Physiological and biochemical aspects of water deficits ~~tolerance in~~ ON Geum ~~urbanum~~ RIVALE L. and Geum ~~rivale~~ URBANUM L. ~~under laboratory and semi field conditions.~~

A thesis submitted by

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I would like to thank Dr. K. Taylor for his help during this study, Dr. J. Graves for many useful discussions and my wife Catherine for her help with the preparation of this thesis, her perseverance and frequent cups of coffee during the composition of this manuscript.

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ABSTRACT.

This study has shown that Geum urbanum can outsurvive Geum rivale during periods of water deficits imposed in the laboratory and in a semi-field situation. Laboratory work showed that there was no inter-population differences in tolerance to water deficits in Geum urbanum and Geum rivale. It was considered that the difference in drought tolerance between the two Geum species was large enough to influence their ecological distributions.

It was found that the major difference contributing to the greater drought tolerance of Geum urbanum over Geum rivale was the formers ability to osmoregulate by accumulating solutes to higher levels than Geum rivale. The osmoregulatory process was then shown to be a three stage process in both species throughout PEG induced water stress.

Of the solutes shown to increase in other species during water deficits the two Geum species only accumulated hexose and diose sugars and a variety of amino acids. However various phenolic compounds were also shown to accumulate during water deficits.

This study also showed that both species could mobilise stored carbohydrate from mature leaves and increase storage of carbohydrate in young leaves, a previously unreported phenomenon.

The primary production of amino acids was shown to be maintained in roots and leaves of both species during water stress. However it was considered that a different mechanism of nitrogen assimilation occurred during water stress in roots as opposed to leaves when compared to unstressed plants.

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INTRODUCTION

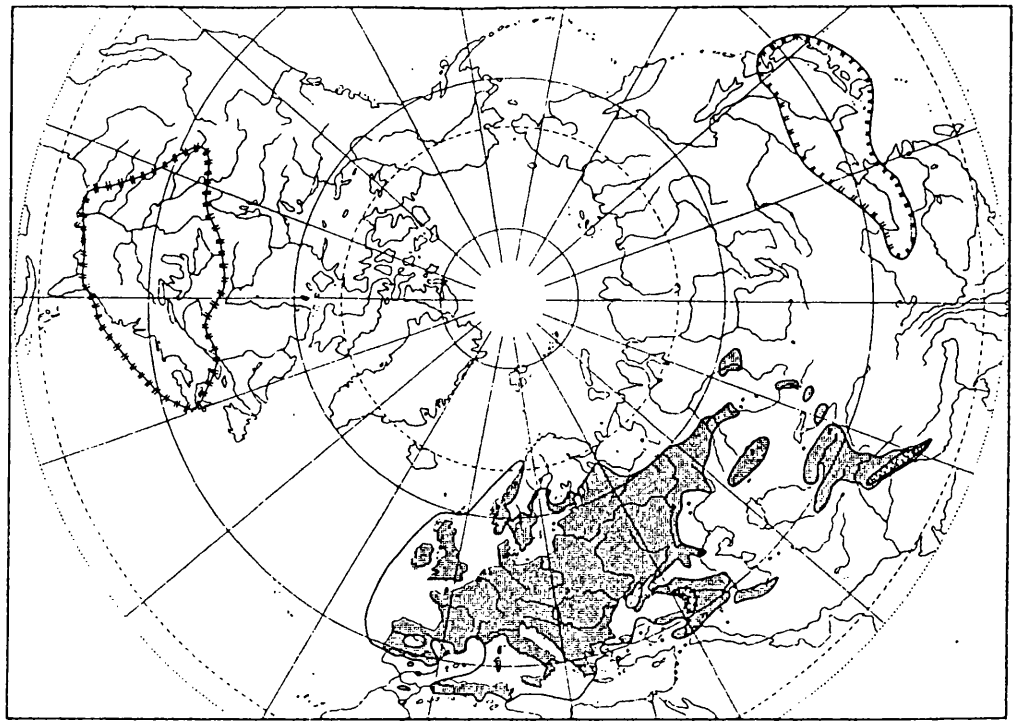
Two closely related, morphologically similar species, Geum urbanum and Geum rivale occur on neutral to basic soils in many parts of Britain and Europe (Graves 1984). They are perennials which reproduce sexually and vegQtatively by means of rhizomes. Where they occur in close proximity the fertile hybrid Geum intermedium may be found with many associated back crosses. Though the two species appear to be very similar morphologically, and their geographical distributions overlap considerably (see Maps 1.1, 1.2 and 1.3), they differ markedly in their ability to exploit different habitats.

Geum rivale is mainly found on wet and even waterlogged ground in deciduous woodland, grasslands, marshes and streamsides. In woodland it is frequently associated with species such as Deschampsia caespitosa, Filipendula ulmaria and locally Primula elatior. On the other hand Geum urbanum prefers somewhat drier sites in deciduous woodland, where it is commonly associated with species such as Mercurialis perennis, Glechoma hederacea and Primula vulgaris. It also occurs on disturbed ground.

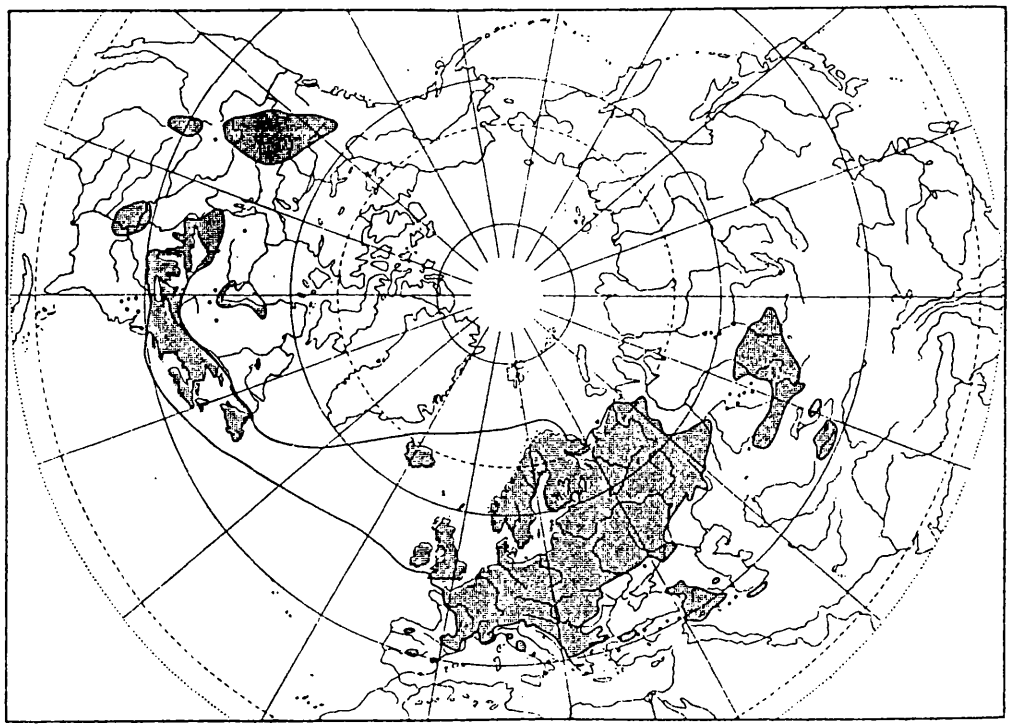
In the British isles Geum rivale occurs at high altitudes (up to 960 metres), while Geum urbanum is rarely found over 504 metres. Though both species are found in the open and in deciduous woodland sites Geum urbanum prefers shaded areas, and Geum rivale is generally only found in woodland where trees are well spaced or in rides.

The southerly and northerly limits of each species distribution also provides some evidence of their differing ecological amplitudes. Geum urbanum is able to extend it's range to the warmer, drier, southern areas of Britain and Europe, while Geum rivale is more prolific in the cooler, wetter, north and can extend its

Map 1.1.0 To show the worldwide distributions of Geum urbanum and Geum rivale. (Ref. Meusel, Jager and Weinert 1958).

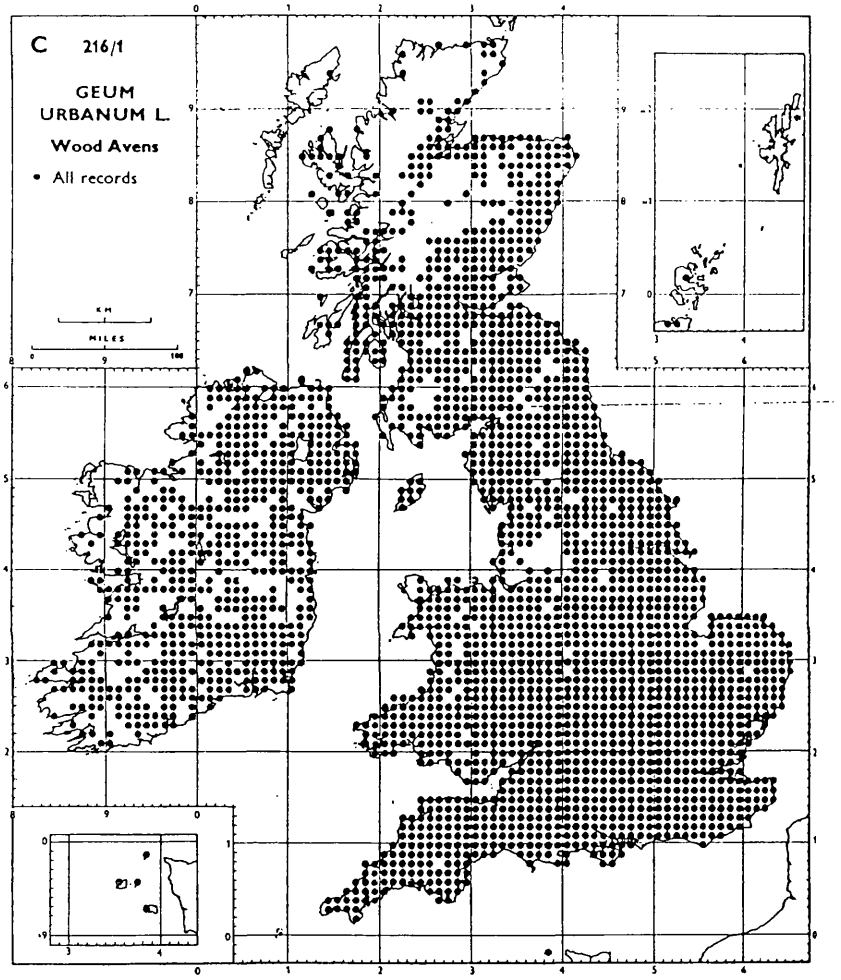


Geum urbanum L.
G. latilobum SOMM. et LEV.
G. roylei WALLICH
G. japonicum THUNB.
G. canadense JACQ.

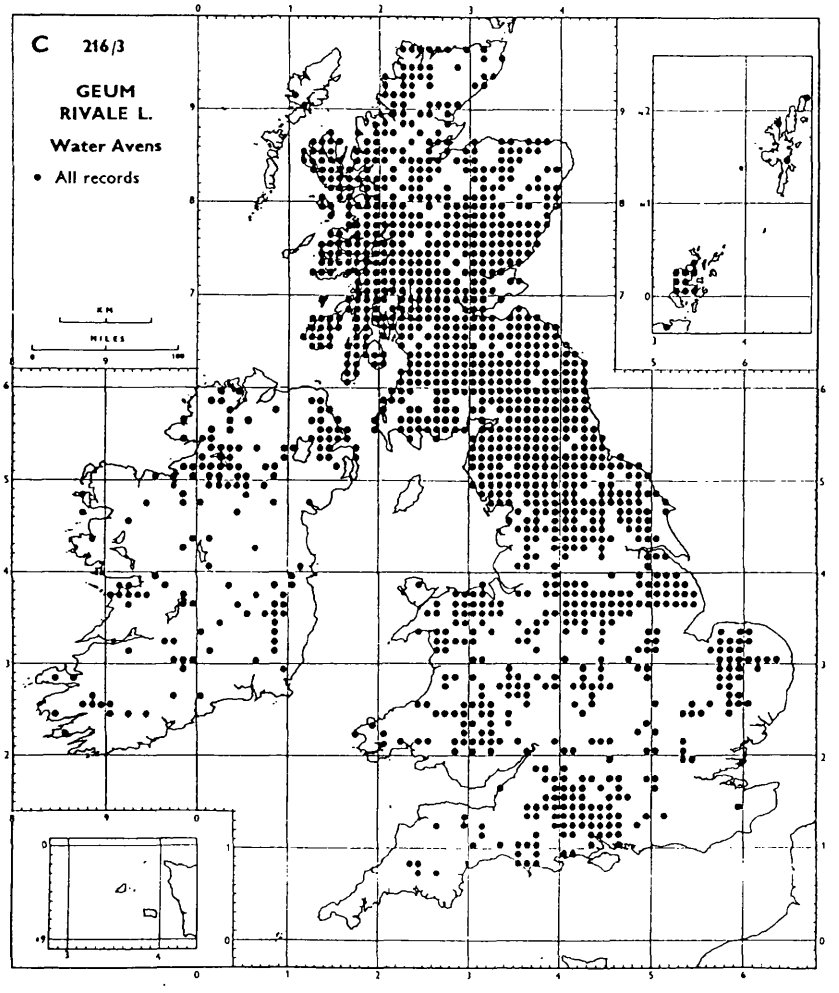


Geum rivale L.

Map 1.2.0 To show the distribution of Geum urbanum in the British Isles. (Ref. Perring and Walters 1962).



Map 1.3.0 To show the distribution of Geum rivale in the British Isles. (Ref. Perring and Walters 1962).



range beyond that of Geum urbanum in northerly latitudes (See maps 1.1 and 1.2).

From the description above it appears that Geum urbanum and Geum rivale may be significantly different physiologically and / or biochemically enabling them to exploit these different habitats. Alternatively, competitive pressures, disease and grazing preferences account for these observed ecological differences. From the available literature there is little evidence to suggest that the biotic pressures could account for such a marked difference in the two Geum species ability to exploit such widely varying habitats (Graves 1984; Brotherton 1974). However, some physiological and biochemical differences between Geum urbanum and Geum rivale have been discovered by various workers (most of which were tested in the field) which have gone some way to explaining some of the habitat preferences of the two species.

Waldren et al (1987 a,b, 1988) found that Geum rivale was able to withstand periods of waterlogging far better than Geum urbanum and explained this in terms of metal tolerance, rooting characteristics and root respiration. Thus providing physiological evidence which may explain why Geum rivale is able to exploit wetland environments in which Geum urbanum does not proliferate.

Graves and Taylor (1986; 1988 a,b) suggested that the germination responses of Geum urbanum and Geum rivale could provide some explanation as to why Geum rivale is able to exploit higher altitude habitats than Geum urbanum. They showed that Geum rivale seeds germinated at higher temperatures than Geum urbanum. It was then argued that Geum rivale seedlings would germinate later in the year at high altitude and avoid spring frosts which may kill early germinating seedlings such as those of Geum urbanum. Differences in growth and photosynthetic rates at different temperatures proved

minimal and did not provide an explanation of their different upper altitudinal range. The work of Waldren may also be of value in explaining altitudinal differences as higher areas tend to have a greater precipitation often producing wet and waterlogged ground. These data may provide an explanation as to why Geum rivale is able to exploit the colder, wetter northerly latitudes which Geum urbanum appears incapable of colonising.

Smith (1975) showed that the phytochrome response of Geum urbanum was consistent with that of a shade species, and Graves (1990) in an eco-physiological study provided further evidence to explain the shade preference of Geum urbanum by virtue of it's photosynthetic response throughout the year. However, there are a lack of data concerning the response of Geum rivale to shade environments and so comparisons cannot be made in this area.

From the above it is apparent that some progress has been made towards determining the reasons why one Geum species can survive and exploit one particular habitat in which the other performs poorly or from which it is absent altogether. In particular, the absence of Geum urbanum from wetland areas and the more northerly distribution of Geum rivale. However, the questions remain as to why Geum rivale appears to be intolerant of shade, why it does not exploit the drier habitats available to Geum urbanum and why Geum urbanum flourishes in more southerly latitudes than Geum rivale.

In this study an attempt has been made to clarify the latter two problems. It appeared that the problems were synonymous, in that the ability to cope with some form of water deficit was the central issue. It was hypothesised that G. rivale could not flourish and survive as a population out of a wetland situation where water supply was periodically limited and G. urbanum

was capable of surviving a period of water stress in order to extend it's southerly range beyond that of Geum rivale.

Plant water deficits in the field can be caused by many factors. Excessive wind speeds can cause water stress in plants, as can extremes of temperature. Plant crowding can also cause water deficits by increasing competition for available water. Of all factors, lack of rainfall causing soil to dry and / or lack of ground water are probably the most important and most frequent cause of drought, especially in the areas of Europe where Geum urbanum and Geum rivale are found. Successful attempts have been made to correlate variations in water availability to plant distributions (Woodell et al. 1969). Jarvis (1961) backed up observed distributions of tree species growing in areas with different water availability in Sheffield with physiological data; as more recently did Whale (1983) with Primula species. It therefore became apparent that studying plant water stress caused by limited soil water may be a profitable line to pursue. Though a large ammount of data concerning ecology and water deficits in native species exists prior to the mid seventies, more recent studies have mainly concentrated on crop species. The present study may give a greater insight as to why water defecits play such a large role in determining a plants ecological distribution by applying discoveries made since the mid 1970's with crop plants.

The strategies which plants utilise to survive periods of drought can broadly be put into three categories:

1. Stress escapers.
2. Stress avoiders.
3. Stress tolerators.

Stress escapers are typified by desert annual species. These plants avoid desiccation by only

germinating after thunder storms when the soil solution becomes dilute and then complete their life cycle in a very short time before drought ensues (Went 1979). In temperate Europe winter annuals which germinate in autumn and set seed before the summer months such as Stellaria media and Phacelia purshii are other examples. Geum urbanum and Geum rivale having a perennial habit and a seed germination character which does not require any after ripening (Graves and Taylor 1988b) do not therefore avoid drought by this strategy.

Stress avoiders fall into two main categories: those which store water such as succulents (typified by cacti) and to a lesser extent sclerophylls (eg. evergreens); and those plants which have extensive or deep root systems in order to reach water supplies deep underground. Examples of such plants are tamarisk which has roots which extend up to 30 meters away from the plant body and mesquite which has a root system which has been found at a depth of 53 meters (Drew 1979), also lilac is a prime example in the British Isles. The two species studied here do not fit into this category either because they are not succulent, or do not have a sclerophyllous leaf structure and do not posses such extensive root systems (Waldren et al. 1987a; Graves and Taylor 1988b).

Stress tolerance is a very wide category which includes the so called resurrection plants such as Ceterach officinarum (Rouschal 1937, 1938), mesophytic plants which have limited tolerance to transient water deficits eg. barley and at the other extreme various Primula species. Geum urbanum and Geum rivale from their ecological distributions appear to fit into the lower end of this category along with the Primula species with which they are ecologically associated. The stress tolerance of plants varies with plant age and growth stage (Morgan 1984), with young plants being less

stress tolerant than older plants. Thus, the early growth stages of stress tolerent plants, normally during establishment proves to be their most vunerable period.

I therefore decided to test my hypothesis making the assumption that Geum urbanum and Geum rivale would be marginally drought tolerant and to concentrate on the early part of the plants life cycle. The research was divided into two parts. The first involved a comprehensive survey in the laboratory assessing the two species ability to resist periods of water deficit. If differences were apparent these were to be identified, and their contributions assessed (see Chapters 1 to 6). The second part of the study was to determine whether any difference in the species ability to resist water deficits in the laboratory was expressed in a field situation. If a difference were to be found in the field a prediction was then to be made as to how this may affect the distribution of the two species.

Water deficits develop slowly in the field, generally over weeks and months. The reduced water supply inhibits mineralisation rates which in turn reduces mineral availability to the plant and drying soils also invoke physical stresses on plants at root level. Any experimental system in the laboratory can only approximate to these effects and is generally a compromise between reproducing such effects and ease of use for further experimentation. Because of these compromises the method and rate at which plants are water stressed in the laboratory has received much criticism in recent years. Research has shown that the rate at which a plant is stressed is perhaps the most important criteria to consider when designing an experiment. For example Jones and Rawson (1979), and Morgan (1980) demonstrated that plants undergoing a rapid water deficit exhibited an altered stress tolerance when compared to a plant which is gradually

stressed.

There were two methods of stressing plants in general use available to me and both had some drawbacks. The plants could either be stressed in pots containing compost by witholding water, or they could be stressed osmotically by increasing the strength of an osmoticum when grown in water culture. An increased stress is produced by gradually increasing the concentration of an osmoticum in the growth media which progressively reduces the water potential of the growth media. As the osmotic potential of the solution becomes lower the osmotic gradient for water flow into the plant increases and net water uptake will be reduced. Further increases in osmoticum concentrations will cause this gradient to become steeper and net water uptake is further reduced and thus the plant is gradually deprived of water.

Plants stressed in a soil-based system have the advantages of being grown in their natural growth medium which may reproduce some mechanical forces the plant experiences in drying soil and also produces a reduced supply of minerals during stress. However, soil drying rates in the laboratory are erratic, often rapid and almost impossible to monitor accurately. An exact level of stress can not be attained, maintained or repeated with ease confidence or reliability. Root measurements are also awkward to make and full root recovery is difficult to achieve without soil contamination.

The use of an osmoticum in water culture solves some of the above difficulties. This system has the advantages of being easily manipulated,any degree of stress can be achieved, monitored and repeated and any required rate of stress can be produced. Root measurements are easy to make at any stage during an experiment and complete uncontaminated root recovery is possible. Osmotic methods do have disadvantages in that the plants are not grown in their natural media and they

can not reproduce any mechanical stresses produced in drying soil. On reflection it was decided that the control over the experimental system the osmotic method gave (especially that over the rate of stress imposition) outweighed the advantages provided by a soil based system. Finally the implications of the field and laboratory results are discussed.

CHAPTER 1

THE GROWTH OF GEUM RIVALE AND GEUM URBANUM EXPOSED TO WATER DEFICITS IMPOSED BY POLYETHYLENE GLYCOL UNDER CONTROLLED CONDITIONS.

INTRODUCTION

The growth of plants is dependent on several factors. They require inputs of carbon, nitrogen and other mineral salts to increase biomass and maintain metabolism, but perhaps most important of all (as with all living things) is a requirement for water. Water is essential to the plant to facilitate all metabolic processes by either direct involvement, providing an environment for the process, or by hydrating macro-molecules required in metabolism. Water is also needed to drive expansive growth, maintain turgor and in many cases provides structural support for the plant. Thus when a plant undergoes a period of water deficit all aspects of growth and metabolism may be affected.

A plant undergoing a period of water deficit may loose water to its environment at a faster rate than water can be collected, this will reduce the water content of the plant. If the loss of plant water is large enough a reduction in biomass will occur (Stanhill 1957, Slatyer 1961) due to disruption of metabolism. A reduced water uptake will also limit the water available to maintain turgor and hence expansive growth will be reduced (Boyer 1968). This will reduce leaf expansion (Boyer 1968, Ashenden 1978), and the plant may become more flaccid and eventually wilt. Thus, in order to asses the ability of Geum urbanum and Geum rivale to withstand periods of water deficit, biomass increase, expansive growth and wilting point would give some indication as to their tolerance of water deficits. In addition, and as a final indicator of the two species ability to withstand water stress the plants would be

stressed to their fullest extent to determine their death points. Similar growth parameters have been used to determine a plants ability to resist drought (Richards and Thurling 1978, Ashenden et al. 1975). Other indices of drought tolerance have been proposed which include leaf rolling, internal water relations, stomatal closure, water use efficiency and many others. However, in an ecological situation a plants distribution is determined by the ability of a plant species to flourish and survive as a population in a particular environment. Thus, the growth of the two Geum species in response to various water deficits and differences in their ultimate ability to survive water deficits, is of greater significance than the previously determined drought tolerance index mentioned above.

Though species may differ in their ability to resist drought, ecotypic differences may also occur (Young 1967, Ashenden et al. 1975). Thus populations of both species from different sites with differing water availabilities were also investigated.

I explained in the introduction that an osmotic stress was to be used in the laboratory to determine the plants' abilities to resist periods of water deficit. Various osmoticums have been used by previous workers such as mannitol (Slatyer 1961),polyethylene glycol (PEG) 4000 (Taylor et al. 1982) and PEG 6000 (Pearson and Stewart 1987). In this study PEG 6000 was chosen as it is not metabolised by the plant like mannitol is and it is less osmotically active than PEG 4000, which makes gradual stresses much easier to achieve. PEG does however need to be decontaminated from aluminium and phosphate prior to use and the culture solution has to be aerated as PEG has a low affinity for oxygen.

In these experiments the plants were stressed in a gradual manner in 5% PEG increments. The plants were also grown in a 20% PEG solution without undergoing any

stress acclimation to determine whether this had any affect on the performance of Geum urbanum and Geum rivale when subject to a water deficit, as had occurred with other plants subjected to rapid water deficits (Pearson and Stewart 1987).

MATERIALS AND METHODS

A: Seed Population Collection Sites

Seeds had previously been collected by my supervisor Dr. Ken Taylor from many sites in Britain and Europe from discrete populations of Geum urbanum and Geum rivale. The form of collection had insured that the seed was free from hybrid contamination. Geum urbanum seeds were available from several populations in England at Helbeck Hall in the northern Pennineswhich is a moist site; Spain at Sierra da Gudar, a dry site; Finland near Turku, a wet northern site; and from northern Iran at Galand a-rud which is probably the driest site exploited by Geum urbanum. Seeds of Geum rivale were collected in England at Moor House and Leadgate which are wet sites in the northern Pennines; from a moist site on Rhum in Scotland; and from Tromso in arctic Norway a moist northern site.

B: Plant Culture

Seeds from the above populations were germinated on damp filter paper at room temperature. When the first true leaves had emerged the seedlings were planted in washed sharp sand, fed with half strength Long Ashton Solution (LAS) pH 7.0 and grown in a greenhouse at 20 ^{o}C /15^{o}C day /night temperatures for 16hr days. Lighting was supplied by a bank of fluorescent tubes, irradiance was maintained at 120 Wm-2 and humidity at 80%. At the 9th leaf stage the plants were transferred into blackened 600 ml plastic pots containing LAS pH 7.0. Three plants were grown in each pot and held in place by black plastic discs into which six holes had been drilled. Plants were placed in alternate holes and secured with foamed rubber. Each pot was aerated and the plants were left to equilibrate for one week (the LAS was changed after three days), growth conditions were maintained as above. After this period a gradual or rapid water deficit was imposed with polyethylene glycol

(PEG).

C: Polyethylene glycol 6000 (PEG) Culture

PEG purchased from BDH was made up as 50% solution in distilled water and passed through a Duolite MB 50 indicator mixed bed resin column to remove aluminium and phosphorus contaminants. PEG solutions were made from this clean PEG with the final working solutions containing LAS and the desired PEG concentration, pH was then adjusted to 7.0 with KOH. The process of gradually stressing the plants proceeded according to the following regime.

After the period of equilibration all solutions were changed, two pots were replenished with LAS and the rest transferred to 5% PEG solutions. After a further three days all the solutions were changed, the pots originally containing LAS were replenished with LAS, two pots were replenished with 5% PEG and the rest filled with 10% PEG. This process continued in 5% PEG increments until the desired stress level had been achieved. Rapidly stressed plants were not acclimated in 5% increments but were grown as control plants until day 15 when they were transferred to 25% PEG culture solutions and treated as such thereafter. See Table 1.1.0 on the following page for clarification of the imposition of gradual stress.

D: Plant Growth Analysis

When the desired final stress level had been achieved all the plants were harvested in order to determine the growth of the plants at each stress level for the duration of the experiment. After fresh weights leaf area and root lengths were measured the plant material was dried in an oven at 80 °C for 1hr and at 60 °C after this for 48 hours. After drying, leaf and root dry weights were determined. These measurements enabled the calculation of the following: total fresh and dry matter accumulation; root fresh and dry matter

Table 1.1.0 to show the method used to create a slow rate of water deficit imposition on plants of Geum urbanum and Geum rivale.

DAY	POT NUMBER AND PERCENTAGE PEG 6000									
	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
3	0	5	5	5	5	5	5	5	5	5
6	0	5	10	10	10	10	10	10	10	10
9	0	5	10	15	15	15	15	15	15	15
12	0	5	10	15	20	20	20	20	20	20
15	0	5	10	15	20	25	25	25	25	25
18	0	5	10	15	20	25	30	30	30	30
21	0	5	10	15	20	25	30	35	35	35
24	0	5	10	15	20	25	30	35	40	40
27	0	5	10	15	20	25	30	35	40	45
30	END OF EXPERIMENT AND PLANT HARVEST									

accumulation; leaf fresh and dry matter accumulation; whole plant, leaf and root fresh to dry weight ratios and leaf area ratio all expressed as a percentage of control plants. The root to shoot ratio was also calculated using these data.

Specific leaf expansion was also determined this involved measuring the leaf area of expanding leaves at the start of the experiment, marking them with plastic bag ties and measuring them again at the end of the experiment. All leaf area measurements were done using a Li- Cor leaf area meter.

E: Wilting And Death Point Determination

Wilting point was considered to have occurred when the newest expanded leaf had become flaccid and non-erect. Death point was deemed to be the time at which all leaves and buds had wilted beyond the point of recovery.

F: Measurement of Relative Water Content (RWC)

At the end of the experiments during the harvests small amounts of root and leaf were excised, weighed (approximately 200mg), placed in a petri dish of water for 24hrs, removed, blotted dry and re-weighed. Finally they were dried in an oven at 80^{o}C and the dry weight determined. The RWC was calculated by the following equation:

$$RWC = \frac{FW1 - DW}{FW2 - DW} \cdot 100$$

Where : FW1 = original fresh weight.
FW2 = turgid fresh weight.
DW = dry weight.

RESULTS

Figs. 1.1.0 to 1.1.3 show the total dry weights of the various plant populations expressed as a percentage of unstressed control plants. These figures show that relative total biomass in all populations of Geum urbanum and Geum rivale fell in response to water deficits imposed by polyethylene glycol (PEG). This decrease was greater in all populations of Geum rivale when compared to Geum urbanum at most PEG concentrations. It is also apparent that there is little difference in biomass reduction between populations of either species when expressed as a percentage. These results indicated that all populations of Geum urbanum were able to maintain their growth throughout a period of water deficit closer to their maximum rates than any population of Geum rivale. It must be stated however, that the biomass did vary between populations and species (Table 1.2.0). Geum rivale populations did have a higher dry weight than Geum urbanum on the whole, but the Spanish population of Geum urbanum had the highest biomass of all.

The dry matter allocated to leaf (Figs. 1.2.0 to 1.2.3) and root (Figs. 1.3.0 to 1.3.3) expressed as a percentage of control dry weights also declined as water deficits increased. However, root dry matter was maintained in preference to leaf in both species. Again populations of Geum urbanum were able to maintain their dry matter closer to its maximum in both leaves and roots than those of Geum rivale with no inter population differences within the species.

The total fresh weights of whole plants (Figs. 1.4.0 to 1.4.3), leaves (Figs. 1.5.0 to 1.5.3) and roots (Figs. 1.6.0 to 1.6.3) again expressed as a percentage of non-stressed plants all declined as water stress developed. Populations of Geum urbanum were able to maintain their total, leaf and root fresh weights closer

to control values than Geum rivale, inter population differences did not occur. The fresh weight to dry weight ratio of leaves (FW:DW) (Figs. 1.7.0 to 1.7.3) and root (Figs. 1.8.0 to 1.8.3) also declined in response to water stress when compared to unstressed plants. Again all populations of Geum urbanum could maintain this ratio closer to maximum values than could Geum rivale with no inter population differences.

Figs. 1.9.0 to 1.10.3 show the decline in relative water content (RWC) in leaf and root in response to water deficits of both species. All populations of Geum urbanum could maintain their RWC to higher levels throughout stress than those of Geum rivale. As reported previously no inter population differences occurred within either species in either roots or leaves.

The leaf area ratio (LAR) cm^2 leaf/g dry weight (Figs. 1.11.0 to 1.11.3) also decreased in both species as water deficits increased and again the reductions were greater in populations of Geum rivale. This indicates that Geum urbanum could maintain a higher leaf area per mg plant dry weight as stress developed than Geum rivale.

The decrease in specific leaf expansion (Figs. 1.12.0 to 1.12.3) shows that the reduction in leaf expansion was largest in populations of Geum rivale, highlighting the ability of Geum urbanum to sustain leaf expansive growth at higher levels than Geum rivale during water deficits.

Figs. 1.13.0 to 1.13.3 show the increase in root to shoot ratio (R:S) in response to stress of the two species. Populations of Geum urbanum were able to increase their R:S and root length (Figs. 1.14.0 to 1.14.3) to a higher levels than Geum rivale, no significant inter population differences were recorded.

Table 1.3.0 shows that the wilting point and death points of Geum urbanum populations occurred at higher

PEG concentrations than those of Geum rivale. These data show unequivocally that Geum urbanum can ultimately survive a higher water deficit than Geum rivale.

The final table (1.4.0) compares the growth of the various populations when stressed gradually and rapidly to 20% PEG when grown over the same time scale. This indicates that all populations of both species had a higher relative biomass production, greater expansive growth and a larger water content when gradually stressed than when rapidly stressed.

Figure 1.1.0

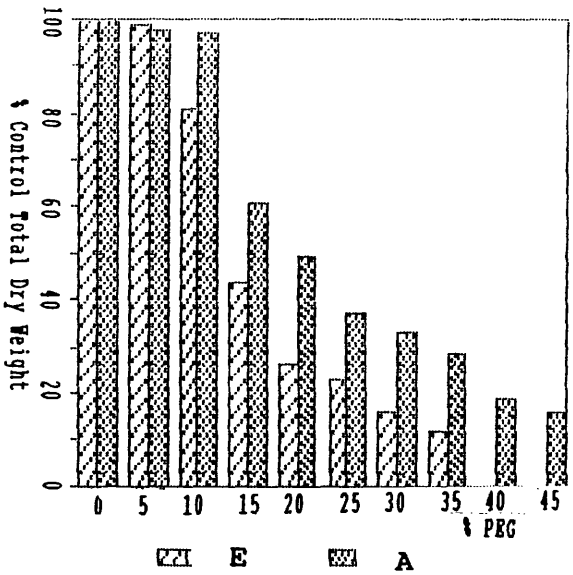


Figure 1.1.1

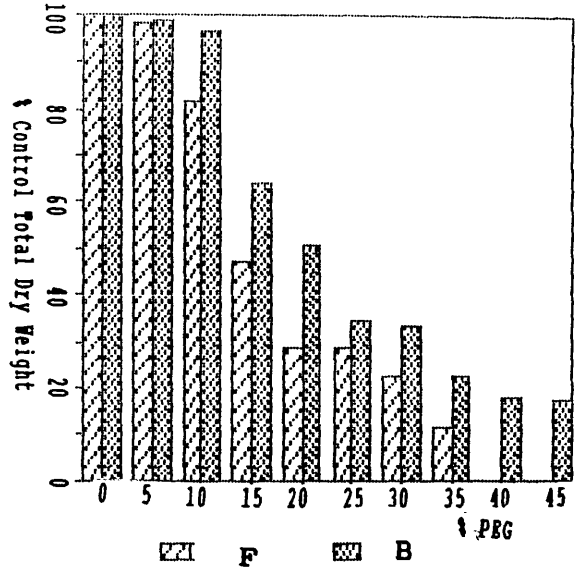
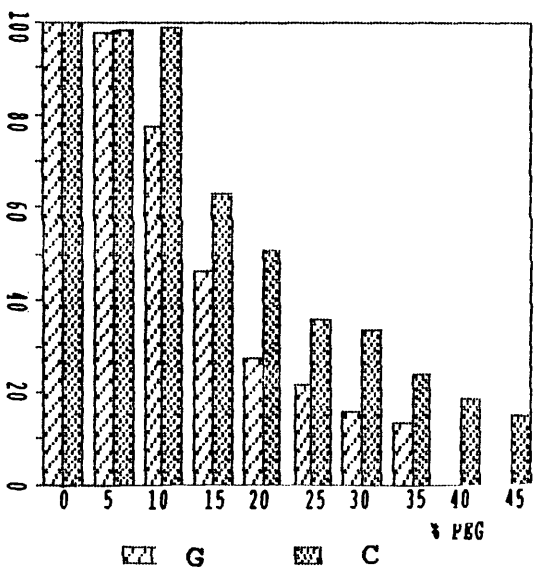


Figure 1.1.2



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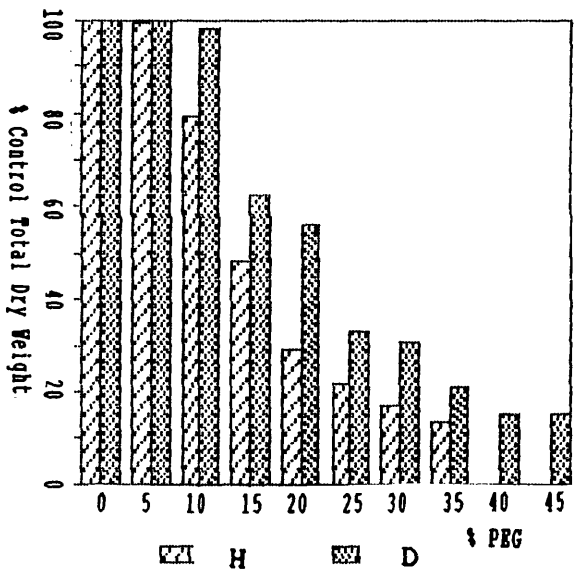


Figures 1.1.0 to 1.1.3 show the decline in total plant dry matter accumulation during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Total dry weight 100% values for figs. 1.1.0 to 1.1.3

G. urbanum populations	G. rivale populations
A 2.402 g	E 2.584 g
B 2.455 g	F 2.637 g
C 2.800 g	G 2.691 g
D 2.275 g	H 2.671 g

Figure 1.2.0

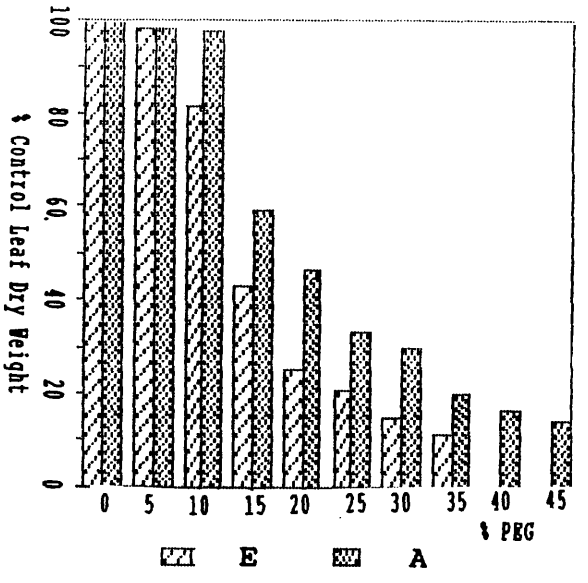


Figure 1.2.1

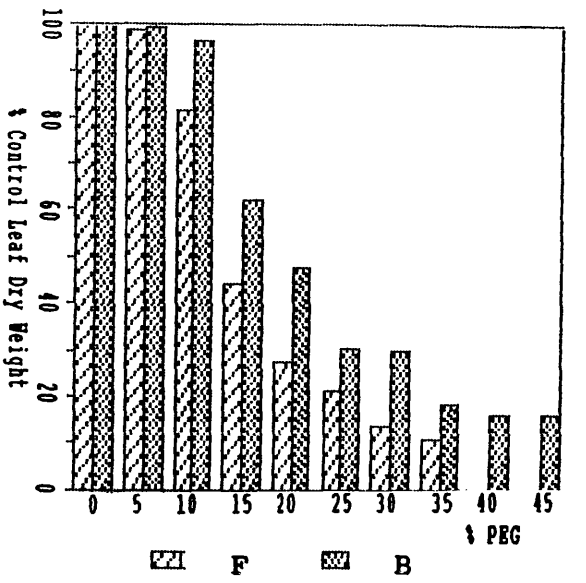


Figure 1.2.2

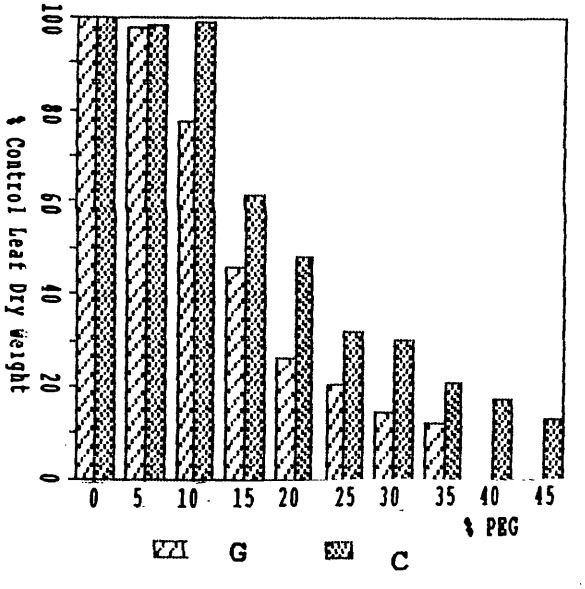
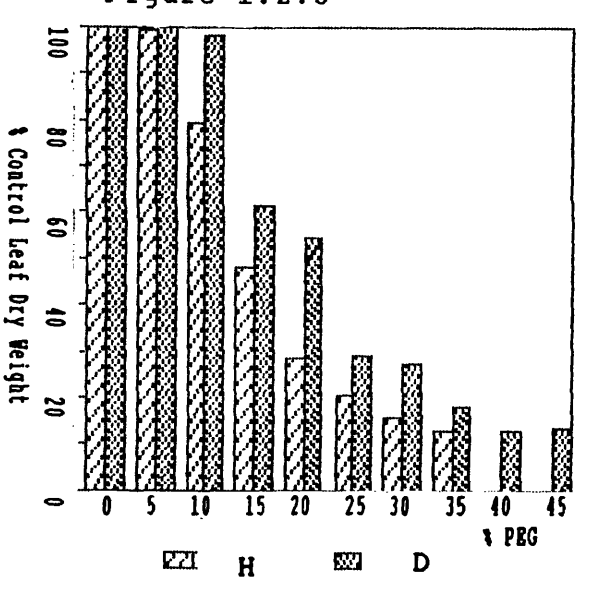


Figure 1.2.3



Figures 1.2.0 to 1.2.3 show the decline in leaf dry matter accumulation during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Leaf dry weight 100% values for figs. 1.2.0 to 1.2.3

G. urbanum populations	G. rivale populations
A 2.188 g	E 2.380 g
B 2.246 g	F 2.424 g
C 2.564 g	G 2.496 g
D 2.072 g	H 2.473 g

Figure 1.3.0

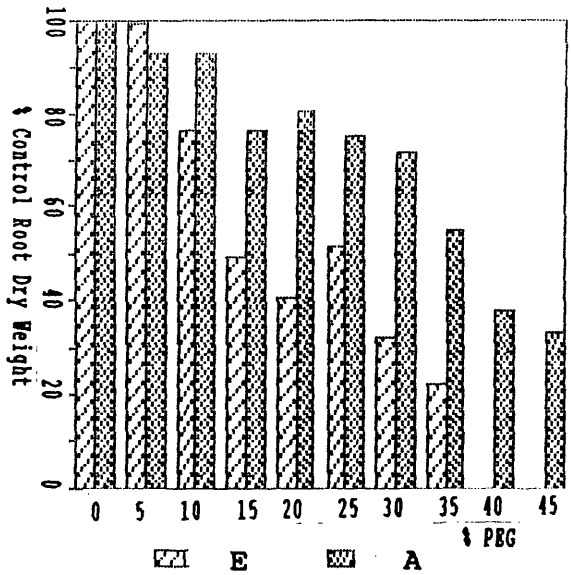


Figure 1.3.1

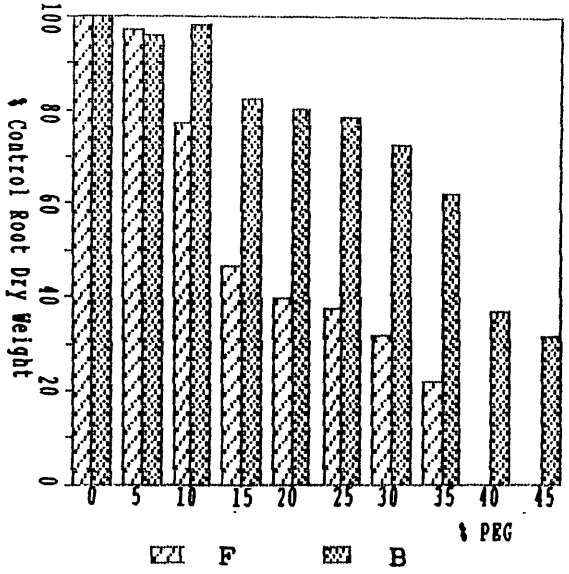


Figure 1.3.2

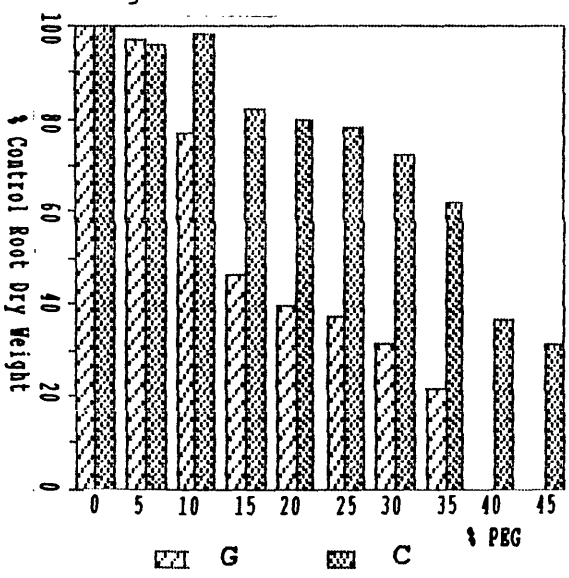
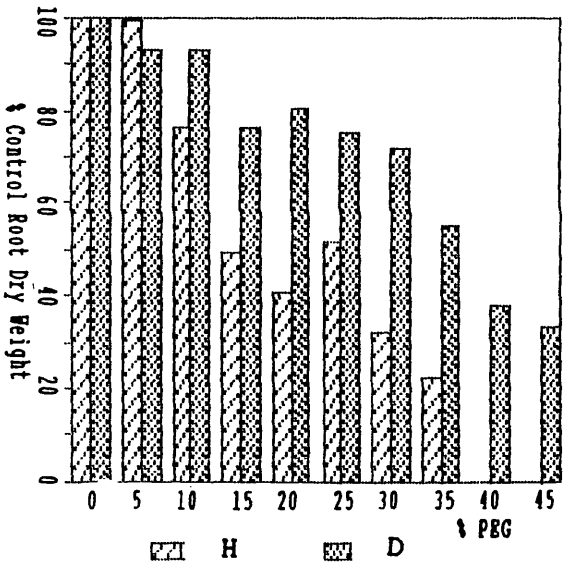


Figure 1.3.3



Figures 1.3.0 to 1.3.3 show the decline in root dry matter accumulation during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Root dry weight 100% values for figs. 1.3.0 to 1.3.3

G. urbanum populations	G. rivale populations
A 0.214 g	E 0.204 g
B 0.209 g	F 0.213 g
C 0.236 g	G 0.222 g
D 0.203 g	H 0.218 g

Figure 1.4.0

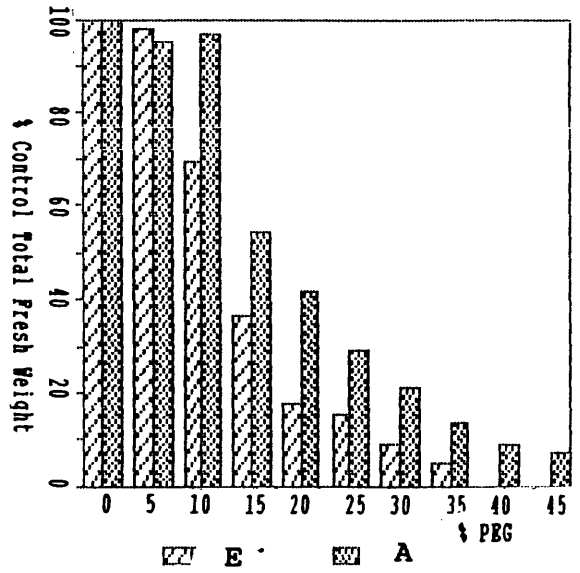


Figure 1.4.1

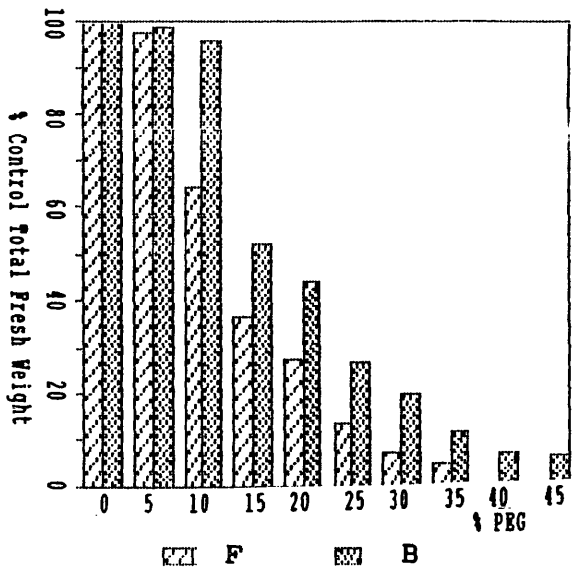


Figure 1.4.2

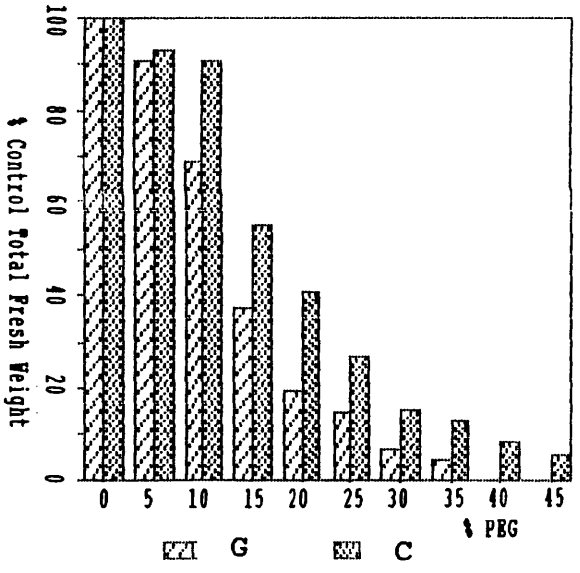
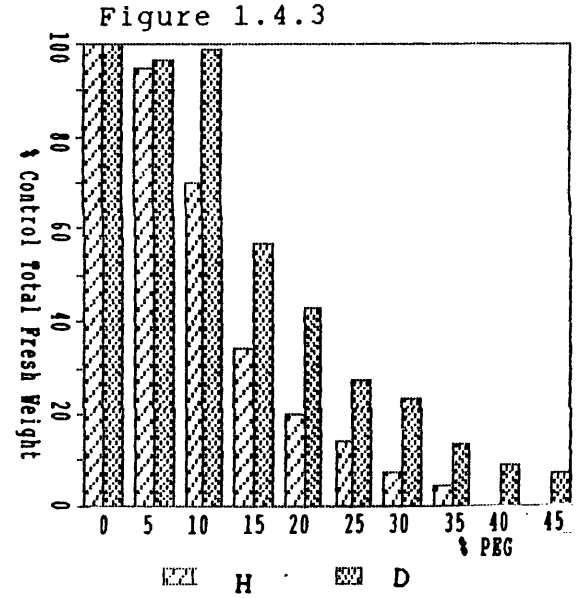


Figure 1.4.3



Figures 1.4.0 to 1.4.3 show the decline in total plant fresh weight during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Total fresh weight 100% values for figs. 1.4.0 to 1.4.3

G. urbanum populations		G. rivale populations	
A	8.870 g	E	11.523 g
B	9.565 g	F	11.878 g
C	11.783 g	G	13.371 g
D	8.670 g	H	12.302 g

Figure 1.5.0

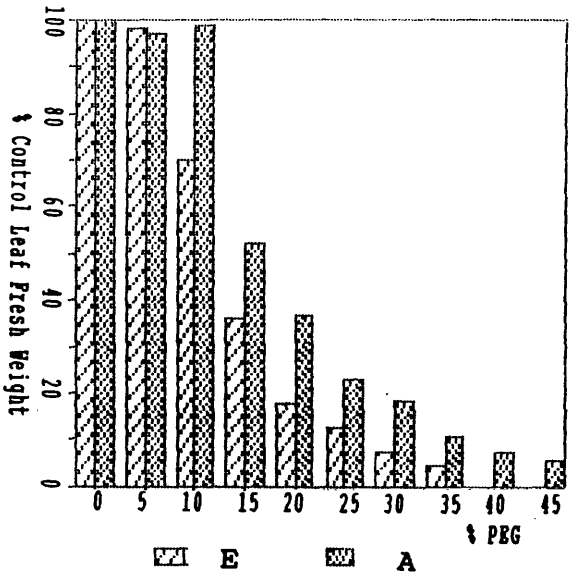


Figure 1.5.1

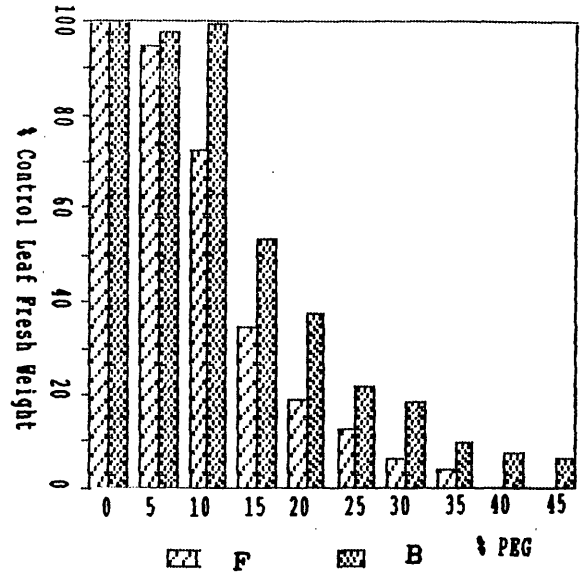


Figure 1.5.2

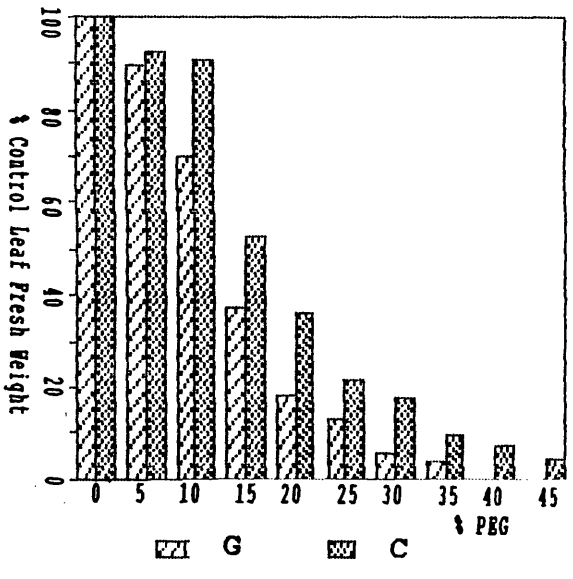
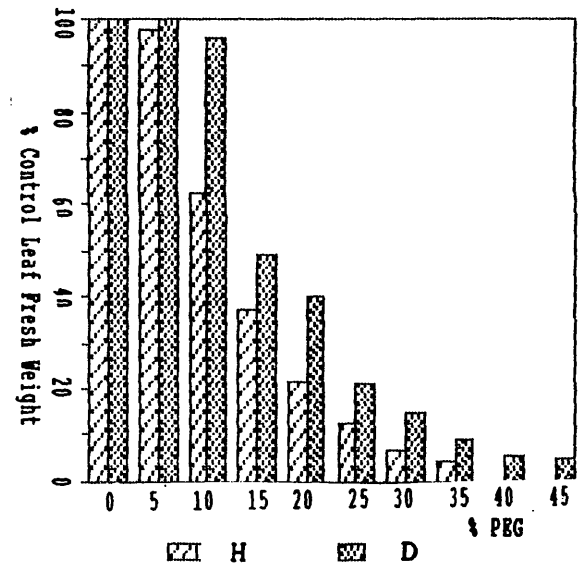


Figure 1.5.3



Figures 1.5.0 to 1.5.3 show the decline in leaf fresh weight during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Leaf fresh weight 100% values for figs. 1.5.0 to 1.5.3

G. urbanum populations	G. rivale populations
A 7.045 g	E 9.496 g
B 7.636 g	F 9.744 g
C 9.718 g	G 11.011 g
D 7.066 g	H 10.139 g

Figure 1.6.0

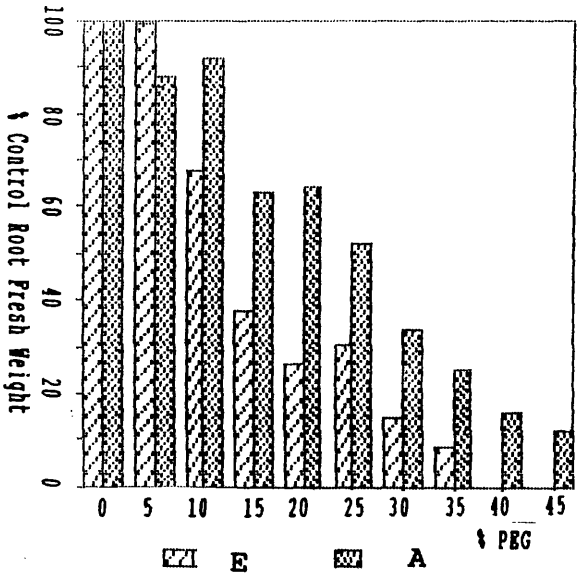


Figure 1.6.1

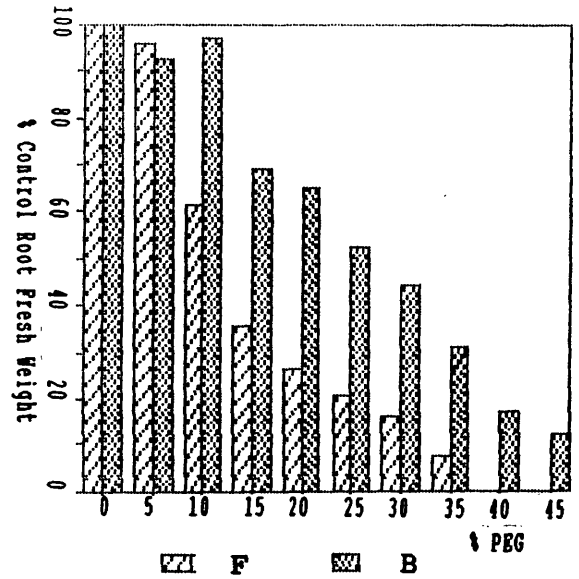


Figure 1.6.2

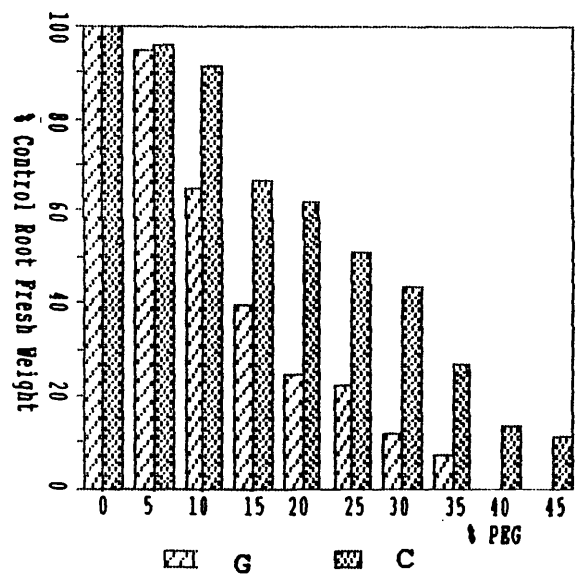
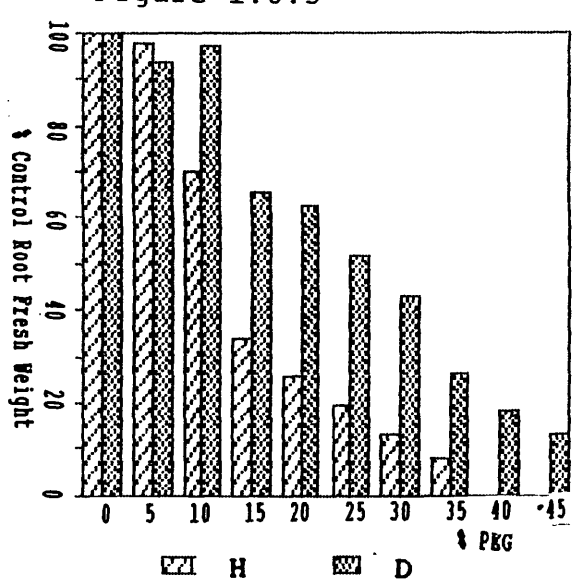


Figure 1.6.3



Figures 1.6.0 to 1.6.3 show the decline in root fresh weight during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Root fresh weight 100% values for figs. 1.6.0 to 1.6.3

G. urbanum populations	G. rivale populations
A 1.825 g	E 2.027 g
B 1.929 g	F 2.134 g
C 2.065 g	G 2.360 g
D 1.604 g	H 2.163 g

Figure 1.7.0

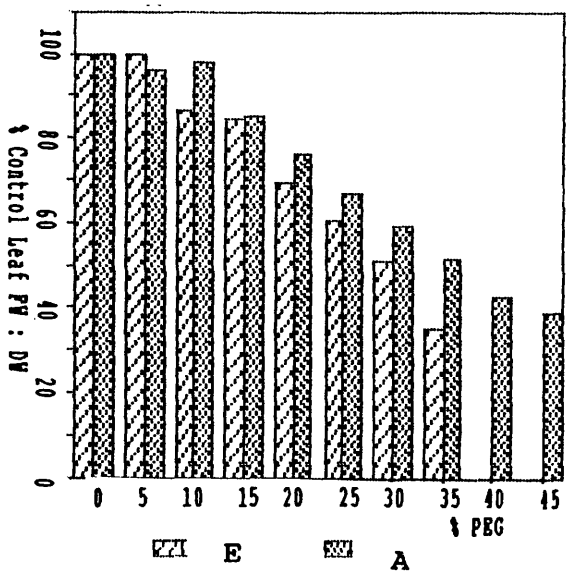


Figure 1.7.1

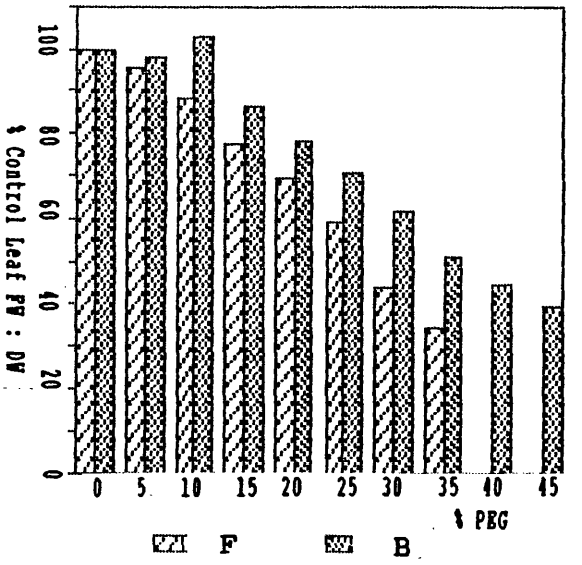


Figure 1.7.2

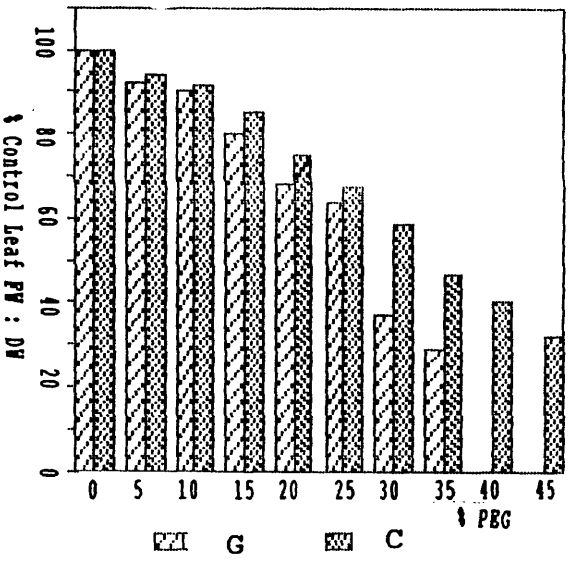
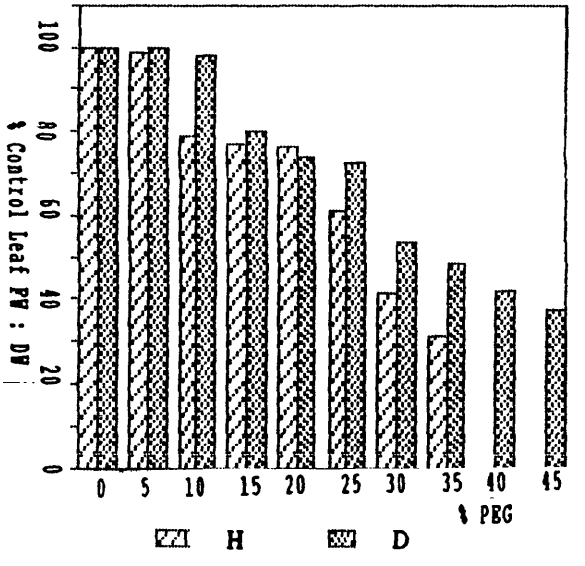


Figure 1.7.3



Figures 1.7.0 to 1.7.3 show the decline in leaf fresh to dry weight ratio during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (**A**); Finland (**B**); Spain (**C**); Iran (**D**). Geum rivale Leadgate (**E**); Moor House (**F**); Norway (**G**); Scotland (**H**).

