

**EFFECTS OF WATER STRESS AND PARTIAL SOIL-DRYING ON
SENESCENCE OF SUNFLOWER PLANTS**



by

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SUMMARY

The present thesis aimed to investigate the symptoms of leaf senescence in response to plant shoot water stress and explore the possibility of the involvement of a non-hydraulic root signal in the senescence response of mature leaves of sunflower.

The effect of plant leaf water status on mature leaf senescence of sunflower was evaluated from changes in the leaf water potential and soluble protein, total free amino acid and chlorophyll contents, and chlorophyll a/b ratio, following a regime of a single water stress cycle (15 days) and resumption of adequate water supply. The fall in leaf water potential accelerated the rate of loss of leaf soluble protein content compared to that occurring in non-stressed senescing leaves. In corresponding to the breakdown of soluble protein there was an accumulation of free amino acid, including a presumed proline accumulation. This phenomenon is different from that which occurred in attached naturally senescing leaves, in which the total amino acid content was found to decrease in company with the fall in leaf soluble protein content.

The rate of loss of chlorophyll content was also accelerated by water stress. Chlorophyll degradation in response to water stress, however, was slower than the protein response. A similar pattern of response to that in total chlorophyll content was also achieved in the chlorophyll a/b ratio.

Upon re-watering, there was a recovery in the protein content to the level found in control leaves coupled with a rapid disappearance of the total free amino acid accumulated. Total chlorophyll content and chlorophyll a/b ratio, on the other hand, did not recover to the control levels after watering was resumed. However, there was a change in the rate of decline of both total chlorophyll content and chlorophyll a/b ratio to the normal senescence rate, subsequent to the recovery in leaf water potential.

To examine the possibility of an effect of a root-sourced signal on leaf senescence, plants were grown with the root system divided equally between two containers. One half of the root system was exposed to drying soil by withholding water from this half of the soil, whereas the other half of the soil was well-watered to maintain the leaf water status. Metabolic changes in mature leaves of these plants following six weeks of the partially soil-drying imposition were determined in comparison to those in well-watered plants. The loss of leaf soluble protein content showed a significant response to the effect of treatment from week 4 of exposing the plant to soil-drying in part of the root system. However, there was no significant response in leaf total chlorophyll, free amino acid and proline contents, but a slight response, when summed across time and leaf position, in chlorophyll a/b ratio compared to that in the control leaf.

To investigate whether this increase in the leaf protein loss rate in response to soil-drying could be attributed to reductions in nutrient supply or undetectable changes in leaf water status as a result of the loss of half the nutrient or water gathering capacity, the half of the root system in drying soil was excised after four weeks of exposure to drying soil. This treatment was followed by an increase in leaf protein content, indicating relief from the effects of roots growing in dry soil.

The results are interpreted to demonstrate an effect of a non-hydraulic root signal on senescence of the leaves. The alleviation of the leaf soluble protein loss rate by the excision of the root system in drying soil also indicates that this signal originates in roots in dry soil and acts to promote protein loss.

STATEMENT

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

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

(Sathaporn Wongareonwanakij)

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CHAPTER I

GENERAL INTRODUCTION



Water stress eventually causes leaf senescence (O'Neill 1983). This is manifested by chlorophyll loss (Alberte *et al.* 1977; Radin 1981; Mayoral *et al.* 1981) and protein loss (Radin 1981; Mayoral *et al.* 1981). Losses of chlorophyll and protein also occur in well-watered aged leaves, and are commonly used as indices of natural senescence (Thimann 1980; Nooden 1980). It has been assumed that water stress-induced and natural senescence are both the same universal senescence process (Radin 1981; O'Neill 1983). However, senescence syndrome is a complex, multi-step process, and it appears that the sequence of events can vary, since certain symptoms can be disengaged from the rest (Woolhouse 1986). Thus, one aim of this thesis was to characterise the sequence of metabolic changes occurring in leaves of different insertion to the plant during natural senescence and the inter-effect of water stress on these changes.

A second aim was to examine the possibility of a direct effect of a non-hydraulic signal from the root system exposed to dry soil on leaf senescence. Several reports have demonstrated that leaf stomatal conductance (Blackman and Davies 1985; Gollan *et al.* 1986; Zhang *et al.* 1987; Zhang and Davies 1989; Neales *et al.* 1989) and growth (Passioura 1988; Saab and Sharp 1989; Gowing *et al.* 1990) can be limited by a fall in soil water status independently of the leaf water status. It is likely that the non-hydraulic signals involved with changes in hormones originate in the root system, are translocated and control the shoot responses (Davies and Zhang 1991). A decrease in the cytokinin that is normally supplied to the shoot (Blackman and Davies 1985) and an increase in the ABA supply to the shoot (Zhang *et al.* 1987; Zhang and Davies 1989) have been suggested as signals from the roots to the shoot. Such hormonal changes are known to promote senescence. Thus, in long-term exposure of part of the root system to dry soil, it is possible that such signals can also have an effect on leaf senescence. In addition, there is also a hypothesis that stomatal closure is an initiator of leaf senescence (Thimann and Satler 1979a, 1979b).

Finally, excision the part of the root system in dry soil has been shown to eliminate the effects of root signals on shoot responses (Gowing *et al.* 1990). Such treatments can provide some information on the nature of the signal from roots to shoots.

CHAPTER II
LITERATURE REVIEW

1 Leaf senescence

1.1 Introduction

Woolhouse (1978) terms senescence as the deteriorative changes in living organisms which lead to death. As a plant phenomenon, however, it need not mean death of the whole plant. Leopold (1961, 1980) classifies the patterns of plant senescence into four categories:

(1) Overall senescence, in which the whole plant dies. Many seasonal or annual herbaceous species are typical of this category.

(2) Top senescence, where the shoot dies off seasonally, but the root remains viable. This category includes perennial and bulbous plants.

(3) Deciduous senescence, in which only the leaves die, as in deciduous trees.

(4) Progressive senescence, where the older leaves die while younger ones remain viable. Evergreen perennials are good examples of this type.

However, in relating to flower and/or fruit development, a further categorisation classifies whole plant senescence behaviour into two types: monocarpic and polycarpic senescence (Nooden 1980; Woolhouse 1986). Monocarpic is a term for senescence and death that occurs following a single reproductive phase. In the second type, polycarpic, senescence occurs only after more than one reproductive phase.

For the various organs of one plant, however, the events are more complicated. Different organs of the same plant may exhibit different rhythms of senescence. Within a plant, the study of the senescence of leaves in particular has an advantage over that of

other plant organs. This is because the changes that occur in senescing leaves are especially obvious, coupled with the experimental convenience of leaves and their importance to the plant. Hence, the scope of senescence in this thesis will emphasise leaves, which is addressed by Thomas and Stoddart (1980) as "the series of events concerned with cellular disassembly of the leaf and the remobilisation of materials released during this process".

1.2 The general metabolic changes in leaf senescence

The senescence syndrome is not necessarily the same in all plant species. The so-called "stay-green senescence" varieties (Thomas and Smart 1993) are good examples of this variation. Even the sequence of events is not identical in all cases. It appears that certain symptoms can be disengaged from the rest. There are several examples from experiments to support this phenomenon. In senescing wheat leaves, French (1985) found that protein catabolism was similar in response in natural, dark-induced and water stress-induced senescence, but chlorophyll degradation was more sensitive in water stress-induced senescence than in natural senescence. Another example of this phenomenon is the observation of Mae *et al.* (1993) with mature leaves of *Lolium temulentum*. In these leaves the CO₂ fixation rate and the RuBPCase declined similarly in shaded and unshaded leaves, whereas the levels of chlorophyll, LHCP II and the 65 kDA protein of photosystem I remained higher in shaded than unshaded leaves until late in senescence. Moreover, three cDNAs from dark senescence-induced excised barley leaves have been isolated by Becker and Apel (1993), two of them are found in wounded and in droughted tissues but are not detectable in natural senescence, while the third is found in natural senescence but not in the stress conditions. Despite these variations, the metabolic changes which are normally used to indicate leaf senescence are as follows:

1.2.1 Decline in photosynthetic rate

As reviewed by Thimann (1980), a wide range of species has been reported to decline in photosynthetic activity during senescence. However, the factors limiting photosynthetic rate are not cleared. Several changes occurring during senescence have been linked with the response and claimed to be causal. This suggests that the limiting factors may be varied depending on conditions and stages of senescence.

A decrease in leaf conductance to CO₂ diffusion apparently restricts photosynthesis during senescence (Osman and Milthrope 1971). Stomatal and mesophyll conductances have been shown to decrease during senescence, in parallel initially, but more rapidly in the mesophyll during later stage of senescence (Woodward and Rawson 1976).

The activities of Calvin cycle enzymes are important determinants of mesophyll conductance. Ribulose 1,5-bisphosphate carboxylase (RuBPCase) activity is closely correlated with photosynthetic rate of pea (Smillie 1962) and *Perilla frutescans* (Woolhouse 1967) leaves. The amount and activity of RuBPCase has been observed to be high in mature leaves and decline during senescence in wheat (Wittenbach 1979), barley (Friedrich and Huffaker 1980) and soybean (Wittenbach *et al.* 1980). However, photosynthetic rate is not solely limited by RuBPCase activity, since the enzyme and its activity had begun to decline before photosynthesis was affected both in wheat (Wittenbach 1979) and barley (Friedrich and Huffaker 1980), although not in soybean (Wittenbach *et al.* 1980).

Loss of chlorophyll during senescence has also been thought to be the cause of the reduction in photosynthesis (Sestak and Catsky 1962). Camp *et al.* (1982) found that the change in photosynthetic rate of senescing wheat leaves was more closely coincident with the decline in chlorophyll content than with the reduction in RuBPCase activity. Electron transport rate of chloroplasts was also observed to decline as senescence

proceeded in wheat (Camp *et al.* 1982), barley (Holloway *et al.* 1983) and beans (Jenkins and Woolhouse 1981a, 1981b). However, Friedrich and Huffaker (1980) found that photosynthesis was more strongly correlated with RuBPCase activity than chlorophyll content. Mutant plants that retain their chlorophyll but lose their photosynthetic activity (non-functional "stay-green") during senescence (Thomas and Smart 1993; Smart 1994) are also good examples of plants that do not fit the correlation between chlorophyll content and photosynthetic rate.

1.2.2 Loss of chlorophyll

The most visually obvious symptom of leaf senescence is yellowing due to chlorophyll loss. Chlorophyll disappearance has been used as the principal index of senescence since the earliest studies in senescence (Thimann 1980). However, the mechanism of chlorophyll loss remains elusive (Smart 1994). In dark-induced senescence of detached oat leaves, the loss of chlorophyll content begins a few hours after protein loss (Martin and Thimann 1972a; Thimann *et al.* 1977). In natural senescence of attached leaves, however, it is often found that chlorophyll loss begins several days later than protein loss (Camp *et al.* 1982; Peoples *et al.* 1983), although occasionally they begin simultaneously (Wittenbach *et al.* 1980). Nevertheless, thereafter, the decline in these two cell components is usually in close parallel (Wittenbach *et al.* 1980; Camp *et al.* 1982; Peoples *et al.* 1983). However, this is not always the case, as a mutant plant of *Festuca pratensis* retains its chlorophyll while losing its protein content during senescence (Thomas and Stoddart 1975).

During senescence, measurements on the amount of chlorophyll per chloroplast indicates that the fall in leaf chlorophyll content is entirely due to a loss of chlorophyll from individual chloroplasts rather than to a reduction in chloroplast number (Jenkins and Woolhouse 1981a; Jenkins *et al.* 1981). This is consistent with the finding that chlorophyll levels decline more rapidly than total numbers of chloroplast (Martinoia *et*

al. 1983; Wardley *et al.* 1984). Moreover, a pool of fluorescent compounds derived from the breakdown of chlorophyll has been observed to be located in the chloroplasts (Duggelin *et al.* 1988). However, the mechanism(s) responsible for the loss of chlorophyll are controversial.

Isolated chlorophyll is rapidly bleached by light due to photo-oxidation (Brown *et al.* 1980). Photo-bleaching can also occur in leaves under excessive irradiation (Smart 1994). Maunders and Brown (1983) have shown that the loss of chlorophyll in attached senescing leaves of sycamore is accelerated by light. However, photo-oxidation cannot account for dark-induced senescence (Thimann 1980).

The finding that, in isolated spinach chloroplasts, pigment bleaching is due to thylakoid membrane ageing which can be accelerated under anaerobic conditions or by heating the membrane in both light and dark conditions, and that two radical scavenger components (salicyl-hydroxamic acid and 8-hydroxy-quinoline) reduce the rate and the extension of pigment bleaching, led Dupont and Siegenthaler (1986) to propose a free radical (or singlet oxygen) concept of chlorophyll loss. The concept of a free radical mediated pathway is that the onset of pigment degradation results from the failure of the scavenging processes that normally protect against free-radical attack. In contrast, Thomas and Matile (1988) demonstrated that pigments are normally broken down by a different pathway which does not involve free radicals. Photo-bleaching of a non-yellowing mutant of *Festuca pratensis* is not different from that in the normal genotype.

Several enzymic activities have been suggested to bring about chlorophyll break down. These include chlorophyllase (EC 3.1.1.14) (Purvis and Barmore 1981; Kuroki *et al.* 1981; Purohit 1982a, 1982b), lipoxygenase (Holden 1970), peroxidase (Matile 1980; Martinoia *et al.* 1982; Huff 1982), fatty acid-dependent oxidase (Martinoia *et al.* 1982; Luthy *et al.* 1984) and Mg-dechelataase (Schoch and Vielwerth 1983; Ziegler *et al.* 1988; Shioi *et al.* 1991). However, the behaviour of these enzymes remains obscure

(Thomas *et al.* 1989; Smart 1994). Possibly, membrane properties are involved in the activity of these enzymes. The association of enzymes such as chlorophyllase, peroxidase and oxidase with the thylakoid membrane in normal conditions (Terpstra 1977; Martinoia *et al.* 1982) suggests that the enzymes are not normally in contact with their substrates. Hence, a change in membrane structure is necessary to activate these enzymes.

The chlorophyll molecule is bound *in vivo* to lipoprotein complexes situated in the chloroplast thylakoid membrane. Differences in pigment aggregation and the type of protein in the chlorophyll-protein complexes determine their specific properties and functions in the different reactions of photosynthesis (Sestak 1977). Two main chlorophyll-protein complexes have been isolated and characterised by Thornber (1975). One of them is P700-chlorophyll a-protein (CP I), which contains the reaction centre of photosystem one (PS I). The other is the light-harvesting chlorophyll a/b-protein complex (LHCP), which acts as an antenna mainly for photosystem two (PS II). During natural senescence, the loss of CP I component is more rapid than that of LHCP (Bricker and Newman 1980; Jenkins *et al.* 1981). This results in the common observation that the chlorophyll a/b ratio declines during natural senescence (Sestak 1977), since chlorophyll b is located only in the LHCP complex. The more rapid loss of the CP I complex also suggests that the environment of the chlorophyll molecule has an effect on its degradation. This implies the possibility that the lipoprotein complexes must be altered in some way before the chlorophyll molecules can be broken down by catabolic mechanisms.

In isolated chloroplasts from oat leaves, it was found that treatment with one of several hydrolytic enzymes including protease and lipase had no effect on chlorophyll loss, but a mixture of protease, ribonuclease, lipase and amylase markedly increased the rate of chlorophyll loss. Acidity of the suspension medium also affected the chlorophyll loss rate. At pH 5, chlorophyll loss was faster than at pH 7.4. The results led Choe and

Thimann (1975) to propose that the loss of chlorophyll is dependent on at least three sites of attack: the thylakoid membrane lipid, the chlorophyll-protein complex, and the chlorophyll molecule itself which is partly modified by acidity. In support of a modification by acidity, leaf internal CO₂ concentration has been observed to increase during senescence (Ticha *et al.* 1988). This increase in CO₂ may result from respiration especially when the stomata are closed, and could result in an increase in acidity (Thimann 1980).

In the non-yellowing mutant of *Festuca pratensis* mentioned earlier, several hydrophobic membrane proteins including CP I and LHCP as well as chlorophyll are maintained during senescence (Thomas 1982a). However, this is not due to any difference in the leaf soluble peptide hydrolase composition compared to the normal plant (Thomas 1982b). However, there is a difference in membrane lipid composition and degradation during senescence between these leaves (Harwood *et al.* 1982). Dhindsa *et al.* (1981) noticed that lipid peroxidation is one of the earliest metabolic changes that occurs in leaf senescence. This results in a decrease in the membrane phospholipid/sterol ratio (McKersie and Thompson 1978; Chia *et al.* 1981). Hence, this alteration in membrane properties might be an early significant event in chlorophyll catabolism.

More recently, however, Thomas *et al.* (1989) found an accumulation of dephytylated derivatives of chlorophyll a in LHCP but not in CP I of the thylakoid membrane during senescence of the non-yellowing *Festuca pratensis* mutant. This was not observed in the normal species of *Festuca* genotype. They proposed that the dephytylated derivatives result from the removal of the phytol side chain of chlorophyll, occurring while the chlorophyll molecule remains associated with protein complexes in the thylakoid. The accumulation of these products in the mutant plant is then due to a metabolic lesion which blocks pigment-proteolipid degradation after the dephytylation step. In the normal plant, removal of magnesium from chlorophyllides may next take place yielding phaeophorbides (Ziegler *et al.* 1988; Shioi *et al.* 1991). Open-chain

tetrapyrrol derivatives (Schellenberg *et al.* 1990) might be the further step of chlorophyll breakdown. However, the complete sequence remains elusive (Thomas *et al.* 1989; Smart 1994).

1.2.3 Loss in protein content

A decline in leaf protein content during senescence has been observed from some of the early studies (Vickery *et al.* 1937, 1939; Wood and Cruikshank 1944). This occurs in both detached and attached leaves. In detached leaves, free amino acid levels rise as the protein content falls (Martin and Thimann 1972a, 1972b). In senescing attached leaves, however, the free amino acid levels also fall, following a slight rise, as they are translocated to other parts of the plants (Thimann *et al.* 1974). If the plants are flowering or fruiting, the liberated amino acids are transported to these reproductive organs (Cockshull and Hughes 1967; Dalling *et al.* 1976; Peoples *et al.* 1983), while if they are growing rapidly the amino acids are translocated to the apices or young leaves (Williams 1955; Hopkinson 1966; Simon 1967). The amides glutamine and asparagine are the major forms in which the liberated amino acids are translocated from the leaves (Simpson and Dalling 1981; Kar and Feierabend 1984; Kamachi *et al.* 1991).

It is generally accepted that protein content is a truer indication of the stage of leaf senescence than the degree of chlorophyll loss (Woolhouse 1986). The fall in protein content is thought to be the most basic of all senescence-related events that could underlie all other aspects of the physiological decline in the leaves during senescence (Nooden 1980). Choe and Thimann (1975) observed that chlorophyll degradation of isolated chloroplasts, maintained in a sterile medium in darkness, is relatively less than in detached leaves from the same plant placed under identical conditions. The results were interpreted as demonstrating that there is a cytoplasmic protein factor in the intact leaves which regulates chlorophyll content and that isolation of the chloroplasts liberated them from this controlling factor.

During senescence there are differential changes in the rate at which individual proteins are synthesised and degraded (Smart 1994). In the initial stages of senescence the predominant soluble protein lost is RuBPCase, since it represents up to 50% of the leaf soluble protein (Woolhouse 1967; Peterson and Huffaker 1975; Wittenbach 1978; Friedrich and Huffaker 1980). This is in accord with the imputed nitrogen storage function of this protein (Kleinkopf *et al.* 1970). Other soluble proteins are also lost, but their loss could be masked by the high proportion of RuBPCase (Friedrich and Huffaker 1980; Woolhouse 1986). Membrane-bound proteins are also lost during senescence, including those from the chloroplast membrane such as chlorophyll-binding proteins (Thomas 1977; Jenkins *et al.* 1981). Thylakoid proteins of soybean have been resolved into 20 bands on SDS-polyacrylamide gels of which 11 were quantified (Bricker and Newman 1980). One of these bands increased dramatically as senescence proceeded, but all of the remaining bands decreased, although at different rates.

In reality, the control of senescence by protein loss has two aspects (Woolhouse 1986). On the one hand there can be a lowering of protein turnover, resulting from ageing of the protein synthesis machinery, and on the other, protein content may decrease as a result of enhanced proteolysis. In all living cells, proteins are continually synthesised and degraded, a process known as protein turnover (Goldberg and Dice 1974; Huffaker and Peterson 1974). The current protein content results from the balance between these two processes. Hence, the terminal decline in protein content during senescence could be the consequence of an increased degradation above normal turnover rates, decreased synthesis, or a combination of both.

Different proteins are normally turned over at different rates. RuBPCase has been reported to be turned over at a very slow rate in barley leaves under normal conditions (Peterson *et al.* 1973), and has a half-life of 7-8 days in maize plants (Simpson *et al.* 1981). Nitrate reductase in cultured tobacco cells, in contrast, is turned over rapidly and has a half-life of only 6 hours (Zielke and Filner 1971). In barley leaves prior to

senescence, Dungey and Davies (1982a) noted that the half-lives of total, soluble and insoluble proteins are about 9, 7 and 20 days, respectively.

During senescence, cessation of synthesis has been reported to be responsible for the decline in fraction I protein (which is predominantly RuBPCase) content in *Perilla* leaves (Woolhouse 1967). A rapid decline in the synthesis of RuBPCase has been demonstrated in wheat leaves at a stage after full expansion (Brady 1981). However, Lamattina *et al.* (1985) estimated, with full expanded detached wheat leaves placed in darkness, that over five days complete cessation of protein synthesis would account for only a 13% loss of protein content, far lower than the observed loss of about 70%.

A clue to the cause of the decline in protein synthesis during senescence may be provided by the observation that nucleic acid content also declines in senescing leaves (Smillie and Krotkov 1961; Watanabe and Imaseki 1982). In senescing attached leaves of bean, Makrides and Goldwaite (1981) observed that, although DNA content remained constant, rRNA and ribosome contents fell after reaching a maximum level before full expansion, rapidly at first then more slowly during senescence. Large polysomes began to break first, and later smaller ones began to disappear, indicating a reduction in the rate of transcription.

Protein synthesis does not cease altogether in senescing leaves, but declines continually at a progressive rate (Brady 1981; Naito *et al.* 1981). Moreover, the observation that treatment with protein synthesis inhibitors retards both chlorophyll and protein loss from leaf segments held in darkness (Martin and Thimann 1972a, 1972b; Thomas 1976; Yu and Kao 1981) suggests that senescence requires continuing synthesis of some proteins. The synthesis of particular proteins on the cytoplasmic ribosomes is implicated, since an inhibitor of cytoplasmic protein synthesis (cycloheximide) is more effective in retarding senescence than inhibitors of chloroplast protein synthesis (Yu and

Kao 1981). It is proposed that these proteins of cytoplasmic origin are proteolytic enzymes (Martin and Thimann 1972a, 1972b).

Another aspect of the decline in protein content during senescence is the regulation of protein degradation by proteolytic enzymes. Peptide hydrolase activity has been observed to increase as protein content falls during leaf senescence in several plants. This has been observed in dark-induced senescence, of detached leaves of oat (Martin and Thimann 1972a, 1972b) and barley (Peterson and Huffaker 1975) and intact plants of wheat (Wittenbach 1978) and in natural senescence of wheat (Dalling *et al.* 1976; Peoples and Dalling 1978; Wittenbach 1979; Peoples *et al.* 1980; Waters *et al.* 1980), barley (Friedrich and Huffaker 1980), corn (Feller *et al.* 1977), soybean (Wittenbach *et al.* 1980), bean (Weckanmann and Martin 1981) and apple (Kang *et al.* 1982).

However, the triggering mechanism for the observed rise in peptide hydrolase activity is not known. Several alternative factors such as synthesis of more peptide hydrolase enzymes, activation of existing enzyme, removal of inhibitors or access to the substrate could account for the rise in peptide activity (Frith and Dalling 1980).

It has been suggested that peptide hydrolase enzymes are always present but recognise proteins only when they are not integrated in complexes or protected by their substrates or prosthetic groups. This is supported by evidence from the stay-green mutant of *Festuca pratensis*, in which abnormal stability of Mg-porphyrin and Fe-porphyrin is associated with retention of their apoproteins (Davies *et al.* 1990).

