, 1991). In a slightly different study to the one carried out here, using two pine species Jackson *et.al.*, (1995) showed that ABA flux into shoots of these plants remained unchanged during drought treatment while stomata closed in response to soil drying. Although they argue that it was increased ABA concentration in xylem, not flux, which caused stomatal closure, it could well be that the rise in ABA concentration in xylem sap was due to a lack of dilution of the xylem sap caused by stomatal closure, therefore it could be the effect and not the cause of stomatal closure. Measurements were made every 2 to 4 days which makes it difficult to affirm whether increase in ABA concentration in the xylem preceded stomatal closure.

The sequence of events which occurs in *Ricinus* plants when the upper roots are droughted shows cause and effect. That is to say that when upper roots are dried with no loss in shoot hydration, ABA concentration and delivery rates increase within 12 h. This is accompanied by small amounts of stomatal closure which may or may not be influencing shoot hydration during root drying. Although the order implies a causal sequence, that is to say that root drying increases ABA delivery which is followed by a reduction in stomatal conductance and inhibition of leaf expansion, the delay is

uncomfortably long. This suggests that if ABA from the roots is the controlling mechanism for stomatal closure and inhibition of leaf expansion, the mechanism is insensitive and heavily dampened. In fact xylem sap of well-watered plants already contains more ABA than that required for stomatal closure in isolated guard cells but, in spite of this, stomata of leaves from well-watered plants remain open (Trejo *et. al.*, 1993). This in itself points to some mechanism within the leaves that prevents apoplastic ABA levels increasing, thus preventing stomatal closure in these plants. The capacity of leaves to metabolise ABA has been studied. The half-life of xylem-derived, exogenously supplied ABA was found to be 36 minutes in hydrated cherry leaves (Gowing *et. al.*, 1993), 42 minutes in maize leaves and 64 minutes in *Commelina* leaves (Jia *et. al.*, 1996). Immediately after feeding detached leaves with ABA for 30 minutes, when ABA content of the leaves was analysed, Jia *et. al.* (1996) found that only 60% remained, suggesting very rapid metabolism. The process of xylem-derived ABA metabolism in leaves appears to match the kinetics of enzymatic catalysis reactions where the reaction rate is related to substrate concentration, provided the catalysis capacity of the enzyme is larger than the substrate (Jia *et. al.*, 1996). In studies by Gowing *et. al.* (1993), Trejo *et. al.* (1993), and Jia *et. al.* (1996), although the upper ABA concentration ranges fed to the detached leaves were much higher than those found occurring in plants, the leaves were still able to metabolise this ABA. Metabolic rates increased with increasing levels of xylem-derived ABA. This shows the tremendous capacity of leaves to dampen the intensity with which ABA arrives into the leaves. In *Ricinus* also, this metabolism may be occurring and

helps to achieve homeostasis with regards to free ABA levels If this is the case, ABA from drying roots may be a weak signal and unlikely to influence shoot physiology during the early stages of drought stress.

The presence of an antitranspirant other than ABA in the xylem sap of *Ricinus* plants after 48 h of drying upper roots may also be a possible explanation for the delayed response. Munns and King (1988) suggested the presence of such an antitranspirant in xylem sap of droughted wheat plants the activity of which remained in xylem sap derived from the plants after ABA was removed using an immunoaffinity column..

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Chapter 6 : COMPARING THE CONCENTRATION AND DELIVERY OF SOLUTES IN TRANSPIRATION FLUID ENTERING THE BASE OF THE SHOOT WITH THAT ENTERING THE LAMINA OF A FULLY OPEN LEAF

Introduction

The delay between increase in ABA output by roots (after 12 h of drying upper roots) and the response of stomata and leaf expansion (2 -3 days later) questions root-sourced ABA as a signal for these responses. One possible reason for the delay could be that ABA is lost from the transpiration stream as it moves up the plant. Changes in pH might have such an effect through redistribution of ABA between xylem sap and surrounding tissues. However, this is ruled out here since no change in xylem sap pH was found as a result of drying upper roots (Chapter 5), although it is possible that pH change may develop as sap ascends the shoot. Xylem sap of *Ricinus* plants also failed to show any changes in delivery rates of nitrate which could perhaps influence ABA activity (Radin *et. al.*, 1982). This chapter examines, experimentally, the possibility that ABA is lost from xylem sap between leaving drying roots and arriving at a target leaf several centimetres away. To test this idea of weakening signal as sap moves away from drying roots, modifications to the existing method of obtaining xylem sap were required, which are explained below.

The xylem sap in an intact, well-watered transpiring plant, is under tension (negative xylem water potential, Ψ_{xy}), generated by a combination of a high

resistance to radial water flow into roots and evaporation from the leaves. As xylem elements connect the two sites, water tension in the xylem is generated as a function of evaporation rate and root resistance. A simple Ohm's Law analogy shows the approximate relationship where the magnitude of tension in the xylem can be calculated by multiplying the resistance of the root (R) by the rate of transpirational flow within the plant (Q), (Ψ_{xy} = R x Q). The negative tension in xylem makes sampling xylem sap for concentration and delivery rate measurements almost impossible at positions in the canopy using direct means. This is because as soon as a vessel is punctured to gain access to sap, the tensions that drive transpiration are released as air enters the vessels. Flow then stops except for a slow, osmotically driven component. In Chapter 5, and in earlier publications (Else *et. al.*, 1994; Jokhan *et. al.*, 1996), an alternative, indirect method was described that involves removing the shoot and applying enough pneumatic pressure to the roots to induce discharge from exposed xylem vessels at rates of whole plant transpiration. Xylem sap obtained in this way is considered to contain solutes that would be entering the base of the shoots from the roots of whole plants. Thus, measurements of concentrations and delivery rates in this sap would give good estimates of output from the roots. However, this technique does not help determine if chemical signals that leave the root system arrive at potential target sites higher in the shoots. This is normally very difficult and only xylem feeding insects (Mittler, 1967) offer this possibility. In addition, to obtain good estimates of delivery rates of solutes into the leaf lamina, the rate of sap flowing into the leaf must also be known. Such sap flow rates are required

for two reasons. Firstly, for concentrations of solutes in sap entering the leaf of intact plants to be realistic the analysed sap may need to flow at rates close to those which occur in the petiole of the intact plant. If these flows are not similar, concentrations in the sap could differ from those in the transpiration fluid because of the dilution effect resulting from any associated changes in water flow into the roots. Secondly, since sap flow rate is needed to calculate solute delivery rates (multiplying flow rate by concentration), incorrect flow rates would lead to inaccurate calculations of delivery rates of solutes into the leaf, even if concentrations were correct.

This chapter describes a sequence of tests and changes in methodology that led to the development of a satisfactory method for examining ABA delivery into a chosen leaf, and comparing it with that entering the base of the shoot from the root system. The experiments used split-top pressure chambers similar in design to those devised by Passioura (1980). These chambers allow the soil around the roots of intact plants to be pressurized to a point where a small cut in the leaf vein is on the verge of exuding sap, making the pressure of that sap zero. The pneumatic pressure around the roots needed to achieve this zero Ψ_{xy} is the so-called 'balancing pressure'. This approach offers the possibility of sampling sap at almost any chosen position in the canopy by severing a leaf or small part of it, and collecting sap emerging from the cut. Once the balancing pressure is set, if a little more pneumatic pressure is applied to the root system or alternatively if humidity of air around the plant is increased, Ψ_{xy} becomes slightly positive, and sap flows slowly out of the cut surface. Xylem sap was

first sampled in this way by Munns and Passioura (1984) and solute content in the sap was considered to reflect that of the transpiration stream. Similarly, Munns (1990) used this method for sampling xylem sap of wheat for ABA. Schultze and co-workers used the same approach to measure a range of solutes dissolved in xylem sap (Gollan *et. al.*, 1992; Schurr *et.al.*, 1992; Schurr and Schulze, 1995; 1996; Heckenberger, *et. al.*, 1996). Despite the increasing sophistication with which such experiments have been conducted using the Passioura-type pressure chamber since 1984, there are still uncertainties concerning how closely the measurements of sap flow and solute concentrations match those of the true transpiration stream. There are also uncertainties concerning sap flow rates at which sap is sampled. The difficulties have been discussed in detail in Chapter 1. In light of these problems, modifications to existing methods for adopting the balancing pressure approach were required before reliable comparisons could be made between the concentrations and delivery rates of solutes at the base of the shoot and at entry into the lamina of a fully expanded leaf. In the present work, solutes examined were either total osmolality, measured by a depression of freezing point method, or ABA concentration, measured using RIA. Subsidiary goals in the work included: (i) assessing the impact on sap solute levels of the wounding that takes place when a leaf lamina is excised to expose the xylem; (ii) assessing the effect of setting a balancing pressure on leaf water potentials; (iii) testing the extent to which extracting sap samples from freshly excised leaves, using a pressure chamber of the type designed for leaf water potential measurements, gives a quick and reliable estimate of the concentration of solutes in the transpiration

stream; and (iv) determining sap flow rates into the shoot base and individual leaves using electronic flow sensors.

Methods

Plants were grown as described in Chapter 2 with some modifications. After gemination, plants were kept in a growth room at 25°C and 80% RH and with a light intensity of 320 μmol m^{-2} s^{-1}, instead of 540 μmol m^{-2} s^{-1}, for 5 days to encourage hypocotyl lengthening. This extra length allowed the plants to be placed in split-top pressure chambers while leaving sufficient hypocotyl protruding for a sap flow gauge (SGA10), to be attached. The plants used were between 35-40 days-old. The use of Dynamax flowgauges to measure flows and the split-top pressure chamber to obtain xylem sap has been described in Chapter 2.

Results and Discussion

Examining the effect of setting a balancing pressure on leaf water potential

A comparison of water potential of leaves from normal well-watered *Ricinus communis* plants and those subjected to a balancing pressure (Table 8) shows that leaf water potential was influenced by pressurising the roots. In theory, applying pneumatic pressure around the roots of the plants raises pressure in the xylem to zero and inevitably raises turgor in the leaf cells by a related amount (Passioura, 1980). This, in turn, renders leaf water potentials less negative. This is confirmed in Table 8 which shows increases in leaf water potentials of 0.15 - 0.20 MPa resulting from the application of a balancing pressure.

Table 8: Effect of applying balancing pressure on leaf water potentials (Ψ_L) of the youngest fully expanded leaves of 5-week-old *Ricinus communis* plants with twin root systems. Well-watered plants were placed in a Passioura-type pressure chamber and balancing pressures of between 0.25 - 0.325 MPa were applied in each case.

	Leaf					
	1	2	3	4	5	6
	Leaf water potential (MPa)					
Without balancing pressure	-0.450	-0.350	-0.500	-0.450	-0.475	-0.400
With balancing pressure	-0.275	-0.200	-0.300	-0.250	-0.325	-0.250

Testing the possibility that when a leaf lamina is severed from a plant previously set at its balancing pressure, sap flows from the cut petiole at the rate of transpiration of the excised lamina

Using split-top chambers Munns and King (1988) and Munns (1990) suggested that when a leaf, or part of a leaf, is removed from a plant previously set to a balancing pressure, sap flows out from the cut petiole or part of the remaining leaf at a rate similar to that which would have flowed into the severed part. If this could be confirmed, sampling sap for authentic assay of transpiration fluid would be straightforward and solute concentrations and deliveries obtained would be similar to those of leaves of intact plants. To test the assumption, 5-week-old plants were placed in split-top pressure chambers and a balancing pressure set using a sighting tube placed at the distal end of leaf 6. The stomatal conductance of leaf number 5, immediately below, was taken and then the leaf removed. The detached lamina was placed at once into a vial of de-ionized water which was weighed at intervals to measure its transpiration rate while stomatal conductance continued to be monitored.

along with that of leaf 4 which remained on the plant. During this time, sap flow out of the cut petiole was measured by collecting the drops and weighing at intervals recorded with a stop watch. It was shown in Chapter 4 that stomatal conductance of the youngest fully grown leaf (leaf 5) and the second youngest leaf (leaf 4) did not differ significantly in well-watered plants.