Leaf FW:DW ratio 100% values for figs. 1.7.0 to 1.7.3

G. urbanum populations	G. rivale populations
A 3.32	E 3.99
B 3.40	F 4.02
C 3.79	G 4.46
D 3.41	H 4.10

Figure 1.8.0

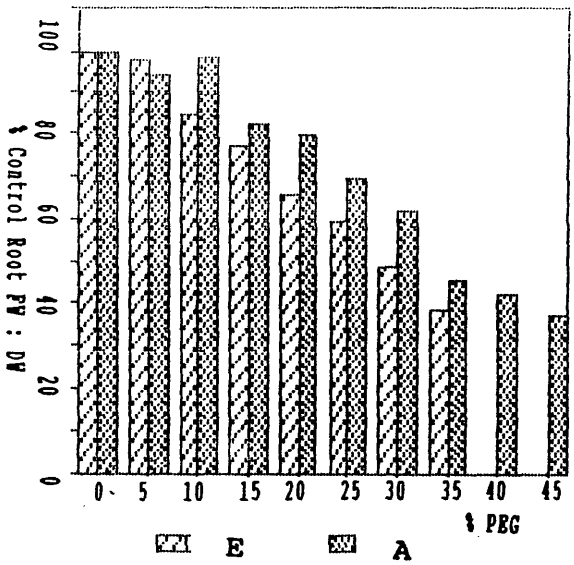


Figure 1.8.1

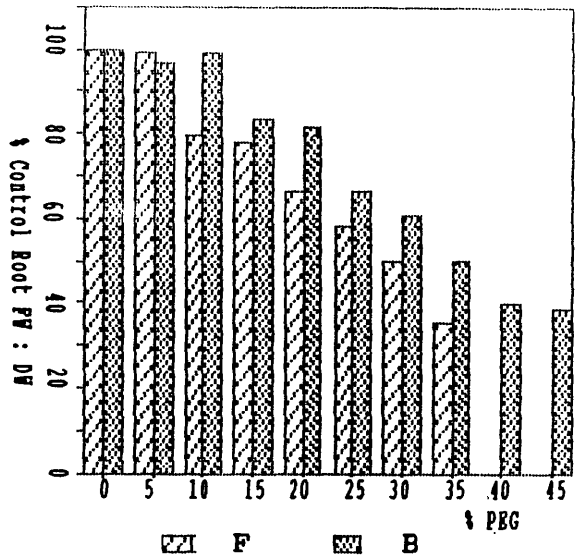


Figure 1.8.2

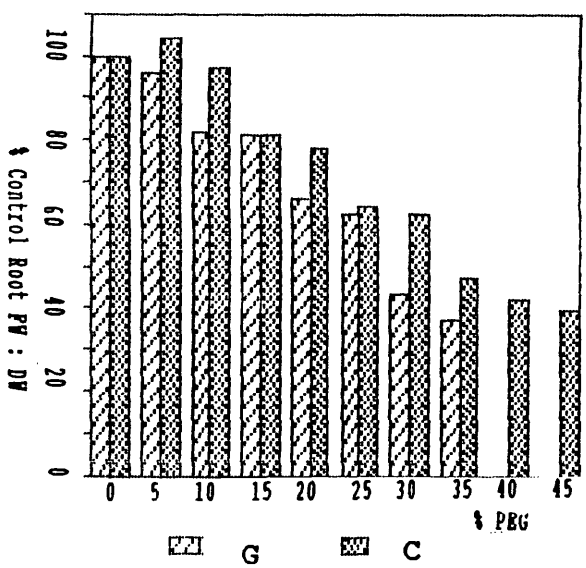
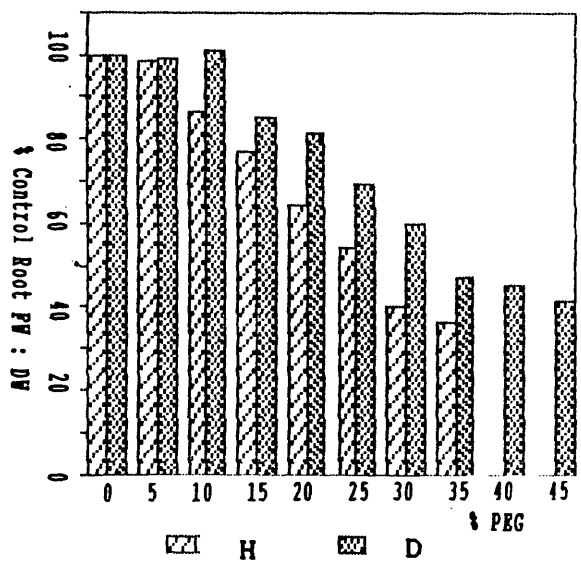


Figure 1.8.3



Figures 1.8.0 to 1.8.3 show the decline in root fresh to dry weight ratio during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Root FW:DW ratio 100% values for figs. 1.8.0 to 1.8.3

G. urbanum populations		G. rivale populations	
A	8.53	E	9.94
B	9.23	F	10.02
C	8.75	G	10.63
D	7.90	H	9.92

Figure 1.9.0

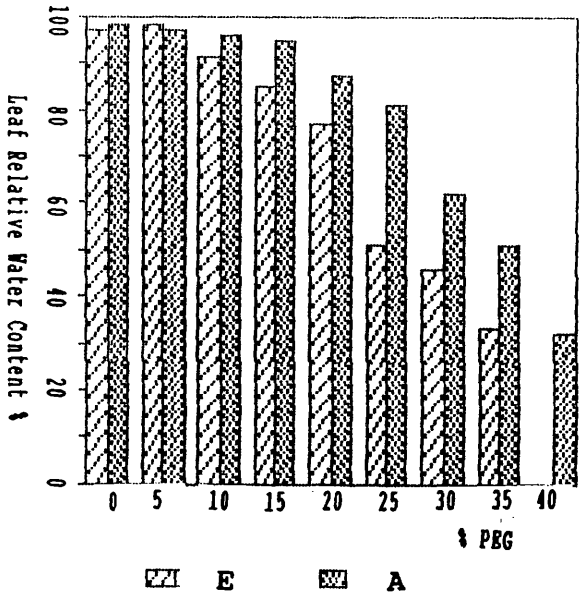


Figure 1.9.1

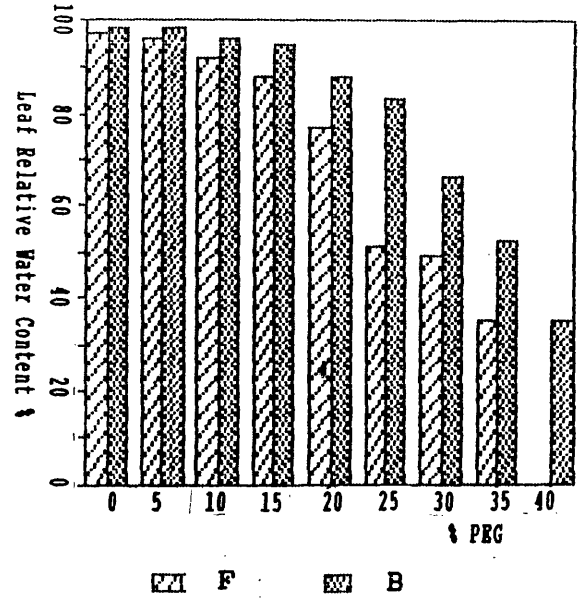


Figure 1.9.2

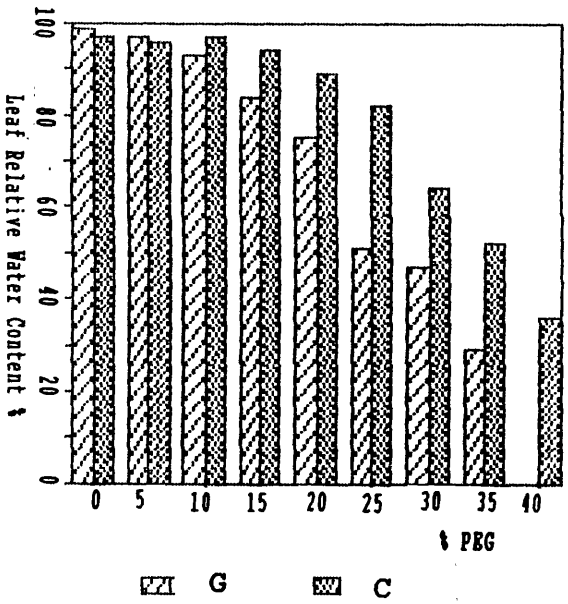
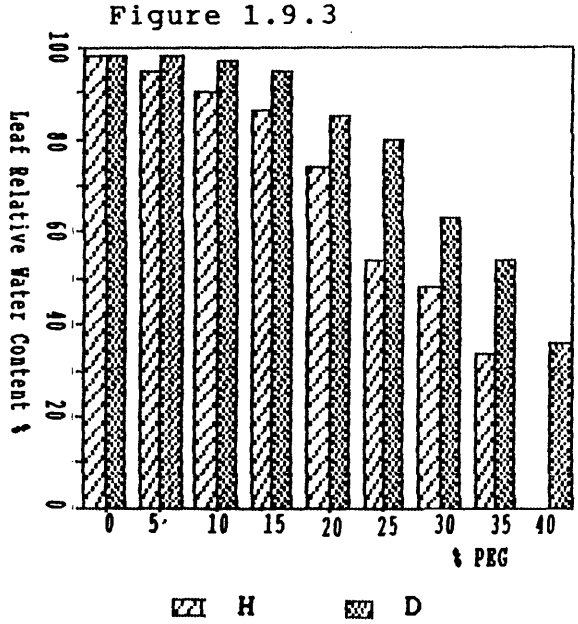


Figure 1.9.3



Figures 1.9.0 to 1.6.3 To show the changes in relative water content during water deficits imposed by PEG 6000. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Figure 1.10.0

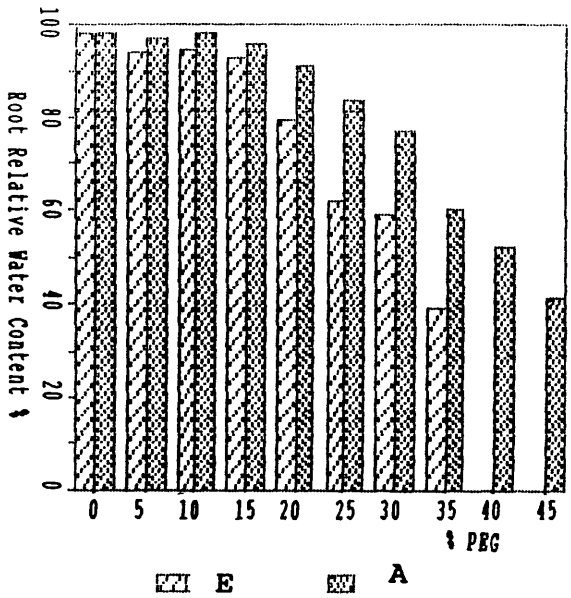


Figure 1.10.1

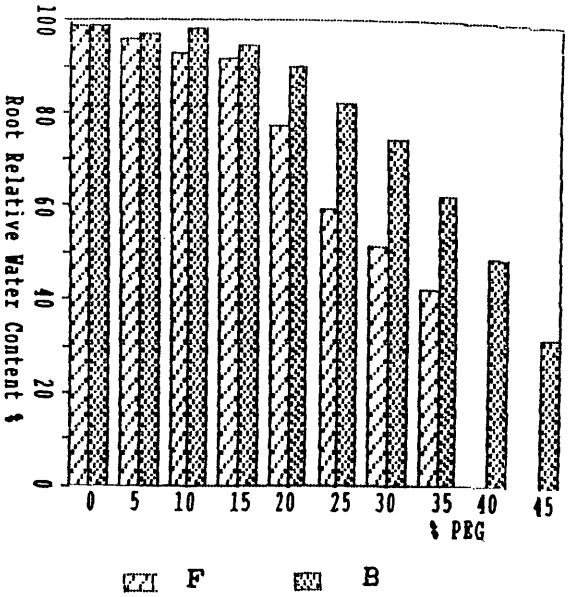


Figure 1.10.2

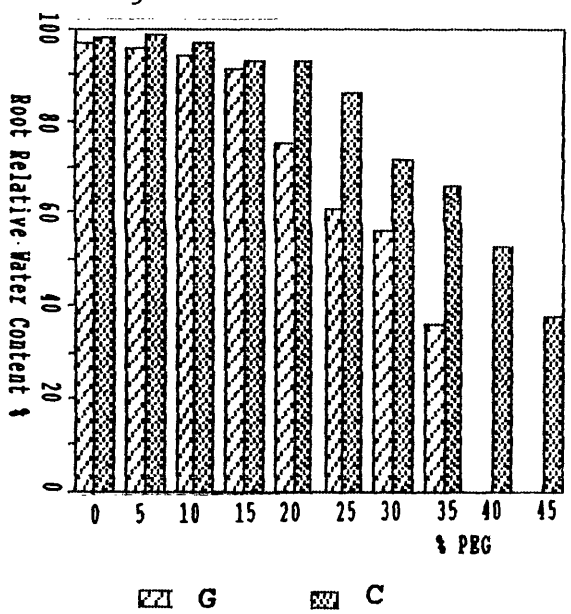
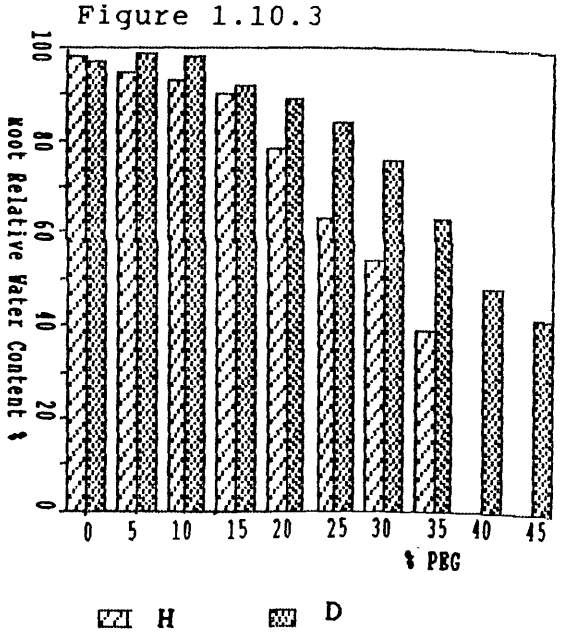


Figure 1.10.3



Figures 1.10.0 to 1.10.3 show the decline in root relative water content (RWC) during water deficits imposed by PEG 6000. Plant populations are: Geum urbanum Helbeck Wood (**A**); Finland (**B**); Spain (**C**); Iran (**D**). Geum rivale Leadgate (**E**); Moor House (**F**); Norway (**G**); Scotland (**H**).

Figure 1.11.0

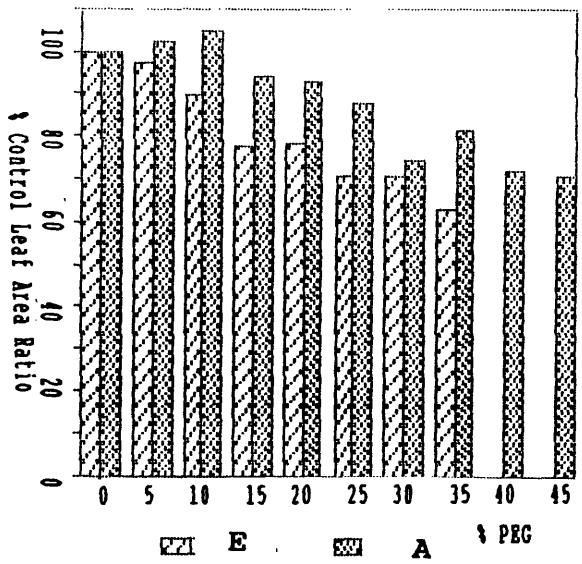


Figure 1.11.1

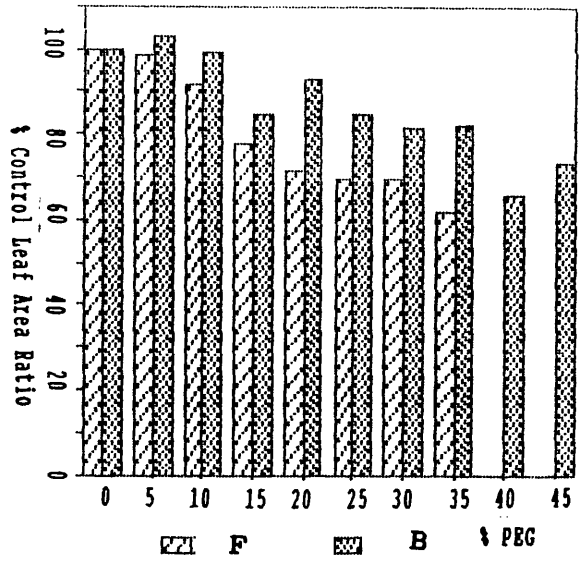


Figure 1.11.2

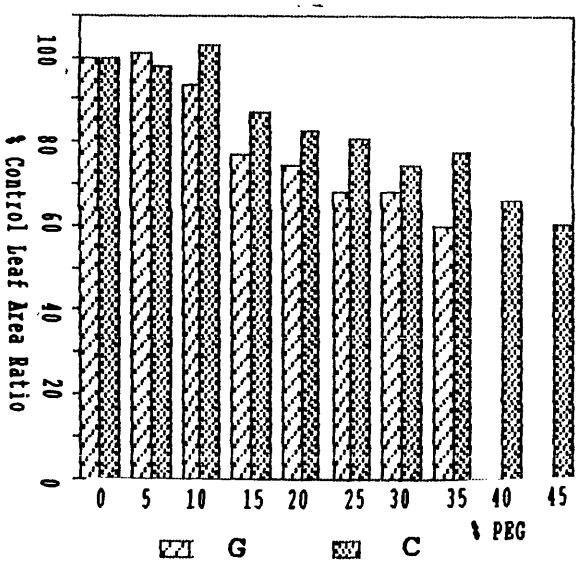
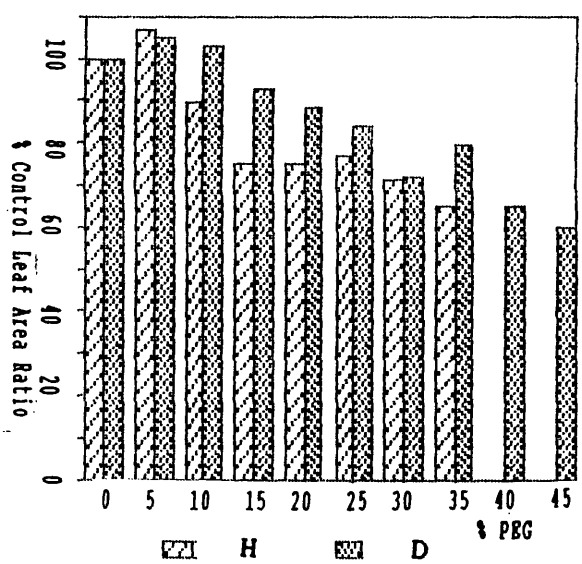


Figure 1.11.3



Figures 1.11.0 to 1.11.3 show the decline in leaf area ratio (cm^2/g dry weight) during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

LAR (cm^2 /g dwt) 100% values for figs. 1.11.0 to 1.11.3

G. urbanum populations	G. rivale populations
A 149	E 172
B 136	F 180
C 151	G 185
D 142	H 175

Figure 1.12.0

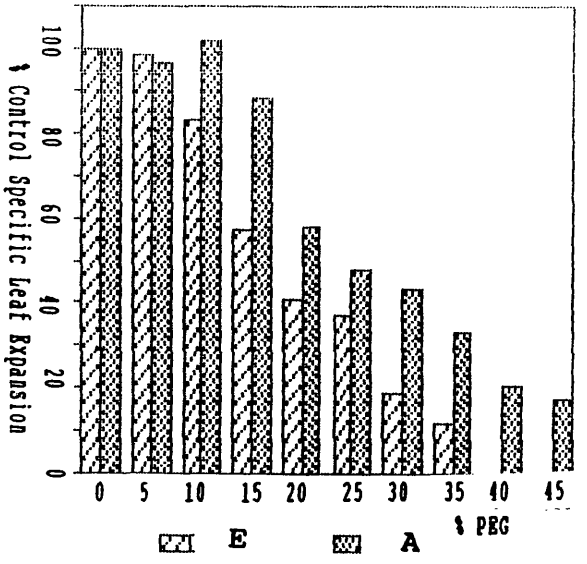


Figure 1.12.1

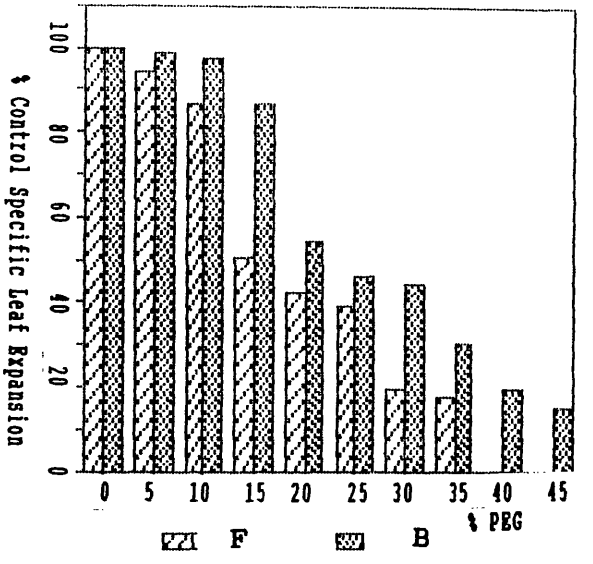


Figure 1.12.2

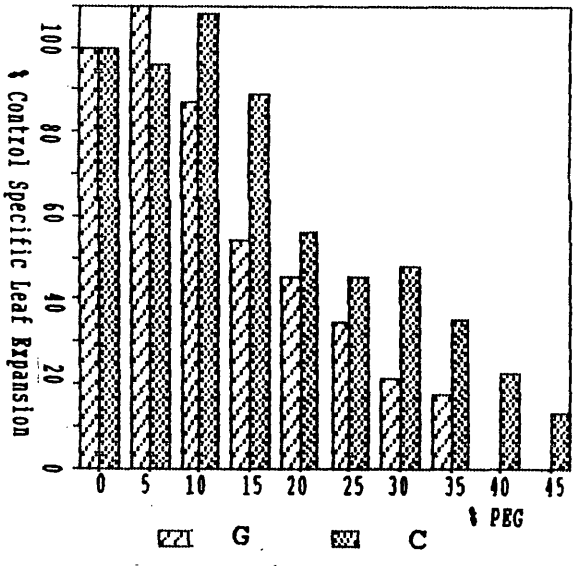
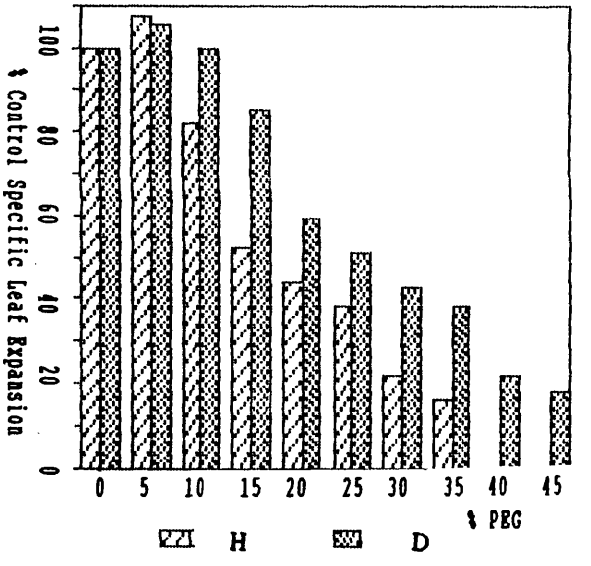


Figure 1.12.3



Figures 1.12.0 to 1.12.3 show the decline in specific leaf expansion during water deficits imposed by PEG 6000. Results are expressed as a percentage of control values. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Specific leaf expansion (cm^2) 100% values for figs. 1.12.0 to 1.12.3.

G. urbanum populations	G. rivale populations
A 11.0	E 13.1
B 10.7	F 12.8
C 12.9	G 14.1
D 10.1	H 12.2

Figure 1.13.0

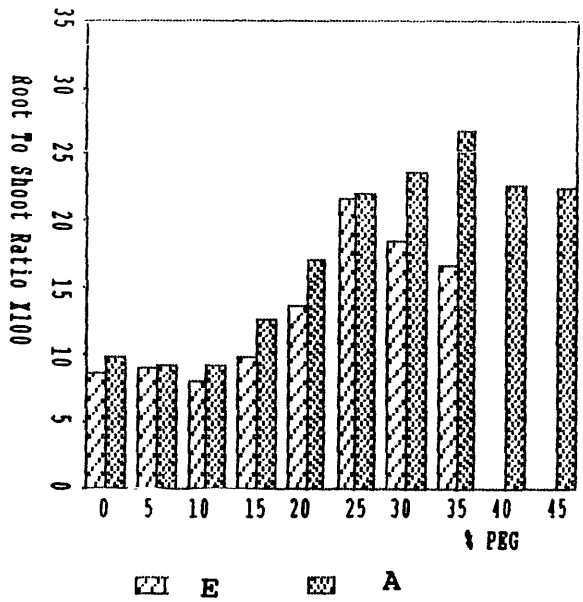


Figure 1.13.1

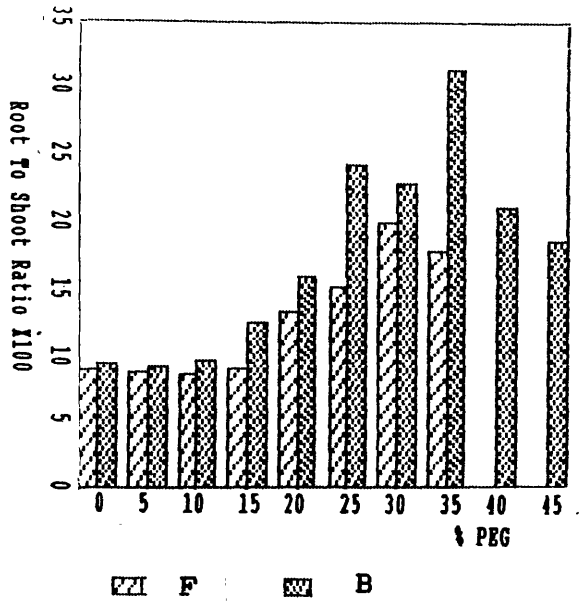


Figure 1.13.2

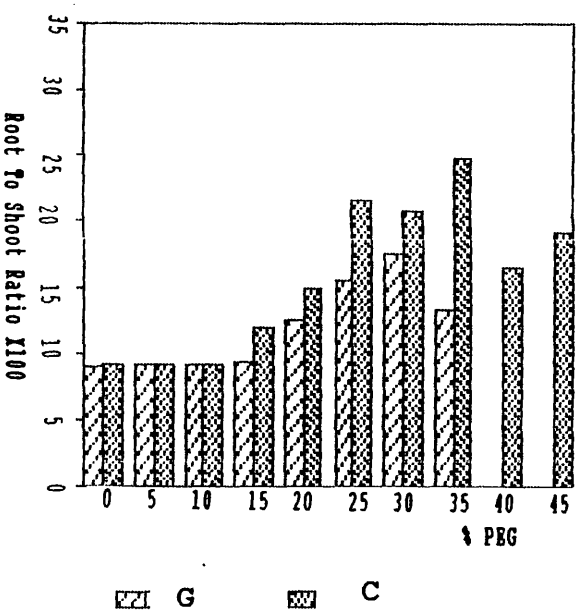
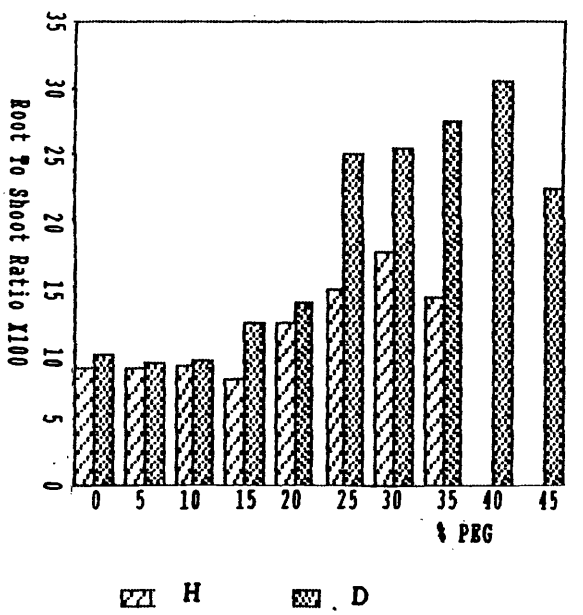


Figure 1.13.3



Figures 1.13.0 to 1.13.3 show the changes in root to shoot ratio during water deficits imposed by PEG 6000. Plant populations are: Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D). Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Root to shoot ratio 100% values for figs. 1.13.0 to 1.13.3

G. urbanum populations	G. rivale populations
A 0.098	E 0.086
B 0.093	F 0.088
C 0.092	G 0.090
D 0.098	H 0.088

Figure 1.14.0

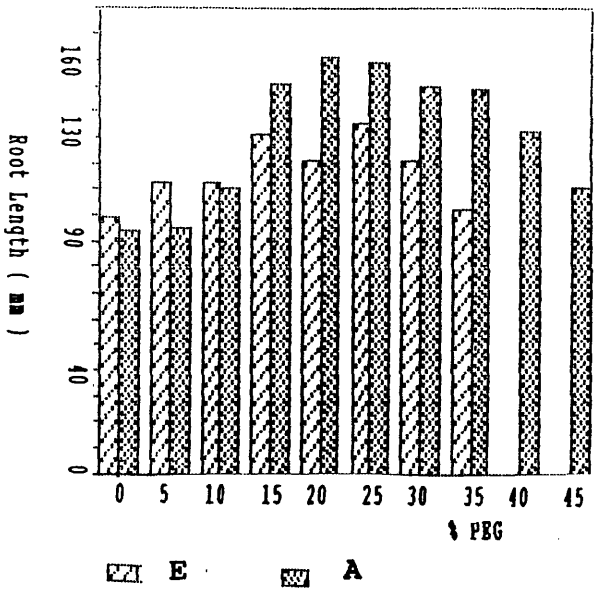


Figure 1.14.1

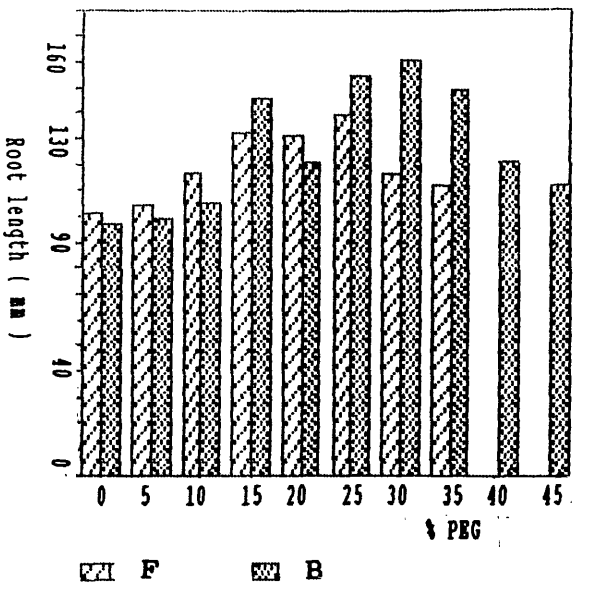


Figure 1.14.2

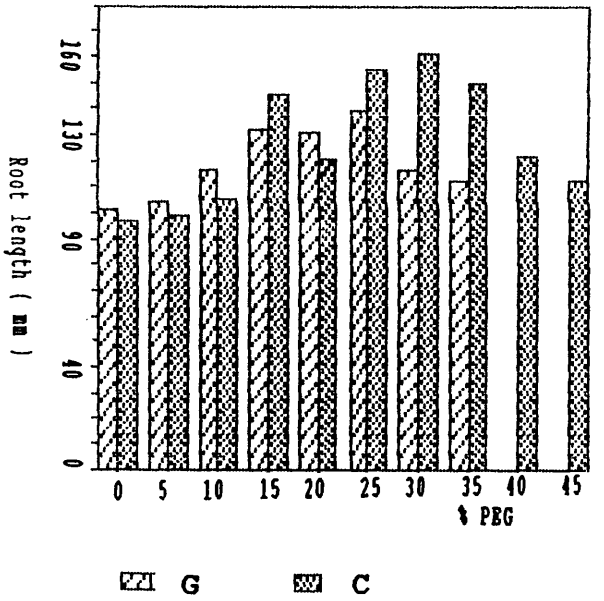
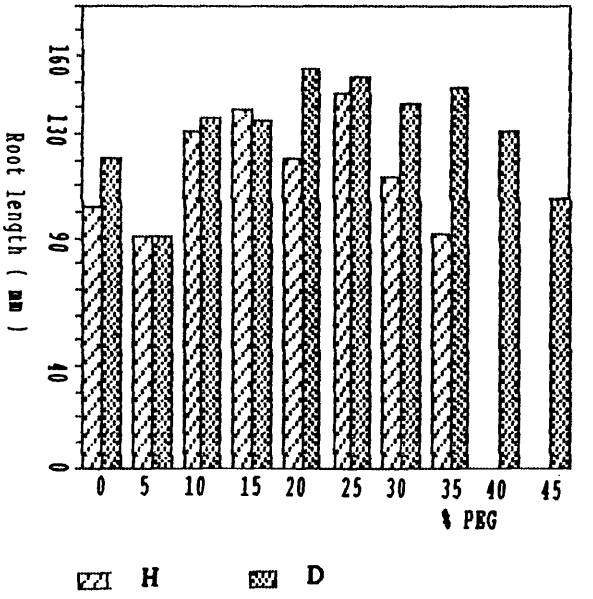


Figure 1.14.3



Figures 1.14.0 to 1.14.3 show the changes in root length during water deficits imposed by PEG 6000. Plant populations are:
Geum urbanum Helbeck Wood (A); Finland (B); Spain (C); Iran (D).
Geum rivale Leadgate (E); Moor House (F); Norway (G); Scotland (H).

Table 1.2.0. To show the total biomass production in control plants of the populations of G. urbanum and G. rivale studied in this investigation

Population	Dry weight accumulation
G. urbanum Helbeck	2.402 grams
G. urbanum Finland	2.455 grams
G. urbanum Spain	2.8 grams
G. urbanum Iran	2.275 grams
G. rivale Leadgate	2.584 grams
G. rivale Moor house	2.637 grams
G. rivale Norway	2.691 grams
G. rivale Scotland	2.671 grams

Table 1.3.0. To show the wilting points and death points of the populations of *G. urbanum* and *G. rivale* during water deficits imposed by PEG 6000

Population	Wilting point	Death point
G. urbanum Helbeck	35 %PEG	45 %PEG
G. urbanum Finland	35 %PEG	45 %PEG
G. urbanum Spain	35 %PEG	45 %PEG
G. urbanum Iran	35 %PEG	45 %PEG
G. rivale Leadgate	30 %PEG	40 %PEG
G. rivale Moor house	30 %PEG	40 %PEG
G. rivale Norway	30 %PEG	40 %PEG
G. rivale Scotland	30 %PEG	40 %PEG

Table 1.4.0. To show how rapid stress can alter a plants reaction to water stress at the 20% peg stress level

Population	% dry weight from control		% fresh weight from control		% leaf expansion from control	
	slow	rapid	slow	rapid	slow	rapid
G. urbanum Helbeck	49	38	42	35	57	42
G. urbanum Finland	50	42	43	32	54	39
G. urbanum Spain	51	42	40	37	56	36
G. urbanum Iran	56	44	45	34	59	41
G. rivale Leadgate	26	20	18	16	40	27
G. rivale Moor house	28	19	20	14	42	22
G. rivale Norway	22	16	19	13	45	31
G. rivale Scotland	22	17	22	17	44	26

DISCUSSION

Table 1.3.0 in this chapter demonstrates that Geum urbanum can survive at higher stress levels than Geum rivale (45% and 35% PEG respectively) and could hold off wilting for a longer period and at higher stress levels than Geum rivale . This demonstrated that Geum urbanum was more tolerant of water deficits imposed by PEG 6000 than Geum rivale .

The reduction in total plant growth of Geum urbanum and Geum rivale to increasing water deficits (Figs. 1.1.0 to 1.1.3) is consistent with that found in many other species eg. Stanhill (1957) and this reduction at mild water deficits was documented by the same author. The reduction of both leaf growth (Figs. 1.2.0 to 1.2.3) and root growth (Figs. 1.3.0 to 1.3.3) to increasing water deficits has also been documented by other authors (Etherington and Rutter 1964) and the maintenance of root biomass in preference to shoot biomass is also well known (Gales 1979). It is then apparent from the above figures that Geum urbanum could maintain growth nearer to its maximum than Geum rivale at all stages of water deficit and maintain growth at higher stresses. Thus the relative reduction in growth in Geum urbanum and Geum rivale is correlated with their ability to resist water deficits. This maintenance of growth however is not always correlated with drought resistance particularly under mild water deficits as Blum et al. (1983) showed in similar experiments with other species. As a general trend however, the maintenance of growth near to control values is generally associated with tolerance of water deficits especially when water availability becomes more limited (Blum et al. 1983; Etherington and Rutter 1964).

Differences in biomasssimilar to those between Geum populations (Table 1.2.0) have previously been correlated in other species with the ability to resist

periods of water deficit (Ashenden *et al.* 1975). However, unless the difference in growth rates is very large little advantage will be conferred and is not the case with these *Geum* species, as *G. rivale* had a slightly higher growth rate than *G. urbanum* and a lower resistance to water deficit.

The reduction in fresh weight of leaves and roots (Figs. 1.4.0 to 1.6.3) undergoing periods of water deficit has been known for many years. This occurs because the plant loses water to its environment *via* transpiration and evaporation combined with a reduced water uptake at root level. The results presented here show *Geum urbanum* could maintain a higher water content than *Geum rivale* in both leaves and roots, when compared on a fresh weight to dry weight basis (Figs. 1.7.0 to 1.8.3) this trend is still apparent. However, the fact that a plant loses more water than another plant is not a proof of a lower water stress tolerance as one plant may have a higher water content to start with. Figs. 1.9.0 to 1.10.3 show that *Geum rivale* lost more water at a given stress level than *Geum urbanum* when expressed as RWC which is consistent with their tolerances to water deficits. Such a fall in RWC has been correlated with the drought tolerance in other species both in ecological situations and in the laboratory (Bannister 1964 a,b) and supports the view that *Geum urbanum* is more water deficit tolerant than *Geum rivale*. As there is less water contained in the plants during a period of water deficit, water available to drive expansive growth may be limited and hence expansive growth may be restricted (Hsiao *et al.* 1976).

The reduction in LAR (Figs. 1.11.0 to 1.11.3) exhibited by both species indicates that such a reduction in expansive growth may occur and has been documented in other species (Ashby and Wolf 1948; McCree and Davis 1974). Figs. 1.12.0 to 1.12.3 confirm this as

there was a decrease in expansive growth in leaves of both species. However, expansive growth in populations of Geum rivale were lower than those of Geum urbanum and occurred at lower stress levels. This is again consistent with the plants ability to resist water deficits and is regarded as the most stress sensitive process in a plant (Hsiao et al. 1976). Indeed, a reduction in expansive growth was apparent in all populations of both species before reductions in biomass occurred.

Drought tends to be transient in a mesophytic environment (especially where these species grow) and the maintenance of biomass production and expansive growth during a period of water deficit may confer an advantage to Geum urbanum over Geum rivale after a period of water deficit. As leaf biomass, LAR and leaf expansion were maintained at higher levels in populations of Geum urbanum as opposed to Geum rivale the potential for total plant photosynthesis would be nearer to that of a previously unstressed plant in Geum urbanum. The potential for greater mineral and water uptake by Geum urbanum over Geum rivale is also apparent as root biomass was maintained closer to control levels by Geum urbanum. Such differences may thus aid the further establishment of Geum urbanum over Geum rivale after a period of water stress.

Figs. 1.13.0 to 1.13.3 show Geum urbanum could increase its root to shoot ratio higher than Geum rivale and also attain a greater root length (Figs. 1.14.0 to 1.14.3) than Geum rivale in response to water deficits. A similar response has been noted by many previous authors and has been suggested as a mechanism by which plants survive water deficits (Hsiao and Acevedo 1974). It was argued that such increases would aid the absorbtion of water due to a higher root area and increases in root length would enable the plants to

exploit water supplies lower in the soil profile. However, it is not considered that such differences could account for the large observed differences in stress tolerance reported here as the difference in R:S and root length though significant were only small. The difference in root length may however have a larger role in a field situation where rooting depth is not restricted as it was in these experiments and may even exacerbate the difference in water deficit tolerance shown here.

To put the two species in the spectrum of water stress tolerance one would describe Geum rivale as a drought intolerant comparable to lettuce (Behboudain and van Holsteijn 1977) and Primula elatior (Whale 1983) with which Geum rivale is associated. Geum urbanum could be described as marginally stress tolerant somewhat below that of Barley (Pearson and Stewart 1987) which could survive up to 60% PEG in similar experiments. In an ecological context Geum urbanum is similar in water deficit tolerance to Primula vulgaris with which it is associated (Taylor and Markham 1978).

Only slight differences occur between populations of either species collected from dry and wet areas of Europe. This is unusual in the field of water deficits as many ecotypes of other species (Young 1967; Ashenden et al. 1975) have shown wide differences in their ability to resist periods of water deficit. It may be however that as the two Geum species have low water deficit tolerance that such wide differences could not occur.

The final table (1.4.0) supports the work of other authors (Jones and Rawson 1979; Pearson and Stewart 1987) that rapid stress produces a detrimental affect on a plants ability to resist a period of water deficit as the fresh weights, RWC, dry weights were all lower than the acclimated plants at the end of the experiments.

However, whether rapid stress impairs a plants ability to withstand a period of water stress or gradual stress acclimates a plant to further water deficits is open to question.

The data presented in this chapter show that all populations of Geum urbanum could out survive all populations of Geum rivale when undergoing water deficit. However, these experiments did not show that populations from dry areas out performed populations from wetter areas in either species. Moreover, there is little difference in the tolerance of populations within either species. Geum urbanum can, without doubt, not only maintain growth during a period of water deficit better than Geum rivale but can also survive at higher water deficits. This is consistent with my hypothesis stating that Geum urbanum would be able to withstand periods of water deficit better than Geum rivale.

CHAPTER 2

THE METHODS EMPLOYED BY GEUM URBANUM AND GEUM RIVALE TO COMBAT WATER DEFICITS IMPOSED BY PEG 6000.

INTRODUCTION

In Chapter 1 it was demonstrated that Geum urbanum had a higher tolerance of water deficits than Geum rivale. Moreover, it was proposed that Geum urbanum was marginally tolerant of water deficits whereas Geum rivale was intolerant of water deficits. However, the study at that stage could not explain why this difference occurred. In this chapter an attempt is made to explain this difference in physiological terms.

Mesophytic plants exhibiting some degree of water deficit tolerance have been shown to have varied methods by which they resist such deficits. Such plants have been shown to increase their root to shoot ratio (R:S) (Etherington 1962) and increase root length (Sharp and Davis 1979) in order to obtain water further away from the plant and to exploit water supplies deeper in the water table. However in the previous chapter this was not considered to be a major factor in determining the water deficit tolerance of these two species. There are other methods by which plants can reduce water loss which have been shown to affect a plants drought tolerance.

Water loss to the environment can be reduced by stomatal closure in the leaves which reduces water loss due to a lowered transpiration rate. This adaption does however have a cost to the plant in that stomatal closure will reduce CO_2 uptake for photosynthesis (Shearman et al 1972). Plants can also alter their internal water relations in order to maintain a favorable gradient for water uptake,this is termed osmoregulation. Plants obtaining water by root absorbtion do so because the water potential of the root

is lower than that of its root environment ie.

soil water potential > plant water potential.

In order to reduce the water potential of the plant it must alter a component which determines water potential. Plant water potential (Ψw) is governed by solute potential (Ψs), the potential generated by dissolved solutes within the cell; the matrix potential (Ψm), the retentive properties of the cell surface; and the turgor pressure (Ψp), which resists the influx of water via the resilience of the cell wall. The water potential of the plant can thus be described by the following equation:

Equation 2.1. $\Psi w = \Psi s + \Psi p + \Psi m$.

Ψw and Ψs are negative, whereas Ψp is positive. Ψm is also negative but is little understood. Ψm acts over small distances and on solid surfaces, it is only significant below a relative water content of 40% and is probably not related to the symplast (Tyree 1976), it is also difficult to measure. For these reasons Ψm is generally ignored during discussions and investigations concerning plant water deficits. The plant can then alter either Ψp or Ψs to reduce Ψw in response to a fall in soil water potential (Ψ soil). The turgor pressure, Ψp drives expansive growth and helps support the plant. Thus, a reduction in Ψp in order to reduce Ψw would cause a reduction in leaf area, root surface area and decrease structural support for the plant. These effects may then reduce photosynthesis, root water absorbtion and cause the plant to become flaccid. If Ψp were to be reduced to zero the plant would then wilt. A plant undergoing water deficits must then maintain Ψp as long as possible in order to continue expansive growth,

maximise photosynthesis and water absorbtion and to prevent wilting. The plant could therefore reduce Ψs which, provided the reduction was large enough would maintain turgor and reduce Ψ w. To reduce Ψs, solutes within the cell could be concentrated by reducing the cell size and also maintain turgor; or the cell can reduce Ψ s by increasing osmotically active solutes within the cell and maintain cell size. The major solutes which have been shown to rise during water deficits are carbohydrates (Meyer and Boyer 1981), inorganic ions (Munns et al. 1979), amino acids (Singh et al. 1973b) and quaternary ammonium compounds (Hanson and Nelson 1978). In reality plants utilise both these methods and often in combination (Morgan 1980). The age of a plant, the age of plant tissue and the rate of stress can all influence the degree of osmoregulation that plant or plant part exhibits (Jones and Rawson 1979). It is therefore very important when undertaking experiments to ensure all external parameters are constant both during the experiment and between one experiment and another. Thus the use of controlled environmental facilities and a stress regime giving a controlled and repeatable stress is essential. The use of a controllable system such as PEG therefore becomes more apparent with such an experiment.

The experiments conducted here used the same experimental design and same populations as in chapter 1 but stomatal closure and osmotic parameters were also measured. In addition to this the levels of the main classes of solute were also determined.

MATERIALS AND METHODS

A: Plant Culture

The culture, stress regime and growth analysis of Geum urbanum and Geum rivale were as described in Chapter 1.

B: Measurement Of Water Potentials And Relative Water Content (RWC)

Water and solute potentials of leaves and roots were measured by the method of (Morgan 1980) using a Wescor HR 33 T psychrometer. Turgor pressure potential was derived from Equation 2.1. Assuming Ψ m is negligible, then water potential is approximately equal to the sum of the turgor pressure and the solute potential.

$$\text{ie.} \Psi w = \Psi s + \Psi p$$
$$\text{thus:} \Psi p = \Psi s - \Psi w$$

RWC was measured as described in Materials And Methods in Chapter 1. Values of Ψ s and RWC obtained by the above method were used to construct Figs 2.10.0 TO 2.13.0 after Morgan (1980). These graphs were designed to show the relative contributions a rise in solute levels and concentration effects made to the decrease in Ψw. However, it assumes that Ψ s is a good approximation of osmotic potential in the symplasm. It compares the observed response to that of an ideal osmometer where the amount of solute does not change but Ψ s is decreased by a concentration of solutes only. A concentration of solutes can be described by the equation: S= So.Vo/V, in the plant this can be approximated by S= So.RWCo/RWC. Here V is the osmotic volume and o is a reference state such as full or zero turgor. A linear form of this equation is log S= log (So.RWCo) -log RWC with an intercept at Log (So.RWCo) and a slope of one. Thus, if the observed data is plotted (log ΨS versus log RWC) it is possible to determine the role concentration effects play in the lowering of Ψ s. Again a slope of one

indicates a concentration of solutes. A slope below one indicates an accumulation of solutes, the lower this figure is the more solute accumulation is responsible for Ψ_s reduction. A slope above one indicates a loss of solutes, the higher the figure the more solutes are lost.

C: Measurement of Polyethylene glycol 6000 (PEG) Osmotic Potential

PEG 6000 osmotic potentials were determined at the end of an experimental run using the Wescor HR 33 T psychrometer used for determining the other osmotic parameters above. See Figs. 2.3.0 and 2.4.0 for results.

D: Stomatal And Photosynthesis Measurements

Stomatal conductance and photosynthetic rate were measured using an A.D.C. portable Infra Red Gas Analyser (IRGA) on newly fully expanded, photosyntheticaly competent, non- senescing leaves. For details of the use of this machine see the appendix.

E: Determination Of Solute Levels

Plant material, either expanded leaves, expanding leaves or roots were dried in an oven at 80°C for 1hr and then at 60°C for 48 hrs. After this period the respective parts were ground with a mortar and pestle. The tissue was then split into 0.5 to 1g portions to which 10ml 95% methanol was added. This was then whirly mixed sealed and placed in a 0 to 5°C fridge over night and whirly mixed the following morning. This process was repeated three times when the extract was ready for use. Previous experimentation had shown that the tissue to solvent ratio and extraction time were sufficient for complete extraction of methanol/water soluble compounds. The following assays were undertaken with aliquats taken from this extract.

Total amino acids were detected by the ninhydrin method described by Pearson and Stewart (1987) using leucine as a standard.

Quaternary ammonium compounds were measured using the periodide method of Story and Wyn Jones (1977) glycine betaine being used as a standard.

Total carbohydrates were determined by the anthrone method of Plummer (1978), carbohydrate levels being expressed in glucose equivalents.

Total hexose sugars were determined by the method of Jensen and Ashton (1960) glucose being used as a standard.

Total pentose sugars were measured by the method of Jensen and Ashton (1960) arabinose was used as a standard.

Samples of dried plant material (see Chapter 1 materials and methods section D) were subjected to sulphuric acid - hydrogen peroxide digestion as described by Allen (1974). Sodium, potassium, calcium and magnesium were analysed on an atomic absorbtion spectrophotometer.

F: Calculation of the contribution the rise in solutes made to Ψs reduction

This calculation was based on that of Jones et al. (1980) and is made on the following assumptions: 1. That Ψs is a good approximation of solute osmotic pressure in the symplast. 2. That plant water behaves as an ideal solute. 3. When carbohydrate levels were considered, total hexose sugars were subtracted from the total carbohydrate results and the remainder divided by two as this was assumed to be di-saccharides, and 4. That solutes measured are present in the cytoplasm. This has not been proven unequivically but Okazaki et al. (1987) showed amino acids and some sugars were prefferentially located in the cytoplasm during salt stress.

If 1 mole of a solute exerts an osmotic pressure of 219 kPa in 1 litre of water, it follows that 1 mole of a solute would exert an osmotic pressure of 219 MPa in 1 ml of solute. Thus, 1 micro-mole of solute would exert

an osmotic pressure of 2.19x 10-4 MPa per ml of water. Thus 40 micro-moles of solute would exert an osmotic pressure of approximately 0.02 MPa of pressure. If we now assume symplastic water represents 10% of total plant water (Pulich 1986) then 40 micro-moles of solute in 1ml symplastic water would exert an osmotic pressure of approximately 0.1 MPa. Though this is by no means an accurate figure it gives a rough approximation of solute contributions to reduced Ψs and can be used to illustrate trends found in experimentation.

RESULTS

The same populations were also investigated in this study as in chapter 1 but only the data for Geum urbanum from Helbeck Wood and that of Geum rivale from Moor House are presented. This is because no significant difference occurred between populations within the species which could account for their tolerances to water defects and presenting them here would unnecessarily complicate this chapter.

Fig. 2.1.0 shows that Geum urbanum and Geum rivale closed their stomata in response to increasing PEG concentrations and no significant difference occurred in stomatal conductance between species in response to increasing water deficits. Photosynthetic rate (Fig.2.2.0) also fell in response to increasing PEG concentrations in both species, however there was no significant difference in net photosynthetic rate between the species.

Figs. 2.3.0 to 2.6.0 show the decline in Ψw and Ψs in response to decreasing PEG in young and old leaves of Geum urbanum and Geum rivale. These show that Ψw and Ψs fell in mature and young leaves during stress imposition in both species. Also young leaves achieved a higher Ψw and Ψs than mature leaves and could maintain a higher RWC (Figs. 2.7.0 t0 2.9.0) than mature leaves in both species. The roots of both species maintained a higher RWC than young and mature leaves. Geum urbanum could, however, maintain turgor (the difference between Ψw and Ψs) at higher stress levels and for a longer period than Geum rivale, in young and mature leaves. Geum urbanum was also able to maintain a larger gradient for water uptake ($\Psi w - \Psi peg$) than Geum rivale mainly by greater reduction of Ψs at more severe water deficits an adjustment which Geum rivale was unable to achieve. Geum urbanum could also maintain a higher RWC in response to lower PEG indicating that Geum urbanum

could maintain a greater osmotic volume throughout stress, again in all plant parts.

Figs. 2.10.0 to 2.13.0 show the relative proportion which solute accumulation makes to the increase in Ψ_s in young and mature leaves. In young and mature leaves of both species a three phase process occurred. Firstly a stage of high solute accumulation, followed by a stage when a concentration of solutes became a larger factor in Ψ_s reduction and a third stage at severe, damaging stress when a loss of solutes was apparent. Geum urbanum was able to reduce Ψ_s by the accumulation of solutes to a greater extent and for a longer length of time than Geum rivale in stage 1 (see Tables 2.1.0 to 2.1.3) in young and mature leaves. Geum urbanum could also reduce Ψ_s by maintaining solute accumulation at stage 1 in expanding leaves for a longer period than Geum rivale. In stage 2 young and mature leaves portrayed a similar trend to that described earlier in stage 1 by both species. However, a reduction in solute accumulation occurred, and in Geum rivale Ψ_s was reduced almost totally by a concentration of solutes. In the third stage where a loss of solutes occurred, the slope of the graph for Geum rivale was much steeper, which indicates relatively more solutes were lost from the plants at the higher stress levels than Geum urbanum.

The classes of solutes that the two species accumulated in all the plant parts measured were almost identical. Neither species accumulated inorganic ions or pentose sugars in response to water stress at any stage (Table 2.2.0).

Quaternary ammonium compounds (Table 2.2.0) were not accumulated by Geum rivale in response to water deficits, and only trace amounts were accumulated by Geum urbanum.

However, both species did accumulate hexoses (Figs. 2.15.0 to 2.15.3) and other carbohydrates (Figs. 2.14.0

to 2.14.3) as well as amino acids (Figs 2.16.0 to 2.16.3) in all plant parts at most stress levels. Of these solute classes carbohydrates accumulated before amino acids in all the plant parts measured in both species.

Solute accumulation in both species was generally coincidental with the trends found in the pressure / volume curves of Figs. 2.10.0 to 2.13.0 and consistent with the data presented in Tables 2.1.0 to 2.1.3 There was a period of rapid solute accumulation, followed by a rise in solute levels at a slower rate and finally a loss of solutes in both species. Geum urbanum accumulated more solutes in all plant parts throughout stress when compared to Geum rivale. However, expanding leaves accumulated more solutes than expanded leaves which in turn accumulated more solutes than roots.

The final tables (Table 2.2.0 to 2.2.3) shows the percentage contributions the rise in solutes made to the reduction in Ψ s. This figure shows that percentage reduction of Ψ s by solutes was greater in Geum urbanum than Geum rivale at most stress levels in all plant parts. The percentage reduction fell in both species in all plant parts and appeared to be in three stages which were comparable to the stages in the log/log plots.