However, the finding that the fall in protein content during senescence can be retarded by protein synthesis inhibitors (Martin and Thimann 1972a, 1972b; Thomas 1976; Yu and Kao 1981) indicates that new enzyme synthesis is still necessary in senescence. Another evidence of synthesis of new enzyme is that an *in vivo* change in the

pattern of protein synthesis during senescence can be demonstrated after pulse labelling with ^{35}S -methionine (Kawakami and Watanabe 1988; Thomas *et al.* 1992).

Recent research

1.3 The regulation of senescence

1.3.1 Nutrient redistribution

In monocarpic plants, leaf senescence follows flowering or fruiting. This was originally thought to be due to a diversion or drain of nutrients from leaves to the growing reproductive organs, causing leaf death by starvation. The idea was supported by the observation that removing flowers or fruits can delay senescence of leaves in many species. These include cotton (Dale 1959), pea (Malik and Davies 1976), common perilla (Beever and Woolhouse 1975) and capsicum (Hall and Brady 1977). However, as pointed out by Thimann (1980) such a redirection of nutrients is more likely to be resultant than causal. Nutrient exchanges (import of water, salts and organic compounds and export of sugars, vitamins and amino acids) are always going on in leaves and must therefore be continually modifying the leaves' vitality. It is more likely that change in control of these nutrient exchanges occurs after senescence is initiated or at least is regulated by the mechanism that triggers leaf senescence. Nooden (1980) suggested that senescence hormones produced in developing fruits cause the rest of the plant to degenerate. There is evidence for the ability of developing fruits to export materials, including hormones, as they accumulate massive amounts of assimilates (Antoszewski and Lis 1968; Grochowska 1968). Various candidates for this death hormone have been advanced, the most popular being jasmonic acid and chlorinated auxin derivatives (Engvild 1989).

1.3.2 Hormonal control

In respect to their role in senescence control, plant hormones can be characterised into two categories. One class can be characterised as senescence promoters. These generally increase during leaf senescence, and accelerate senescence if applied externally. The other group are senescence retardants, which decrease in concentration during leaf senescence, and retard senescence when applied to the leaves. Leaf senescence appears to involve a shift in the balance of senescence promoters and retardants rather than a change in concentration of any individual hormone (Nooden 1980). In contrast, hormone studies have generally been concerned with one category or the other alone.

1.3.2.1 Senescence promoter hormones

The most studied is abscisic acid (ABA). Applied ABA has been shown to promote senescence of leaf discs and detached leaves of a wide range of species (El-Antably *et al.* 1967; Aspinall *et al.* 1967; Beevers 1968; Back *et al.* 1972). However, the response is genotype-dependent, since different cultivars of soybean show a range of responses from very sensitive to completely insensitive following a single application (Sloger and Cardwell 1970). The effect of ABA on attached leaves is comparatively less pronounced. In several cases, ABA shows no effect on attached leaves of the same species for which it promotes the senescence of excised leaves (El-Antably *et al.* 1967; Sloger and Cardwell 1970). Translocation, inactivation, poor uptake and competition from endogenous hormones are all possible reasons for this difference between attached and detached leaves (Nooden 1980). In addition, the physiological status of the leaf material is also important. For instance, ABA shows an effect on mature cotton leaves but not on young ones (Smith *et al.* 1968) and on *Rhoeo* leaves grown under low light but not on healthy ones (De Leo and Sacher 1970). In some cases, ABA retards senescence, such as on leaf discs of radish (Colquhoun and Hillman 1972) and on attached leaves of wheat plants (Hall and McWha 1981).

Endogenous ABA content generally increases in senescing leaves (Even-Chen and Itai 1975; Lindoo and Nooden 1978). However, the endogenous levels of ABA do not always correlate well with senescence (Dumbroff *et al.* 1977; Samet and Sinclair 1980).

Ethylene, although very effective on fruit ripening which can be regarded as a senescence response, generally shows less effect on leaf senescence (Thimann 1980; Nooden 1980). However, in segments of oat leaf it has been shown that an increase in the evolution of ethylene is closely correlated with the rate of chlorophyll loss, and inhibition of ethylene evolution results in retarding the chlorophyll loss (Gepstein and Thimann 1981).

1.3.2.2 Senescence retardant hormones

In this group, cytokinin is the most prominent. With few exceptions, applied cytokinin has a substantial effect in retarding senescence, although again the effect is comparatively less pronounced in attached leaves than in leaf segments or intact detached leaves (Thimann 1980; Nooden 1980). Yellow leaves of bean have been observed to even regreen after an application of cytokinin (Venkatarayappa *et al.* 1984).

Endogenous cytokinin generally falls with progress in senescence. Singh *et al.* (1992) found that young tobacco leaves can synthesise their own cytokinin, but not mature and senescent leaves, and demonstrated that this difference is a factor in controlling the sequential leaf senescence observed in the tobacco plant. However, in general, it is widely held that root apices are the major site of endogenous cytokinin synthesis and that the root-produced cytokinin is translocated to the shoot via the xylem (Kende 1965; Itai and Vaadia 1971; Skene 1972; Nooden *et al.* 1990). It is further suggested that decreasing cytokinin flux from the root system initiates leaf senescence. This concept is supported by the finding that treatments which decrease cytokinin production by the roots such as derooting, salt stress, water-logging or mineral

deficiency promote leaf senescence (Van Staden *et al.* 1988). Further actions that increase the export of cytokinin from the roots such as disbudding or decapitation delay leaf senescence (Colbert and Beever 1981; Crafts-Brandner 1991).

Gibberellin (GA) and auxin also exhibit some senescence-retarding effect, but at a lesser efficiency than cytokinin (Thimann 1980; Nooden 1980).

1.3.3 Stomatal closure

A decline in stomatal conductance has been observed at an early stage in natural senescence of attached leaves (Wittenbach *et al.* 1980; Friedrich and Huffaker 1980). In barley plants, Friedrich and Huffaker (1980) found that, leaf stomatal conductance fell more rapidly during leaf senescence than leaf mesophyll conductance and accounted for about 24% of the total increase in resistance to CO₂ diffusion. However in soybean plants, Wittenbach *et al.* (1980) found that a decline in photosynthetic rate occurred earlier than the fall in stomatal conductance and attributed the lowering of the photosynthetic rate to a fall in mesophyll resistance.

However, studies with oat leaf segments led Thimann and Satler (1979a, 1979b) to conclude that stomatal closure is the principal controlling agent in leaf senescence. This is because of the observation that treatments which cause stomatal closure in light promote senescence, and treatments that induce stomatal opening in darkness delay senescence of the leaf segments. Stomatal closure may initiate leaf senescence by stimulating ABA production, since ABA content in the treatments with stomata closed rose significantly (Gepstein and Thimann 1980). Another possibility is that the closure of stomata in light limits intracellular CO₂ concentration and the lowered in CO₂ partial pressure initiates leaf senescence by stimulating ethylene evolution (Gepstein and Thimann 1981; Satler and Thimann 1983).

2 Water stress and its effect on leaf senescence

2.1 Introduction

Although certain plant tissues are able to endure periods of almost complete desiccation, they are metabolically inactive while dry. When in an active state, most plant tissues contain approximately 70-95% water. The amount of water present in a plant cell (or tissue or whole plant) at a given time will be determined by a balance between water uptake and water loss. Whenever the loss of water exceeds uptake, water stress develops. Under field conditions, water stress in a plant usually develops when the soil water content is limited, but it may occur when low atmospheric humidity, high irradiance and temperature conspire to raise the transpiration rate above the capacity of the roots to extract water.

2.1.1 Water status parameters

The severity of water stress is indicated by the water status of certain organs. This can be characterised in a number of ways ranging from visual wilting, leaf thickness, tissue water content (percent of fresh weight) to more rigorous parameters of relative water content (RWC) and water potential (Ψ). RWC is the water content relative to the water content of the same tissue at full turgor, which can be expressed alternatively as water saturation deficit (WSD), where:

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

$$\text{WSD} = \frac{\text{TW}-\text{FW}}{\text{TW}-\text{DW}} \times 100\%$$

$$\text{WSD} = 100 - \text{RWC}$$

where: FW = fresh weight of tissue
 DW = dry weight of tissue
 TW = weight of tissue at full turgid

RWC is generally well related to Ψ of the same tissue, but the relationship can vary between species or stages of growth (Connor and Tunstall 1968). A major shortcoming of RWC is that it is less sensitive than Ψ in circumstances where water stress is very mild (Hsiao 1973).

Ψ is the chemical potential of water in the system relative to that of pure free water and expressed in units of energy per unit volume.

$$\Psi = \frac{\mu_w - \mu_w^\circ}{V_w}$$

where: μ_w = chemical potential of water in the system
 μ_w° = chemical potential of pure water
 V_w = partial molal volume of water in the system

Ψ in plant tissue is determined by three kinds of forces, expressed in the formula:

$$\Psi = \Psi_p + \Psi_\Pi + \Psi_m$$

where: Ψ_p = turgor pressure, arising from the hydrostatic pressure generated by the elasticity of the cell walls.
 Ψ_Π = osmotic potential, arising from the colligative effects of cell solutes.
 Ψ_m = matric potential, arising from surface effects of cell structures.

However, Ψ_m is usually ignored due to its relative unimportance in highly vacuolated cells and doubts concerning its meaningfulness. It has been claimed by Passioura (1980) to disappear under rigorous thermodynamic analysis of tissue water.

2.1.2 Possible mechanisms underlying plant responses to water stress

As water stress develops, almost all aspects of plant physiology and metabolism could be altered, depending on how severe and how prolonged the stress. Many plant responses to water stress are reviewed in the review of Hsiao (1973) and the volumes edited by Turner and Kramer (1980) and Paleg and Aspinall (1981). However, how the effects of water stress are transduced into the responses is rarely known. Hsiao (1973) lists possible physical and chemical changes, that may occur as direct effects of water stress as follows:

(1) Reduced water activity causing changes in cell metabolic reactions in which water is involved. However, this is very unlikely, since the changes in activity of water that are associated with water stress normally encountered in mesophytes are extremely small. The relationship between Ψ and activity of water is expressed as:

$$\Psi = (RT/V_w) \ln a_w$$

where:

R	=	the gas constant
T	=	absolute temperature
V_w	=	partial molar volume of water
a_w	=	activity of water

From this equation, the calculated a_w is 0.986 at 25 °C for a Ψ of -2.0 MPa. This shows that there is only a slight reduction (1.4%) in a_w when the stress is extremely severe.

(2) Reduced turgor pressure. This can be certainly evident even with mild stress and at least partially reduces leaf growth and stomatal opening (details will be discussed in section 3).

(3) Increased concentration of molecules within the cell as cell volume is reduced by water stress. This may affect cell metabolism. Hsiao (1973) considers that the increase in concentration is not sufficient to cause significant enzyme inhibition. However, evidence that increased concentrations of ionic solutes are largely responsible for the non-stomatal inhibition of photosynthesis (as a result of limitations to RuBP regeneration capacity) in water stress has been reported (Kaiser and Heber 1981; Kaiser 1982; Kaiser *et al.* 1983).

(4) Spatial relations of cell component being altered by volume changes as the cell dries out.

(5) Macromolecular structure being altered by hydration changes. Protein conformation is sensitive to changes in water structure, which may occur when concentrations change. However, this possibility is mainly speculative, with little concrete *in vivo* data support.

The primary effects of water stress may initiate a long series of subsequent effects. For examples, changed in hormone levels may induce many metabolic changes or stomatal closure may reduce photosynthetic rate.

2.2 Metabolic changes in response to water stress

2.2.1 Photosynthesis

As water stress develops, the fall in leaf water potential eventually lead to a decline in the rate of photosynthesis (Boyer 1971; Ludlow and Ng 1976; Dietz and Heber 1983). Decrease in stomatal conductance for CO₂ diffusion is generally thought to be the main impedance to photosynthesis during water stress, since the closure of stomata is obvious and it is observed to be closely correlated with the fall in photosynthetic rate. (Ludlow and Ng 1976).

However, this is not always the case. Wong *et al.* (1979) observed no reduction in the intercellular CO₂ concentration, despite a fall in CO₂ uptake in water stressed maize leaves, indicating a fall in the photosynthetic capacity of the chloroplasts. In *Primula palinuri* leaves dehydrated with or without the epidermis, Dietz and Heber (1983) found that CO₂ assimilation was lost in both but at a higher RWC in leaves with epidermis, than in ones without. This suggests that both stomatal and mesophyll inhibition occur. Both components of photosynthetic inhibition were also observed in dehydrated *Sinapsis* leaves (Cornic *et al.* 1983).

The observed mesophyll-based inhibition of photosynthesis is partly due to photosynthesis being more susceptible to water deficit than photorespiration (Lawlor 1976; Mawson and Coleman 1983). Thus, the CO₂ compensation point rises when leaves are stressed (Lawlor 1976; O'Toole *et al.* 1977; Dietz and Heber 1983).

Electron transport activity of chloroplasts of wilted leaves has been reported to be reduced (Keck and Boyer 1974; Mayoral *et al.* 1981). In *Oleander* leaves, however, this occurs only in high light intensity (Bjorkmann *et al.* 1981; Powles and Bjorkmann

1982). Grapevines also show similar responses (Downton 1983). This inhibition has, therefore, been termed photoinhibition due to its dependence on photon flux.

Mesophyll inhibition is not solely due to effects on electron transport, however. Sharkey and Badger (1982), using intact mesophyll cells osmotically stressed *in vitro*, found no decline in electron transport activity, but a considerable decline in photophosphorylation activity. Nevertheless, cell ATP content did not change. The results led these authors to conclude that the inhibition of photosynthesis was due to RuBP regeneration (which uses ATP). Kaiser *et al.* (1981) and Berkowitz and Gibbs (1982) also came to the same conclusion with experiments using an osmotic stress imposed *in vitro*.

Most of the studies referred here were made during short periods of water stress. In long-term water stress, however, other changes which could inhibit photosynthesis may occur. O'Toole *et al.* (1977) and Mayoral *et al.* (1981) have reported a reduction in RuBPCase activity in response to water stress. However, the response is uncertain, since Huffaker *et al.* (1970) and Sharkey and Badger (1982) observed no such response.

2.2.2 Chlorophyll catabolism

Loss of chlorophyll during water stress is frequently observed (Huffaker *et al.* 1970; Mayoral *et al.* 1981; Mukherjee and Choudhuri 1981; Radin 1981). However, Jones (1973) and Vapaavuori and Nurmi (1982) found no such response. The difference in the responses is likely to be due to differences in the severity and duration of the stress, since chlorophyll molecules are known to be well protected by their association with the thylakoid membrane.

Little is known about the nature of chlorophyll catabolism during water stress. In maize plants, Alberte *et al.* (1977) found that the proportion of extracted LHCP, CP I and

free pigment was decreased, stable and increased respectively when the leaves were stressed, suggesting that the major loss of chlorophyll content in response to water stress was from the LHCP. This resulted in a reduction in photosynthetic unit size, and an increase in chlorophyll a/b ratio, since chlorophyll b is known to locate only on this chlorophyll-protein complex. In stressed willow leaves, Vapaavuori and Nurmi (1982) observed no significant change in the total chlorophyll content, but a change in the state of the chlorophyll present. When the stress was imposed under high light intensity, LHCP and CPa (chlorophyll a protein-complex) (both LHCP and CPa associated with PS II) were lost while CP I was retained. However, under low light intensity little LHCP was lost, but pigment was lost from CP I and CPa. Possibly, the high light intensity-induced loss of the complexes associated with PS II is a continuation of photoinhibition.

2.2.3 Protein metabolism

Water stress is commonly accompanied by a loss in protein content (Petrie and Wood 1938). Both soluble and insoluble protein are found to be lost, so the effect is fairly general (Shah and Loomis 1965). However, different proteins may respond differently to water stress. Stutte and Todd (1969) observed that iron-containing proteins rose, despite a fall in the content of other proteins during water stress. High molecular weight proteins are found to be more sensitive to water stress than low molecular weight ones (Stutte and Todd 1967; Botha 1979).

The fall in protein content of water stressed *Lemna minor* plants has been reported to be due to both a decrease in protein synthesis and an increase in protein hydrolysis (Cooke *et al.* 1979). This combined response was also observed in detached and attached leaves of barley (Dungey and Davies 1982a, 1982b).

That water stress causes a rapid reduction in protein synthesis of young growing tissues is well-known. The effect is usually concluded from the observation that water

stress causes a rapid fall in polyribosome content with a concomitant rise in free ribosomes (Shah and Loomis 1965). Alternatively, the effect is also achieved by following the incorporation of radioactive amino acids into protein (Dhindsa and Cleland 1975). As review by Bewley (1981) these effects of water stress observed are not restricted to leaves, but also occur in stem apices, root tips and cotyledons.

In respect to protein degradation during water stress, it has been observed that large proteins begin to disappear first and the content of smaller proteins rise, apparently due to the appearance of new polypeptides, before ultimately declining (Botha 1979). The new polypeptides that appear during the initial stage are likely to be endoproteolytic cleavage products of larger proteins, which accumulate as the larger proteins are hydrolysed, and are ultimately hydrolysed themselves.

It has also been observed with barley leaves that the hydrolysis of long half-life proteins (8 days or more) is much more affected by water stress than that of short half-life proteins (2.5 days), which was found not to be faster than that in unstressed leaves (Dungey and Davies 1982a, 1982b). Perhaps, stress-induced protein loss is a means by which nitrogen sequestered in long-lived storage proteins (such as RuBPCase) is salvaged by the plants when leaf death becomes likely.

2.2.4 Proline accumulation

During a period of water deficit, plant cell solute concentration increases. This can be passive, but often involves active accumulation, either by uptake of solutes from outside the cell or by synthesis of new solutes. Such active accumulation lowers Ψ_{π} which in turn maintains Ψ_p at a lower Ψ . This process is known as osmoregulation. The solutes which accumulate in water stressed plants fall broadly into three groups:

- (i) inorganic ions such as K^+ , Na^+ and Cl^- ,

- (ii) polyhydroxy organic solutes such as sugars and glycerol, and
- (iii) nitrogen-containing organic solutes such as betaines and amino acids

Since many enzyme activities are adversely affected by low concentrations of inorganic ions (Flowers 1972; Stewart and Lee 1974; Pollard and Wyn-Jones 1979; Billard and Boucaud 1980), it has been suggested that where these ions are accumulated, they are restricted to the vacuole, with their osmotic effects being countered in the cytoplasm by organic "compatible solutes". Brown and Simpson (1972) define a compatible solute as one which, at high concentration, allows enzymes to function effectively. They are generally neutral compounds of high solubility, they can be zwitterionic (Borowitzka 1981).

Among the compatible solutes which accumulate in leaves during water stress, proline is the most universal one. With barley plants Singh *et al.* (1973a) have concluded that concentration of leaf proline content at a given time is a response to the water potential of the leaves, the length of exposure to water stress and the amount transported from the leaves to other organs. However, studies on the progressive accumulation of proline with a fall in water potential are usually confounded with time of stress exposure (Aspinall and Paleg 1981). The response of proline accumulation to water status is relatively rapidly (Singh *et al.* 1973a; Rajagopal *et al.* 1977), and the lower limit to the threshold water potential at which proline begins to accumulate varies between species and growth conditions (Aspinall and Paleg 1981). However, in most species the response is large. In some halophytic plants, proline can accumulate to as high as 10% of tissue dry weight, constituting 70% of the total amino acid pool, and undergoes 10-100 fold changes in amount in response to osmotic stress (Stewart and Lee 1974). Wilted sunflower leaves have been reported to accumulate proline to around 30 fold more than the control content (Wample and Bewley 1975).

When the leaves regain turgor, however, the accumulated proline disappears rapidly (Stewart 1972; Singh *et al.* 1973b, 1973c; Blum and Ebercon 1976; Parameshwara and Krishnasastry 1980) due to oxidation to glutamic acid (Stewart *et al.* 1977). Although at the beginning the rate and the extent of this lowering of proline levels varies depending on the species and the severity of the stress, eventually the concentration of proline declines to the control level (Aspinall and Paleg 1981).

Apart from the role in the osmotic adjustment of stressed leaves (Munns *et al.* 1979) proline has also been reported to act as an enzyme protectant in stressed tissues (Paleg *et al.* 1984). Other roles of proline in cell metabolism are that it serves as a substrate for respiration (Stewart *et al.* 1977), as a protein precursor (Zhang *et al.* 1982) and as a source of nitrogen for plant metabolism (Britikov *et al.* 1970).

Proline is synthesised in the leaves and can be translocated to other parts of the plant (Singh *et al.* 1973a; Tully *et al.* 1979). Proline or precursors thereof, appear to be translocated from old leaves to younger leaves and if the stress is prolonged or severe enough, the materials may move to the tissues enclosed in the leaf sheaths (Munns *et al.* 1979). Phloem exudates from stressed barley leaves contain only minor quantities of proline, but considerable quantities of glutamine and glutamic acid (Hanson and Tully 1979; Tully and Hanson 1979). These suggest that the translocated entity of proline could be a precursor.

In water stressed leaves, a main cause of proline accumulation is the loss of feedback inhibition of the first step of proline synthesis from glutamate, hence the synthesis of proline increases (Boggess *et al.* 1976). Proline accumulation is also aided by an inhibition of proline oxidation under stress conditions (Stewart *et al.* 1977; Stewart and Boggess 1978). Inhibition of proline utilisation in protein synthesis (Stewart *et al.* 1977) or the release of proline from protein hydrolysis (Singh *et al.* 1973a) may also contribute to proline accumulation, although in a comparatively lesser

proportion. It is likely that the precursors for proline synthesis are nitrogen from amino acids released by protein hydrolysis (Fukutoku and Yamada 1981), and sugars and reducing power from photosynthesis, since light is necessary for the accumulation (Aiyar 1981).

2.3 Comparison of water stress-induced senescence and natural senescence

2.3.1 Senescence induced by water stress

Although the gross changes in response to water stress are not identical to those which occur in naturally senescing leaves (such as stomatal closure and proline accumulation), it is generally assumed that the events occurring in water stressed leaves are a manifestation of the universal senescence syndrome. This is because many of the symptoms normally used as indications of senescence also occur in stressed leaves, and both eventually lead to leaf death.

Considering the imputed regulation of natural senescence, there are theoretical reasons to suspect that water stress-induced senescence and natural senescence are similar. Firstly water stress stimulates the production of the hormones ABA and ethylene and depresses cytokinin level (Aspinall 1980; Milborrow 1981). Such hormonal changes have been proposed as a most likely regulatory system for senescence (Thimann 1980; Nooden 1980). Secondly, water stress causes stomatal closure which has been implicated as the initiator of senescence, both in darkness and the natural environment (Thimann and Satler 1979a, 1979b). Stomatal closure may initiate similar changes in water stressed leaves.

In general, the acceleration of senescence by water stress is well known. Nilson and Muller (1981) observed that exposing *Lotus scoparus* plants to continuous stress

using PEG caused early leaf senescence, and at any given time the proportion of senescent leaves on the plant was increased by the stress. Water stress has also been reported to initiate senescence of the old leaves of strawberry plants (O'Neill 1983).

2.3.2 Comparison of the symptoms of senescence in water stressed and naturally senescing leaves

As mentioned earlier, the gross changes in water stressed and natural senescing leaves are different. This difference reflects that the mechanisms responsible for senescence in these two leaves may be different. However, to compare the senescence syndromes it is necessary to compare on the basis of each symptom, since the senescence syndrome comprises of several symptoms and certain of these symptoms can be disengaged from the rest.

2.3.2.1 Photosynthesis

An obvious difference between water stress-induced and natural senescence is that stomatal inhibition of photosynthesis tends to be much greater in water stressed leaves. However, stomatal resistance does not always limit photosynthesis in stressed leaves (Wong *et al.* 1979).

