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ACCUMULATION OF LOW MOLECULAR WEIGHT SOLUTES IN WATER-STRESSED TROPICAL LEGUMES

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Key Word Index—Tropical legumes; water stress; solute accumulation; inorganic ions; sugars; organic acids; *O*-methyl-inositols; amino acids; proline; betaines.

Abstract—Fourteen species of tropical legumes, representing 10 genera, were subjected to water stress. Youngest fully expanded leaves of stressed and unstressed plants were analysed for inorganic ions, sugars, inositols, organic acids, betaines and amino acids. The major compounds which accumulated with water stress were *O*-methyl-inositols (14 species), 2-methyl-2,3,4-trihydroxybutanoic acid-1,4-lactone (10 species) and proline (9 species). Concentrations of inorganic ions, sugars and organic acids decreased or were unchanged in the majority of the stressed species. The betaines, glycinebetaine, trigonelline and stachydrine were detected in low concentrations in most of the legumes but did not accumulate to any degree during water stress. All the legumes which tolerated low leaf water potentials accumulated the *O*-methyl-inositol, pinitol. The other species, with the exception of Siratro, contained ononitol or *O*-methyl-scyllo-inositol but no pinitol. It is suggested that pinitol accumulation may indicate a legume able to tolerate low leaf water potentials.

INTRODUCTION

Tropical crop and pasture species, used in the dryland farming systems in the summer rainfall areas of northern Australia, frequently experience water stress because of the variability in the amount and frequency of rainfall. The success or failure of species growing in semi-arid tropical areas depends on their ability to survive water stress. Several mechanisms have been suggested to explain the varied physiological responses of several tropical pasture legumes when subjected to water stress [1]. Those legumes which had a high tolerance of water stress and recovered from low water potentials, were found to exhibit osmotic adjustment. Some of this adjustment was accounted for by a change in the water-holding capacity of the cells, measured as a change in the turgid weight/dry weight-ratio, while the remaining adjustment was attributed to the accumulation of osmotically active solutes [1, 2].

So far, little is known about phytochemical changes induced in tropical legumes subjected to water stress. Accumulation of proline with water-stress has been reported in the tropical annual legume *Stylosanthes humilis* [3]. In contrast, studies on the tropical perennial pasture legume Siratro (*Macroptilium atropurpureum*) indicated low proline levels in both water-stressed and well-watered leaves, but found accumulation of D-pinitol (1D-3-*O*-methyl-chiro-inositol) in water-stressed tissue [4]. Accumulation of other *O*-methyl-inositols, ononitol (1D-4-*O*-methyl-myo-inositol) and *O*-methyl-scyllo-inositol, have been reported in a recent study of water-stressed *Vigna* species [5]. Leaves of water-stressed chickpea (*Cicer arietinum*) have been found to accumulate a novel lactone [6]. Thus, it is possible that there is considerable species variation in the types and quantity of solutes which may accumulate during water stress.

This paper investigates the effect of water stress on the levels of a range of solutes in the leaves of several tropical crop and pasture legumes. Identification and quantitative determinations are reported for inorganic ions, organic acids, carbohydrates, amino acids and quaternary ammonium compounds. The wide range of analyses was necessary to include the varied types of compounds which have been reported to occur in the Leguminosae, and to indicate metabolic changes which may explain the varying degrees of osmotic adjustment, found for these legume species, in terms of phytochemical changes.

RESULTS

The legumes investigated were divided into two groups, crops and pastures, based on usage in agriculture (Table 1), with no biological differences being implied. The crops were all annual and the pastures all perennial species which had widely differing growth rates. This resulted in the drying cycle commencing at different times for different species. There was also large inter-species variations in their resistance to water stress. The harvested material was green, viable tissue, and the water potentials measured represented values in most cases close to the minimum which could be measured for each species just before the plants died [1].

Water-soluble inorganic ion concentrations, and their changes due to increase in water-stress, varied between species (Table 2). Sodium levels were generally very low (< 1 mg/g) except for species 5 (5 mg/g, wet replicates). Only two species (nos. 9 and 14) had small increases in Na levels, so that quantitatively this element appeared to be of little osmotic importance in legumes. Potassium, the major cation, varied from 12.0 mg/g (species 14, dry) to 40.7 mg/g (species 13, wet). As with Na, levels of K tended to be lower in dry than in wet tissue (eight species), with only three species (5, 6 and 7) showing reasonable

Table 1. Legume species investigated, age at start of water stress, duration of drying cycle and minimum leaf water potential (ψ_l) of the water-stressed plants at the time of harvest