Stomatal conductance of the detached leaf (leaf 5) was much the same as that of the intact leaf (leaf 4) (Fig. 37A). Therefore, transpiration rate of the detached leaf, measured gravimetrically in glass vials over the 80 minutes, was similar to that occurring just before excising it at approximately 60 mm^3 min^{-1}. However, it was found that sap flowed out of the cut petiole stump at almost twice this rate (Fig. 37B). Thus, contrary to previous suggestions in literature, this is unnaturally fast and the sap cannot be used to estimate delivery of solutes and water into the leaf lamina of intact plants. The source of the extra sap flow could not be determined with certainty. One possibility is that increased proportion of total plant flow is directed out of the cut stump. This may be expected because excising the leaf lamina inevitably generates a slightly positive pressure in the xylem and because a resistance has been removed (the lamina), a low resistance pathway to mass flow is opened up. If this diversion hypothesis is true then less sap would be flowing into areas above the severed petiole than before the lamina of leaf 5 was removed.

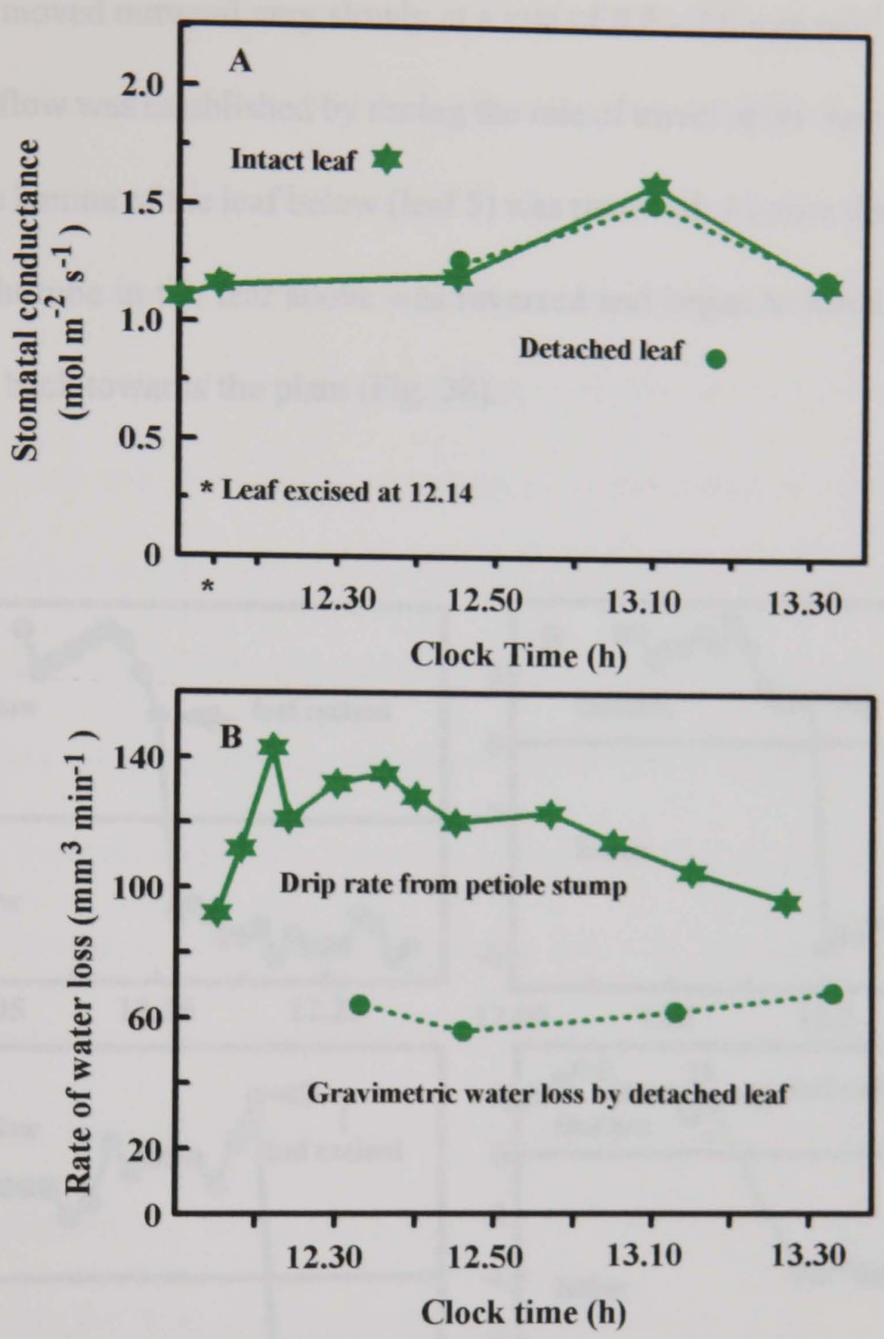

Figure 37 : A comparison of (A) stomatal conductance of an intact leaf (leaf 4) with that of a detached leaf (leaf 5) from the same plant of 5-week-old *Ricinus communis*. Leaf 5 was excised after a balancing pressure of 0.30 MPa was set, and placed in a vial with water, (B) flow rate of sap from the petiole stump of leaf 5 determined by collecting sap dripping in an Eppendorf tube over a time interval, with flow rates in leaf 5 determined by gravimetric method.

This possibility was examined using the following procedure. A sight tube was fitted to a 3-4 mm-long exposed vein of leaf 6 for establishing balancing pressure. This tube was 40 cm long and secured horizontally with the help of clamps. Pressure

around the roots was raised very slightly above balancing pressure so that the meniscus moved outward very slowly at a rate of 0.5 - 7.0 mm min^{-1}. When the rate of sap outflow was established by timing the rate of travel of the meniscus with a stop watch, the lamina of the leaf below (leaf 5) was removed. At once the rate of sap flow in the sight tube in the leaf above was reversed and began to travel in the opposite direction, back towards the plant (Fig. 38).

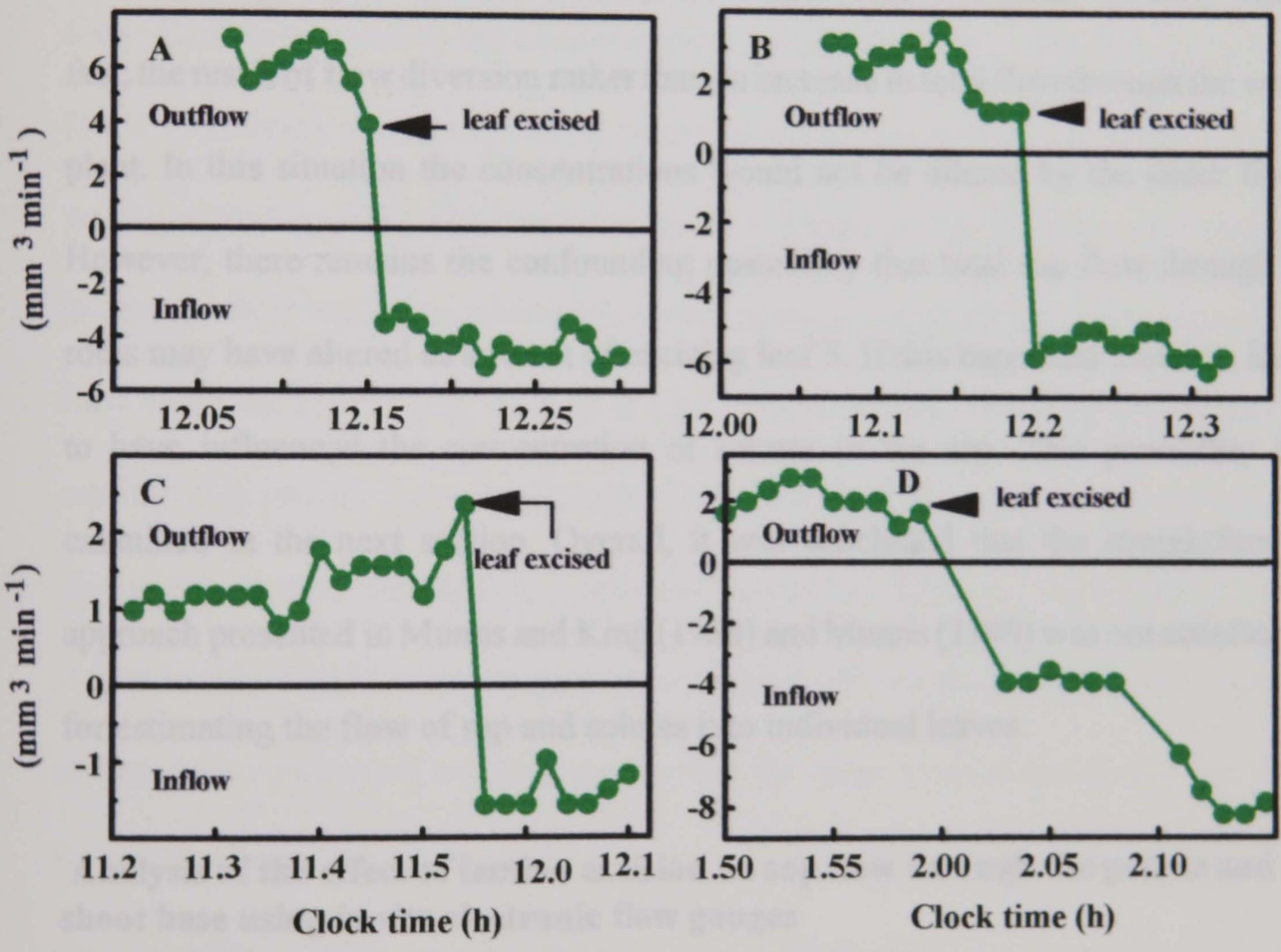

Figure 38 : The effect of removing leaf 5 of 40-day-old *Ricinus communis* plants on the movement of meniscus in a 40-cm-long sight tube attached to an exposed vein in leaf 6 directly above it. Balancing pressure was set in four separate plants to place the meniscus in the sight tube and then increased very slowly to propel the meniscus slowly outwards. Pressures required for these were (A) 0.30 MPa, (B) 0.30 MPa, (C) 0.30 MPa, (D) 0.40 MPa. Flows above the horizontal lines are out from the petiole stump, flows below the horizontal line are towards the petiole stump. Arrows show the times the leaves were removed.

These observations indicate that when leaf 5 was excised the slightly positive Ψ_{xy} in the leaf above it changed to negative because on removing the lamina of leaf 5 the flow of xylem sap was diverted preferentially along a pathway of low resistance in the cut petiole stump. This increased flow rate out of the petiole stump was maintained for some time after excision of the lamina. Clearly, this flow is unsuitable to use in calculating delivery rates of solutes in the leaf. However, sap flowing at this faster rate may still be useful for estimating concentrations of solutes in xylem sap entering the petiole of leaf 5 of intact plants. This would be because the flow was, in fact, the result of flow diversion rather than an increase in total flow through the whole plant. In this situation the concentrations would not be diluted by the faster flows. However, there remains the confounding possibility that total sap flow through the roots may have altered as a result of excising leaf 5. If this happened then it is likely to have influenced the concentration of solutes in the sap. This possibility was examined in the next section. Overall, it was concluded that the straightforward approach presented in Munns and King (1988) and Munns (1990) was not satisfactory for estimating the flow of sap and solutes into individual leaves.