Mesophyll inhibition of photosynthesis due to changes in electron transport activity also appears to be different between water stressed and senescing tissues. Firstly, a decline in electron transport activity occurs as the chlorophyll content declines in senescing leaves (Jenkins and Woolhouse 1981b), but the chlorophyll content remains unaffected in water stressed leaves although electron transport declines (Bjorkmann *et al.* 1981). Secondly, the effects of water stress are more pronounced in PS II than PS I compared to the situation in natural senescence (Keck and Boyer 1974; Bjorkmann *et al.* 1981; Jenkins and Woolhouse 1981b).

However, these differences may not be critical, since the responses to water stress reported were all caused by a short period of water stress. If senescence begins at the imposition of water stress, it would not limit photosynthesis until some time has elapsed. It would be more valuable to compare these responses on the basis of long term effects of water stress on photosynthesis. However, few such data are available. RuBPCase activity has been reported to decline during prolonged stress (O'Toole *et al.* 1977; Mayoral *et al.* 1981; Vapaavuori and Valanne 1982). This may provide a link between water stress-induced and natural senescence, since decline in RuBPCase activity is postulated to be a major limiting factor of photosynthesis during senescence (Friedrich and Huffaker 1980).

2.3.2.2 Chlorophyll catabolism

The difference in chlorophyll responses to water stress and to natural senescence resides in the changes in chlorophyll a/b ratio. In natural senescence the fall in leaf total chlorophyll content is usually paralleled by a fall in the ratio of chlorophyll a to b (Sestak 1977). This is because LHCP is the most stable chlorophyll-protein complex in senescing leaves (Bricker and Newman 1980). In water stressed leaves, however, the chlorophyll a/b ratio displays an inconsistency in response. It was found to increase in stressed maize leaves (Alberte *et al.* 1977), but to decrease in stressed willow (Vapaavuori and Nurmi 1982) and wheat (French 1985) leaves.

Sudden closure of stomata in the water stressed leaf limits energy used for CO₂ reduction. Thus, the light harvesting apparatus would become overloaded with energy that cannot be dissipated, eventually leading to photo-oxidation. Destruction of LHCP would reduce energy transfer to the reaction centres, and thus also reduce the probability of reaction centre damage.

On the other hand, during natural senescence chlorophyll content and electron transport might determine the rate of photosynthesis (Camp *et al.* 1982) and the light harvesting apparatus will not be overloaded, then chlorophyll catabolism can proceed in a more orderly fashion.

2.3.2.3 Protein metabolism

The finding that large proteins (Botha 1979) and long-lived proteins (Dungey and Davies 1982a, 1982b) are most sensitive to water stress implies that RuBPCase (which is both large and long-lived) is one of the first proteins lost in response to water stress, as it is in natural senescence. However, the loss of specific proteins during senescence has not been studied.

Protein synthesis is dramatically reduced in both naturally senescing (Woolhouse 1967; Brady 1981) and water stressed (Bewley 1981; Dungey and Davies 1982a, 1982b) leaves. In both cases the reduction is associated with breakdown of polysomes to ribosomes (Bewley 1981; Makrides and Goldwaite 1981).

Protein hydrolysis is observed to be accelerated in both water stressed (Dungey and Davies 1982a, 1982b) and senescing (section 1) leaves. French (1985) concluded that protein catabolism in water stress-induced, dark-induced and natural senescence had a common mechanism in wheat leaves, since the relationship between protein hydrolysis and soluble peptide hydrolase activities is similar in all of these three types of senescence.

3 Non-hydraulic root-signals regulating shoot responses

3.1 Introduction

A general concept of plant response to drought is that when a plant is growing in a drying soil water uptake is reduced, resulting in a fall in leaf water status. This fall in leaf water status (water potential or turgor) has been taken to be the central regulation of all other plant physiological and biochemical responses to drought. However, leaf stomatal closure and growth rate can be affected by exposing plant roots to soil drying directly, before any change leaf water status (Schulze 1986; Davies and Zhang 1991). These and similar results suggest that plant roots in drying soil may also regulate shoot responses to water deficit via chemical signals independently of the hydraulic signal (Jones 1980).

3.2 Evidence of non-hydraulic root-signals regulating shoot responses

3.2.1 Indirect evidence for non-hydraulic root-signals

With respect to the stomatal response to water deficit, it has been observed that the leaves of unirrigated cow pea plants, with almost closed stomata, can have a water potential identical to that in well-watered plants with open stomata (Bates and Hall 1982). Similar observations are also reported for sugar-cane plants (Meinzer and Grantz 1990). Even where leaf water status does change with water deficit, Turner *et al.* (1985) have shown that changes in stomatal conductance and the rate of net photosynthesis of sunflower plants are more closely correlated to changes in soil water status than to changes in leaf water status.

With respect to the growth response, which has previously been postulated to be regulated by leaf turgor (Sharp and Davies 1979; Turner and Jones 1980; Munns and

Weir 1981), intensive observation confined strictly to elongating regions has raised doubts concerning dependence of growth on turgor. A reduction in growth has been observed to occur before any change in turgor (Matsuda and Riazi 1981; Meyer and Boyer 1981; Michelena and Boyer 1982).

The growing basal regions of barley leaves (Matsuda and Riazi 1981) and the elongating regions of soybean hypocotyls (Meyer and Boyer 1981) showed similar responses to water stress. Stress was induced with nutrient solutions containing NaCl or PEG for barley, while for soybean it was induced by withholding water supply. In both tissues, growth was inhibited immediately but then resumed at a new rate which was reduced in proportion to the degree of stress. However, turgor remained virtually constant, since water potential and osmotic potential fell about equally and in parallel with the growth rate.

In maize, Michelena and Boyer (1982) provided clear evidence of the relationship between turgor and growth rate in the elongating region of the maize leaf (basal region, 2 cm adjacent to ligule enclosed by sheath of lower leaf). In conditions of low water potential, osmotic adjustment in that region maintained turgor constant throughout the experiment in a normal light period. On the other hand, when the night period was prolonged, turgor appeared to decrease. This decrease in turgor was a result of loss of the ability of the tissue to accumulate solutes. However, in both cases the growth rates were reduced and eventually ceased within a short period. Full turgor maintenance in the normal light period only helped to delay the reduction in growth rate slightly, and supported a few longer periods of growth before cessation.

These observations indicate that there are some water stress-induced factors other than solute accumulation and turgor that affect growth and may cause most of the loss in plant cell growth under dry conditions.

Nonami and Boyer (1989, 1990a, 1990b) suggest that soil drying may result in a reduction in the gradient of water potential between xylem and growing cells. The reduction in the gradient of water potential would in turn limit water supply to the cells and eventually limit the growth rate in spite of full turgor maintenance by osmotic adjustment of the cells (Cavaleiri and Boyer 1982; Westgate and Boyer 1985).

Attribution of the growth inhibition to a reduction in water potential gradient was questioned by an observation on orchard apple trees (Jones *et al.* 1983). Over a period of several weeks the xylem water potential in an unwatered tree was higher than that in the irrigated control presumably due to reduction in stomatal conductance. This was interpreted to imply a non-hydraulic signalling response of shoots to soil drying, although the effect on plant growth was not observed in this experiment.

3.2.2 Direct evidence of non-hydraulic root-signals

Experiments using a split-root method or an artificial pressurising technique to maintain plant shoot water status provide opportunities to investigate the direct effects of soil drying on stomatal response. The split-root method, in which the roots from one plant are divided between two containers with the soil in one container allowed to dry out while the roots in the other container are well watered to maintain shoot water status, have been carried out with a range of plants including maize and *Commelina communis* L. plants (Blackman and Davies 1985; Zhang *et al.* 1987). With wheat and sunflower, Gollan *et al.* (1986) maintained leaf turgor despite drying soil by using a pressurising technique. Plants were grown in pots that could be enclosed in pressure chambers with the shoots emerging. The soil was allowed to dry but the leaves were kept turgid by applying an appropriate pressure to the root system. All of these plants showed stomatal closure in responses to the root drying, despite the fact that the leaf water status was stable.

This effect of non-hydraulic root signals on leaf conductance led to a consideration of their effect on plant growth. In wheat plants in which leaf turgor was maintained by applying pneumatic pressure to the roots, Passioura (1988) found that the relative leaf expansion rate (RLER) of the plant in drying soil fell coincidentally with the decrease in leaf conductance, whether or not the leaves were kept turgid. Using a split-root method, Saab and Sharp (1989) also found an effect of this sort of signal on the growth of maize, although here no effect on stomatal closure was detected. When the water potential of the drying soil declined below that of the leaves, the leaf elongation rate decreased by 25% below that of the control plant. Excising half of the roots of a well-watered plant had no effect on leaf expansion, confirming that the treatment did not cause an inadequate water supply to the leaves.

Gowing *et al.* (1990) found direct evidence for the drying root effect on plant growth rate in a split-root experiment with apple plants. Without any change in leaf water status, the growth rate of the plant with half its roots in dry soil decreased progressively over a 24-day period. The expansion of individual leaves, the initiation of new leaves and final leaf length were each reduced. Stomatal aperture also decreased coincidentally with the reduction in growth. If the roots in dry soil were excised, there was a recovery in leaf growth rate similar to that induced by rewatering. However, the recovery in stomatal aperture was less than that in rewatered plants.

3.3 Nature of chemical signals passing from roots to shoot

3.3.1 Negative signals

According to the terminology of Jackson and Kowalewska (1983), a 'negative' signal could occur where a signal only supplied by turgid roots promotes stomatal opening and growth. The production and transport of this signal would decrease as the proportion of roots in drying soil increased. Blackman and Davies (1985) have

produced some evidence for such a mechanism in maize, involving the production of cytokinin. They found that the total ABA content of the leaves on plants with partially closed stomata resulting from drying half the root system was not different from that in a control plant. However, stomatal closure could be reversed by incubating the leaf pieces in kinetin or zeatin, while water incubation could not induce opening. The results led these authors to conclude that absence of cytokinin might be the negative signal from the stressed roots, which is in accord to an early finding that production and transport of cytokinin from plant roots can be reduced by water stress (Itai and Vaadia 1965).

The difficulty with the cytokinin hypothesis or any other negative signal hypothesis, is evidence that excising the roots in a drying soil can eliminate the shoot response (Gowing *et al.* 1990). Such evidences would discount this type of signal. Further, Jones (1990b) has pointed out that as the magnitude of any negative signal would vary with root volume, whether or not the roots were stressed, such a signal by itself would not have the required information content.

Nevertheless, it is possible that in a drying soil a reduction in cytokinin supply may contribute to the response, perhaps acting in concert with other signals. For instance high concentration of cytokinin can override the effects of ABA on stomata (Blackman and Davies 1983; Radin *et al.* 1982; Radin 1984), hence reduction in the cytokinin supply from the roots in drying soil may amplify the shoot response to ABA.

3.3.2 Positive signals

The converse of a negative signal would be a positive signal which would increase in supply from roots in a drying soil. Zhang *et al.* (1987) have produced strong evidence for this type of signal in *Commelina communis* L., with ABA as the signal. The ABA content of the abaxial epidermis was found to increase coincidentally with an increase in the ABA content of the roots in drying soil. The small increase in the epidermal ABA

content correlated to the stomatal response, although bulk leaf ABA content could not be detected to differ from that in well-watered leaves.

Despite early reports to the contrary, many plant species are found to synthesise ABA in their roots (Cornish and Zeevaart 1985; Robertson *et al.* 1985; Hubick *et al.* 1986; Lachno and Barker 1986). It is probable that root-originating ABA is translocated to the shoot via the xylem stream, since the concentration of ABA in xylem sap has been observed to increase in plants in a drying soil before any change in leaf water status or bulk ABA concentration (Munns and King 1988; Zhang and Davies 1989, 1990). This translocation may occur because the roots in drying soil continue to contribute to the transpiration stream and the dry root releases ABA as it rehydrates during the dark period.

However, the amount of root-sourced ABA would be masked in bulk leaf ABA due to a comparatively much higher leaf-sourced ABA content (Zhang *et al.* 1987). One possible explanation for the effects of root-sourced ABA on the leaf response is that the shoot is able to differentiate between root-sourced and leaf-sourced ABA and possibly between ABA arriving in the xylem stream and xylem-derived ABA that arrived earlier (Davies and Zhang 1991). Shoots may be able to do this, since at least in well-watered plants in light, much ABA is effectively sequestered within chloroplasts, which act as anion traps (Cowan *et al.* 1982; Hartung *et al.* 1990). However, a proportion of the transpiration stream may arrive directly at the sites of evaporation in the epidermal cell walls adjacent to guard cells (Meidner 1975), which are also thought to be the sites of action for ABA on the guard cells (Hartung 1983). The epidermis may also be a site for the regulation of leaf growth (Dale 1988). The transpiration stream may therefore provide a direct link between the point in the root tip where soil drying is sensed and the location in the leaf where plant water balance and growth can be regulated.

Nevertheless, the identification of ABA as the positive root signal has been challenged by an experiment of Munns and King (1988) with wheat plants. In this study, xylem sap from leaves of soil-drying plants was collected and fed to a detached leaf of well-watered plants to observe the stomatal response. Stomatal closure of the detached leaf could be induced by this sap even when ABA was removed from the sap by passing it through an immunoaffinity column, suggesting that ABA may not be the positive signal from roots in drying soil for stomatal response in wheat.

3.3.3 Accumulative signals

Jackson and Hall (1987) has proposed this type of signal for the increase in the concentration of ABA in leaves of flooded plants. In this case it is proposed that the accumulated ABA is shoot-sourced and would normally be transported from leaves to roots via the phloem. However, such a mechanism seems unlikely in drying soil, as root activity is often sustained or may even increase (Malik *et al.* 1979). It may be important in cases where soil drying is severe and root activity is severely limited, but there is no evidence for the response.

CHAPTER III
MATERIALS AND METHODS

1 Plant growth, growth environment and treatment application

The studies were conducted in a growth room under artificial lights of 14/10 hours day/night photoperiod. The lights were provided by high-pressure sodium lamps and fluorescent tubes. The light panel was fitted at 140 cm above the base of the plants. During the period of leaf harvesting, the average irradiance at the top of the plants was $250 \mu\text{Em}^{-2}\text{s}^{-1}$, but was around $50 \mu\text{Em}^{-2}\text{s}^{-1}$ at the sample leaves (leaves 3-7 from the base) level, due to self-shading. The temperature was controlled to be 25°C in the light period and 20°C in the dark period. The relative humidity was not controlled, and varied in a range of 70-90%.

Seeds of sunflower (*Helianthus annuus* L. cv. Sunbird Grey Stripe) were germinated in vermiculite. After 10 days, seedlings of uniform size were selected and transferred to grow on in sterilised potting mix or coarse sand media, detailed for each experiment. The planting pots were placed under the light panel area (150 x 260 cm), and circulated to new positions once a week to minimise effects due to gradients of light, temperature and wind velocity within the growth chamber. Plants were allowed to grow until the fifth leaf (from the base) was fully expanded. At this stage of development, treatments (depending on the experiment) were applied to the plants. Under the light panel area, the plants were re-positioned in groups according to replications. Within each replication, the plants were randomly placed, and re-positioned, as before, after every occasion of leaf harvesting.

2 Plant measurements

2.1 Leaf sampling and water potential determination

On every occasion of leaf sampling, sample leaves (leaves 3, 5 and 7 or 5, 7 and 9 from the base, depending on the experiment) of each plant were harvested in the middle of

the light period (7 hours after lights were turned on). The water potential of each leaf was determined with a pressure chamber as described by Scholander *et al.* (1964). The leaf was covered with a plastic sheath before excision to prevent errors arising from rapid water loss (Turner and Long 1980) and loaded rapidly into the pressure chamber. Compressed air was gradually admitted to the chamber. The balancing pressure at which xylem sap just emerged from the cut end of the petiole (viewed through a lens) was recorded as the leaf water potential. The leaf was then placed in a plastic bag on ice to await leaf disc sampling, which was done within 3 hours.

From each leaf, sample discs (0.75 mm diameter) were taken at random from the whole leaf blade (not including veins), using a punch. A number of these discs weighing a total of approximately 0.2 g was collected into a 15 ml centrifuge tube on ice to await extraction of soluble protein, which was carried out immediately after disc sampling (within 2 hours). Other discs to a total weight of approximately 0.2 g were transferred to a sample vial, frozen in liquid nitrogen and maintained at -20°C. This sample was used for total amino acid and proline analysis on the following day. Another disc sample of around 0.1 g was collected into a stoppered centrifuge tube containing 5 ml of 80% acetone, and maintained at -20°C. This sample was used for chlorophyll content analysis during the following two days. The remainder of the leaf blade was then weighed for fresh weight/dry weight ratio determination (oven-dried at 60°C for 48 hours).

2.2 Soluble protein content determination

The sampled discs in the centrifuge tube were ground with 3 ml pre-chilled 0.05 M sodium orthophosphate buffer, pH 7.0, using an Ultraturrex tissue grinder. Another 2 ml of the sodium orthophosphate buffer was used to wash the grinding head, and the combined homogenate was centrifuged at 4°C for 20 minutes at 47,000 g (19,000 rpm in a Beckman JA.-20.1 rotor on a Beckman J2-21 centrifuge). The resulting clear supernatant was transferred to a sample vial for soluble protein determination.

Soluble protein content was estimated using the dye-binding method of Bradford (1976). One volume of the dye-reagent concentrate (Bio-Rad Laboratories, California) was diluted with four volumes of distilled and deionised water. The diluted reagent was filtered through Whatman no.1 paper and the filtrate was stored at room temperature for up to two weeks.

0.1 ml of the extract solution was mixed with 5 ml of the diluted dye-reagent. The combined solution was vortexed and incubated for 5 minutes before absorbance at 595 nm was determined on an Ultraspec 4050 spectrophotometer. The absorbance reading was calibrated against a regression curve for a bovine serum albumin standard (Bio-Rad Laboratories, California). Results were calculated as mg/g leaf dry weight, leaf dry weight being estimated from the fresh weight/dry weight ratio.

2.3 Total amino acid content determination

The leaf sample was ground with 4 ml of a mixture of methanol: chloroform: distilled water (12:5:3) in a 15 ml centrifuge tube, using an Ultraturrex tissue grinder. The homogenate was then centrifuged at 3,000 rpm for 10 minutes. The resulting supernatant was decanted into another centrifuge tube, and 3 ml of distilled water was added to separate chlorophylls into the supernatant chloroform layer. After centrifugation at 3,000 rpm the upper layer, consisting of methanol, water and water soluble solutes, was collected into a sample vial using a volumetric pipette. The pellet was re-extracted with another 2 ml of the methanol-chloroform-water mixture and 2 ml of distilled water. The resulting clear supernatant was pooled in the sample vial.

Total amino acid was determined using a ninhydrin reaction method based on that of Lee and Takahachi (1966). 0.5 M citrate buffer was prepared from trisodium citrate. To reduce the reaction blank, as mentioned by Shannon and Wallace (1979), the solution was adjusted to pH 11.0 with NaOH, and boiled for 5 minutes. After cooling to room

temperature, the buffer was adjusted to pH 5.75 with HCl and stored at 5°C for up to two weeks. 14 ml of the citrate buffer was mixed with 0.1 g ninhydrin and 24 ml glycerol to form the ninhydrin reagent.

0.4 ml of the extract solution was mixed with 5.6 ml of the ninhydrin reagent in a boiling tube. The tube was then heated on a boiling water bath (95-100°C) for 12 minutes. A glass marble was placed onto the tube to prevent the solution evaporating. Amino acid formed a purple-coloured complex with the ninhydrin. After cooling to room temperature, the absorbance at 570 nm was read within an hour, using an Ultraspec 4050 spectrophotometer. The absorbance reading was calibrated against a regression curve for a glycine standard. Results were calculated as $\mu\text{moles/g}$ leaf dry weight.

2.4 Proline content determination

Proline content was estimated using the rapid method developed by Singh *et al.* (1973a) based on Troll and Lindsley (1955). 4 ml of the methanol: chloroform: water extract was shaken with 500 mg of "Amberlite" IR-120(Na), 14-52 mesh resin (regenerated with 1 M NaOH prior to use) in a test tube. The supernatant was decanted to a boiling tube (20 mm x 140 mm). The resin was washed twice, each time with 3 ml distilled water, and the supernatants were pooled in the boiling tube. 5 ml of fresh acidic ninhydrin solution (2.5 g ninhydrin dissolved in 60 ml of acetic acid and 40 ml of 6 M orthophosphoric acid by heating at 70°C) and 5 ml of glacial acetic acid were added to the tube. To prevent bumping, a few glass beads were placed into the tube.

The contents were then thoroughly mixed and covered with a glass marble to prevent evaporation, before heating in a water bath of 90-100°C for 1 hour. After cooling to room temperature, 5 ml of toluene was added to the tube and thoroughly mixed to extract the red colour. The toluene phase was allowed to settle for 1 hour, before absorbance at 520 nm (using a Pye Unicam PU 8600 spectrophotometer) was recorded.

The absorbance value was calibrated against a regression for L-proline standard, assaying at the same time as the samples. Results were calculated as $\mu\text{moles/g}$ leaf dry weight.

2.5 Chlorophyll contents determination

Chlorophylls were extracted by grinding sample tissue with 25 ml of 80% acetone, using an Ultraturrex tissue grinder. After centrifugation at 3,000 rpm for 15 minutes, the supernatant was taken for absorbance readings. The absorbances at 645 nm and 663 nm were recorded, using an Ultraspec 4050 spectrophotometer. Chlorophyll contents were calculated from the following equations, according to Arnon (1949).

$$C_a = 12.7D_{663} - 2.69D_{645}$$

$$C_b = 22.9D_{645} - 4.68D_{663}$$

$$C = 20.2D_{645} + 8.02D_{663}$$

Where C_a , C_b and C are the chlorophyll a, chlorophyll b and total chlorophyll contents in mg/L, D_{645} and D_{663} are the absorbance reading values at the respective wave lengths.

Total chlorophyll content was calculated as mg/g leaf dry weight.

3 Soil water measurements

After leaf sampling, soil samples (125 ml) were taken from the middle of each pot. The soil wet weight and dry weight (oven-dried at 110°C for 48 hours) were determined and water content was calculated. In order to convert soil water content into soil water potential for the potting mix used, a standard curve describing the relationship between soil water content and soil water potential was established. Compressed air of different pressures was applied to sampled soil in a pressure plate apparatus to extract

the soil water. After drainage stopped, the remained soil water content was then determined and plotted against the pressure (Figure 1).

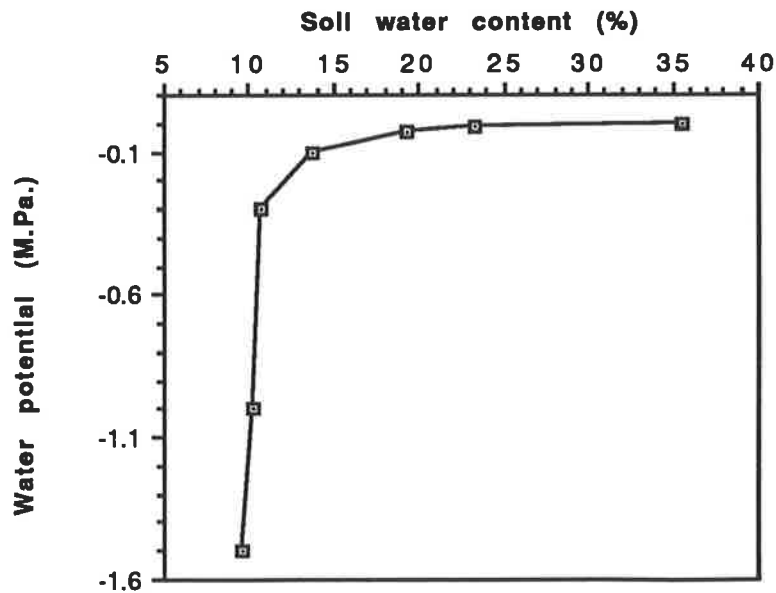


Figure 1 The relationship between soil water potential and soil water content (%). Data of soil water content are means of 3 replications.

4 Data analysis

A split-plot with 2-factor factorial as main-plot and single factor as sub-plot in randomised complete block design was used unless otherwise stated. The combination of treatment and time course was designed as the main-plot, whereas sub-plot was the position of leaves. Analysis of variance was carried out using the Genstat 5 programme. Tukey's wholly significant difference between means values were calculated at the 0.05 level of probability.