Figure 2.2.0

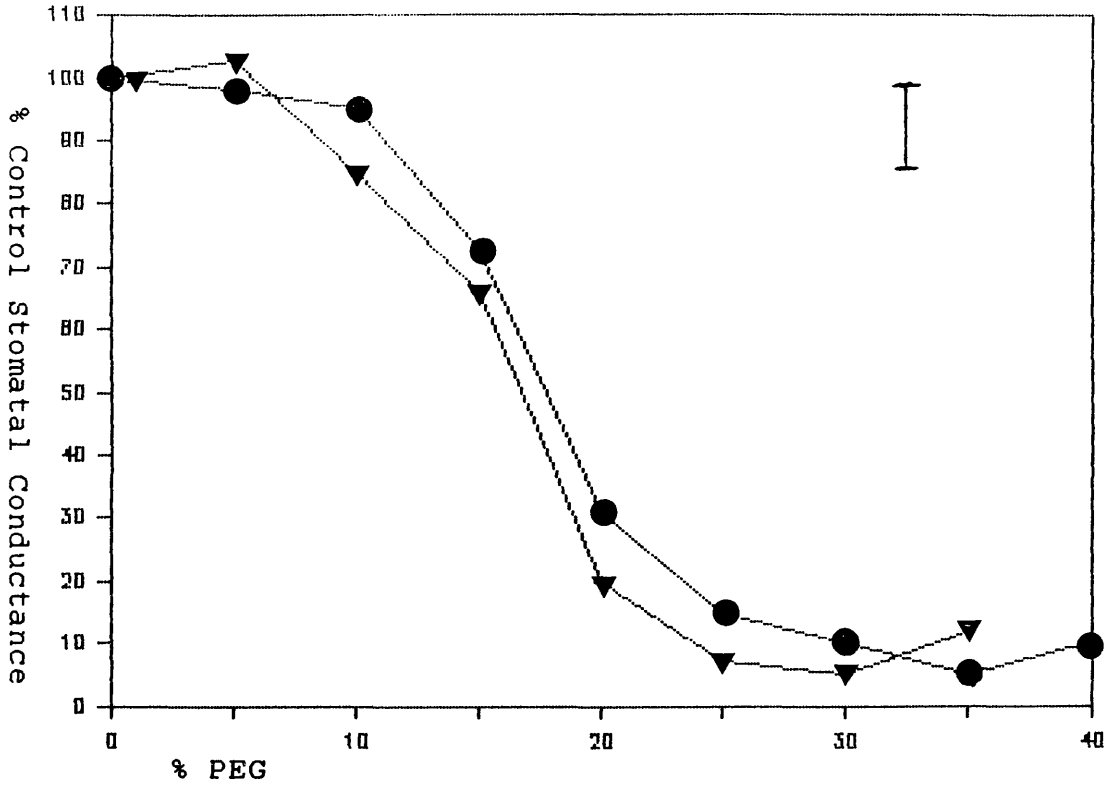


Figure 2.2.0 to show the decline in stomatal conductance as measured by IRGA of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as a percentage of control stomatal conductance.

Bar indicates L.S.D. ($P < 0.05$)

Control stomatal conductance (100%) values :
G. urbanum 0.698 mol m^{-2} s^{-1}
G. rivale 0.721 mol m^{-2} s^{-1}

Figure 2.2.0

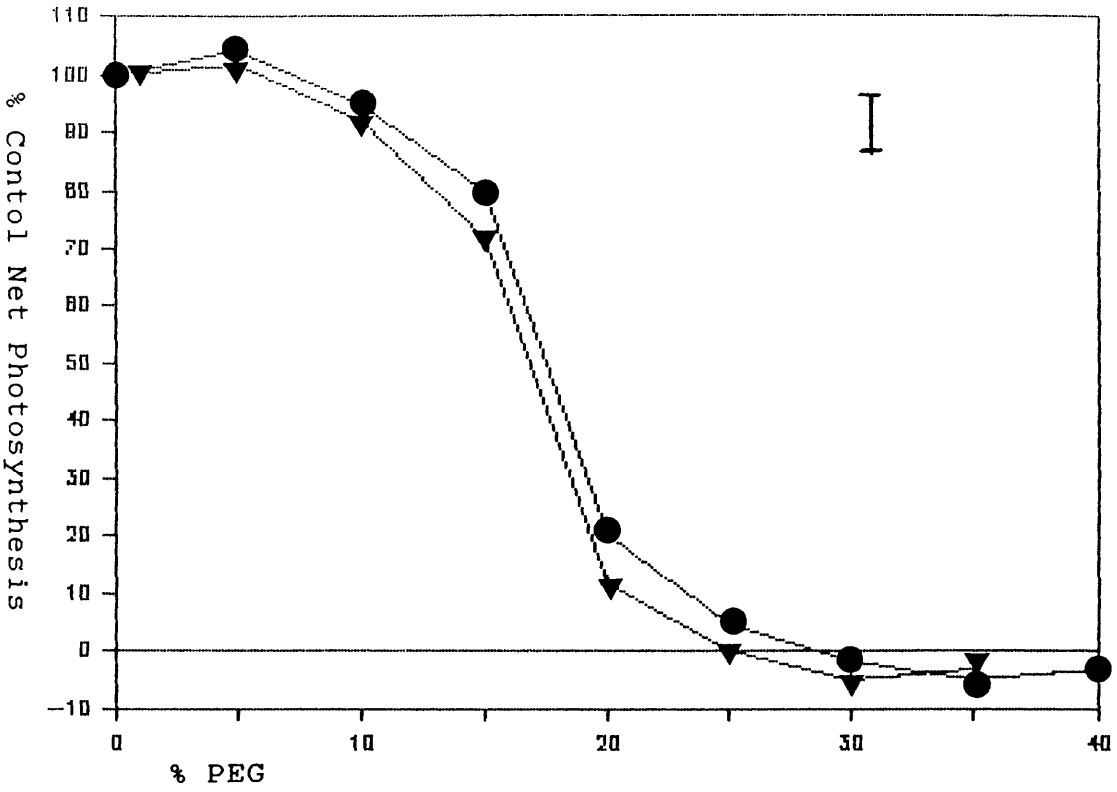


Figure 2.1.0 to show the decline in net photosynthetic rate as measured by IRGA of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as a percentage of control net photosynthesis.

Bar indicates L.S.D. ($P < 0.05$)

Control net photosynthetic rate (100%) values :

G. urbanum	24.26 m mol CO_2 m^{-2} s^{-1}
G. rivale	26.02 m mol CO_2 m^{-2} s^{-1}

Figure 2.3.0

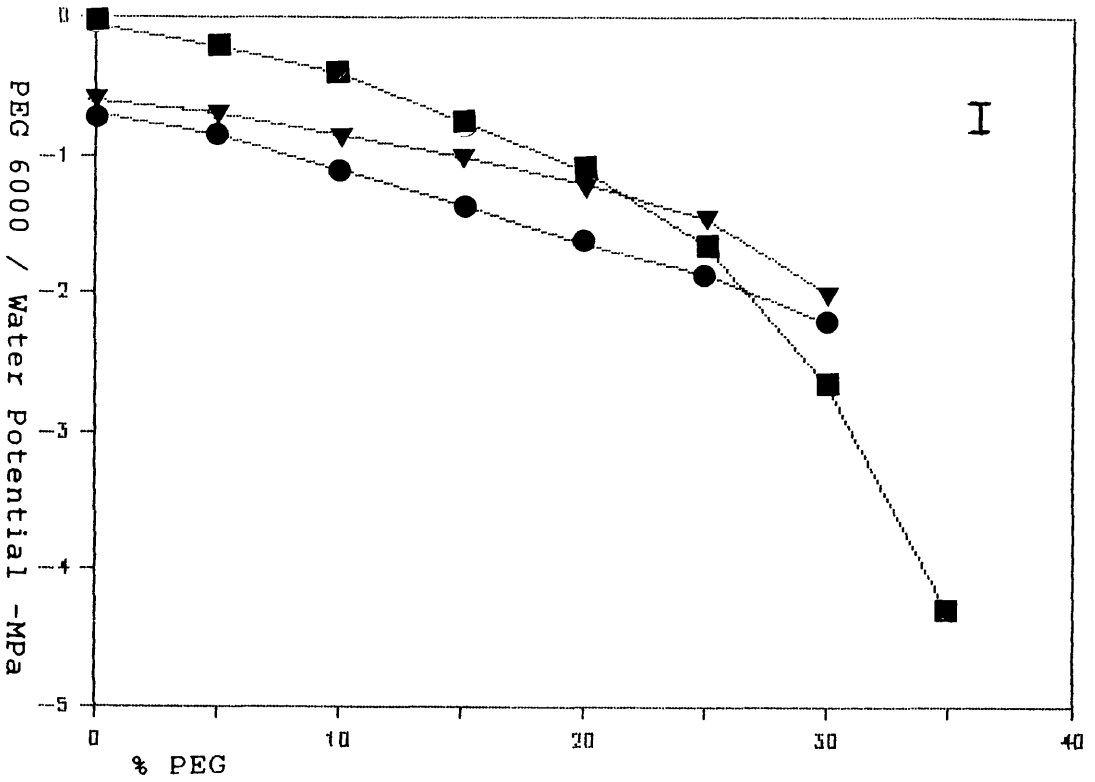


Figure 2.3.0 to show PEG 6000 water potential and the decline in mature leaf water potential of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in -MPa.

Bar indicates L.S.D. ($P < 0.05$)

■ indicates PEG 6000 water potential

Figure 2.4.0

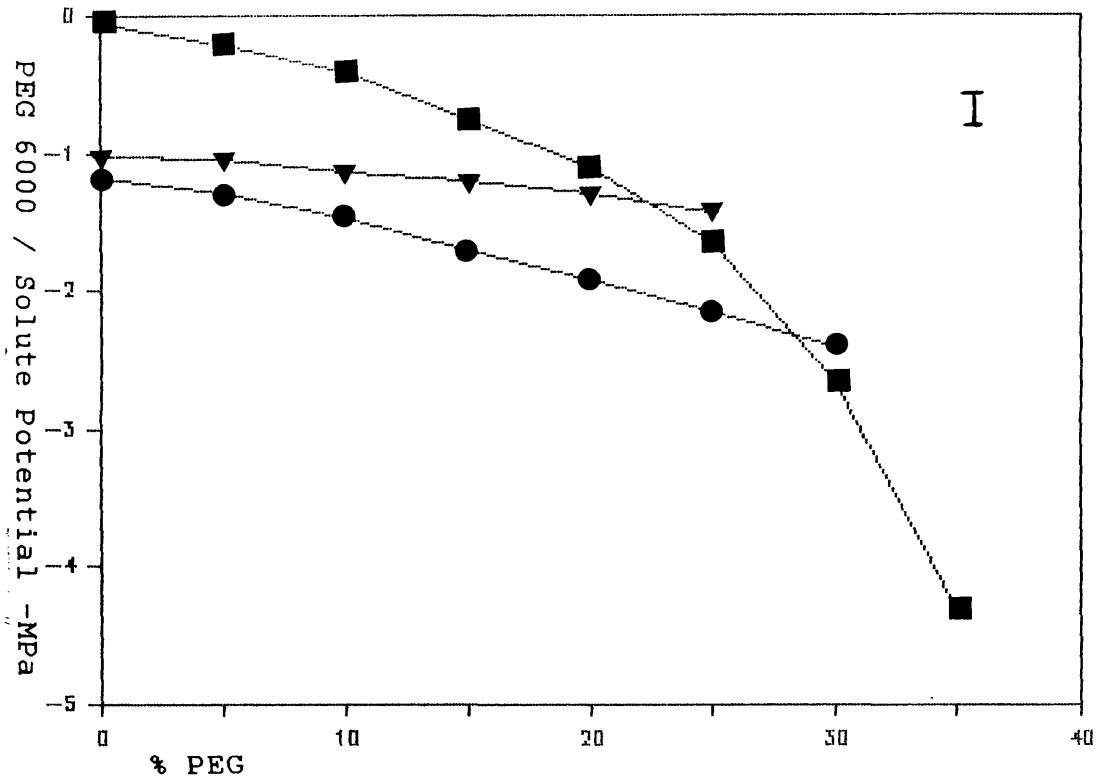


Figure 2.4.0 to show PEG 6000 water potential and the decline in mature leaf solute potential of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in -MPa.

Bar indicates L.S.D. ($P < 0.05$)

■indicates PEG 6000 water potential

Figure 2.5.0

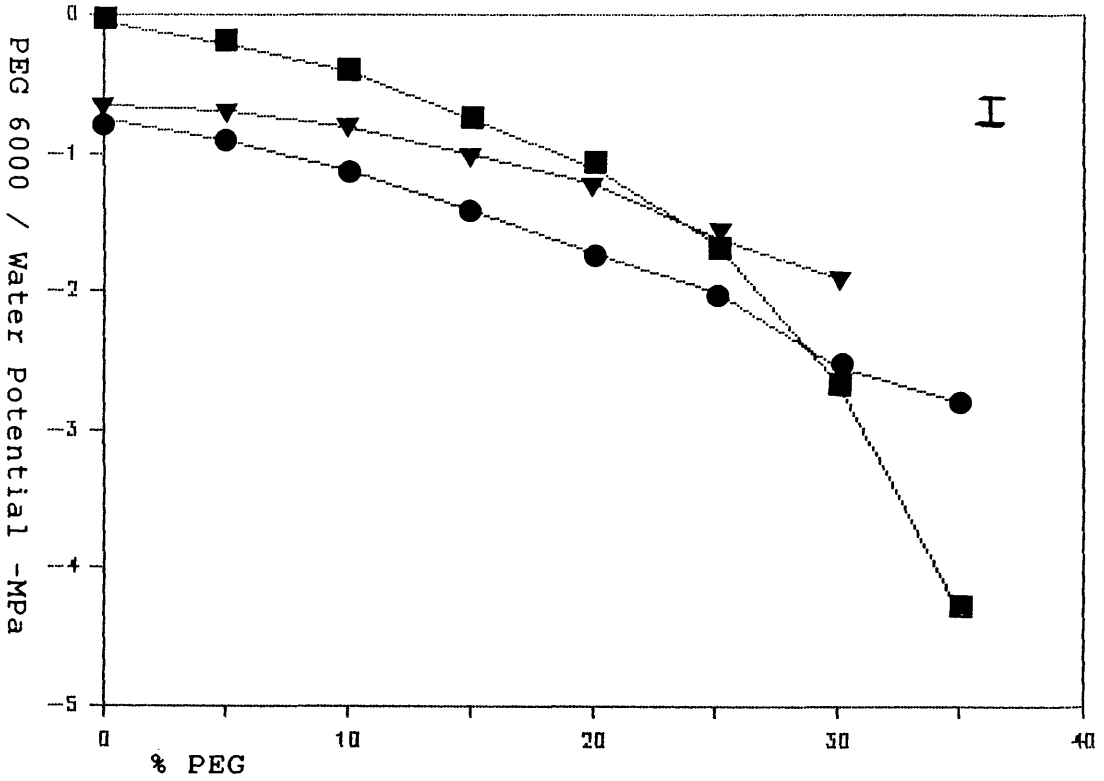


Figure 2.5.0 to show PEG 6000 water potential and the decline in young leaf water potential of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in -MPa.

Bar indicates L.S.D. ($P < 0.05$)

■indicates PEG 6000 water potential

Figure 2.6.0

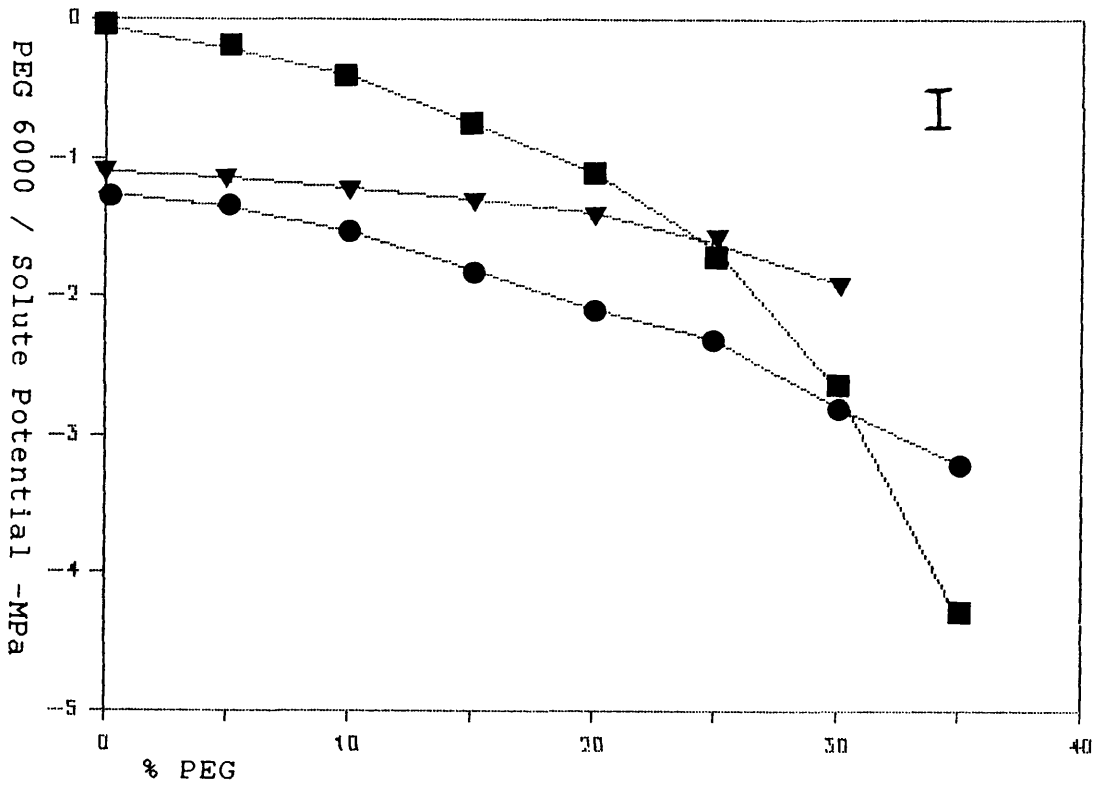


Figure 2.6.0 to show PEG 6000 water potential and the decline in young leaf solute potential of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in -MPa.

Bar indicates L.S.D. ($P < 0.05$)

■indicates PEG 6000 water potential

Figure 2.7.0

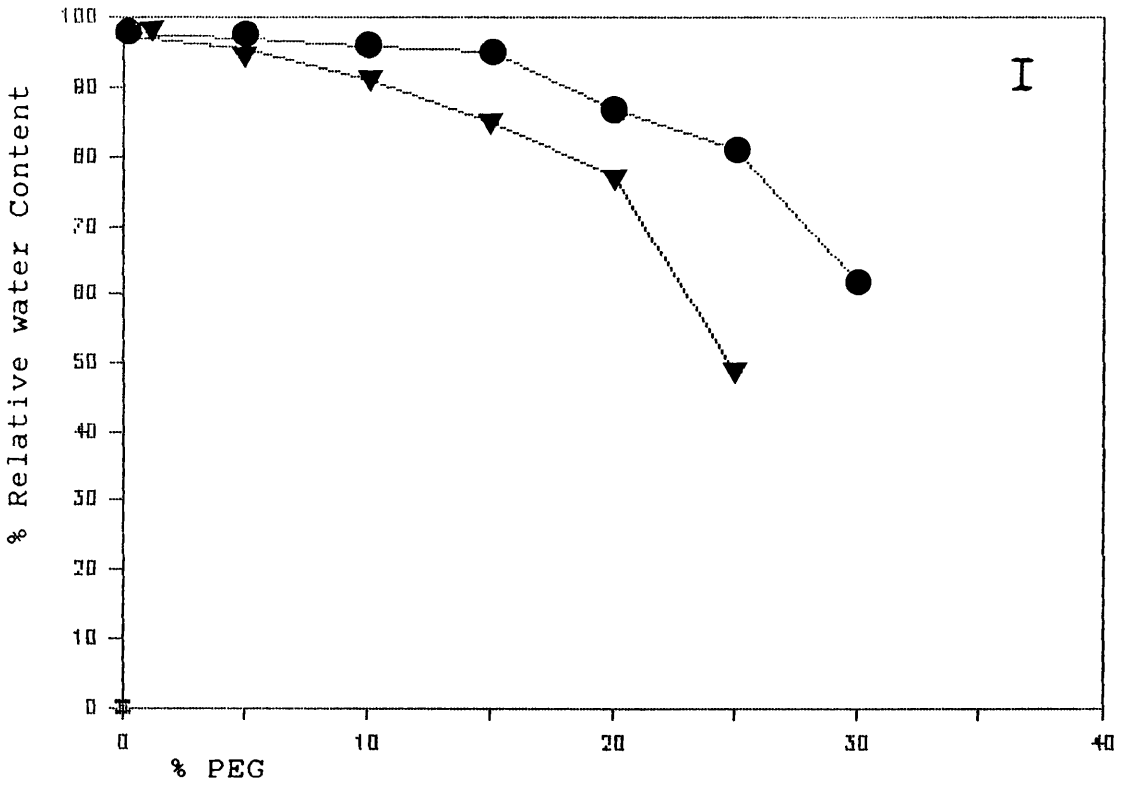


Figure 2.7.0 to show the decline in mature leaf relative water content (RWC) of Geum urbanum● and Geum rivale▼ in response to water deficits imposed by PEG 6000.

Bar indicates L.S.D. ($P < 0.05$)

Figure 2.8.0

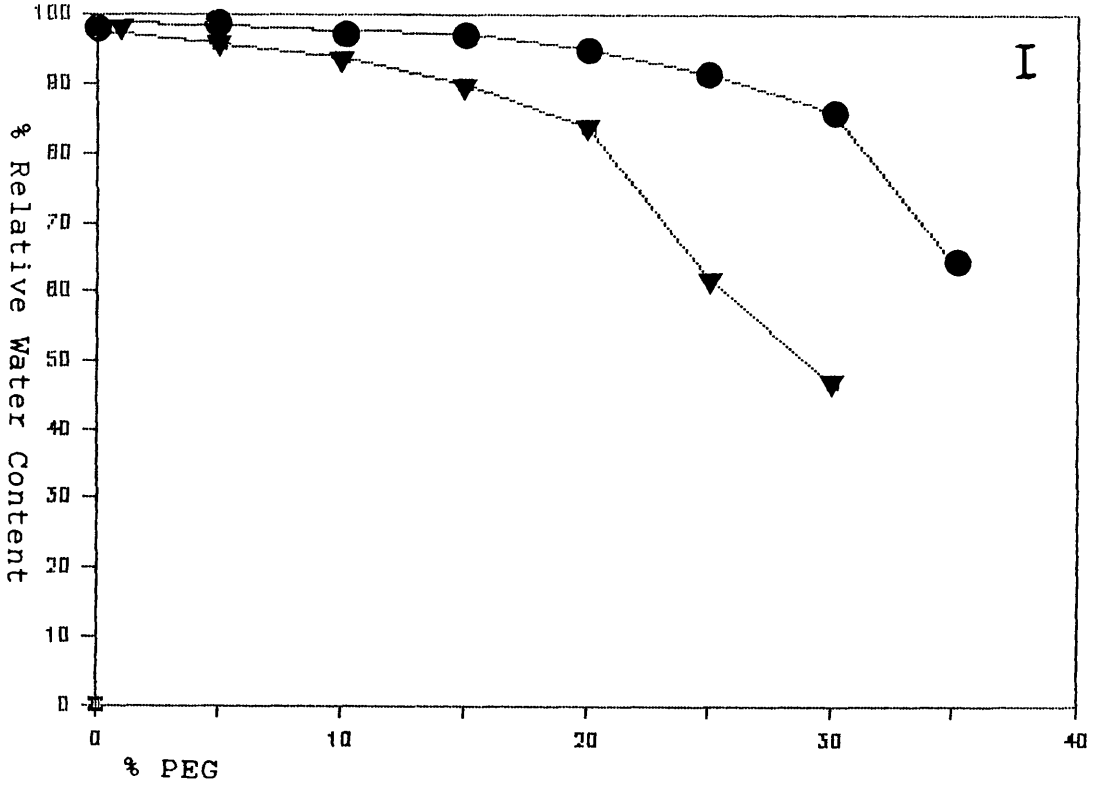


Figure 2.8.0 to show the decline in young leaf relative water content (RWC) of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000.

Bar indicates L.S.D. ($P < 0.05$)

Figure 2.9.0

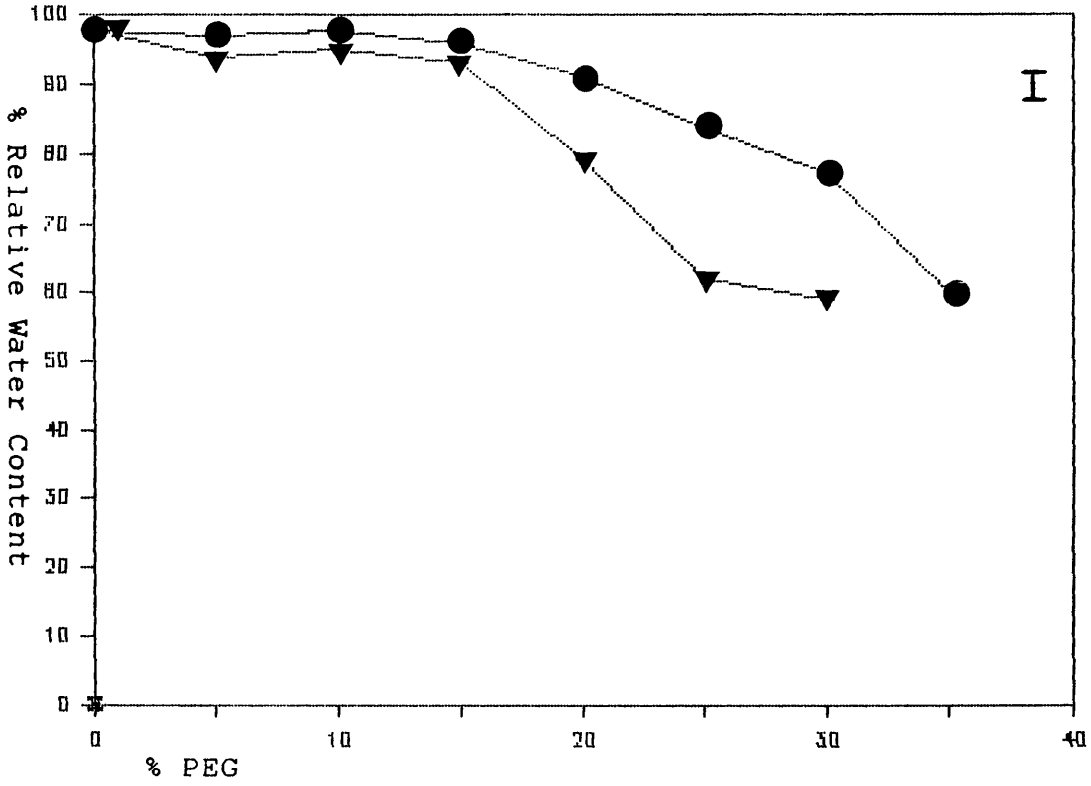


Figure 2.9.0 to show the decline in root relative water content (RWC) of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000.

Bar indicates L.S.D. ($P < 0.05$)

Figure 2.10.0

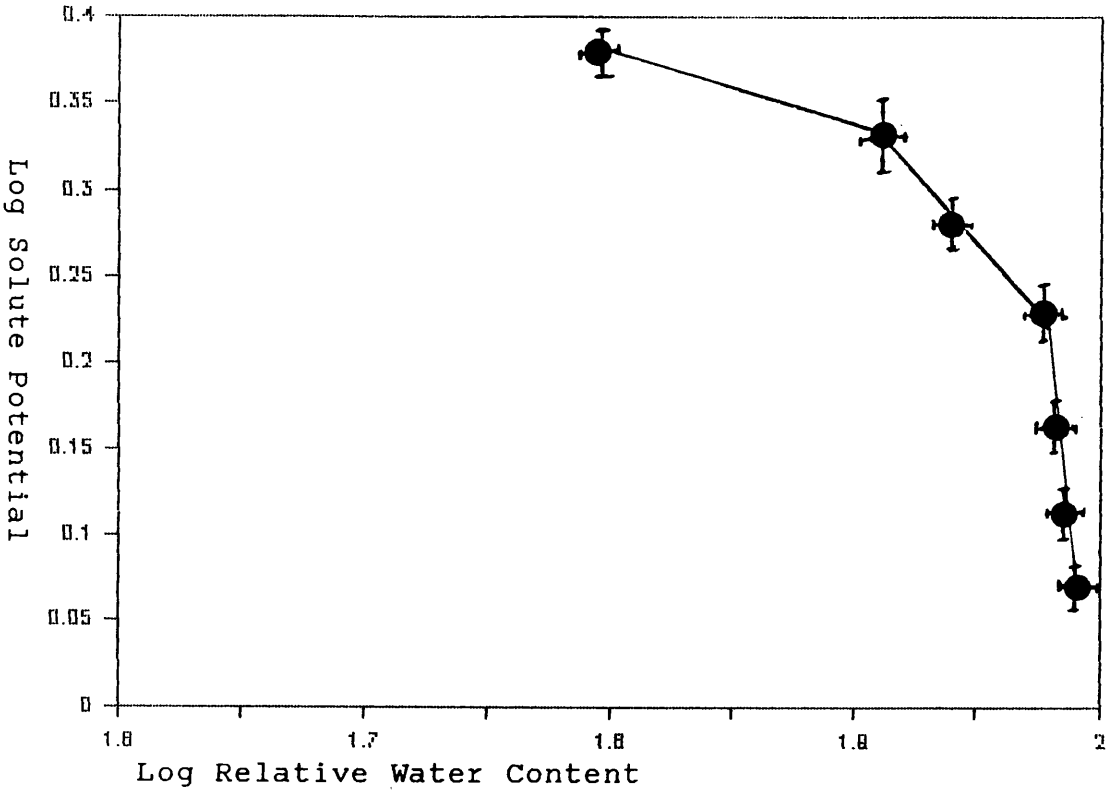


Figure 2.10.0 is a plot of log relative water content against log solute potential in mature leaves of G. urbanum during water deficits.

Bars indicate standard deviation

Table 2.1.0. To show the slopes of the lines produced in stages 1, 2 and 3 in figure 2.10.0.

Stage	Slope
Stage 1	0.110
Stage 2	0.897
Stage 3	3.890

Figure 2.11.0

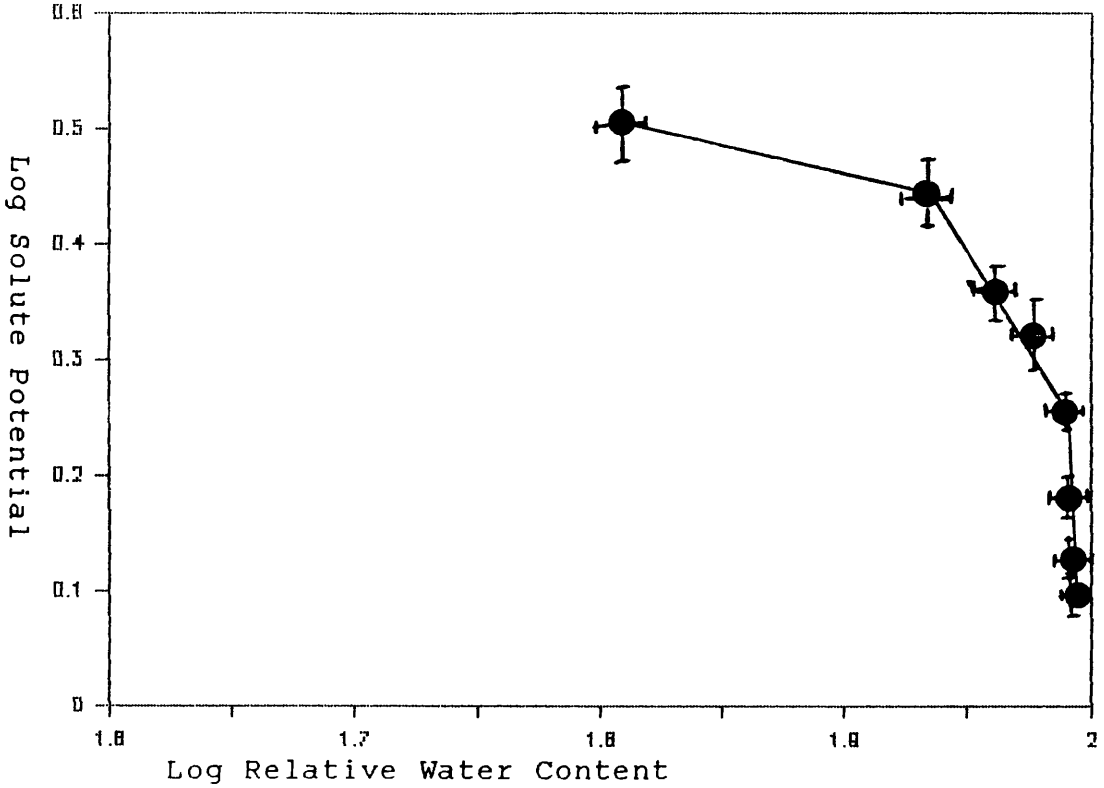


Figure 2.11.0 is a plot of log relative water content against log solute potential in young leaves of G. urbanum during water deficits.

Bars indicate standard deviation

Table 2.1.1. To show the slopes of the lines produced in stages 1, 2 and 3 in figure 2.11.0.

Stage	Slope
Stage 1	0.115
Stage 2	0.392
Stage 3	3.200

Figure 2.12.0

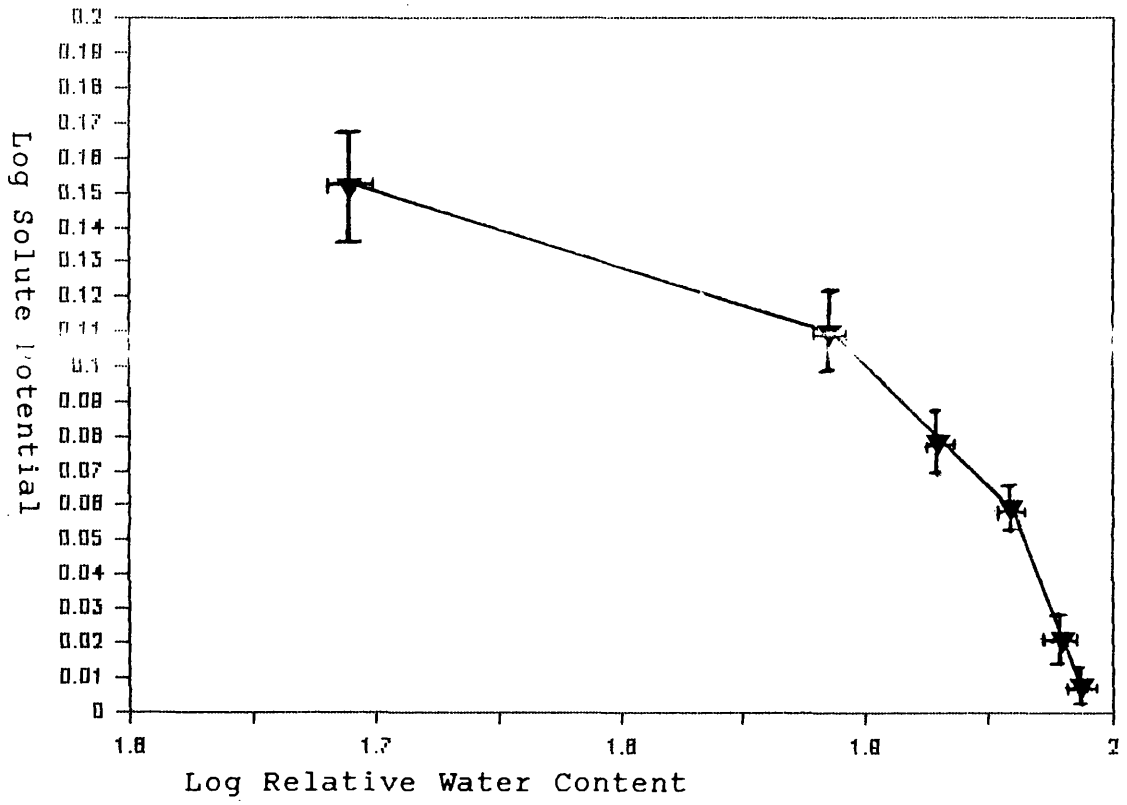


Figure 2.12.0 is a plot of log relative water content against log solute potential in mature leaves of G. rivale during water deficits.

Bars indicate standard deviation

Table 2.1.1. To show the slopes of the lines produced in stages 1, 2 and 3 in figure 2.12.0.

Stage	Slope
Stage 1	0.377
Stage 2	1.090
Stage 3	3.530

Figure 2.13.0

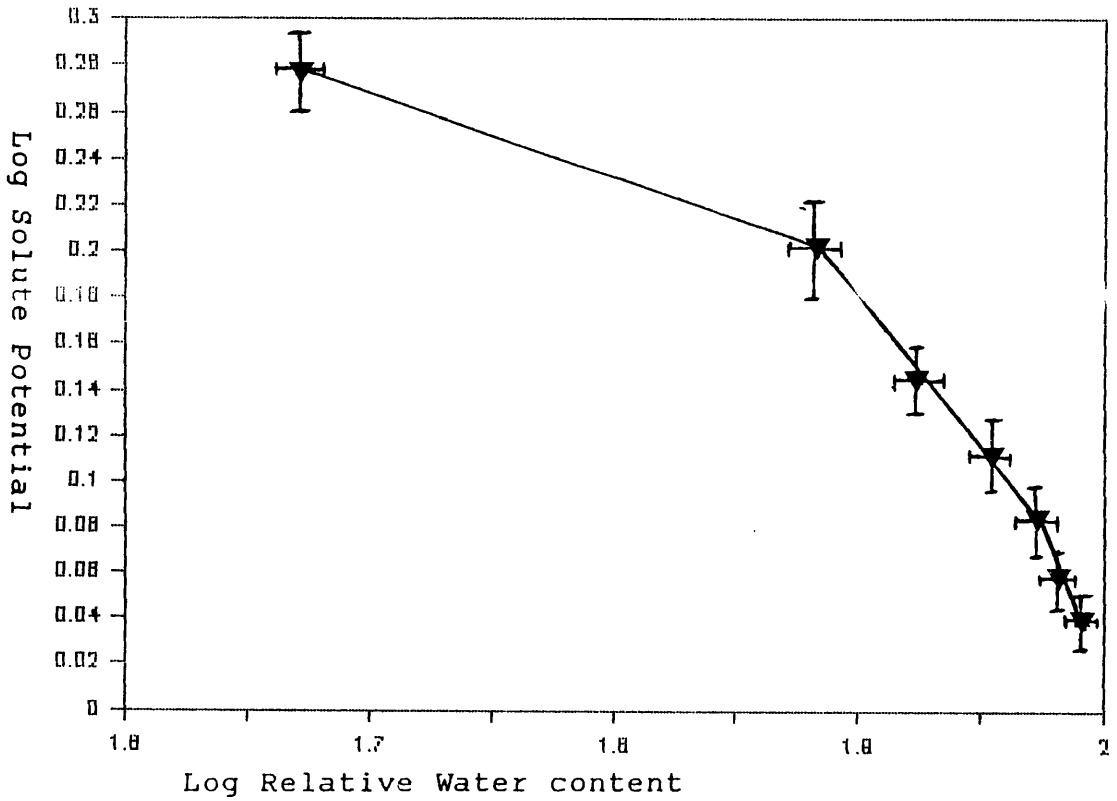


Figure 2.13.0 is a plot of log relative water content against log solute potential in young leaves of G. rivale during water deficits.

Bars indicate standard deviation

Table 2.1.2. To show the slopes of the lines produced in stages 1, 2 and 3 in figure 2.13.0.

Stage	Slope
Stage 1	0.392
Stage 2	0.941
Stage 3	3.040

Figure 2.14.0

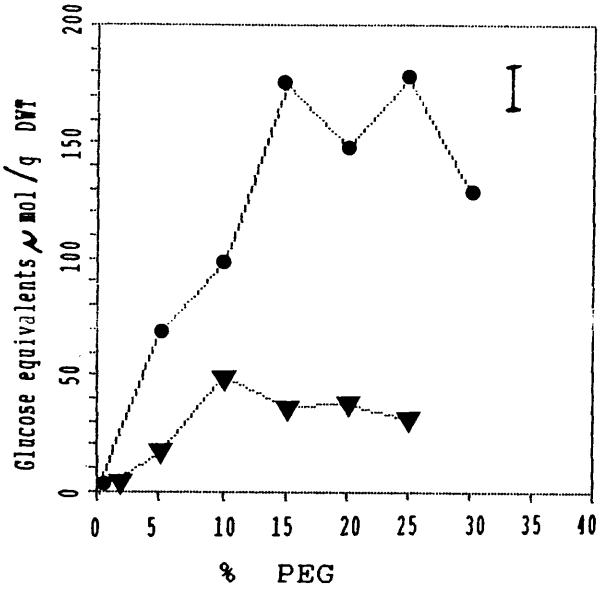


Figure 2.14.1

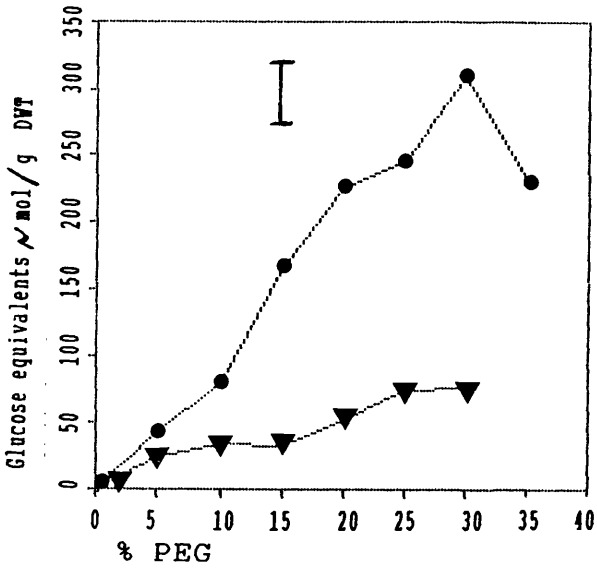
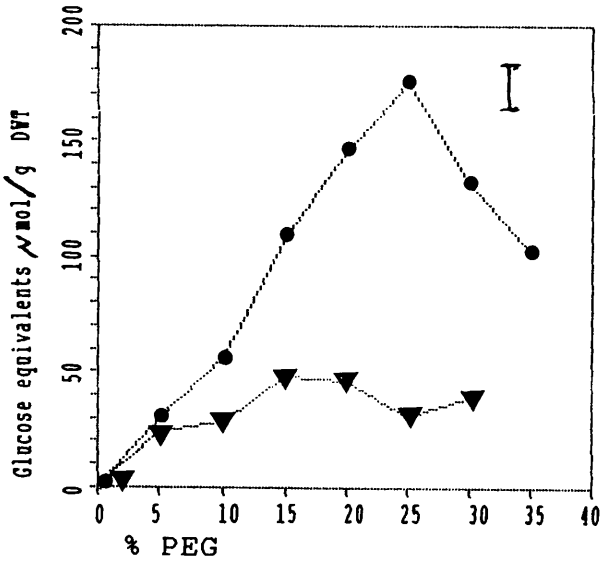


Figure 2.14.2



Bars indicate L.S.D. ($P < 0.05$)

Figures 2.14.0 to 2.14.2 show the increase in free carbohydrates from control levels in mature leaves (fig 2.14.0), young leaves (fig 2.14.1) and roots (fig. 2.14.2) of G. urbanum ● and G. rivale ▼ in response to water deficits imposed by PEG 6000.

Figure 2.15.0

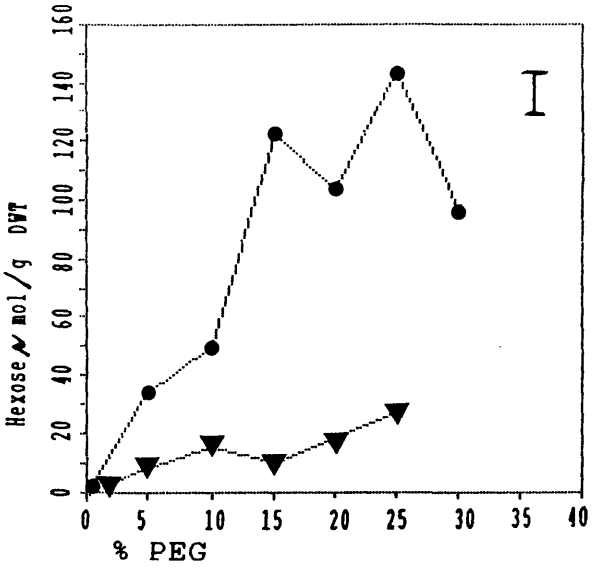


Figure 2.15.1

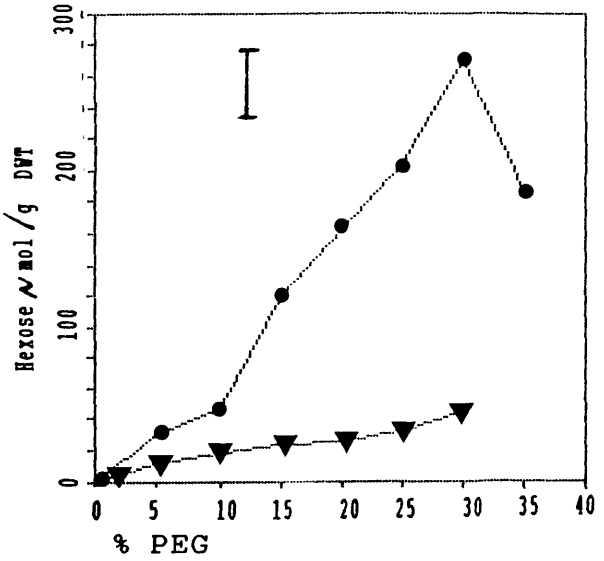
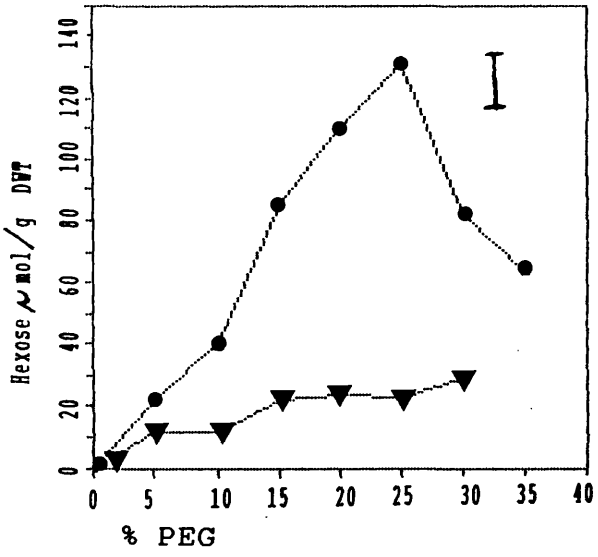


Figure 2.15.2



Bars indicate L.S.D. ($P < 0.05$)

Figures 2.15.0 to 2.15.2 show the increase in free hexose sugars from control levels in mature leaves (fig 2.15.0), young leaves (fig 2.15.1) and roots (fig. 2.15.2) of G. urbanum ● and G. rivale ▼ in response to water deficits imposed by PEG 6000.

Figure 2.16.0

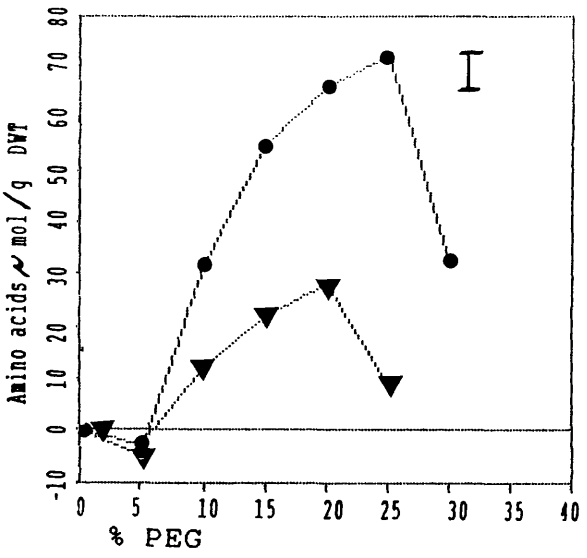


Figure 2.16.1

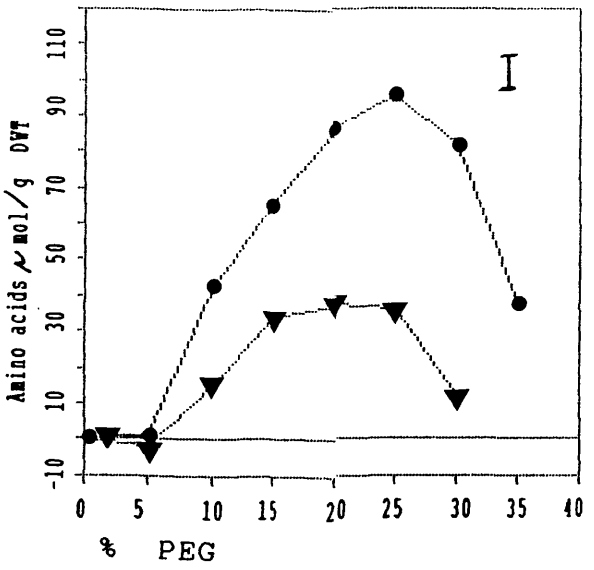
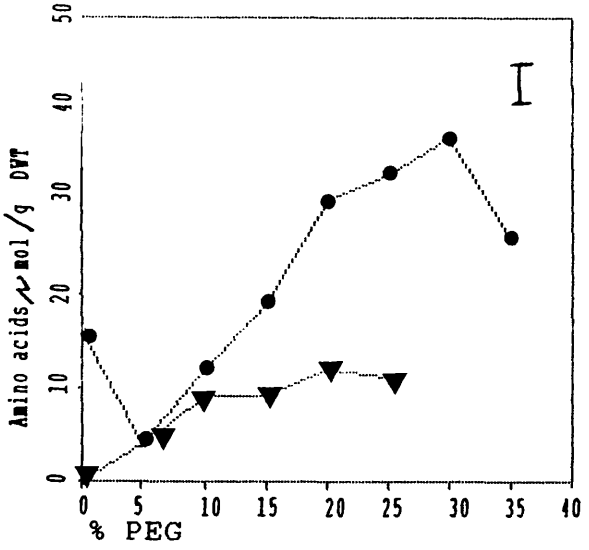


Figure 2.16.2



Bars indicate L.S.D. ($P < 0.05$)

Figures 2.16.0 to 2.16.2 show the increase in free amino acids from control levels in mature leaves (fig 2.16.0), young leaves (fig 2.16.1) and roots (fig. 2.16.2) of G. urbanum ● and G. rivale ▲ in response to water deficits imposed by PEG 6000.

Table 2.2.0. To show the contribution the measured solutes make to Ψ_s reduction in mature leaves of G.urbanum.

%PEG	$\Delta\Psi_s$ -MPa	Δamino acids ν mol	ΔCHO ν mol	Δ total solutes ν mol	Ψ_s solutes -MPa	% contribution
5	0.12	-5	51	46	0.115	96
10	0.28	32	72	104	0.26	93
15	0.52	55	148	203	0.51	98
20	0.74	66	126	192	0.48	65
25	0.97	72	160	232	0.58	60
30	1.22	33	113	146	0.365	30

Table 2.2.1 To show the contribution the measured solutes make to Ψ_s reduction in young leaves of G. urbanum

%PEG	$\Delta\Psi_s$ -MPa	Δamino acids ν mol	ΔCHO ν mol	Δtotal solutes ν mol	Ψ_s solutes -MPa	% contribution
5	0.1	1	37	38	0.095	95
10	0.27	43	63	106	0.265	98
15	0.56	66	144	210	0.525	94
20	0.85	87	194	281	0.702	83
25	1.05	95	224	319	0.797	76
30	1.55	82	290	372	0.93	60
35	1.95	38	208	246	0.615	32

Table 2.2.2. To show the contribution the measured solutes make to Ψ_s reduction in mature leaves of G. rivale

%PEG	$\Delta\Psi_s$ -MPa	Δ amino acids ~mol	ΔCHO ~mol	Δ total solutes ~mol	Ψ_s solutes -MPa	% contribution
5	0.03	-3	12	9	0.022	75
10	0.13	12	32	44	0.11	85
15	0.18	22	18	40	0.1	56
20	0.27	28	28	56	0.14	52
25	0.4	9	31	40	0.1	25

Table 2.2.3. To show the contribution the measured solutes make to Ψ_s reduction in young leaves of G. rivale

%PEG	$\Delta\Psi_s$ -MPa	Δ amino acids ~mol	ΔCHO ~mol	Δ total solutes ~mol	Ψ_s solutes -MPa	% contribution
5	0.05	-2	18	16	0.04	80
10	0.12	15	26	41	0.102	85
15	0.2	34	28	62	0.155	77
20	0.3	38	39	77	0.192	64
25	0.5	36	53	89	0.222	44
30	0.8	12	60	72	0.18	22

DISCUSSION

As root responses have been eliminated as a reason for the differing water deficit tolerances of Geum urbanum and Geum rivale, stomatal closure could have played some part in this difference. Both species closed their stomata in response to increasing water deficits (Fig. 2.2.0) a phenomenon which has been well documented for other species by many other authors (e.g. Boyer 1970). At each stress level there was no significant difference between the stomatal conductances of the two species. Thus, stomatal closure can not account for the differences in stress tolerance between the two species.

There is also no difference in photosynthetic rate between the two Geum species (Fig 2.1.0) which would discount this process as being the key to the different abilities of Geum urbanum and Geum rivale to resist a period of water stress due to increased levels of carbohydrate from photosynthesis. Indeed Graves (1984) also reported Geum urbanum and Geum rivale to have very similar photosynthetic responses in relation to other environmental pressures.

Differences in levels of osmoregulation are however apparent at all stress levels between the two species and between all plant parts. Geum urbanum consistently exhibited a higher Ψ_w (Figs. 2.3.0 and 2.5.0) at all stress levels which enabled a greater potential for water uptake than Geum rivale. The reduction in Ψ_w was mainly produced by a reduction in Ψ_s in both species. It is implicit in Figs. 2.10.0 to 2.13.0 Geum urbanum could decrease Ψ_s by accumulating solutes to a greater extent than Geum rivale which relied on a concentration of solutes to reduce Ψ_s far more than Geum urbanum. Supporting evidence is supplied by the solute data (Figs. 2.14.0 to 2.16.2) where Geum urbanum accumulated more solutes in response to water stress in all plant parts at most stress levels than Geum rivale. Data

presented in Tables 2.2.0 to 2.2.3 also provides further evidence that Geum urbanum reduced Ψ_s by a greater amount of solute accumulation in percentage terms. Geum urbanum could also maintain turgor (Figs. 2.4.0 and 2.6.0) at a higher level than Geum rivale at higher stress levels which implies Geum urbanum could maintain expansive growth at these levels. When these data are compared with the specific leaf expansion data (Figs. 1.12.0 to 1.12.3) presented in Chapter 1 it is apparent that this could be the main reason for the greater expansive growth achieved by Geum urbanum. It is therefore proposed that Geum urbanum can withstand water deficits better than Geum rivale due to its ability to reduce Ψ_s by the accumulation of solutes rather than rely on concentration effects which Geum rivale largely utilises. The degree of osmoregulation a plant exhibits has been correlated with its ability to resist drought (Davis and Mooney 1986, Walter and Stadelmann 1974) and it appears that this view is supported here by the results for the two Geum species.

Osmoregulation has been demonstrated in many species in response to declining water potential in expanding organs such as roots (Sharp and Davis 1979) and leaves (Jones and Turner 1980; Michelena and Boyer 1982) however, this capacity may be lost or decreased on completion of expansion (Ackerson and Hebert 1981b). The ability to osmoregulate can be increased in older leaves however by diurnal fluctuations in humidity and radiation (Morgan 1980). The ability to osmoregulate was not lost in the older leaves of Geum urbanum or Geum rivale, but was reduced in both species when compared to expanding leaves. The reasons for this difference in osmoregulation of leaves is not fully understood but it is possible that declining photosynthesis in older leaves and the higher demand for assimilates in expanding tissues and other plant parts may have some

role in this phenomenon. As expanding leaves rely on imported solutes to generate turgor (Molz and Boyer 1978) the plant may sacrifice older leaves reaching the end of photosynthetic competence in order to maintain new growth, which may be advantageous after stress relief. A second advantage may also be conferred to the plant by this mechanism in that the new leaf will have been expanded later in the ontogeny of the plant and may thus be able to osmoregulate to a higher level than the older leaf (Condon 1982) and hence survive at higher stress levels.

A high level of osmoregulation has been correlated with the maintenance of open stomata and hence a higher photosynthetic rate throughout stress (Wright et al. 1983). However this does not occur in the two Geum species as there is no significant difference in stomatal conductance or net photosynthetic rate during PEG imposed water deficits.

From Figs. 2.14.0 to 2.16.2 it is apparent that carbohydrates are accumulated before amino acids in all plant parts and in greater quantities in leaves. This is consistent with findings of other workers (Munns and Weir 1981; Osonubi and Davis 1978) and probably appears because there are greater storage pools of carbohydrate as opposed to amino acids in the plant and sugars are freely available from photosynthesis. Within the carbohydrate pool, hexoses and larger carbohydrates (assumed to be sucrose) were accumulated in both species. Pentose sugars were not accumulated in either Geum urbanum or Geum rivale, Which is consistent with the results obtained by other workers (Popp 1984). The absence of inorganic ion accumulation in the two species to increasing water deficits is not uncommon (Cutler and Rains 1978; Cutler et al. 1977), neither is an absence of QAC s (Story and Wyn Jones 1977) as exhibited by Geum rivale or little accumulation (Coughlan and Wyn Jones

1980) as shown by Geum urbanum, as this solute tends to be associated with salt tolerance rather than drought stress (Story & Walker et al. 1977). It is also apparent that the accumulation of solutes in the root occurs simultaniously with leaf solute accumulation which agrees with the data of Drossopoulos et al. (1987).

When the pressure log/log plots (Figs. 2.10.0 to 2.13.0) produced by Geum urbanum and Geum rivale are compared with those of Morgan (1980), differences are apparent. Morgan showed that water deficits induced by withholding water from six inch pots produced a monophasic or biphasic response in the leaves of various wheat species. The consistent triphasic response shown by the two Geum species in all plant parts would appear to be at odds with these data. This implies that wheat shows a different response to Geum or there is fault with one or other methodology. As Morgan produced a stress by withholding water from a peat/sand based system, drying rates may have been different between pots which may have produced altered osmoregulation between the treatments as found by other workers (Jones and Rawson 1979). Morgan to his credit noted a difference in drying rates between pots and produced a plant Ψ_w of -5 MPa in 6-10 days. Compared to the experiments here a far faster rate of stress development was used which may have produced such different results. Morgan also noted that each plant was sampled every 2-3 days which in some cases would represent half the drying time of some plants. It is therefore possible that the phases presented in my figures could have been missed by Morgan or that such a rapid drying rate altered the osmoregulatory patterns exhibited by the plants. It is then, my contention that species undergoing water defects produced at a slow rate which reduce Ψ_s by accumulating solutes, would produce a similar three (or possibly more) stage response when stressed to their

fullest extent. Firstly a period of osmoregulation where the accumulation of solutes plays a major role in Ψ_s reduction at mild stress levels. This is then followed by a period of reduced solute accumulation where a concentration of solutes plays a larger role in the reduction in Ψ_s at more severe stresses. Finally a period of solute loss is apparent when the level of water deficit becomes damaging to the plant. It is difficult to find support for this contention in the literature, mainly because studies rarely stress plants to their ultimate limit, where the third stage occurs. It is also difficult to draw conclusions from experiments using a rapid stress regime and comparisons between experiments using different methodologies. However, some support is available from studies which do not stress their plants to the ultimate level and hence show a two phase osmoregulation pattern.

Wilson and Ludlow (1983) demonstrated osmotic adjustment exhibited a two stage trend in the three species they studied which was also apparent in the soluble carbohydrates they measured throughout stress. Pearson and Stewart (1987) also showed the same trend in a variety of barley and wheat cultivars, where total amino acids and total carbohydrates followed such a two stage accumulation.

The loss of solutes at stage three is difficult to reconcile with the plants need to maintain a high Ψ_s. However, at such high stress levels when there is a net loss of carbon from the plant (Fig. 2.1.0) solutes such as carbohydrates and even the carbon skeletons of amino acids may be sequestered for use in respiration or other metabolism essential to the plants survival.

This chapter shows that the major reason why Geum urbanum has a higher water stress tolerance than Geum rivale is the ability of Geum urbanum to osmoregulate to a greater extent than Geum rivale at all stress levels.

It also shows the osmoregulatory process to be a three stage process which involves a period of high solute accumulation followed by a period of reduced solute accumulation and eventually a loss of solutes when stress is severe and damaging.

CHAPTER 3

ASPECTS OF CARBON METABOLISM *GEUM URBANUM* AND *GEUM RIVALE* IN RESPONSE TO WATER DEFICITS.

INTRODUCTION

In the previous chapter the greater water-deficit tolerance exhibited by *Geum urbanum* as compared to *Geum rivale* was attributed to the larger solute accumulation which it achieved throughout stress to reduce Ψs. Of the solutes present, carbohydrates were shown to be accumulated to higher levels in all plant parts in both species with *Geum urbanum* showing the highest accumulation at all stresses. The ability of *Geum urbanum* to accumulate such large amounts of carbohydrate was therefore a major factor contributing to its greater drought tolerance. Thus a study was undertaken to reveal the sources of carbohydrate which each plant species utilised and which might help to clarify the differences in water stress tolerance of the two species.

The availability of carbohydrates in an autotrophic plant is ultimately controlled by the rate of photosynthesis, the levels of stored carbohydrate and the demand for carbohydrate in metabolism. An attempt was therefore made to ascertain the relative proportions these carbohydrate sources made to the increase in carbohydrate in both species and to determine whether one particular source could account for the higher free carbohydrate pool attained by *Geum urbanum*.

It has long been known that photosynthesis declines during water stress and early workers, noting a close correlation between the reduction in photosynthesis and reductions in transpiration thus proposed a primarily stomatal mechanism for the reduction of photosynthesis during water deficits (Barrs 1968). The main mechanism by which stomata control photosynthesis is by reducing the diffusion of CO_2 from the atmosphere to the plant.