Analysis of the effect of lamina excision on sap flow through the petiole and shoot base using *in situ* electronic flow gauges

The uncertainty in taking dependable samples of authentic transpiration fluid entering the leaf is primarily because of a lack of knowledge about sap flow into the shoot base and into the leaf, both before and after excision of the lamina, and about the effects of any changes altered sap flows may have on solute concentrations. These uncertainties

were addressed experimentally using electronic flow sensors positioned on the petiole of the leaf and at the base of the shoot. This allowed changes in sap flow rates at both positions to be detected in response to excision of the leaf lamina. The method and calibration of the gauges are described in Chapter 2. Figure 39 shows three sets of measurements of sap flow along the petiole of leaf 5 and at the shoot base, before and after setting a balancing pressure, and before and after excision of the leaf lamina. In Figure 39 (B,D,F) no change in the total sap flow into the shoot base was observed as balancing pressure was set. However, this was not always found to be the case. In some plants while setting the balancing pressure total sap flow into the shoot base was found to increase transiently before returning to the levels before balancing pressure was applied (Fig. 40B). Figure 40 confirms the results of Fig. 39, that removing a leaf lamina to allow sap to be collected from plants set at balancing pressure increases sap flow along the petiole (Fig. 39 A,B & C). The observation of a generally unchanged sap flow rate into the base of the shoot after removing the lamina of leaf 5 is important because it strongly supports the view that excess sap flow into the petiole stump is redirected flow and hence not subject to dilution effects since there was no marked change in the amount of water drawn through the roots. Thus, although the rate of sap flow into the petiole was faster, it was not likely that this would have decreased solute concentration which would give a good indication of naturally occurring concentrations entering the lamina of the intact leaf. Since sap flow into the leaf before excision is provided by the flow gauges it should be acceptable to multiply this pre-excision flow rate by the concentration in faster-flowing, post-excision sap to obtain estimates of delivery rates of solutes into the intact leaf lamina.

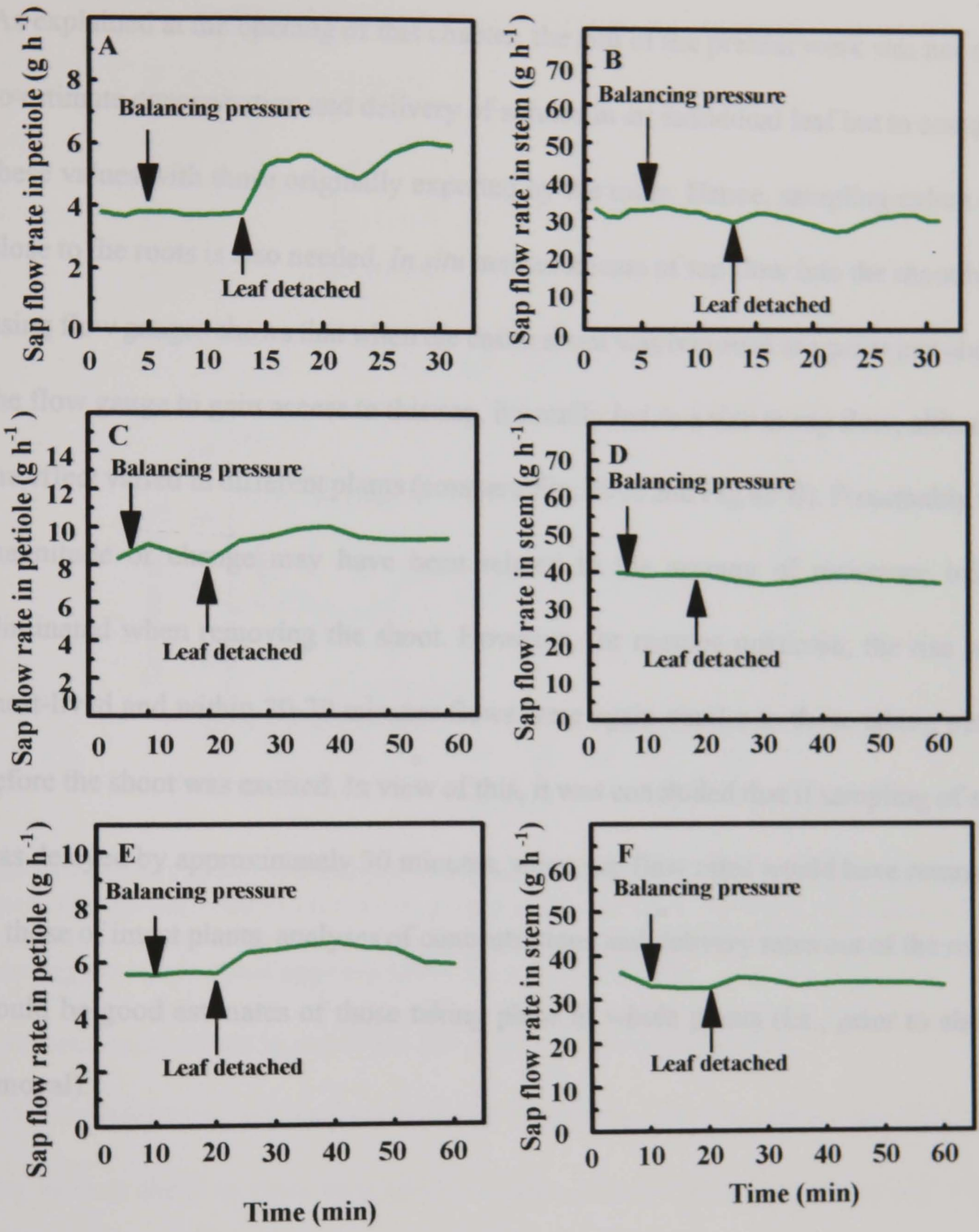

Figure 39 : The effect of removing a leaf lamina (leaf 5) on sap flow rates along the petiole stump (A,C,E,), and also out of the roots into the stem base (B,D,F,) of 40-day-old *Ricinus communis* plants placed in split-top chambers with balancing pressures. *In situ* sap flow rates were measured using electronic sap flow gauges. Arrows show the times of setting a balancing pressure or removing the lamina of leaf 5. Results are from three different plants.

Analysis of the effect of shoot excision on sap flow through the shoot base using *in situ* electronic flow gauges

As explained at the opening of this chapter, the aim of the present work was not only to estimate concentration and delivery of solutes in an individual leaf but to compare these values with those originally exported by the roots. Hence, sampling xylem sap close to the roots is also needed. *In situ* measurements of sap flow into the shoot base using flow gauges shows that when the entire shoot was removed at a point just above the flow gauge to gain access to this sap, it usually led to a rise in sap flow, although the effect varied in different plants (compare Fig. 40 A and Fig 40 B). Presumably the magnitude of change may have been related to the amount of resistance being eliminated when removing the shoot. However, for reasons unknown, the rise was short-lived and within 20-30 minutes flows were again similar to those taking place before the shoot was excised. In view of this, it was concluded that if sampling of sap was delayed by approximately 30 minutes, when sap flow rates would have returned to those of intact plants, analyses of concentrations and delivery rates out of the roots would be good estimates of those taking place in whole plants (i.e., prior to shoot removal).

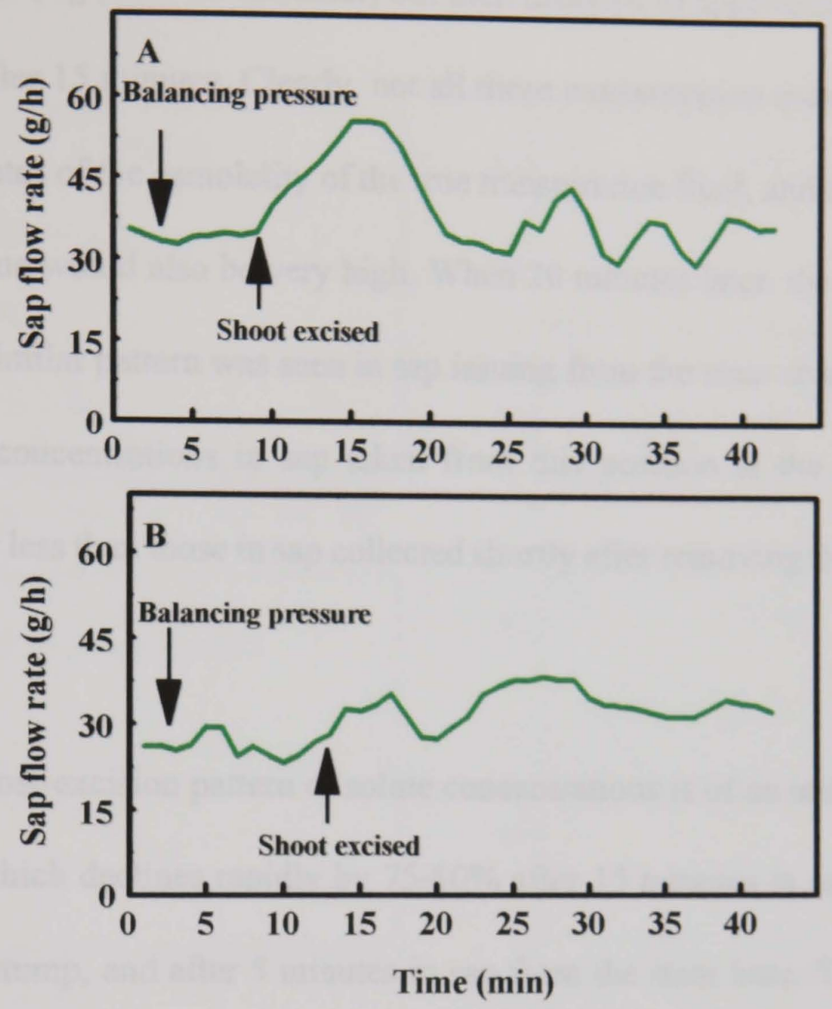

Figure 40 : Effect of removing whole shoot on sap flow rates into the base of the shoot of two, 40-day-old *Ricinus communis* plants (A,B) placed in split-top chambers with balancing pressures. Arrows show the times of setting a balancing pressure or removing the whole shoot approximately 8 cm above the soil.

Solute concentrations in sap from the petiole and root system

So far, consideration has been given to sap flow rates, with the underlying concern being to keep these as close as possible to those of intact plants or to quantify any discrepancy. Attention is now given to the solute levels carried by the sap as it flows out of the petiole of an excised leaf, or flows from near the shoot base when the shoot is excised. On removing the leaf lamina, sap flow into the petiole stump increased and soon became constant. The pattern for osmotically active solute concentration in the

sap was somewhat similar despite the early increased rate of sap flow. Osmolality was initially high (e.g., 55 - 65 mOsmol) but then dropped to approximately one third of this level after 15 minutes. Clearly, not all these concentration measurements can be good estimates of the osmolality of the true transpiration fluid, and delivery estimates over this time would also be very high. When 20 minutes later, the whole shoot was excised, a similar pattern was seen in sap issuing from the stem stump. However, the early high concentrations in sap taken from this position at the shoot base were consistently less than those in sap collected shortly after removing the lamina (Fig. 41 B,D).