CHAPTER IV

RESULTS AND DISCUSSION

1 Effects of leaf water stress on mature leaf senescence

1.1 Introduction

A typical feature of senescence in detached leaves is a fall in protein and chlorophyll levels coupled with an increase in free amino acid content in corresponding to the protein hydrolysis (Martin and Thimann 1972a, 1972b). The accumulation of liberated amino acid, as a measure of proteolysis, and the degradation of chlorophyll have been used as criteria of the progress of senescence in several studies with leaf segments (Thimann and Satler 1979a, 1979b). In attached senescing leaves, however, the protein breakdown products have been observed to be translocated to other parts of the plants (Thimann *et al.* 1974; Wittenbach *et al.* 1980; Friedrich and Huffaker 1980). Hence, progress in natural leaf senescence observed after full leaf expansion is generally measured by losses in protein and chlorophyll contents (Peoples and Dalling 1978). These symptoms of senescence have also been observed in wheat leaves (Mayoral *et al.* 1981) and cotton leaves (Radin 1981) when the plants undergo water stress. However, plants of different species may exhibit different responses to different degrees of stress (Mayoral *et al.* 1981). This study aimed to characterise the sequence of metabolic changes occurring in leaves of different physiological ages during natural senescence and the effect of the leaf water status on these changes.

1.2 Materials and methods

1.2.1 Plant growth and growth environment

The experiment was conducted in a growth room under the environmental conditions detailed in Materials and Methods. Two uniform 10 day-old seedlings (from sowing) were transplanted into each plastic pot containing 10 litres of sterilised potting mix. The pots were weighed daily and watered to soil field capacity whenever the soil

water content of any pot was depleted to 75% of the soil field capacity. After another 16 days, most of the plants were at the stage where the fourth pair of leaves (counting from the base) were expanding and the fifth pair of leaves were emerging. At this stage, the plants were thinned to one uniform plant per pot. The uniformity of the plants was judged visually from the size of the fourth pair of leaves and the stage of emergence of the fifth pair of leaves.

1.2.2 Imposition and relief of water stress

Plants were allowed to grow until the fifth leaf was fully expanded (35 days after sowing), the plants were then divided into two groups: well-watered and water stressed, and re-positioned into 3 groups according to replications. Control plants were maintained on a watering regime in which the soil water content fell to no lower than 75% of field capacity throughout the experiment. For the water stressed plants, water was withheld from this day which was defined as day 0. Water was withheld from this group of plants until the third leaf was severely wilted, 15 days later. The soil in which these plants were growing was then rewatered to field capacity, and the soil water content was maintained to be no lower than 75% of field capacity thereafter.

1.2.3 Plant and soil measurements

The third, fifth and seventh leaves from the base of each plant were sampled to evaluate physical and chemical changes in response to water stress. Measurements were made on 7 occasions during the period of 35 days of water stress imposition and relief (included day 0). On each occasion, the sampling procedure described in Materials and Methods was followed. Soil and leaves water potentials, and soluble protein, total amino acid and chlorophyll contents were determined using the methods described in Materials and Methods.

1.3 Results

1.3.1 Soil water potential

In the soil which was daily watered to field capacity before the end of the light period, the mid-day soil water potential on the following day remained fairly high throughout the experimental period. Although it fell slightly when the plants became larger, it was never lower than -0.25 MPa (Figure 1.1). Withholding water severely reduced the soil water potential. In this treatment, the soil water potential fell gradually to -0.33 MPa on day 7, but quickly thereafter to -0.68 and was lower than -1.5 MPa on days 10 and 15. However, when the soil was returned to the normal watering schedule, it recovered to the control level rapidly. From day 18 onwards, it did not vary from that in the continuously well-watered soil.

1.3.2 Leaf water potential

The pattern of change in leaf water potential in response to treatment and time differed significantly between leaves at different positions (Figures 1.2a, 1.2b, and 1.2c). In well-watered plants, the water potential of the youngest leaf (leaf 7) did not vary significantly throughout the 34 day experimental period. In the older leaves, however, the water potential fell with time, being significantly lower than day 0 on day 18 in leaf 3 and day 23 in leaf 5.

In water-stressed plants, the water potential of leaves at all positions decreased gradually during the first 7 days of withholding water. Thereafter, the water potential fell more rapidly until re-watering on day 15. However, the change in leaf water potential in response to the regime of water stress and resumption differed between leaves at different positions. The oldest leaf responded more rapidly to the imposition of water stress. The water potential of leaf 3 decreased from day 7 to a significantly lower level than either that on day 0 or in the control leaf on the same day. This significant

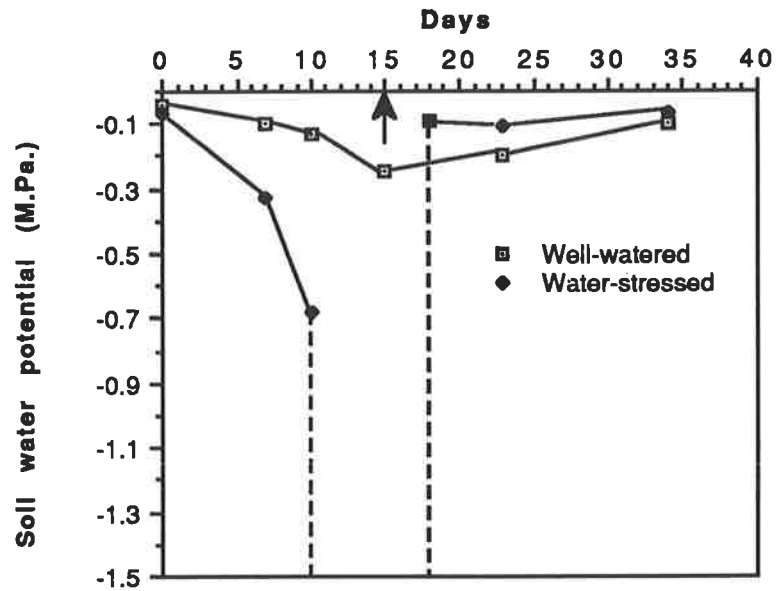
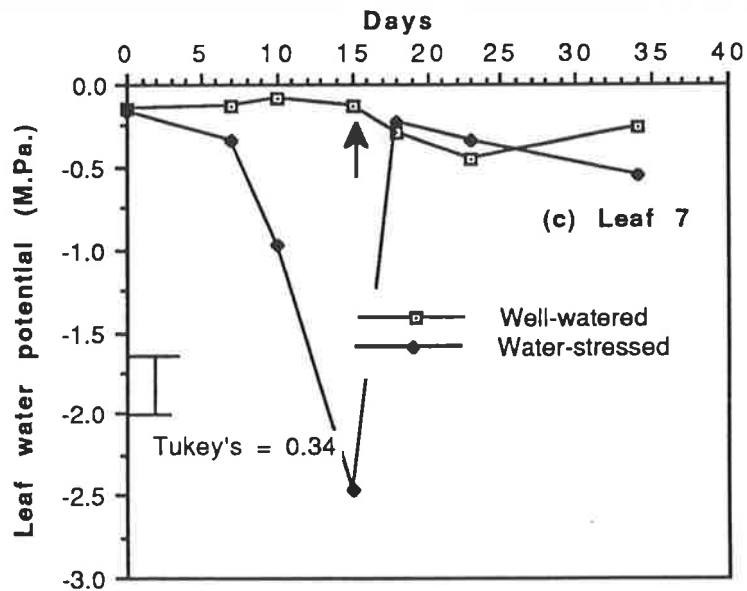
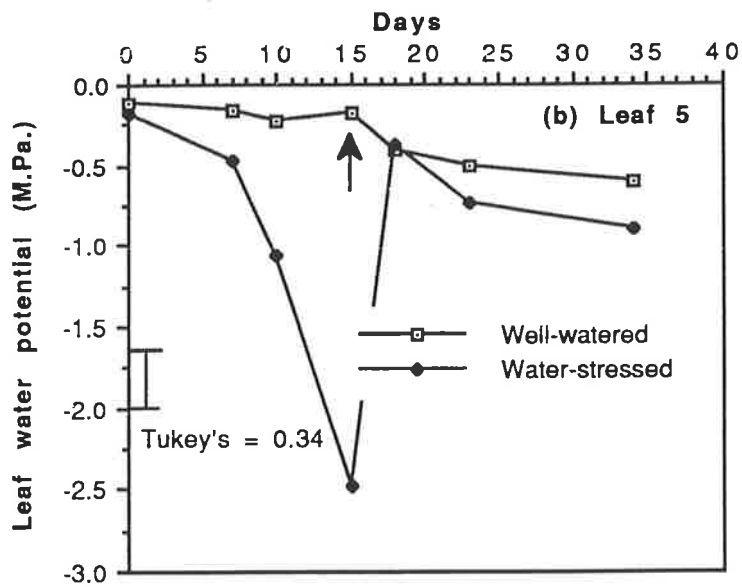
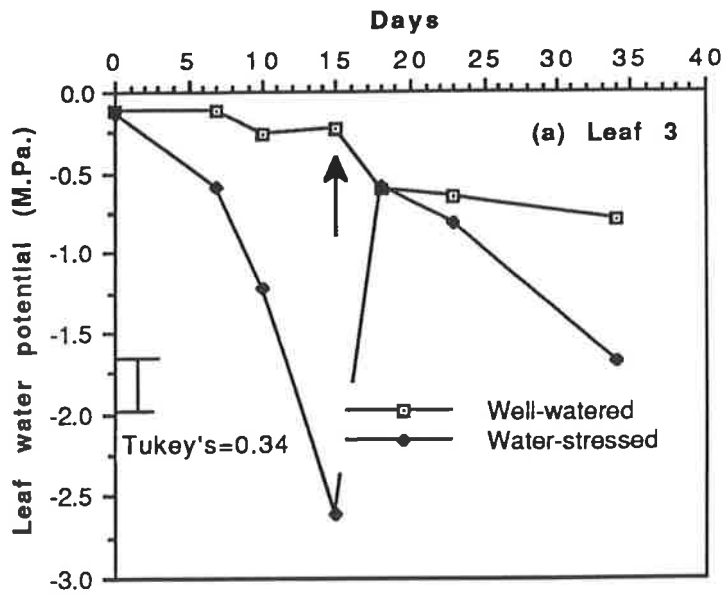


Figure 1.1 Changes with time in water potential of the soil in which well-watered and water-stressed sunflower plants were rooted. The water-stressed plants were not watered from day 0 to day 15, and rewatered from day 15 (arrow) and subsequently. Data presented are means of 3 replications and are converted from soil water content data. Data indicated by dotted lines are lower than -1.5 MPa.

Figure 1.2 Changes with time in leaf water potential of well-watered and water-stressed sunflower plants at 3 different leaf positions: (a) leaf 3; (b) leaf 5; and (c) leaf 7 from the base. The water-stressed plants were not watered from day 0 to day 15, and rewatered on day 15 (arrow) and subsequently. Data presented are means of 3 replications. Vertical bars represent Tukey's wholly significant difference between means at 5% level.



reduction was not evident before day 10 in leaves 5 and 7. Finally, water potential fell to -2.61, -2.48 and -2.46 MPa in leaves 3, 5 and 7 respectively, on day 15 before watering was resumed.

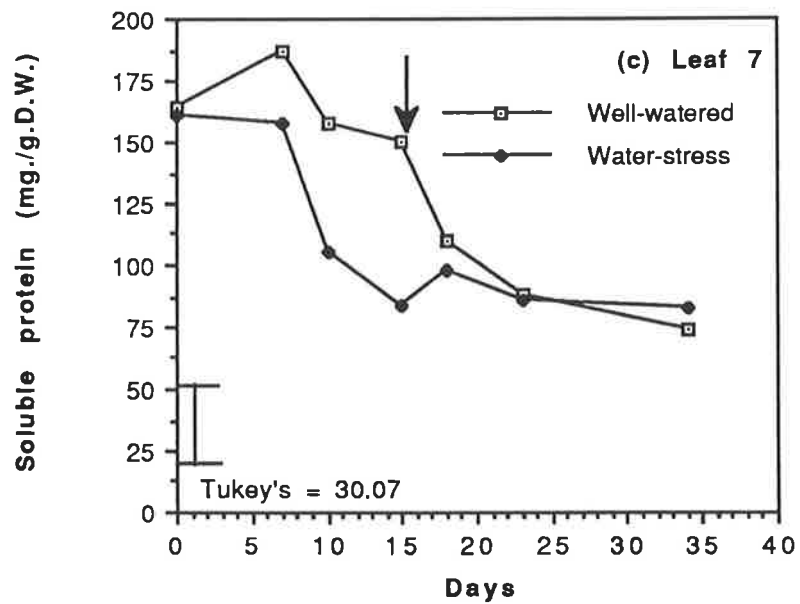
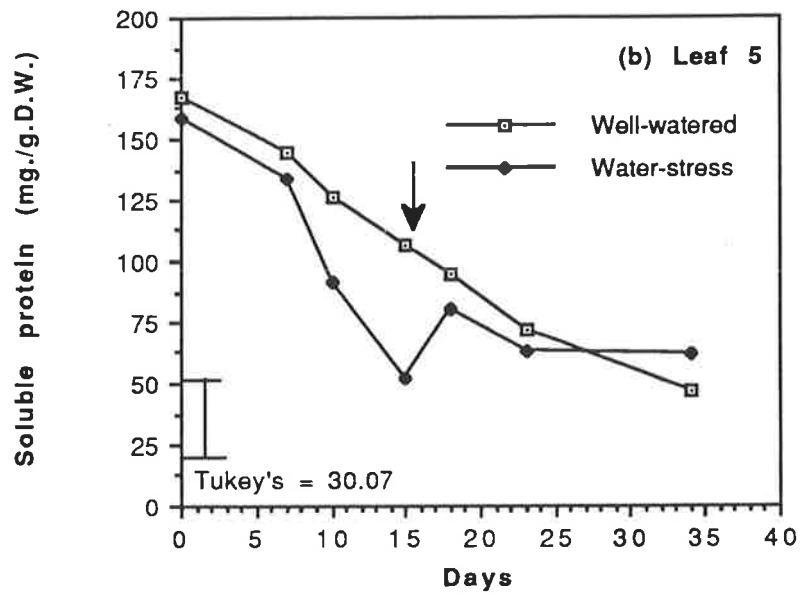
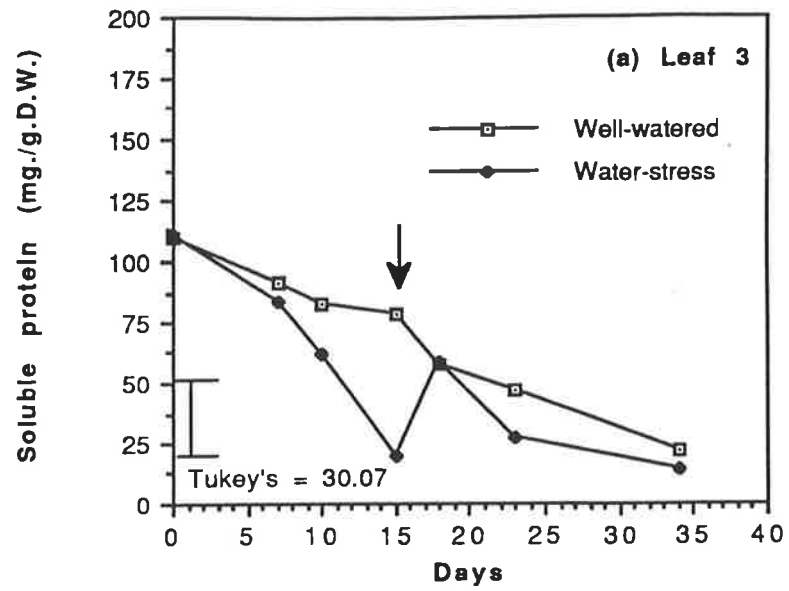
After the plants were re-watered, the water potential of all the leaves increased rapidly, being at a similar level as that in the equivalent leaves of control plants on day 18. Thereafter, the water potential of the stressed leaves fell again, even though the plants were now watered frequently. This fall was slight in the younger leaves, but severe in leaf 3, the water potential of leaf 3 being significantly lower than that of the control leaf on day 34.

1.3.3 Total soluble protein content

At the beginning of the experiment leaf soluble protein content was at a similar level in leaves 7 and 5, but was significantly lower in the oldest leaf (leaf 3) (Figures 1.3a, 1.3b and 1.3c). Thereafter, in control plants, the soluble protein content of leaves 3 and 5 fell drastically and linearly with time throughout the experimental period. Although soluble protein content had fallen significantly by day 15 in both leaves, the percentage loss on this day was higher in leaf 5 (36% of the same well-watered leaf on day 0) than in leaf 3 (28%). In contrast, there was an increase in the soluble protein content of leaf 7 during the first 7 days of the experimental period but thereafter the soluble protein content of this leaf also fell at an approximately linear rate. In this leaf, the percent loss in soluble protein on day 15 was 9% of day 0. These differences in protein content and response reflected the difference in stage of maturity of the leaves.

The protein loss with leaf age was accelerated by the imposition of water stress. By the last day of stress (day 15), the protein content of the stressed leaves had fallen by 82, 67 and 48% (of the same stressed leaves on day 0) in leaves 3, 5 and 7 respectively. When compared with the percent protein loss of the equivalent well-watered leaves of the

Figure 1.3 Changes with time in leaf soluble protein content of well-watered and water-stressed sunflower plants at 3 different leaf positions: (a) leaf 3; (b) leaf 5; and (c) leaf 7 from the base. The water-stressed plants were not watered from day 0 to day 15, and rewatered on day 15 (arrow) and subsequently. Data presented are means of 3 replications. Vertical bars represent Tukey's wholly significant difference between means at 5% level.



same day (day 15), water stress increased the percentage loss by 54, 31 and 39% in leaves 3, 5 and 7 respectively. On a leaf dry weight basis, from day 0 to day 15 the protein content lost by 31, 60 and 15 mg/gDW in well-watered leaves 3, 5 and 7 respectively, but 91, 107 and 77 mg/gDW respectively in the equivalent stressed leaves. Thus, by day 15 the loss of protein content due to water stress was 60, 47 and 62 mg/gDW in leaves 3, 5 and 7 respectively.

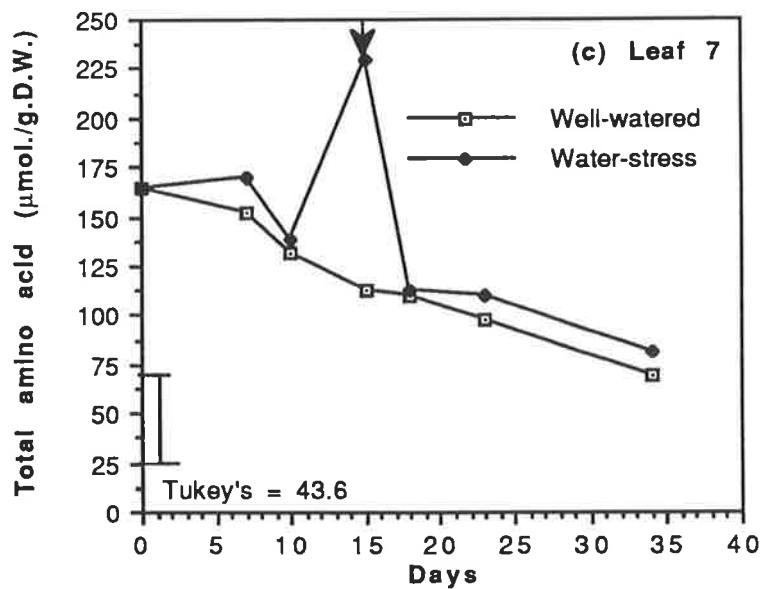
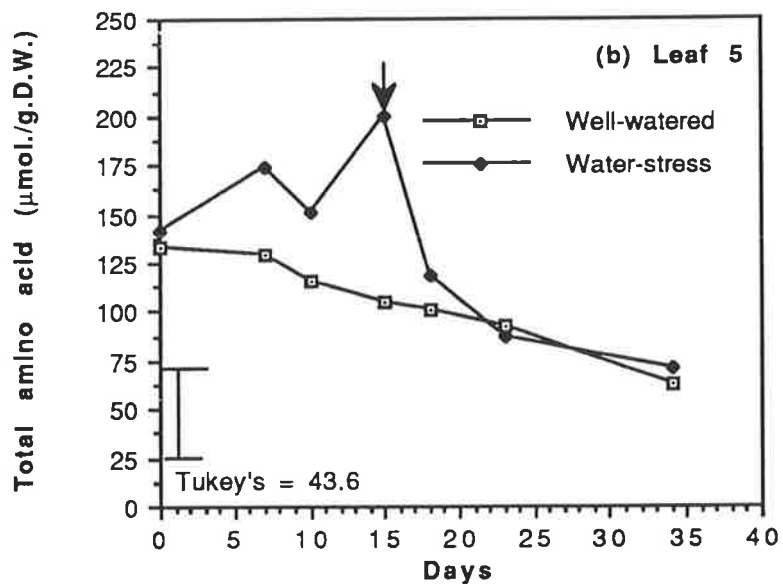
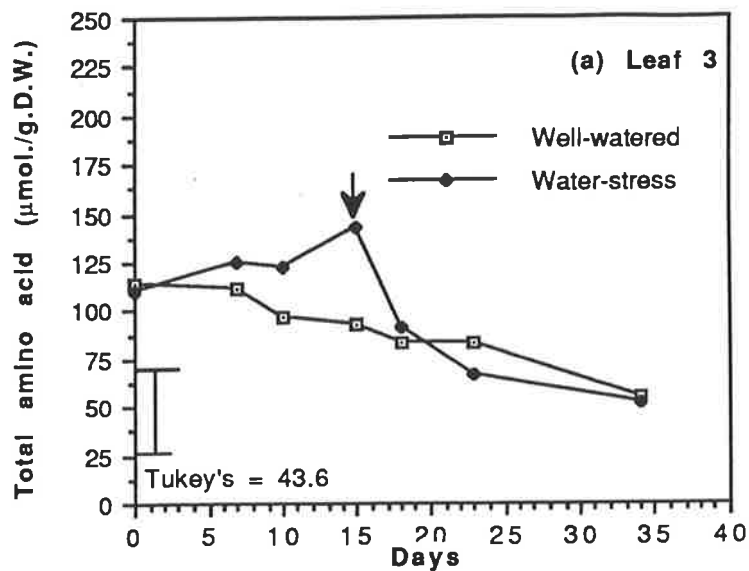
After watering was resumed, the protein content of the stressed leaves increased in all three leaves to the level found in the leaves not subjected to stress. Following day 18, the soluble protein content of the previously stressed leaves fell at a similar rate to that in the equivalent control leaves. There was no apparent influence of the previous exposure to water stress on the rate of subsequent protein loss.

1.3.4 Total amino acid content

The data for leaf free amino acid in leaves 3, 5 and 7 are shown in figures 1.4a, 1.4b and 1.4c respectively. On week 0, the amino acid content was lower in the older leaves than in the younger leaves. Thereafter, in well-watered plants, it fell continuously with time in all three leaves. During the 34 day experimental period, it fell from 114, 133 and 164 to 54, 63 and 70 $\mu\text{moles/gDW}$ in leaves 3, 5 and 7 respectively. In contrast, the amino acid content of all three leaves tended to increase during the 15-day period of water stress. This trend to increase was more evident in younger leaves (leaves 5, 7), although the amino acid level in these two leaves appeared to fall on day 10 (Figures 1.4b and 1.4c). This slight anomalous fall on day 10 may have occurred by chance.