However other resistances to CO_2 diffusion are present. One component is the boundary layer conductance to CO_2 which is largely independent of water stress, the other being what is termed the mesophyll conductance to CO_2. This is not a conductance as such but represents the barriers to photosynthesis caused by the light and dark reactions of photosynthesis. More recently workers have reported reductions in mesophyll conductance during water deficits including: inhibition of the light reactions of photosynthesis (Boyer and Bowen 1970; Boyer 1976); inhibition of photophosphorylation (Keck and Boyer 1974); and inhibition of the dark reactions of photosynthesis (Jones 1973), all of which may cause a reduction in photosynthetic rate during water stress. Much controversy has been created in the literature by such findings (and the lack of them), so it was decided to determine if the mesophyll conductance did vary in the two Geum species and to try and evaluate any contribution this may have in reducing photosynthetic rate in the two plants.

MATERIALS AND METHODS

A: Plant culture conditions.

The culture conditions of Geum urbanum and Geum rivale were identical to those stated in Chapter 1.

B: The Measurement Of Photosynthesis

In order to undertake an investigation concerning stomatal and non-stomatal limitations to photosynthesis two methods of measuring photosynthesis were utilised. Measurements were made using an IRGA under normal atmospheric conditions where stomata control the passage of CO_2 into the plant and thus a measure of photosynthesis can be made which include stomatal limitations to CO_2 fixation. The other method of photosynthesis measurement was by oxygen electrode where the leaf under observation is maintained in an atmosphere saturated with CO_2 . This has the effect of negating the barrier to CO_2 diffusion caused by reductions in stomatal conductance. Thus, while the IRGA provides a measure of net carbon gain the oxygen electrode gives a measurement of net potential photosynthesis by removing the major diffusion barrier for CO_2 uptake. Moreover, the oxygen electrode provides a means by which differences in photosynthetic capacity in the light and/or dark reactions of photosynthesis can be determined. See appendix for the details of IRGA and oxygen electrode use.

C: Measurement of free carbohydrates by gas chromatography

Sugars, organic acids and sugar alcohols were determined using gas chromatography techniques. The details of sample preparation, derivatisation and chromatogram conditions may be found in the appendix.

D: Detection and counting of chloroplasts containing starch.

Thin sections of fresh leaf material were cut using a razor blade, mounted on a microscope slide bathed in

an IKI solution and viewed under a microscope. Amyloplasts appeared black/brown in colour and hence were easy to count. Chloroplasts not containing starch were detected by bringing the image in and out of focus until all chloroplasts were counted.

E: Starch determination

Starch was determined by the anthrone method of Mc Cready *et al.* (1950)

Total starch was then expressed as glucose equivalents.

F: Detection of amylase levels

Amylase was detected by the method of Chrispeels and Varner (1967). Five gram portions of leaf material were pulverised in liquid nitrogen in 15 ml of 10 mM calcium chloride with a mortar and pestle and the extract was then centrifuged at 8000 g for 25 minutes at 2 C. The supernatent was then used for analysis of amylolytic activity.

G: Ribulose bis phosphate carboxylase and phosph- enol-pyruvate assay.

Five to ten grams of leaf tissue were plunged into liquid nitrogen and ground in a mortar and pestle to which 200 mg of polyclar was added. The tissue was then further ground in 20 to 30 ml extraction buffer (see appendix for details). The extract was then centrifuged at 8000 g for 25 minutes at 2 ^{o}C. The resulting supernatent was used to determine RuBP carboxylase and PEP carboxylase rates of reaction.

(1) PEP carboxylase assay. The assay mixture contained 2.5 ml 50 mM MOPS; 0.1 ml 150 mM magnesium chloride; 0.1 ml 30 mM phospho-enol-pyruvate; 0.1 ml 130 mM NADH; 0.1 ml 1:50 dilution malate dehydrogenase and 0.1 ml extract. PEP was used to start the reaction. The rate of reaction was measured by the disappearance of NADH (using a spectrophotometer at 340 nm).

(2) RuBP carboxylase assay. The assay mixture contained 0.2 ml 5 mM phosphocreatin; 0.2 ml 1 mM ribose-5-

phosphate; 0.2 ml 10 mM sodium hydrogen carbonate; 0.1 ml extract; 3 units Creatin phospho kinase; 7.5 units phospho glycerate phospho kinase; 7.5 units NAD linked glyceraldehyde phospho dehydrogenase and 0.2 ml of assay mix. The assay mix was made up previously and contained 10 mM KCl, 15 mM MgCl , 1 mM EDTA, 5 mM DTT, 5 mM ATP, 50 mM HEPES and 330 mM sorbitol at pH 7.9. The enzyme extract was used to start the reaction and rate was determined by the disappearance of NADH (using a spectrophotometer at 340 nm).

RESULTS

Photosynthesis was measured by two methods; by IRGA and Oxygen electrode. Figs. 3.1.0 and 3.2.0 show the fall in net photosynthesis and stomatal conductance in Geum urbanum and Geum rivale when exposed to water stress as measured using the IRGA. These figures show that photosynthesis was reduced rapidly in both species and was coincidental with the reduction of stomatal conductance. No significant difference in photosynthesis or stomatal conductance was apparent between the species as water deficits increased. Fig. 3.3.0 shows the photosynthetic responses of Geum urbanum and Geum rivale in response to water deficits when measured by an oxygen electrode. These data show photosynthesis when measured under a non - limiting CO_2 environment and indicate that both Geum urbanum and Geum rivale could maintain photosynthesis up to the relevant point of stomatal closure in each species. After this point the rate of photosynthesis fell gradually up to the relevant wilting point of each species when the photosynthetic rate fell dramatically. Again no significant difference between the photosynthetic rates of the two species occurred during water deficits.

The dark respiration rates in leaves of both species in relation to water stress are shown in Fig. 3.4.0. These data indicate that the dark leaf respiration was maintained in both species until moderate stress was reached whereupon the respiration rate fell gradually. When the relevant wilting points of each species was reached then respiration declined rapidly.

The levels of phospho - enol - pyruvate carboxylase (PEPcase), Fig. 3.5.0, and ribulose 1,6 bis phosphate, Fig. 3.6.0 declined very gradually up to the wilting point of both species when the rates of both enzymes fell dramatically. Amylase however gradually rose in

older leaves in both species up to their respective wilting points but was maintained in young leaves (Figs. 3.7.0. and 3.8.0.).

There were also differences in the numbers of starch grains contained in chloroplasts between control young and old leaves and stressed leaves (Figs. 3.9.0. and 3.10.0.) which show an opposite trend to that of amylase. The results show that as stress increased the numbers of chloroplasts containing starch decreased in older leaves and increased in young non-photosynthetically competent leaves. There was also a significant difference between the numbers of chloroplasts containing starch remaining in old leaves between Geum urbanum and Geum rivale with Geum urbanum loosing more starch from chloroplasts than Geum rivale. There was an opposite effect in non-photosynthetically competent leaves where Geum urbanum deposited a significantly higher amount of starch in chloroplasts than Geum rivale.

Starch levels (Figures 3.11.0 and 3.12.0) followed a similar pattern to the results gained by light microscopy for chloroplasts containing starch by in that a loss of starch was recorded in older leaves and starch accumulation occurred in younger leaves as water deficits developed. As with starch containing chloroplasts number a significant difference occurred in the amount of starch lost from older leaves of Geum urbanum and Geum rivale and also the levels deposited in younger leaves in the two species.

The individual carbohydrates measured by GC (Table 3.1.0 to 3.1.5) show that fructose, glucose and sucrose were the major carbohydrates accumulated in both species throughout stress, in young leaves, old leaves and roots, with Geum urbanum having significantly higher sugar levels than Geum rivale. It is also apparent that glucose and fructose were the major sugars accumulated

in young and mature leaves of both species. However, Geum rivale accumulated proportionally more sucrose than Geum urbanum. In roots the proportions of glucose, fructose and sucrose were approximately equal in Geum rivale, whereas Geum urbanum accumulated glucose and fructose in equal proportions with sucrose as a minor component. These tables also show that there was no change in the levels or composition of sugar alcohols or organic acids in either species throughout water stress.

Figure 3.1.0

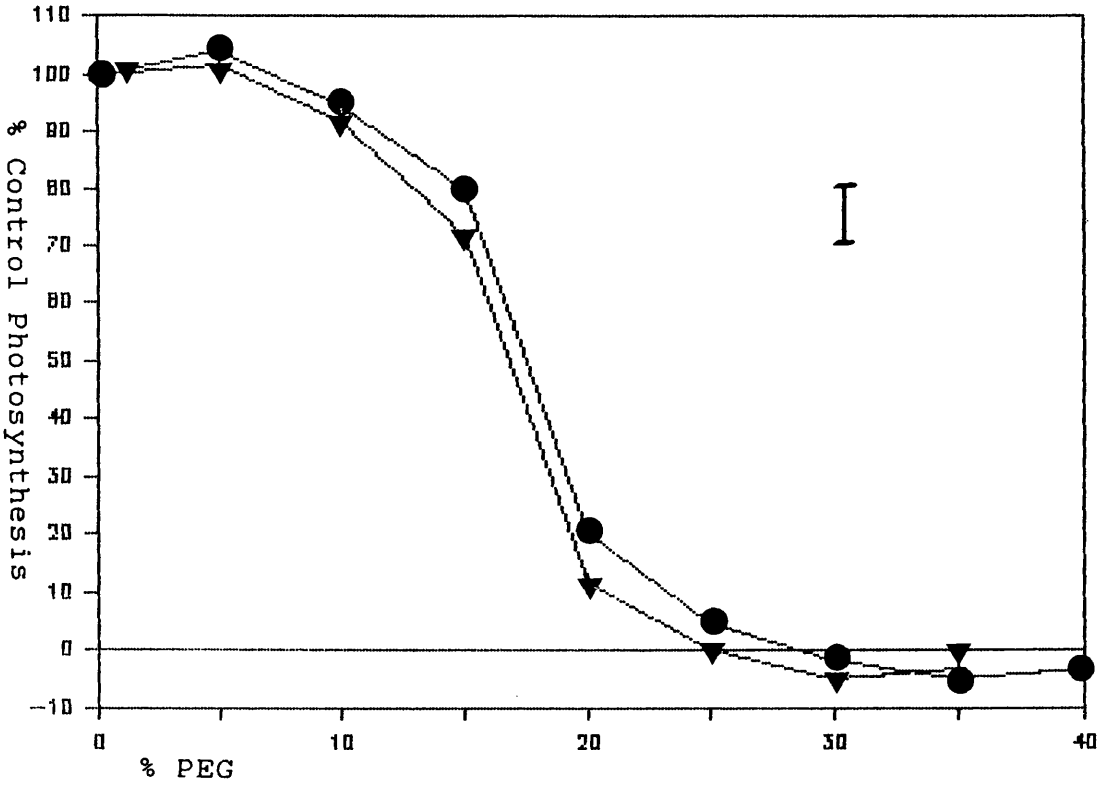


Figure 3.1.0 to show the decline in net photosynthetic rate as measured by IRGA of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as a percentage of control net photosynthesis.

Bar indicates L.S.D. ($P < 0.05$)

Control net photosynthetic rate (100%) values :

G. urbanum	24.26 m mol CO_2 m^{-2} s^{-1}
G. rivale	26.02 m mol CO_2 m^{-2} s^{-1}

Figure 3.2.0

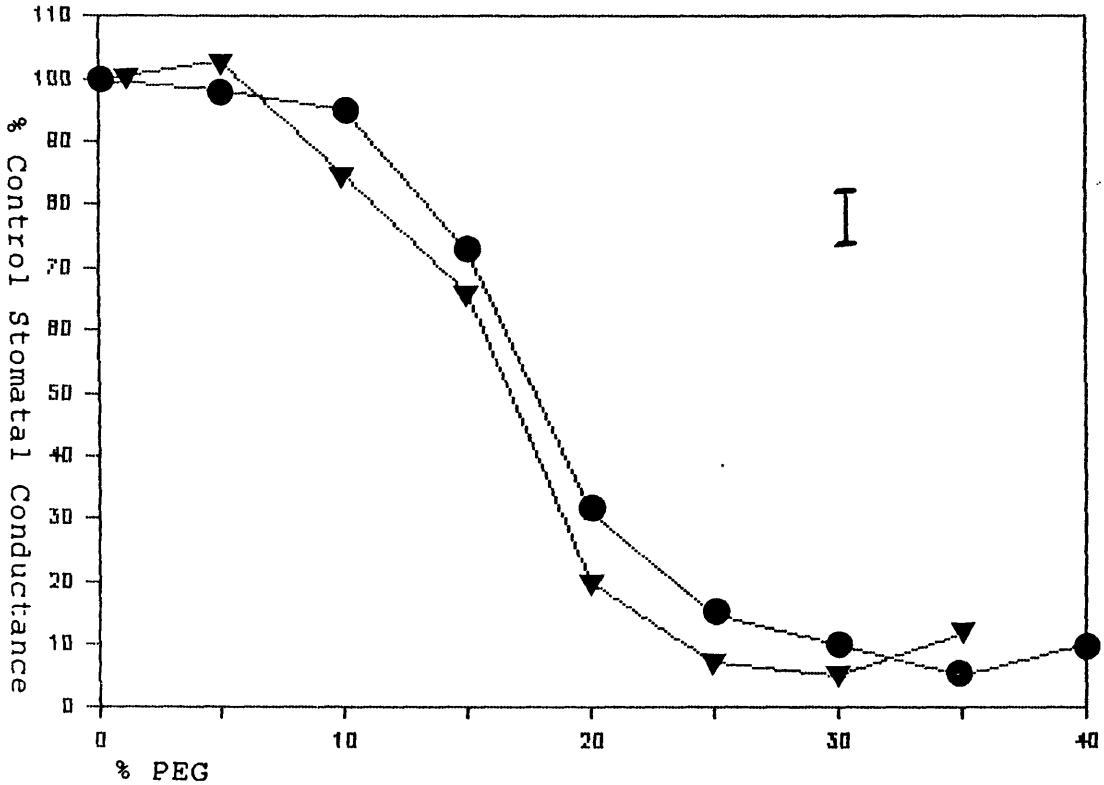


Figure 3.2.0 to show the decline in stomatal conductance as measured by IRGA of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as a percentage of control stomatal conductance.

Bar indicates L.S.D. ($P < 0.05$)

Control stomatal conductance (100%) values :

G. urbanum	0.698	mol $m^{-2} s^{-1}$
G. rivale	0.721	mol $m^{-2} s^{-1}$

Figure 3.3.0

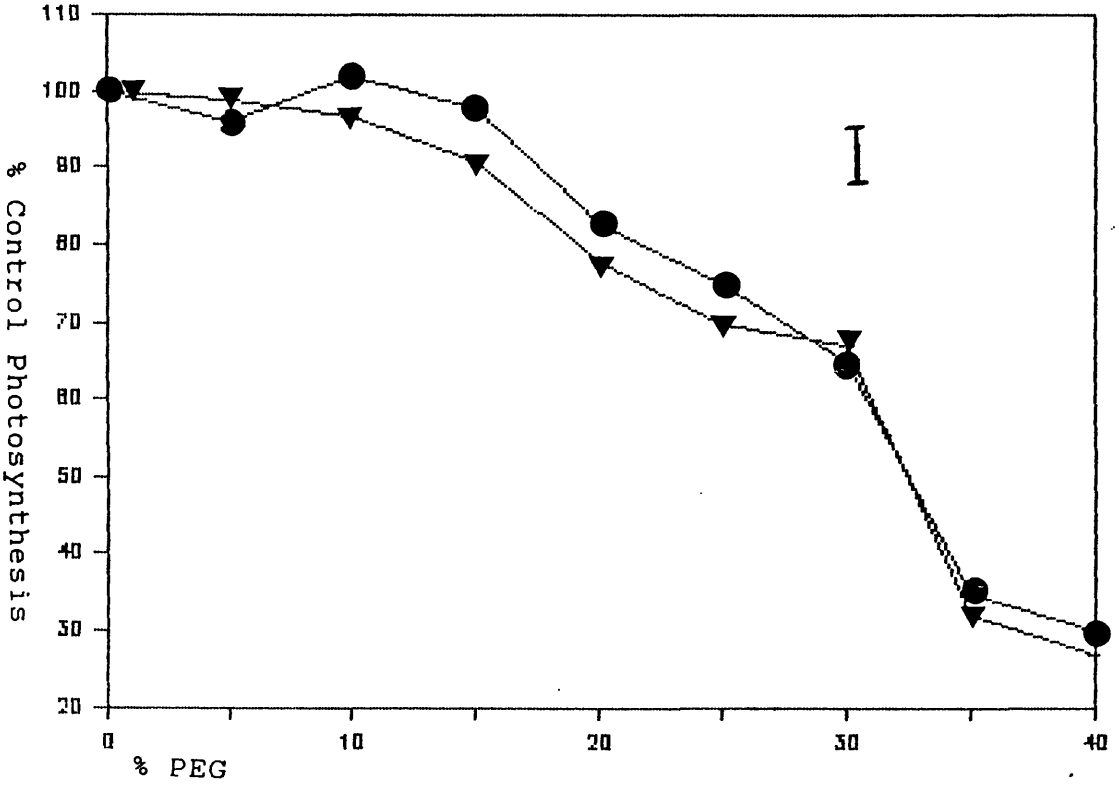


Figure 3.3.0 to show the decline in net photosynthetic rate as measured by oxygen electrode of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as a percentage of control net photosynthesis.

Bar indicates L.S.D. ($P < 0.05$)

Figure 3.4.0

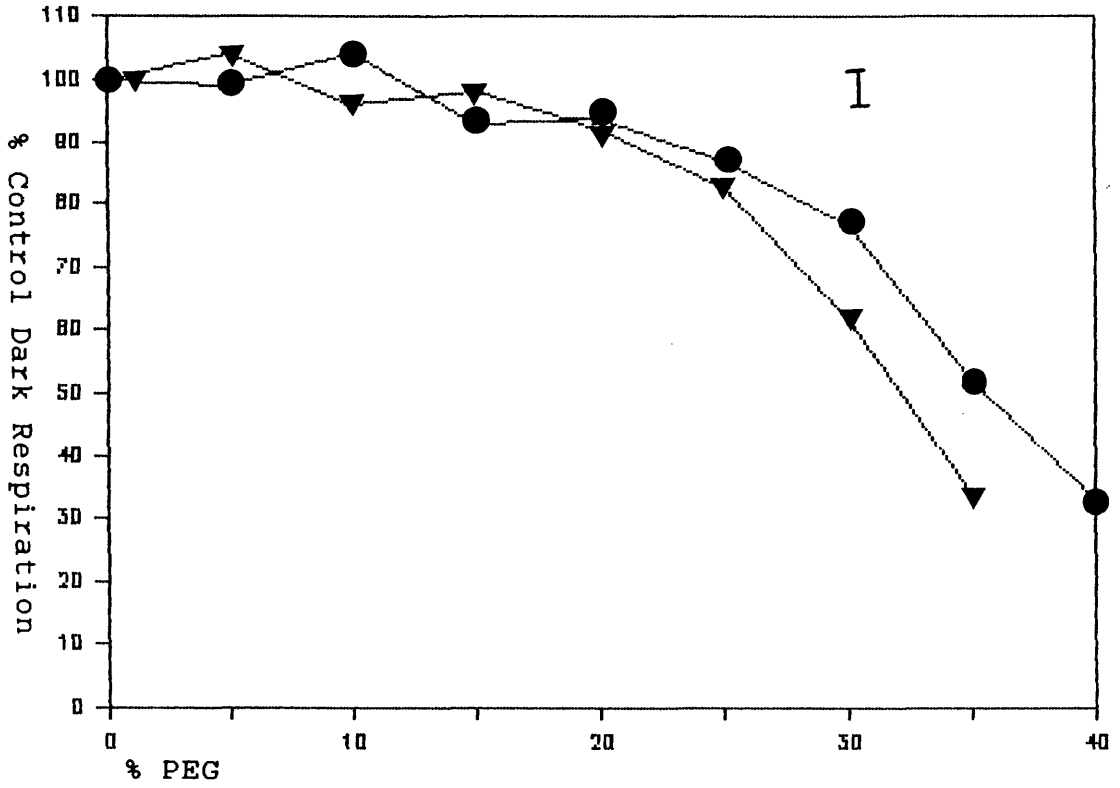


Figure 3.4.0 to show the decline in dark leaf respiration as measured by IRGA of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as a percentage of control dark respiration.

Bar indicates L.S.D. ($P < 0.05$)

Control leaf dark respiration rate (100%) values :

G. urbanum	2.01 m mol CO_2 m^{-2} s^{-1}
G. rivale	2.65 m mol CO_2 m^{-2} s^{-1}

Figure 3.5.0

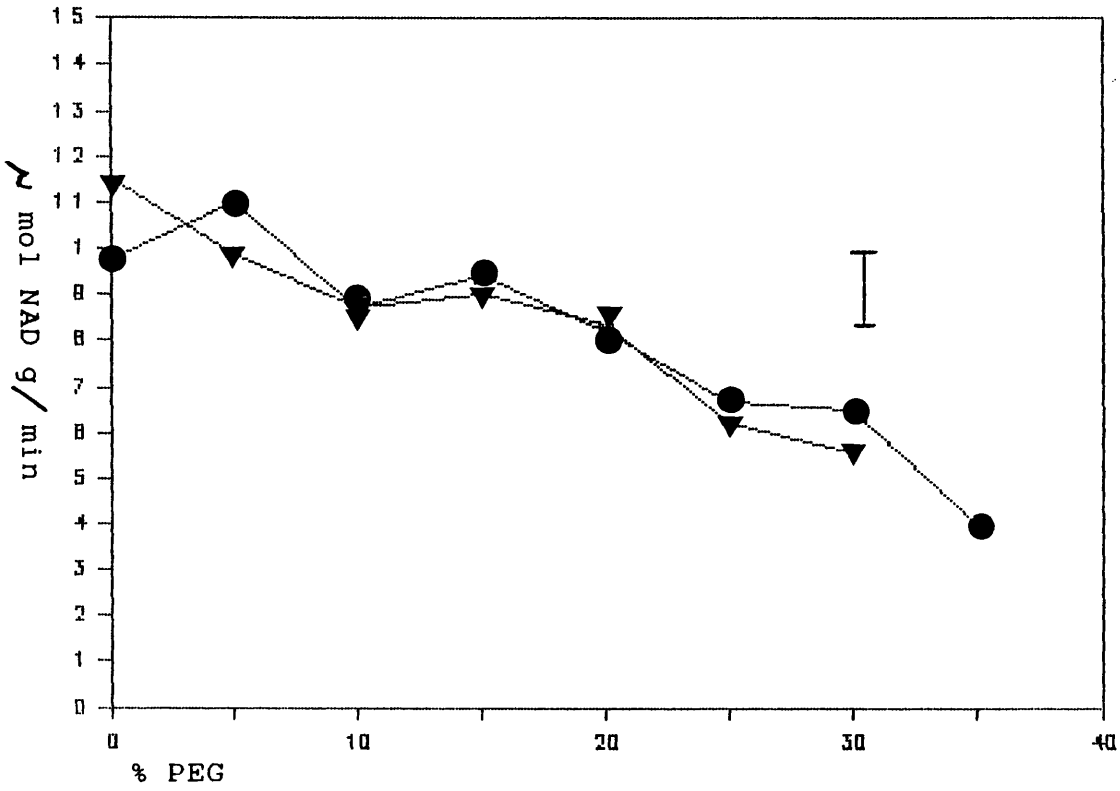


Figure 3.5.0 to show the decline in Phospo-Enol-pyruvate carboxylase activity of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in μ mol NAD produced / g dwt.

Bar indicates L.S.D. ($P < 0.05$)

Figure 3.6.0

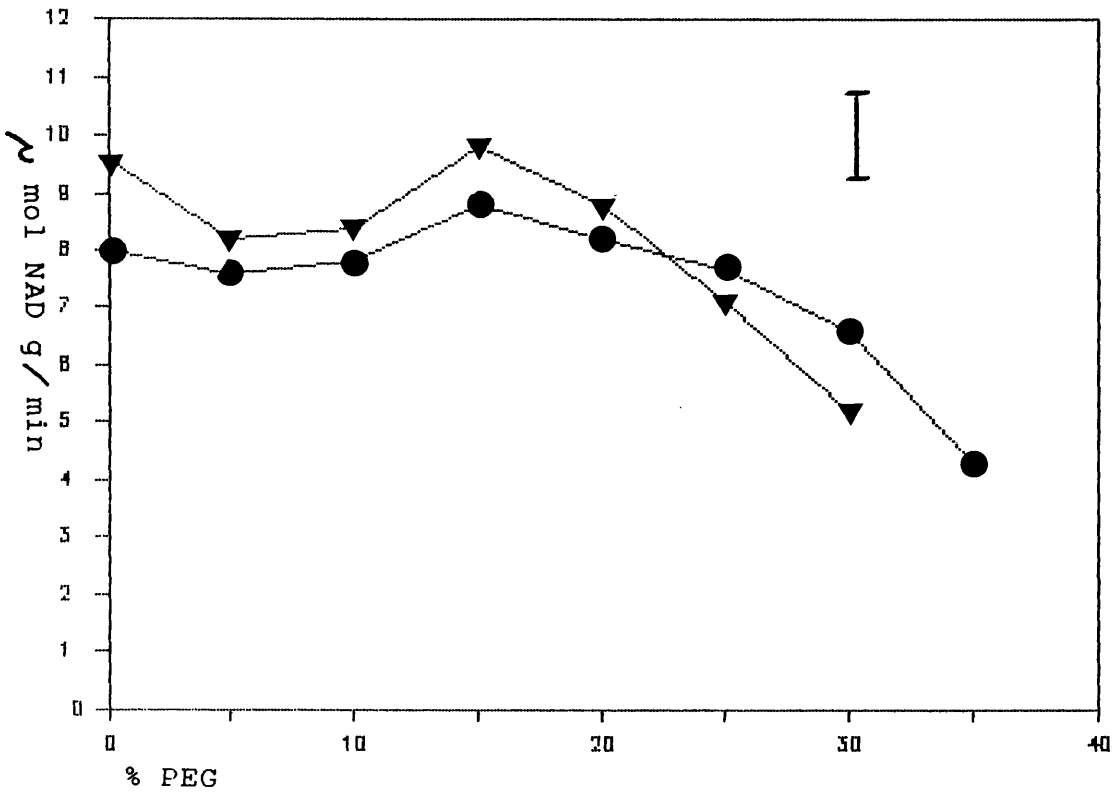


Figure 3.6.0 to show the decline in Ribulse bis-phosphate carboxylase activity of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in ~ mol NAD produced / g dwt.

Bar indicates L.S.D. ($P < 0.05$)

Figure 3.7.0

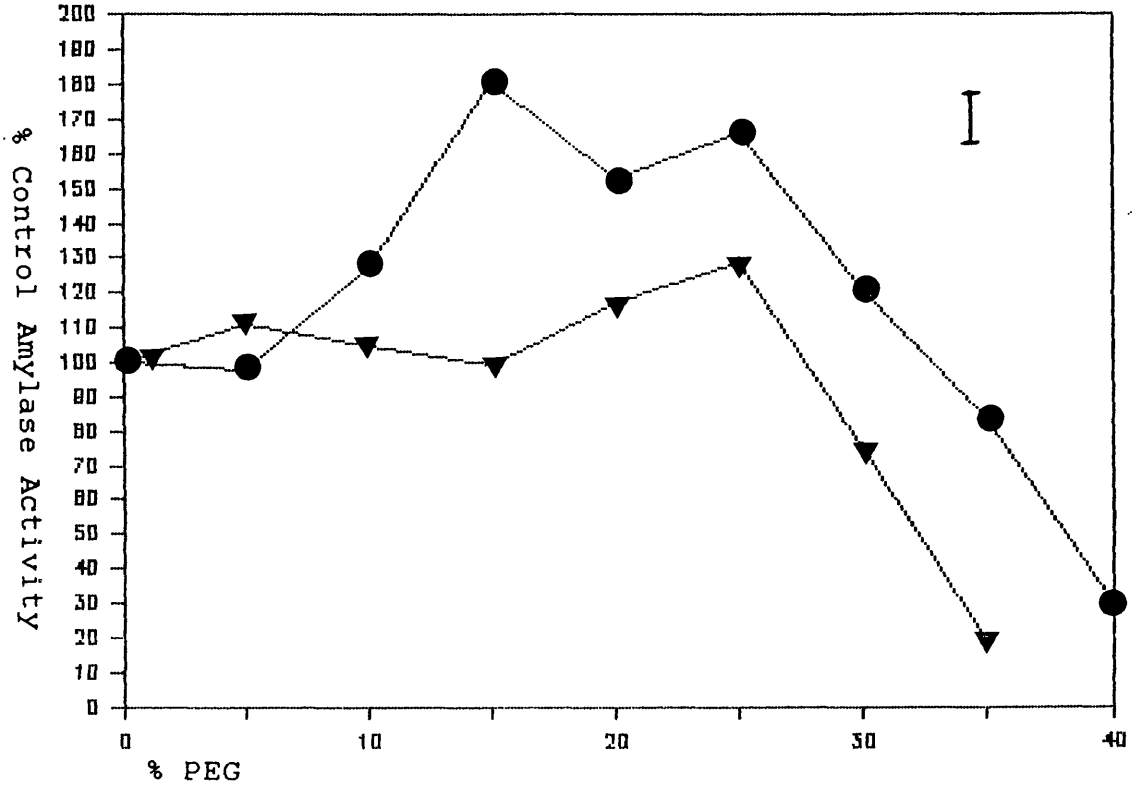


Figure 3.7.0 to show the changes in amylase activity in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as a percentage of control activity.

Bar indicates L.S.D. ($P < 0.05$)

Figure 3.8.0

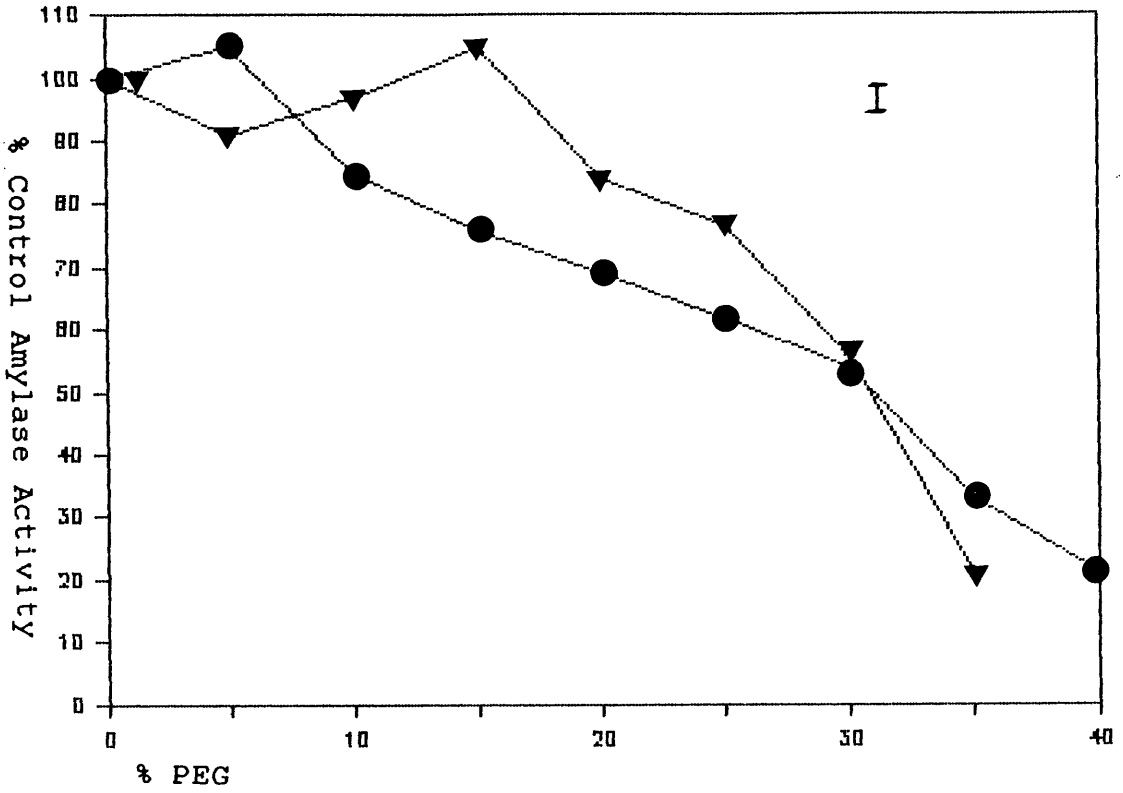


Figure 3.8.0 to show the changes in amylase activity in young leaves of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as a percentage of control activity.

Bar indicates L.S.D. ($P < 0.05$)

Figure 3.9.0

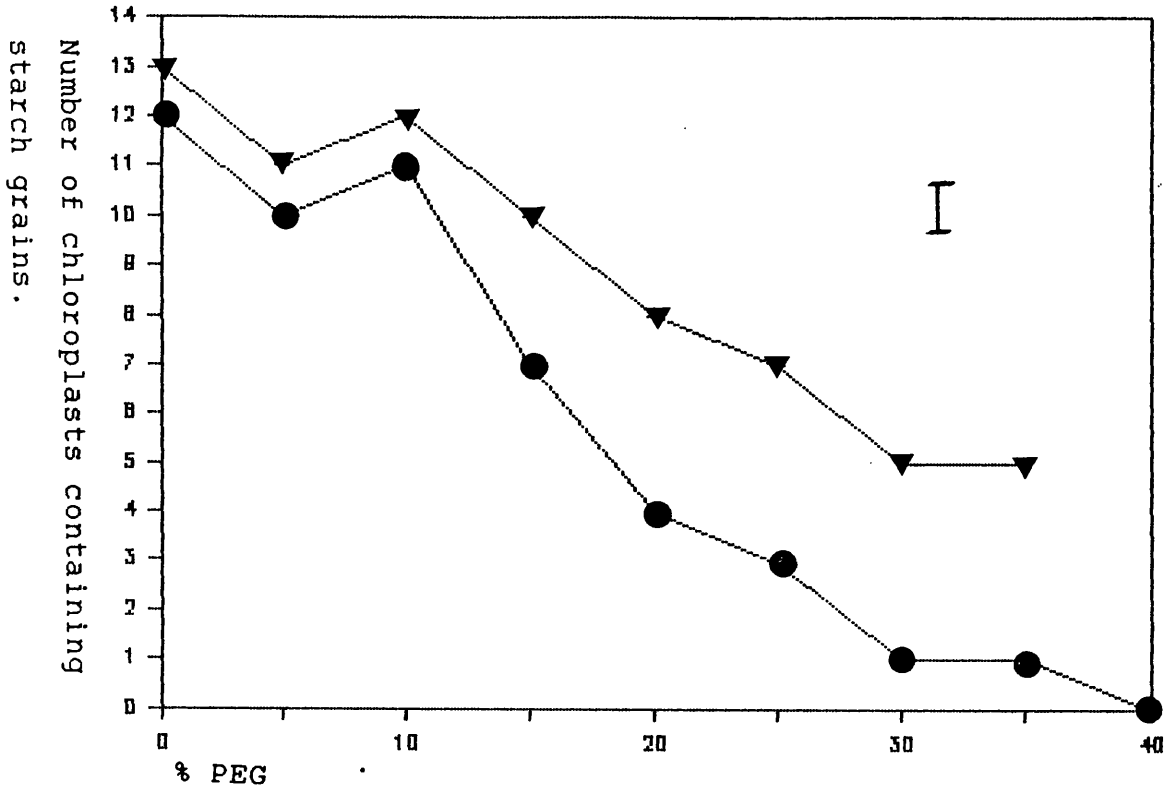


Figure 3.9.0 to show the number of chloroplasts containing starch grains in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000.

Bar indicates L.S.D. ($P < 0.05$)

Figure 3.10.0

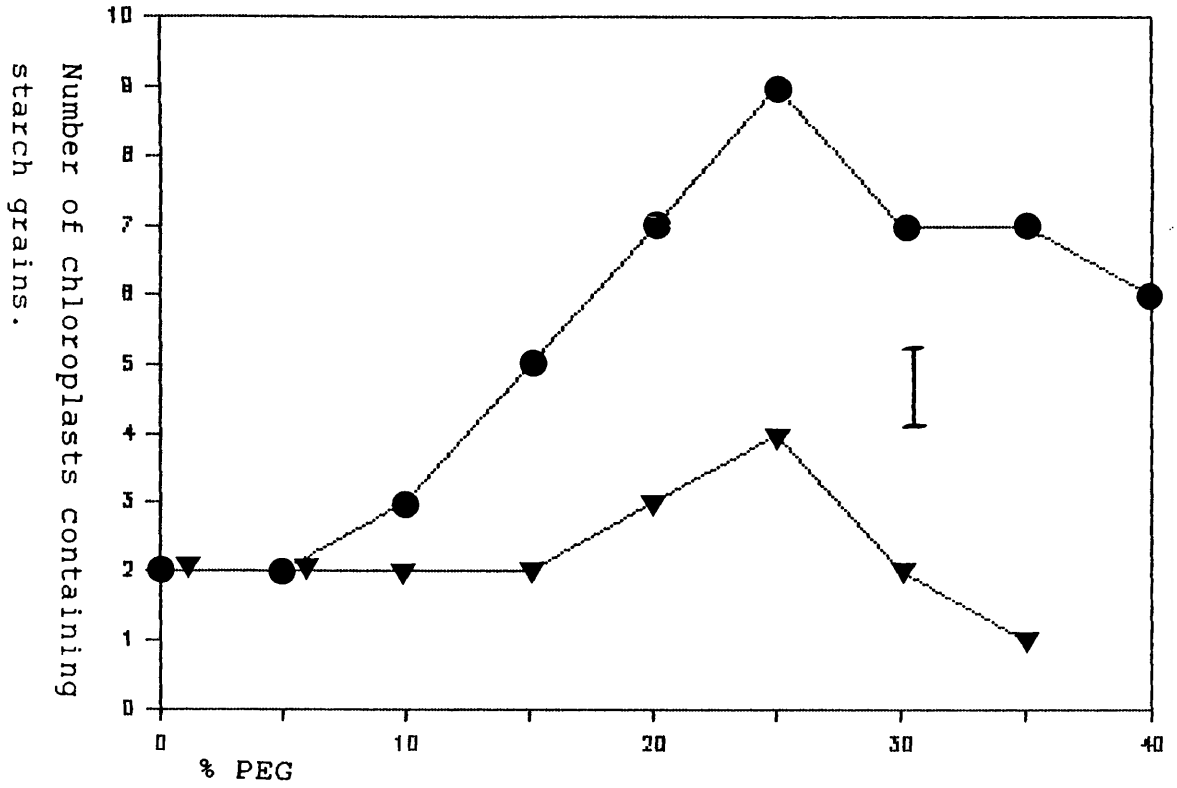


Figure 3.10.0 to show the number of chloroplasts containing starch grains in young leaves of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000.

Bar indicates L.S.D. ($P < 0.05$)

Figure 3.11.0

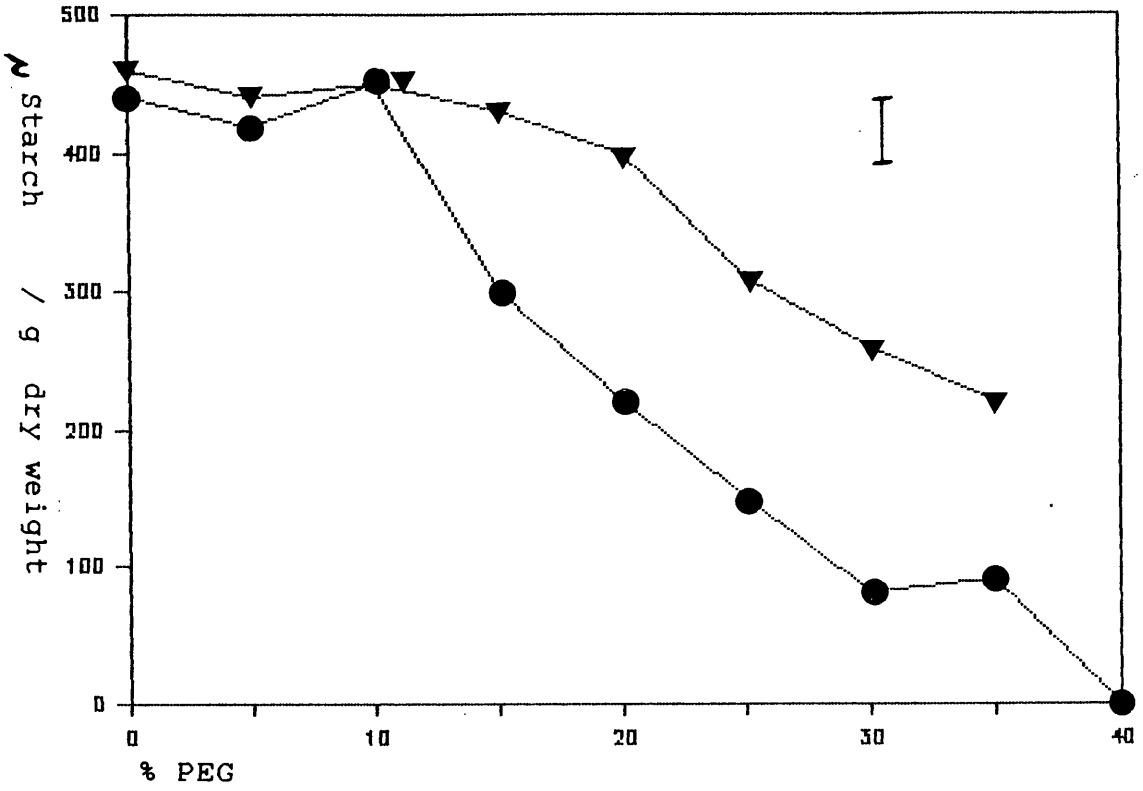


Figure 3.11.0 to show the changes in starch content in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as μgram starch per gram dry weight.

Bar indicates L.S.D. ($P < 0.05$)

Figure 3.12.0

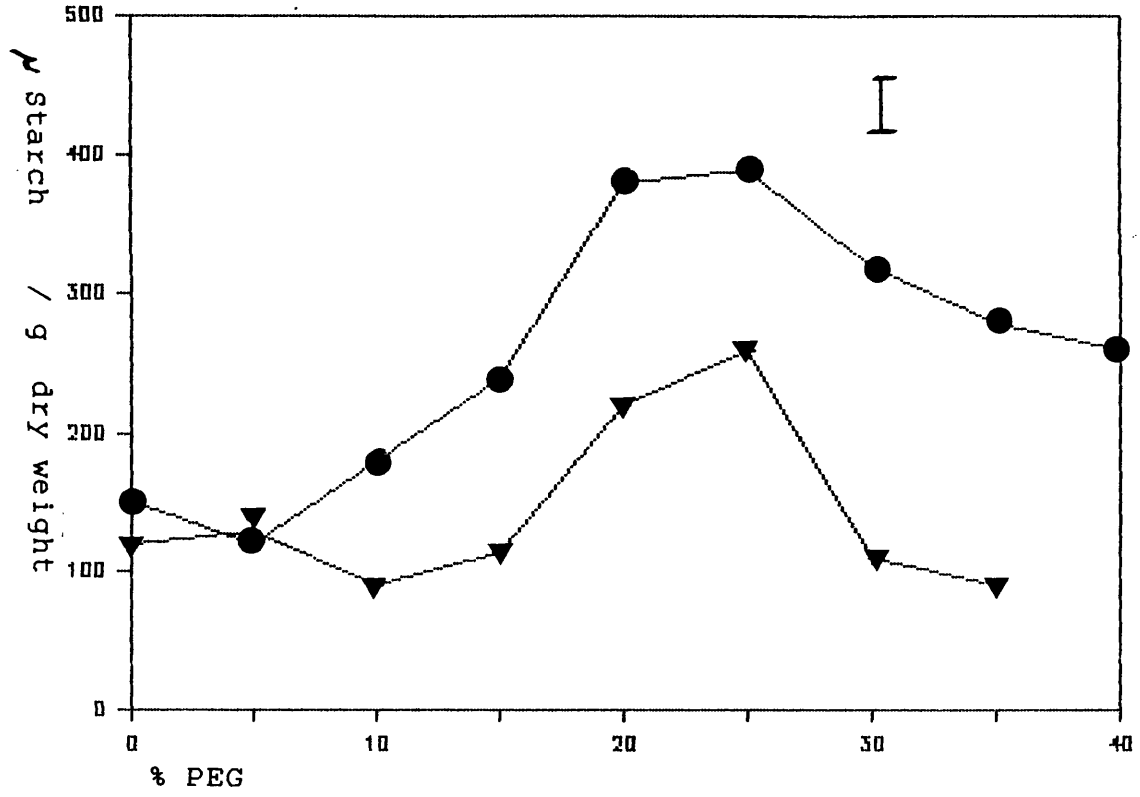


Figure 3.12.0 to show the changes in starch content in young leaves of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as μ gram starch per gram dry weight.

Bar indicates L.S.D. ($P < 0.05$)

Table 3.1 0 to show the rise in individual carbohydrates expressed as a percentage of total carbohydrate during water stress in mature leaves of G. urbanum

%PEG	Fructose	Glucose	Sucrose
10	35	34	31
20	40	42	18
25	46	41	13
30	39	45	16

Table 3.1.1 to show the rise in individual carbohydrates expressed as a percentage of total carbohydrate during water stress in mature leaves of G. rivale

%PEG	Fructose	Glucose	Sucrose
10	35	38	27
15	30	24	46
20	27	26	47
25	29	33	38

Table 3.1.2 to show the rise in individual carbohydrates expressed as a percentage of total carbohydrate during water stress in young leaves of G. urbanum

%PEG	Fructose	Glucose	Sucrose
10	41	45	14
20	44	36	20
25	43	42	15
30	45	40	15

Table 3.1.3 to show the rise in individual carbohydrates expressed as a percentage of total carbohydrate during water stress in young leaves of G. rivale

%PEG	Fructose	Glucose	Sucrose
10	35	29	36
15	40	37	23
20	41	38	21
25	32	33	35

Table 3.1.4 to show the rise in individual carbohydrates expressed as a percentage of total carbohydrate during water stress in roots of G. urbanum

%PEG	Fructose	Glucose	Sucrose
10	36	43	21
20	44	41	15
25	45	42	13
30	44	35	21

Table 3.1.5 to show the rise in individual carbohydrates expressed as a percentage of total carbohydrate during water stress in roots of G. rivale

%PEG	Fructose	Glucose	Sucrose
10	33	31	36
15	35	29	36
20	33	32	35
25	35	27	38

DISCUSSION

It was shown in the previous chapter that although Geum urbanum could osmoregulate at higher levels throughout a period of water deficit than Geum rivale this was not large enough to maintain a significantly higher stomatal conductance or higher photosynthetic rate when measured by IRGA. In this chapter these results are represented (Figs. 3.1.0. and 3.2.0.) along with results gained from an Oxygen electrode (Fig. 3.3.0). Both methods of measurement indicated that photosynthesis was affected by water stress and generally declined with increased water deficit, a frequently reported phenomenon (Hanson and Hitz 1982). The dark respiration of leaves indicate that both species suffered some depression of their dark respiratory rates and when the plants reached their respective wilting points the dark respiratory rates fell to a greater extent in both species. This phenomenon has been shown to exist in most plant species (Lawlor 1976), though some workers have shown dark respiration to be maintained during water stress (Brown and Thomas 1980).

As the rate of photosynthesis was affected to a greater extent than respiration in both Geum species their overall net carbon gain was controlled by water deficit effects on photosynthesis. The net carbon gain produced by Geum urbanum and Geum rivale during mild water deficits could provide a source of free carbohydrate which accumulated during mild water deficits as Munns and Weir (1981) suggested happened in wheat. While free carbohydrate could be produced via photosynthesis in both species and could possibly provide a significant increase in free carbohydrate it is improbable that this is the only source of carbohydrate. This is due to the fact that Geum urbanum accumulated much higher levels of carbohydrate than Geum

rivale throughout the stress period the rates of photosynthesis were not significantly different between the two species. Also at the peak of carbohydrate accumulation in both species net photosynthesis (as measured by IRGA Figs. 3.1.0 and 3.2.0) was severely curtailed.

However, an alternative source of these carbohydrates could come from previously stored starch. From Figs. 3.11.0. and 3.12.0. it is apparent that starch reserves in mature leaves fall while starch reserves in younger leaves increase in both Geum species. Moreover, a greater amount of starch is mobilised from the leaves of Geum urbanum than Geum rivale which is also reflected in the number of chloroplasts containing starch (Fig. 3.9.0) and amylase levels (Fig. 3.7.0). It may then be reasonable to suggest that in mature leaves, starch is degraded by amylase which slowly rises in the mature leaves of both species as water deficits develop. The soluble sugars thus produced enter the carbohydrate pool and may be a major source of the rise in free carbohydrate in the two species during water stress. As carbohydrates have been shown to be transported in the phloem of severely water stressed plants (Sung and Krieg 1979) it seems feasible to suggest that these carbohydrates are also transported to young leaves where they increase the pool of sugars dissolved in these leaves. Starch may also be deposited in the young leaf, if starch deposition is maintained even at normal levels, as amylase levels fall in young leaves (Fig. 3.8.0). However, Nieman et. al (1988) reported that the uridine and/ or adenine nucleotides required for starch formation fall in expanding leaves at least during salt stress. However it is possible that this may not occur in the two Geum species during water deficits.

The degradation of starch has previously been

numerically correlated with the rise in soluble carbohydrates (Stewart 1972a). It is thus possible that the major source of the rise in soluble carbohydrate in Geum urbanum and Geum rivale during periods of water deficit is from starch degradation in mature leaves. Moreover, it may be the ability of Geum urbanum to utilise this source of carbohydrate to a greater extent than Geum rivale that provides it with a larger pool of free carbohydrate in order to raise Ψs. However, it would appear unlikely that starch is the sole contributor to soluble carbohydrate in all plant parts as photosynthetic products may also contribute to this pool considering a net carbon gain is experienced by the plants at least during mild water deficits.

The starch accumulation in the chloroplasts of young expanding leaves and the starch loss in fully photosynthetically competent leaves of both species is a previously unreported phenomenon in plants exposed to water deficits. Though starch loss from leaves has been reported in some species (Stewart 1972a) and accumulation reported in others (Cortes and Sinclair 1987) both accumulation and degradation has never been reported in the same species, though in the salt tolerant Atriplex amnicola this type of deposition has been described when exposed to salt stress (Aslam et al. 1986). This change in starch deposition patterns could be advantageous to the plants during water stress and also after stress alleviation. As starch is degradated in older photosynthetically competent leaves, full photosynthetic potential may be possible as any inhibition to photosynthesis caused by starch in the chloroplast would be alleviated. The deposition of

starch in young leaves which are not fully photosynthetically competent may then help protect the young chloroplasts by reducing the osmotic volume in the chloroplast and water held in the starch grain would then reduce the water content of the chloroplast. It has been argued that these two effects may decrease the solute potential within the chloroplast by having the effect of concentrating any solutes already present (Ackerson and Hebert 1981). When stress is alleviated the young leaf and its chloroplasts would then be relatively undamaged by water stress and thus ready for photosynthesis. The breakdown of starch after water stress would also release the bound water held in the starch grain thus increasing the osmotic potential of the chloroplast partly relieving inhibition of photosynthesis caused by low water potentials.

It was stated in the introduction to this chapter that recently there has been much controversy concerning the relative contributions that reductions in stomatal and non-stomatal effects have on photosynthesis when a plant undergoes a period of water deficit. In order to investigate this problem photosynthesis was measured in the two Geum species by two methods i.e by IRGA (Fig. 3.1.0) which measures net photosynthesis as limited by stomatal conductance; and by oxygen electrode (Fig. 3.3.0) which measures net photosynthesis but overcomes the CO_2 diffusion barrier created by reduced stomatal conductance and in effect is also an indication of the level of mesophyll resistance to photosynthesis.

When Figs. 3.1.0 and 3.3.0 are compared the reduction in net photosynthesis was greater when measured by an IRGA and was coincidental with reductions in stomatal conductance. With the oxygen electrode net photosynthesis was maintained during mild water deficits, gradually fell and eventually fell dramatically at the respective wilting points of the two

species. This pattern of photosynthetic activity was not consistent with reductions in stomatal conductance. Thus during mild water deficits when stomata are closing the potential for photosynthesis remains unchanged. However, net physiological photosynthesis (as measured by the IRGA) declines, thus during mild water deficits the only limitation to photosynthesis is stomatal in Geum urbanum and Geum rivale. As water deficits increase, the potential for photosynthesis declines in both plants (Fig. 3.3.0) and it is therefore possible that stomatal and non-stomatal limitations to photosynthesis contribute to the reduced photosynthetic rates during more severe water deficits. This pattern of maintenance of photosynthetic potential and inferred maintenance in mesophyll resistance followed by an increase in mesophyll resistance (decrease in photosynthetic potential) has been shown to occur in both cotton (Troughton 1969) and maize (Lawlor and Fock 1978). However, other studies have reported an increase in mesophyll resistance or no change at all even in the same plant species (Troughton and Slatyer 1969; Radin and Ackerson 1981). It is possible though that differences in methodology and differing rates of stress development in fact produce such anomalies reported in the literature.

The increase in mesophyll resistance to photosynthesis as measured by the oxygen electrode could manifest itself in two ways, specifically in the light or dark reactions of photosynthesis. As far as the dark reactions of photosynthesis are concerned both PEPcase and RuBPcase enzymic rates did fall during stress, which has also been noted by other workers (O' Toole et al. 1976; Kaiser 1984). However, Plaut (1971) showed isolated enzymes were not affected by low water potential and it has therefore been suggested that the reduction in the activity of such enzymes is a result of

reduced carbon income (Jones 1973; Collatz 1977). It is considered unlikely that the dark reactions of photosynthesis could have caused such a reduction in photosynthetic rates of Geum urbanum and Geum rivale in these experiments. It is therefore possible some inhibition of the light reactions of photosynthesis must have occurred in Geum urbanum and Geum rivale. These could manifest themselves as disruptions of the light harvesting complexes, reduced photophosphorylation, inhibition of the Hill reactions or disruption of the photosynthetic apparatus by photoinhibition. The literature is somewhat contradictory in these areas.

Photorespiration has been proposed as a mechanism by which plants can reduce photoinhibition (Powles 1984). During water stress photorespiration has been shown to decline as photosynthesis declines (Boyer 1971). However, in unstressed plants photorespiration is responsible for 30% of carbon cycling; while in water stressed plants 60% of carbon cycling is accounted for by photorespiration (Lawlor and Fock 1975). This is a clear relative increase which could reduce damage to photosynthetic apparatus by photoinhibition. However, evidence for damage in other parts of photosynthesis appears to contradict this line of reasoning. Thylacoid mediated electron transport has been shown to be stress resistant at low light levels (Sharkey and Badger 1982) but at higher light levels electron transport from water to methylviologen has been shown to be inhibited at light levels likely to be present under drought conditions (Boyer 1976). Again photophosphorylation was shown to be stress resistant at low light levels (Bjorkman and Powles 1984), but not at higher light levels (Keck and Boyer 1974). Thus, it would appear that high light levels likely to be experienced during stress cause damage to the photosynthetic apparatus via photoinhibition. This could certainly occur in these

experiments as the plants were grown under conditions of high light intensity. It would therefore appear that the relative increase in photorespiration is not sufficient to prevent damage via photoinhibition to membrane mediated systems.

After the wilting point of each plant was reached a dramatic rise in mesophyll resistance occurred and hence photosynthesis fell. This fall could have been due to an increase in damage to the photosynthetic membranes *via* the disruption of the internal lamellae of the chloroplast and dissociation of granal stacks caused during severe drought stress as reported by Nir (1969). This latter phase of photosynthetic decline has not been widely studied in the literature. However, most if not all studies in this area have not undertaken experiments whereby plants were stressed to their ultimate death points and thus may have been omitted.

The sugars identified as being contributory to rises in Ψ_s in both species were glucose fructose and sucrose. A pattern of accumulation which has been documented in many other species (Ford and Wilson 1981; Laurie 1988). However, the proportions of individual sugars accumulated appears to be species related. In the two *Geum* species it is apparent that glucose and fructose are the major sugars accumulated during water deficits and it is a type of accumulation which is of advantage to the plants. If the breakdown of starch is a major contributor to carbohydrate accumulation then sucrose will be produced. If this sucrose is then hydrolised by acid invertase, glucose and fructose are formed. Thus two osmotically active particles are accumulated rather than one, if sucrose was mainly accumulated. Circumstantial support for this type of accumulation is given in this study and in other work. As there is a higher proportion of glucose and fructose in younger leaves than mature leaves and acid invertase

activity is higher in young leaves than mature leaves (Munns and Weir 1981), one would expect the proportion of glucose and fructose to sucrose to increase. This indeed does occur in the two Geum species and in Triticum aestivum (Munns and Weir 1981). It is also apparent that Geum urbanum accumulates glucose and fructose in greater proportion to sucrose than Geum rivale (Tables 3.10 to 3.1.5). Thus Geum urbanum may be able to hydrolise sucrose into glucose and fructose during water stress better than Geum rivale. This would then help to increase Ψs in Geum urbanum above that of Geum rivale by the conversion of a single osmotically active partical into two osmotically active particles.

In this study and other studies reported in the literature the levels of glucose and fructose may have been overestimated as the process of separating sugars and amino acids etc. for GC determination involves the use of strong acids and ammonia which may hydrolyse the sucrose molecule and hence give a lower reading (Hendrix and Peelen 1987). However, if the colorimetric carbohydrate results are compared with the proportions of hexose and sucrose determined by GC a fair correlation exists.

In some species sugar alcohols and organic acids have been shown to accumulate during water stress, but not in others (Ford and Wilson 1981). However both Geum urbanum and Geum rivale failed to accumulate sugar alcohols or organic acids in response to water stress. It is interesting to note however that both Geum urbanum and Geum rivale showed significant levels of sorbitol at all stages of experimentation which has been shown to be taxonomically linked with the Rosaceae (Lewis and Smith 1967) of which they are members. The lack of sugar alcohol accumulation during water stress may be linked to sugar accumulation during water deficits. Laurie (1988) noted in a survey of tropical trees subject to

water and heat stress that those species accumulating large amounts of sugars did not accumulate sugar alcohols to any great extent. However, those species which accumulated high levels of sugar alcohols showed a low level of mono and di-saccharide accumulation. Moreover it was suggested that sugar alcohol accumulation in preference to other carbohydrates could be linked to the higher stress tolerances of such plants. As Geum urbanum was found to be only marginally tolerant to water deficits and Geum rivale was found not to be drought tolerant in this study it would therefore fit into Laurie's suggested theory.

It is suggested in this chapter then that the major source of free carbohydrates accumulated in the tissues of Geum urbanum and Geum rivale throughout stress comes from the mobilization of stored carbohydrate in the form of starch. Moreover it is suggested that this is part of the reason why Geum urbanum can attain a higher Ψ_s than Geum rivale and hence out perform Geum rivale in a water stress situation. It is also suggested that the ability of Geum urbanum to convert more sucrose into fructose and glucose than Geum rivale may be significant in the greater reduction of Ψ_s in Geum urbanum.

CHAPTER 4

ASPECTS OF NITROGEN METABOLISM IN *GEUM URBANUM* AND *GEUM RIVALE* EXPOSED TO WATER DEFICITS

INTRODUCTION

It was shown in Chapter 2 showed that amino acids accumulated in response to water stress in both *Geum urbanum* and *Geum rivale*. The level of amino acid accumulation was large enough to significantly contribute to the lowering of Ψs in both species however the accumulation was greater in *Geum urbanum*. As the accumulation of solutes was considered to be the major reason for the differing drought tolerances of the two species the reasons for the difference in amino acid accumulation and the source of these amino acids may give a further clue as to why such a wide difference in water deficit tolerance was exhibited by the two species.

The nitrogen metabolism of plants starts when a nitrogen source such as nitrate enters the plant usually at root level. If nitrate is the only source of nitrogen input, this will be reduced by nitrate reductase (NR) to ammonia usually in the leaves or roots of the plant. The ammonia thus produced can then be assimilated by glutamate dehydrogenase (GDH) or more usually by glutamine synthetase (GS) in combination with glutamine(amide):2-oxoglutarate aminotransferase (oxidoreductase NADP) or as it is more commonly referred to GOGAT. A variety of amino acids can then be produced by transaminations. Though other pathways exist to form different amino acids, the GS/GOGAT pathway is responsible for most ammonia assimilation in the plant. The resultant amino acids can be used in other metabolic processes such as protein synthesis or remain as free amino acids within the cell. It is therefore possible that this pathway could provide amino acids to the free

amino acid pool during water deficits. GS and GDH have previously been shown to be largely insensitive to water stress development though differences between root and shoot have been reported (Taylor *et al.* 1982). However, nitrate reductase (the first step in nitrate acquisition) has been shown to be stress sensitive in many species (Mattas and Pauli 1965; Dusky and Galitz 1977) and thus the primary processes of nitrate assimilation are not considered to be a major source of free amino acids during water deficits. Nevertheless, more recent work by Smirnoff *et al* (1985) has cast some doubt as to the degree of drought susceptibility which nitrate reductase exhibits. Moreover, of the few studies conducted concerning glutamine synthetase and water stress, none have studied the effects on the two isoforms of glutamine synthetase through stress. These two isoforms are compartmentalised either solely in the cytoplasm (GS1) or solely in the chloroplast (GS2). It is therefore possible that some difference in isoform complement occurs and may be important, especially in the case of GS2. This isoform is thought to be involved in the photorespiratory pathway and hence would be involved in the recycling of carbon during stress. Thus some re-evaluation of the contribution that the primary nitrogen assimilation pathway makes to the rise in amino acids throughout water stress is required.