This post-excision pattern of solute concentrations is of an initial high levels of osmolites which declines rapidly by 75-80% after 15 minutes in sap flowing out of the petiole stump, and after 5 minutes in sap from the stem base. This suggests that sap was contaminated by the effects of injury due to handling and wounding of the tissues during excision. If this is correct then re-cutting the tissue should produce a similar temporary rise in osmolite levels in the sap. When this test was carried out, re-cutting either the petiole or stem resulted in a second sharp but short-lived rise in osmolality of sap (Fig. 42), thus supporting the wounding hypothesis. Similar problems have been experienced by others (e.g., Else *et. al.*, 1994).

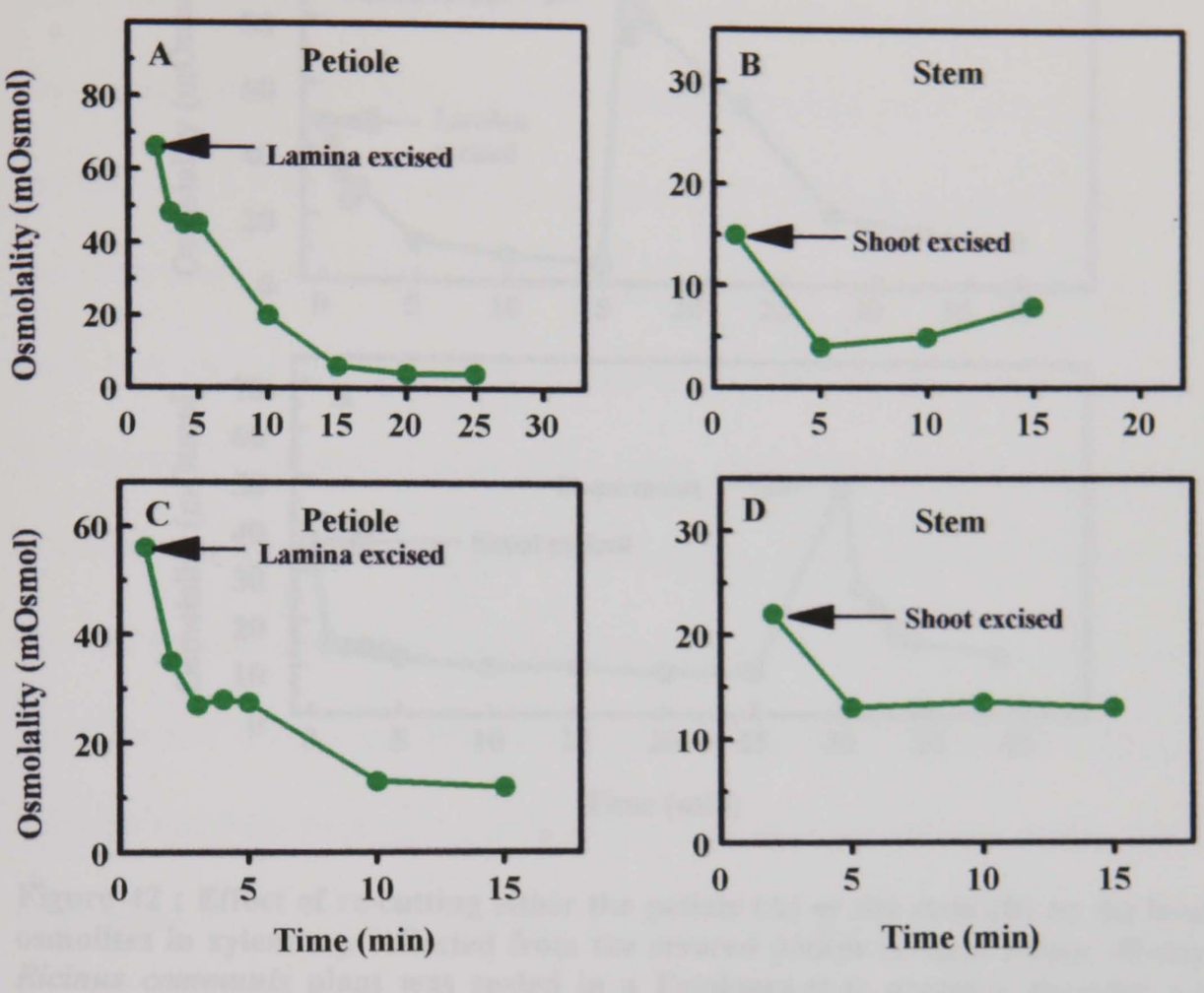

Figure 41 : Total osmolality of xylem sap obtained at different times after the excision of either leaf lamina (A,C) or shoot (B,D) of 40-day-old *Ricinus communis* plants with twin root system. Intact plants were pressurised in split-top pressure chambers with balancing pressures of 0.2 MPa. Arrows show the times at which the lamina of leaf 5 or the whole shoot was detached.

These results provide a second reason for delaying sampling sap following the removal of the lamina or whole shoot. The first reason to delay was to ensure that sap flow rates return to normal after excision, as already discussed. The second reason is that it allows contaminated sap arising from handling and wounding to be discharged from the plant and discarded. Approximately 15-30 minutes appears to be adequate for this.

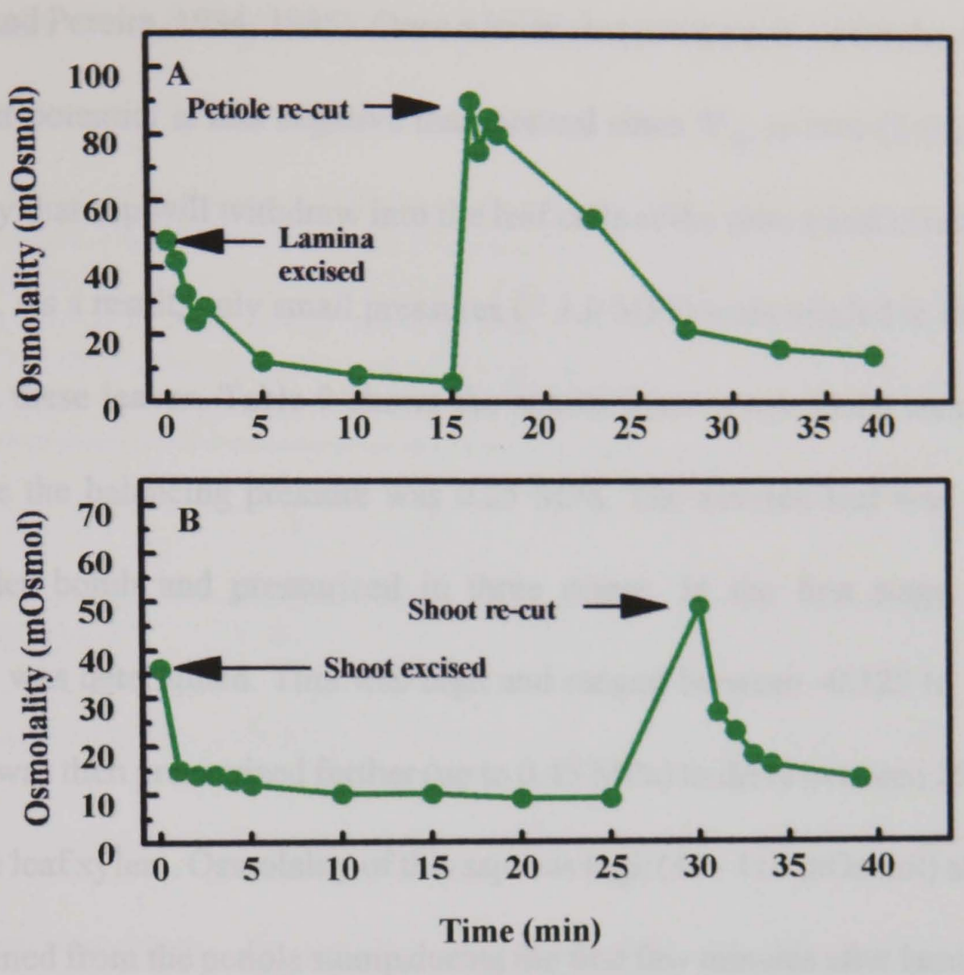

Figure 42 : Effect of re-cutting either the petiole (A) or the stem (B) on the levels of osmolites in xylem sap collected from the severed petiole or stem stump. 40-day-old *Ricinus communis* plant was sealed in a Passioura-type pressure chamber with a balancing pressure of 0.25 MPa. Arrows show the times at which either the shoot or petiole was excised.

Osmolality in sap expressed from detached leaves in a Scholander pressure chamber

Finding that sap samples obtained immediately after excising leaf lamina or stem are highly contaminated with solutes due to wounding, raises the question of whether xylem sap expressed from freshly detached leaves placed in the Scholander bomb would expose the same problem. The Scholander pressure chamber is commonly used to determine leaf water potential of, and express xylem sap from, freshly detached leaves or branches, for estimating solute levels in the transpiration stream (Loveys,

1984; Tardieu *et. al.*, 1991; Tardieu and Davies, 1992; Khalil and Grace, 1993; Correia and Pereira, 1994, 1995). Once a balancing pressure is set for the whole plant, leaf water potential is less negative than normal since Ψ_{xy} is zero (Table 8), so it is less likely that sap will withdraw into the leaf cells at the time a leaf is removed from the plant. As a result, only small pressures (< 1.0 MPa) were needed to obtain xylem sap from these leaves. Table 9 shows the results from seven plants using leaf 5. In each case the balancing pressure was 0.25 MPa. The severed leaf was placed in a Scholander bomb and pressurized in three stages. In the first stage, leaf water potential was determined. This was high and ranged between -0.125 to -0.25 MPa. The leaf was then pressurized further (up to 0.45 MPa) to drive between 25 - 40 µl sap out of the leaf xylem. Osmolality of this sap was high (44 - 110 mOsmol) and matched that obtained from the petiole stump during the first few minutes after lamina excision from plants set at a balancing pressure. The leaves were pressurised a second time (maximum pressure 0.85 MPa) to expel a further 25 - 50 µl xylem sap. Osmolality of this second sample of sap was lower (30-66%) than the first (Table 9) but still comparable with osmolality of sap from corresponding petiole stump remaining on the plant. Initially it was expected that sap expressed the second time might have more osmolites since using greater pressure to expel more sap might be expected to drive more ions out of the symplastic and apoplastic compartments where ions may accumulate. However, the fact that the level of osmolites in this sap decreased indicates that the sap expressed by the first pressurising was heavily contaminated through wounding caused by leaf excision. These results indicate that sap obtained

from detached leaves pressurised in a Scholander bomb are likely to seriously overestimate the true solute levels in the transpiration fluid as it enters the leaves of intact plants.

Table 9: Effect of expressing different volumes of xylem sap from leaves taken from seven individual plants in a Scholander bomb on levels of total osmolites in the sap. Lamina of leaf 5 of 40-day-old *Ricinus communis* plants were excised after setting balancing pressure in split-top chambers and immediately sealed in the Scholander bomb. After determining water potentials of the leaves, two sequential samples of sap were expressed by applying overpressures.