In addition, the high amino acid reading in stressed leaves on day 15 may have been exaggerated by interference with the ninhydrin reaction in these samples. The purple-coloured complex of the ninhydrin reaction in the samples from these leaves combined

Figure 1.4 Changes with time in leaf total amino acid content of well-watered and water-stressed sunflower plants at 3 different leaf positions: (a) leaf 3; (b) leaf 5; and (c) leaf 7 from the base. The water-stressed plants were not watered from day 0 to day 15, and rewatered on day 15 (arrow) and subsequently. Data presented are means of 3 replications. Vertical bars represent Tukey's wholly significant difference between means at 5% level.



with a brown-coloured complex of unknown origin. The contamination with the brown-coloured complex may have caused an unusually high absorbance reading and resulted in an erroneously high amino acid reading, when plotting against the glycine standard curve. It is possible that this brown-coloured complex arose from reaction of the ninhydrin reagent with proline in these samples. A high level of proline is to be expected to accumulate in severely stressed sunflower leaves (Wample and Bewley 1975). The ninhydrin reaction with 1.0 mM L-proline standard solution in the conditions of this test resulted in a yellow-brown coloured complex. Moreover, the complex resulting from a combined solution of proline and standard glycine gave a combination of purple and brown colour. Nevertheless, since proline is also an amino acid, it can be presumed that the total amino acid of stressed leaves was high on day 15.

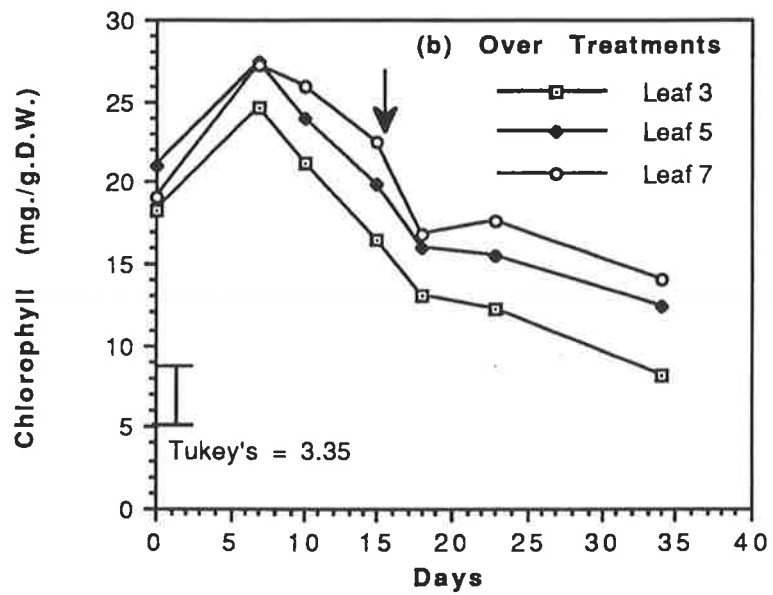
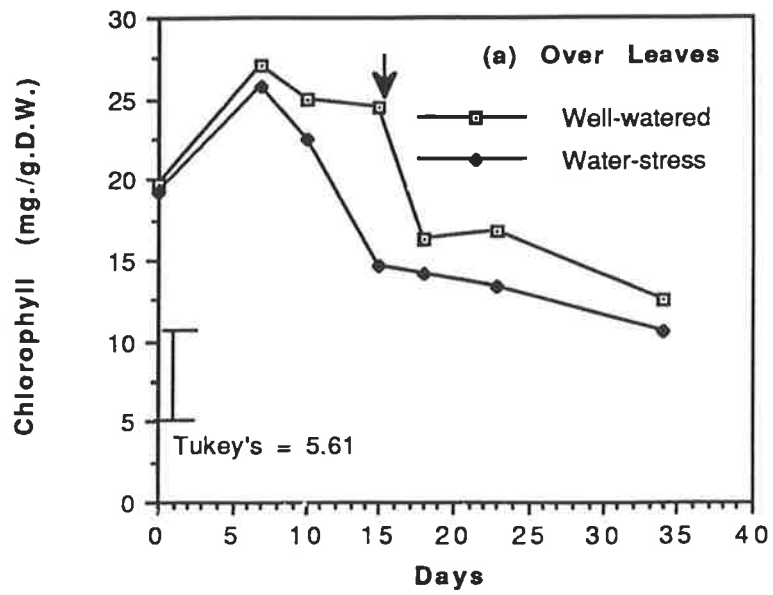
As with the leaf soluble protein content, the amino content of these previously stressed leaves returned rapidly to the control level after re-watering. It was at a comparable level to that of the equivalent control leaves from day 18 onwards.

1.3.5 Total chlorophyll content

There was no significant difference in the pattern of change in chlorophyll content with increasing time between leaves at different positions in response to the effect of different treatments. So, results are illustrated in terms of mean change in chlorophyll content with time in response to treatment (Figure 1.5a), and of different leaves in response to time but summed across treatments (Figure 1.5b).

In the absence of water-stress, mean leaf chlorophyll content rose to a maximum on day 7, and fell rapidly to a content of around 16 mg/gDW on day 18 but gradually thereafter. Water stress enhanced chlorophyll loss by accelerating the rate of chlorophyll degradation. However, during the first 7 days, chlorophyll content in the stressed plants increased significantly as in the control plants. Thereafter, it fell at a

Figure 1.5 Changes with time in leaf total chlorophyll content of sunflower: (a) in well-watered and water-stressed plants, data are means of 3 replications calculated over 3 leaf positions; and (b) at leaves 3, 5 and 7 from the base, data are means of 3 replications calculated over 2 treatments. The water-stressed plants were not watered from day 0 to day 15, and rewatered on day 15 (arrow) and subsequently. Vertical bars represent Tukey's wholly significant difference between means at 5% level.



faster rate than that in control plants to below 15 mg/gDW on day 15, before turning to a gradual loss rate, comparable to the later phase of control plants, after watering was resumed.

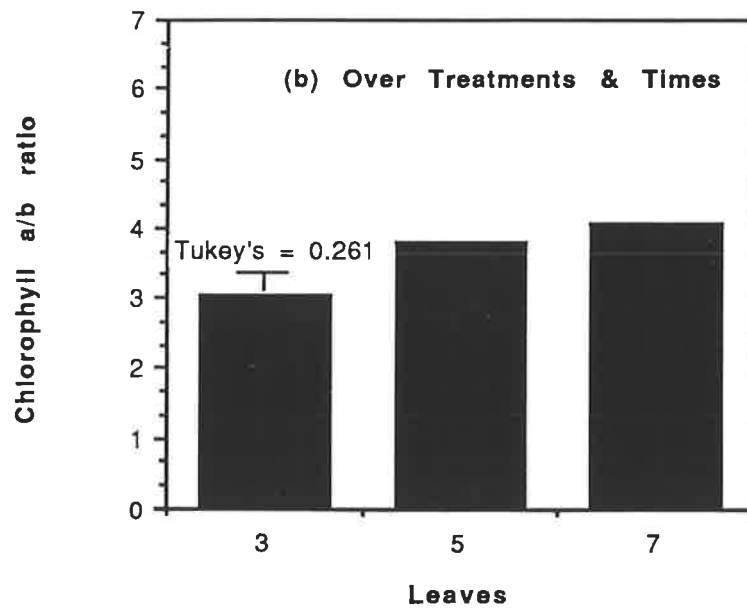
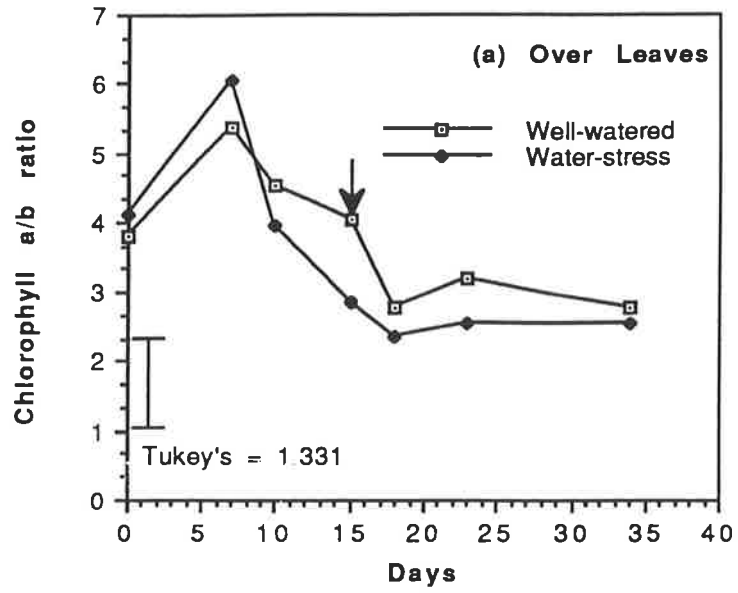
Regardless of treatment, chlorophyll content of leaves at all positions changed with time in a similar pattern. It increased to the maximum level on day 7, but fell rapidly thereafter and changed to a more gradual loss rate from day 18 onwards. There was no significant difference in the chlorophyll content of leaves 5 and 7 throughout the experimental period. However, the chlorophyll content of leaf 3 was significantly lower than that of leaf 7 or 5 from day 10 and 15 onwards.

1.3.6 Chlorophyll a/b ratio

As in the total chlorophyll content, no significant interaction of the 3 factors was found in the leaf chlorophyll a/b ratio response. Regardless of insertion of the leaf (Figure 1.6a), the chlorophyll a/b ratio of both well-watered and stressed leaves increased to a maximum value on day 7. However, thereafter the ratio fell in both treatments, faster in the stressed leaves than in the well-watered leaves. Following re-watering on day 15 the ratio stabilised close to 2.5 in previously stressed leaves. The ratio continued to fall in the well-watered leaves before stabilising at a similar level.

Despite the similarity in the responses of leaves at different points of insertion on the plant, there was a difference in mean chlorophyll ratio between these leaves. The pooled data over treatments and time periods (Figure 1.6b), showed that the mean chlorophyll a/b ratio of the younger leaves was significantly higher than that of the older leaves.

Figure 1.6 (a) Changes with time in leaf chlorophyll a/b ratio of well-watered and water-stressed sunflower plants. Data are means of 3 replications calculated over 3 leaf positions. (b) Average chlorophyll a/b ratio of leaves 3, 5 and 7 from the base. Data are means of 3 replications calculated over 2 treatments and 7 time periods. The water-stressed plants were not watered from day 0 to day 15, and rewatered on day 15 (arrow) and subsequently. Vertical bars represent Tukey's wholly significant difference between means at 5% level.



1.4 Discussion

1.4.1 Water potential responses

In the single water stress cycle used in this experiment, the use of a large volume of soil (10 litres) allowed the soil to dry out relatively slowly after withholding water. It then provided a period of plant water stress long enough to investigate leaf responses, before any occurrence of leaf permanent wilting. The observation that the water potential of the older leaves fell more rapidly than that of the younger leaves may indicate a sequential response of the plant leaves to soil drying (Zhang and Davies 1989). However, water potential recovered completely and rapidly to the control level in leaves at all positions upon re-watering.

The rapid recovery in water potential of the previously stressed leaves also demonstrated that the plants were supplied with adequate water after the stress regime. Nevertheless, there was a fall in leaf water potential in both control and previously stressed plants in the later period of the experiment. This fall was more marked in older or previously stressed leaves. This was unlikely to be due to a lack of water supply, since the data showed that the fall in leaf water potential during this period did not correlate to the soil water status. On the other hand, it may indicate the effect of senescence on leaf water uptake efficiency. In addition, a loss of stomatal function with age and consequent lowering of the ability to conserve leaf water potential would be expected (Atkinson *et al.* 1989).

1.4.2 Senescent responses

1.4.2.1 Changes in soluble protein

The continuous fall in soluble protein content in leaves 3 and 5 of control plants (Figures 1.3a and 1.3b) suggests that the natural process of senescence had begun for these leaves at the beginning of the experiment. Although the chlorophyll content of these leaves continued to increase for a further 7 days (Figure 1.5a), chlorophyll loss is not an obligatory part of the senescence process (Thomas and Stoddart 1975). In leaf 7, however, the protein content increased coincidentally with the rise in chlorophyll content during the first 7 days, but thereafter it also fell before the chlorophyll content decreased (Figure 1.3c and 1.5a). This apparent earlier loss of protein than of chlorophyll has also been observed in senescing leaves of oat (Thimann *et al.* 1974), barley (Friedrich and Huffaker 1980), wheat (Camp *et al.* 1982) and cowpea (Peoples *et al.* 1983). This suggests that the mechanism responsible for losses in leaf protein content and in chlorophyll content are not the same (Friedrich and Huffaker 1980; French 1985; Smart 1994). The later decline in protein content of leaf 7 compared to the other leaves also indicates a sequential natural senescence of the plants.

Water stress accelerated the rate of protein loss from leaves at all positions. The observation that leaf 5 lost slightly less protein than leaves 3 and 7 on both comparative amount per gDW and percent loss bases in response to water stress suggests that leaves of different physiological ages may exhibit a different degree of protein loss response to water stress. This is in accord to that found in attached leaves of barley (Dungey and Davies 1982a). These authors noted a more marked loss in protein content of the older region (the tip which was at a stage of the beginning of protein loss), compared to the younger region (the base which was at a stage of increasing in protein content) of the primary leaf blade, when barley plants were subjected to water stress (using PEG.).

Despite a difference between the leaves in the degree of protein loss in response to water stress, the recovery in protein content to the natural senescence level during leaf water potential recovery indicates that all the leaves were alleviated from the effects of water stress, and were able to respond by net protein synthesis. Recovery in leaf protein content was also observed in detached leaves of tobacco (Vonshak and Richmond 1975) and attached leaves of wheat (Wittenbach 1977) after reillumination of leaves induced to senesce by a period in darkness.

1.4.2.2 Changes in total amino acid

The lack of increase in leaf total amino acid concentration at the time of soluble protein loss from naturally senescing leaves indicated total translocation of the resulting free amino acid from the leaves or rapid metabolic conversion to other products. Younger leaves may not have been the major translocation sinks at this time, since the plants were at the stage of flowering. The continuous fall in leaf amino content agrees with that found in attached senescing leaves of soybean (Wittenbach *et al.* 1980) and barley (Friedrich and Huffaker 1980). In attached oat leaves, although Thimann *et al.* (1974) detected a slight increase in the amino acid content during the first 3 days of senescence, thereafter it also fell with time.

In water-stressed leaves, however, there was an accumulation in the amino acid content corresponding approximately to the increment in protein breakdown, induced by water stress. As mentioned earlier, it seems that proline accumulation involved in the increase in total amino acid content during water stress, particularly when the stress was more severe on day 15 (Figures 1.2a, 1.2b, 1.2c, 1.4a, 1.4b and 1.4c). Upon re-watering the interference in the ninhydrin reaction also disappeared with the disappearance of amino acid accumulation (Figures 1.4a, 1.4b and 1.4c). The disappearance in the interference supports the possibility that proline was the contaminant, since proline accumulation is known to disappear rapidly after water stress

relief (Aspinall and Paleg 1981) due to oxidation to glutamic acid (Stewart *et al.* 1977). The rapid return to the non-stressed amino acid level coincided with an increase in the soluble protein content of these previously stressed leaves (Figure 1.3a, 1.3b and 1.3c). This may support a putative role of proline as a protein precursor after water stress recovery (Zhang *et al.* 1982).

1.4.2.3 Changes in chlorophyll

Loss of chlorophyll content and the consequent visible yellowing is the most recognised symptom of natural leaf senescence. However, this began later than the loss of soluble protein content in this experiment. This was also evident in the response to water stress. The stressed leaves showed a significantly lower chlorophyll content than well-watered leaves only when they were severely stressed, which was evident only on day 15 in leaves at all insertions. The slower response of chlorophyll to adverse environment may result from a temporary protection from hydrolysis due to an association with the chloroplast thylakoid membrane (Friedrich and Huffaker 1980). Upon re-watering, the chlorophyll loss rate of previously stressed leaves changed rapidly to the normal senescence rate in parallel with the recovery in leaf water potential. This suggests that the processes responsible for the control of chlorophyll content were relieved from the influence of water stress. In juvenile leaves of maize plants recovering from water stress, a rapid recovery in chlorophyll content to the normal level has been reported (Alberte *et al.* 1977). This was also observed in young wheat leaves, after the leaves were induced to senesce by a period of darkness and then reilluminated (Wittenbach 1977).

A decline in chlorophyll a/b ratio with the fall in total chlorophyll content is typical of natural senescence (Sestak 1977; Gordon *et al.* 1978; Jenkins *et al.* 1981; Camp *et al.* 1982; French 1985). In water stress-induced senescing maize leaves, however, Alberte *et al.* (1977) observed an increase in the ratio of chlorophylls a to b.

This is in contrast to the observation for water stressed leaves of willow (Vapaavuori and Nurmi 1982) and wheat (French 1985), where the ratio declined. In the present experiment, the chlorophyll a/b ratio of sunflower leaves changed in a similar manner to the total chlorophyll change in response to both natural and water stress-induced senescence. The results suggest that in sunflower leaves, the chlorophyll a-protein complex (CP I) was more sensitive than the light-harvesting complex (LHCP) to treatments inducing chlorophyll loss.

2 Effects of partial soil-drying on mature leaf senescence

2.1 Introduction

When plants experience soil-drying, the falling water status of the soil eventually induces stomatal closure. This is commonly interpreted as being due to a fall in leaf water status (leaf water potential or turgor) which arises because the roots fail to extract adequate water from the drying-soil to maintain the leaf water status (Gollan *et al.* 1986). Other plant physiological modifications or morphological adaptations have also been interpreted as being correlated to shoot water status. These include a sensitive response in cell expansion and the subsequent process of leaf senescence (Hsiao 1973).

Stomatal closure in response to soil-drying has been observed despite the fact that leaf water status was stable during the experiment (Bates and Hall 1982; Meinzer and Grantz 1990). Indeed, several plants have shown an apparently direct stomatal response to soil-drying attributed to a signal originating in the drying root (Blackman and Davies 1985; Gollan *et al.* 1986; Zhang *et al.* 1987). Manipulation of shoot and root environments also suggests that this source of root signals also affects leaf initiation and expansion (Passioura 1988; Saab and Sharp 1989; Gowing *et al.* 1990; Davies and Zhang 1991). Stomatal closure in sunflower leaves has also been reported in response to a probable root-derived signal (Turner *et al.* 1985; Gollan *et al.* 1986; Neales *et al.* 1989; Zhang and Davies 1989). However, the effects of this phenomenon on leaf senescence have so far not been assessed. This study was designed to investigate any direct effect of soil-drying on the plant leaf senescent responses, independent of changes in leaf water status.

2.2 Materials and methods

2.2.1 Plant growth and growth environment

The experiment was conducted in a growth room in the same environmental conditions as in Materials and Methods (Chapter III). 10 days after sowing, uniform seedlings of sunflower (cv. Sunbird Grey Stripe) were selected and transferred to grow in a sterilised coarse sand media. The plants were daily watered to excess and supplied with 100 ml Hoagland's solution (Hoagland and Arnon 1938) each on every fourth day.

2.2.2 Root division and treatment application

After another 16 days, plants at a uniform stage of development similar to that described in the previous experiment, were selected and removed from the sand medium by rinsing in water. The plants were then grown on, dividing the root system equally between two potting bags containing sterilised potting mix (5 litres each) with a layer of granite gravel at the bottom to facilitate water-drainage. The primary root was placed in the side that would eventually be allowed to dry, and this side consequently contained a slightly higher root mass. The two potting bags were held together with staples and supported by placing in a single container. To avoid water flowing between the two potting bags, the bottom of the container was punched with many holes to allow rapid drainage.

Both potting bags were watered to excess each day until the plants were at the stage when the fifth leaf was fully expanded, as in the previous experiment. This took 40 days to this stage in this experiment. The 5-day delay in attaining this stage of leaf development, compared to the previous experiment, might be due to the disturbances to the root system at transplanting. Hence, week 0 in this experiment refers to 40 days after sowing. On this day, the plants were divided into two groups; well-watered (control) and partial soil-drying, and re-positioned into 3 groups according to

replications. In control plants, daily watering to excess was continued in both halves of the root zone. Only one half of the root zone (one bag) was maintained on this daily watering regime in the split-root drying treatment, the other half was not watered from day 0 through to the end of the experiment.

2.2.3 Plant and soil measurements

Sample leaves (leaves 3, 5 and 7 from the base) were harvested each week for 7 weeks (included 0) to measure water status and chemical changes. On each occasion, the sampling procedure and methods of leaf water potential, soluble protein, total amino acid, proline and chlorophyll contents determination described in Materials and Methods were followed. The same methods of soil sampling and soil water potential estimation described in Materials and Methods were also carried out for the soil in both potting bags in the well-watered and soil-drying treatments following each time of leaf harvesting.

2.3 Results

2.3.1 Soil water potential

The water potential of the well-watered soil in both potting bags for control plants (data not shown) and the single well-watered bag the partial soil-drying treatment did not fall lower than -0.01 MPa over the experimental period (Figure 2.1). This confirmed that both groups of plants had access to an adequate water source from the soil throughout the experiment.

In the unwatered half of the soil, however, there was a fall in water potential. The soil water potential in this bag fell gradually to -0.03 MPa on week 1, rapidly to -0.7 MPa on week 2 and to lower than -1.5 MPa thereafter. The severe fall in water potential in this half of the soil supported the conclusion that it was not contaminated with water

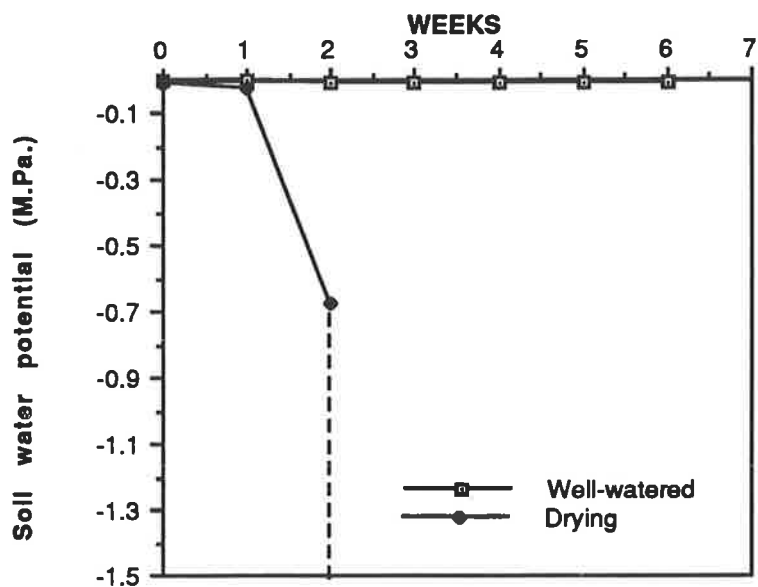


Figure 2.1 Changes with time in water potential of well-watered and drying halves of the soil in which sunflower plants were grown with divided root systems. The well-watered half was watered to excess daily, meanwhile the drying half was not watered from week 0 onwards. Data presented are means of 3 replications, which were converted from the soil water contents. Data beyond the dotted line are lower than -1.5 MPa.

flowing from the well-watered half. The result also showed that the part of the plant root system in this half of the soil was certainly exposed to a condition of soil-drying.

2.3.2 Leaf water potential

Figures 2.2a, 2.2b and 2.2c show that throughout the 7 week experimental period the leaf water potential of the soil-drying plants did not vary from that in equivalent control leaves. The data supported the conclusion that the roots in the well-watered soil were able to supply adequate water to maintain plant shoot water status at the level found in control plants. Regardless of treatment or position of leaf, however, there was a fall in mean leaf water potential with time (Figure 2.2d). The mean water potential began to fall from week 3, but was not significantly reduced until week 5. Nevertheless, it did not fall to a water stress level. At the end of the experiment it remained around -0.5 MPa.

Although the water potential pattern of change with time was similar between leaves at different insertions, mean water potential summed over time differed between leaves at different positions. It was lower in older leaves (leaf 3) compared to younger leaves (leaves 5 and 7) (Figure 2.2e). These patterns of change with time in water potential of all three leaves of both treatments are similar to those of control plants of the previous experiment (Figures 1.2a, 1.2b and 1.2c).

2.3.3 Total soluble protein content

Changes in leaf soluble protein content with time in leaves 3, 5 and 7 of control and the soil-drying plants are shown in Figures 2.3a, 2.3b and 2.3c. Since the inter-effects of treatment, position of leaf and time course were not statistically significant, the data are plotted for each pair of factors that interacted significantly.