In this study it was decided to follow the aspects of nitrogen metabolism outlined above in order to try and determine the source of amino acid accumulation in the two *Geum* species. In the light of the findings of Smirnoff *et al* (1985), experiments were conducted investigating nitrate reductase and for the reasons outlined above glutamine synthetase isoforms were also to be investigated. In addition glutamate dehydrogenase levels in the two plants were studied together with the levels of individual nitrogen containing compounds

during water deficits. These include amounts of individual amino acids, nitrate, ammonia, and soluble protein contained in the plants during water deficits. Thus the trends in enzyme activity in the root and shoot were to be determined during water deficits as well as levels of nitrogen containing compounds to determine the significance of such biochemical aspects on the stress tolerance of Geum urbanum and Geum rivale.

MATERIALS AND METHODS

A: Plant Growth And Conditions

The growth conditions and stress regimes were as reported in chapter 1. However, only populations of *Geum urbanum* from Helbeck Hall and populations of *Geum rivale* from Leadgate were used in these experiments.

B: Analysis of Nitrate Reductase (EC 1.6.6.2)

At the end of a stress regime nitrate reductase was measured in the leaves and roots of both *Geum* species. The method used was similar to that of Stewart and Orebamjo (1979). Approximately 1 gram of leaf tissue was cut into fine strips and placed in a 1/3 pint milk bottle containing 20 ml of assay mixture (assay mixture contained 100 mM potassium phosphate buffer pH 7.5, 50 mM potassium nitrate and 0.01 ml n-propanol). The mixture was then vacuum infiltrated into the tissue covered and placed in a darkened water bath at 30 °C for one hour after which time aliquats were assayed as follows: 0.5 ml incubated assay mix was mixed with equal volumes of 1% sulphanilic acid in 3 M hydrochloric acid and 0.02% alpha-napthyletheylene diamine diHCl (NEDD). This was incubated for 20 minutes and absorbance measured at 540 nm. Root nitrate reductase was measured in a similar way. 100 mg of root tissue was cut into approximately 0.5 cm lengths and placed in a Tunberg tube with 5 ml of the assay mix above. The tissue was vacuum infiltrated and the tubes sealed while still under vacuum. Incubation and assay procedure were as reported for leaves.

C: Investigations Concerning Glutamine Synthetase (EC 6.3.1.2)

Approximately 1 to 5 grams of leaf or root tissue were ground in liquid nitrogen to which polyclar had been added at a rate of 40 mg / 1 g plant tissue. Extraction buffer was then added (5 ml/1 g plant tissue), this contained 10 mM magnesium sulphate

heptahydrate; 5 mM glutamic acid; 1 mM EDTA disodium salt; 1 mM dithiothreitol; 1 mM glutathione; 2 mM mercaptoethanol and 2% w/v soluble polyvinyl-pyrrolidone; in 25 mM TRIS-HCl pH 8.0. This extract was centrifuged at 8000 g for 25 minutes at 2 °C. The supernatent was then assayed for glutamate synthetase activity by the transferase and synthetase methods of Rhodes, Rendon and Stewart (1975).

Some leaf extracts were to undergo column chromatography in order to determine the GS isoform complement of the two species throughout the stress period. 1.5 x 15 cm columns of DEAE-Sephacel were prepared equilibrating with 25 mM TRIS-HCl buffer for 1 hour before use. The extract was loaded onto the column and washed with 75 ml 25 mM TRIS-HCl buffer. The proteins were eluted using a linear KCl gradient of 0 to 600 mM salt (KCl was dissolved in 25 mM TRIS-HCl buffer) over 16 hours and 2 ml fractions collected over this period. The fractions were then assayed for glutamine synthetase activity using the aforementioned assay systems.

D: Assay Of Glutamate Dehydrogenase (EC 1.4.1.2)

Glutamate dehydrogenase was assayed and extracted by the method of Taylor and Havill (1981).

E: The measurement Of Soluble Protein

Soluble protein was measured by the method of Lowry (1951)

F: Tissue Nitrate Determination

Tissue nitrate was extracted from oven dried tissue in methanol as previously described in chapter two for organic solutes. 0.5 ml aliquats of extract were added to 2.0 ml ammonia buffer pH 9.6. To this was added approximately 0.5 grams of previously prepared spongy cadmium. The extract was left for three hours while the cadmium reduced the extracted nitrate to nitrite. This was then assayed by the sulphanilic acid / NEDD method

described in the nitrate reductase section.

G: Ammonia determination in plant tissues

Plant tissue was boiled in deionised water for 20 minutes. The ammonia extracted was then assayed by the colourimetric technique of McCullough (1967).

H: Detection of individual amino acids by gas chromatography

Gas chromatography was used to detect individual amino acids present in Geum urbanum and Geum rivale at various stress levels. For details of extraction, derivatisation and detection see the appendix.

RESULTS

Nitrate reductase levels in mature and young leaves (Figs. 4.1.0 and 4.1.1) of both species remained constant between 0 and 5% PEG at higher concentrations levels fell rapidly in Geum rivale but were maintained up to 15% PEG in Geum urbanum . Root nitrate reductase levels however rose in both species as stomata closed, reaching a peak until rapidly falling sometime before the wilting point of each plant (Fig. 4.1.2). Nitrate reductase activity was always higher in mature leaves than in young leaves in both species and furthermore at moderate to high stress levels, Geum urbanum could maintain a significantly higher nitrate reductase activity in all plant parts.

Figs. 4.2.0. to 4.2.2. show the changes in glutamine synthetase activity in mature leaves, young leaves and roots of Geum urbanum and Geum rivale. These curves show the activity of glutamine synthetase in mature and young leaves of both plants to increase slowly until the wilting point of the plants was reached when glutamine synthetase activity fell. Root glutamine synthetase activity however decreased gradually in both species up to the wilting point and again declined sharply after this point. Again mature leaf glutamine synthetase activity was consistently higher than that of young leaves and Geum urbanum could maintain a significantly higher glutamine synthetase activity in all plant parts at moderate to high stress levels.

The GS profiles of both plants (Table 4.1.0) are identical throughout stress with both species having a complement of around 65% GS1 and 35% GS2 activity in leaf tissue at all stages of stress development.

A different pattern emerged for glutamate dehydrogenase activity in both plant species. Activity decreased in young and mature leaves (Figs. 4.3.0 and 4.3.1), but activity increased in the roots of both

species (Fig. 4.3.2). Again Geum urbanum was able to maintain significantly higher glutamate dehydrogenase activity over Geum rivale at moderate to high stress levels in all plant parts.

The changes in individual free amino acids of Geum urbanum and Geum rivale in young leaves, mature leaves and roots in response to water deficits are shown in Tables 4.2.0. to 4.2.5. They show that Geum urbanum accumulated more free amino acids than Geum rivale throughout water deficits and the levels are comparable to those estimated by colourimetric means (see Figs. 2.16.0 to 2.16.2 in Chapter 2). However a distinct pattern of accumulation occurs in all plant parts in both species. At low water deficits a general rise in all amino acids is seen with proline increasing massively from control levels. However at moderate water stress only proline, asparagine, glutamine, aspartate and glutamate levels rose significantly from those seen at low stress levels. Under more severe stresses again only proline, asparagine and glutamine levels rose in roots, young and mature leaves to any great extent. The other amino acids were either maintained at previous levels or fell. Of all the amino acids proline was accumulated to the highest degree in both species with the highest accumulation occurring in Geum urbanum.

The fall in nitrate levels is shown in Figs. 4.4.0 and 4.4.1 in both species. Fig. 4.4.0 shows that nitrate levels are initially maintained in all plant parts in Geum urbanum during mild water deficits then they fall sharply in all plant parts until a steady state is reached. In Geum rivale however there is acontinual fall in free nitrate in all plant parts.

Figs. 4.5.0 and 4.5.2 show the changes in ammonia levels in roots leaves and mature leaves in both Geum species. These figures show ammonia levels initially fell then remained constant throughout stress in all

plant parts in both species. However after the wilting point ammonia levels rose in all plant parts in both Geum urbanum and Geum rivale.

Levels of soluble protein in both species contained in roots, young and mature leaves are shown in Figs. 4.6.0 to 4.6.2. these graphs show an initial maintenance of soluble protein followed by a steady decrease in protein levels in all plant parts in both species. Geum rivale however lost significantly more protein than Geum urbanum.

Figure 4.1.0

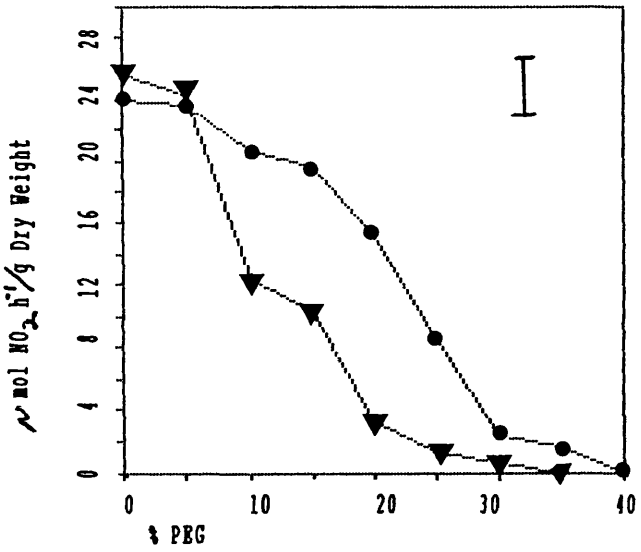


Figure 4.1.1

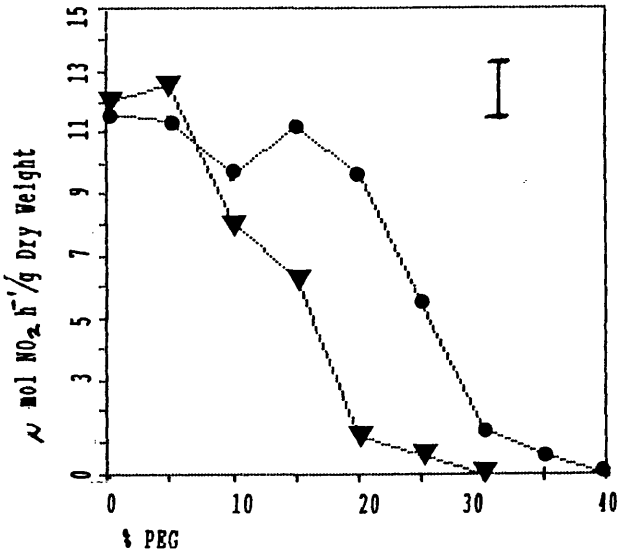
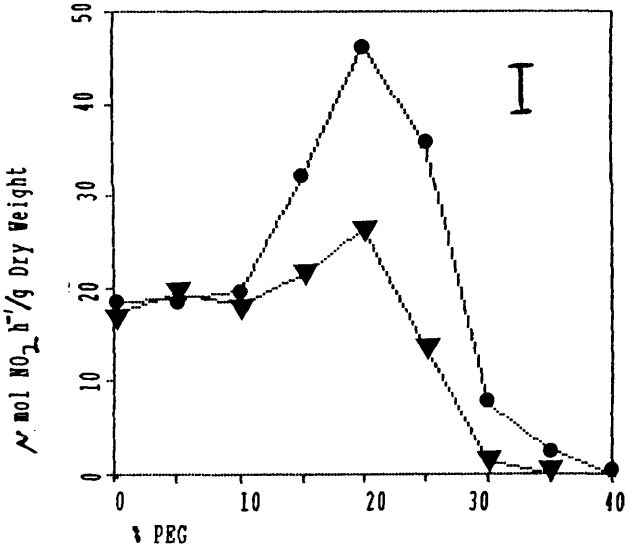


Figure 4.1.2



Bars indicate L.S.D. ($P < 0.05$)

Figure 4.1.0 to figure 4.1.2 show the changes in nitrate reductase activity in mature leaves (4.1.0), Young leaves (4.1.1), and roots (4.1.2) of G. urbanum ● and G. rivale ▼ during water deficits imposed by PEG 6000. Results are expressed as ~ mol NO_2 produced per hour per gram dry weight.

Figure 4.2.0

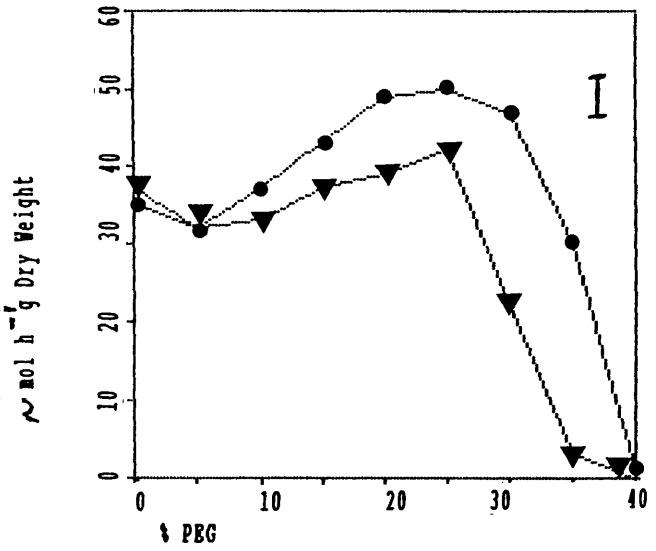


Figure 4.2.1

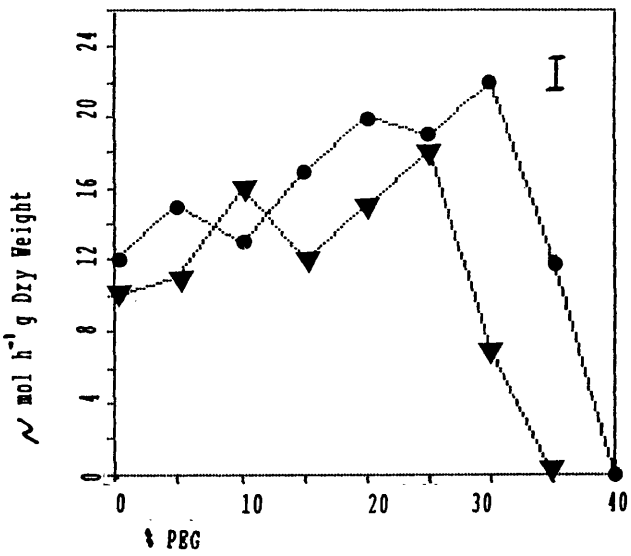
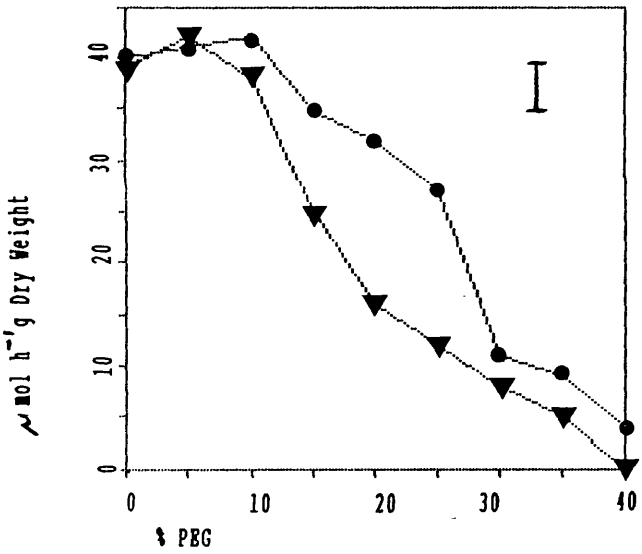


Figure 4.2.2



Bars indicate L.S.D. ($P < 0.05$)

Figure 4.2.0 to figure 4.2.2 show the changes in glutamine synthetase activity in mature leaves (4.2.0), Young leaves (4.2.1), and roots (4.2.2) of G. urbanum ● and G. rivale ▼ during water deficits imposed by PEG 6000. Results are expressed as ~ mol gamma glutamyl produced per hour per gram dry weight.

Figure 4.3.0

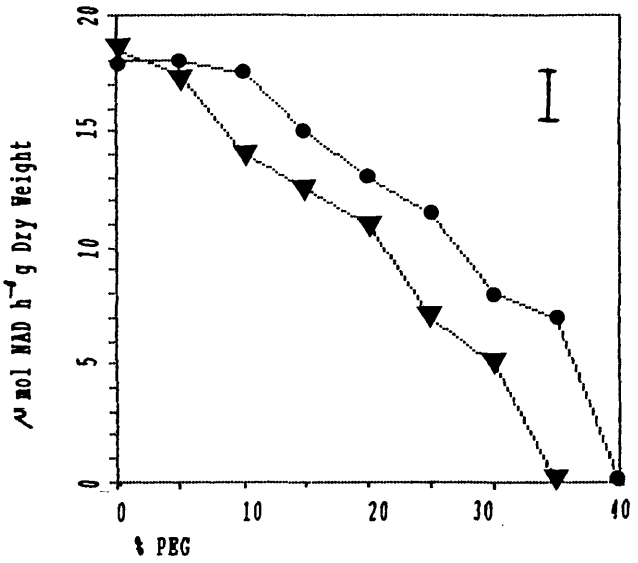


Figure 4.3.1

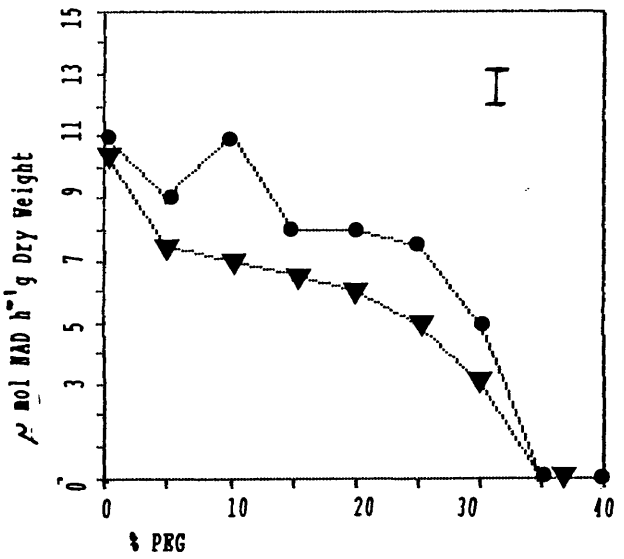
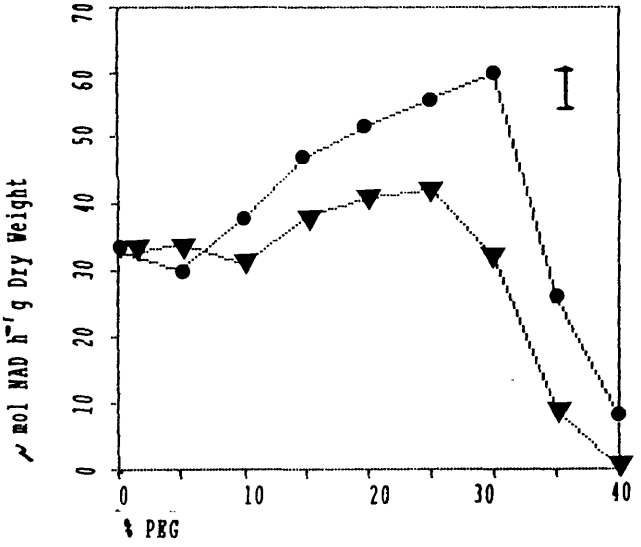


Figure 4.3.2



Bars indicate L.S.D. ($P < 0.05$)

Figure 4.3.0 to figure 4.3.2 show the changes in glutamate dehydrogenase activity in mature leaves (4.3.0), Young leaves (4.3.1), and roots (4.3.2) of G. urbanum ● and G. rivale ▼ during water deficits imposed by PEG 6000. Results are expressed as ~ mol NADH oxidised per hour per gram dry weight.

Figure 4.4.0

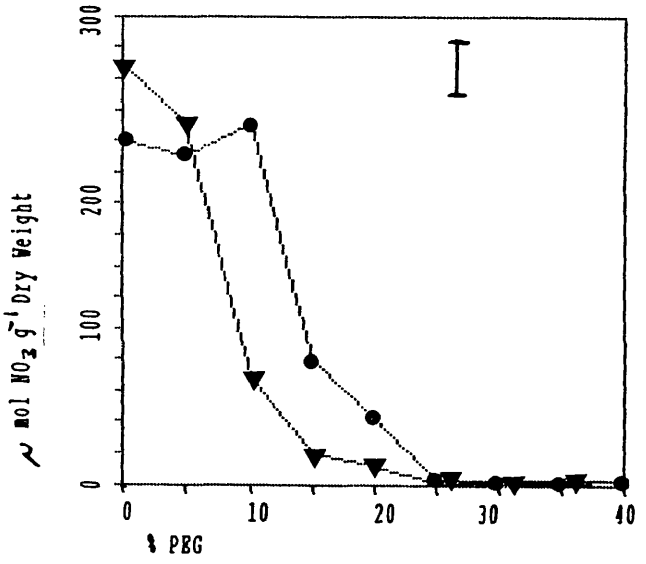


Figure 4.4.1

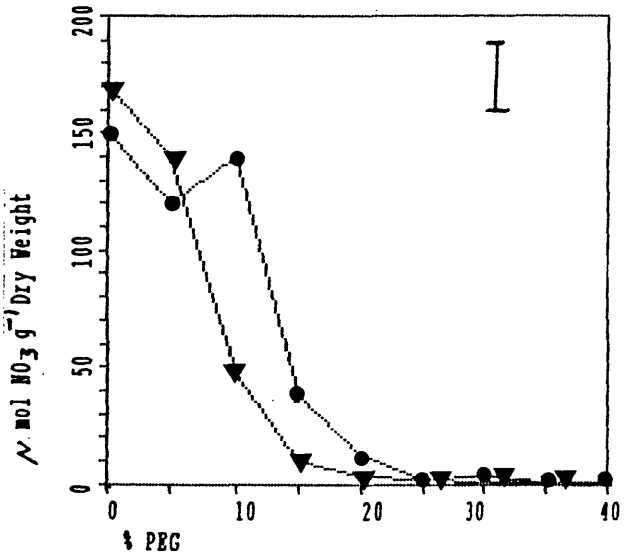
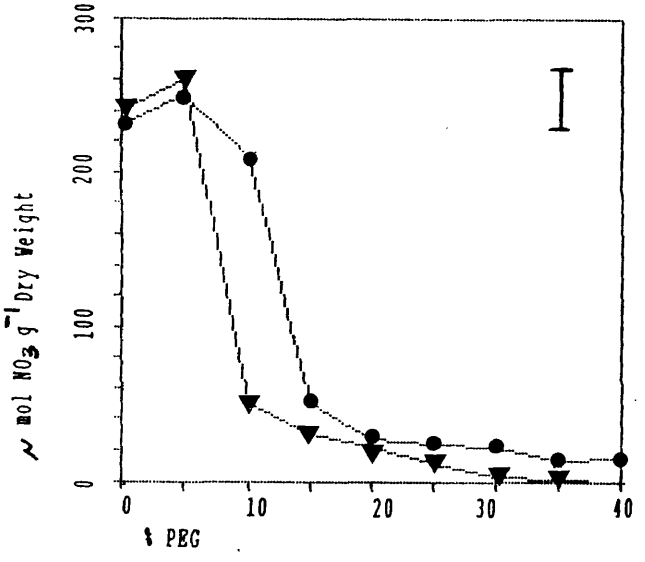


Figure 4.4.2



Bars indicate L.S.D. ($P < 0.05$)

Figure 4.4.0 to figure 4.4.2 show the reduction in free nitrate in mature leaves (4.4.0), Young leaves (4.4.1), and roots (4.4.2) of G. urbanum ● and G. rivale ▼ during water deficits imposed by PEG 6000.

Figure 4.5.0

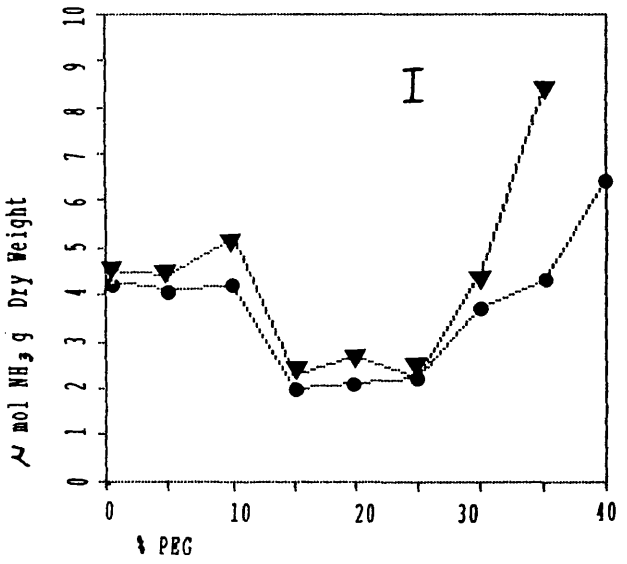


Figure 4.5.1

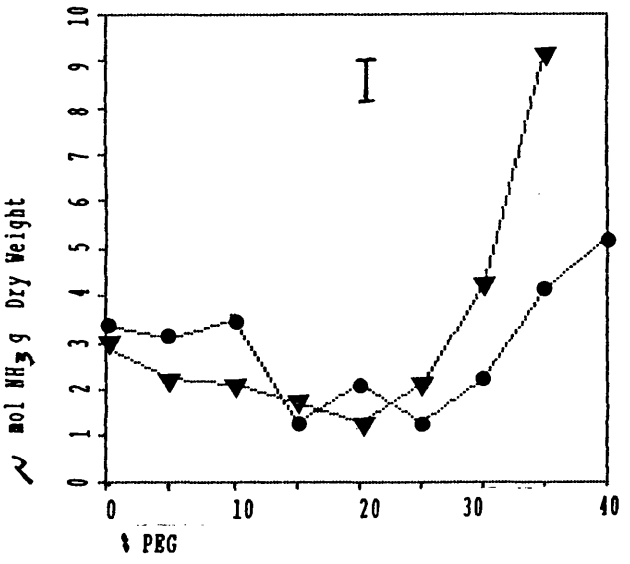
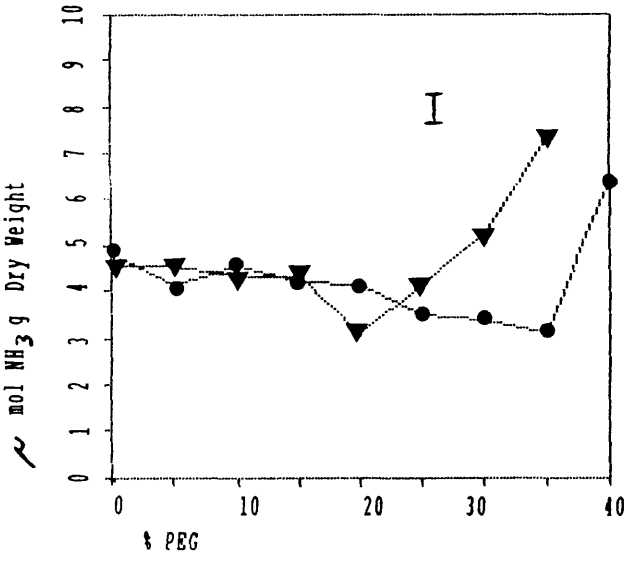


Figure 4.5.2



Bars indicate L.S.D. ($P < 0.05$)

Figure 4.5.0 to figure 4.5.2 show the changes in free ammonia in mature leaves (4.5.0), Young leaves (4.5.1), and roots (4.5.2) of G. urbanum ● and G. rivale ▼ during water deficits imposed by PEG 6000.

Figure 4.6.0

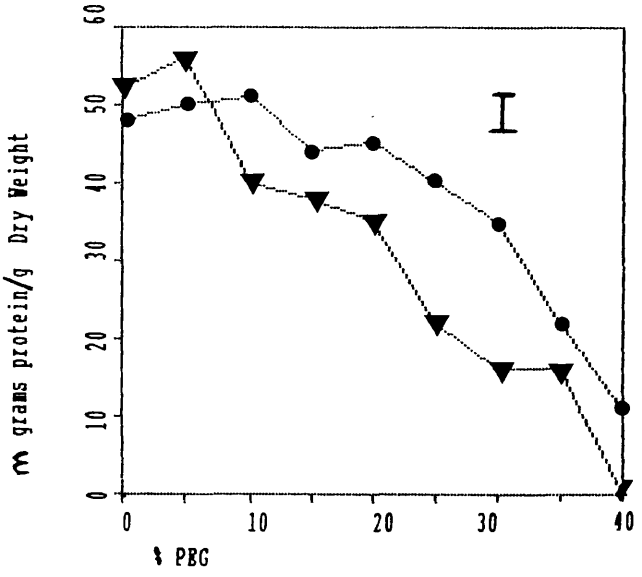


Figure 4.6.1

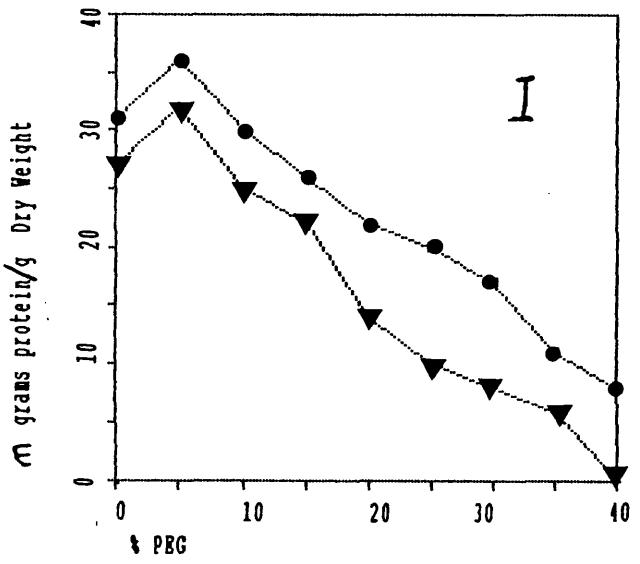
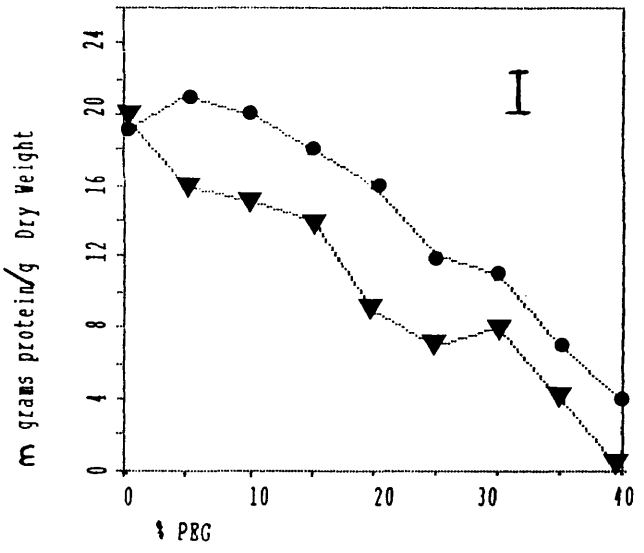


Figure 4.6.2



Bars indicate L.S.D. ($P < 0.05$)

Figure 4.6.0 to figure 4.6.2 show the changes in soluble protein in mature leaves (4.6.0), Young leaves (4.6.1), and roots (4.6.2) of G. urbanum ● and G. rivale ▼ during water deficits imposed by PEG 6000.

Table 4.1.0. To show the change in glutamine synthetase isoforms in *G. urbanum* and *G. rivale* during water stress

	G. urbanum		G. rivale	
%PEG	GS1	GS2	GS1	GS2
0.0	65.0	35.0	68.0	32.0
5.0	63.0	37.0	62.0	38.0
10.0	67.0	33.0	66.0	34.0
15.0	62.0	38.0	65.0	35.0
20.0	64.0	36.0	63.0	37.0
25.0	63.0	37.0	64.0	36.0
30.0	62.0	38.0	67.0	33.0
35.0	66.0	34.0	nd	nd
40.0	nd	nd	nd	nd

Results indicate % glutamine synthetase isoforms as a function of total recovered activity

All recoveries were above 92%

Table 4.2.0. To show the amino acid accumulation in mature leaves of G. urbanum during water stress

%PEG	0.0	10.0	20.0	25.0
Amino acid				
Proline	0.3	11.4	28.0	34.4
Alanine	2.1	4.1	4.2	4.1
Glycine	0.5	1.2	1.4	0.5
Valine	1.7	2.8	3.0	3.0
Threonine	0.3	0.4	0.3	0.3
Serine	2.2	3.4	3.5	2.3
Leucine	0.8	1.5	0.9	0.4
Isoleucine	1.2	1.8	1.5	0.9
Methionine	0.3	0.4	0.3	0.4
Asparagine	1.1	3.3	10.0	17.3
Aspartate	4.1	7.1	8.6	5.3
Glutamine	1.6	3.1	6.1	15.4
Glutamate	4.0	4.3	8.7	5.7
Cysteine	0.2	0.3	0.4	0.3
Histidine	0.7	2.6	3.3	2.7
Arginine	1.7	2.7	2.0	1.6
Phenylalanine	0.7	1.3	1.3	0.9
Total	23.5	54.0	88.8	96.5

Results expressed in μmol / g dwt

Table 4.2.1. To show amino acid accumulation in young leaves of G. urbanum during water stress

%PEG	0.0	10.0	20.0	30.0
Amino acid				
Proline	0.2	12.4	34.9	39.2
Alanine	2.4	2.9	3.2	4.1
Glycine	0.7	0.8	1.1	0.7
Valine	1.4	2.3	2.6	1.2
Threonine	0.2	0.3	0.3	0.3
Serine	2.2	4.1	4.3	3.7
Leucine	1.3	1.5	1.9	1.2
Isoleucine	1.0	1.9	1.9	0.8
Methionine	0.3	0.3	0.3	0.2
Asparagine	5.7	14.3	25.9	27.7
Aspartate	1.6	4.7	8.1	5.1
Glutamine	1.3	3.4	7.1	8.2
Glutamate	4.4	6.9	7.5	4.9
Cysteine	0.3	0.3	0.4	0.3
Histidine	0.2	0.2	0.5	0.4
Arginine	1.9	3.3	3.6	2.9
Phenylalanine	0.4	0.5	0.5	0.3
Total	25.8	60.4	104.5	101.5

Results expressed in μ mol / g dwt

Table 4.2.2. To show the amino acid accumulation in the roots of *G. urbanum* during water stress

%PEG	0.0	15.0	20.0	30.0
Amino acid				
Proline	0.3	5.0	11.3	21.1
Alanine	1.1	1.7	2.0	2.3
Glycine	0.3	0.3	0.3	0.3
Valine	0.7	1.6	1.1	1.0
Threonine	0.3	0.2	0.4	0.3
Serine	1.8	2.8	2.4	2.1
Leucine	0.6	1.4	0.8	0.6
Isoleucine	0.6	1.8	1.3	0.8
Methionine	0.3	0.6	0.4	0.4
Asparagine	0.8	2.8	4.6	7.3
Aspartate	3.2	4.9	5.3	4.2
Glutamine	0.6	2.0	4.8	3.2
Glutamate	0.9	2.7	5.1	6.2
Cysteine	0.2	0.3	0.3	0.3
Histidine	0.4	0.8	0.9	0.6
Arginine	1.3	1.4	1.0	0.9
Phenylalanine	0.8	0.7	1.3	1.1
Total	14.1	31.7	43.4	52.7

Results expressed in μ mol / g dwt

Table 4.2.3. To show the amino acid accumulation in mature leaves of *G. rivale* during water stress

%PEG	0.0	10.0	15.0	20.0
Amino acid				
Proline	0.2	4.8	15.8	18.3
Alanine	1.9	2.6	2.7	3.4
Glycine	0.4	0.6	0.6	0.4
Valine	1.9	2.3	1.9	1.7
Threonine	0.3	0.3	0.3	0.3
Serine	1.8	2.4	2.5	1.8
Leucine	0.9	1.2	1.0	0.9
Isoleucine	0.9	1.1	1.0	0.8
Methionine	0.4	0.3	0.3	0.3
Asparagine	1.6	2.1	4.1	5.7
Aspartate	3.8	4.4	3.2	2.9
Glutamine	1.9	2.8	4.4	5.6
Glutamate	3.0	3.6	5.0	3.2
Cysteine	0.3	0.3	0.3	0.2
Histidine	0.5	0.7	1.4	1.1
Arginine	1.5	1.8	1.4	1.2
Phenylalanine	0.8	1.0	1.0	0.9
Total	22.1	32.3	46.9	48.7

Results expressed in μmol / g dwt

Table 4.2.4. To show the amino acid accumulation in young leaves of *G. rivale* during water stress

%PEG	0.0	10.0	15.0	25.0
Amino acid				
Proline	0.2	6.7	18.6	24.1
Alanine	2.3	2.9	3.4	3.7
Glycine	0.3	0.4	0.8	0.3
Valine	2.4	2.7	2.9	1.4
Threonine	0.2	0.3	0.3	0.2
Serine	2.2	2.6	2.9	1.9
Leucine	1.3	1.4	1.1	0.8
Isoleucine	0.9	1.0	1.3	1.2
Methionine	0.2	0.2	0.3	0.2
Asparagine	4.9	6.1	9.1	11.7
Aspartate	1.5	3.3	4.1	2.9
Glutamine	1.7	2.9	6.9	7.3
Glutamate	3.7	4.4	4.9	2.2
Cysteine	0.2	0.2	0.3	0.2
Histidine	0.2	0.4	0.8	0.3
Arginine	1.7	2.4	2.5	1.8
Phenylalanine	0.4	0.6	0.5	0.4
Total	24.3	38.6	60.7	61.6

Results expressed in μmol / g dwt

Table 4.2.5. To show amino acid accumulation in the roots of *G. rivale* during water stress

%PEG	0.0	10.0	15.0	25.0
Amino acid				
Proline	0.3	3.6	8.1	11.1
Alanine	0.8	1.4	1.7	1.6
Glycine	0.3	0.5	0.3	0.2
Valine	0.4	0.3	0.3	0.3
Threonine	0.3	0.3	0.2	0.2
Serine	1.9	2.1	1.9	1.2
Leucine	0.8	0.9	1.1	0.6
Isoleucine	0.7	0.9	0.9	0.4
Methionine	0.3	0.2	0.3	0.3
Asparagine	1.1	2.1	3.5	3.5
Aspartate	2.9	3.4	3.7	1.9
Glutamine	0.9	1.5	1.8	1.2
Glutamate	1.2	2.3	2.9	3.1
Cysteine	0.3	0.2	0.3	0.2
Histidine	0.7	0.9	0.8	0.6
Arginine	0.8	0.9	1.0	0.6
Phenylalanine	0.5	0.7	0.8	0.6
Total	18.8	22.2	27.0	27.6

Results expressed in μmol / g dwt

DISCUSSION

It is apparent from Figs. 4.1.0 to 4.3.2 that the enzymes of nitrogen assimilation show different responses in root and shoot of both species.

Nitrate reductase activity in the mature and young leaves was maintained initially until moderate water deficits were reached in Geum urbanum wherupon levels fell drastically. In Geum rivale however a much shorter period of maintenance was exhibited followed by a sharp fall. This maintenance of nitrate reductase activity is in opposition to that previously reported for other species where only a continual sharp fall in nitrate reductase activity has only been reported in laboratory experiments (Venkataramana et al. 1987; Mattas and Pauli 1965). Moreover, root nitrate reductase increased during water deficits (Fig. 4.1.3), a previously unreported phenomenon.

The maintenance and following fall of nitrate reductase activity in leaves of both species seams curious. As nitrate reductase activity can be induced by nitrate this pattern seems even more implausible as the amount of nitrate contained in the leaves of both species (Figs. 4.4.0 and 4.4.1) appears unrelated to the nitrate reductase activity recorded. However, Shaner and Boyer (1976 a) demonstrated that when the transpiration stream of maize was blocked by antitranspirants, nitrate reductase activity in the leaf remained high despite lowered nitrate content in the leaves. Shaner and Boyer (1976 a) then demonstrated that the flux of nitrate through the blocked transpiration stream remained high and therefore nitrate reductase activity depended on nitrate flux rather than the amount of nitrate present. In a follow up experiment, Shaner and Boyer (1976 b) showed leaf nitrate reductase activity was reduced during water deficits and nitrate flux also decreased. At first site these data may appear at odds with each

other, however in the water stress experiments of Shaner and Boyer (1976 b) stress was induced by withholding water from 11 cm pots which, as explained earlier in this thesis, can produce a rapid stress and exacerbated stress reactions. Thus the initial maintenance and following slow decline of nitrate reductase activity in the experiments of Shaner and Boyer (1976 b) may have been lost due to the high rate of stress development. Thus, the maintenance of nitrate reductase activity exhibited by the Geum species may be due to maintained nitrate flux through the xylem when the transpiration rate is reduced. At higher stress levels xylem flux of nitrate to the leaves may be reduced during water stress as the xylem vessels are distorted by low water potentials (Hsiao 1973; Pearce and Beckett 1987).

Though this line of reasoning holds true for leaf nitrate reductase activity it does not follow that the flux of nitrate through the root increases during water deficits to produce increased nitrate reductase activity (Fig. 4.1.2). On the contrary during water deficits ion uptake into the roots would diminish as water became limiting (Pitman 1981). However, under conditions of nitrogen deficiency Chapin et al. (1988) showed for various species that the potential for nitrate uptake was increased in the roots by up to five times under mild nitrogen stress and nitrate flux through the root actually increased. Moreover, nitrate accumulated in the root in preference to being transported to the shoot. Thus as the flux of nitrate increased one would expect nitrate reduction to increase (this was not measured), as nitrate reductase activity can be controlled by nitrate flux. Furthermore Butz and Jackson (1977) proposed that nitrate transport was mediated by nitrate reductase itself and the more membranes the nitrate passed through, the more nitrate was reduced. Thus after passing through four membranes 80% of nitrate would have

been reduced by nitrate reductase.

The reason for the higher nitrate reductase activity in the roots of both Geum species during water deficits now becomes clear. It is proposed that a similar sort of system happens in drought stress as during nitrogen stress. As water deficits increase and the availability of nitrate from the media is reduced a form of nitrogen starvation is produced. The plant then compensates by increasing the flux of nitrate through the roots and hence nitrate reductase activity increases. However, nitrate accumulation does not occur in the root as it is reduced while passing through the cell membranes. Increased flux of nitrogen does not then follow as the plant is attempting to maintain root nitrate levels in preference to shoot levels. Thus reduced nitrate reductase activity in the leaf may be caused by this method in combination with disrupted xylem vessels.

Hence the pattern of nitrate reductase activity shown by the two Geum species is as a result of nitrate flux through the root and shoot and the increased demand of nitrate by the root and could be considered an adaptive response to water deficits.

Though this system operates in culture solution it is doubtful whether this could happen to the same extent in a field situation as nutrients would become even more limited in the field. However field results are presented and discussed in Chapter 6.

The reduction in shoot assimilation of nitrate previously reported has been seen as advantageous during water deficits as the high energy requirement for uptake and requirement of reducing power for reduction of nitrate would be decreased. As photosynthesis and respiration are both reduced during water stress this would appear an advantageous plant strategy. However, it has been shown in Chapter 2, that reductions in

respiration and photosynthesis as measured by oxygen electrode do not fall until moderate water stress is reached and only fall rapidly at the wilting point of each species. Thus nitrate reduction follows a similar pattern to that exhibited by the energy and reductant producing processes and may not therefore create problems of energy balance in the two species.

In roots however, nitrate reductase levels increase by up to two times that of control levels which may cause problems in the supply of reducing power from the mitochondrion, as root respiration rates fall when nitrate reduction increases. However, in the root the nitrate reductase complex is thought to be more efficient at gaining NADH from the mitochondrion (Oaks and Hirel 1985). Thus this increased efficiency in NADH collection by root nitrate reductase may aid nitrate reduction if NADH production from the TCA is lowered.

As well as problems created by energy balance being created with the rise in nitrate reductase activity in roots, a pH imbalance may also occur. Raven and Smith (1976) calculated that an excess of hydroxyl ions would be produced when nitrate is reduced in plants and these ions can then be neutralised by strong acids such as malate. However from chapter 3 it was noted that organic acid levels did not rise in root tissue in either species. Raven and Smith (1976) also argued for a process whereby in effect hydroxyl ions were passed into the soil by the root and hence a biophysical rather than biochemical pH buffering mechanism was utilised. As there is little evidence for a biochemical pH buffering mechanism in the roots of Geum urbanum and Geum rivale it is suggested the biophysical system operates during stress in the two Geum species.

The ammonia produced by the reduction of nitrate by nitrate reductase, can then be assimilated into amino acids by either glutamine synthetase or glutamate

dehydrogenase. Glutamine synthetase activity rose slightly during stress in the young and mature leaves of both species (Figs. 4.2.0 and 4.2.1)but decreased in the root of both species (Fig. 4.2.2). This is consistent with the literature to date as increases in glutamine synthetase levels have been reported in leaves (Taylor et al 1982) and decreases in root glutamine synthetase activities have previously reported (Taylor et al 1982; Kaur et al. 1985). Glutamate dehydrogenase activity fell in both species in young and old leaves (Figs. 4.3.0 and 4.3.1) and increased in roots (Fig. 4.3.2). However, increases in the activity of this enzyme has previously been reported in leaves and decreases in roots (Taylor et al. 1982). Thus in the two Geum species a totally different nitrate assimilating pattern occurs during water deficits than previously reported in other plant species.

The ammonia produced by nitrate reduction in leaves could then be assimilated into amino acids by glutamate dehydrogenase however the activity of this enzyme declined in leaves during water deficits in both species. Alternatively and perhaps more importantly the ammonia could be assimilated by glutamine synthetase (the activity of which increases during stress) which is considered to be the major assimilation point for ammonia.

The decrease in glutamine synthetase activity and the increase in glutamate dehydrogenase activity in the root may indicate that ammonia assimilation in the root is preferentially undertaken by glutamate dehydrogenase. Support for this apparent differential ammonium assimilation between roots and shoots during water stress is provided by de Lourdes Miranda-Ham and Loyola-Vargas (1987). These workers extracted glutamate dehydrogenase from leaves and roots of maize and subjected them to PEG treatment. The results showed that

glutamate dehydrogenase activity was reduced drastically in the leaves but was maintained in the root. However glutamine synthetase activity showed the opposite trend, with root glutamine synthetase activity being drastically reduced in the root and leaf glutamine synthetase activity showed itself to be more stress tolerant than leaf glutamate dehydrogenase. Moreover, when proline was added to the medium the Km for ammonia of root glutamate dehydrogenase was reduced by a factor of 100. Thus, the ability of glutamate dehydrogenase to assimilate ammonia may be increased in roots when proline is accumulated. As proline levels in the roots of both species (Tables 4.2.1 and 4.2.5) this provides futher circumstantial evidence for an increased role for glutamate dehydrogenase in the assimilation of ammonia in root tissue during water stress.

Thus in Geum urbanum and Geum rivale, ammonia may be fixed by glutamine synthetase in the leaves while in the roots glutamate dehydrogenase may preferentially fix ammonia into amino acids.

In the introduction it was argued that although glutamine synthetase activity had been studied during water stress, the proportion of the activity exhibited by GS1 and GS2 had not been investigated. Furthermore as leaf glutamine synthetase activity increases during stress in both species and decreases in root tissues it is attractive to think that the chloroplastic form of glutamine synthetase is less stress sensitive than the cytosolic form. Thus one may expect the chloroplastic form of glutamine synthetase to increase relative to that of the cytoplastic form during water deficits. It was indeed found that Geum urbanum and Geum rivale contained both glutamine synthetase isoforms in the same proportions in each species (Table 4.1.0) in unstressed plants. i.e. GS1 (the cytosolic isoform) accounted for around 65% of glutamine synthetase activity while GS2

(the chloroplastic isoform) accounted for approximately 35% of glutamine synthetase activity. From Table 4.1.0 however it is apparent that though glutamine synthetase activity increased in the leaf during water deficits the proportions of the isoforms did not change. Thus another reason for such differential effects has to be sought. The answer may lie in the structural properties of the glutamine synthetase isoenzmes. Hirel and Gadal (1980) and Hirel et al. (1984) demonstrated that GS1 and GS2 extracted from leaves of other species were not identical with each other and root glutamine synthetase was different again from the two isoforms present in the leaf. Thus during water stress leaf glutamine synthetase isoforms are more stress resistant than root glutamine synthetase and this may be related to the differences in protein structure between root and shoot isoforms.

A different reason may apply to the differential stress tolerance of glutamate dehydrogenase. Loyola-Vargas and Sanchez de Jimenez (1984) found that additions of NH_4^+ to tissue cultures derived from maize roots increased glutamate dehydrogenase activity. However additions of NH_4^+ to cultures derived from maize leaves did not result in an increase in glutamate dehydrogenase activity. Moreover Stewart, Mann and Fentem (1980) also found increased glutamate dehydrogenase activity in roots when plants were fed ammonium chloride. Thus the literature indicates that root glutamate dehydrogenase is induced by increased ammonia levels while leaf glutamate dehydrogenase is not. From Fig. 4.5.2 it is apparent that ammonia levels in the root do not increase during water stress. However, glutamate dehydrogenase is associated with the mitochondrion where NH_4^+ levels are high thus preferential accumulation of ammonia may occur in the root mitochondrion particularly as the nitrate reductase complex is closely associated with the mitochondrion in

roots.

It is thus apparent that the rise in free amino acids produced by Geum urbanum and Geum rivale could be produced at least in part by continued nitrogen assimilation in the leaf and the increased assimilation in the root.

Protein synthesis and protein degradation were not followed during these experiments, however levels of soluble protein were monitored during water stress (Figs. 4.6.0 to 4.6.2). This showed a decrease after a short period of maintenance at mild water deficits in both species through mild and severe stress. This fall in soluble protein could arise from a fall in protein synthesis or an increase in protein degradation. Data available concerning protein degradation shows there is an increase in protein degradation during stress (Paleg and Aspinall 1981). Indeed Fukutuku and Yamada (1984) showed 54% of ^{15}N labelled protein was liberated as free amino acids during stress. Protein synthesis on the other hand has been shown to decline at even mild water deficits (Hasio 1973; Dhindsa and Bewley 1976). It is therefore considered then that the rise in amino acids is caused by a lack of utilization in protein synthesis and a rise in protein degradation. However in this study soluble protein was reduced to a greater extent in Geum rivale than Geum urbanum which implies protein synthesis was more affected in Geum rivale or protein degradation increased. A larger rise in free amino acids would then occur in Geum rivale, which does not happen. Though some amino acids may be used for their carbon skeletons in other parts of metabolism it seems improbable that such a large amount of amino acids should be used in this way when free carbohydrates are in such profusion within the plant. Other workers have noted such anomalies on a more quantitative basis for individual amino acids (Jones et al. 1980; Singh et al. 1973b). The present study has

shown however that nitrogen reduction can be maintained at significant levels in both Geum species during stress, and ammonia assimilation is largely unaffected. Moreover, Geum urbanum was able to maintain primary nitrogen assimilation at higher levels than Geum rivale at most stress levels. Thus a major source of free amino acids could be produced by primary nitrogen fixation in Geum urbanum and Geum rivale in combination with reduced protein synthesis. Thus a more prominent role for amino acid synthesis and lack of utilization in protein synthesis is proposed in the accumulation of amino acids during stress.

The fact that Geum urbanum was able to accumulate a larger amount of free amino acid during water deficits than Geum rivale and these accounted for significant reduction in Ψ_s leads to a further proposal. That the ability of Geum urbanum to maintain nitrate reduction and assimilation of ammonia into amino acids at higher levels than Geum rivale throughout stress is of relevance to the observed resistance to water deficits of the two species and contributes to the higher stress tolerance of Geum urbanum.

It is interesting to note within the amino acid pool that a general rise in almost all amino acids occurs during mild water deficits in both species in all plant parts. However at moderate and more severe water deficits certain patterns occur which are often associated with the metabolic derivation of such amino acids and their use in plant metabolism.

Of those amino acids derived from pyruvate, levels of valine, leucine and isoleucine either fall or are maintained at previous levels. Those amino acids derived from oxaloacetate exhibit a similar pattern with methionine, threonine and lysine levels all falling or being maintained after the initial rise. Only the levels of asparagine and aspartate are seen to rise

consistently during stress from this group in the two species. However under severe stress the levels of aspartate decline while those of asparagine increase. The two amino acids derived from aspartate show a similar trend with lysine and alanine both falling after the initial rise.

The amino acids derived from alpha keto gluterate however show a different pattern. Glutamate and glutamine levels increase and a massive increase in proline occurs. However a similar pattern in the accumulation of glutamate and glutamine occurs to that of aspartate and asparagine. In root tissue however glutamate levels continue to rise when glutamine levels fall.

When stress levels are low a general rise in amino acids occurs probably due to lack of utilization in protein synthesis and maintenance of amino acid synthesis and transamination. However as stress rises to moderate levels those amino acids derived from pyruvate do not increase and those derived from oxaloacetate do not increase (apart from asparagine and aspartate). The compounds they are derived from are present in the TCA cycle or glycolysis and it may therefore show a reflection of the need for these compounds in this type of metabolism. In other words the maintenance of the TCA cycle and glycolysis is of greater importance than providing amino acids not required in protein synthesis. Thus a rise does not occur in these amino acids. The only amino acids which do rise from these two sources are asparagine which has a proposed role in nitrogen transport in some plants (Peoples et al. 1987) and aspartate. However there is a distinct shift in accumulation between aspartate and asparagine during more severe stress as aspartate levels fall and asparagine levels increase. Thus there may be an accumulation of asparagine at the expense of aspartate

from which asparagine is derived which provides some evidence for the role in amino acid transport for asparagine. Moreover, the continued rise in asparagine in young leaves adds more weight to this hypothesis as nitrate reduction and ammonia assimilation are lower in these leaves than mature leaves. This is apparent when amino acid levels in young leaves are above those of mature leaves. Thus transport of amino acids to young leaves may be occurring. As amino acids have been shown to be transported in the phloem during water stress (Singh et al. 1973; Sung and Krieg 1979) this appears to be an attractive hypothesis. Furthermore alanine and lysine levels fall and are derived from aspartate and may therefore reflect the increased requirement of aspartate for asparagine synthesis. The production of alanine and lysine also require pyruvate for their synthesis. Pyruvate as previously mentioned is required in glycolysis and thus the fall in the levels of these two amino acids during moderate and severe water deficit could also be linked to demand of glycolysis for pyruvate.

The amino acids derived from alpha keto-glutérate (also present in the TCA cycle) show another trend consistent with their role in metabolism. Glutamine and glutamate levels rise during moderate water stress in leaves of both species and during severe stress their glutamine levels continue to rise when glutamate levels fall. These compounds are involved in ammonia assimilation by glutamine synthetase and as nitrate reduction is maintained at these stresses it would be advantageous to the plant to maintain levels of these amino acids. The reduction in glutamate during severe stress may be a reflection of its increased utilisation in the production of proline. In roots however, the opposite occurs as glutamate levels increase in roots while glutamine levels decrease. This may be due to the

increase in glutamate dehydrogenase activity as the product of the ammonia assimilated by this method is glutamate.

Of the other amino acids derived from alpha keto glutérate in roots and leaves, arginine levels fall to pre stress levels and proline levels increase dramatically. The increase in proline levels is a common response to water stress (Aspinall and Paleg 1981) and it has been assigned roles significant to plant survival in water stress (Schobert and Tschesche 1978; Pollard and Wyn-Jones 1979), this will be discussed in Chapter 7.

Thus the amino acid content of the plants during stress can be related to their need and use in stress metabolism. Although the above explanation argues from the point of veiw of amino acid production from primary sources the appearance of amino acids from protein degradation could suffer a similar sort of fate *via* transamination reactions in the cell or the reversal of their biosynthetic pathways. It would therefore appear unlikely that such a pattern of amino acid accumulation is a passive result of water deficits but rather a positive adaption to water stress.

Of the studies completed on the accumulation of amino acids during water stress few have shown details of individual amino acid accumulations. Of those that have some agreement with the accumulation of amino acids accumulated by the two *Geum* species can be seen. Drossopoulos *et al.* (1985) working on wheat cultivars showed glutamine, glutamate and asparagine were all accumulated during moderate water stress in the field, however these accumulations were cultivar specific. Furthermore an excellent correlation exists when the results here are compared with those of Taylor *et al.* (1982) who showed glutamine and asparagine rose during severe stress in shoot tissue while glutamate and

aspartate levels fell. Binzel *et al.* (1987) in their study with tobacco cells which were acclimated to osmotic stress by NaCl at low, moderate and high stress levels inicated that the individual amino acids all rose at low stress levels, while at moderate and high stress levels virtually the same pattern of amino acid accumulation was produced. Thus this type of amino acid accumulation is not unique to the two *Geum* species and may be a general phenomenon linked to water stress metabolism.

This chapter has shown that the previously reported massive declines in root and leaf nitrate reductase activity do not occur in *Geum urbanum* and *Geum rivale*. This may indicate that the two *Geum* species are a special case or that differences in the rate of stress production cause such differences. It is considered that differences in methodology can produce such effects. Furthermore it is suggested that the ammonia produced by nitrate reductase activity is assimilated by glutamine synthetase in the leaf and a more prominent role for glutamate dehydrogenase in the primary assimilation of ammonia is proposed in the root.

It has also been demonstrated in this chapter that primary amino acid production can contribute greatly to the free amino acid pool during water stress (in conjunction with reduced utilization in protein synthesis) and the ability of *Geum urbanum* to maintain this at higher levels than *Geum rivale* could be a major factor determining the drought tolerance of the two species.

CHAPTER 5

THE EFFECTS OF WATER STRESS ON SOME ASPECTS OF PHENOLIC METABOLISM IN GEUM URBANUM AND GEUM RIVALE

INTRODUCTION

The phenolic metabolism of plants received much attention in past years but was generally restricted to studies concerning chemotaxonamy (eg Bate-Smith and Lerner 1954), lignification (Goldschmid 1954), and biosynthetic pathways (Gross 1981). However the vast range of phenolic compounds identified was ignored by main stream plant physiologists for many years. In more recent years some phenolic compounds have been assigned important roles as plant growth regulators (Kefeli and Kadyrov 1971) and as defence mechanisms during pest attack (Harborne et al. 1976).

However water deficit effects on phenolic metabolism have received little attention. Some work has been undertaken which points to a significant role of such compounds in water stress.

Pizzi and Cameron (1986) suggested that polyflavenoid tannins linked via secondary forces to proline rich proteins or by covalent bonds to hemicelluloses may result in increased cell wall elasticity and hence decreased cell collapse and these are accumulated when proline levels are low and thus could greatly influence a plants ability to resist periods of water deficit.

It has long been known that anthocyanin pigments become more visible during water stress (Onslow 1916). Further work concerning anthocyanin formation during water stress was carried out by Spyropoulos and Mavrommatis (1978). These workers showed that anthocyanins were accumulated in various Quercus species and accumulation was linked to the xerophytic character of such species.

Pirie and Mullins (1976) showed that sucrose and ABA which accumulate during water stress could induce the formation of anthocyanins and increase the levels of phenolic compounds in unstressed leaf discs.

It has also been suggested that such pigments and other glycosides could act as osmoticums (Wasicky 1921) and thus if these compounds increase during water stress they could have some significance in plant survival during water deficits. Indeed some glycosides have been shown to accumulate in unstressed leaves to levels of 100 mg/g dry weight and above (Lindroth and Pajutee 1987).

It was noticed during the PEG experiments that the leaves of the two Geum species became reddened which is a typical sign of anthocyanin production and so some investigation into phenolic metabolism was required. It was therefore decided to follow phenolic acid, tannin and anthocyanin levels during water deficits. In addition to this a further study were to be undertaken; glycoside formation was to be followed. Although no glycosides of Geum rivale had previously been identified the glycoside gein had been shown to be present in Geum urbanum (Psenak et al 1969).

Production of phenolic acids requires, as a first step, the de-amination of phenylalanine by the activity of Phenylalanine ammonia lyase (PAL). Bardzik et al. (1971) showed PAL activity decreased during water deficits until a steady state was reached.

However PAL activity has been shown to be unrelated to phenolic acid accumulation and is not considered to be the rate limiting step in phenolic biosynthesis (Hanson and Havir 1981) and it was eventually decided not to follow PAL activity throughout stress in either species.

MATERIALS AND METHODS

A: The growth and culture of Geum urbanum and Geum rivale

The growth, culture and experimental regime were as stated in the materials and methods section in chapter 1. However only the population of Geum urbanum from the Leadgate site and the population of Geum rivale from Moor House were used in this study.

B: The extraction of phenols,tannins and glycosides from plant material

The method of drying plant material was as described in chapter 2. Methanolic extracts of were then made by grinding between 1 and 2g of tissue in a mortar and pestle in liquid nitrogen. 20 ml of ice cold methanol was then added and the mixture was reground. This was then sonicated and centrifuged at 8000 g for 20 minutes at 3 °C. the supernatent was then used to assay phenolic compounds, phenolic glycosides and tannins.

C: The determination of free phenols

Phenols were determined by the method of Swain and Hillis (1959) using the Folin-Denis reagents described in the A.O.A.C. Methods of Analysis. Results were obtained by reading the absorbence at 660 nm.