Legume	Age before stress (days)	Drying cycle (days)	ψ_l (−MPa)
Crop species			
1. *Cicer arietinum* cv Tyson	31	20	3.8±0
2. *Cajanus cajan* ICP 7035	29	22	5.3±0.2
3. *Lablab purpureus* cv Highworth	23	50	1.8±0
4. *Vigna radiata* cv Berken	29	21	1.7±0.1
5. *Vigna mungo* cv Regur	29	24	2.3±0.3
6. *Vigna unguiculata* CPI 28215	23	49	1.6±0.1
7. *Glycine max* CPI 26671	20	31	6.2±0
Pasture species			
8. *Stylosanthes hamata* cv Verano	45	29	9.5±0
9. *Stylosanthes scabra* cv Seca	57	30	10.6±0.7
10. *Psoralea eriantha*	57	34	4.7±0.5
11. *Rhyncosia minima*	64	50	3.0±0.8
12. *Macroptilium atropurpureum* cv Siratro	35	51	2.0±0
13. *Glycine tomentella*	42	28	5.3±0.3
14. *Centrosema* sp. aff. *pubescens* cv Belalto	12	45	6.0±0

Table 2. Variation of water-soluble inorganic ion concentrations with increase in water-stress in leaves of tropical legumes (mg/g dry weight)

Species		Na	K	Ca	Mg	Cl
1	W	1.5±0.3	27.9±0.2	0.2±0	2.9±0.1	5.3±0.9
	D	0.5±0.1	12.6±0.7	12.2±1.3	6.1±0.1	3.7±0.1
2	W	0.2±0	26.6±0.3	0.2±0	2.0±0.1	0.7±0
	D	0.2±0	24.8±0.1	0.2±0	2.2±0.1	0.6±0
3	W	0.5±0	32.8±0.8	6.6±4.1	2.5±0.8	3.6±0.3
	D	0.3±0	20.4±1.8	2.3±0.1	2.1±0.1	4.7±0.3
4	W	0.4±0	20.2±0.7	11.5±0.5	6.4±0.2	2.6±0.1
	D	0.3±0	18.6±0.8	18.5±1.8	6.0±0.7	4.8±0.2
5	W	5.0±0.8	14.5±0.5	11.5±0.2	7.3±0.4	2.4±0.4
	D	2.0±0.3	22.9±0.6	5.7±0.4	4.3±0.4	3.4±0.4
6	W	0.5±0.1	21.8±1.6	9.5±1.3	4.1±0.4	5.0±2.8
	D	0.4±0.1	26.6±1.3	7.8±1.4	4.0±0.2	6.0±2.0
7	W	0.3±0	19.2±2.2	2.4±1.0	2.5±0.2	2.8±1.5
	D	0.3±0	26.9±0.5	7.1±1.3	3.7±0.3	0.7±0
8	W	0.6±0.1	25.3±0.3	2.1±0.1	1.9±0.1	3.9±0.3
	D	0.5±0.1	28.4±1.6	5.3±0.5	2.7±0.1	6.4±0.2
9	W	0.5±0.1	21.9±0.7	0.7±0.1	1.6±0.2	6.8±0.8
	D	0.8±0	23.7±1.1	3.9±0.3	1.9±0.1	6.3±0.4
10	W	0.8±0.1	28.5±1.8	2.6±0.4	3.4±0.2	2.4±1.0
	D	0.6±0	29.0±0.2	1.5±0.6	3.3±0.6	3.3±1.3
11	W	0.8±0.3	33.6±1.3	0.5±0.2	2.3±0.1	4.5±0.9
	D	0.4±0	26.3±1.6	2.3±1.6	2.8±0.6	6.7±1.1
12	W	0.6±0.2	20.2±0.7	10.1±0.9	9.5±1.0	5.7±2.1
	D	0.3±0	12.4±0.4	8.5±0.5	9.1±0.6	7.0±0.1
13	W	0.3±0	40.7±1.4	0.9±0.2	2.9±0.2	2.3±1.1
	D	0.3±0	29.5±0.9	1.1±0.1	3.1±0.1	1.1±0.4
14	W	0.3±0	17.7±0.2	1.9±0.5	4.1±0.5	2.8±0.1
	D	0.8±0.5	12.0±1.1	1.9±0.3	4.5±0.5	3.6±1.6

W, Wet; D, dry.

increases in K. Six species had increased calcium levels in water-stressed tissue, two species had lower calcium levels, and the other six species showed no significant change. The most noticeable variation in calcium levels was in species 1 where a change from 0.2 to 12.2 mg/g was measured. Magnesium levels, generally less variable with water-stress than the other cations, were unchanged in ten species. Water-stressed tissue of three species (1, 7 and 8) had small increased magnesium levels, while species 5 had 3 mg/g less magnesium than wet tissue. Compared to wet tissue, chloride levels in water-stressed material were higher in five species, lower in three species and did not change in six species. The changes that were measured were variable but generally small (max. 2.5 mg/g in species 8).

No consistent pattern could be seen in the variation of fructose, glucose and sucrose concentrations, with increase in water-stress (Table 3). Overall, only six species had increased total sugar levels in the dry tissue, while three species had lower values. Levels of the individual sugars varied considerably among the species both quantitatively and in the direction of change of levels of the individual sugars. The maximum increase of total sugar levels measured was 24.5 mg/g (113 μmol/g) in species 11, and maximum decrease was 28.8 mg/g (137 μmol/g) in species 4.