Figure 2.2 Comparison of water potential of leaves 3, 5 and 7 (from the base) at different ages of well-watered and partial soil-drying sunflower plants growing with divided root systems. Both halves of the root systems of the well-watered plants and one half of the root system of the soil-drying plants were watered to excess daily. The other half of the root system of these plants was not watered from week 0 onwards. Data presented are means of 3 replications for (a, b and c), which were calculated over 2 treatments and 3 leaf positions for (d) or over 2 treatments and 7 time periods for (e). Vertical bars represent Tukey's wholly significant difference between means at 5 % level.

Analysis of variance (F , $\leq 0.01^{**}$, 0.05^* or not significant: ns):

Source of variation	Df	Vr.	Sig.
Treatment	1	0.06	ns
Time	6	24.44	* *
Treatment*Time	6	0.39	ns
Leaf	2	38.02	* *
Leaf*Treatment	2	0.02	ns
Leaf*Time	12	1.82	ns
Leaf*Treatment*Time	12	0.48	ns

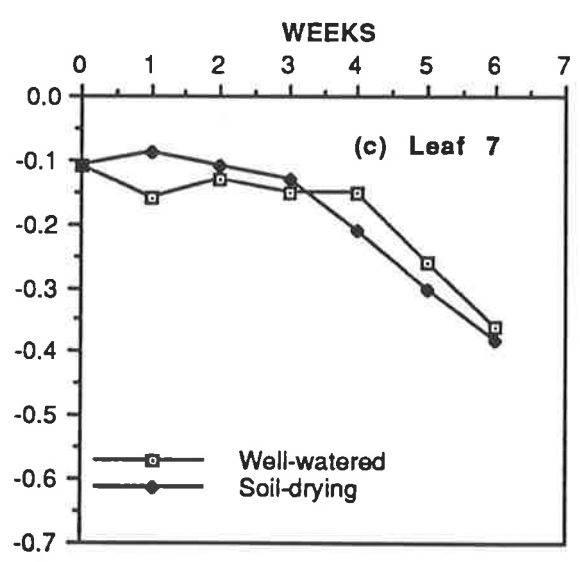
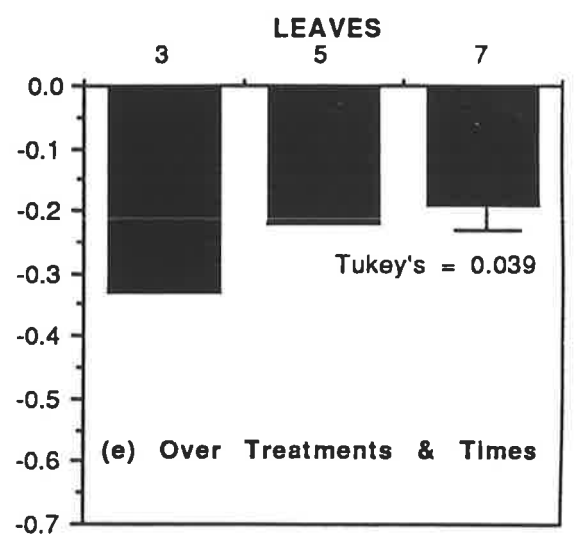
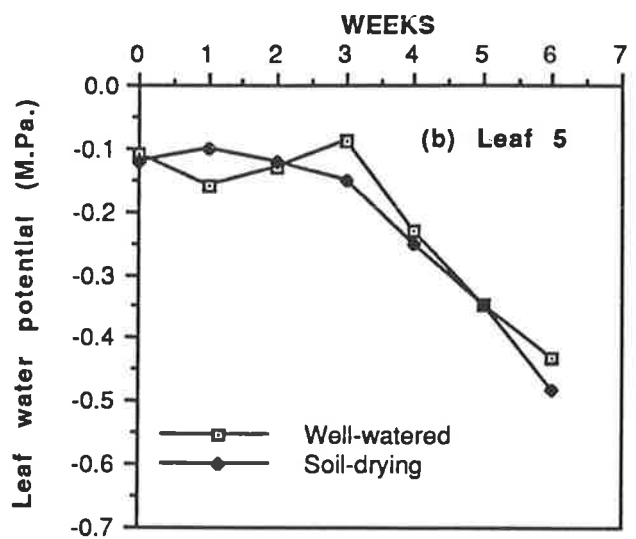
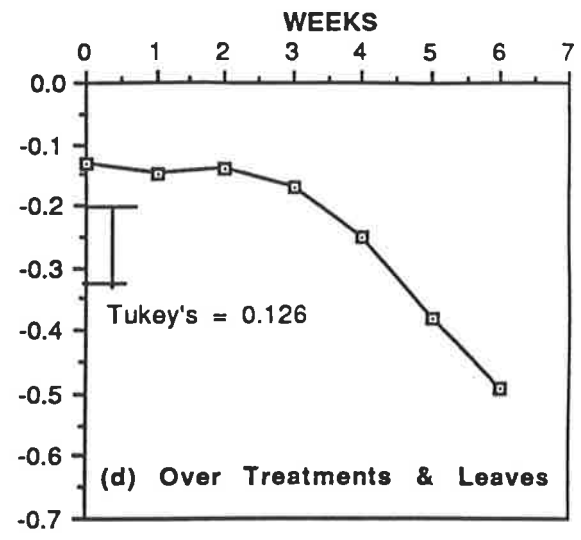
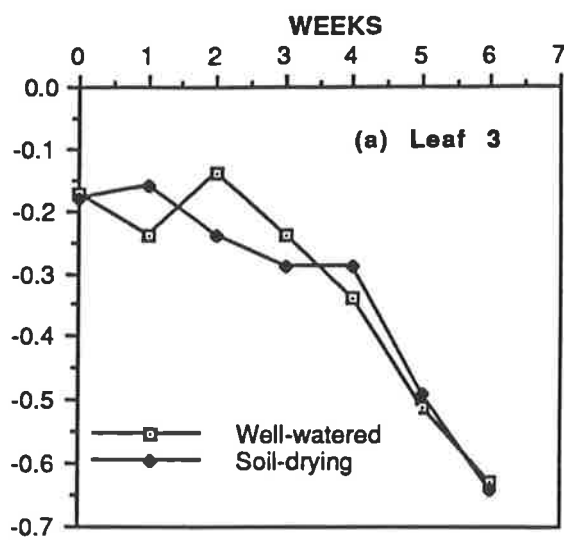
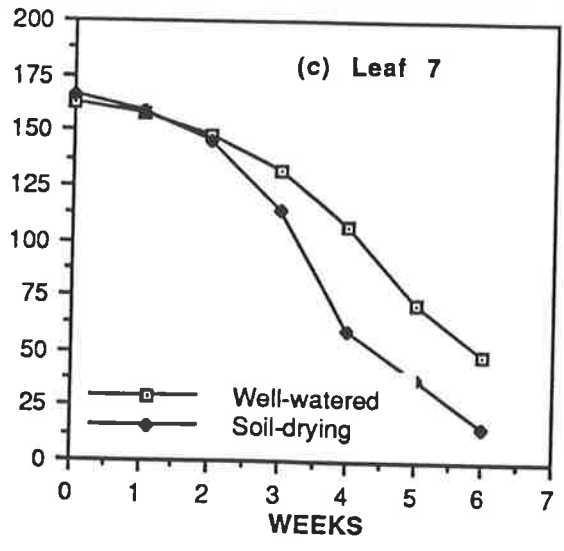
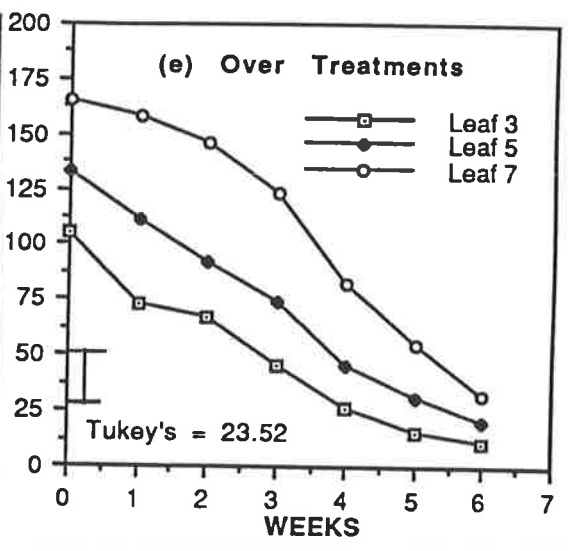
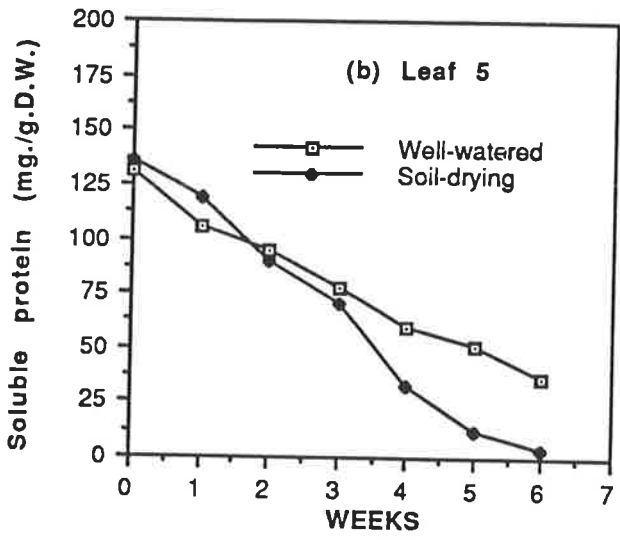
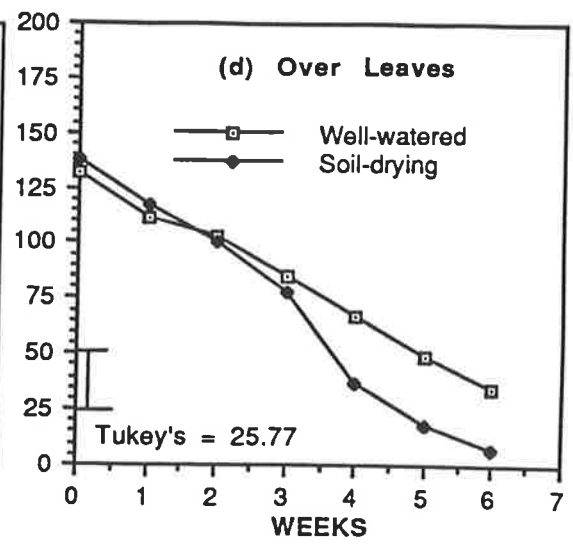
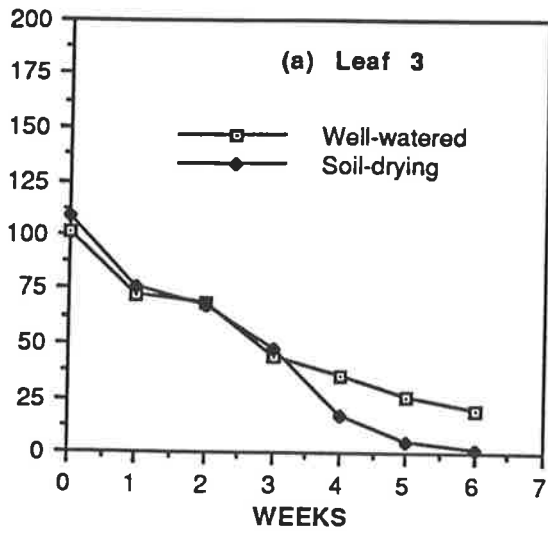


Figure 2.3 Comparison between the soluble protein contents of leaves 3, 5 and 7 (from the base) at different ages of well-watered and partial soil-drying sunflower plants growing with divided root systems. Both halves of the root system of the well-watered plants and one half of the root system of the soil-drying plants were watered to excess daily. The other half of the root system of these plants was not watered from week 0 onwards. Data presented are means of 3 replications for (a, b and c), which were calculated over 3 leaf positions for (d) or over 2 treatments for (e). Vertical bars represent Tukey's wholly significant difference between means at 5 % level.

Analysis of variance (F, $\leq 0.01^{**}$, 0.05^* or not significant: ns):

Source of variation	Df	Vr.	Sig.
Treatment	1	21.34	* *
Time	6	143.60	* *
Treatment*Time	6	5.48	* *
Leaf	2	315.98	* *
Leaf*Treatment	2	3.02	ns
Leaf*Time	12	7.15	* *
Leaf*Treatment*Time	12	0.59	ns



The pooled data over treatments are shown in Figure 2.3e. At the beginning, soluble protein content was higher in the younger leaves than in the older leaves. In addition to this difference between leaves, the soluble protein of leaves at all positions declined continuously from the beginning throughout the experimental period. In leaf 3, the decline in protein content was approximately linear with time from week 0 until week 5. During the last week, however, the rate of loss was slightly lower. By week 6, 90% of the initial protein content had been lost. Similarly, the protein content of leaf 5 declined linearly with time until the end of the experiment. Although the decline in protein content was slightly faster in this leaf, final percent protein loss was less than in leaf 3 (85%). In leaf 7, the loss of protein content was gradual during the first 2 weeks, before changing to a higher loss rate thereafter. Finally, on week 6, the protein content of this leaf had declined by around 81%. As in the previous experiment, the results implied a sequential maturing of the plant leaves.

Despite the difference in stage of maturity of the leaves, the pooled data over leaves at all positions (Figure 2.3d) indicated a significant effect of the soil-drying treatment on the rate of loss of protein. In the treatment with one half of the plant roots in drying-soil, the protein loss was faster than in the control plants at week 4. On that week, the percent protein loss of the control leaf was around 49%, and of the soil-drying treatment leaf 73%. Thereafter the rates of loss of protein in the leaves of control and partial soil-drying plants were comparable with the difference in protein content maintained.

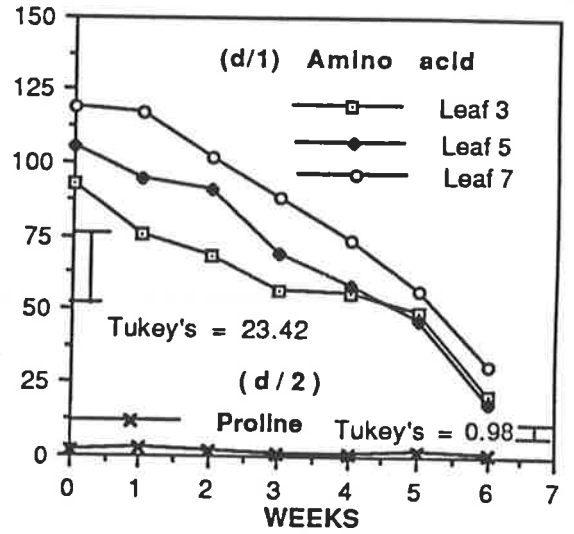
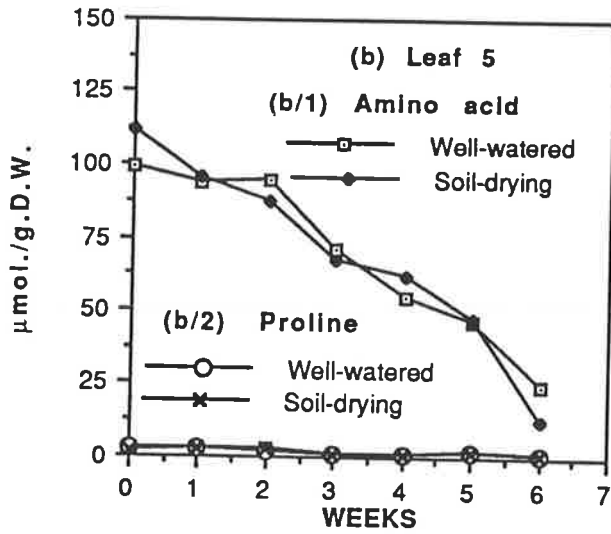
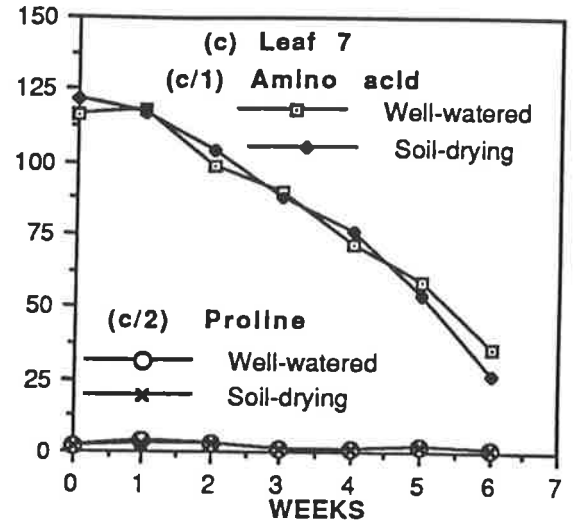
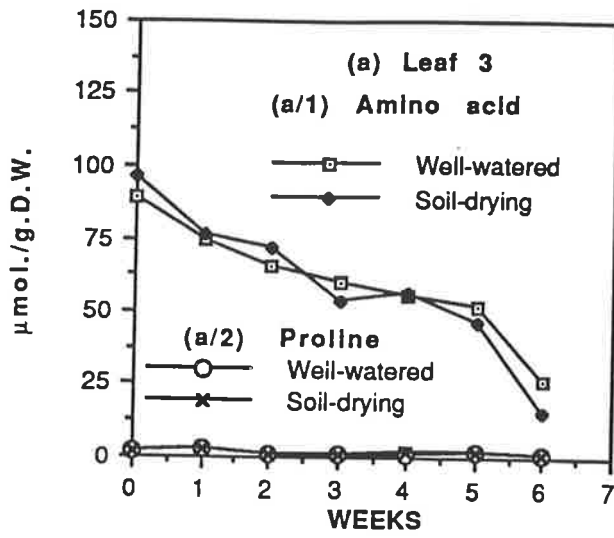
2.3.4 Total amino acid and proline contents

Unlike leaf soluble protein, there was no significant difference in leaf free amino acid content between treatments (Figures 2.4a/1, 2.4b/1 and 2.4c/1). Regardless of treatment, amino acid content declined with time in leaves at all positions, despite a higher initial (week 0) amino acid level of the higher insertion leaves (Figure 2.4d/1). In leaf 3, the decline in amino acid content was obvious from the beginning. However, it

Figure 2.4 Comparison on total amino acid and proline contents in leaves 3, 5 and 7 (from the base) of different ages of well-watered and partial soil-drying sunflower plants growing with divided root systems. Both halves of the root system of the well-watered plants and one half of the root system of the soil-drying plants were watered to excess daily. The other half of the root system of these plants was not watered from week 0 onwards. Data presented are means of 3 replications for (a, b and c), which were calculated over 2 treatments for (d/1) or over 2 treatments and 3 leaf positions for (d/2). Vertical bars represent Tukey's wholly significant difference between means at 5 % level.

Analysis of variance (F , $\leq 0.01^{**}$, 0.05^* or not significant: ns):

Source of Variation	Amino acid			Proline		
	Df	Vr.	Sig.	Df	Vr.	Sig.
Treatment	1	0.01	ns	1	0.20	ns
Time	6	36.07	* *	6	12.26	* *
Treatment*Time	6	0.43	ns	6	0.66	ns
Leaf	2	51.63	* *	2	1.79	ns
Leaf*Treatment	2	0.04	ns	2	0.07	ns
Leaf*Time	12	2.43	*	12	1.46	ns
Leaf*Treatment*Time	12	0.23	ns	12	0.51	ns



was slower from week 3 to week 5 before changing to a rapid rate during the last week. In leaf 5, however, the decline in amino acid content was at an approximately linear rate from week 0 to week 5, but thereafter it also changed to a more rapid rate. The amino acid content of leaf 7 did not vary during the first week. Thereafter, it also declined linearly with time through to the end of the experiment.

As in the previous experiment, no increase in leaf amino acid content was found in association with leaf protein loss, indicating either a rapid translocation of the protein breakdown products out of the leaf or de-amination. However, the differences in level and decline rate of amino acid content between leaves at different positions may indicate that the leaves were in a different stage of senescence, although these differences were not observed significantly between treatments.

Unlike amino acid, leaf proline content did not vary between treatments or between leaves (Figures 2.4a/2, 2.4b/2 and 2.4c/2). However, regardless of treatment or leaf position, there was a slight decrease in leaf proline content from around 3 $\mu\text{moles/gDW}$ on week 1 to around 1.5 $\mu\text{moles/gDW}$ on week 2 and thereafter (Figure 2.4d/2). The maintenance of a low proline level of both control and the soil-drying leaves supports the conclusion that the leaves did not experience a fall in water potential below a level critical for proline accumulation throughout the experimental period.

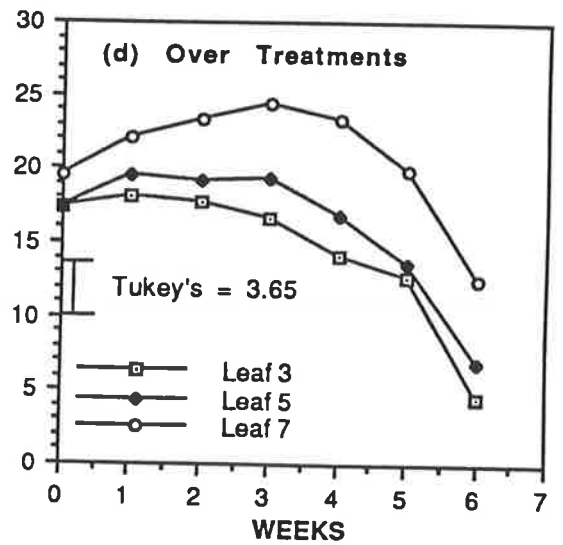
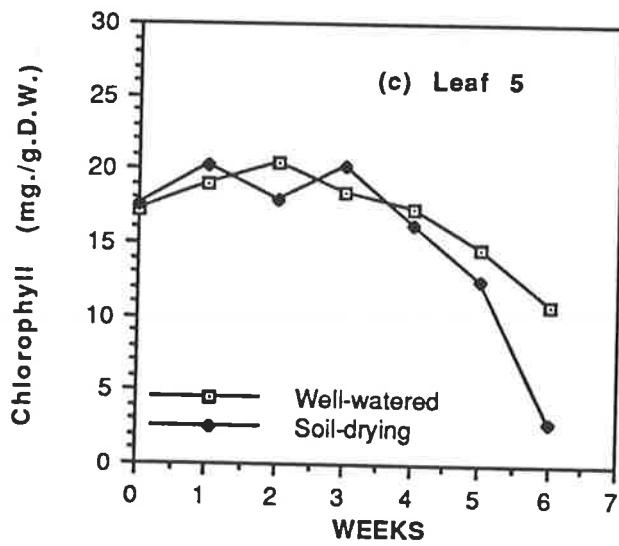
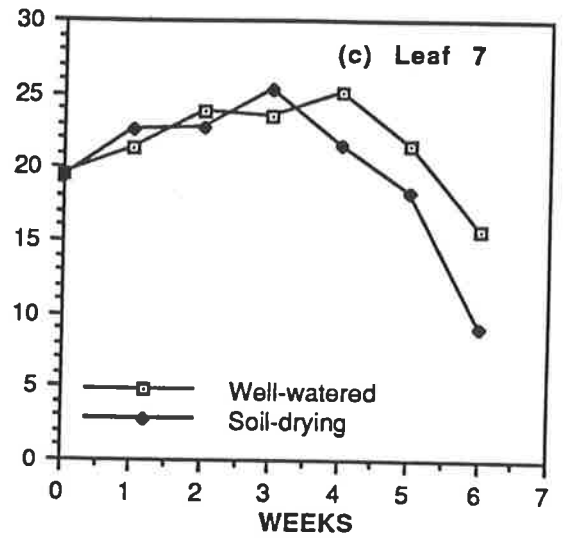
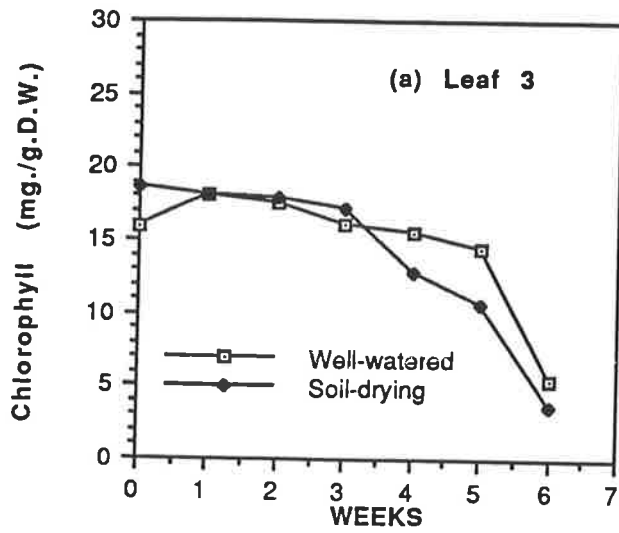
2.3.5 Total chlorophyll content

As in the amino acid response, the treatment with one half of the plant roots in drying-soil also showed no statistically significant effect on the level of leaf total chlorophyll content (Figures 2.5a, 2.5b and 2.5c). The pooled data for control and soil-drying plants are plotted in Figure 2.5d. In leaf 3, the chlorophyll content remained at around 18 mg/gDW for 3 weeks, before falling thereafter. A similar pattern of chlorophyll change was also evident in leaf 5. Although there was a slight increase in the

Figure 2.5 Comparison on total chlorophyll content in leaves 3, 5 and 7 (from the base) of different ages of well-watered and partial soil-drying sunflower plants growing with divided root systems. Both halves of the root system of the well-watered plants and one half of the root system of the soil-drying plants were watered to excess daily. The other half of the root system of these plants was not watered from week 0 onwards. Data presented are means of 3 replications for (a, b and c), which were calculated over 2 treatments for (d). Vertical bars represent Tukey's wholly significant difference between means at 5 % level.