D: The measurement of Glycoside content

The 5 ml portions of the extracts from B (above) were acidified with 1ml HCl and boiled in a water bath for 15 minutes these were then cooled and assayed for phenolic content as above. The amount of glycosides present was then calculated by the rise in phenolic levels after hydrolysis.

E: The determination of tannins

Tannins were determined by the modified vanillin method of Price et al. (1978). Results were obtained by measuring the absorbence at 500 nm.

F: Determination of anthocyanins

Anthocyanins were extracted by macerating the tissue in a polytron for 2 minutes in 10 ml ice cold acidified methanol (methanol 50%, water 49.5 %, HCl 0.5 %). The supernatent was then decanted off and the tissue was re - extracted for a further minute. The supernatent was again decanted and added to the previous supernatent. A final wash was given and pH was adjusted to 1 with HCl. The supernatent was then centrifuged at 8000 g at 3 ^{o}C for 20 minutes. The supernatent was then made up to a volume of 50 ml and the absorbence was then measured at 530 nm in a spectophotometer.

RESULTS

It is apparent from Figs. 5.1.0 to 5.1.2 that Geum urbanum and Geum rivale accumulated phenolic acids in young leaves, old leaves and roots in response to water stress up to 20% PEG when levels fell gradually and then fell again more rapidly at the respective wilting points of both species. However Geum rivale accumulated more phenolics in all plant parts than Geum urbanum. It is also apparent that phenolics were accumulated to higher levels in mature leaves as opposed to young leaves and roots. Figs. 5.2.0 to 5.2.2 show the inferred glycoside levels in the two species and indicate that Geum urbanum accumulated more glycosides than Geum rivale in roots, but similar levels were seen in young and mature leaves of both species. In all plant parts in both species, a fall in glycoside levels was recorded during severe stress.

The levels of tannins (Figs. 5.3.0 to 5.3.2) in the two species show a curious trend, with both species exhibiting a rapid fall in tannin content during mild water deficits followed by a steady state during moderate water deficits, and then a large increase in tannins during severe water stress.

Figs. 5.4.0 and 5.4.1 show the increase in anthocyanin content in mature leaves and young leaves in Geum urbanum and Geum rivale and indicate that Geum urbanum was able to accumulate more anthocyanin than Geum rivale in both young and mature leaves.

Figure 5.1.0

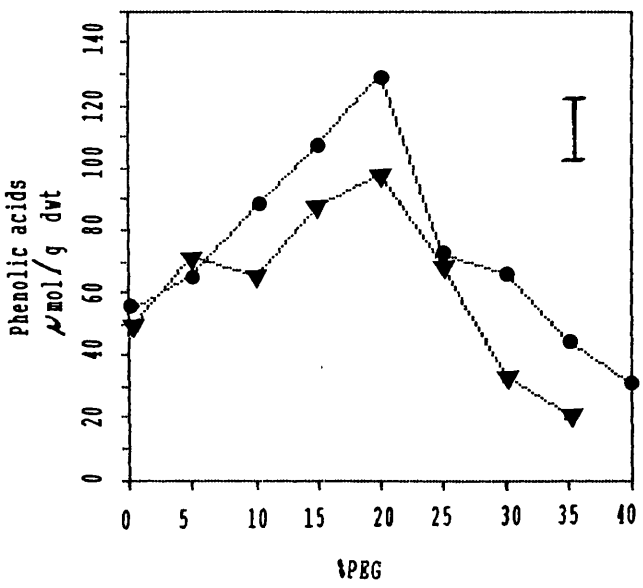


Figure 5.1.1

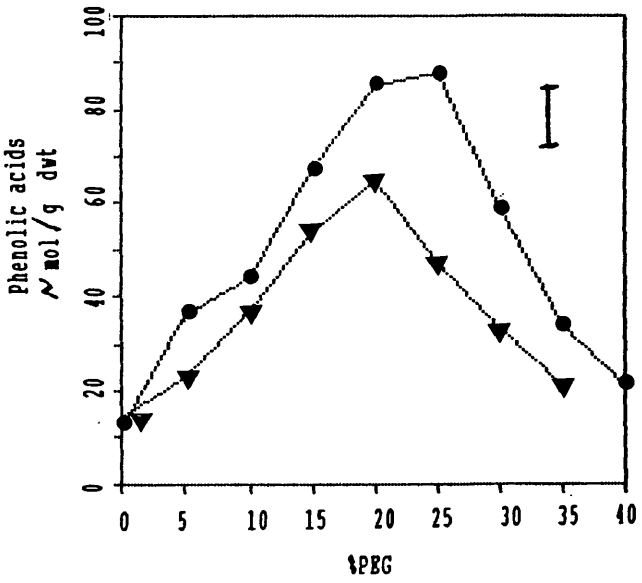
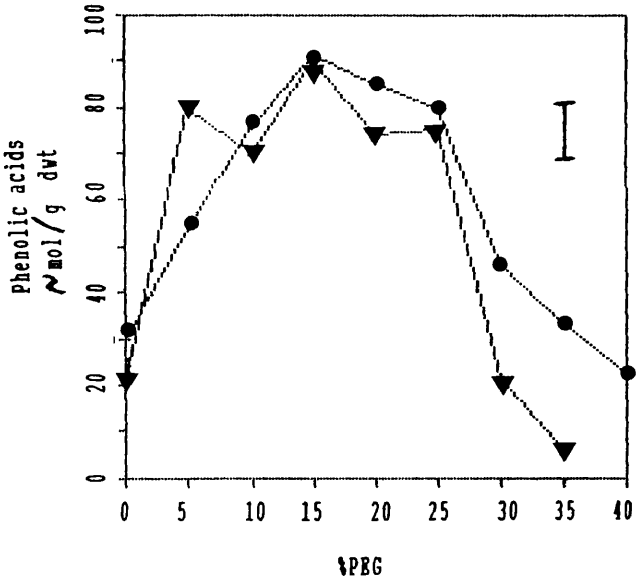


Figure 5.1.2



Bars indicate L.S.D. ($P < 0.05$)

Figures 5.1.0 to 5.1.2 to show the pattern of phenolic acid accumulation in mature leaves (fig.5.1.0), young leaves (fig. 5.1.1) and roots (fig 5.1.2) of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in μmol per gram dry weight.

Figure 5.2.0

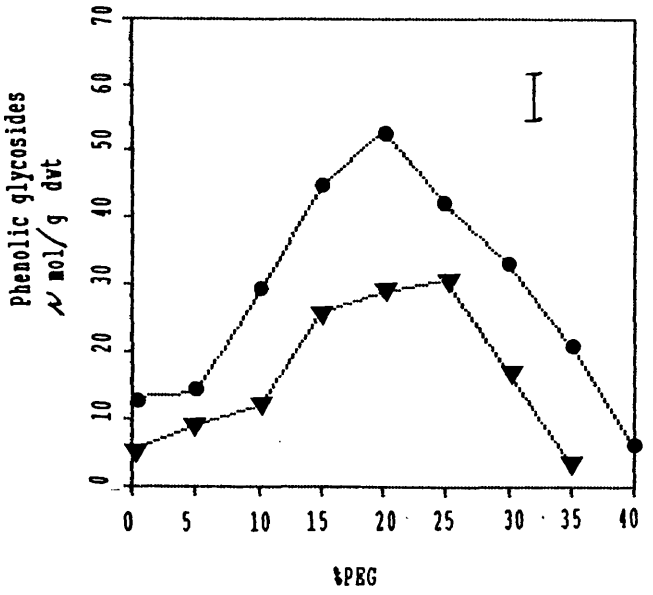


Figure 5.2.1

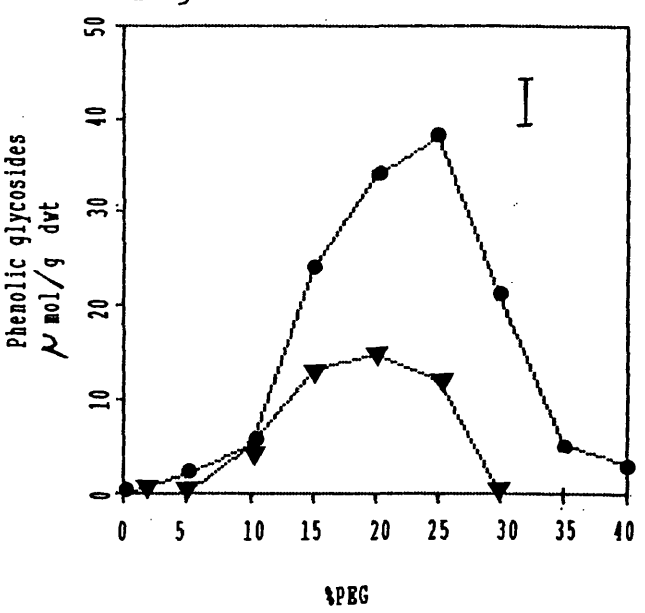
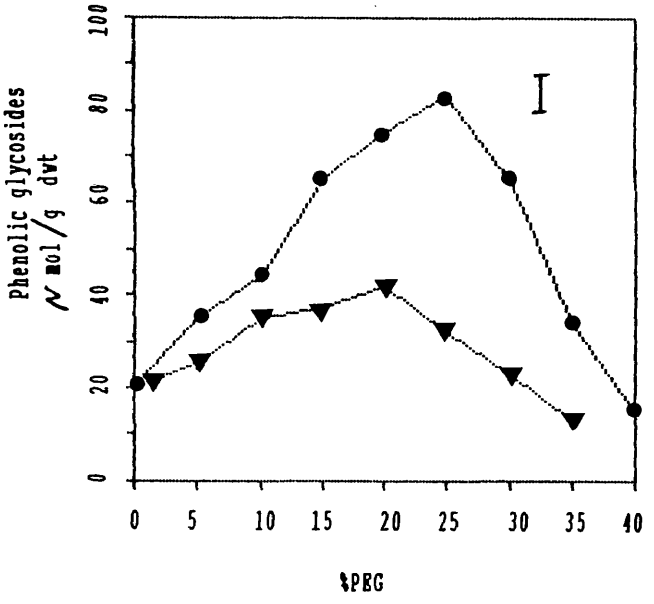


Figure 5.2.2



Bars indicate L.S.D. ($P < 0.05$)

Figures 5.2.0 to 5.2.2 to show the pattern of phenolic glycoside accumulation in mature leaves (fig.5.2.0), young leaves (fig. 5.2.1) and roots (fig. 5.2.2) of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in μ mol per gram dry weight.

Figure 5.3.0

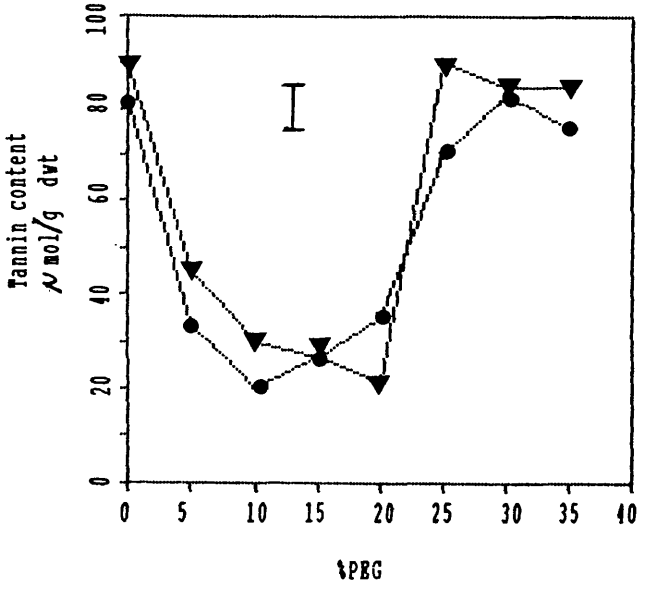


Figure 5.3.1

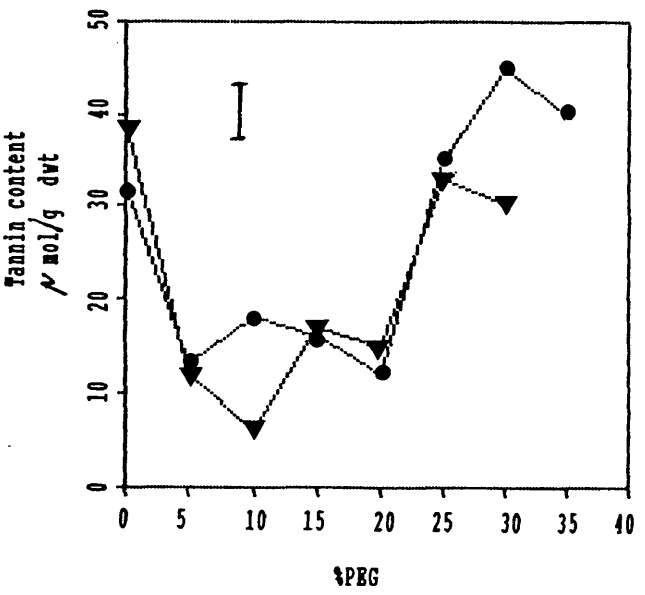
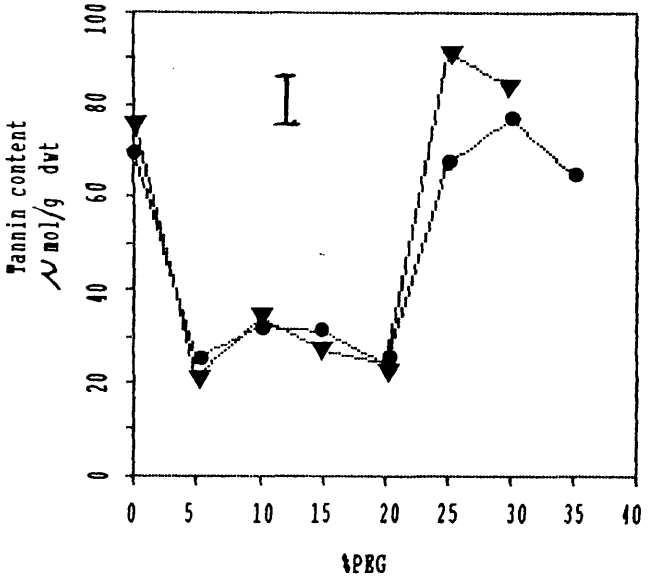


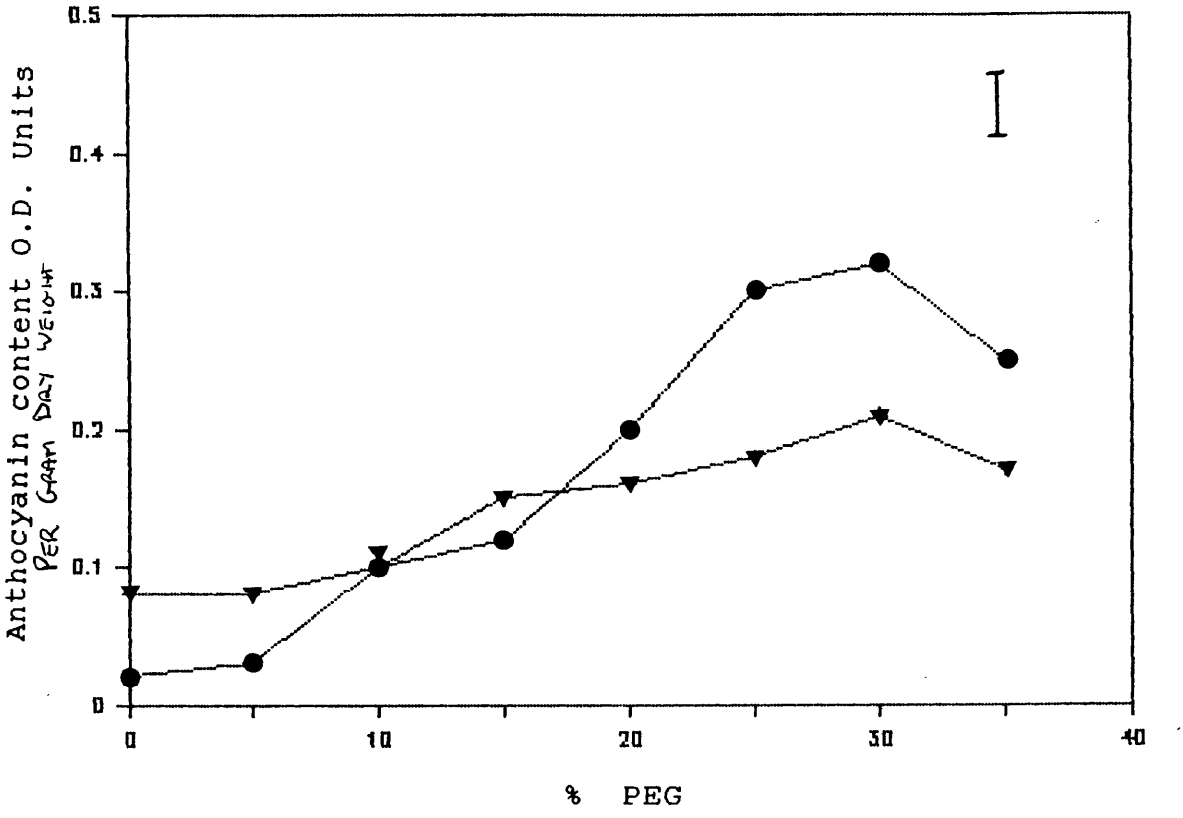
Figure 5.3.2



Bars indicate L.S.D. ($P < 0.05$)

Figures 5.3.0 to 5.3.2 to show the levels of total tannins in mature leaves (fig.5.3.0), young leaves (fig. 5.3.1) and roots (fig. 5.3.2) of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in ~ mol per gram dry weight.

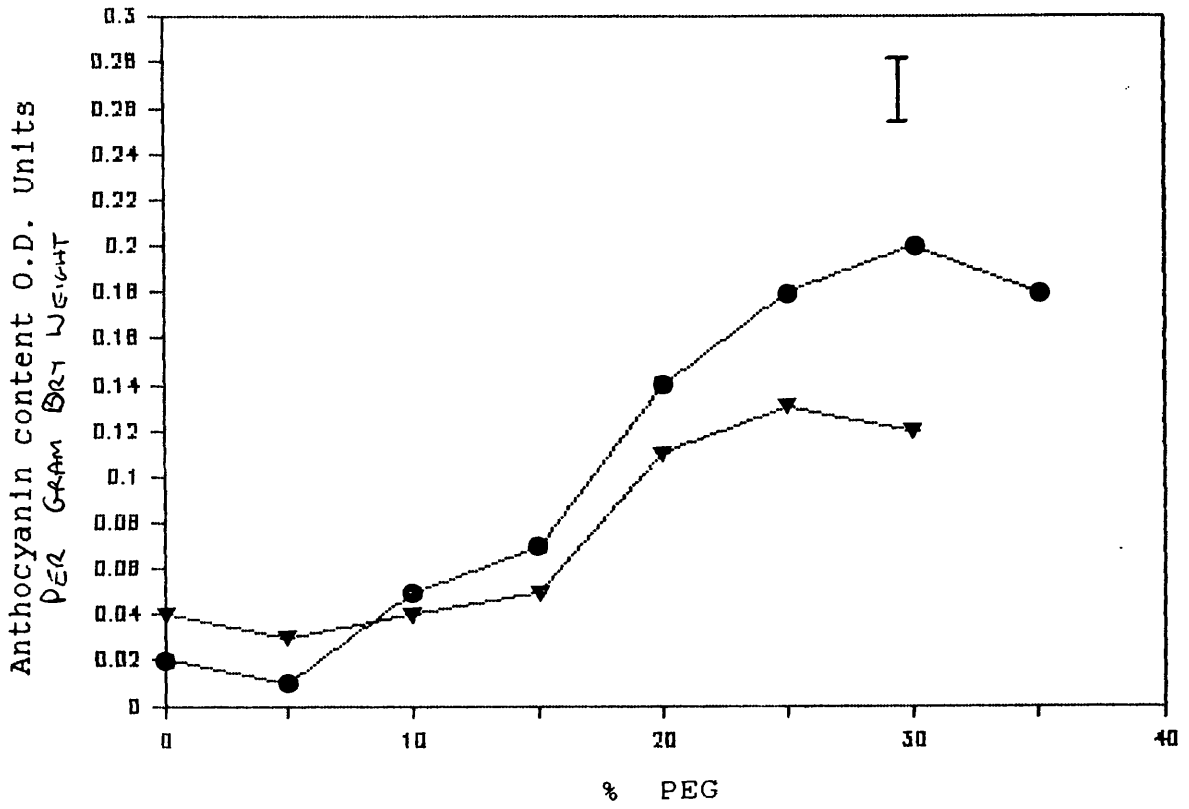
Figure 5.4.0



Figures 5.4.0 to show the increase in anthocyanin content in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as optical density units.

Bar indicates L.S.D. ($P < 0.05$)

Figure 5.4.1



Figures 5.4.1 to show the increase in anthocyanin content in young leaves of Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed as optical density units.

Bar indicates L.S.D. ($P < 0.05$)

DISCUSSION

The rise in total phenolic acids, up to 20% PEG in the various plant parts (Figs. 5.1.0 to 5.1.2), shows that mature leaves in both species accumulated more phenolics than young leaves and roots. After this initial rise there was a gradual fall in phenolic content until severe stress were reached when a further fall occurred in all plant parts in both species. This may be explained by the results gained by Pirie and Mullins (1976). These workers showed that sucrose and ABA could increase the levels of phenolic compounds in unstressed mature and young leaf discs. However the induction of accumulation was higher in mature leaves than in young leaves. They also showed that the induction effects could be reversed by nitrate. In the present study sugar levels increase rapidly up to this point (Figs. 2.14.0 to 2.15.2 in Chapter 2) and nitrate levels show a sharp decrease (Figs. 4.4.0 to 4.4.2). It has also been shown by many workers that the levels of ABA increase during water deficits (Pierce and Rascke 1980; Hsiao 1973). Thus the rise in phenolic levels in the two Geum species may be explained in these terms.

What is the significance of this accumulation of phenolic compounds to the plant? The answer may lie in the photosynthetic apparatus itself. Some phenolic acids and their derivatives have been shown to be excellent antioxidents, particularly caffeic and chlorogenic acids (Larson 1988; Dhindsa et al. 1981); ie they are able to neutralise photochemically produced reactive oxygen species which can damage plant proteins and membranes. Thus this type of accumulation may be advantageous to the plant during water stress by reducing damage by highly reactive oxygen when the plant is already under pressure from the effects of reduce water potential. Indeed Geum urbanum has previously been shown to have significant levels of caffeic and chlorogenic acids

(Gstir.er and Huch 1964). Thus the high level of phenolics in leaves may protect enzyme and membrane systems during water stress. Moreover a synergistic effect was noted by Hayase and Kato (1984) between phenolic acids and amino acids which also accumulate rapidly during water deficits (Figs. 2.16.0 to 2.16.2).

If the decline in photosynthesis measured by the oxygen electrode (Fig. 3.3.0) is compared with the accumulation of phenolic compounds some similarities can be seen between the two. Photosynthesis is maintained up to 20% PEG after which a slow decline occurs followed by a rapid decline under severe stress conditions. Although this is not evidence for the protection of the photosynthetic apparatus by phenolic compounds, it is possible they may be involved in reducing the effects of photoinhibition which may occur in these species.

It could be inferred from Figs. 5.2.0 to 5.2.2 that Geum urbanum produced more phenolic glycosides than Geum rivale in all plant parts particularly in roots where the increase in levels was in the region of 80 micro moles. This figure may also be an under estimate of glycoside content as glycosides may be lost during the drying of plant material and subsequent extraction in methanol (Lindroth and Pajutee 1987). The accumulation of glycosides has not previously been reported during water stress though Newton et al. (1986) did detect traces of dhurrin in water stressed cultured sorghum cells. However Perry et al. (1987) did not find increases in the glycoside betanin in excised storage tissue in red beet during sucrose mobilisation and salt accumulation. However, glycoside formation is known to increase during times of increased carbohydrate mobilisation (Ribereau 1972) and the results presented here are consistent with this observation. Of the many roles that have been ascribed to phenolic glycosides, Wasisky (1921) proposed a role in osmoregulation for

plant phenolic glycosides. However, Kojoma et al. (1979) and Oba et al. (1981) have shown glycosides to be located in the vacuole and thus cannot be involved in cytoplasmic solute maintenance. However the levels of nitrate which is stored in the vacuole of higher plant fall during water deficits. Thus glycosidic accumulation in the vacuole during water deficits may help restore the imbalance created by the reduction in nitrate levels during stress. The glycoside geniposidic acid has been shown to quench reactive oxygen species (Takahama 1983) in a similar way to phenolic acids. However it is unlikely that glycosides undertake this role in a physiological system as glycosides are contained in the vacuole and are thus unavailable to the cytosol. As reported in the introduction the O-glycoside, gein, has been isolated in Geum urbanum and consists of the reactive phenolic compound eugenol and the diose sugar vicianose (glucose and arabinose). Thus this compound may have been accumulated in Geum urbanum in the present experiments. However glucose transferase has been isolated in Geum urbanum (Psenak et al. 1969) which mediates the transfer of sugar residues between glycosides and thus the potential for the production of other glycosides is present in Geum urbanum. Attempts were made to synthesise gein and thus try to positively identify at least one of the glycosides produced by Geum urbanum, but this was unsuccessful and identifications could not be made. It is unfortunate that no information exists on glycoside formation in Geum rivale.

As glycosidic formation and accumulation has only been reported in the roots of Geum urbanum, transport of glycosides to leaves may occur during water deficits. Although transport of glycosides has not been studied during water stress, Pridham (1965) showed transport of glycosides and simple phenols did occur in Vicia faba and phloem transport has been shown to be relatively

water stress resistant (Sung and Krieg 1979; Johnson and Moss 1976). Thus it is possible glycosides could be transported during water stress in the two Geum species. Geum urbanum has also been shown to have glucose transferase activity in leaves as well as roots (Psenak et al. 1969) and thus glycosides other than gein may be produced in leaves and roots of Geum urbanum.

Another role for the formation of glycosides was suggested by Pridham (1958). He suggested that glycoside formation could be responsible for the detoxification of compounds harmful to the plant. This may be true in the case of gein, as eugenol is a highly reactive plant phenolic which may be detrimental to the plant particularly in leaves where it may form a reactive oxygen radical in the para position of the aromatic ring.

From Figs. 5.3.0 to 5.3.2 it is apparent that there is a fall in tannins in all plant parts during mild water stress, followed by a period of stabilization. Rises only occurred in tannin levels during more severe stress in all plant parts. Under severe stress tannin levels in young and mature leaves of both species only returned to pre - stress levels. However in roots tannin levels increased two fold at the higher water deficits from pre - stress levels. Thus during mild and moderate water deficits tannins play no role in increasing cell wall elasticity proposed by Pizzi and Cameron (1986) in Geum urbanum and Geum rivale. However, during severe stress when solute levels are being reduced some increase in cell wall elasticity may result especially in the roots of the two species.

The source of these tannins could arrive from the utilisation of free phenolics which fall during water stress and also from phenolic acids liberated when glycoside levels decrease. This may have a two fold value in that carbohydrates previously stored in the

vacuole as glycosides may then be liberated and available to the cytosol, at a time when free carbohydrates are declining. Thus glycosides may also act as a store of carbohydrate during periods of high carbohydrate mobility and then this stored carbohydrate may be mobilised when free carbohydrates begin to decline.

The rise in tannins was not correlated with the water stress tolerance of the two species as both species showed similar tannin levels at all periods. Prior,Tuohy and Whiting (1987) in a study of subtropical trees found little correlation between tannin levels and soil water status. However some trees did accumulate more tannins in the leaves when grown in dry sites as opposed to wet sites. This reaction to water deficits may then be more related to specific woody species and herbaceous plants such as the two Geum species may only utilise this mechanism as a last resort in an attempt to maintain cell elasticity. Other more tolerant plants may however use this mechanism, only further research will tell.

The rise in anthocyanin levels in mature leaves (Fig. 5.4.0) during water stress has previously been documented in various Quercus species (Spyropoulos and Mavrommatis 1978) and was consistent with the observed water deficit tolerances of the species studied. As Geum urbanum contained more anthocyanin in mature leaves than Geum rivale this is also consistent with the water deficit tolerance of these species. Anthocyanin levels in young leaves (Fig. 5.4.1) has not previously been studied during water stress but followed a similar pattern to that of mature leaves in Geum urbanum and Geum rivale, though levels were lower.

The appearance of anthocyanins during water deficits is not surprising as increased carbohydrate levels are known to increase anthocyanin content in

strawberry leaf discs (Creasy et al. 1965) and increases in anthocyanins have been recorded during starch mobilisation (Ribereau-Gayon 1972). However the carbohydrate levels are higher during water stress in young leaves and one may expect a larger rise in this tissue. This may not be apparent as work by Pierie and Mullins (1976) suggests. These workers showed both sucrose and ABA could induce anthocyanin production in leaf discs but when the two were added together a synergistic effect on anthocyanin accumulation was apparent in old leaves and an additive effect occurred in young leaves. As mentioned earlier in this chapter both sucrose and ABA have been shown to accumulate during water deficits and this may then explain why more anthocyanins are accumulated in mature rather than young leaves in the two species.

Anthocyanins are reported to be contained in the vacuole of the plant cell (Hrazdina et al. 1978) as are the glycosides produced in the plant. Thus anthocyanins may also contribute to the osmotic potential of the vacuole when nitrate levels decrease.

The rise in anthocyanins in mature leaves was fairly large and approached the levels of the xerophytic species Quercus coccifera in the experiments of Spyropoulos and Mavrommatis (1978). However, in their experiments a single shock stress of -4.8 MPa (approximately 45% PEG in my experimental system) was used to stress detached leaves. This is clearly a very rapid form of stress development and the levels of anthocyanin produced may have been much reduced in their experiments.

This chapter has shown that phenolic metabolism is greatly affected by water deficits imposed by PEG 6000. It has been proposed that phenolic glycosides and anthocyanins could be involved in the maintenance of vacuolar osmotic pressure during water stress. Moreover,

phenolic glycosides may also provide a store of carbohydrate during high carbohydrate mobility, which can be mobilised during severe water deficits. It has also been proposed that rises in phenolic acids may confer some protection to the photosynthetic apparatus during mild to moderate water deficits in Geum urbanum and Geum rivale.

CHAPTER 6

THE ROLE OF PROLINE IN GEUM URBANUM AND GEUM RIVALE IN WATER DEFICITS IMPOSED BY PEG 6000

INTRODUCTION

Low molecular weight solutes such as proline, glycine betaine and polyols have been shown to accumulate during various environmental stresses but particularly in salt and drought stress (Aspinall and Paleg 1981; Storey and Wyn-Jones 1977). The rise in these solutes from pre - stress levels is very large and in the case of proline rises in the order of 40,000% have been documented during water stress (Tyankoua 1967). Indeed it was shown in chapter 4 that proline increased markedly in Geum urbanum and Geum rivale during water deficits in all plant parts studied.

The significance of such large accumulations of proline during water deficits has created much controversy among the scientific community in recent years. Some authors have suggested an adaptive role for proline accumulation during drought stress (Boyer and Meyer 1979) and others disagree (Aspinall 1980).

Nevertheless, there is some evidence to suggest that there is a role for proline of adaptive significance during water stress in that proline may be able to confer stability to proteins by interactions with the hydrophobic residues on proteins, thus causing increased solubility on such proteins. Proline has also been shown to protect Bovine serum albumen from denaturation by ammonium sulphate and ethanol (Schobert and Tschesche 1978). At a more physiological level proline has been shown to protect enzyme systems in isolated organelles (Nash et al. 1982). However few investigations have been undertaken which determine the effects of proline on enzyme stability when solute concentration is increased. Franks (1977) showed that

even minor increases in solute concentration can cause major changes in protein conformation and hence destabilise proteins. Thus during water stress such effects on proteins may occur and proline may be involved in a protective way during water stress. Paleg, Stewart and Starr (1985) showed proline could reduce glutamine synthetase precipitation by PEG and thus maintain glutamine synthetase in solution. Moreover this was shown to be related to proline concentration ie the greater the concentration of proline the more protection was afforded. However before any protection can be conferred on enzyme systems proline must be shown to occur in the areas of the cell where enzyme systems occur, namely in the cytosol and not in the vacuole. There is some circumstantial evidence to suggest proline does indeed occur in the cytosol as proline concentrations rose towards the root tips of salt stressed corn where cells are poorly vacuolated (Goring et al 1977). More concrete evidence has been provided as Pahlich, Kerres and Jager (1983) showed proline concentrations were higher in extravacuolar fractions than vacuolar fractions in protoplasts subjected to water stress. Supportive evidence was provided by Okazaki, Sakano and Tazawa (1987) who also showed that proline accumulated during salt stress was primarily located in the cytosol. Thus proline appears to be preferentially accumulated in the cytosol where it is possible some enzyme stabilisation may be afforded by proline during water stress. Proline has also been demonstrated to accumulate in significant quantities in chloroplasts (Demmig and Winter 1986) during salt stress and may thus be able to confer some protection to the photosynthetic apparatus during water deficits. Thus on balance an adaptive role for proline during water deficits can be proposed.

As Geum urbanum could accumulate more proline than

Geum rivale a greater degree of protection may be conferred on Geum urbanum by proline than Geum rivale and thus may have some significance in the water deficit tolerance of the two species. It was therefore decided to determine whether proline could protect enzyme systems in Geum urbanum and Geum rivale by using glutamine synthetase as an example after Paleg, Stewart and Starr (1985). Additionally proline was to be fed to excised leaves and subsequently the leaves were to be stressed and photosynthesis was to be determined by the oxygen electrode in order to discover if proline had any protective properties on the photosynthetic apparatus of the two Geum species.

MATERIALS AND METHODS

A: Plant culture conditions

Plants were grown and stressed as described in chapter 1. Plant material for the experiments with proline were however grown in four inch pots in John Innes No 2 compost and continually watered with tap water. Plant material was collected at the twenty leaf stage.

B: Proline determination

Proline was extracted in methanol as described in chapter 2 and was determined by the method of Singh et al. (1973). Some interference occurred with samples containing high amounts of chlorophyll. In these cases the methanol was partitioned with methylene chloride in a separating funnel which removed chlorophyll from the methanoic solvent but proline remained in the methanoic fraction.

C: Amino acid determination

Amino acids were extracted in methanol as described in chapter 2 and assayed by the method described by Pearson and Stewart (1987).

D: Glutamine synthetase extraction and assay

Glutamine synthetase was extracted purified and assayed as described in chapter 4. PEG 6000 induced precipitation was achieved by the method of Paleg, Stewart and Starr (1985) and proceeded as follows: 0.5 ml GS1 was placed in a 1.5 ml Eppendorf tube on ice to which 0.5 ml PEG was added or 0.5 ml PEG and proline. All reagents were made up in 50 mM Tris-HCl at pH 6.5. The tubes were then agitated and left for 30 minutes when they were centrifuged for 10 min at room temperature. The supernatant was then removed and the tubes wiped dry. The remaining pellet was redissolved in 200 1, 50 mM Tris-HCl at pH 6.5 and assayed as previously described.

E: Proline effects on Photosynthesis

PEG 6000 solutions were made up in distilled water and adjusted to pH 7 with KOH. In some solutions proline was added to give a final solution of the desired PEG concentration and 1M proline. In experiments with a proline pre-treatment a 1M proline solution was made in distilled water and adjusted to pH 7 as before. Mature leaves were then cut with a razor blade under the various solutions so as to maintain xylem flow. Some leaves were pre-incubated in 1M proline for 1 hour before being transferred to PEG solutions or PEG/proline solutions.

RESULTS

The dramatic rise in proline levels during water deficits is shown in Figs. 6.1.0 to 6.1.2. This shows that Geum urbanum could accumulate more amino acids than Geum rivale in all plant parts throughout water stress. The following Figs. (6.2.0 to 6.3.2) show the rise in total amino acids and the percentage of proline in the amino acid pool. The percentage of proline in root tissue was always higher than that in young and old leaves in both species. Also when proline and total amino acids fell proline levels were maintained when expressed as a percentage of total amino acids.

The experiments undertaken to investigate the reputed protective properties of proline are shown in Figs. 6.4.0 and 6.5.0 and show that proline could reduce PEG induced precipitation of glutamine synthetase isolated from Geum urbanum and Geum rivale. However the protection conferred on glutamine synthetase was dependent on proline concentration. The figures also show that there was no difference in protection of glutamine synthetase isolated from either species.

Table 6.1.0 indicates proline was accumulated to greater levels in proline fed treatments when compared to the relevant controls. However proline had no effect on photosynthesis in detached leaves of Geum urbanum or Geum rivale.

Figure 6.1.0

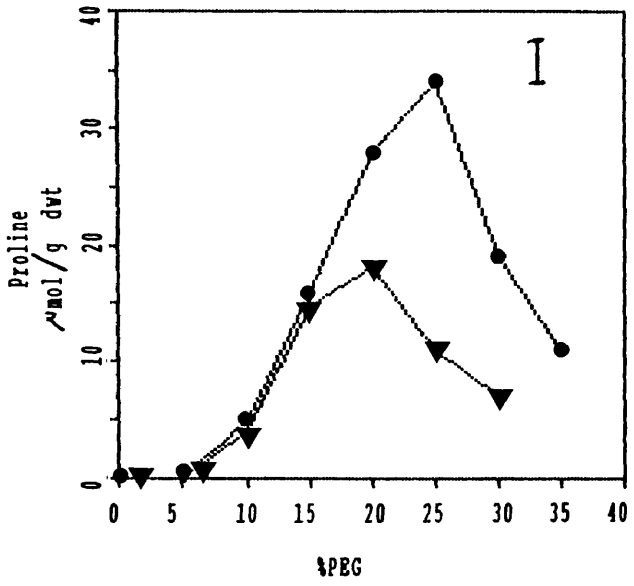


Figure 6.1.1

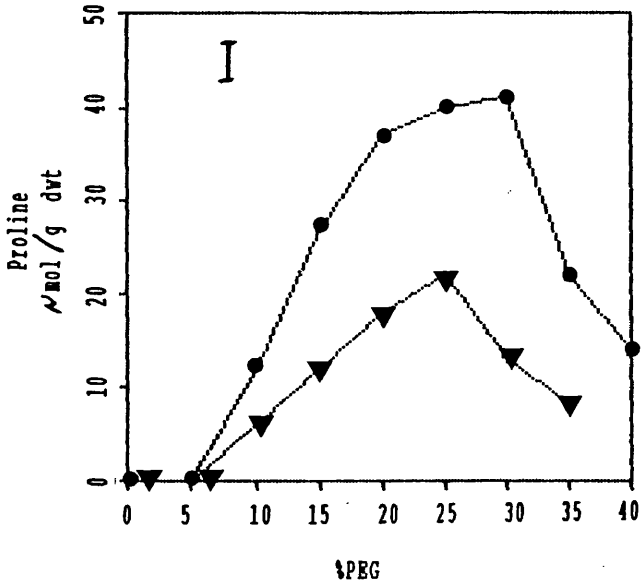
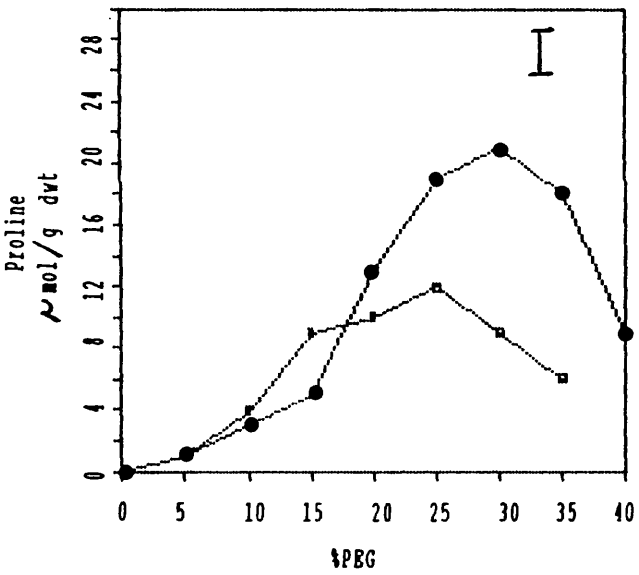


Figure 6.1.2



Bars indicate L.S.D. ($P < 0.05$)

Figures 6.1.0 to 6.1.2 show the changes in proline levels in mature leaves (6.1.0), young leaves (6.1.1), and roots (6.1.2) in Geum urbanum ● and Geum rivale ▼ in response to water deficits imposed by PEG 6000. Results are expressed in µmol/g dwt.

Figure 6.2.0

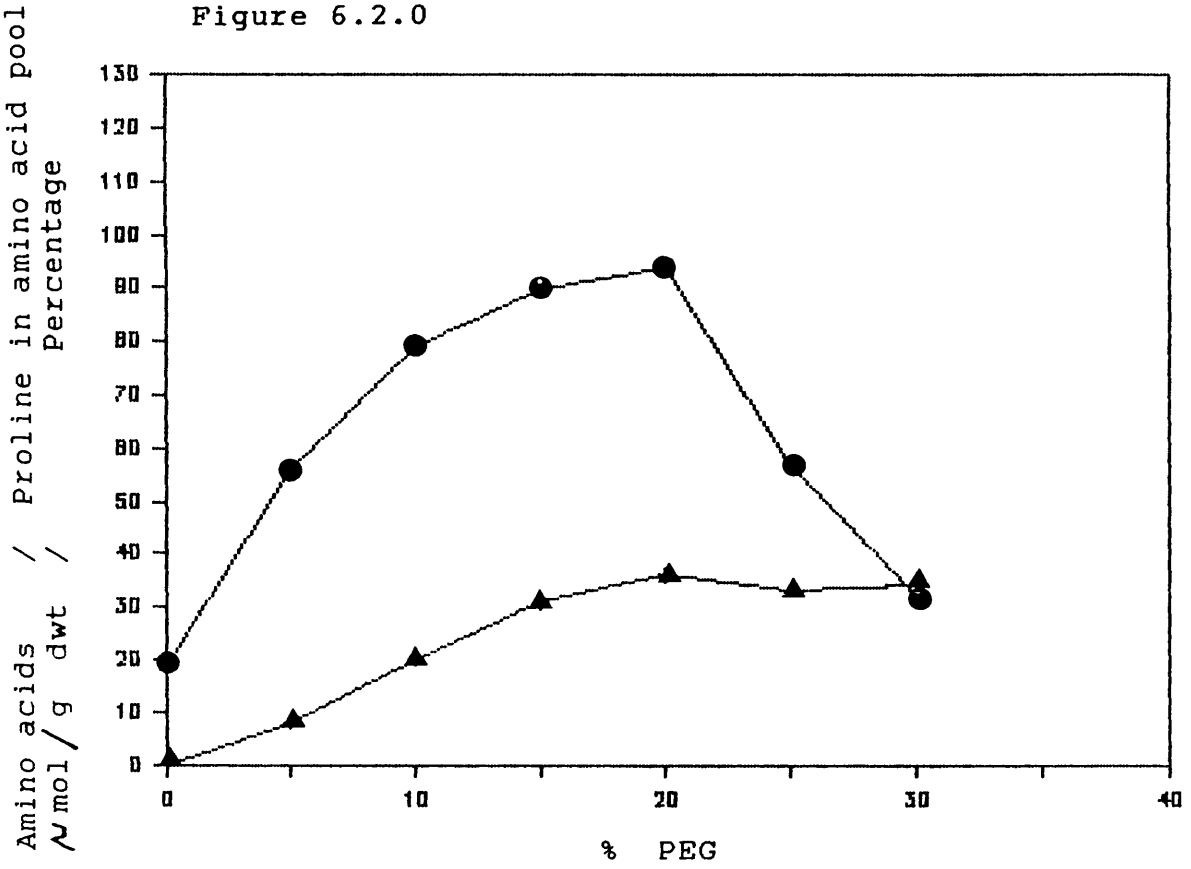


Figure 6.2.0. To show the changes in amino acid levels in mol / g dwt ● and the percentage of proline within the amino acid pool ▲ in mature leaves of Geum urbanum plants exposed to water deficits imposed by PEG 6000.

Figure 6.2.1

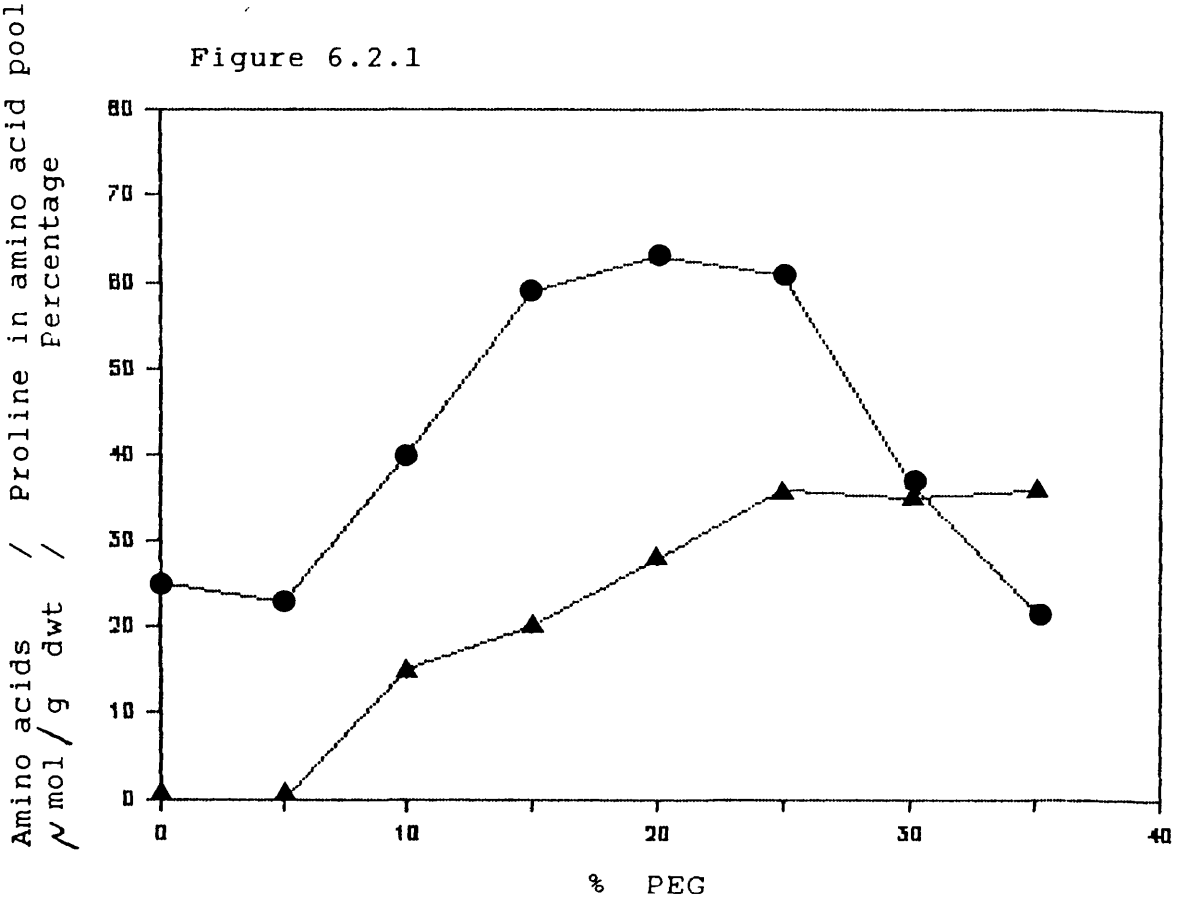


Figure 6.2.1. To show the changes in amino acid levels in mol / g dwt ● and the percentage of proline within the amino acid pool ▲ in young leaves of Geum urbanum plants exposed to water deficits imposed by PEG 6000.

Figure 6.2.2

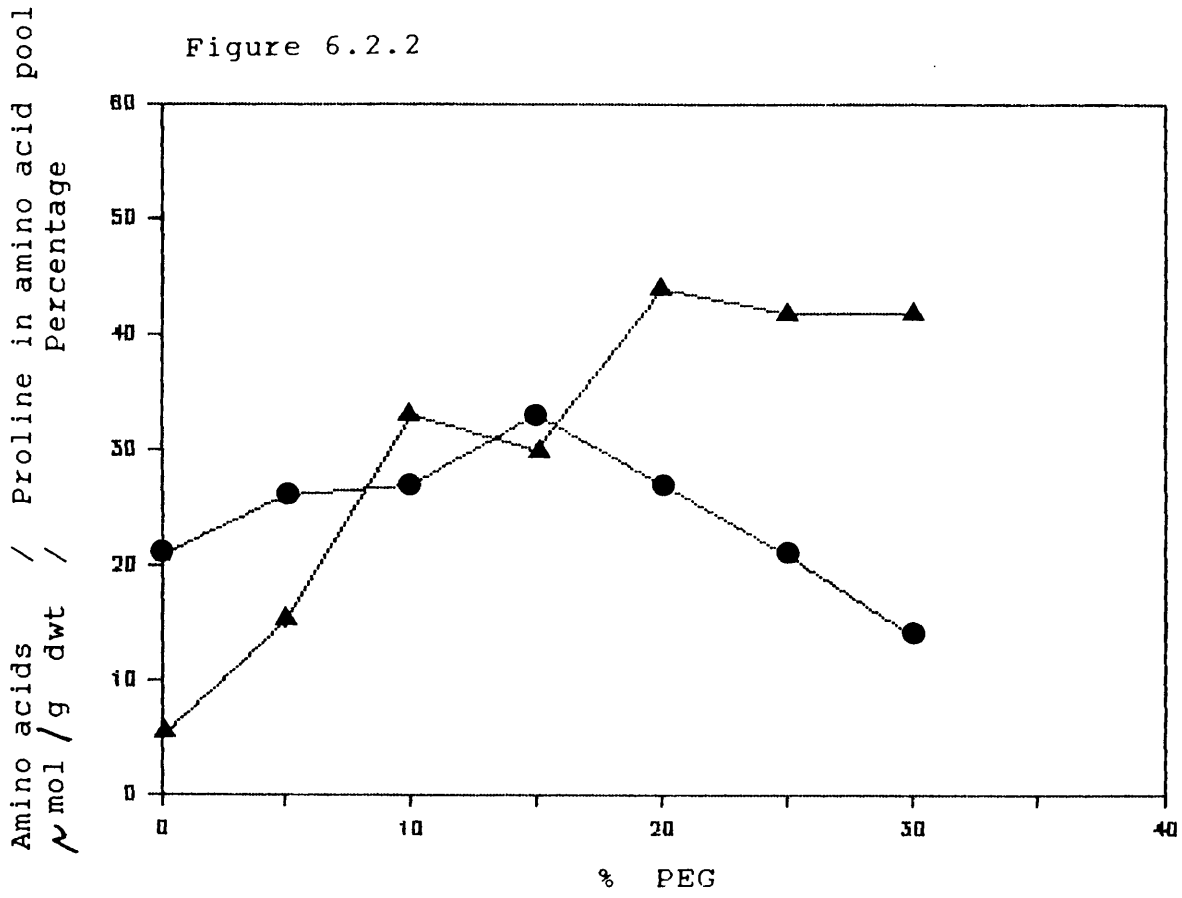


Figure 6.2.2. To show the changes in amino acid levels in mol / g dwt ● and the percentage of proline within the amino acid pool ▲ in roots of Geum urbanum plants exposed to water deficits imposed by PEG 6000.

Figure 6.3.0

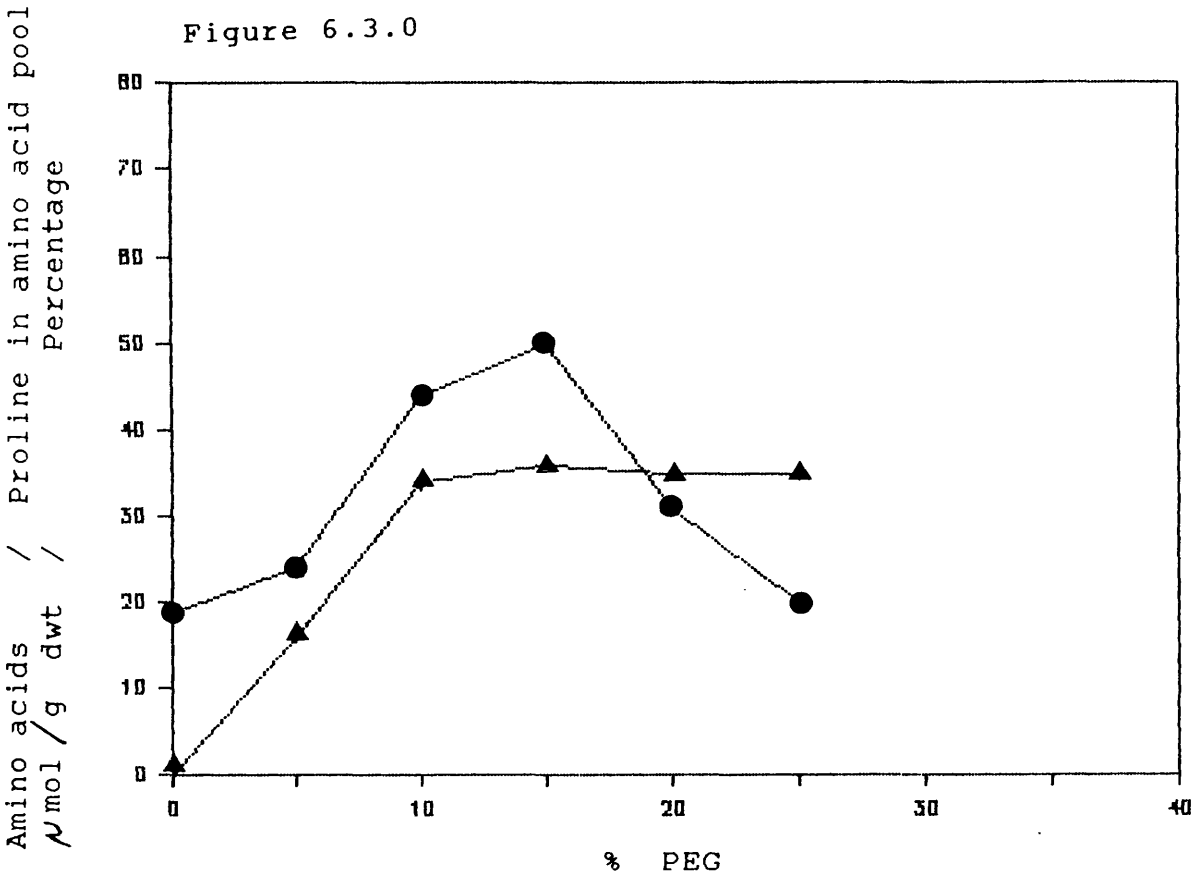


Figure 6.3.0. To show the changes in amino acid levels in mol / g dwt ● and the percentage of proline within the amino acid pool ▲ in mature leaves of Geum rivale plants exposed to water deficits imposed by PEG 6000.

Figure 6.3.1

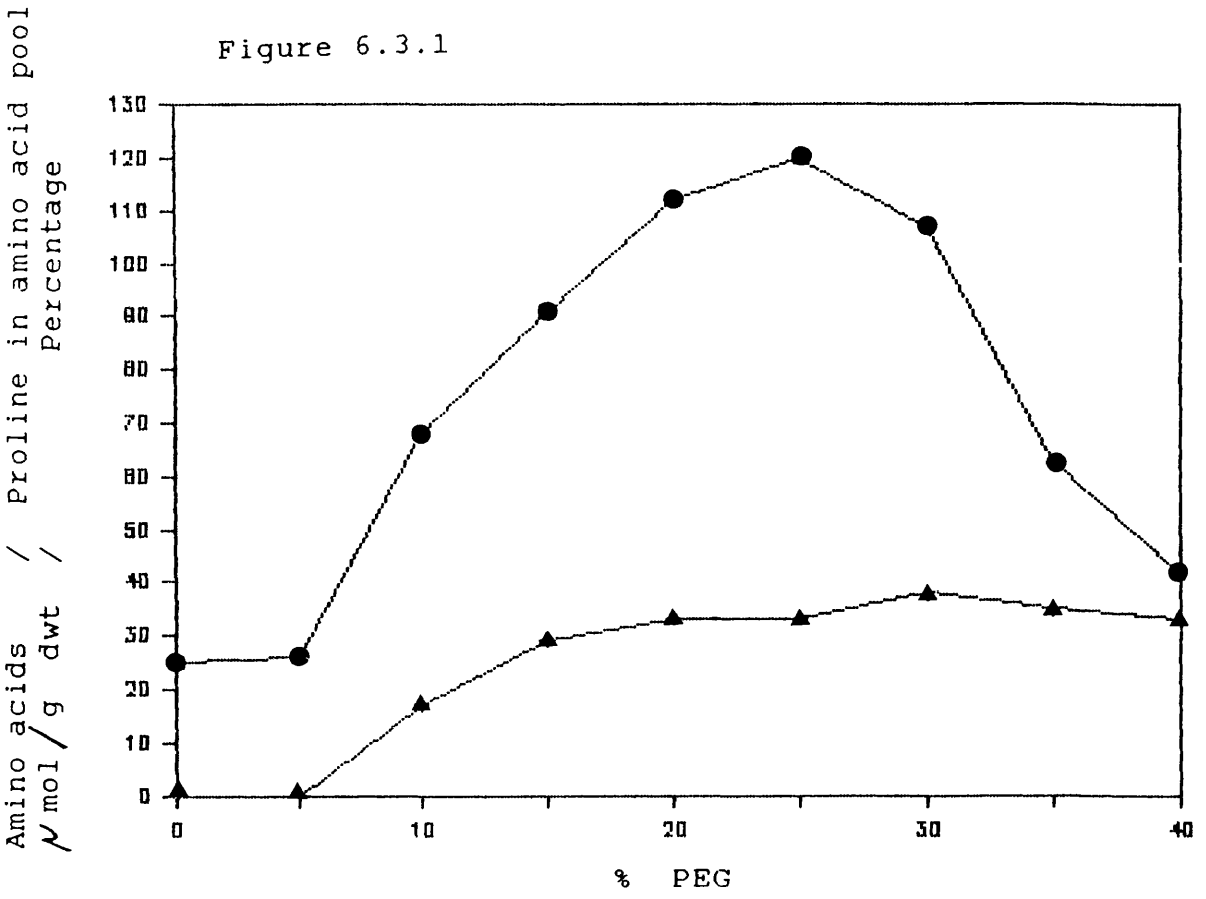


Figure 6.3.1. To show the changes in amino acid levels in mol / g dwt ● and the percentage of proline within the amino acid pool ▲ in young leaves of Geum rivale plants exposed to water deficits imposed by PEG 6000.

Figure 6.3.2

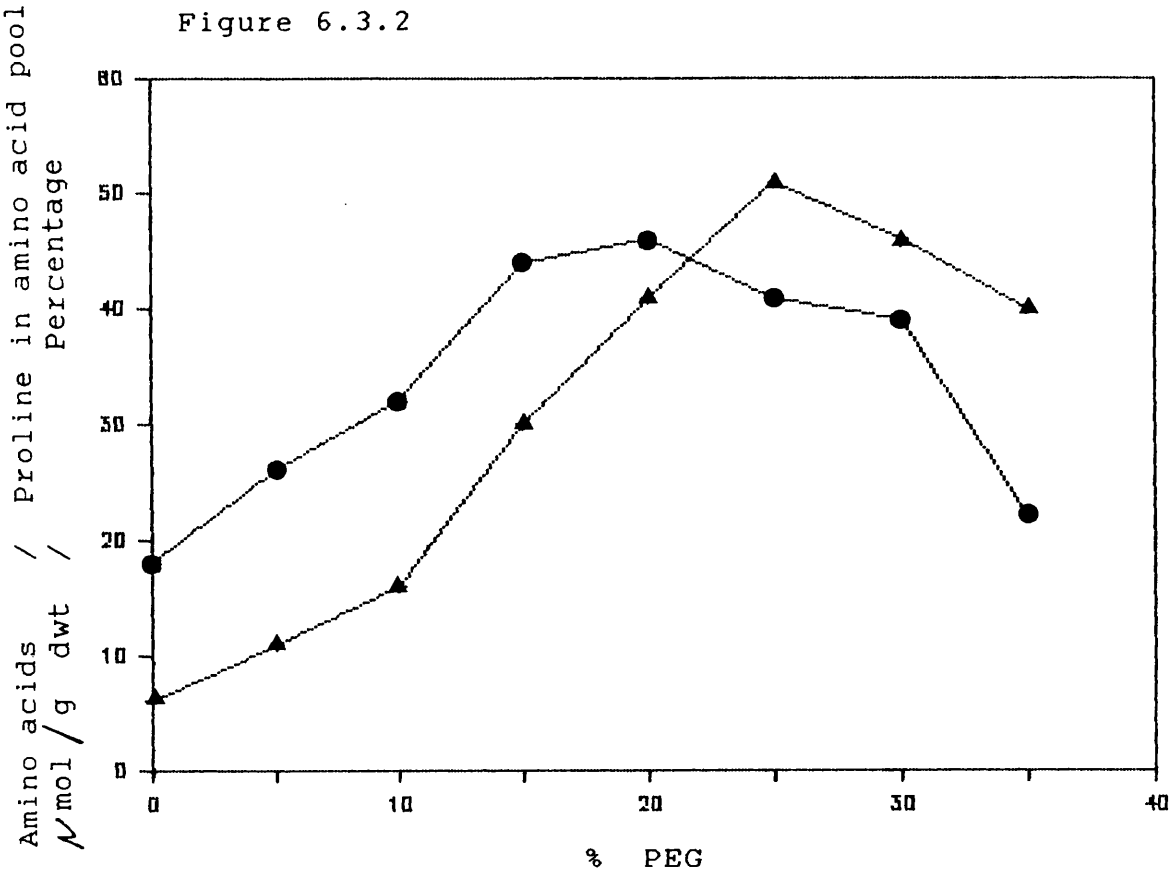


Figure 6.3.2. To show the changes in amino acid levels in mol / g dwt ● and the percentage of proline within the amino acid pool ▲ in roots of Geum rivale plants exposed to water deficits imposed by PEG 6000.