	Leaf						
	1	2	3	4	5	6	7
Balancing pressure (MPa)	0.25	0.25	0.25	0.24	0.25	0.25	0.25
Leaf water potential (MPa)	-0.15	-0.15	-0.15	-0.15	-0.25	-0.175	-0.125
First overpressure (MPa)	0.45	0.25	0.25	0.35	0.45	0.45	0.45
First volume expressed (µl)	30	30	25	40	30	25	30
Osmolality (mOsmol)	110	63	44	82	43	64	50
Second overpressure (MPa)	0.85	0.50	0.65	0.60	0.70	0.70	0.70
Second volume expressed (µl)	25	50	50	40	45	25	40
Osmolality (mOsmol)	48	28	24	40	26	32	35

Comparison of concentration and delivery of osmolites and ABA from roots into the shoot base and into the 5th oldest leaf

Levels of overall solutes and ABA delivery into the shoot base and into leaf 5 situated approximately 20 cm above were compared, after taking the precautions indicated above. Accordingly, concentrations were measured only in sap collected after a delay

of 20-30 minutes to allow for the effects of contamination from wounding to subside. The flow rates used to calculate delivery into the lamina of the leaf were those measured with sap flow gauges before excision. Flow rates used to calculate delivery into the base of the shoot were those of the actual sap since these flows were shown earlier to be close to those of the whole plant (Fig. 39 B,D F). The delivery values were calculated on a unit leaf area basis (m^2 or cm^2) to take account of the large difference in total area between the whole shoot and a single leaf.

Transpiration fluid from six plants was analysed for osmolites in a first experiment (Table 10 (i)). The mean osmolality in sap from the petiole stump was 11.0 mOsmol which estimates the true osmolality of the transpiration fluid entering this leaf lamina. Osmolality of sap from the shoot base was 12.67 mOsmol, slightly higher than in the leaf. This small difference in sap osmolality was statistically insignificant. However, calculated delivery rates of osmolites on a leaf area basis, evidently showed a smaller osmolite delivery per unit area into leaf 5 compared to that delivered into the whole shoot by the roots. The reason for smaller delivery rate into leaf 5 compared with shoot base is not a consequence of any major loss of solutes, which was small (13%) and statistically not significant. The difference arises because the rate of water loss per unit area of leaf was slower for leaf 5 than for the canopy as a whole (31% slower), leading to less total solutes being drawn into each unit area of the leaf. Similar observations were made in a second experiment (Table 10 (ii)).

Table 10: A comparison between sap flow rates, total osmolite levels and delivery rates of osmolites into the shoot base and petiole of leaf 5 in well-watered plants of 5-week-old *Ricinus communis*. Means of 6 replicates in experiment (i) and 4 replicates in experiment (ii) are shown. Results of t-tests are presented as either significantly different (sd) or not significantly different (nsd) at 5 percent significance level and marked with *. Values in brackets show values for petiole sap as a percentage of sap at the shoot base.

	Sap flow rate ($mm^3\ cm^{-2}\ s^{-1}$)		Concentration (mOsmol)		Delivery rate ($mOsmol\ cm^{-2}\ s^{-1}$)	
	Shoot base	Petiole	Shoot base	Petiole	Shoot base	Petiole
(i)	0.0107	0.0074 (31%)	12.67	11.0 (13%)	0.134	0.083 (38%)
	± 0.0005	± 0.0006	± 0.76	± 0.07	± 0.012	± 0.012
	*sd (0.05)		*nsd (0.05)		*sd (0.05)	
(ii)	0.0094	0.0065 (30%)	9.25	8.25 (11%)	0.088	0.055 (37%)
	± 0.0012	± 0.0004	± 0.09	± 0.62	± 0.015	0.003
	*sd (0.05)		*nsd (0.05)		*sd (0.05)	

The picture for ABA was different from that for total solutes. Comparison of ABA delivery into the shoot base and into the lamina of leaf 5 was made using four plants grown in each of two experiments with twin root systems. The lower roots were kept well-watered while water was withheld from the upper pots of the treated plants for 48 h. As shown previously (Chapter 5) ABA delivery from the drying roots increases within the first day of droughting the upper roots and is maintained over the next few days. On the basis of this observation, collecting xylem sap for ABA analysis after 48 h would ensure increased output by the dried roots. Table 11 shows that in the two separate experiments carried out drying the upper root system for 48

h, raised ABA delivery (as measured by the homogeneous immunoassay) into the shoot base. This was also true for delivery into the lamina of leaf 5.

In the well-watered plants some attenuation of ABA message was seen as xylem sap travelled out of the shoot base and into the lamina of leaf 5. However, this reduction was not statistically significant in the first experiment (Table 11 (i) whereas in the second experiment (Table 11 (ii)) significant reduction in the mean delivery rate of ABA into the lamina of leaf on a unit area basis was recorded. This decrease in delivery rate per unit area of leaf 5 was partly a consequence of slower transpiration rate than for the shoot as a whole. However, this was not sufficiently large to account for all of the smaller delivery rate. The substantial contribution to this smaller delivery rate was a loss of ABA concentration entering the leaf. In droughted plants there was a clear indication of loss in delivery of the ABA message in both the experiments (Table 11 (iii), (iv)). Here, also, lower delivery rates of ABA into the lamina of leaf 5 resulted primarily from a decreased concentration of ABA in the transpiration stream entering this leaf.

Table 11: A comparison between sap flow rates, ABA concentration and delivery rates of ABA into the shoot base and petiole of leaf 5 in well-watered and droughted, 5-week-old *Ricinus communis*. Means of 4 replicates in each of two experiments are shown. Results of t-tests are presented as either significantly different (sd) or not significantly different (nsd) at 5 percent significance level and marked with *.

	Sap flow rate ($mm^3\ m^{-2}\ s^{-1}$)		Concentration ($\mu mol\ m^{-3}$)		ABA delivery rate ($nmol\ m^{-2}\ s^{-1}$)	
	Shoot base	**Petiole**	**Shoot base**	**Petiole**	**Shoot base**	**Petiole**
			Sap from well-watered plants			
(i)	80.171 ± 10.882 *nsd (0.05)	69.088 (13%) ± 4.810	48.193 ± 8.733 *nsd (0.05)	39.151 (18%) ± 9.255	3.576 ± 0.554 *nsd (0.05)	2.798 (22%) ± 0.776
(ii)	96.645 ± 7.302 *nsd (0.05)	79.011 (18%) ± 2.577	35.272 ± 4.243 * sd (0.05)	11.815 (66%) ± 2.074	3.399 ± 0.375 * sd (0.05)	0.923 (73%) ± 0.128
			Sap from droughted plants			
(iii)	93.186 ± 8.902 * nsd (0.05)	82.219 (12%) ± 12.326	151.527 ± 34.992 *sd (0.05)	54.285 (64%) ± 11.923	13.496 ± 2.548 *sd (0.05)	4.735 (65%) ± 1.593
(iv)	83.636 ± 1.536 *nsd (0.05)	91.354 (8%) ± 4.532	96.991 ± 12.983 *sd (0.05)	42.796 (56%) ± 1.253	8.043 ± 0.930 *sd (0.05)	4.236 (74%) ± 0.478

Unlike osmolality where clearly there was little change in total osmolite levels in sap originating from the roots and that entering the lamina of leaf 5, ABA was lost *en route* to leaf 5.

Conclusions

Extracting authentic transpiration fluid from intact plants presents problems that have been addressed in this chapter. When a leaf is removed higher up in the canopy to sample xylem sap, sap flow rates can change. It was found that removing a leaf from the plant at balancing pressure increased sap flow along the petiole stump. It was also shown that the increased flow out of the petiole stump was redirected flow and thus unlikely to change solute concentrations. However, calculation of solute delivery would be in error if the fast, post-excision flow rate was used. Instead, flow rates prior to excision were used to calculate deliveries. Contamination of xylem sap obtained after removal of leaf lamina has been found here and previously by others (Else *et. al.*, 1995). Unless this contaminated sap is avoided, levels of solutes in the transpiration stream will be seriously overestimated. This error was avoided by allowing enough time for contaminated sap to be discarded. Attention is also drawn in this chapter to the drawbacks of collecting xylem sap from freshly detached leaves using a Scholander bomb, since this fluid too is likely to be contaminated.

When deliveries are calculated on a whole plant basis, the entire shoot is considered as a unit. However, transpiration of different leaves will vary depending upon their position on the plant, age, orientation relative to light, temperature and relative humidity around the leaf and their boundary layer resistance (Ansley *et. al.*, 1994; Smith and Allen, 1996). As a result of this, solute delivery rate per unit leaf area of the individual leaf will not necessarily be the same as that calculated for the whole

plant since rates of sap flow per unit leaf area will be different. Therefore, when comparing the fate of root signals as transpiration fluid travels in the plant, estimating delivery rates must be expressed on an area basis and their interpretation based on sap flow rates and solute concentrations.

A comparison of the components of xylem sap from the base of the shoot of *Ricinus communis* plants with that entering petiole of leaf 5 showed that while delivery of total osmotically active solutes per unit leaf area was smaller than that calculated for the whole plant, this was almost entirely the result of a slower transpiration rate per square centimetre. However, ABA deliveries were reduced as transpiration fluid moved up the plant as a consequence of mainly a loss of concentration between the shoot base and leaf 5 with a contribution from slower transpiration rate of leaf 5 than for the shoot as a whole. At $P = 0.05$ there was no statistically significant difference between rates of transpiration at the shoot base and of leaf 5. However, at $P=0.10$ these differences were significant. There appears to be a net loss of ABA from the xylem sap to the surrounding cells and vessels thus depleting xylem sap of the hormone, although where this loss takes place is unclear since xylem vessels are lightly lignified for most of the length. ABA metabolic rates are known to be rapid in shoot tissues (Trejo *et. al.*, 1993; Jia *et. al.*, 1996). It is conceivable that metabolism of ABA may take place in xylem sap itself, thus lowering the intensity of the message as sap moves up the plants. Overall, the results show the need to investigate hormone levels close to target to be sure that putative

messages from the roots do actually arrive at the proposed target sites. Comparisons between signal levels at the shoot base and in foliar targets will also eliminates the possibility that solute changes in sap samples taken from leaves occurred *after* the sap left the roots.

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Chapter 7 : A STUDY OF ANTITRANSPIRANT ACTIVITY IN XYLEM SAP OBTAINED FROM *RICINUS COMMUNIS* PLANTS

Introduction

The findings so far suggest that ABA from drying roots is unlikely to be a highly effective positive message influencing shoot responses such as stomatal closure. There is no doubt that drying roots of *Ricinus communis* produce more ABA, some of which is loaded into the transpiration stream within 12 h (Chapter 5). However, stomatal aperture and leaf expansion are markedly influenced only after a 2-day delay following the onset of drought. This raises the question of how these processes are induced 48 h later. One possible explanation could be that a certain level of ABA needs to be accumulated within the leaf, and around the stomatal guard cells, to influence leaf growth and stomatal aperture. Leaf dehydration is unlikely to cause ABA levels within the leaves to increase during this time since leaf water relations remain unaltered (Chapter 4). Thus, ABA originating from the drying roots could be accumulating slowly in the leaves. However, according to Jia *et. al.* (1996) the half-life of ABA metabolism appears to be about one hour in the two species they studied, and the rate of metabolism increased with increasing amounts of ABA moving into the leaves. If this is so then there would be very little accumulation over the two days, despite increased ABA delivery into the shoot of the droughted plants. In an earlier study Trejo *et. al.* (1993) suggested that the ability of the mesophyll to rapidly metabolise ABA moving into the leaves controls ABA levels in the mesophyll and

epidermis which in turn influences stomatal behaviour. Stomatal guard cells have been shown to be capable of ABA metabolism (Grantz *et. al.*, 1985), although Trejo *et. al.* (1993) were unable to show this. Thus, it may be that in *Ricinus*, because of fast ABA metabolism in the shoots it takes at least 48 h before sufficient ABA is accumulated in the leaves to levels which close stomata. A second possibility is that a shift in pH towards alkalinity would influence compartmentalization of ABA within the leaves (Hartung *et. al.*, 1988; 1990). Increased pH of xylem sap of droughted plants can play an important role, not only in compartmentation of ABA already present in leaves as well as that arriving into the leaves in xylem sap, but it may also influence partitioning of ABA in leaves between apoplast and symplast (Wilkinson and Davies, 1997). These authors found that there was more bulk ABA in leaf tissue at pH 7.0 compared to at pH 6.0 when ABA was fed into leaves *via* the transpiration stream, suggesting reduced uptake from xylem sap when in alkaline conditions. However, since drying the upper roots of *Ricinus communis* did not cause significant changes in pH of the xylem sap it could not have influenced the compartment distribution of ABA, or its metabolic rate within the leaf during this time or even deposition in phloem. A third possibility is that a second antitranspirant, present in the transpiration stream after 48 h of droughting, could have caused partial stomatal closure and inhibited leaf expansion. The first report suggesting that transpiration fluid of droughted plants may contain a substance other than ABA which inhibits transpiration was by Munns and King (1988). Munns (1990) used a leaf elongation assay and showed that a growth inhibitor was present in the xylem sap of droughted

wheat and barley. In *Ricinus communis,* the presence of an antitranspirant in the transpiration stream after 48 h of drying upper roots may explain the delayed stomatal response. Therefore, epidermal strips of *Commelina communis* were used in a bioassay to detect stomatal closing activity in xylem sap obtained from *Ricinus communis*. Although epidermal strips will behave somewhat differently to intact leaves they are frequently used because there is a direct interaction between stomatal guard cells and the chemical treatment dissolved in the bathing medium.