Analysis of variance (F , $\leq 0.01^{**}$, 0.05^* or not significant: ns):

Source of variation	Df	Vr.	Sig.
Treatment	1	2.64	ns
Time	6	18.11	* *
Treatment*Time	6	1.59	ns
Leaf	2	151.10	* *
Leaf*Treatment	2	1.20	ns
Leaf*Time	12	3.54	* *
Leaf*Treatment*Time	12	1.33	ns



chlorophyll content of this leaf on week 1, it was not significant. In contrast, the chlorophyll content of leaf 7 rose significantly from week 0 to week 3 and remained at this level for a week before also falling thereafter.

2.3.6 Chlorophyll a/b ratio

Changes with time in chlorophyll a/b ratio of leaves 3, 5 and 7 in control and the soil-drying plants are shown in Figures 2.6a, 2.6b and 2.6c. Since there was no interaction of the effects on the response, pooled data are plotted. The pooled data over treatments and time periods indicated a higher chlorophyll a/b ratio in younger leaves compared to older leaves (Figure 2.6e). However, it declined with time in a similar manner in all leaves. The mean chlorophyll a/b ratios across treatment and leaf position are plotted against time periods in Figure 2.6d. Although drying one half of the root system had no significant effect on individual leaves and at specific times, the ratio of leaf chlorophyll a/b across leaf position and time was significantly lowered (Figure 2.6f).

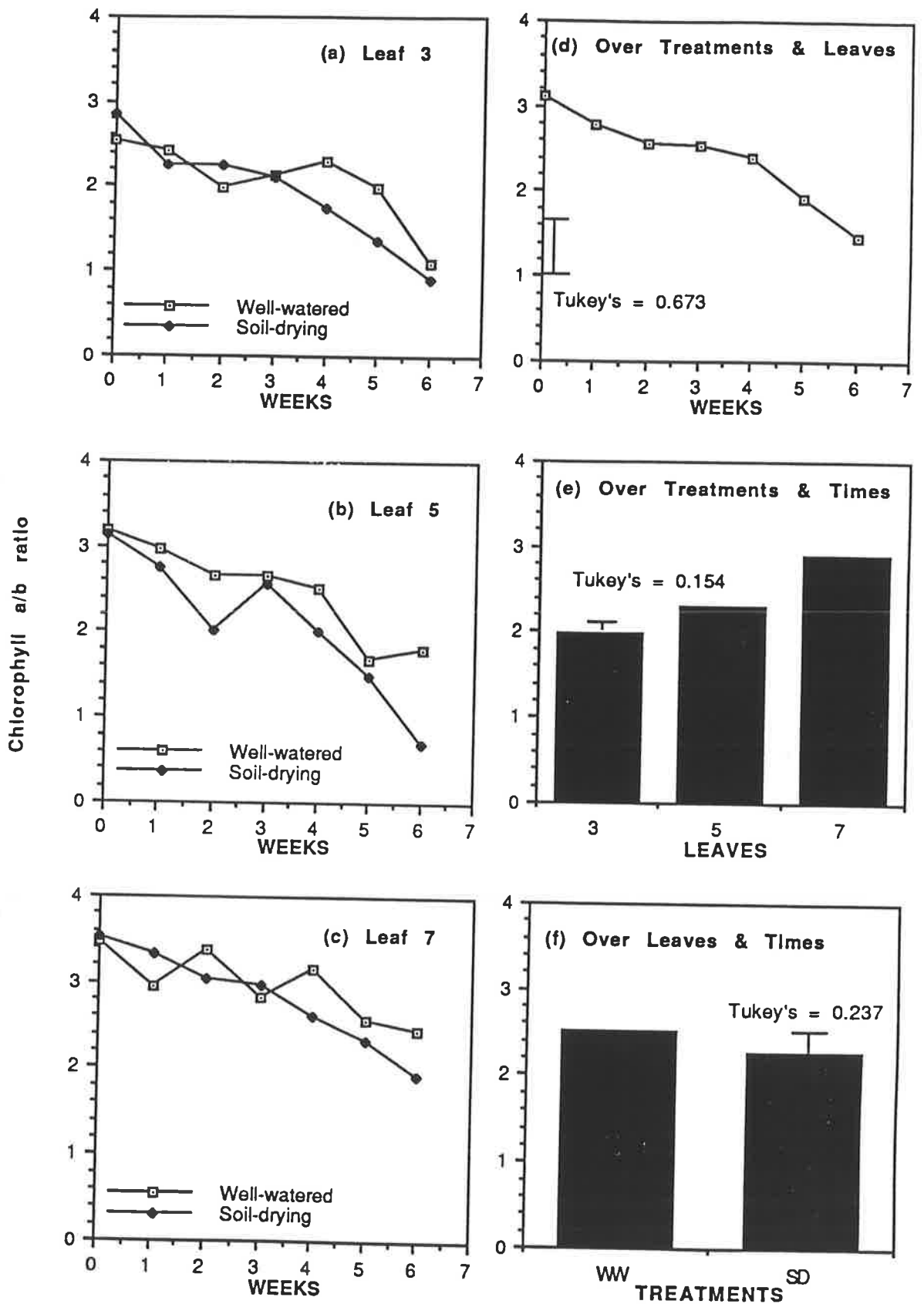
2.4 Discussion

The data for soil water potential in the unwatered root zone demonstrated that the soil was substantially dry from week 2 onwards. However, the part of the root system in this soil was observed to be apparently not as dry as the surrounding soil even at the end of the experiment. Although the root water potential was not determined, it was visually observed that almost all parts of the root system in the dried soil remained turgid except the root tips, which were more shrunken compared to those in well-watered soil. This is different from those of water stressed plants in the previous experiment in which most parts of the root system shrunk or dried out except some main roots. A similar observation was made by Saab and Sharp (1989) in a similar split-root drying experiment with maize plants. They noted that after 15 days of withholding water, while the water potential of the dried soil fell to -1.0 MPa, the midday water potential of the

Figure 2.6 Comparison on chlorophyll a/b ratio in leaves 3, 5 and 7 (from the base) of different ages of well-watered and partial soil-drying sunflower plants growing with divided root systems. Both halves of the root system of the well-watered plants and one half of the root system of the soil-drying plants were watered to excess daily. The other half of the root system of these plants was not watered from week 0 onwards. Data presented are means of 3 replications for (a, b and c), which were calculated over 2 treatments and 3 leaf positions for (d), over 2 treatments and 7 time periods for (e) or over 3 leaf positions and 7 time periods for (f). Vertical bars represent Tukey's wholly significant difference between means at 5 % level. WW: well-watered and SD: soil-drying plants.

Analysis of variance (F , $\leq 0.01^{**}$, 0.05^* or not significant: ns):

Source of variation	Df	Vr.	Sig.
Treatment	1	4.36	*
Time	6	13.77	* *
Treatment*Time	6	0.90	ns
Leaf	2	103.93	* *
Leaf*Treatment	2	2.62	ns
Leaf*Time	12	1.93	ns
Leaf*Treatment*Time	12	1.44	ns



part of maize root system in this soil remained as high as -0.29 MPa. As proposed by Saab and Sharp (1989), the portion of the root system in drying soil was probably supplied with water from the root system in damp soil or from the plant shoot, possibly overnight. Despite these differences, the remained high leaf water potential of the partial soil-drying plants indicates that any metabolic change in the leaves was not mediated through the leaf water status.

The results of the leaf soluble protein assays demonstrated an effect of the partial soil-drying treatment on leaf senescence, but no responses in the leaf total chlorophyll and amino acid, and proline contents. As mentioned earlier, chlorophyll loss is not an obligatory part of the senescence process and frequently occurs later than protein loss (which was also observed in the previous experiment). Change in total amino acid content also does not give a precise indication of senescence in attached turgid leaves as it can be translocated out from the leaves, although can accumulate in water stressed leaves at least partially in the form of proline (Section 1.3.4 and Figures 1.4a, 1.4b and 1.4c of the previous experiment), particularly when the leaves are detached from the plant. Nevertheless, the ratio of leaf chlorophyll a/b was also lower in the soil-drying treatment. This may be attributed to a slight decrease in the chlorophyll a component (of CP I), but the degree of loss was not sufficient to cause a statistically significant change in the total chlorophyll value.

There are several possibilities as to the nature of the message, passing from the plant roots in drying-soil to the shoot. These include hydraulic and chemical signals (Davies *et al.* 1987). Since the leaf response occurred in the absence of any change in leaf water status, hydraulic signals can be ruled out (Jones 1990a). As to the nature of chemical signals, there are three possible types; positive, negative and accumulative (Gowing *et al.* 1990; Davies and Zhang 1991). The positive message refers to an increase in the supply of some physiologically active substance from the roots in drying-soil to the shoot. ABA is the most prominent candidate for this type of signal in the leaf stomatal and

growth responses (Zhang *et al.* 1987; Zhang and Davies 1989; Neales *et al.* 1989; Davies and Zhang 1991). The negative signal consists of a reduction in supply of some product normally transmitted from roots to shoot. The production of cytokinin has been demonstrated to act as this source of signal in maize plants (Blackman and Davies 1985). The accumulative signal is an accumulation in the shoot of some shoot product normally transmitted to the roots. Jackson and Hall (1987) have proposed some evidence for this type of signal in flooded plants, involving an increase in leaf ABA concentration due to a reduction in phloem transport of ABA from the shoot to the roots.

All of these three types of signal are also possible for the leaf senescent response, since an increase in ABA and a decrease in cytokinin levels are widely known to accelerate leaf senescence. The results in the present experiment demonstrate evidence for a non-hydraulic root signal which promotes leaf senescence. This is indicated by a faster rate of loss of protein content and a lower chlorophyll a/b ratio for the soil-drying leaves than for the control leaves. However, this experiment does not provide evidence as to the nature or identity of the signals involved.

3 Alleviation of the effects of a root-signal on leaf senescence by root excision

3.1 Introduction

The results from the previous experiment demonstrate an effect of exposure of part of the plant root system to drying soil on the rate of leaf soluble protein loss and, to a lesser extent, the chlorophyll a/b ratio. The responses were similar to the senescent responses of sunflower leaves to whole-plant water deficit. They provide evidence for a role for the roots in stress-accelerated leaf senescence. However, since a shoot response to signals from the roots depends on the presence or absence of factors in the xylem stream, attributing the responses to a direct effect of a translocated root signal is premature. Reductions in nutrient supply or undetected falls in leaf water potential as a result of the loss of half the nutrient or water gathering capacity may arise and be involved in the effect of the soil-drying treatment. The present experiment was designed to investigate whether the responses can indeed result from root-signals rather than from changes in nutrient supply or leaf water status. In addition, with a treatment in which that part of the root system in drying-soil eventually would be excised, the results can be used to provide some evidence as to the nature of the signals (Gowing *et al.* 1990).

3.2 Methods

Four replications of sunflower plants were grown and stress was imposed on one half of the root system under environmental conditions and a management as described in the previous experiment. Four weeks after the plants had been placed in a split-root watering withheld treatment, sample leaves (leaves 5, 7 and 9 from the base) were harvested from a set of well-watered and the partial soil-drying plants to determine the effect of the soil-drying treatment. Data of leaf water status and biochemical contents were determined and analysed as a split-plot in randomised complete block design.

Treatment (comprised of well-watered and the partial soil-drying plants) was regarded as the main-plot and position of leaves as the sub-plot.

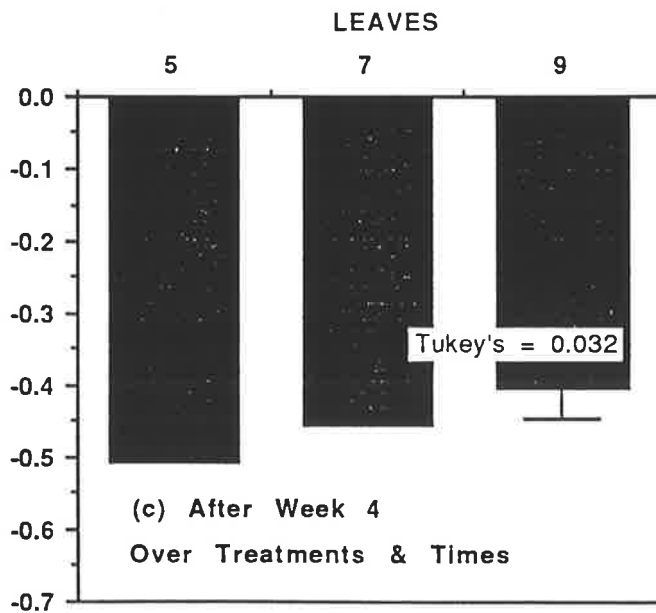
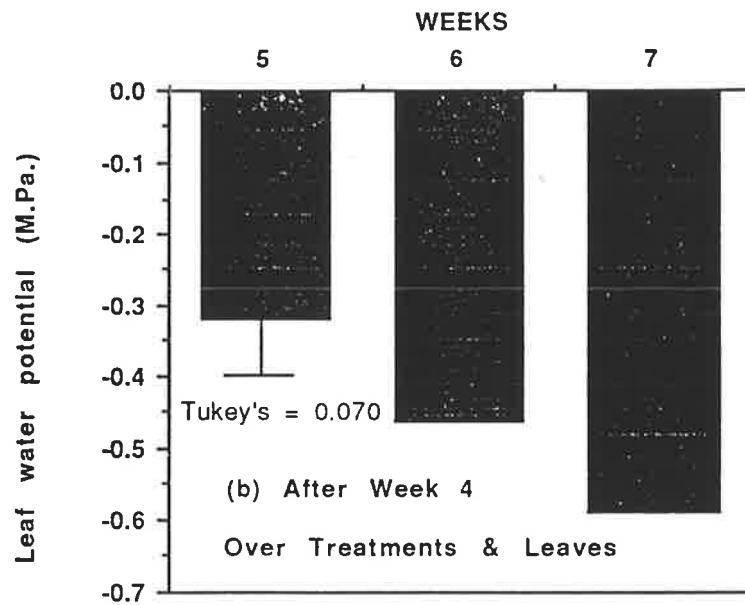
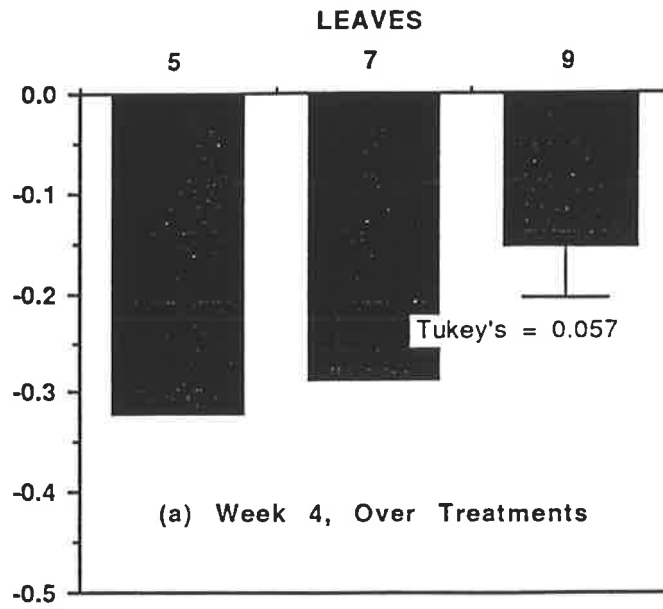
The remainder of the soil-drying plants were then divided into two groups. In one group, the plants were maintained with part of the roots in drying soil as before. In the other group, the half of the root system in drying soil was excised. A daily watering to excess schedule was maintained in the other half of the root zone of these two groups of plants throughout the experimental period (as well as in both halves of the root zone of well-watered plants). Thus, in this second set of analyses, treatments were comprised of well-watered, partial soil-drying and partial root-excised plants. Sample leaves were harvested each week for a further three weeks. On each occasion (including on week 4), the procedure of leaf sampling and water potential and soluble protein, proline and chlorophyll contents determination followed that described in Materials and Methods (Chapter III).

3.3 Results

3.3.1 Leaf water potential and proline content

On week 4, there was no difference in the leaf water potentials of well-watered and the partial soil-drying plants ($V_r = 0.82$, $F_{pr} = 0.431$). Regardless of treatment, however, the water potential of the youngest leaf (leaf 9) was higher than that of the older leaves (leaves 5, 7) (Figure 3.1a). These were sustained through to the end of the experiment. Excision of half of the root system in drying soil (on week 4) also did not vary the leaf water potential during the following weeks 5-7 ($V_r = 0.05$, $F_{pr} = 0.947$). Figures 3.1c and 3.1b show that the summed leaf water potential was higher in leaves of higher insertions on the plants, regardless of treatment or time period, and fell with time, regardless of treatment or position of leaves. The results obtained agree with the previous experiment that the well-watered part of the root system was able to extract

Figure 3.1 Water potential of leaves 5, 7 and 9 (from the base) at different ages of well-watered, partial soil-drying and partial root-excised sunflower plants growing with a divided root system. Both halves of the root system of the well-watered plants and one half of the root system of the soil-drying and the root-excised plants were watered to excess daily. The other half of the root system of these plants was not watered from week 0 onwards. In the root-excised plants, the half of the root system in drying-soil was excised on week 4. Data presented are means of 4 replications, which were calculated over (a) well-watered and soil-drying treatments, (b) 3 treatments and 3 leaf positions or, (c) 3 treatments and 3 time periods. Vertical bars represent Tukey's wholly significant difference between means at 5 % level.



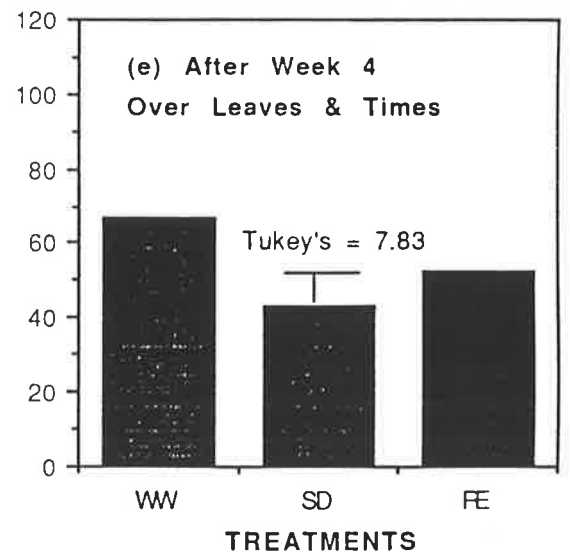
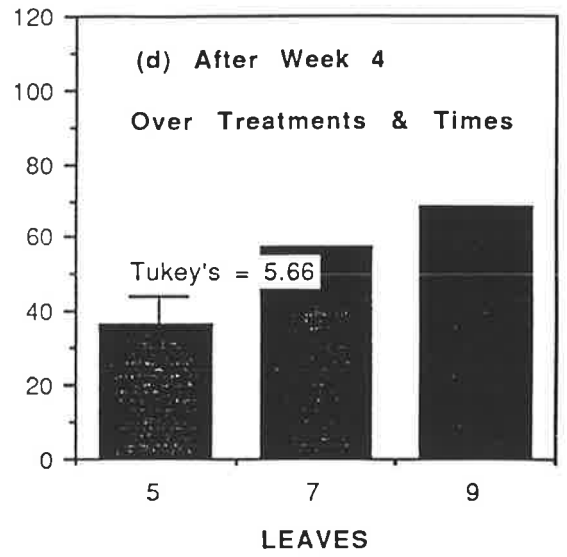
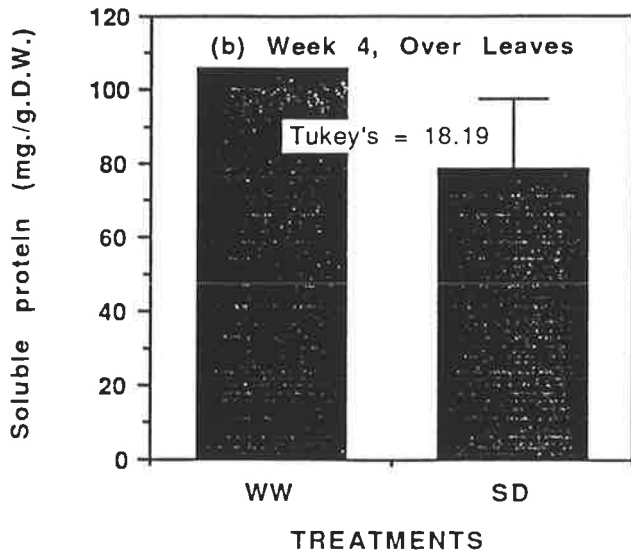
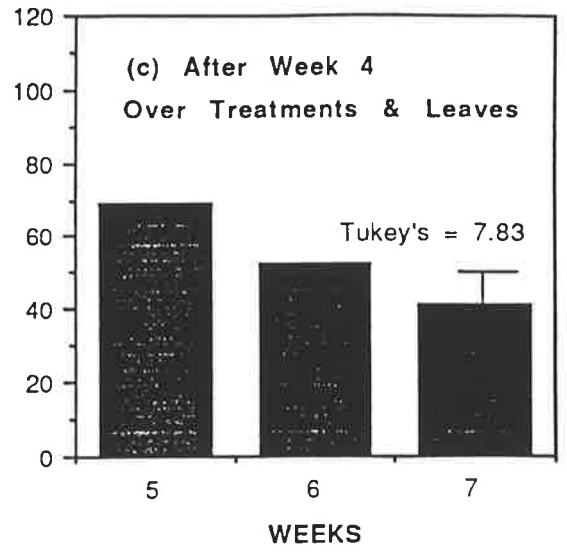
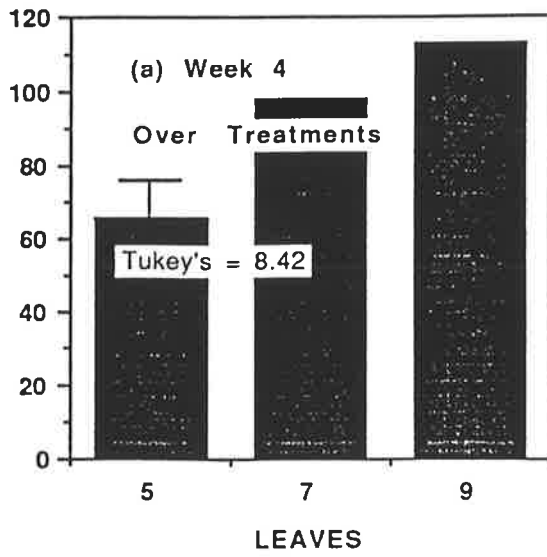
adequate water to maintain the plant leaf water status. This is also supported by the fact that proline content remained at a low level (2-4 $\mu\text{moles/gDW}$) and did not statistically vary between leaves ($V_r = 0.32$, $F_{pr} = 0.732$ for week 4 analysis, and $V_r = 0.98$, $F_{pr} = 0.383$ for weeks 5-7 analysis), treatments ($V_r = 0.15$, $F_{pr} = 0.722$ for week 4 analysis, and $V_r = 0.40$, $F_{pr} = 0.675$ for weeks 5-7 analysis) and times during weeks 5-7 ($V_r = 0.21$, $F_{pr} = 0.811$) (data not shown).