Figure 6.4.0

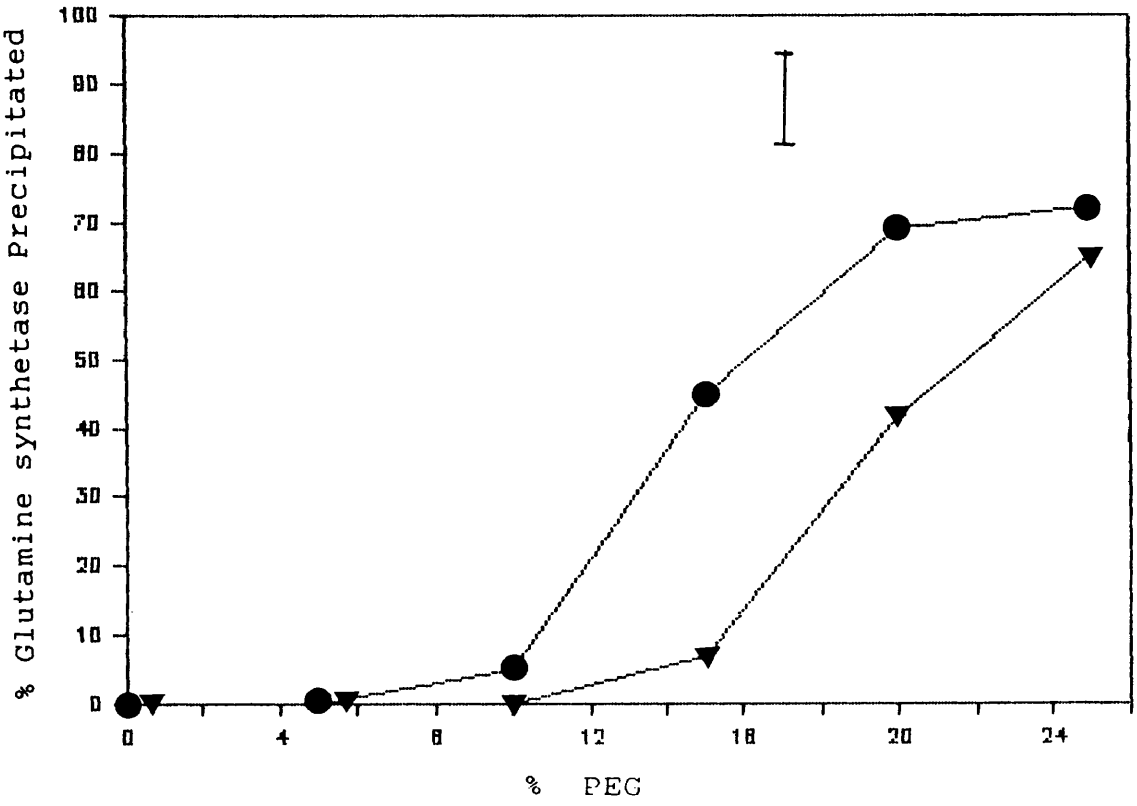


Figure 6.4.0 to show the effect of 1M proline on the PEG 6000 induced precipitation of glutamine synthetase. ▼ = 1M proline + PEG 6000; ● = PEG 6000. Results are expressed as a percentage of control glutamine synthetase activity.

Bar indicates L.S.D. ($P < 0.05$)

Figure 6.5.0

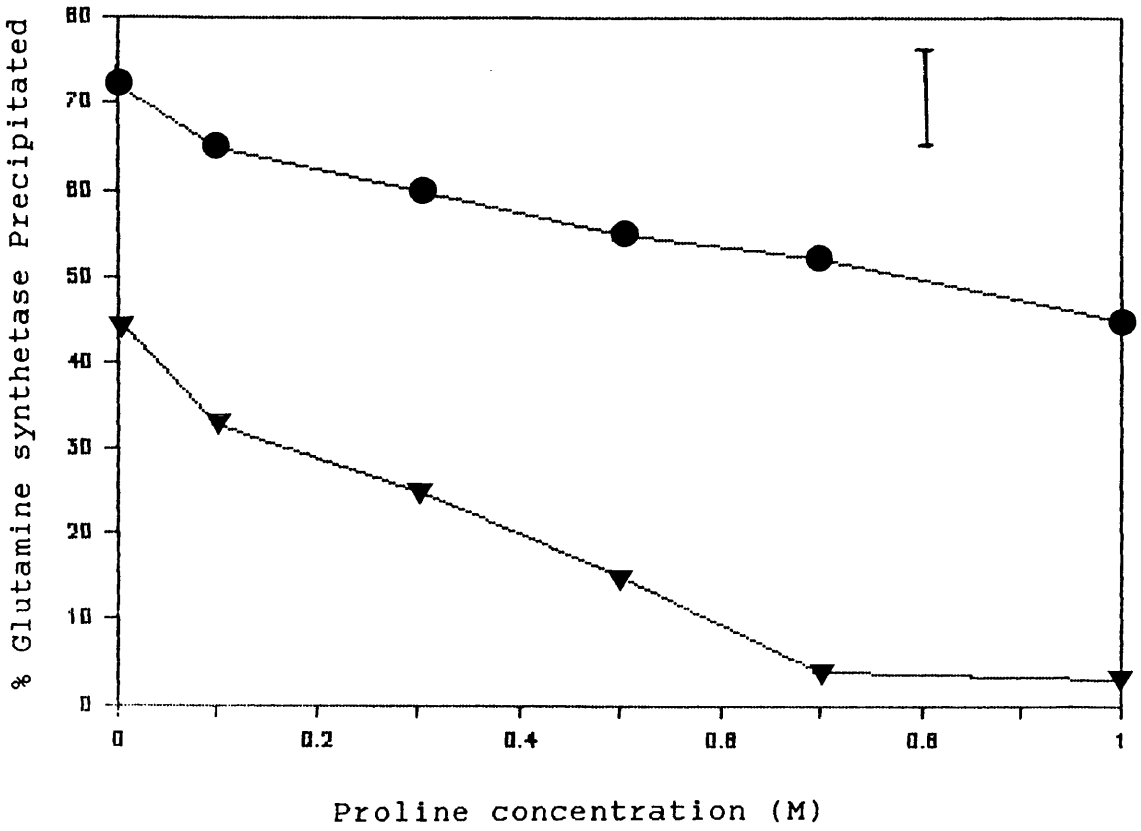


Figure 6.5.0 to show the effect of proline concentration on PEG 6000 induced precipitation of glutamine synthetase. ▼ = 15% PEG; ● = 20% PEG. Results are expressed as a percentage of control glutamine synthetase activity.

Bar indicates L.S.D. ($P < 0.05$)

Table 6.1.0 to show the effect of exogenous proline on net photosynthesis on excised leaves of Geum urbanum and Geum rivale during water stress imposed by 25% PEG Results are expressed as a percentage of the relavent control photosynthesis. Proline in μmol g dwt

Treatment	G. urbanum	G. rivale	Proline
1	100	100	0
2	100	103	6
3	47	49	1.5
4	49	51	2.4
5	46	47	7.2

Treatment 1. Leaves incubated in distilled water for 3 hrs prior to measurement

Treatment 2. Leaves incubated in 1M proline for 3 hrs prior to measurement

Treatment 3. Leaves incubated in distilled water for 1 hr followed by incubation in 25% PEG for 2hrs prior to measurement

Treatment 4. Leaves incubated in distilled water for 1hr followed by incubation for 2 hrs in 25% PEG and 1M proline before measurement

Treatment 5. Leaves incubated in 1M proline for 1 hr followed by incubation in 25% PEG and 1M proline for 2 hrs before measurement

DISCUSSION

The rise in proline accumulation during water stress exhibited by Geum urbanum and Geum rivale has been well documented in the literature in other plant species subject to water deficits both in the field and under laboratory conditions (Aspinall and Paleg 1981; Poljakoff-Mayber et al. 1987). Rises have also been shown to occur in root and leaves with rises in leaves occurring prior to accumulation in roots as in these experiments (Singh, Paleg and Aspinall 1973 a). These rises in proline accumulation have led many workers to attempt to correlate such rises with the drought tolerance of various species and cultivars. The results gained have shown correlations between leaf proline accumulation and water stress tolerance of individual leaves (Singh, Paleg and Aspinall 1973 b) and whole plants, (Singh and Gupta 1983). However other studies have shown no correlation between proline accumulation and water deficits (Al oni and Ro senshtein 1984; Waldren, Teare and Ehler 1974; Pearson and Stewart 1987). However, differences in methodology and the need to correlate drought resistance and yield may account for some anomalies reported. Waldren and Teare (1974) concluded proline was related to the drought tolerance of species but was not a sensitive indicator of drought tolerance itself. However, in Geum urbanum and Geum rivale proline accumulation is related to the stress tolerance of the two species during more severe stresses but not during mild water deficits.

The rise in proline during water stress could occur by reduced incorporation into protein or increased proteolysis. However, calculations have revealed that only 5-15% of the rise in proline could occur by this means (Stewart, Morris and Thompson 1966). Reduced proline oxidation could also provide the plant with an increase in proline levels. However there have been

conflicting results quoted in the literature concerning proline oxidation. Stewart and Boggess (1978) showed proline oxidation was reduced during water deficits and Stewart (1972b) showed proline oxidation was maintained during water stress. Despite these contradictory results even when proline oxidation was shown to fall, the rise in proline could not be accounted for by reduced proline oxidation. However, Boggess et al. (1976) showed ^{14}C incorporation into free proline from glutamate increased during water deficits and was correlated closely with proline accumulation without label in tobacco leaves. These workers also found incorporation of ^{14}C label from the ornithine /arginine cycle into proline but only after an initial rise in proline from glutamate. Kueh et al. (1984) working on barley confirmed proline could be produced from glutamate in unstressed plants and proposed the ornithine pathway to be a catabolic pathway. Thus it is thought that proline is synthesised during water stress primarily from glutamate itself. Proline production is however controlled by end product inhibition ie proline inhibits its own production. Thus this end product inhibition must be removed for proline synthesis to occur. This was demonstrated to occur in barley by Boggess, Aspinall and Paleg (1976) using radio tracers. Fukutoku and Yamada (1984) showed that a large proportion of proline was synthesised during water stress and the source of this proline was leaf protein. However earlier in chapter 4 it was stated that Geum urbanum and Geum rivale could produce amino acids due to continued primary production and thus a significant amount of proline may be produced by such a method. Circumstantial evidence for glutamate being a precursor of proline synthesis is provided in chapter 4 when glutamate levels fall dramatically in the leaves of both species under severe stress when proline levels continue to rise.

The results presented in Figs. 6.2.0 to 6.3.2 show proline levels to be maintained during severe water stress as a proportion of the total amino acid pool in both species in all plant parts measured. Hence, some mechanism must exist whereby proline is preferentially maintained at high levels within the plant, possibly by the conversion of glutamate to proline. However the increase in proline and its maintenance at high levels within the amino acid pool do not necessarily indicate adaptive significance, but if proline is not adaptively significant then the production of proline would represent a considerable waste of energy and reducing power during water stress.

The protection afforded to glutamine synthetase by proline during Peg 6000 precipitation and the proline concentration dependent protection (Figs. 6.3.0 to 6.5.2) are consistent with those reported by Paleg, Stewart and Starr (1985). Though proline can reduce precipitation by PEG 6000 it is not clear whether proline affects the ability of PEG to precipitate the enzyme or protects the enzyme from the action of PEG precipitation (Paleg, Stewart and Starr 1985). However it was considered by these authors that the protection afforded by proline during PEG induced precipitation was analogous to conditions that appear during water deficits. Thus provided proline is preferentially accumulated in the cytosol of Geum urbanum and Geum rivale during water stress some protection to enzyme systems may arise during water stress in the two species. From these figures the protection afforded to glutamine synthetase was not significantly different between glutamine synthetase isolated from Geum urbanum and Geum rivale which indicates that proline could afford similar protection to this enzyme in both species. Yet in chapter 2 it was seen that glutamine synthetase activity in Geum urbanum was maintained at

higher levels in roots and leaves during stress than Geum rivale. It is therefore attractive to consider these differences occur because of the increased protection higher proline accumulation confers on the glutamine synthetase of Geum urbanum. Indeed it is possible that this type of protection could be conferred on other enzymes during water stress as Geum urbanum generally could maintain higher enzyme activities than Geum rivale. Moreover this was particularly when a significant difference in proline levels was apparent between the two species. Thus the levels of proline accumulated in the two Geum species could partially determine the ability of the two species to osmoregulate and hence may contribute to the difference in water deficit tolerance of the two species.

The experiments conducted concerning the protection of photosynthesis by proline (Table 6.1.0) show that proline did accumulate to significant levels in proline fed leaves however photosynthetic rate was not affected by any of the treatments undertaken during water stress when compared to the relevant controls. Thus it may be concluded that proline had no significant protective or detrimental effect on photosynthesis during rapid water deficits under such experimental conditions. However such an experiment does not confirm or deny a role for proline in the protection of photosynthesis during water stress as such a rapid stress may alter the availability of proline to the chloroplast and proline levels in the chloroplast itself were not determined.

It has been shown that other low molecular weight solutes other than proline can provide protection for enzymes in vitro such as glycine betaine and various polyols (Larkum and Wyn-Jones 1979; Laurie 1988) which have been shown to accumulate in other species during drought stress (Ford 1984). Moreover these have been shown to afford greater protection to enzyme systems

than proline (Laurie 1988; Larkum and Wyn-Jones 1979) and occur in more water stress tolerant plants than the two Geum species, often in combination with each other and proline (Ford 1984). Thus drought resistance could be determined, at least in part, not only by the level of such protectant accumulation but also by the types of solute accumulated. Thus the frustrated attempts at defining a metabolic marker for drought tolerance in plants such as occurred with proline (Pearson and Stewart 1987) may have been thwarted by incomplete examination of such solutes described above as well as differences in methodology.

This chapter has demonstrated that proline can confer some protection to glutamine synthetase isolated from Geum urbanum and Geum rivale during PEG induced precipitation. Moreover it was shown that there was no difference in protection of glutamine synthetase isolated from either species, but the protection conferred was dependent on proline concentration. It was then tentatively suggested that the higher proline accumulation exhibited by Geum urbanum over Geum rivale during water stress may be influential in determining the water deficit tolerance of the two species.

CHAPTER 7

THE IMPOSITION OF FIELD WATER DEFICITS ON *GEUM URBANUM* AND *GEUM RIVALE*. SOME ASPECTS DETERMINING THE DROUGHT TOLERANCE OF THE TWO SPECIES

INTRODUCTION

From previous chapters it is apparent that *Geum urbanum* could out survive *Geum rivale* during water deficits imposed by PEG 6000. However laboratory induced stresses do not necessarily apply directly to the field situation (Begg and Turner 1976). Thus these differences in water deficit tolerance of the two species found in the laboratory must be demonstrable in the field if their contribution in determining the ecological distributions of the two species is to be assesed. Due to pressures of time it would have been impossible to follow all the aspects of water stress physiology and metabolism discussed in chapters 1 to 6 in this thesis, therefore only the most important aspects were to be followed in the field.

Chapter 2 showed that the main reason for the difference in water deficit tolerance of the two species was that *Geum urbanum* could reduce Ψ_w and Ψ_s to a higher extent than *Geum rivale* and hence maintain turgor and water uptake nearer to pre - stress levels. This was achieved because *Geum urbanum* could accumulate a greater amount of free solutes than *Geum rivale* and could thus lower Ψ_s by a greater degree than *Geum rivale*. The solutes accumulated in the two species were identical in that both species accumulated free carbohydrates and free amino acids with carbohydrate forming the greater contribution to Ψ_s reduction. Chapter 3 showed that *Geum urbanum* was capable of mobilising stored carbohydrate reserves during water deficits in greater amounts than *Geum rivale* and this was a major reason for the larger increase in free carbohydrate during water deficits in

Geum urbanum. It was proposed in chapter 4 that the primary production of amino acids could significantly contribute to the rise in free amino acids as well as increased protein degradation.

These aspects outlined above were considered to be the most important aspects of physiology and metabolism which affected the water deficit tolerance of and Geum rivale in the laboratory and thus these were to be followed in a natural situation.

Water potential, solute potential and relative water content were to be determined in order to asses the changes in plant water relations. The levels of free carbohydrate and free amino acids including proline were also to be determined as these were the only solutes shown to increase under laboratory conditions. Photosynthesis and transpiration were also to be followed to assess their effect on the plants ability to resist periods of drought in the field. The mobilisation of stored carbohydrate was also to be assessed in the form of starch grain disappearance and appearance in the chloroplasts of mature and young leaves to evaluate the results gained in the laboratory and determine whether or not starch forms a major input to free carbohydrate in the field. Primary nitrogen assimilation was also to be assessed by determining the nitrate reductase activity in roots and leaves in order to evaluate the contribution this process could make to any rise in amino acid levels during drought.

MATERIALS AND METHODS

A: Field Site

For field work a site had to be found in the south of England which could potentially satisfy the particular habitat requirements of both species, but at the same time provide a range of wet and dry sites. It was not good enough to find a dry site and plant the two species only to find that Geum rivale suffered complete mortality. The argument may then be made that the site could not have supported a Geum rivale population in the first place, and the experiment would be of no value. A suitable site was found at Hailey Wood Cambridgeshire. This wood has a perched water table during the winter months but frequently becomes lochally droughted in the summer. This gave ample scope for selecting a range of wet and dry sites and had the added advantage in that the wood had an indigenous population of Geum urbanum. This coppiced wood, owned by the Cambridge Wildlife Trust would provide adequate protection for the experiment. Coppicing takes place every two years in different blocks and so a range of shade environments could be found. Work was attempted in Hayley Wood in the final two years of the study. Nine sites were chosen with differing water availabilities and shade environments. Fifteen plants of each species were planted in each site in late march of each year when they had reached the eighth leaf stage. An automatic weather station was placed in the wood to record air and soil temperatures and levels of irradience in both open and shaded environments. Soil moisture blocks were also buried in the areas where the plants were placed.

B: Outdoor site in Nuffield Gardens

In the final year of the project a site was selected in the College gardens to carry out a stress experiment on both Geum urbanum and Geum rivale. The

plants were planted out at the ten leaf stage in March 1988 and allowed to grow for one month before drought was imposed. To insure that drought took place the plants were enclosed in a framework covered with polyethylene sheeting. In periods of fine weather the plastic sheeting was removed to prevent excessive heating of the plants. Eighty plants of each species were planted alternately with a six inch spacing between plants. One third of the plants were watered while the rest were allowed to dry out naturally. Samples were taken weekly between midday and 2 pm. Photosynthesis and transpiration were measured *in situ* and other plants were returned to the laboratory in plastic bags for further experimentation. Relative water content was used as a measure of plant water status.

D: Determination Of Solute Levels

Extraction of free solutes was undertaken by adding 10ml methanol to 0.5 to 1.0g of dried plant material (dried in the same way as described in Chapter 1) this was then whirly mixed and placed in a fridge over night and whirly mixed the following morning. This process was repeated three times when the extract was ready for use. The following assays were undertaken with this extract:

Total amino acids were detected by the ninhydrin method described by Pearson and Stewart 1987 using leucine as a standard.

Total hexose sugars were determined by the method of Jensen and Ashton (1960) glucose being used as a standard.

Total carbohydrates were determined by the anthrone method of Plummer 1978, carbohydrate levels being expressed in glucose equivalents. Total hexose was then subtracted from this figure and the remainder divided by two to give inferred sucrose levels.

Proline was measured by the method of Singh *et al.* (1973)[b] and interfering chlorophyll removed as described

in chapter 6.

E: Wilting point and death point determination

The wilting point of the plant was considered to occur when all mature leaves had reached permanent wilting. The death point was reached when all leaves and visible buds were desiccated.

F: Measurement of other parameters

Relative water content was determined as described in chapter 1. Water potential and solute potential were determined as in chapter 2. Photosynthesis and stomatal conductance were measured using the ADC portable IRGA, again see chapter 2 for details. Starch grains in chloroplasts were determined as detailed in chapter 3.

RESULTS

In Hayley Wood both species became established and flourished in both years. Unfortunately during 1987 and 1988 when the field work was undertaken there was too much rainfall and no water deficits were experienced by the plants in this field situation. As a result of this no data are presented for this site.

Results were however gained from the College Gardens in London and were as follows. Table 7.1.0 shows the points at which 50% of the droughted plants wilted or died during the experimental period. This table shows that Geum urbanum was able to hold off wilting for approximately 20 days longer than Geum rivale and could eventually outsurvive Geum rivale by 38 days. Thus the clear difference in water deficit tolerance exhibited in the laboratory was shown to exist in a semi-field situation.

Fig. 7.1.0 shows the decline in Relative Water Content (RWC) during the stress period and shows that Geum urbanum could maintain a higher RWC throughout the stress period than Geum rivale, indicating that Geum urbanum could maintain a higher water status than Geum rivale during the stress period.

Fig. 7.2.0 shows the decline in photosynthesis as measured by IRGA in the two species. The results are expressed as a percentage of control unstressed photosynthesis on the particular day in question so as to negate the effects of temperature and light fluctuations between sampling dates. The results show that as stress ensued photosynthesis declined in both species and from halfway through the experiment Geum urbanum could maintain photosynthesis at a significantly higher level than Geum rivale.

The transpiration rates of Geum urbanum and Geum rivale throughout the stress period are shown in Fig. 7.3.0 and are presented in the same manner as above.

This follows the same trend exhibited by photosynthesis in the two species with Geum urbanum maintaining a higher rate of transpiration than Geum rivale during the later stages of the experiment.

The decline in water potential, solute potential and turgor pressure in the two species are shown in Figs. 7.4.0 to 7.6.0 and show that Geum urbanum could reduce water and solute potential far lower than Geum rivale as drought developed and could maintain turgor for a longer period than Geum rivale.

Figs. 7.7.0 to 7.7.2 show the accumulation of free hexose sugars in mature leaves, young leaves and roots in Geum urbanum and Geum rivale during the experimental period and show that Geum urbanum was able to accumulate more hexose sugars than Geum rivale in all plant parts as drought conditions progressed. Geum urbanum also accumulated more sucrose in all plant parts measured than Geum rivale as drought progressed (Figs. 7.8.0 to 7.8.1).

The accumulation of amino acids in mature leaves, young leaves and roots occurred later during drought than carbohydrate accumulation, but again Geum urbanum was able to accumulate a greater amount of amino acids than Geum rivale as water became more limiting in the soil (Figs. 7.9.0 to 7.9.2).

Geum urbanum was also able to accumulate higher levels of proline than Geum rivale throughout the stress period (Figs. 7.10.0 to 7.10.2) in mature leaves, young leaves and roots throughout drought stress. Table 2.2.0 shows the increase in proline during drought imposition as a percentage of total amino acid accumulation and shows both Geum urbanum and Geum rivale maintained high levels of proline during water deficits and could maintain these levels when amino acids and proline levels declined.

Figs. 7.11.0 to 7.11.1 show the changes in starch

grain number in the chloroplasts of mature and old leaves of Geum urbanum and Geum rivale during the stress period. These data show that starch grains were lost from chloroplasts of mature leaves and deposited in young leaves of both species. However Geum urbanum lost more starch grains from chloroplasts than Geum rivale in mature leaves and also starch grain deposition in chloroplasts increased to a greater extent in young leaves of Geum urbanum.

Nitrate reductase activity in mature leaves and roots of both species is shown in Figs. 7.12.0 to 7.12.1. It is apparent from these figures that nitrate reductase activity was maintained in the leaves of both species until more severe stress was reached whereupon activity fell. However, nitrate reductase activities rose slightly in the roots of both species during mild to moderate water deficits. When water stress began to affect the water status of the plant significantly Geum urbanum could maintain nitrate reductase activity at higher levels than Geum rivale in both roots and leaves.

Figure 7.1.0

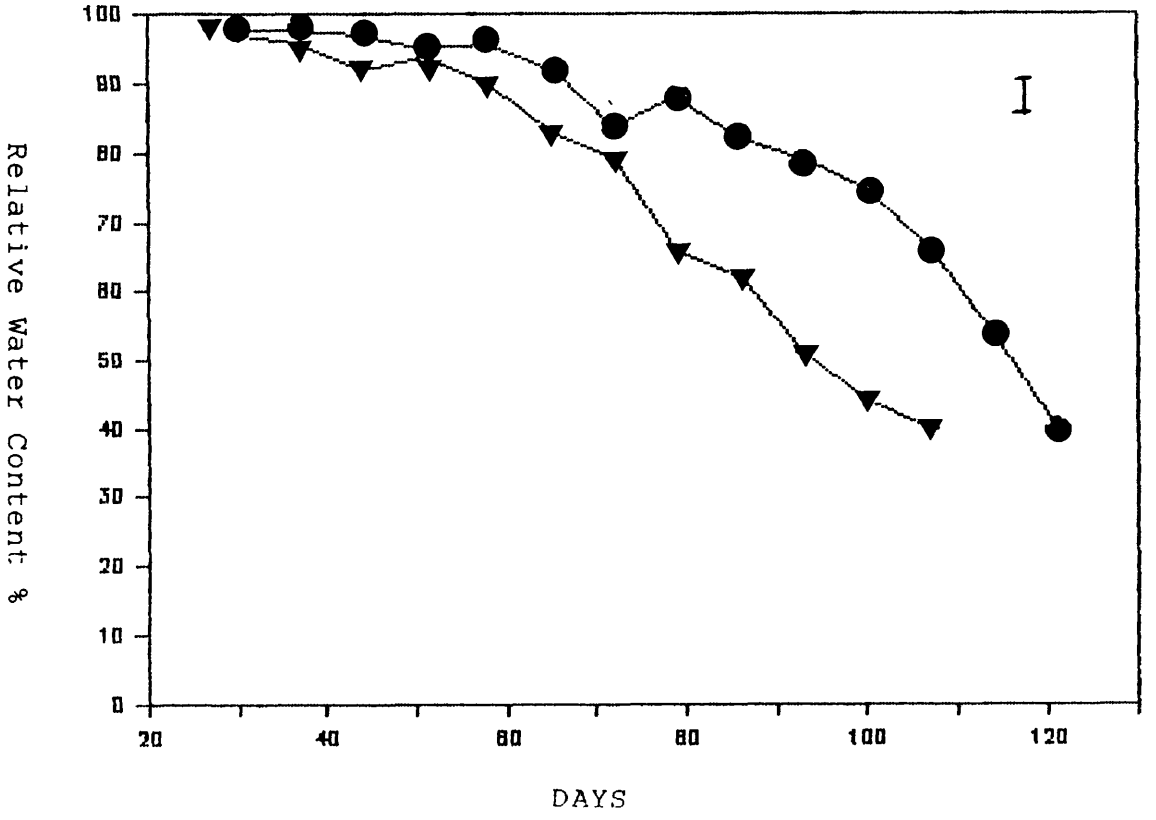


Figure 7.1.0. To show the decline in relative water content in Geum urbanum ● and Geum rivale ▼ during drought in a semi-field situation.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.2.0

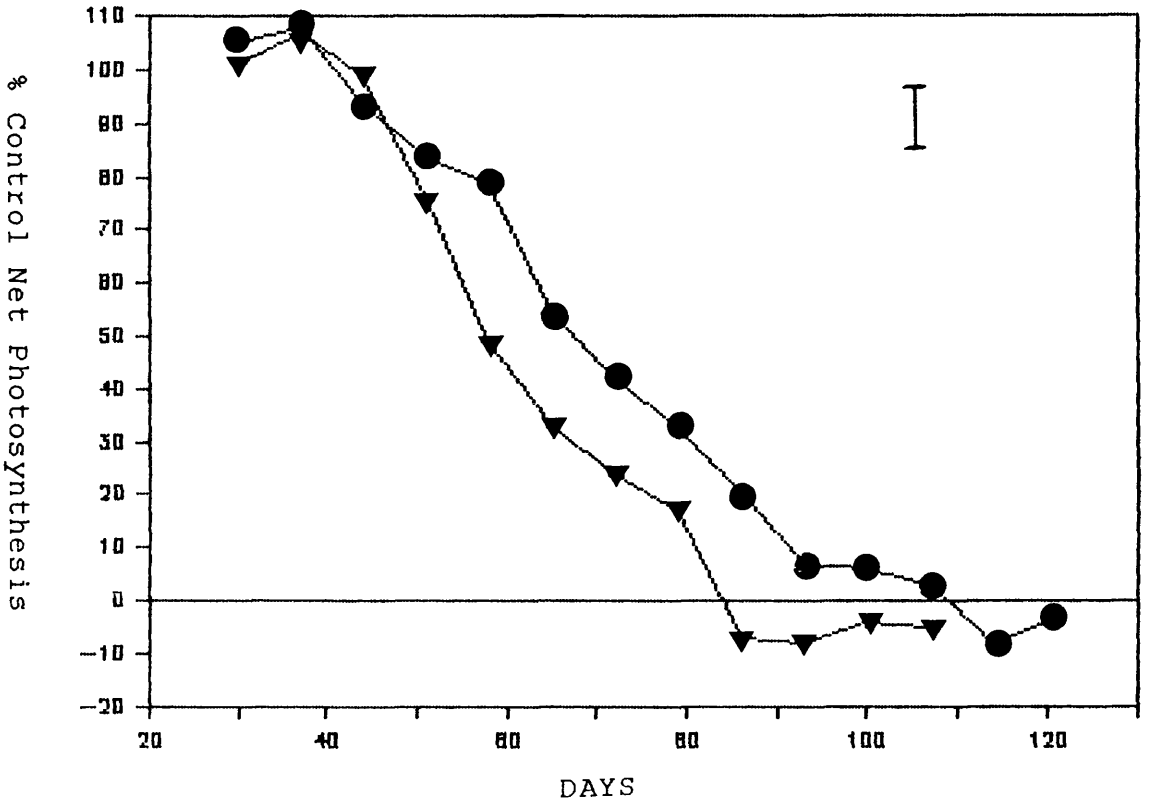


Figure 7.2.0. To show the decline in net photosynthesis in Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.3.0

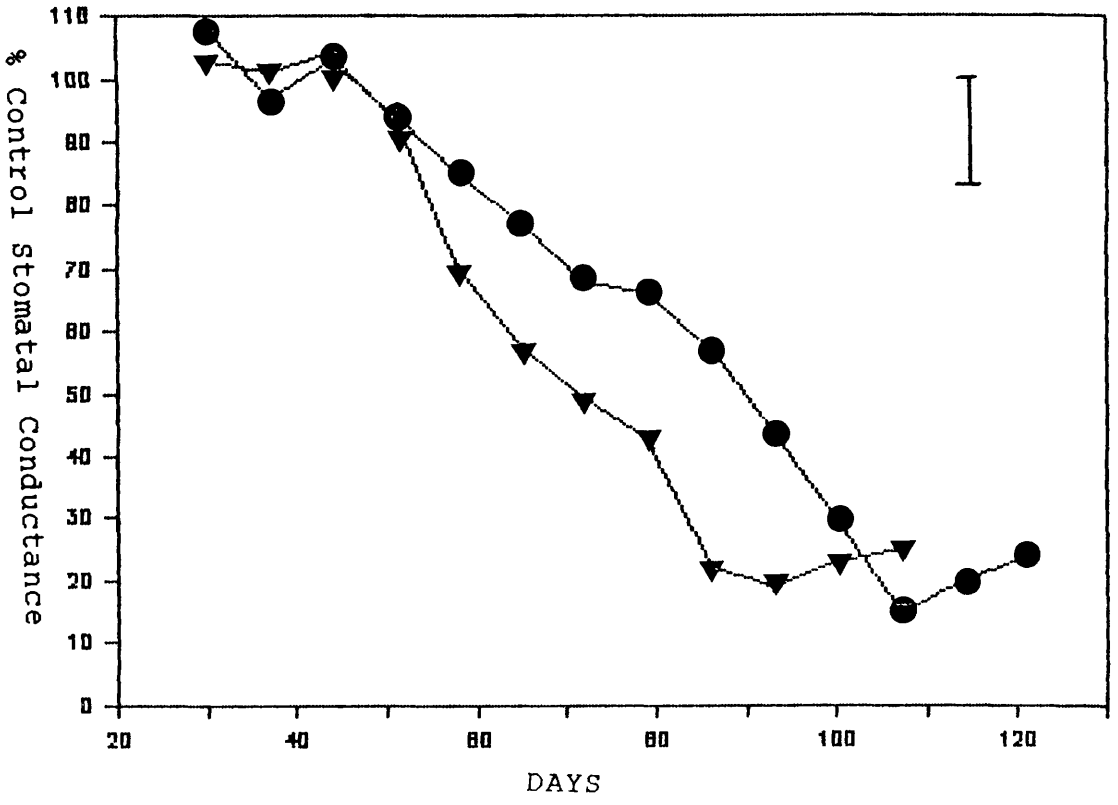


Figure 7.3.0. To show the decline in stomatal conductance in Geum urbanum ● and Geum rivale ▼ in response to drought in a semi field situation.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.4.0

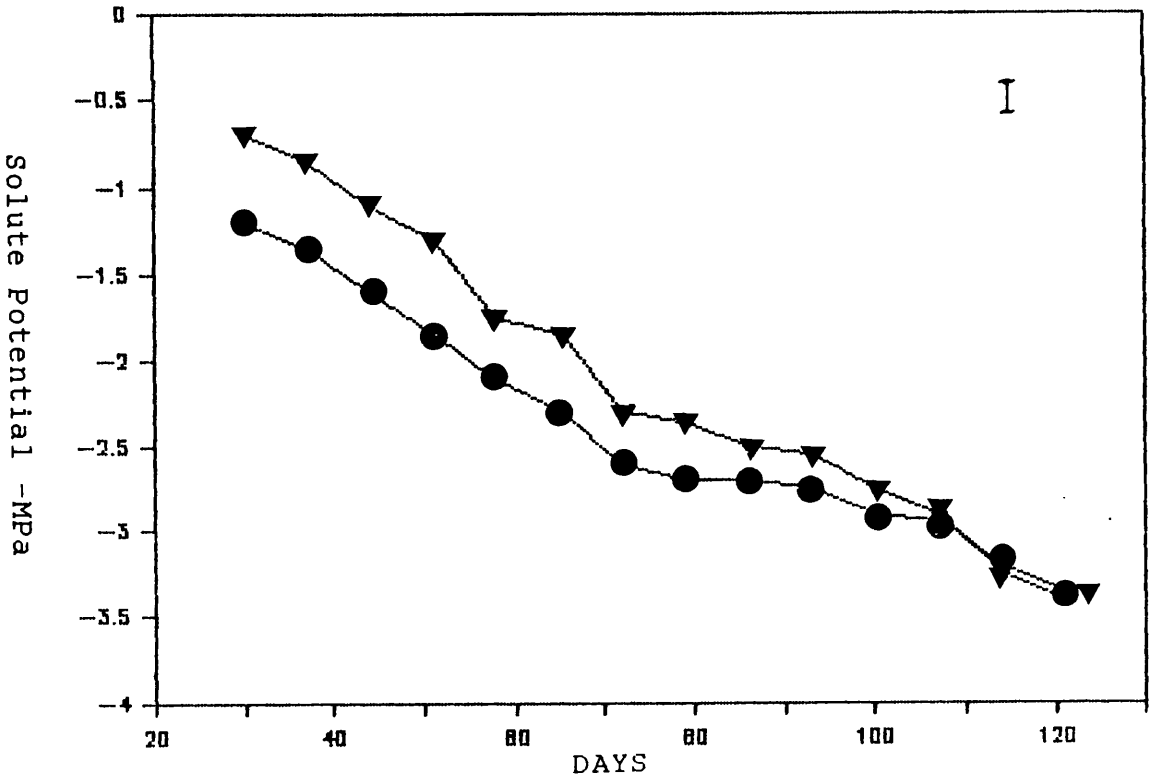


Figure 7.4.0. To show the decline in solute potential in Geum urbanum ● and Geum rivale ▼ during drought in a semi-field situation.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.5.0

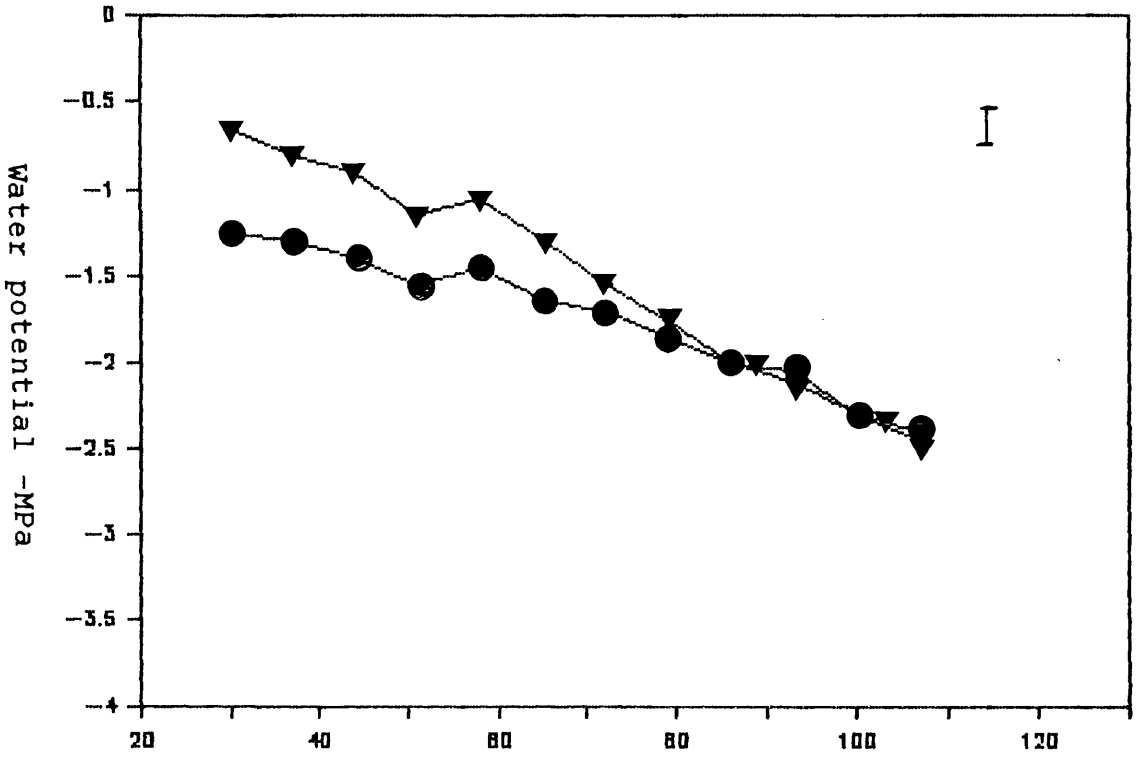


Figure 7.5.0. To show the decline in water potential in Geum urbanum ● and Geum rivale ▼ during drought in a semi-field situation.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.6.0

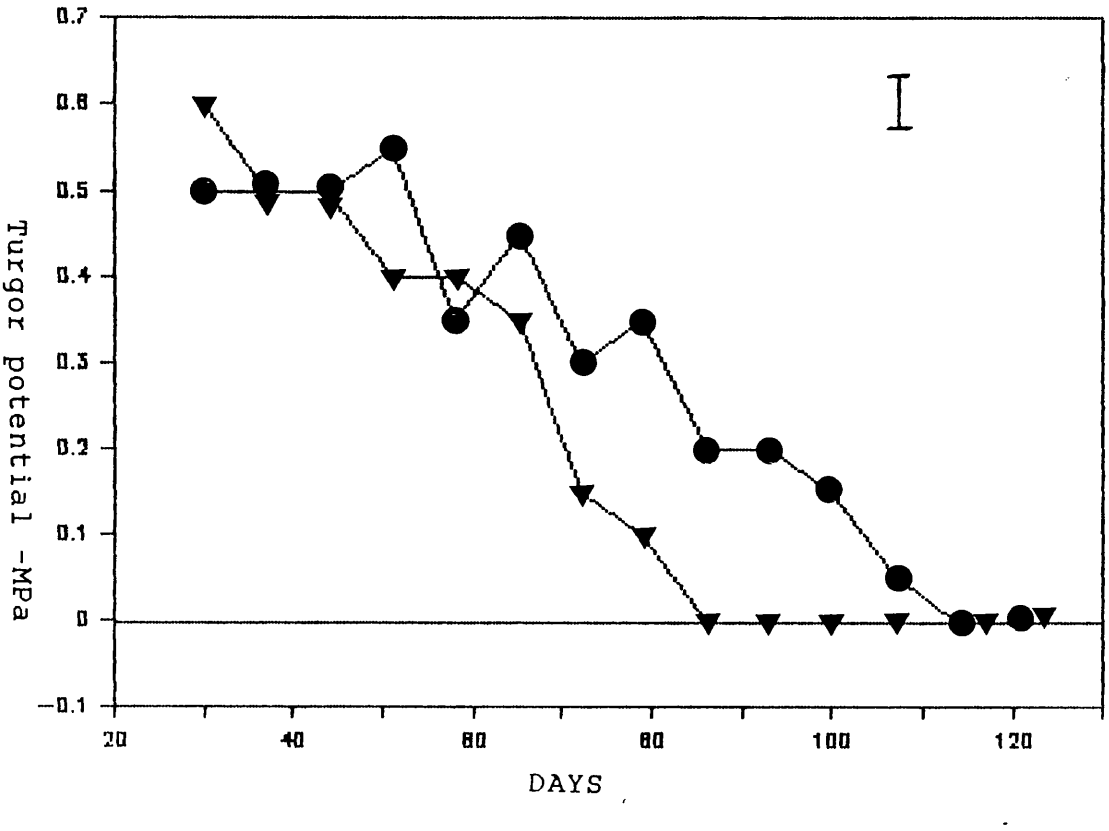


Figure 7.6.0. To show the decline in turgor potential in Geum urbanum ● and Geum rivale ▼ during drought in a semi-field situation.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.7.0

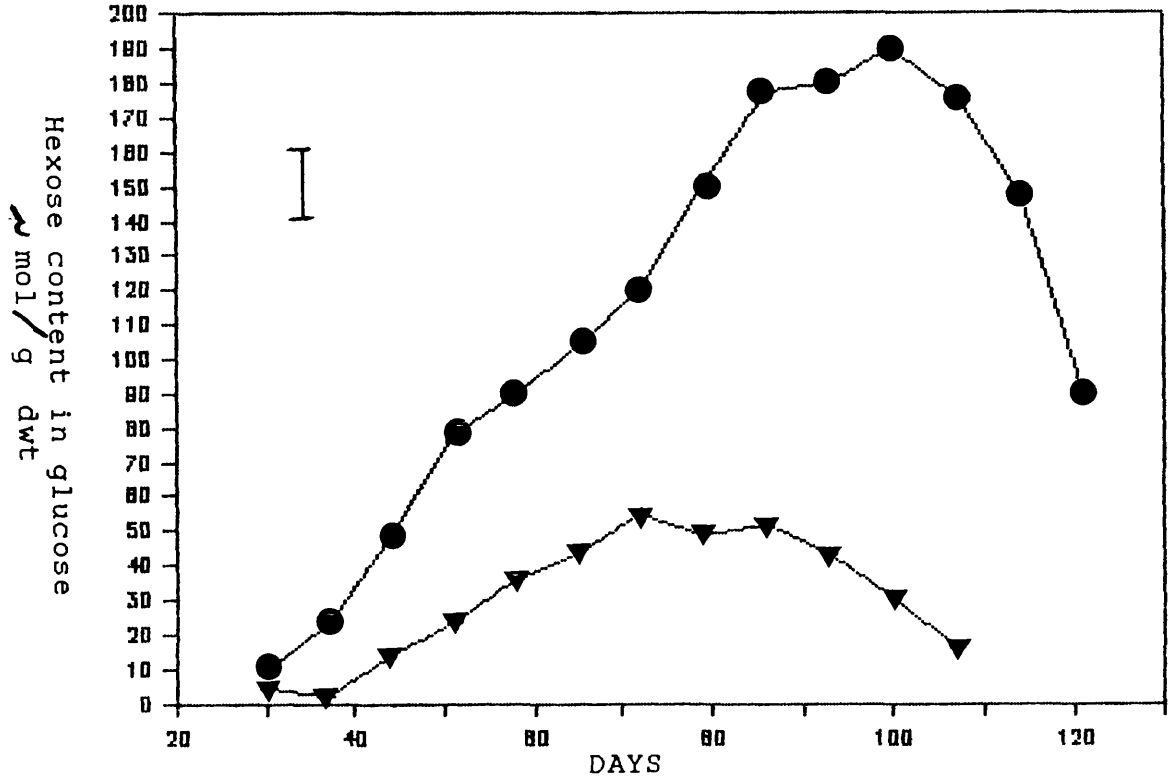


Figure 7.7.0. To show the changes in hexose sugars in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation. Results are expressed in glucose equivalents and represent increases from control levels.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.7.1

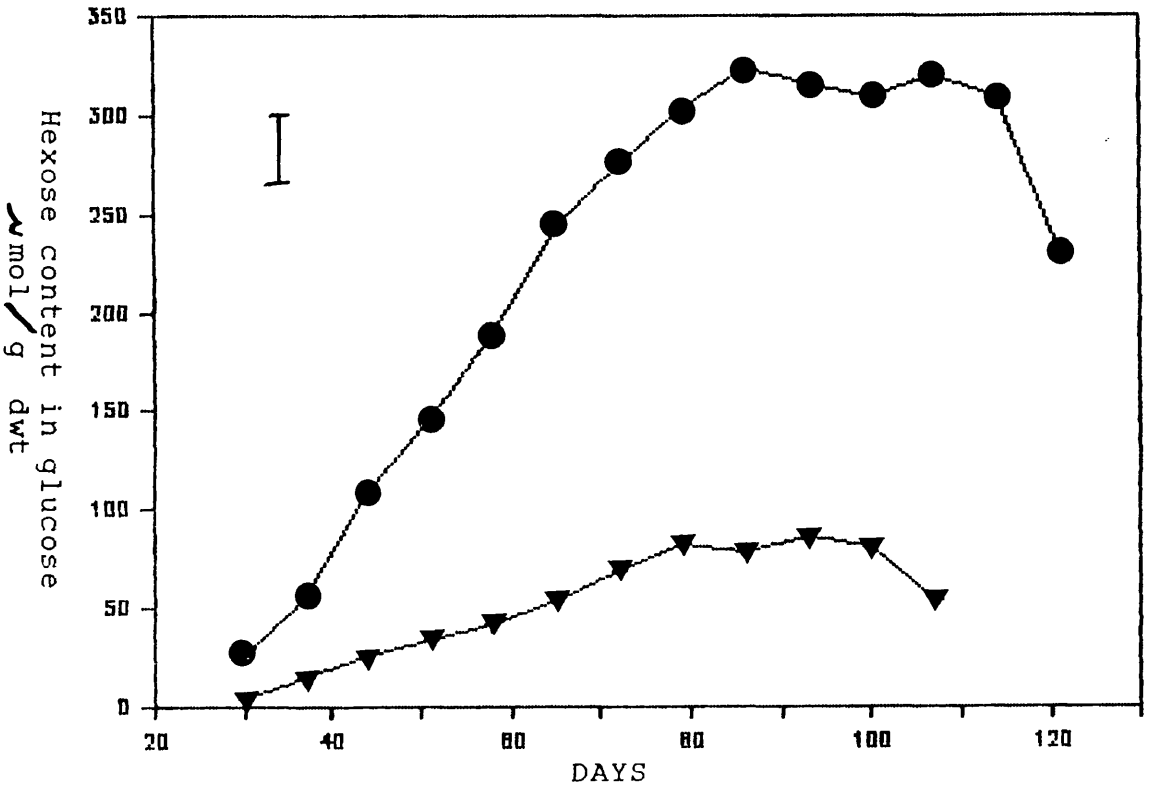


Figure 7.7.1. To show the changes in hexose sugars in young leaves of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation. Results are expressed in glucose equivalents and represent increases from control levels.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.7.2

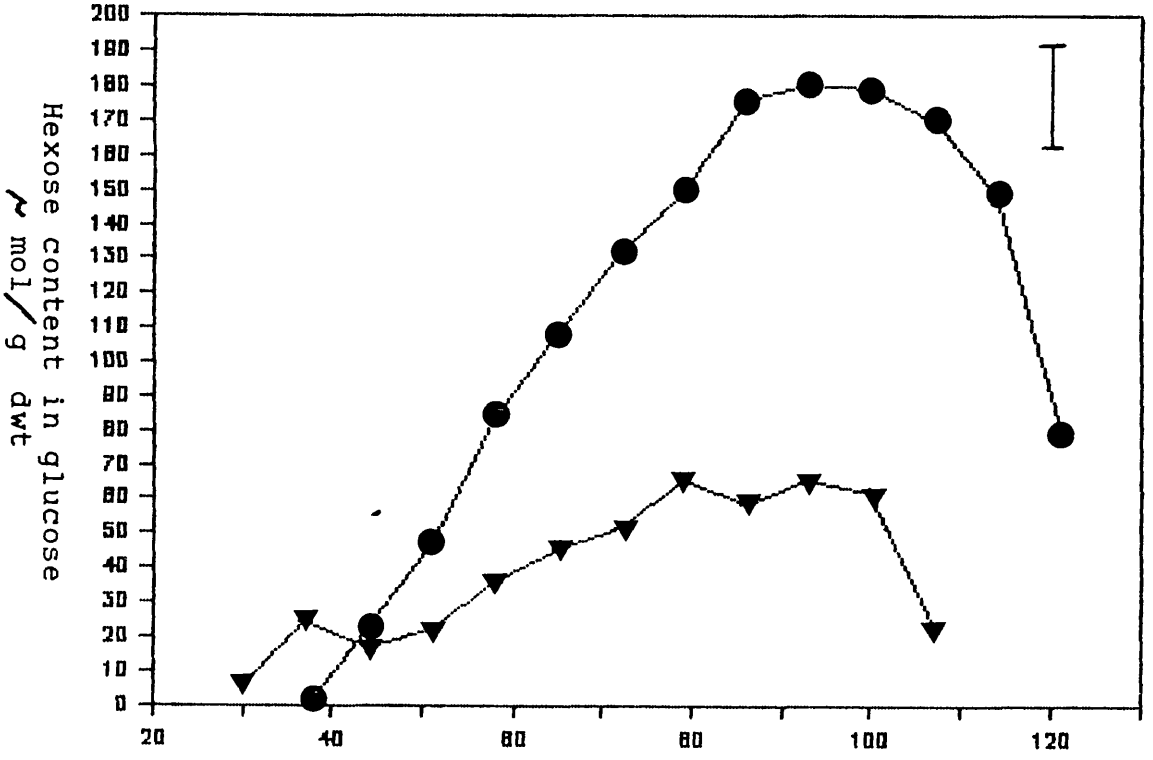


Figure 7.7.2. To show the changes in hexose sugars in roots of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation. Results are expressed in glucose equivalents and represent increases from control levels.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.8.0

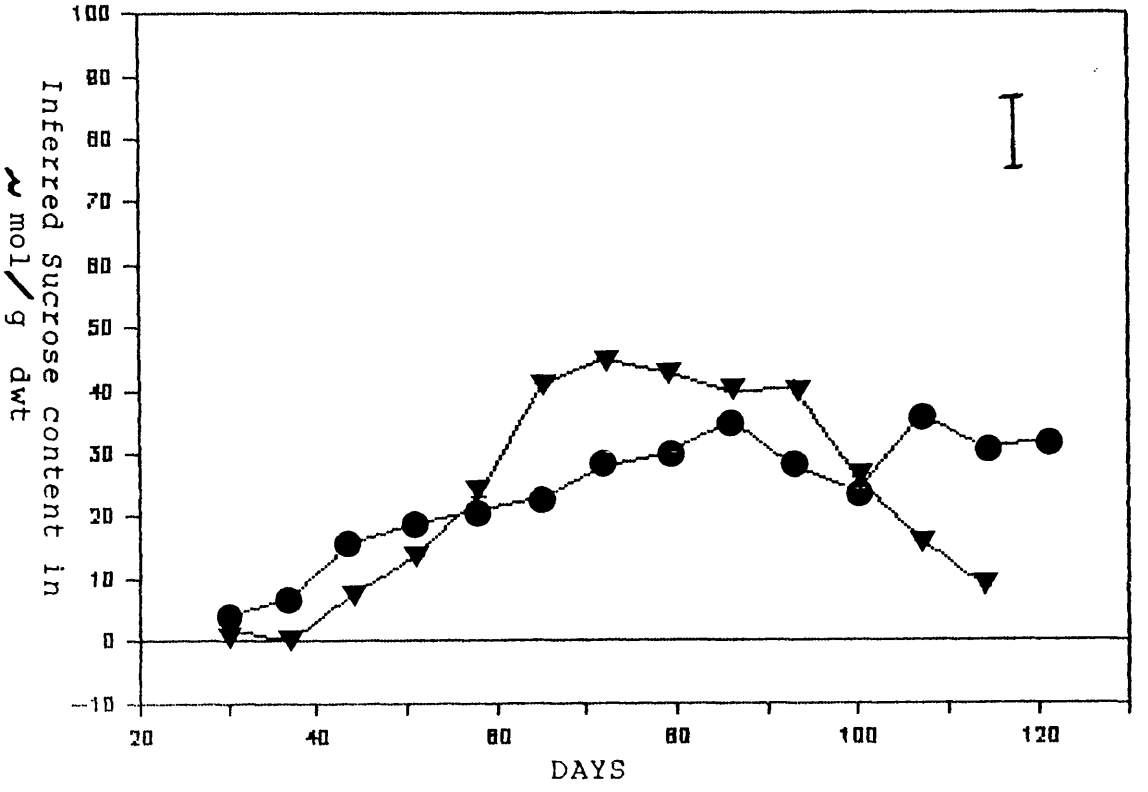


Figure 7.8.0. To show the changes in inferred sucrose levels in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation results are expressed in ~ mol/g dwt and are converted from glucose equivalents.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.8.1

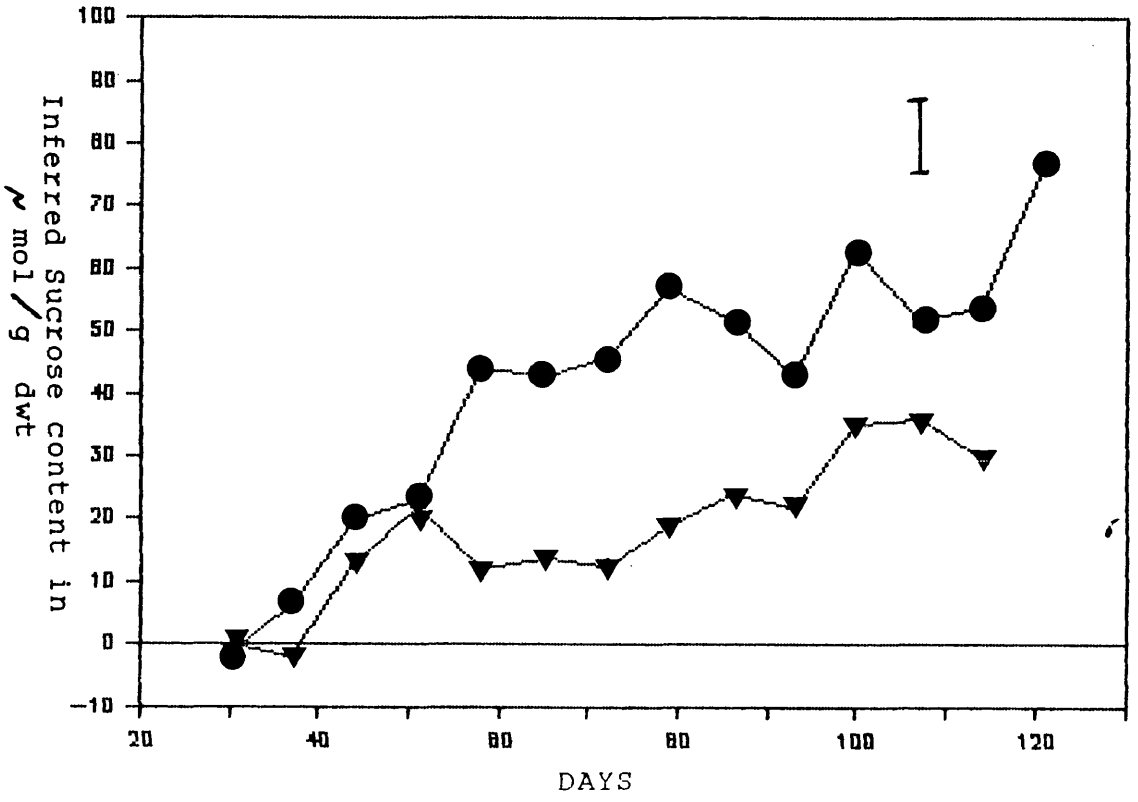


Figure 7.8.1. To show the changes in inferred sucrose levels in young leaves of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation results are expressed in ~mol/g dwt and are converted from glucose equivalents.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.8.2

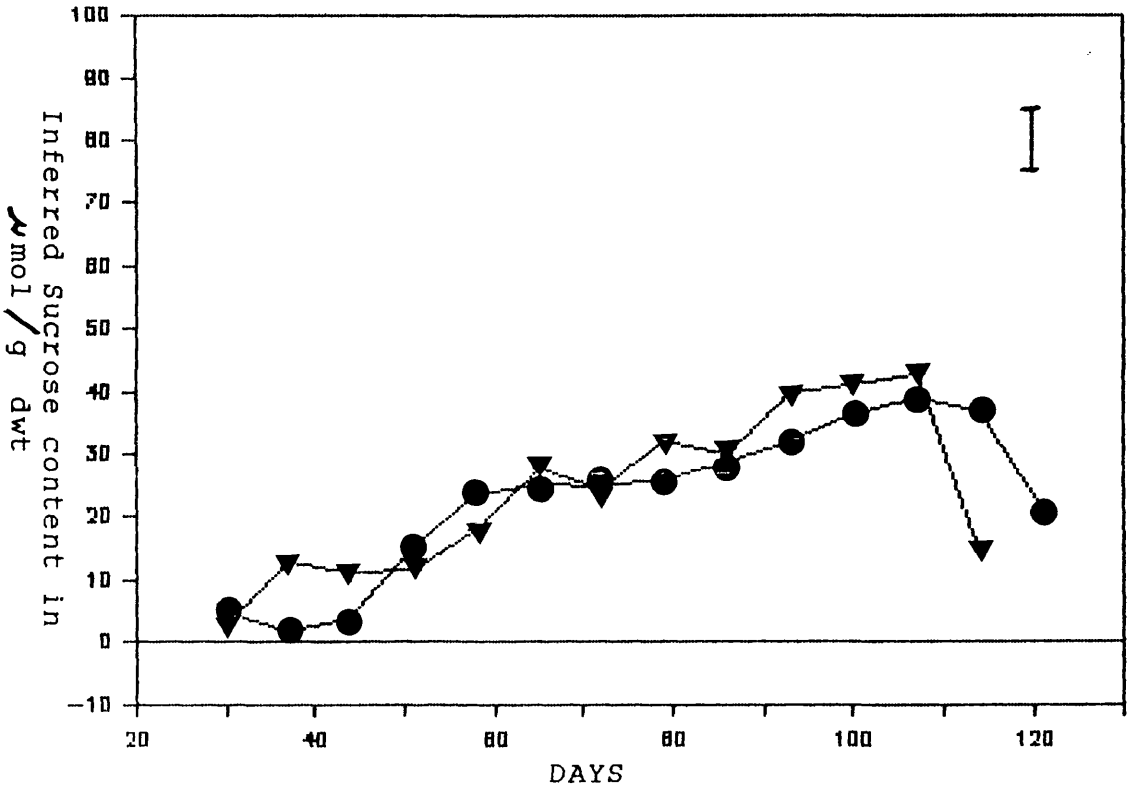


Figure 7.8.2. To show the changes in inferred sucrose levels in roots of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation results are expressed in ~mol / g dwt and are converted from glucose equivalents.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.9.0