Method

The youngest, fully grown leaves of 3-week-old *Commelina communis* plants were used as a source of epidermal strips for the bioassay as described in Chapter 2. In two separate experiments it was found that although stomatal apertures in epidermal strips from the older leaves were initially wider than those from younger leaves, apertures were not stable, and after an initial increase over the first 2 h, apertures decreased (Fig. 43 A&B). Apertures in epidermal strips from the youngest fully expanded leaves were more stable (Fig. 43 C&D). Hence, epidermal strips from these leaves were used. Incubation of the strips in the MeS buffer for 90 - 120 minutes was adequate to allow complete stomatal re-opening following stripping-off of the epidermis prior to incubation in treatment solutions. A further 60 minute incubation in the treatment solution was then allowed for stomata to respond to the solution. Prior to incubating the epidermal strips in xylem sap collected from either well-watered or droughted *Ricinus communis*. ABA concentrations in the sap samples were determined using

RIA. The solutions were then diluted so that a range of natural ABA concentrations in the dilutions were within the active range established using synthetic ABA diluted in orders of magnitude from 10^{-6} M (1000 nM) to 10^{-10} M (0.1 nM). Since a number of different experiments were carried out and stomatal pore sizes varied between experiments (the size of fully open stomata ranged between 13 - 18 μm), effects on apertures are presented as percentages of apertures seen in MeS buffer (i.e., apertures in MeS buffer were taken as 100%).

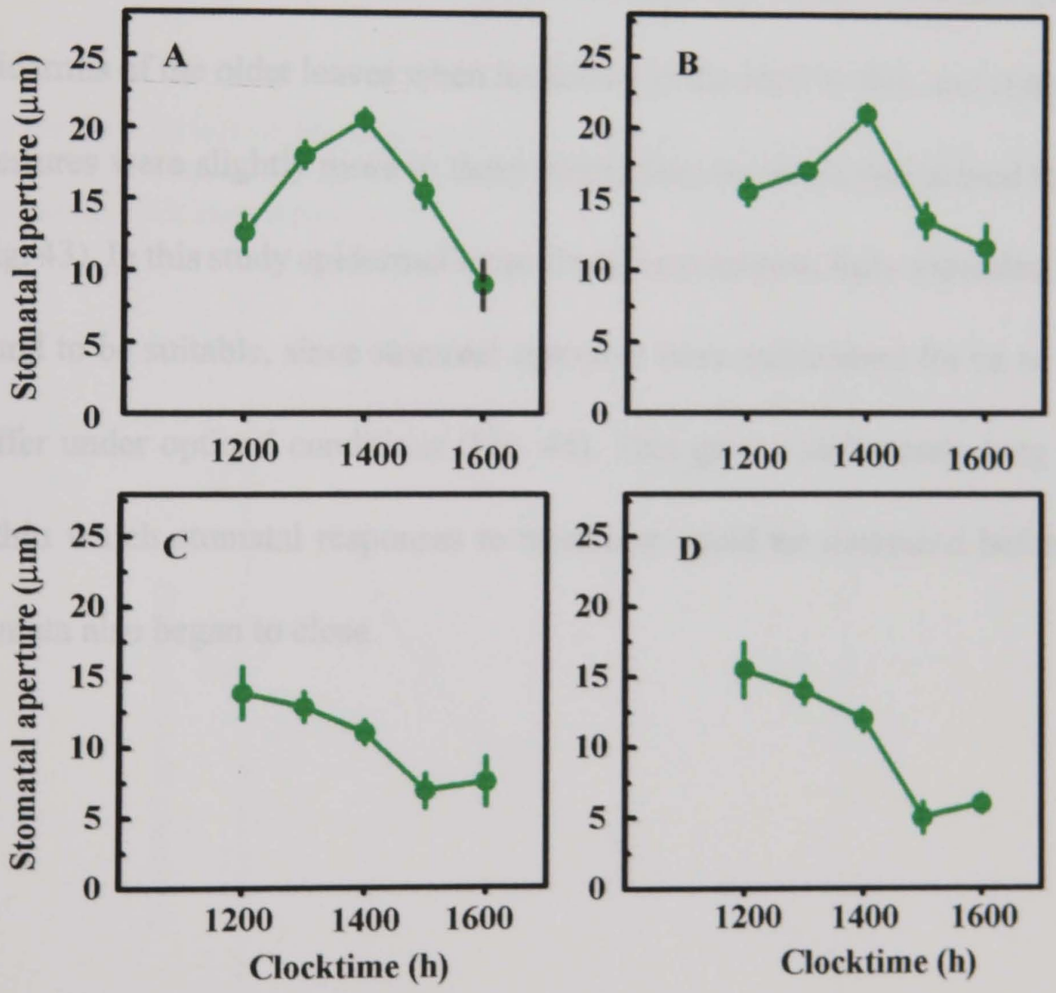

Figure 43 : Effect of incubating lower epidermal strips from 3-week-old *Commelina communis* in MeS buffer for 5 h on stomatal aperture. (A) and (B) show apertures in epidermal strips obtained from the 3rd oldest leaf, (C) and (D) show apertures in epidermal strips obtained from the youngest fully expanded leaves. Means of 50 replicates with standard errors.

ABA induces stomatal closure in isolated epidermal strips. To measure this response, the ionic composition, pH, and temperature of the incubation buffer need to be optimal for stomatal opening. The buffer used for incubation of epidermal strips of *Commelina communis* induced maximum stomatal opening with 100 mol m^{-3} KCl and a pH of 6.1 at 25°C (Else *et. al.*, 1996). In an earlier experiment, using epidermal strips from leaves of two different ages from the same plants (youngest fully expanded leaf, and third oldest leaf), it was found that stomata opened more slowly in epidermis of the older leaves when incubated in the MeS buffer, and although overall apertures were slightly more in these strips, they were not maintained for very long (Fig. 43). In this study epidermal strips from the youngest, fully expanded leaves were found to be suitable, since stomatal apertures were maintained for up to 5 h in MeS buffer under optimal conditions (Fig. 44). This gave a sufficiently long time frame within which stomatal responses to treatment could be measured before untreated stomata also began to close.

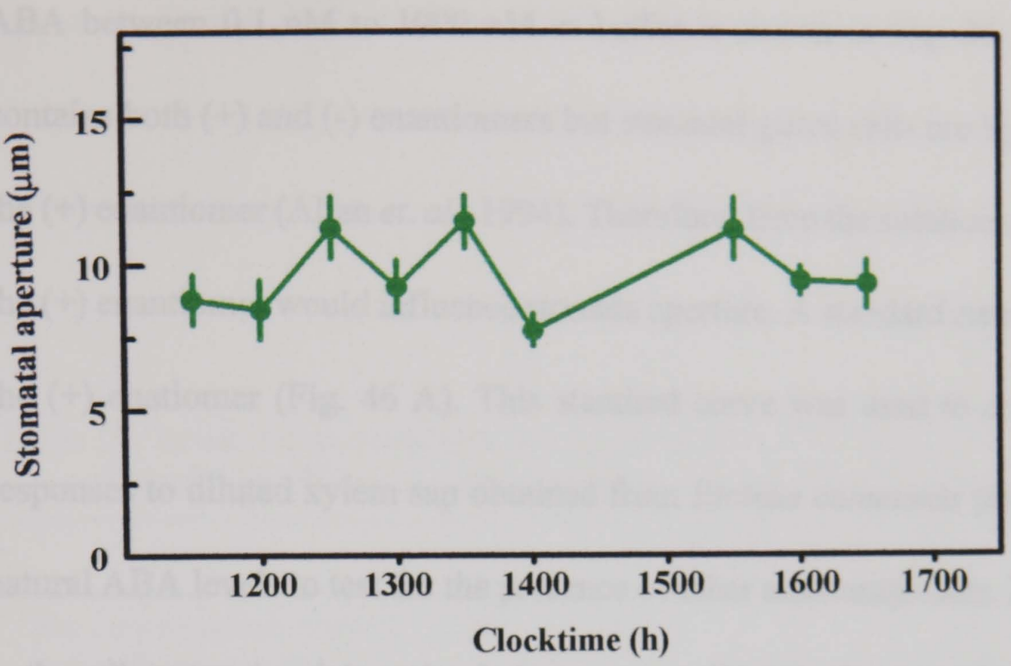

Figure 44 : Effect of incubating lower epidermal strips of the youngest, fully grown leaves of 3-week-old *Commelina communis* plants in MeS buffer for 5 h. Means of 50 replicates with standard errors.

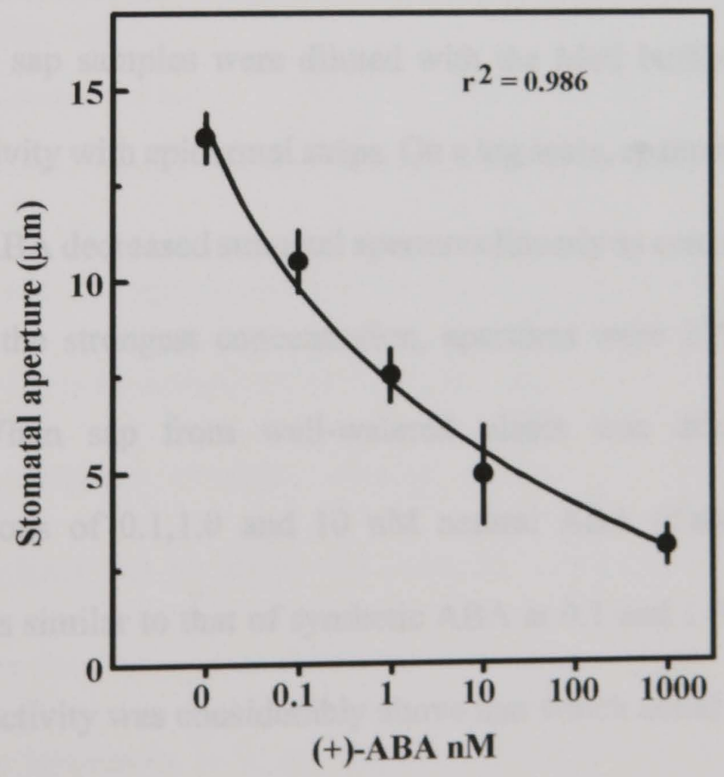

Figure 45 : Effect of increasing concentrations of synthetic (+)-ABA in buffer on stomatal aperture in lower epidermal strips from the youngest fully grown leaves of 3-week-old *Commelina communis*. Means of 50 replicates with standard errors