3.3.2 Soluble protein content

The results obtained are in agreement with those from the previous experiment, although the sampled leaves in this experiment were leaves 5, 7 and 9 from the base rather than 3, 5 and 7. On week 4, the pooled data over well-watered and the soil-drying treatments indicated a lower soluble protein content of the lower leaves (from the base) compared to leaves of higher insertions (Figure 3.2a). This was also evident during weeks 5-7, despite differences between the three treatments and three times of sampling (Figure 3.2d). The protein content of all leaves also fell continuously with time (Figure 3.2c). The pooled data over leaf positions (on week 4), and over leaf positions and time periods (weeks 5-7) demonstrated an effect of the soil-drying treatment in accelerating the rate of loss in leaf protein content compared to the rate in well-watered leaves (Figures 3.2b and 3.2e, respectively), confirming the indication of a root-derived response evident in the previous experiment.

Following root-excision on week 4, the pooled data over leaf positions and times (weeks 5-7) (Figure 3.2e) show that the leaf soluble protein content of the treatment with the half of the root system excised was higher than that in the plants where half of the root system was maintained in dry soil. The result indicates a reduction in the rate of protein loss, induced by a drying soil, following the removal of those roots. The soluble protein content of all leaves, including the control, continued to decline during this period

Figure 3.2 Changes in soluble protein content in leaves 5, 7 and 9 (from the base) at different ages of well-watered, partial soil-drying and partial root-excised sunflower plants growing with a divided root system. Both halves of the root system well-watered plants and one half of the root system of the soil-drying and the root-excised plants were watered to excess daily. The other half of the root system of these plants was not watered from week 0 onwards. In the root-excised plants, the half of the root system in drying-soil was excised on week 4. Data presented are means of 4 replications, which were calculated over (a) well-watered and soil-drying treatments, (b) 3 leaf positions, (c) 3 treatments and 3 leaf positions, (d) 3 treatments and 3 time periods, or (e) 3 leaf positions and 3 time periods. Vertical bars represent Tukey's wholly significant difference between means at 5 % level. WW: well-watered; SD: soil-drying and RE: root-excised plants.



and root excision did not return protein content to the control level nor to the level in the root drying treatment before root excision.

3.3.3 Total chlorophyll content and chlorophyll a/b ratio

Leaf total chlorophyll content was not affected by the root treatments. Figures 3.3a, 3.3b and 3.3c show that chlorophyll content was higher in leaves of higher insertions on the plants and fell with time in a similar pattern, but did not vary between treatments ($V_r = 1.46$, $F_{pr} = 0.314$ for week 4 analysis, and $V_r = 1.33$, $F_{pr} = 0.282$ for weeks 5-7 analysis).

In contrast to the previous experiment (Figure 2.1f), the treatment with one half of the plant root system in drying soil showed no effect on the leaf chlorophyll a/b ratio response on week 4 ($V_r = 1.95$, $F_{pr} = 0.257$). This was also obtained on the following weeks 5-7 analyses, including the treatment in which the soil-drying part of the root system was excised ($V_r = 0.12$, $F_{pr} = 0.892$).

The chlorophyll a/b ratio in different leaves and at different times showed some slight differences from the total chlorophyll responses. On week 4, the oldest leaf (leaf 5) had a much lower chlorophyll a/b ratio than the other leaves, but the ratio for leaf 7 was not significantly different from that of leaf 9 (Figure 3.4a). However, in the summed later time period (weeks 5-7), differences in the ratio of chlorophyll a/b between leaves at different positions were similar to those in total chlorophyll content (Figure 3.4c). The chlorophyll a/b ratio also fell from weeks 5 to 7, as did the chlorophyll content. The fall was significant from week 6 onwards in the total content (Figure 3.3b), but not in the ratio until week 7 (Figure 3.4b).

Figure 3.3 Changes in total chlorophyll content in leaves 5, 7 and 9 (from the base) at different ages of well-watered, partial soil-drying and partial root-excised sunflower plants growing with a divided root system. Both halves of the root system of the well-watered plants and one half of the root system of the soil-drying and the root-excised plants were watered to excess daily. The other half of the soil of these plants was not watered from week 0 onwards. In the root-excised plants, the half of the root system in drying-soil was excised on week 4. Data presented are means of 4 replications, which were calculated over (a) well-watered and soil-drying treatments, (b) 3 treatments and 3 leaf positions, or (c) 3 treatments and 3 time periods. Vertical bars represent Tukey's wholly significant difference between means at 5 % level.

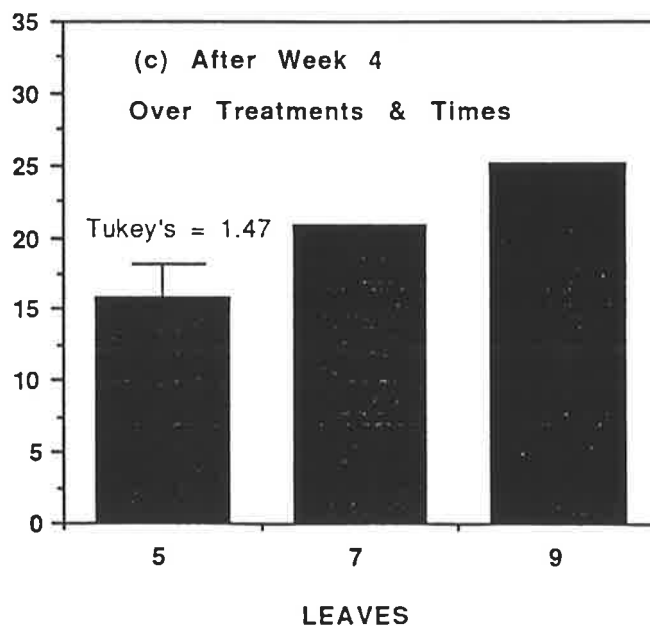
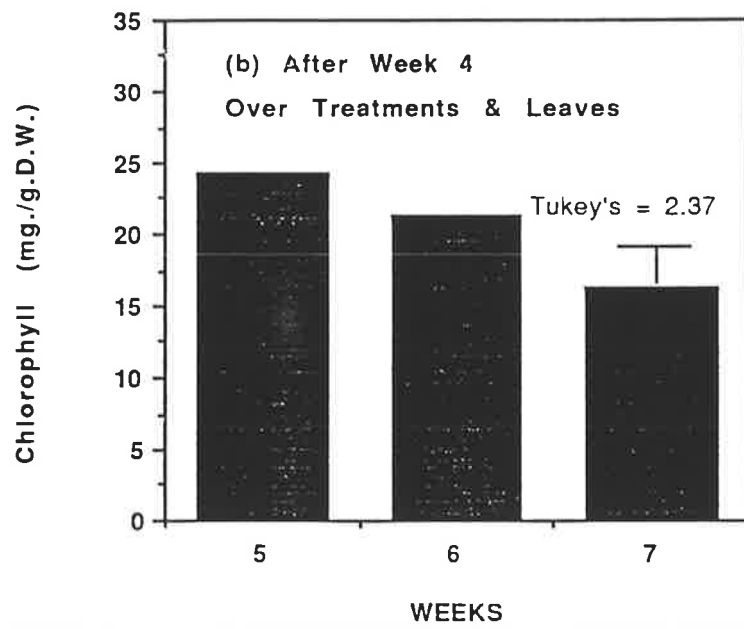
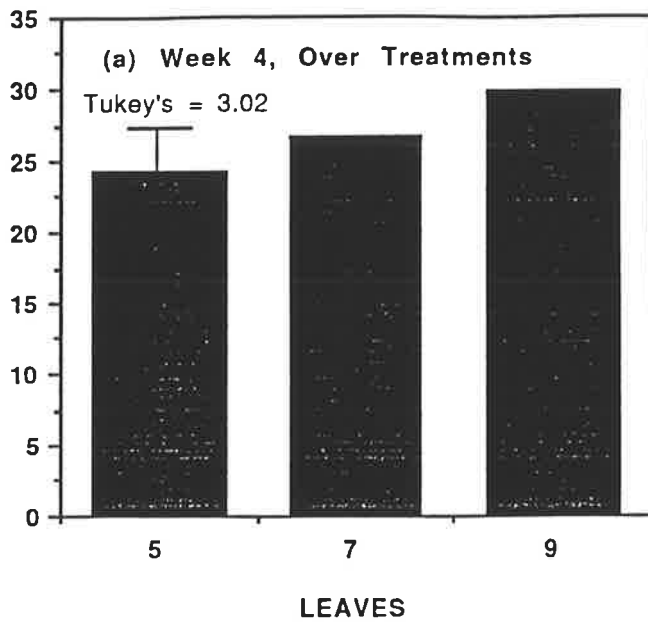
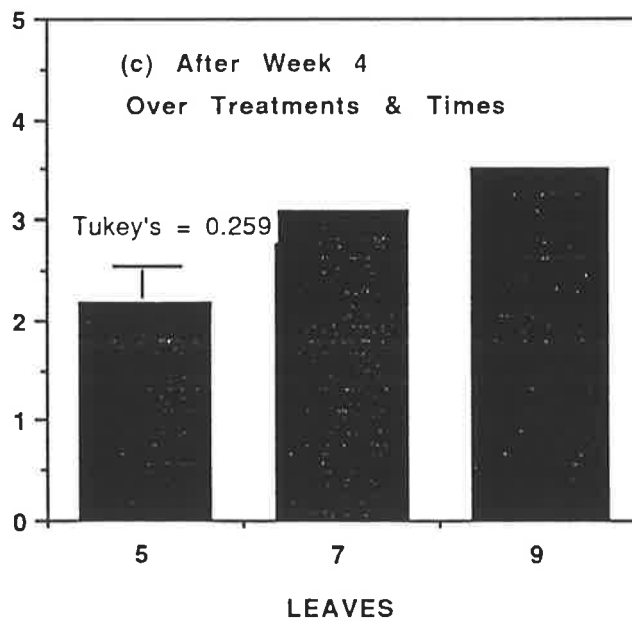
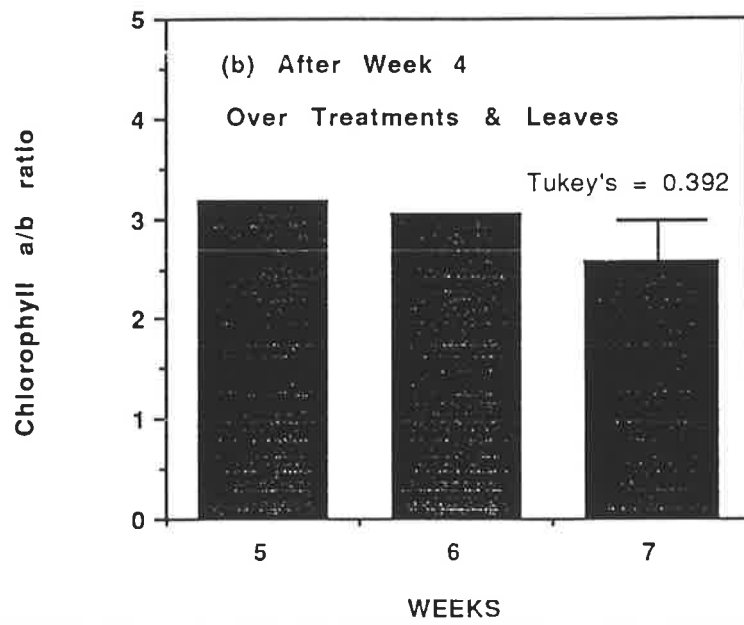
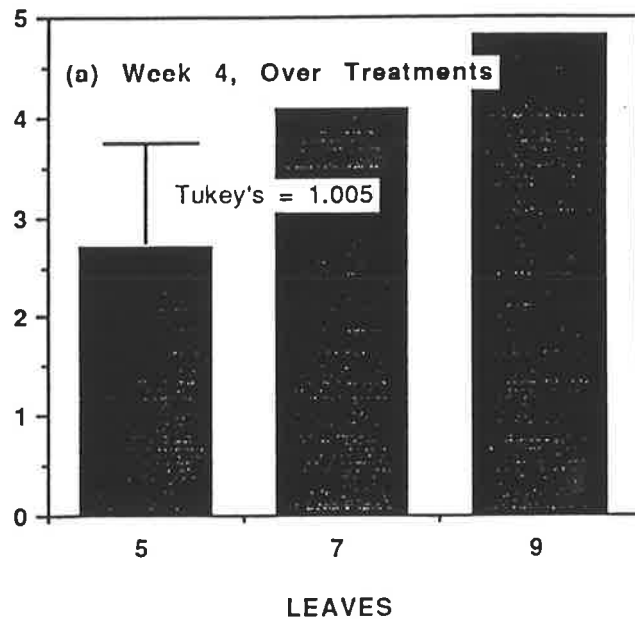


Figure 3.4 Changes in chlorophyll a/b ratio in leaves 5, 7 and 9 (from the base) at different ages of well-watered, partial soil-drying and partial root-excised sunflower plants growing with a divided root system. Both halves of the root system of the well-watered plants and one half of the root system of the soil-drying and the root-excised plants were watered to excess daily. The other half of the root system of these plants was not watered from week 0 onwards. In the root-excised plants, the half of the root system in drying-soil was excised on week 4. Data presented are means of 4 replications, which were calculated over (a) well-watered and soil-drying treatments, (b) 3 treatments and 3 leaf positions, or (c) 3 treatments and 3 time periods. Vertical bars represent Tukey's wholly significant difference between means at 5 % level.



3.4 Discussion

The results obtained from the week 4 analysis confirm the previous conclusion in demonstrating an effect of soil-drying on leaf senescence. This is evident by an acceleration in the rate of loss of leaf soluble protein in both experiments (Figures 2.3d and 3.2b), since protein loss is observed in all of the experiments it was here the most sensitive symptom of leaf senescence. A similar result was also achieved on the following weeks 5-7 analysis, indicating a continuation of the effect. But the critical feature of the present results is the reduction in the rate of protein loss after removal of the roots in dry soil (Figure 3.2e). This result strengthens the hypothesis of a non-hydraulic root source signal on the response by ruling out possible involvement of a reduction in nutrient supply or an undetected fall in leaf water status, as a result of the loss of half the nutrient or water gathering capacity in the soil-drying plants. Removal of the dried part of the root system would not restore the normal supply of nutrients or water from the roots to the shoot, but would be predicted to remove any supply of these nutrients from the dry soil.

The reduction in the rate of protein loss following root excision also provides evidence for a positive root signal which would be a senescence promoter produced in the dried roots and translocated to the shoot. Evidence that positive root signal control the shoot responses has also been reported from a similar experiment with maize (Saab and Sharp 1989) and apple (Gowing *et al.* 1990) plants, with respect to limitation of the leaf stomatal opening and growth rate, respectively. As discussed earlier, ABA is the most prominent candidate for this type of root signal. This may also be applied to the present experiment as ABA is known to promote leaf senescence.

Nevertheless, since the protein content of the root-excised plants did not recover completely to the control level, an additional negative root signal remains a possibility in the control of the total response. Alternatively, the incomplete recovery to the control

protein level may have been because root-excision changed the potential rate of protein turnover to the control rate, but synthesis of further proteins to compensate for previous losses was restricted by lack of precursors. This is in accord with the observation that there was no accumulated proline (presumed to be an important precursor of protein synthesis) in the soil-drying leaves. Hence, recovery to the control protein content would not be expected, since at root-excision the protein content in these plants was lower than that in control plants. This may be a difference from plants in leaf water stress conditions, in which a complete recovery in protein content to the control level was observed following a resumption of water supply (Figures 1.3a, 1.3b and 1.3c).

In the present experiment, the results for the chlorophyll a/b ratio showed no statistical response to soil-drying. This differs from the previous observation, where there was a slight decrease in the chlorophyll a/b ratio in response to a similar treatment (Figure 2.6f). This difference is most likely due to low sensitivity of chlorophyll a/b ratio to soil-drying, and it is possible that variation in the plant materials caused this difference in the statistical result. This possibility is supported by the previous conclusion that chlorophyll a/b ratio is less sensitive than loss of soluble protein content in both natural and water stress-induced senescence (section 1.4.2 of Chapter IV).

CHAPTER V
GENERAL DISCUSSION

The primary aim of the present thesis was to examine the possibility that a non-hydraulic root signal is involved in the control of water stress-induced leaf senescence. Such a signal has been demonstrated in the limitation of leaf stomatal opening and growth rate by water stress and proposed to involve translocation of ABA originating in the roots in drying-soil to the shoot (Davies and Zhang 1991). This suggests that a similar control of leaf senescence is possible, since ABA is known to promote senescence in many species (Thimann 1980; Nooden 1980).

However, the senescence syndrome is not an invariable sequence of events. Plants of different species exhibit different patterns of response to different degrees of the inductive effects (Mayoral *et al.* 1981). This is because senescence is subject to direct genetic control (Thomas and Stoddart 1980) and genetic variation exists for symptoms of senescence (Thomas and Smart 1993). Also, senescence response to external factors are not directly related to natural senescence (Smart 1994). As reviewed earlier, cDNAs isolated from naturally and drought-induced senescing leaves of barley are different (Becker and Apel 1993). The effect of ABA on leaf senescence is also genotype-dependent (Sloger and Cardwell 1970) and can vary depending on the physiological status of the leaves (Smith *et al.* 1968; De Leo and Sacher 1970). Therefore, characterisation of the sequence of events occurring during natural senescence and in response to leaf water stress seemed to be necessary as a guide for the senescence syndrome of sunflower plants.

In the natural senescence of leaves on well-watered plants in these experiments, loss of soluble protein content preceded the other changes recorded (sections 1 and 2 of Chapter IV). This observation agrees with that often found in the natural senescence of attached leaves (Camp *et al.* 1982; Peoples *et al.* 1983) and supports the idea that loss of protein content is the most basic of all senescence-related events (Nooden 1980). Whether the net loss of protein content resulted from a decline in protein synthesis or an increase in protein degradation or both responses is unknown.

Subsequent to the fall in soluble protein content, there was a gradual fall in the total amino acid content. No accumulation of liberated free amino acids, including proline (section 2 of Chapter IV), in the leaves as a consequence of the protein breakdown was observed. This suggests a rapid total translocation out of the leaves or *in situ* conversion of the resulting free amino acids to other products. The amides glutamine and asparagine are the major forms in which liberated amino acids are translocated from senescing leaves of cereals including wheat (Simpson and Dalling 1981; Kar and Feierabend 1984) and rice (Kamachi *et al.* 1991). Plant flowers and seeds may have been the major translocation sinks in the present studies, since the plants were in stages of flowering and seed development during the period of leaf senescence (Cockshull and Hughes 1967; Dalling *et al.* 1976; Peoples *et al.* 1983).

The loss of chlorophyll content during natural senescence appeared to begin later than the fall in soluble protein content. This may be because chlorophyll molecules are temporarily protected by thylakoid membranes, to which they are bound. Simultaneous with the fall in total chlorophyll content, there was a fall in the chlorophyll a/b ratio. A fall in chlorophyll a/b ratio is normally observed during natural senescence (Sestak 1977) due to a more rapid loss of CP I (which contains only chlorophyll a molecules) than LHCP (which contains both chlorophyll a and b molecules) (Bricker and Newman 1980; Jenkins *et al.* 1981).

In the water stressed plants (section 1 of Chapter IV), the rate of fall in soluble protein content, total chlorophyll content and chlorophyll a/b ratio increased during the severe fall in leaf water potential experienced by the plants. Again, the effect of water stress was more pronounced in accelerating the rate of loss in soluble protein content, suggesting that protein loss is also the most sensitive symptom of senescence in response to water stress. Water stress-induced senescence of attached leaves of cotton plants also exhibits an earlier decline in the protein content than the fall in chlorophyll content

(Radin 1981). In wheat plants, however, both symptoms occurs simultaneously in response to water stress (Mayoral *et al.* 1981).

In these stressed leaves, however, total α -amino acid content increased together with an assumed proline accumulation (Wample and Bewley 1975). These are the major observed metabolic differences of water stress-induced senescence from those occurring in natural senescing leaves.

Upon recovery in the leaf water potential, the accumulated total α -amino acid, including proline, disappeared rapidly, and coincidently with an increase in the soluble protein content to the non-stressed level. It is possible that these amino acids, including proline, were re-synthesised to proteins. This attribution is in accord with a putative role of proline as a conserved nitrogen source in a readily available form for protein synthesis (Aspinall and Paleg 1981; Zhang *et al.* 1982). The increase in protein content of the previously stressed leaves also indicates recovery of protein synthesis, suggesting that a decrease in protein synthesis was at least partly responsible for the fall in protein content during water stress.

Total chlorophyll content and chlorophyll a/b ratio, however, did not recover to the non-stressed levels when the stress was eliminated, but their rates of decline returned to a comparable rate to those in the non-stressed leaves. These phenomena imply that the effect of water stress on the loss of chlorophyll content was mainly if not totally due to an enhancement of the rate of chlorophyll degradation.

In conclusion, it seems that loss of protein content is the most sensitive symptom of the senescence syndrome in sunflower leaves both when induced by ageing and by water stress. Hence, this symptom was used as the first criterion of senescence in sunflowers leaves rather than the loss in total chlorophyll content or change in chlorophyll a/b ratio.

A direct effect of soil-drying on leaf senescence was assessed by exposing one half of the plant root system to drying soil, while the other half was well-watered to maintain the leaf water potential. In these plants, the rate of loss of soluble protein content was eventually accelerated (Figure 2.3d), but there were no significant responses in the total amino acid, proline and total chlorophyll contents. The chlorophyll a/b ratio, however, showed a slight statistically significant reduction in the summed value across leaf position and time in response to root drying (Figure 2.6f).

The results obtained from these split-root drying plants indicate a direct effect of soil-drying on leaf senescence independent of the leaf water status. Similar to the response in the natural or water stress-induced senescence, loss of protein content is the most sensitive symptom in the response. In this treatment, however, there was no accumulation of total amino acid or proline content corresponding to the increased loss of protein (section 2.3.4 of Chapter IV), which was found in water stressed leaves. It seems that the liberated amino acids were eventually de-aminated or translocated from the leaves as occurred in naturally senescing leaves.

Excision of the part of the root system in drying soil resulted in a higher leaf soluble protein content compared to that in plants where half of the root system was maintained in dry soil (figure 3.2e). The result indicates that the effect of soil-drying on the loss of protein content can be eliminated when those roots were removed, suggesting that the effect was not involved with any reduction in nutrient or water supply. This confirms the effect of soil-drying on leaf senescence, and provides evidence for the nature of a non-hydraulic signal as a positive root-sourced signal. However, the exact signal involved is not known, although ABA is the most likely (Davies and Zhang 1991).

The critical feature of the present studies is focused on the apparent effect of a positive root signal on leaf senescence. In field conditions, this would occur before a substantial effect of water stress. However, it seems that the influence of root signal

requires a comparatively long time lag before there is a measurable response. Thus, in the case where shoot water stress eventually developed, this effect of a root signal would be masked. But in the case where the soil surface is dried but the shoot is supplied with adequate water from deeper roots, this effect may be more obvious. Indeed, it is possible that the root signal can also affect chlorophyll content or chlorophyll a/b ratio if the exposure period is prolonged. This may be investigated by using leaf materials which normally have a long life-span on the plant such as perennial trees.

The effect of positive root signal on leaf senescence may be a general phenomenon, however, examination of other species is needed. If it is found to be a common phenomenon, then applied cultural practices such as mulching or top soil root pruning to eliminate the signal may be required to give a higher level of leaf protein content and a longer life-span of the leaves. This is particularly important in crops where leaf material is the economic product (e.g.. mulberry cultivars for silk-worm rearing).

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