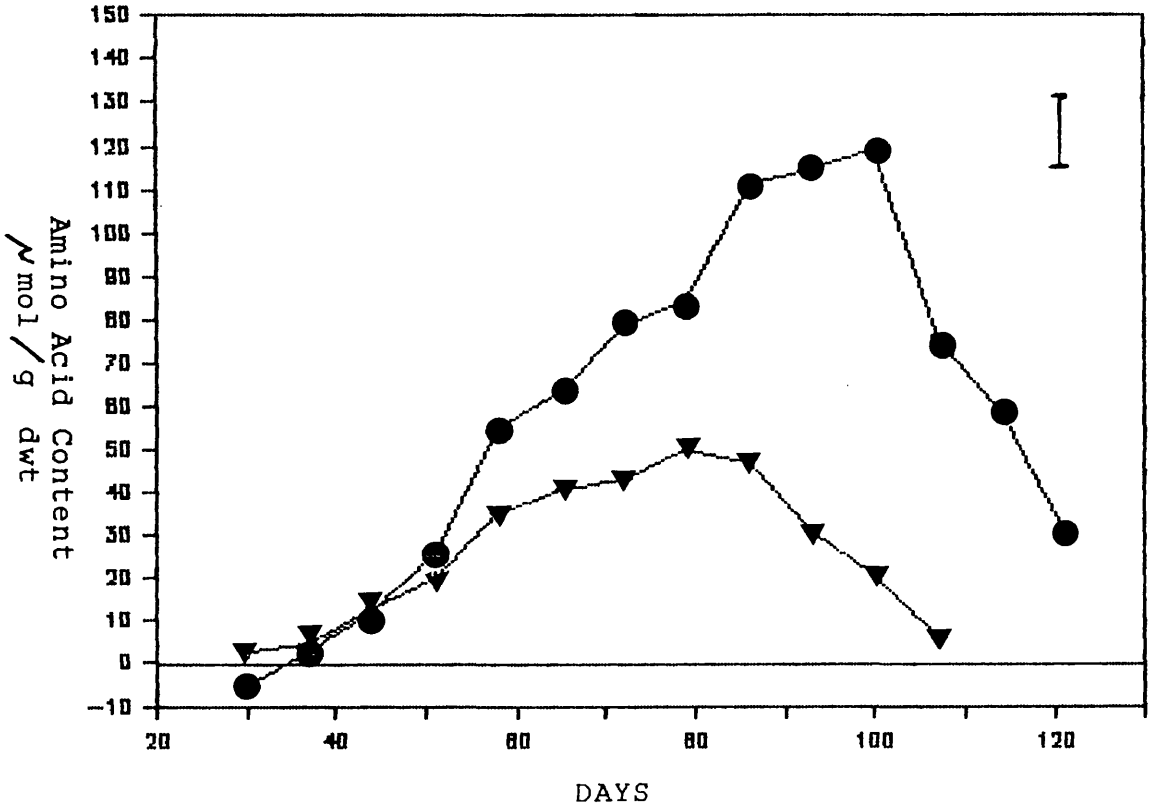


Figure 7.9.0. To show the changes in amino acid levels from control plants in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation results are expressed in ~mol/g dwt.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.9.1

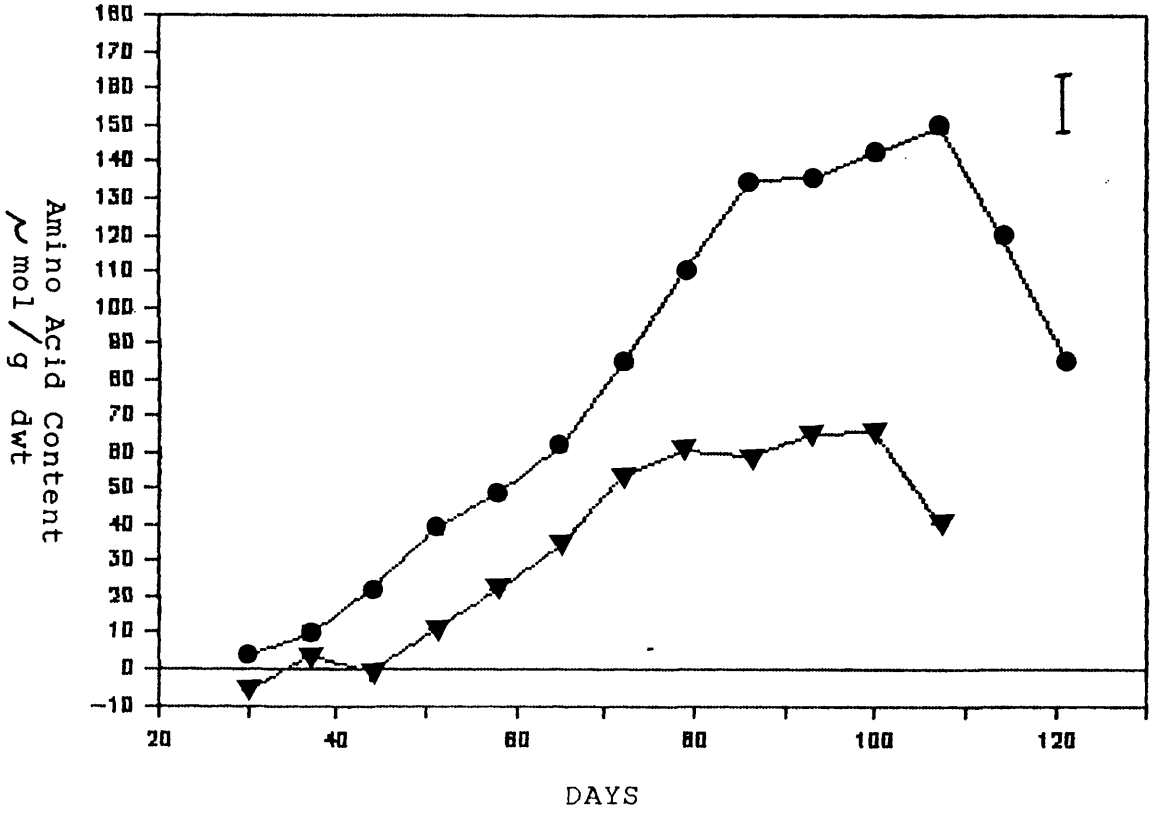


Figure 7.9.1. To show the changes in amino acid levels from control plants in young leaves of Geum urbanum● and Geum rivale ▼ in response to drought in a semi-field situation results are expressed in ~mol/g dwt.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.9.2

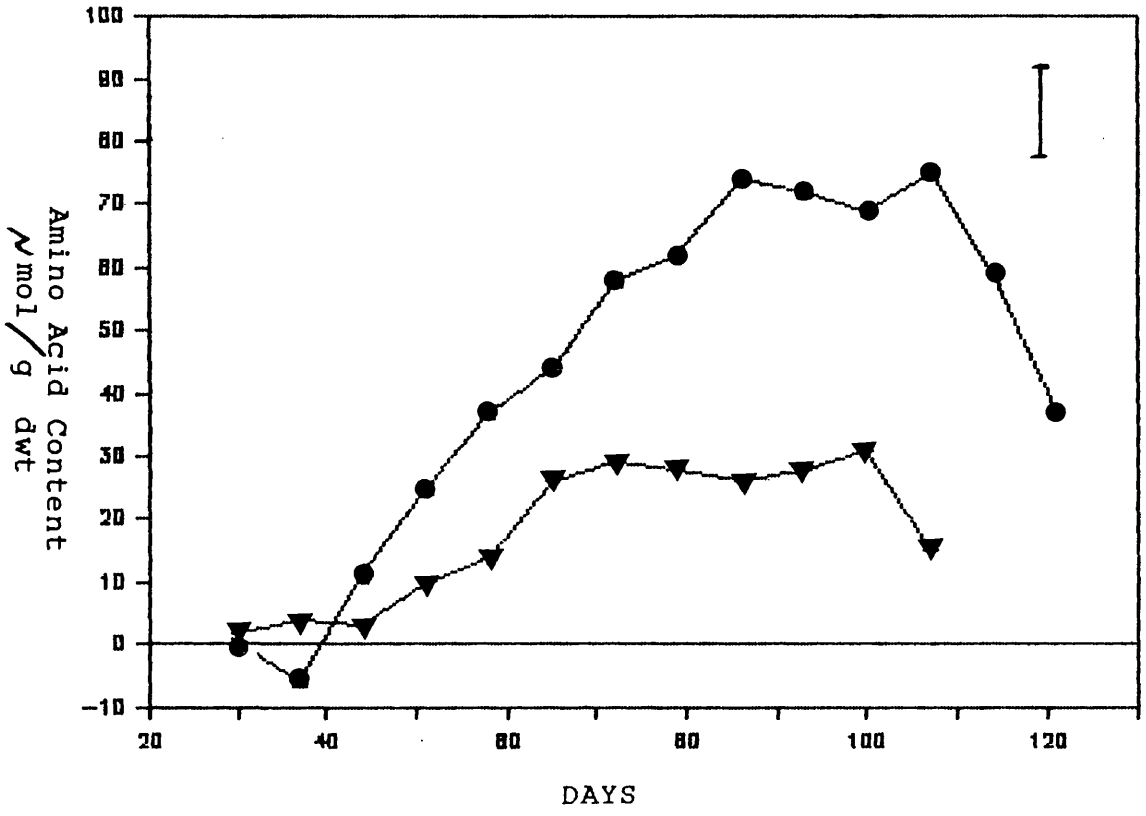


Figure 7.9.2. To show the changes in amino acid levels from control plants in roots of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation results are expressed in μmol/g dwt.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.10.0

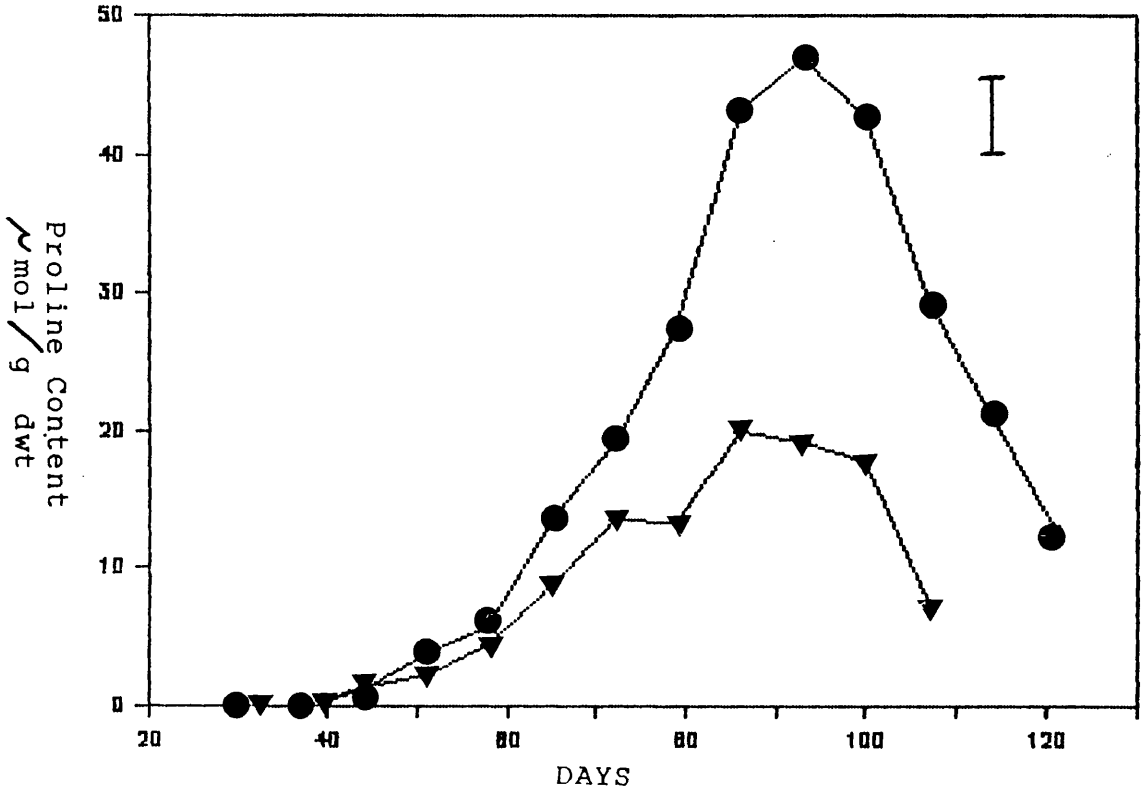


Figure 7.10.0. To show the changes in proline levels from control plants in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation results are expressed in ~mol/g dwt.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.10.1

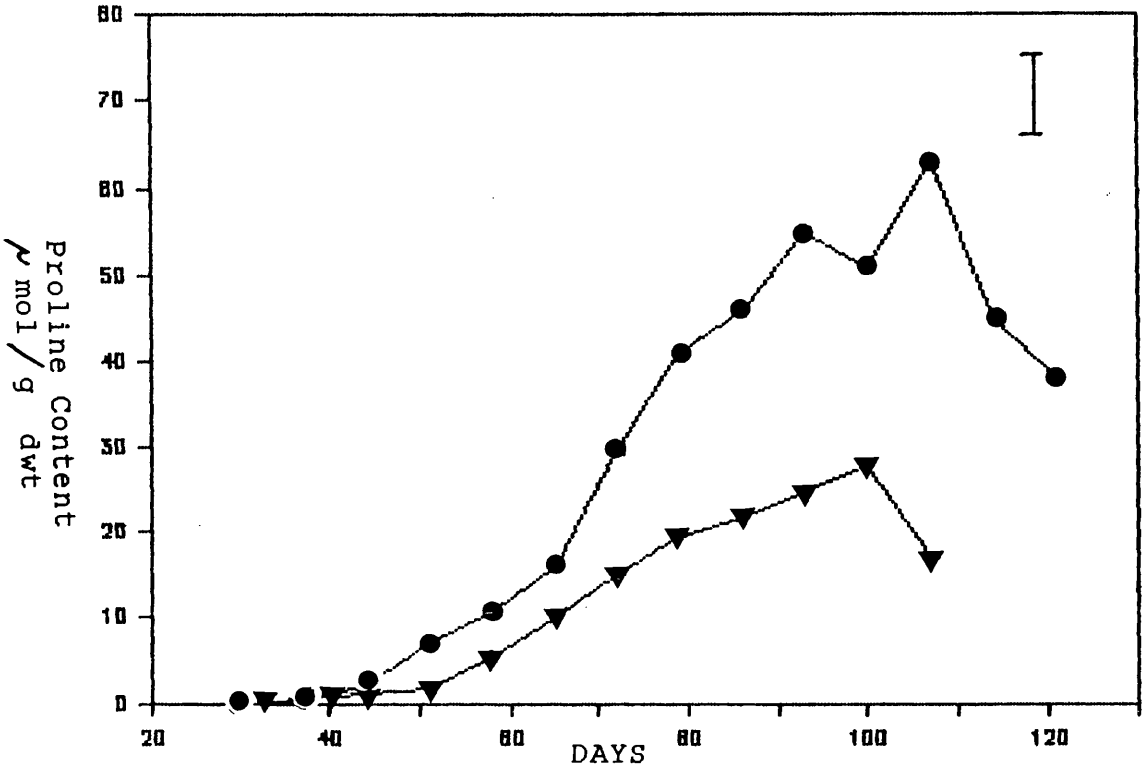


Figure 7.10.1. To show the changes in proline levels from control plants in young leaves of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation results are expressed in μ mol / g dwt.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.10.2

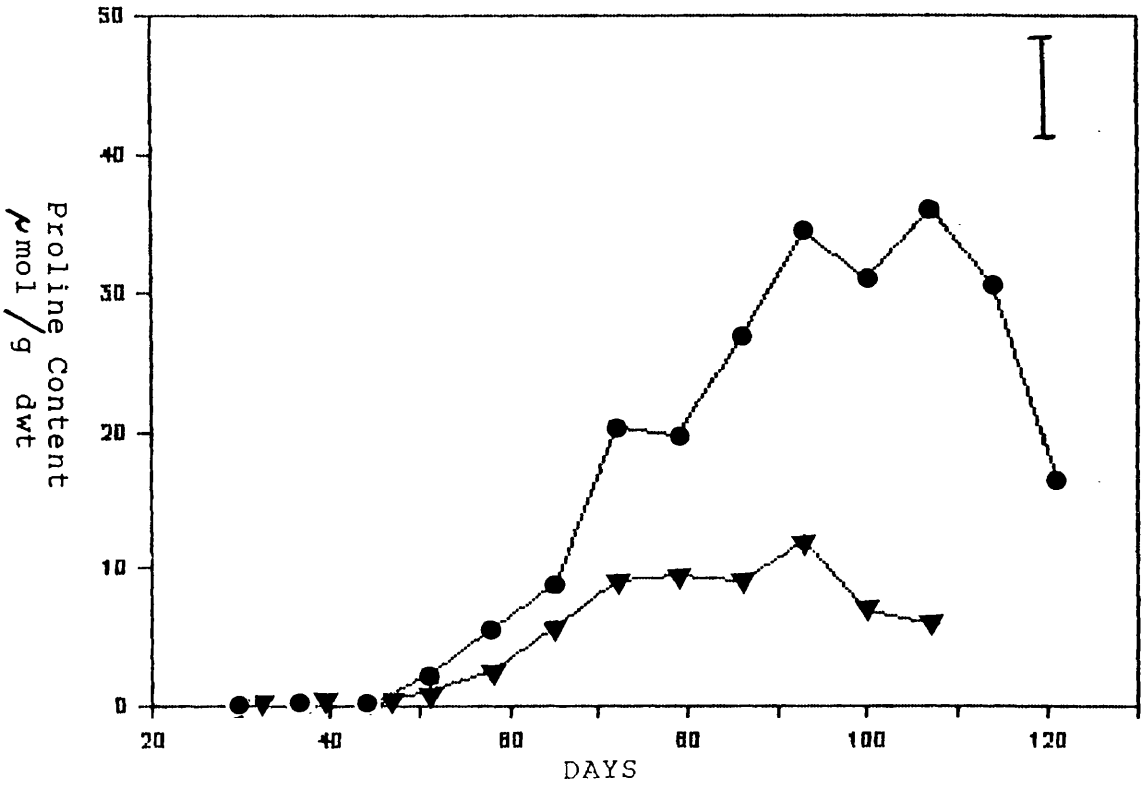


Figure 7.10.2. To show the changes in proline levels from control plants in roots of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation results are expressed in µmol/g dwt.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.11.0

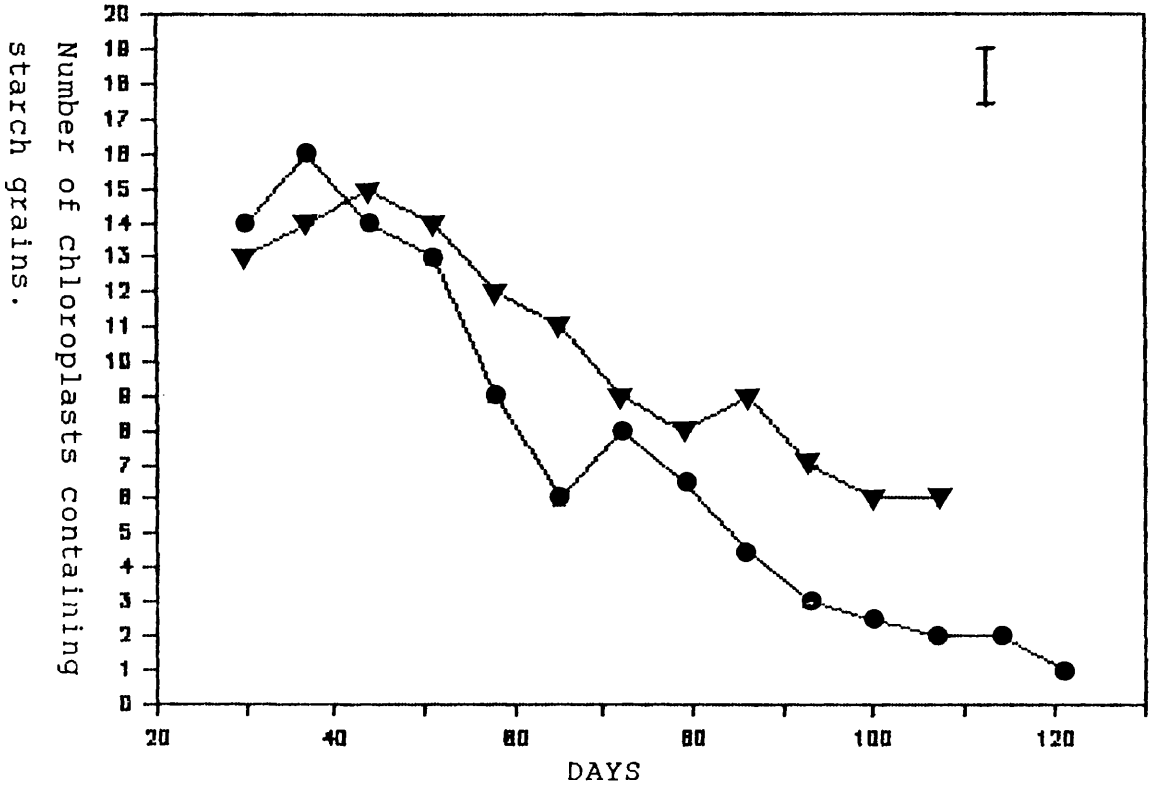


Figure 7.11.0 to show the number of chloroplasts containing starch grains in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to field water deficits.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.11.1

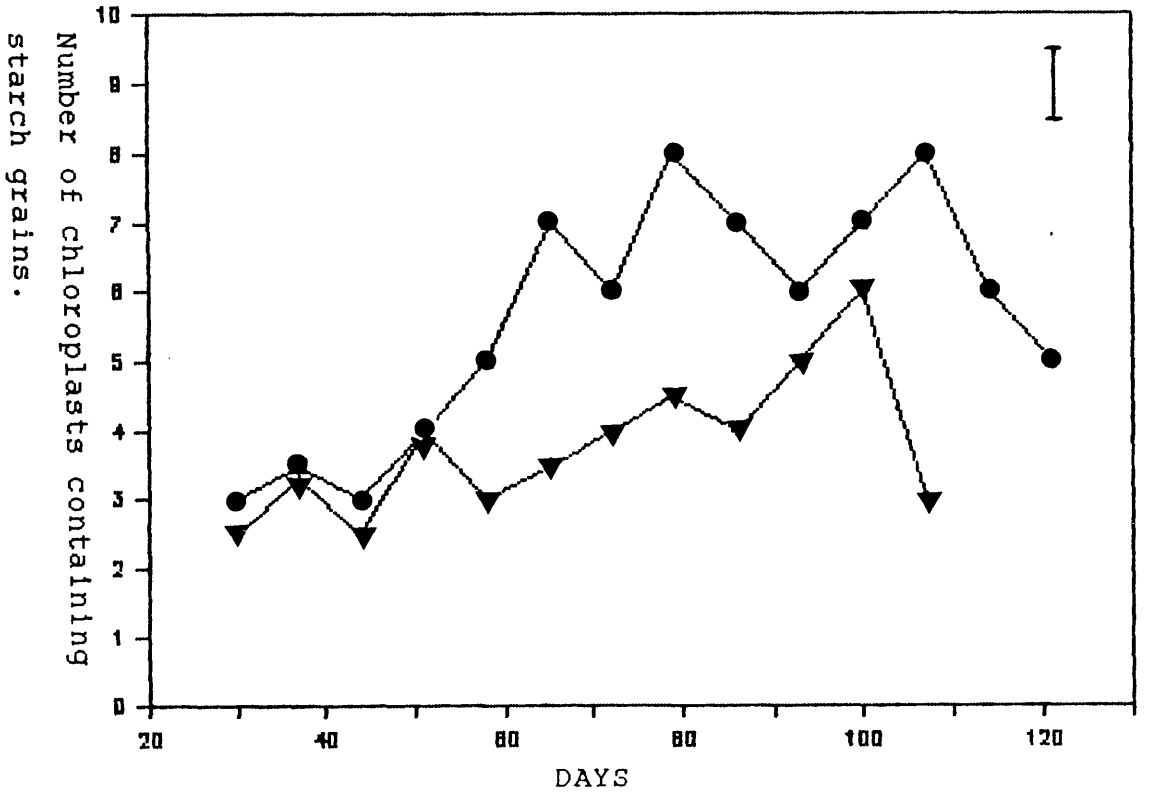


Figure 7.11.1 to show the number of chloroplasts containing starch grains in young leaves of Geum urbanum● and Geum rivale ▼ in response to field water deficits.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.12.0

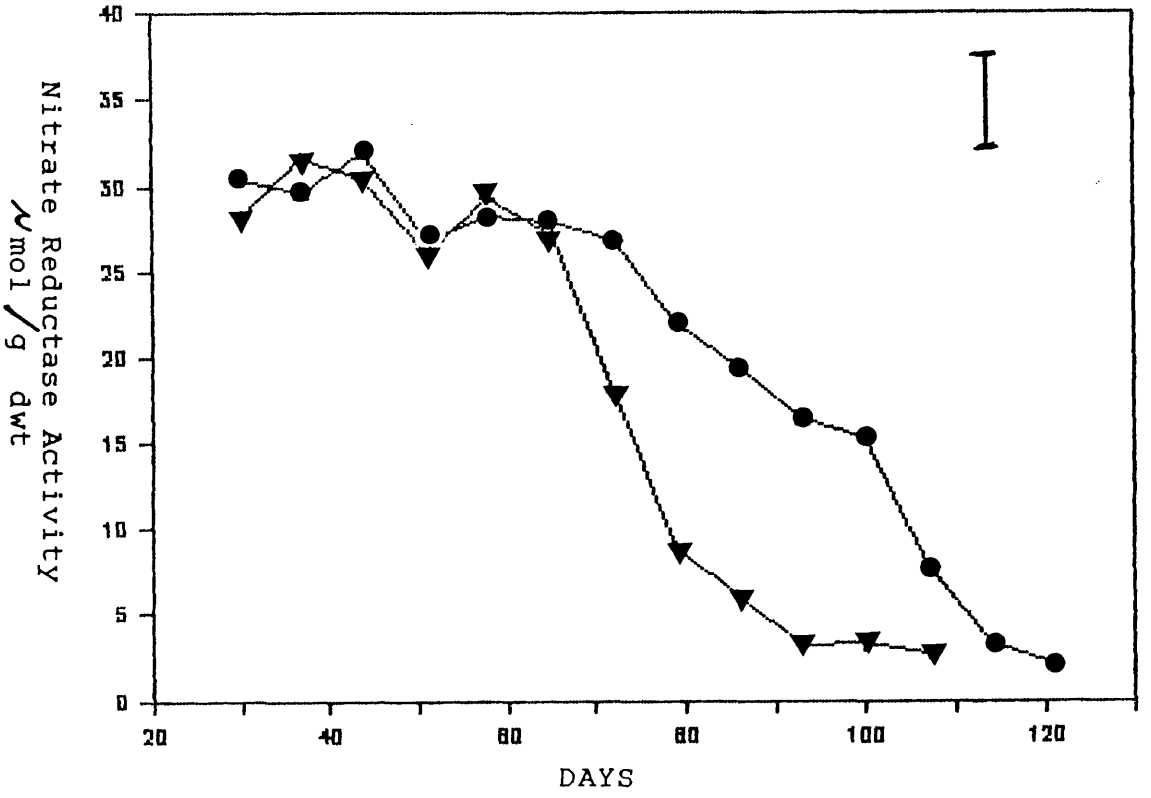


Figure 7.12.0. To show the changes in nitrate reductase activity from control plants in mature leaves of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation.

Bar indicates L.S.D. ($P < 0.05$)

Figure 7.12.1

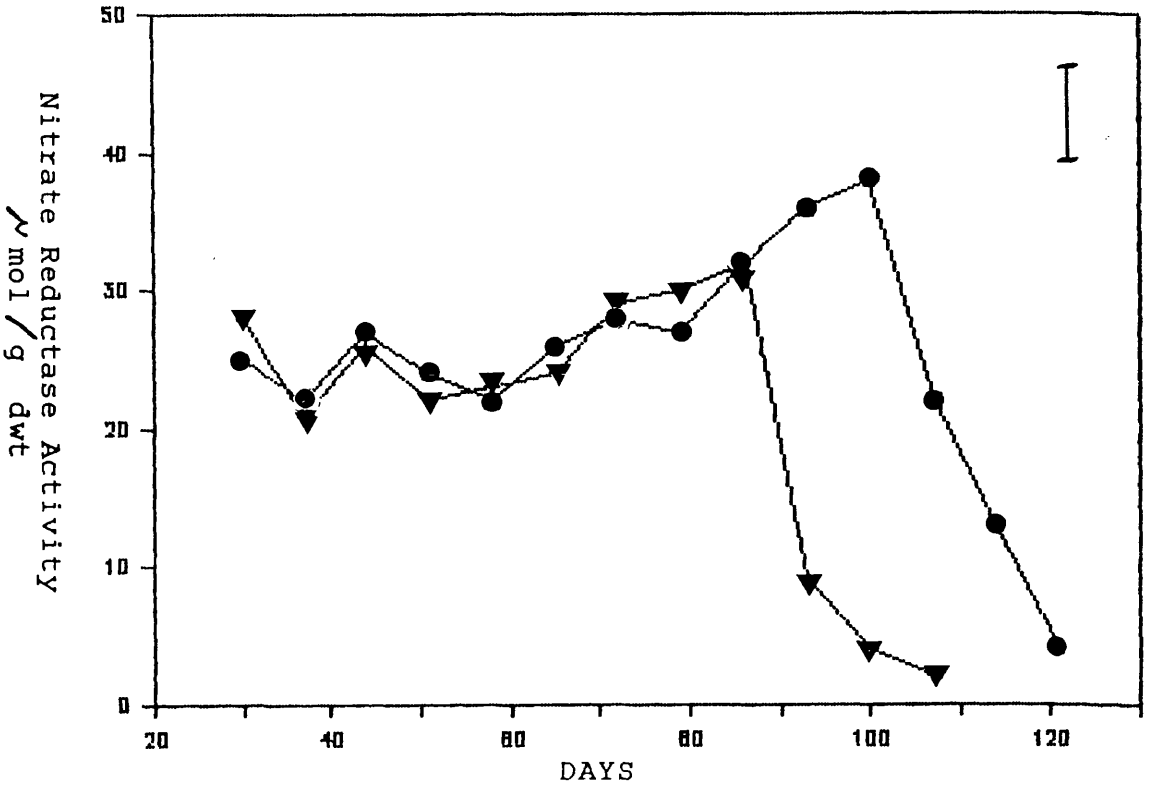


Figure 7.12.0. To show the changes in nitrate reductase activity from control plants in the roots of Geum urbanum ● and Geum rivale ▼ in response to drought in a semi-field situation.

Bar indicates L.S.D. ($P < 0.05$)

Table 7.1.0 to show the wilting point and death points of *Geum urbanum* and *Geum rivale* in response to field water deficits

Species	Wilting Point (Days)	Death point (Days)
G. urbanum	103	124
G. rivale	82	103

Table 7.2.0 to show the change in the proportion of proline expressed as a percentage of the total amino acid pool throughout field water stress in *Geum urbanum* and *Geum rivale*.

Day	*Geum urbanum*			*Geum rivale*		
	Mature leaf	Young leaf	Root	Mature leaf	Young leaf	Root
30	0	1	0	0	0	0
37	0	5	0	0	0	0
44	8	10	0	10	0	0
51	15	18	9	17	15	10
58	22	22	15	20	25	17
62	25	26	20	25	29	22
69	30	35	35	33	28	31
77	35	37	32	31	32	34
84	39	34	37	40	37	32
91	41	41	48	41	38	40
98	36	36	45	38	42	47
105	39	42	49	35	38	42
112	36	38	52	nd	nd	nd
119	41	45	45	nd	nd	nd

DISCUSSION

It is apparent from table 7.1.0 that Geum urbanum could hold off wilting and out survive Geum rivale in a semi natural situation during periods of water deficit. This difference was not merely a few days but was a matter of weeks and at a soil drying rate of approximately 0.1 MPa per day in a field situation it is therefore proposed that drought tolerance could affect the ecological distributions of Geum urbanum and Geum rivale. Though a drought of such duration does not occur frequently, particularly in the areas where Geum urbanum and Geum rivale are found, when such droughts do occur they may then reduce the range in which a plant species can colonise and be successful at the population level. Thus under non - drought conditions which exist over a period of years, when water deficits occur infrequently and are of short duration, populations of Geum rivale may extend the areas in which they grow and even colonise areas which were previously uncolonisable. However a sustained period of drought may be catastrophic to populations of Geum rivale growing in such areas, when water deficits last for longer periods and are more severe and thus may limit the spread of Geum rivale to areas where Geum urbanum is able to survive.

The fall in transpiration rate during field water deficits exhibited by Geum urbanum and Geum rivale (Fig. 7.3.0) has been well documented in crop species (eg Ackerson et al 1980) and in wild species (Hinckley et al. 1980). However some plants which have been shown to close stomata under controlled conditions have not been shown to close stomata under field conditions (Davies 1977). However, this did not occur in the two Geum species. Stomatal closure was not a means by which Geum urbanum could achieve a higher water deficit tolerance over Geum rivale in the laboratory and is also not a means by which Geum urbanum can achieve this in

the field.

The fall in photosynthesis during periods of field drought in Geum urbanum and Geum rivale (Fig. 7.2.0) has been documented during the past in crop species (Ackerson et al 1977; Garrity et al. 1984) but studies are limited with wild species due to technical difficulties in measuring photosynthesis in situ. However the development of the ADC portable IRGA has enabled such studies to be undertaken. In the present study it can be seen that there is a close relationship between photosynthetic rate and stomatal conductance just as there was during the laboratory experiments (see chapter 2). As with stomatal conductance, Geum urbanum had a higher photosynthetic rate than Geum rivale in the later stages of drought. However, in the laboratory there was no significant difference between stomatal conductance or photosynthesis between the two species (Fig. 2.2.0) yet in the field Geum urbanum could maintain a higher stomatal conductance and photosynthesis than Geum rivale.

It has been proposed for other species that higher osmoregulation can maintain open stomata and hence a higher rate of photosynthesis (Richardson and McCree 1985). It is apparent from Figs. 7.4.0 and 7.5.0 that Geum urbanum can reduce Ψ_w and Ψ_s to a greater extent than Geum rivale in the field and it is also apparent that Ψ_w and Ψ_s reduction in Geum urbanum and Geum rivale is greater than that in the laboratory (see Figs. 2.3.0 to 2.6.0) especially when more severe stresses are imposed. Thus in a field situation Geum urbanum is able to osmoregulate at higher levels than Geum rivale which may then enable Geum urbanum to maintain open stomata and photosynthesis in the field. The reason for this could lie in differences in the rate of stress development between laboratory and in field experiments which can reduce the ability of a plant to osmoregulate

(Jones and Rawson 1979). During stress development imposed by PEG 6000 the rate of water deficit development increased as PEG concentration increased due to the exponential relationship of PEG 6000 concentration and PEG water potential. The larger fluctuations in humidity and in radiation experienced in the field as opposed to greenhouse grown plants may also contribute to the increased osmoregulatory capacity in the field (Morgan 1980). Thus in the field as opposed to the laboratory, photosynthate may contribute to the increased water deficit tolerance of Geum urbanum over Geum rivale by either contributing to the rise in free carbohydrate or decreasing the demand for stored carbohydrate in metabolism.

Osmoregulation has been shown to occur in many plant species to field water deficits (eg Tunstall and Connor 1975; Maxwell and Redmann 1978) and it has been proposed that plants which exhibit a smaller reduction in relative water content for a given reduction in leaf water potential are more drought tolerant (Jarvis and Jarvis 1963). Though this is not a universal occurrence (eg Sanches-Diaz and Kramer 1971), drought tolerance may broadly be categorised in this way. Indeed if the decrease in water potential and relative water content are compared in Geum urbanum and Geum rivale (Figs. 7.1.0. and 7.4.0) this certainly appears to be true.

It is apparent from Figs. 7.7.0. and 7.8.1 that Geum urbanum could accumulate more hexose and other carbohydrate than Geum rivale in the field, as indeed it could in the laboratory. Carbohydrate accumulation has been previously recorded during osmoregulation by plants in the field (Drossopoulos et al. 1987), and during field water deficits (Ford and Wilson 1981). In particular hexose sugars and sucrose have been shown to increase in leaves of water stressed plants. However root accumulation has not been studied in the field. In

chapter 3 it was argued that a large proportion of the free carbohydrate accumulated during water stress was mobilised from stored carbohydrate in the form of starch in mature leaves of both species. From Figs. 7.11.0 and 7.11.1 it can be seen that starch grains are lost from the chloroplasts of mature leaves of both species and deposition occurs in young leaves as water deficits increased. This pattern of deposition was reported in chapter 3 during PEG 6000 imposed stress and has not previously been reported in field produced drought. Thus a similar mechanism of carbohydrate mobilisation exists in field droughted plants as during PEG imposed stress. However a much more significant contribution to the rise in carbohydrates which Geum urbanum can achieve over Geum rivale could be provided by photosynthate. The rise in free carbohydrates was however greater than that achieved in the laboratory in both Geum urbanum and Geum rivale and may then contribute to the lower Ψ_w and Ψ_s achieved by both species in the field. It was also considered during PEG imposed water deficits that Geum urbanum could reduce Ψ_s by the conversion of sucrose into glucose and fructose, thus creating two osmotically active molecules rather than one. Although individual sugars were not identified in the field experiments, it can be seen that Geum urbanum did indeed contain a larger amount of free hexose in proportion to other sugars accumulated when compared to Geum rivale. Though this proportion may be higher due to contributions from photosynthate it is possible such a mechanism exists in field droughted plants.

Amino acids accumulated during field drought in both species, with Geum urbanum accumulating more amino acids than Geum rivale (Figs. 7.9.0 and 7.9.1) in leaves and roots, as they did under PEG imposed stress. The accumulation of amino acids has previously been reported during field water deficits (Drossopolus et al. 1985)

and occurred later in the field than carbohydrate accumulation as happened during PEG imposed water deficits. The amino acid accumulation was also higher during field stress in both species as opposed to PEG imposed stress again consistent with the higher Ψ_s achieved by both species in the field.

Nitrate reductase activity in the leaves of both species was maintained during field water stress (Fig. 7.12.0) However, the activity was maintained in the leaves of Geum urbanum for longer periods than Geum rivale. Similar results are reported for other species by Smirnoff et al. (1985) but contrasts with that of Havill et al. (1977). This same pattern of leaf nitrate reductase activity was recorded in the PEG based system (Fig. 4.1.0) in Geum urbanum and Geum rivale. However the maintenance of increased activity was recorded during much higher water deficits in the field than in the PEG system especially for Geum rivale. Such differences between field and laboratory nitrate reductase activities were recorded by Smirnoff et al. (1985) and were explained by the differential rates of stress development between field and laboratory experiments. From the present results however it is apparent that the rate of stress development affects Geum rivale more than Geum urbanum and could therefore be related to the drought tolerance of the species being studied ie the less drought tolerant a species the more damaging a high rate of stress imposition can be. Nitrate reductase activity in roots however has not previously been studied in a field situation and activity was shown to increase during moderate water deficits and then fall during more severe drought (Fig. 7.12.1) as it was in the laboratory (Fig. 4.1.2). This is somewhat surprising as the supply of nitrate would be reduced as water supply was limited. However, the rate of nitrate uptake by plant roots has been shown to be

relatively independent of nitrate concentration (Clement et al. 1979). Thus, it is possible that nitrate uptake from the soil is not affected during moderate water deficits and a similar mechanism of nitrate reduction occurs in the field as in the laboratory (see chapter 4).

Proline accumulation has been demonstrated in many species during field drought (eg Blum and Ebercon 1976; Ford and Wilson 1981). Proline accumulation in field stressed plants of Geum urbanum and Geum rivale is shown in Figs. 7.10.0 and 7.10.1 which is consistent with results reported in the literature. The results show that Geum urbanum could accumulate more proline in roots and leaves than Geum rivale but accumulation was greater in the leaves of both species than in roots. Also proline levels were maintained as a percentage of the total amino acid pools even when stress became severe (Table 7.2.0). These results again show the same trend as in the PEG system however a greater amount of proline was accumulated during field water deficits but was maintained in the same proportions as in the laboratory system (see chapter 6). It is thus possible that the proposed protective properties of proline occur in the field as well as in the laboratory and thus influence the drought tolerance of the two species as previously suggested.

It is evident from this chapter that Geum urbanum had a higher drought tolerance than Geum rivale. It is also apparent that the major differences in water deficit tolerance between Geum urbanum and Geum rivale shown to exist in the laboratory are expressed during drought in a semi-field situation. Geum urbanum was able to raise Ψ_s and Ψ_w in leaves above that of Geum rivale which was considered the main reason for the difference in water deficit tolerance of the two species in the laboratory. This was achieved in the field by Geum

urbanum accumulating more solutes than Geum rivale and hence reducing Ψ_s by accumulating solutes rather than relying more on a concentration of solutes as in Geum rivale. However in the semi-field situation this difference was amplified and thus the tolerance of Geum urbanum to water deficits was greater in the field than in the laboratory. It is also apparent that the mechanisms by which Geum urbanum and Geum rivale accumulate carbohydrates in the field are similar to those reported in the PEG based system with stored carbohydrate being mobilised in both species. The differences in free carbohydrate accumulation between the two species are also similar to the laboratory situation with Geum urbanum mobilising more stored carbohydrate than Geum rivale. However in the field photosynthate could have contributed to the rise in free carbohydrate as there was a significant difference in photosynthetic rate during moderate water deficits in the field which did not occur during PEG imposed water stress. In the laboratory results suggested that primary nitrogen assimilation could be a source of amino acids which were accumulated during water deficits. In the field amino acids were accumulated and primary nitrogen assimilation was shown to be maintained during drought imposition in a similar manner to that in the laboratory. It is thus proposed that Geum urbanum is more tolerant of drought than Geum rivale and is more tolerant due to the ability of Geum urbanum to accumulate a greater amount of solutes than Geum rivale during periods of limited water supply. Moreover it is suggested that this difference is so great that it may affect the ecological distributions of the two species in areas where water availability becomes limiting.

CHAPTER 8.

GENERAL DISCUSSION

This study set out to determine whether the drought tolerance of Geum urbanum and Geum rivale could affect their ecological distributions. The study was to be laboratory based during the first year to identify basic mechanisms by which each species coped with water deficits and identify any differences which may affect their drought tolerance in the field. The second and third years of the study were to be mainly field based to determine whether differences found in the laboratory were expressed in the field and thus determine the reasons for any differences in drought tolerance and its possible effects on ecological amplitude. Unfortunately neither mild water deficits nor drought conditions were encountered in the field site during 1987 and 1988, which were exceptionally wet years. The study then became more laboratory based and a greater insight into the biochemical aspects of water deficits in Geum urbanum and Geum rivale was gained. As results were not available in the field in 1987, a back up experiment was set up at the College Gardens in London whereby a soil drought could be imposed in a large area (Approx 7 m) and some indication of the two species drought tolerance in the field could be determined. However, the study did show that Geum urbanum was more tolerant to water deficits than Geum rivale both in the laboratory and in a semi-field situation. Moreover, this difference was large and it is possible to suggest with some confidence that the drought tolerance of Geum urbanum and Geum rivale could indeed affect their ecological distributions.

With the results gained from these experiments and those gained by Waldren it may now be possible to give some explanation as to the distributions of Geum urbanum

and Geum rivale in an area described by Marsden-Jones (1930). Firstly it must be assumed that drought tolerance and waterlogging tolerance are heritable factors which appears to be the case in water deficit tolerance (see Morgan 1984), and in waterlogging tolerance (Waldren, Etherington and Davies 1988). Secondly it must be assumed that the differences in the two species tolerance to water deficits and waterlogging is significant in an ecological situation.

Marsden-Jones described populations of Geum urbanum, Geum rivale, and various crosses in a wood by the river Pang in Berkshire. The wood was split into two areas one wet and one dry. At the wet end were populations of Geum rivale and Geum rivale crossed with Geum intermedium. In the driest areas the main populations were Geum urbanum, Geum urbanum crossed with Geum intermedium, Geum intermedium and some individuals were Geum intermedium crossed with Geum rivale. In the intermediate areas many more crosses were found. Thus Geum rivale and crosses which were closely related to it only occurred in the wet areas where Geum urbanum was totally absent. this is consistent with the waterlogging tolerance of the two species and the heritability of waterlogging tolerance as no closely related crosses of Geum urbanum were present. In the driest areas Geum urbanum was found together with crosses which were in general more closely related to Geum urbanum than Geum rivale. This is consistent with the drought tolerance of the two species and with the heritability of water deficit tolerance.

The present study has gained some insight into how Geum urbanum and Geum rivale react to the development of water stress which may relate to other species. The study here utilised a stress system which delivered a slow rate of stress which was not previously produced in a great deal of earlier work. As the rate of stress

development has been shown to alter a plants reaction to water stress it is possible that some of the results presented here are more relevant to the field situation than results previously gained by other stress regimes. This is particularly apparent in chapter 7 where a fair correlation exists between laboratory results and results gained in a semi-field situation which was not apparent previously (Begg and Turner 1976; Smirnoff et al. 1985).

One of the most striking differences between the results gained here and other studies (with the notable exception of Smirnoff et al. 1985), is the maintenance of nitrate reductase activity in leaves and the rise in nitrate reductase activity in roots in the laboratory and in the field. This occurrence has many ramifications throughout drought development. Firstly, the maintenance of nitrate reduction and continued low levels of ammonia in plant parts indicate that the primary production of amino acids is not reduced during water stress and in fact increases at least up to moderate water deficits. Thus primary amino acid production could contribute to the rise in free amino acids during water stress. Secondly, it is possible that such an increase in these amino acids in plants such as the Geum species exhibiting low drought tolerance will be amplified in other mesophytic plants with higher drought tolerance. Finally, this phenomenon also brings into question the role of protein synthesis in the accumulation of amino acids during water deficits. Though I am not suggesting protein synthesis and degradation do not have such a role during mild and moderate water deficits; I am suggesting that the maintenance and rise in primary amino acid production does not necessitate such a prominent role for these previously hypothesised sources of amino acids (Hasio 1973; Dhindsa and Bewley 1976).

Another previously unreported phenomenon is the

mobilisation of stored carbohydrate in mature leaves and the deposition of carbohydrate in young leaves. Though this has been reported in salt stress (Aslam et al 1986), both accumulation and deposition has not been recorded during water stress in the same species. As discussed in chapter 3 this type of accumulation can be of advantage to the plant. However the main point which can be gained from such mobilisation and accumulation patterns is the differential pattern which exists between mature and young leaves. The mobilisation of starch occurs throughout stress and is apparent when carbohydrates decline in mature leaves. Thus export of carbohydrate must occur previous to leaf death. This type of mobilisation of stored carbohydrate occurs during leaf senescence (Thomas and Stoddant 1980) and is considered an advantageous occurrence and even an adaptive response in deciduous trees. Thus such an advantageous differential response to water stress in carbohydrate storage could be interpreted as an adaptive response to water stress.

Other differential responses were recorded during water stress between root and shoot in the enzymes of nitrogen assimilation, with glutamine synthetase activity rising slightly in leaves and reducing in roots, with glutamate dehydrogenase exhibiting the opposite trend. This was explained by differential effects of high osmotic potential in the leaves and roots having different effects on the proteins in these enzymes. It is also possible that compatible solutes such as proline may also affect different proteins in different ways due to the different nature of the proteins in roots and leaves. As mentioned in chapter 6 small rises in solutes can have large effects on enzyme perturbations. This may then cause some proteins to be altered in such a way as to predispose them to protection by compatible solutes and hence some enzyme

activities rise and fall due in part to such differences. However any changes in enzyme levels require changes in gene expression. Changes in gene expression have been shown to occur in response to many environmental stresses (see Sachs and Ho 1986) including water stress (Heikkila et al. 1984). It is therefore possible that genetic control in the production of enzymes produces such differential plant responses to water deficits. The orchestration of events durin water deficits remains unclear, but is thought to be mediated from the root (Blackman and Davies 1985) by changes in membrane status produced by changes in turgor (Sinclair and Ludlow 1985).

It has long been known that plants can alter their internal water relations during periods of water deficit and that the accumulation of solutes in the cytoplasm can be linked to the ability of a plant species to survive periods of water deficit. However, few studies have attempted to link the two phenomenon and solute contributions to reductions in solute potential. Morgan (1982) showed periods of water deficit could produce different degrees of osmoregulatory mechanisms in different plant parts at different stress levels in wheat cultivars. However no studies to date could link all the stages of osmoregulation to one plant part throughout gradual water stress. The present study showed that there were three stages of osmoregulation, as identified by Morgan (1980), in Geum urbanum and Geum rivale and that these were related to the solutes accumulated during water deficits and to the stress which the individual species experienced. Though it was not suggested that a three stage osmoregulatory mechanism universally exists within species which osmoregulate during water stress, it is suggested that a similar mechanism does exist in most plants and is related to the solutes accumulated during water deficits

and hence to plant metabolism. For solutes to accumulate, plant metabolism must alter and several workers have demonstrated and proposed mechanisms by which this can occur (Pierce and Raschke 1980; Hsiao 1973; Jones et al 1981) and have therefore linked solute accumulation to changes in metabolism.

It was shown that solute accumulation was rapid during mild water deficits, the rate of solute accumulation then declined during more severe stresses and solute accumulation eventually fell. Solute accumulation has been shown to exist in many plant species (Morgan 1984), but again few studies have shown all stages of solute accumulation and have concentrated on one or two particular stages of water deficit. Thus an empirical model for the osmoregulatory responses of plants throughout water deficits up to their individual wilting points has never been proposed. This is probably due to the fact that most studies have been confined to water deficits imposed on crop plants and the need to relate water deficit tolerance to yield and growth prior to harvest. It is thus proposed from the results gained in these experiments that a similar type of osmoregulatory pattern exhibited by Geum urbanum and Geum rivale exists in most plant species.

These stages when compared between the two species differed in the length of time they were maintained and differed in the contributions solute accumulation made to Ψ_s reduction. The study also showed that these stages were related to the drought tolerance of the two species. Previous workers had attempted to relate such mechanisms to the drought tolerance of various plant species with varying degrees of success (Boyer and Meyer 1979; Aspinall 1980; Morgan 1980; Wright et al 1983). It is not surprising that much confusion has arisen concerning drought tolerance indices in drought stress due to the wide variety of mechanisms utilised by plants

to combat periods of water deficit. Thus in the Geum species this is achieved almost solely by osmoregulatory means. However other species utilise a far wider range of mechanisms which may be as important or more important than the osmoregulatory mechanism employed by the Geum species. Thus any drought indices relying on a single feature or mechanism will not take into account other tolerance methods utilised by different species. Though the osmoregulatory mechanism is related to drought tolerance in some species, increased water uptake from roots penetrating deeper into the soil profile or reduced water loss from early stomatal closure will not be taken into account. It will become apparent that osmoregulation will not be related to the drought tolerance of a group of plants. If we take the case of enzyme protectants and assume such a mechanism does exist, the formation of a metabolic indicator during water stress is even more implausible as there is a wide variety of protectants with differing enzyme protectant properties (Morgan 1984). Some of these solutes have been shown to accumulate in combination (Laurie 1988) or alone (this study). Thus the correlation of one particular solute to water deficit tolerance is complicated by the presence of other solutes and other drought resistance mechanisms. A further complication to this problem is the rate at which plants are stressed as mentioned earlier. For any drought tolerance indices to be developed at all an empirical basis for drought tolerance must be produced before indicators can generally be developed.

Thus it is most unlikely that a metabolic (or other) marker for drought resistance can be found unless a large number of species are stressed to their ultimate limit in a standard system. All aspects of metabolism and physiology must then be followed due to the many different mechanisms plant species utilise to combat

drought even between closely related species and cultivars. The requirement of plant breeders to have a metabolic marker for drought resistance in order to screen plants rapidly, appears to be an impossible goal until such a system is developed or decided upon. One such laboratory based system could be a modification of the PEG system used here. It has long been known that different rates of stress imposition produce different plant responses to water deficit (Jones and Rawson 1979). The slower the rate of stress development the more able a plant is to survive water stress, as illustrated in these experiments. Moreover, the rate of stress imposition and plant reaction to water deficits can be species dependent. This is illustrated when nitrate reductase activities between the laboratory and field are compared between Geum urbanum and Geum rivale. In laboratory experiments Geum rivale could only maintain nitrate reductase activity in mature leaves between 0 and 5% PEG at a relative water content representing more or less full turgor (98%). However in the field, Geum rivale could maintain nitrate reductase activity at a relative water content below 85%. Thus, though the rate of stress development between 0 and 10% PEG was approximately -0.12MPa /day and did not affect nitrate reduction in Geum urbanum the rate was too high for Geum rivale, as a reduction in nitrate reduction in the region of 50% was apparent at a relative water content of 96%. Thus any attempt to screen plants for drought tolerance must take account of the proposed drought tolerance of a species prior to screening. This method of screening would however be very long winded and require a large amount of time and technical back up.

Another quicker method which may relate to the drought tolerance of plants may be to stress a range of plants at different rates of stress development and

determine at which rate a 50% reduction in nitrate reductase activity is produced. By this method any or most metabolic/ osmotic mechanisms by which mesophytic plants survive drought will be taken into account as it does not rely on the production of any particular metabolites, but relies on the effect drought mechanisms have on a sensitive plant metabolism. This would require precise formulation of PEG solutions and leaf choice and would not take into account non leaf assimilating species or yield concerns. However, provided a general knowledge of the plants growth habits and yield are known this could be a system by which is sensitive to drought tolerance of the plant and may also be adapted to screening for salt tolerance. Though such a system requires an empirical basis for drought tolerance before such a proposal can be evaluated, it is an attractive system which is both cheap and rapid and enables a large number of plants to be screened.

This thesis has shown the need to stress plants in a slow manner in order to recreate water deficits in the laboratory which reflect the metabolic events during drought in the field. However during the later stages of stress the rate of stress development may have still been too high due to the exponential relationship of PEG concentration and PEG. eg. between 0 and 10% a rate of 0.12MPa per day was experienced by the plants. However between 25 and 30% PEG a rate of 0.3MPa per day was experienced by the plants. As this produced a different plant reaction between field and laboratory experiments this rate may have been too high. Thus, smaller stress increments may be required to produce a slower rate of stress. Therefore particularly at the higher PEG concentrations increments of 2.5% may be more appropriate. Thus it is proposed that in future experiments that laboratory experiments utilising PEG 6000 as a stress medium should use 2.5% increments

rather than 10% increments used in other studies (Pearson and Stewart 1987) or the 5% increments used here.

The two Geum species have also shown themselves to be excellent plants to work with concerning tolerance to water deficits. They may also have a further role to play in studies concerning drought. As they are closely related cross fertile and genetically well characterised they could be used in studies concerning the inheritance of osmoregulation. These species are easy to cross and as has been shown in this study, survive periods of drought mainly by osmoregulatory means. They also have a biochemical marker in the phenolic glycoside, gein, which may be utilised in the characterisation of any progeny produced during crossing. They also have the advantage that the osmoregulatory process is the major factor in determining the difference in drought tolerance between the two species. Thus, any difference in drought tolerance found in the progeny could be accounted for by the osmoregulatory process and its value in drought tolerance can be critically assessed.

This thesis has also followed the effects of water stress on secondary plant metabolism and has shown this to be much affected by water deficits. In particular the accumulation of phenolic compounds and phenolic glycosides. The effect of water stress on such metabolism has been ignored in most studies concerning drought stress in the literature. However, such metabolism can have a profound effect on plant growth and metabolism. The individual phenolic acid accumulations were not studied during this study but with the reported profound effects of different phenolic acids on different parts of metabolism Nitsch and Nitsch (1962) and stomatal function (Rai et al. 1986) further work is required. This work must also take into account the inactivation of phenolic acids by glycoside

formation and their accumulation and possible role in as vacuolar osmoticums and their roles as neutralisers of reactive oxygen species. Other methods of protection against damage to membrane systems have also been reported and some have been followed during water stress. Such mechanisms include ascorbic acid systems, glutathione, catalase peroxidase and super oxide dismutase (Salin 1987), as well as other more obscure compounds. Thus the secondary metabolism of plants could be influential in determining the drought resistance of plants and a much more rigorous characterisation of drought effects on secondary metabolism is required both in the laboratory and in the field.

In conclusion then this study has shown Geum urbanum to be more drought tolerant than Geum rivale which is probably related to the ecological distributions of the two species. The study has also highlighted the need for slow rates of stress development when studying the reactions of plants to water stress, if such results gained in the laboratory are to be related to a field situation.

APPENDIX 1.

A: Separation of amino acids, sugars and organic acids.

50 to 500mg of oven dried plant material was ground to a powder in liquid nitrogen and extracted in 5ml methanol,dichloromethane water extraction media (12:5:3 v/v). The slurry was decanted into a boiling tube and the remaining slurry washed into the tube with a further 5ml of extraction media. At this point internal standards were added to the media, S-carbomethylcysteine was used as an internal standard for acidic amino acids and hydroxyproline for neutral to basic amino acids. The media was then placed in a fridge overnight when and whirlymixed the following morning. Phase separation was then induced with the addition of 5ml dichloromethane and 5ml distilled water. The extracts were then left overnight in a fridge to complete phase separation. The upper methanoic phase which contained amino acids, sugars, polyols and organic acids was then drawn off and rotor-evaporated to dryness. The residue was then redissolved in 1ml distilled water and stored at -20^{o}C.

Amino acids were then separated from the other constituents on Dowex 50+ acidic ion exchange resin (50x8 400 mesh on 0.5 x 5cm columns). Amino acids were eluted with 6M NH OH and the other compounds with 7.5ml distilled water. The eluents were then frozen in liquid nitrogen and lyophilized. The residues were then resuspended in 1ml distilled water. Neutral and basic amino acids were then separated from acidic amino acids on Dowex 1 acetate strongly basic ion exchange resin (1x8 400 mesh on 0.5 x 5cm columns). Neutral to basic amino acids were eluted with 7.5ml distilled water and acidic amino acids were eluted with 7.5ml 2M acetic acid. The eluents were again frozen in liquid nitrogen, lyophilized and the residues resuspended in 1ml distilled water. These fractions were then stored at -20

°C until required.

Sugars were separated from organic acids on Dowex 1 formate strongly basic ion exchange resin (1x8 400 mesh 0.5 x 5cm columns). Sugars and sugar alcohols were eluted with 7.5ml distilled water and organic acids with 7.5ml 8M formic acid. The eluents were again frozen in liquid nitrogen and lyophilized redissolved and stored as before.

B: Derivatisation of amino acids.

Amino acids were transformed into N (O-S), heptaflourobutyl isobutyl esters (n-HFBI esters). 0.25 to 0.5ml of the purified amino acid extracts were placed in 1ml reactivials and dried at room temperature under a steady stream of gaseous nitrogen. 0.2ml dichloromethane was then added to the dry residue and re dried under nitrogen. When dry a further 0.2ml dichloromethane was added and again dried under a stream of nitrogen. The residues were then resuspended in an acetyl chloride/isobutyl alcohol mixture (3:10 v/v), the vials were then sealed and heated at 200°C for 30 mins. The mixture was then allowed to cool and was then evaporated to dryness under gaseous nitrogen. 0.05ml heptaflourobutyric anhydride was then added, the vials re-sealed and heated at 250°C for 10 mins. The vials were again allowed to cool and evaporated to dryness under nitrogen. The residue was finally resuspended in 0.025 to 0.5ml ethyl acetate/acetic anhydride mixture (1:1 v/v).

C: Derivatisation of sugars and sugar alcohols.

Trimethylsilyl (TMS) derivatives were produced of sugars and sugar alcohols. 0.1ml of the sugar sample prepared earlier was dried under gaseous nitrogen and 0.1ml Stox solution (Pierce-Warriner UK Ltd.) was added, this contains pyridine, 25mg/ml hydroxy ammonium chloride and 6mg/ml phenyl-B-D-glucopyranoside as an internal standard. The mixture was then heated at 75°C

for 30 mins and then allowed to cool. 0.1ml trimethylsilylimidazole (TMSI) was then added and the vial shaken at room temperature for one hour prior to GC determination.

D: Derivatisation of organic acids.

Organic acids were derivatised with N, O-bis (trimethylsylyl) triflouroacetamide (BSTFA). 0.1ml of the organic acid fraction was dried as before and 0.1ml pyridine was added followed by 0.05ml BSTFA and pantothenic acid as an internal standard. The mixture was then shaken and left at room temperature for 30 mins prior to injection.

E: Gas Chromatography (GC).

A 1µl sample of the derivatised products was injected into a Varian gas chromatograph (model 3700). GC conditions were as follows: for amino acids the column temperature remained at 80 ^{o}C for 4 mins and was raised to 250^{o}C at a rate of 8 ^{o}C /min for neutral to basic amino acids and 16^{o}C/min for acidic amino acids. For sugar and sugar alcohol determination the column starting temperature was 150 ^{o}C for 10 mins and increased to 300^{o}C at a rate of 13 ^{o}C/min. Organic acids start temperature was 80 ^{o}C for 4 mins and raised to 280^{o}C at a rate of 16^{o}C/min. Detection was by a flame ionisation detector on coated phase vitreous silica capillary column which had an internal diameter of 0.22mm, an outer diameter of 1mm and a length of 25m. Column pressure was 20 psi and the helium carrier gas flow was 30 cm^{3} /min. Detector gas flow rates were 30 cm^{3}/min for hydrogen and 300cm^{3} for air. A temperature of 280^{o}C was employed for the injector port and the detector.

APPENDIX 2

A: Oxygen electrode

A Hanseatech LD2 leaf disk oxygen electrode was used to determine oxygen evolution from leaf disks of Geum urbanum and Geum rivale. Mature fully expanded leaves were chosen for experimentation.

Teflon membranes were used with the electrode and the instrument was zeroed by introducing a sodium dithionate crystal. Carbon dioxide was supplied from a saturated sodium bicarbonate solution applied to a sponge situated above the electrode.

The leaf disks were placed upon an aluminum mesh disk and the whole chamber was then sealed.

Temperature was maintained at 25 ^{o}C via water flowing from a water bath around the external water jacket of the electrode. The electrode was then linked to a chart recorder and readings were taken over a six minute period.

B: Measurement of photosynthesis by IRGA

Photosynthesis measured by carbon dioxide gas exchange were made in the laboratory and in the field by an ADC LCA2 portable gas analyser and recorded on a data logger.

This machine is capable of measuring CO_2 partial pressures entering and leaving the cuvette, cuvette air temperature, relative humidity and photosynthetically active radiation. From this stomatal conductance and photosynthetic rate can be calculated by the equations described by von Caemmerer and Farquhar (1981).

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