A standard curve showing the response of stomata to concentrations of synthetic ABA between 0.1 nM to 1000 nM in buffer is shown in Fig. 45. Synthetic ABA contains both (+) and (-) enantiomers but stomatal guard cells are highly selective to the (+) enantiomer (Allan *et. al.*, 1994). Therefore, from the solution of (±)-ABA only the (+) enantiomer would influence stomata aperture. A standard curve was drawn for the (+) enatiomer (Fig. 46 A). This standard curve was used to compare stomatal responses to diluted xylem sap obtained from *Ricinus communis* plants with known natural ABA levels to test for the presence of other antitranspirants. Xylem sap from both well-watered and droughted plants were collected after inducing sap flow in the detopped plants to flow at rates of whole plant transpiration. ABA concentration in xylem sap from well-watered *Ricinus communis* plants (determined using RIA) was found to be 20 nM, and that in sap from plants with upper roots dried for 48 h was 140 nM. These sap samples were diluted with the MeS buffer and tested for stomatal closing activity with epidermal strips. On a log scale, spanning the range 0 - 1000 nM, synthetic ABA decreased stomatal apertures linearly as concentrations increased (Fig. 46 A). At the strongest concentration, apertures were 20% of those in ABA-free buffers. When sap from well-watered plants was diluted in steps to create concentrations of 0.1,1.0 and 10 nM natural ABA (Table 12), stomatal closing activity was similar to that of synthetic ABA at 0.1 and 1.0 nM. However, at the 10 nM level, activity was considerably above that which could be explained by its ABA content alone since stomatal closure was almost complete (<5%). A similar picture emerged when testing sap of plants where the upper roots were droughted for 48 h.

Sap from these plants at each natural ABA concentration was less active than sap from well-watered plants but statistically similar to the activity of synthetic ABA at 0.1, 1.0 and 10.0 nM. However, the least diluted sap, which contained 100 nM natural ABA was considerably more active than 100 nM synthetic ABA.

Table 12: Xylem sap concentrations and natural (+)-ABA concentrations in the sap collected from *Ricinus communis* plants used to incubate epidermal strips.

	Well-watered plants				
Sap concentration relative to that flowing at the transpiration rate (%)	100	50	5	0.5	
(+)-ABA concentration (nM)	20	10	1	0.1	
Sap concentration relative to that flowing at the transpiration rate (%)	100	71.4	7.14	0.714	0.071
	Droughted plants				
(+)-ABA concentration (nM)	140	100	10	1	0.1

These results suggest that an antitranspirant other than ABA is present in sap but that its activity is diluted away more quickly than activity due to ABA. Before a true comparison between stomatal closing activity in sap from well-watered and droughted plants can be made, the level of stomatal closing activity needs to be corrected for two sources of differential dilution. The first differential dilution was made in the laboratory to bring natural ABA levels to those which were equal to synthetic (+)-ABA. When data were re-plotted (Fig. 46 B), using dilution levels used in the solutions tested in Fig. 40A, there was little difference between the two curves.

The second differential dilution took place *in the plant* because transpiration in droughted plants was 24% slower than in well-watered plants. Only when these corrections due to slower transpiration in droughted plants are made can any difference in antitranspirant activity be ascribed to an increase in output from drying roots. When this was done and data re-plotted (Fig. 46 C), it was found that the curves obtained for sap from both droughted and well-watered plants were the same. This implies that there was no difference in the amount of antitranspirant activity exported from roots of drying and well-watered plants.

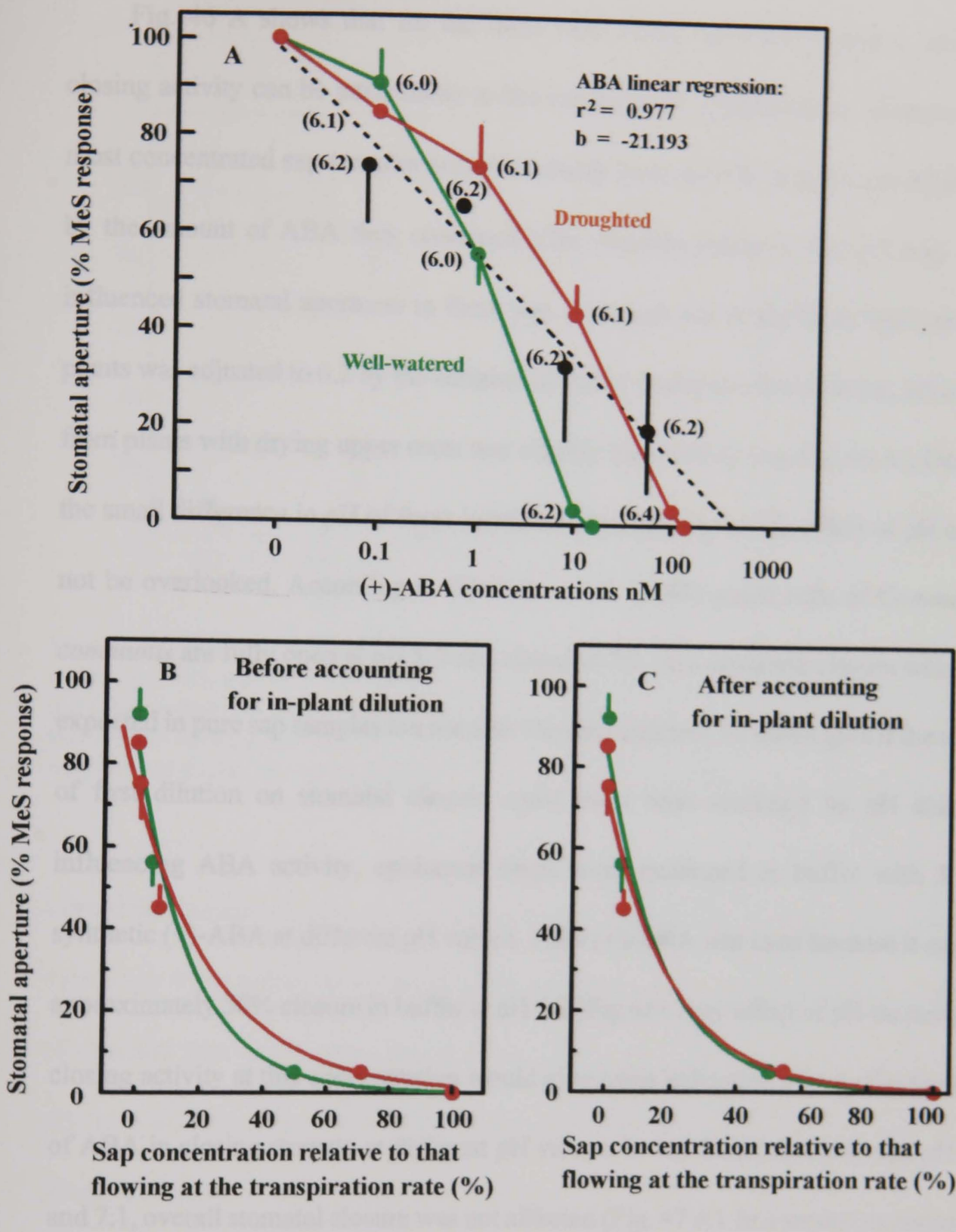

Figure 46 : Response of stomata of lower epidermal strips of fully-expanded *Commelina communis* to synthetic ABA and to xylem sap of well-watered and droughted plants. This sap was collected while flowing from roots at rates close to those of whole plant transpiration (10.254 $mm^3 s^{-1}$, 7.79 $mm^3 s^{-1}$ respectively) and diluted as shown in Table 12. (A) shows the activity of synthetic ABA over 0 to 1000 nM and of diluted sap plotted against its natural ABA concentration. (B) shows the activity of diluted sap plotted against the level of dilution in relation to that of sap flowing at the rate of transpiration. (C) shows the activity of diluted sap plotted against the level of dilution after arithmetically increasing the dilution levels in droughted plants to compensate for slower transpiration.

Fig. 46 A shows that for the three most dilute sap concentrations, stomatal closing activity can be attributable to the natural ABA concentration. However, the most concentrated sap samples possessed much more activity than can be explained by the amount of ABA they contained. One possible reason is that pH may have influenced stomatal apertures in these sap. Although pH of sap from well-watered plants was adjusted to 6.2 by the addition of buffer during the first dilution, pH of sap from plants with drying upper roots was slightly higher (6.4) (see Fig. 46 A). Despite the small difference in pH of these incubation media the possible effect of pH could not be overlooked. According to Edwards *et. al.* (1988) guard cells of *Commelina communis* are fully open at pH 5.5 and closed at 7.5, thus complete closure would be expected in pure sap samples but not after the first dilution. To investigate if the effect of first dilution on stomatal closure could have been mediated by pH changes influencing ABA activity, epidermal strips were incubated in buffer with 5 nM synthetic (+)-ABA at different pH values. 5 nM (+)-ABA was used because it caused approximately 50% closure in buffer at pH 6.2 (Fig 45). Any effect of pH on stomatal closing activity at this concentration would give some indication of the effectiveness of ABA in closing stomata at different pH values. It was found that between pH 6.2 and 7.1, overall stomatal closure was not affected (Fig. 47 A). In a second experiment, the effect of 5 nM (+)-ABA on stomatal aperture increased when pH was 7.2 or above, but this was far more alkaline than any of the diluted sap samples tested in Fig. 46 (Fig. 47 B).

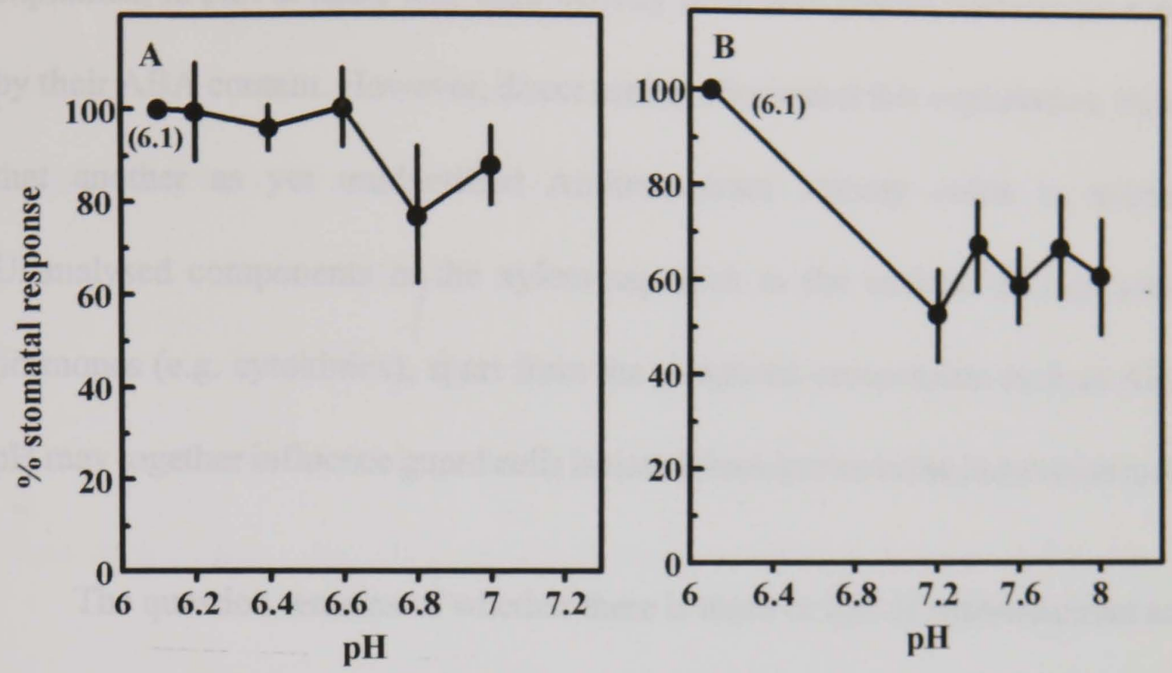

Figure 47 : Effect of increasing pH of the incubation buffer on stomatal aperture in epidermal strips from the lower epidermis of the youngest fully grown leaves of 3-week-old *Commelina communis* plants. A constant 5 nM (+)-ABA solution was maintained in the buffer throughout the experiment. A and B are the results of two experiments carried out under the same conditions. Means of 50 replicates with standard errors.

Discussion

Complete stomatal closure in epidermal strips of *Commelina* in pure (i.e., undiluted) xylem sap from well-watered and droughted plants demonstrates the high sensitivity of stomatal guard cells to antitranspirants of xylem sap. These could include ABA, pH, ions and possibly some unidentified antitranspirant. Synthetic (+)-ABA alone reduced stomatal apertures by a maximum of 75% in the concentration range found in xylem sap of these plants. This indicates that some other component of the xylem sap of these plants caused the additional stomatal closure. The only other characterized component, apart from ABA, was pH. In undiluted sap, pH was 7.6 in well-watered plants and 7.5 in droughted plants, compared to pH of the MeS buffer

which was 6.1 - 6.2. Hence this increased alkalinity of undiluted xylem sap could have explained, in part at least, why their activity level was greater than can be explained by their ABA content. However, direct testing eliminated this explanation, indicating that another as yet unidentified Antitranspirant activity exists in xylem sap. Unanalysed components in the xylem sap such as the cocktail of ions and other hormones (e.g. cytokinins), apart from the measured components such as ABA and pH may together influence guard cells isolated from leaves in the incubation medium.

The question remains of whether there is more or less of antitranspirant activity exported from drying roots than from well-watered roots. To avoid being misled by differential dilutions between the two treatments in the analysis, account was taken of dilutions made in the laboratory after sap collection and that taking place within the plant before sap collection. The resulting activity curves revealed that at similar levels of overall dilution there was a similar level of overall antitranspirant activity and no evidence that drying roots export more such activity. This suggestion is based on results of only a single experiment of this kind carried out using xylem sap from *Ricinus communis*. Clearly, more experiments are needed and a closer look at stomatal response to more gradual dilution is also required.

The results in this chapter re-enforce those of other studies on stomatal response to exogenous ABA (Trejo *et. al.*, 1993; Roelfsema and Prins, 1995; Else *et. al.*, 1996), showing that even to bring about modest changes in stomatal conductance in epidermal strips, ABA concentrations in the bathing medium needed to be increased

in steps of approximately 10-fold. Even with xylem sap, dilution of 10-fold caused only about 20% stomatal closure in epidermal strips. These increments are large compared to increases in ABA concentrations and deliveries out of drying roots of droughted plants, which are comparatively small (Bano *et. al.*, 1993; Khalil and Grace, 1993; Masia *et. al.*, 1994; Schurr and Schulze, 1996, Shashidhar *et. al.*, 1996). This, combined with the evidence presented by Trejo *et. al.* (1993) and Jia *et. al.* (1996) that leaves have a high capacity to metabolise ABA implies that ABA output from drying roots is too small to have a marked effect on stomatal apertures.

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Chapter 8 : SUMMARY AND FUTURE WORK

General Summary

This project investigated root-to-shoot communication in *Ricinus communis* plants in response to drying part of the root system, a stress that can be expected to influence shoot behaviour through chemical rather than hydraulic signalling. Much of the project was concerned with establishing reliable methods for quantifying possible root signals, particularly ABA, transported in the transpiration stream to the shoots. A particular concern was developing a suitable method of sap collection from intact plants by first addressing the shortcomings in methods used previously. *Ricinus communis* was chosen because it is endemic to regions where summers are dry. Thus, it could be expected to possess distinct mechanisms of adaptation to soil drying. A second attribute of *Ricinus communis* is the ease with which phloem sap can be obtained from this plant, making it possible to study phloem sap in addition to xylem sap.

Chapter 3 described the development of a twin-root system for growing *Ricinus communis* in a way that allowed half of the roots (in the upper pots) to be dried while the lower roots remained fully hydrated. This partial drying treatment led to slower leaf growth and stomatal closure. Shoot water content was not affected by this drying of upper roots. The stomatal closure and inhibition of leaf expansion were strongly established 2-3 days after droughting began.

In Chapter 4, water relations of the shoot were studied as roots in the upper pots were dried. It was found that during the early stages of root drying (up to 4 days) shoot water relations (Ψ_w, Ψ_s, Ψ_p) remained unchanged. This confirmed that stomatal closure and inhibited leaf expansion after the second or third day of droughting was not dependent on hydraulic signals from the drying upper roots.

In Chapter 5, ABA transported out of the drying roots was quantified. After detopping the plants, xylem sap was sampled after inducing it to flow through the roots at rates close to those of whole plant transpiration. ABA concentration in the sap was determined by a radioimmunoassay that used a proximity scintillation reagent. Delivery rates (concentration x flow rate) were calculated which took into account the different rates of transpiration in the well-watered and treated plants. This delivery term was modified to include the relative sizes of roots and shoots of the two sets of plants. The most integrating delivery term developed was in terms of moles of ABA transported out of a kg of root into a m^2 of leaf per second. Delivery of ABA out of roots was found to increase within 12 hours of the start of withholding water, and this increased rate was maintained for a further 4 days. Delivery rates of nitrate and ACC from the roots did not change during this time and the pH of the xylem sap also remained unaltered by drying the upper roots. The principal conclusion is that if ABA was acting as a root-sourced signal which controlled stomatal conductance and leaf expansion, it was a weak signal since these processes did not respond strongly to it until at least two days later. This possibility prompted work described in the next chapter.

In Chapter 7 the presence of an antitranspirant other than ABA was present in xylem sap was sought. Xylem sap was obtained from detopped plants after inducing sap to flow at rates close to whole plant transpiration. Stomatal closing activity of the sap was tested on epidermal strips of *Commelina communis* against stomatal closing activity of synthetic ABA. Adjustments were made to activity levels for differential dilutions carried out in the laboratory and *in planta* as a result of different rates of transpiration between droughted and well-watered plants. Two features were clear. Firstly, stomatal response to natural ABA in xylem sap was similar to that in synthetic ABA. Secondly, although antitranspirant activity other than ABA was found in sap from both well-watered and droughted droughting the upper roots did not increase its delivery out of the roots. Failure to find any evidence for an increase in this alternative antitranspirant to ABA in xylem sap of droughted *Ricinus* plants led to the study of the fate of root-sourced ABA as sap travelled up the plant.

Comparison of concentration and delivery of ABA in transpiration stream entering the base of the shoot with that entering the lamina of leaf 5 was described in Chapter 6. A technique for collecting transpiration fluid entering the lamina of leaf 5 was used that involved measuring sap flow rates into the leaf lamina before and after its excision, and investigated the possible effect of leaf excision on concentration of solutes in the sap collected from the cut petiole stump. A promotion of sap flow into the cut stump of leaf 5 was seen after removal of the leaf lamina. This was shown to be redirected sap since no consistent change in total sap flow at the shoot base was

measured during this time. Thus, it was assumed that concentration of solutes would not be decreased by the faster sap flow caused by leaf excision. This was confirmed by showing that total solute concentrations entering shoot base was similar to in sap expelled from the excision point on the petiole. However, there was a definite reduction in concentration of ABA entering lamina of leaf 5 compared to that coming out of the roots. When delivery rates of total solutes and ABA into the whole plant was compared to that into leaf 5 on a unit area basis, these were found to be lower in the leaf than at the shoot base. This could not always be explained in terms of slower transpiration rates into the leaf compared to the whole canopy (on a unit leaf area basis). Instead, it was mainly the result of a loss in ABA concentration in sap as it travelled from the shoot base to the leaf. This was especially clear for droughted plants. Thus, some ABA is removed from the transpiration stream as it travels up the plant. This may be a possible mechanism of dampening the signal in the early stages of drought stress and may be part of the explanation of why it appears to take 2-3 days before enough ABA is accumulated around the guard cells to promote stomatal closure. It may also take a similar length of time for ABA to accumulate to levels which inhibit leaf expansion. An additional point to consider is that this work showed that drying upper roots of *Ricinus* plants increased ABA delivery out of the drying roots by 3-4-fold. Similar increases in delivery rates and concentrations have been shown by others. This increase appears small compared to the 10-fold and more increase required to cause significant stomatal closure when added directly to guard cells of *Commelina* epidermal strips. This casts further doubt on the role of increased

delivery of ABA as a root-sourced message that is responsible for closing stomata of *Ricinus communis* during soil drying. The role of increased ABA as a root signal requires further investigation before it can be seen to be a convincing component of the adaptive responses to soil drying.

Future Work

- Antitranspirant activity in xylem sap of *Ricinus communis* plants needs to be re-examined. Preliminary studies (Chapter 7) failed to show an increase in antitranspirant activity in droughted plants other than that attributable to ABA. This was the result of findings of a single experiment and clearly more experiments are needed to be sure of this result.
- Leaf elongation was inhibited after a delay of 2 days of soil drying, suggesting the possible involvement of an inhibitor of leaf expansion that is not ABA. This possibility could be checked using leaf expansion assays. The presence of leaf expansion inhibitors other than ABA has been reported in some other species (e.g., wheat) in response to soil drying.
- The activity of the ABA delivered to leaves of intact plants as soil dries requires more direct testing. Excised leaves could be fed with exogenous ABA at the same rates as those being delivered in well-watered and droughted plants. Stomatal response to this exogenous ABA measured it will show if stomata of intact plants are responding to ABA alone or if some other component of the xylem sap may be required to influence its effects on stomatal closure. Since pH of xylem sap did

not change with drought treatment it is not likely that intact stomata respond to changes in pH caused by drought. However, it may still be worthwhile to test pH effects on stomatal sensitivity, provided the pH range tested was that occurring naturally in transpiration fluid.

- Using excised leaves, their capacity to metabolise ABA could be studied. This will quantify the capacity of these leaves to metabolise or conserve xylem-borne ABA for over at least 2 days after the plants start to experience drought stress.
- ABA concentration and delivery rates into lamina of leaf 5 was studied in this project. A study of concentration and delivery into other leaves in the canopy will show if ABA is being loaded preferentially into some leaves and not others.
- The effect of root drying on photosynthesis needs to be examined. The possibility cannot be ignored that in some way drying part of the root system reduces photosynthesis in leaves. This in turn, would increase internal carbon dioxide concentrations in leaves, which would close stomata.
- Cytokinin is believed to cause stomatal opening and to sustain the photosynthetic apparatus. Stomata may be responding to a balance between ABA and cytokinin. Drought stress has been found to inhibit cytokinin production by roots in some systems. Drying upper roots of *Ricinus* may change cytokinin/ABA balance within xylem sap in favour of ABA. ABA output was influenced within the first day of droughting. It is possible that cytokinin production and or export by roots may be depressed later, influencing the hormonal balance, resulting in the delayed response in stomatal closure and leaf expansion. Concentrations and deliveries of the appropriate cytokinins in the transpiration stream are therefore required.

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