In contrast to the sugars, inositol levels changed in a more predictable manner (Table 3). Myo-inositol levels decreased with water-stress in 13 species. The exception was Siratro in which a small increase of 1.3 mg/g (7.2 μmol/g) was measured. The maximum value found was in species 14 (6.4 mg/g) which also gave the largest decrease for myo-inositol of 4.2 mg/g (23.3 μmol/g). All 14 species investigated had increased levels of one or other of three mono-*O*-methyl-inositols in the dry tissue (Table 3). Pinitol occurred in 10 of the species, ononitol in two species, and *O*-methyl-scyllo-inositol in two species. Quantitatively, the largest accumulations were found for pinitol, and increases around 40 mg/g (222 μmol/g) were measured for several species. The greatest level found was 57.1 mg/g in dry tissue of species 11.

The quantitatively important organic acids present in all the species investigated were malonic, malic and citric acids (Table 4). The changes in the levels of those acids with increase in water-stress were variable. Malonate levels increased in only two species (6 and 12) and decreased in four species, and were thus largely unaffected by water-stress. Malate levels were higher in six species

Table 3. Effect of water-stress on ethanol-soluble carbohydrate levels in leaves of tropical legumes (mg/g dry wt)

Species		Fructose	Glucose	Sucrose	Myo-inositol	*O*-methyl-inositol*
1	W	6.8 ± 2.6	9.4 ± 3.6	26.7 ± 1.4	2.6 ± 0.6	18.7 ± 0.2
	D	13.2 ± 1.7	10.3 ± 0.7	30.3 ± 0.7	1.2 ± 0.1	48.8 ± 1.7
2	W	5.5 ± 1.0	3.9 ± 1.2	19.2 ± 1.2	4.4 ± 0.1	9.6 ± 0.8
	D	2.0 ± 0.1	2.0 ± 0.1	11.6 ± 0.6	0.8 ± 0	52.6 ± 2.2
3	W	11.0 ± 4.0	13.4 ± 5.7	8.3 ± 7.9	3.4 ± 0.1	3.1 ± 0.5†
	D	4.5 ± 0.3	13.9 ± 1.6	15.6 ± 3.7	1.6 ± 0.1	27.0 ± 0.3
4	W	6.6 ± 0.5	16.9 ± 0.6	14.7 ± 1.7	2.8 ± 0.3	9.2 ± 0.3‡
	D	0.9 ± 0.9	2.5 ± 0.9	6.0 ± 0.1	0.9 ± 0.1	18.6 ± 0.2
5	W	4.2 ± 1.1	5.8 ± 0.5	8.1 ± 0.4	2.1 ± 0.1	8.1 ± 0.9‡
	D	11.4 ± 4.4	13.8 ± 2.9	0.6 ± 0.3	1.7 ± 0.2	27.9 ± 2.6
6	W	10.8 ± 6.8	24.3 ± 7.3	9.6 ± 9.6	4.1 ± 1.1	2.1 ± 1.2†
	D	12.8 ± 2.8	17.8 ± 3.7	20.3 ± 3.3	1.5 ± 0.1	26.5 ± 3.0
7	W	7.1 ± 2.4	7.5 ± 1.4	16.1 ± 6.0	4.2 ± 0.3	10.9 ± 0.5
	D	2.0 ± 2.0	4.3 ± 1.8	20.2 ± 1.5	0.6 ± 0.2	51.3 ± 0.1
8	W	0.8 ± 0	0.8 ± 0	10.0 ± 0.5	3.2 ± 0.4	4.0 ± 0.4
	D	4.4 ± 0.1	2.8 ± 0.2	21.0 ± 1.9	1.2 ± 0	40.4 ± 0.1
9	W	7.4 ± 1.1	7.6 ± 1.1	0	2.2 ± 0	11.9 ± 2.3
	D	4.8 ± 0.9	3.8 ± 0.8	4.5 ± 0.8	1.1 ± 0	38.2 ± 1.2
10	W	6.6 ± 0.7	9.6 ± 0.9	20.0 ± 0.8	3.3 ± 0.2	13.0 ± 0.7
	D	7.3 ± 1.5	22.9 ± 2.8	25.6 ± 11.4	1.4 ± 0.3	55.8 ± 0.7
11	W	4.6 ± 0.5	5.2 ± 0.2	16.1 ± 1.6	4.1 ± 0.2	19.7 ± 1.4
	D	11.9 ± 3.1	13.6 ± 2.6	24.9 ± 11.5	2.0 ± 0.6	57.1 ± 8.1
12	W	1.7 ± 0.5	3.9 ± 0.2	13.0 ± 2.2	2.9 ± 0.4	13.9 ± 3.5
	D	3.2 ± 0.2	13.9 ± 3.3	3.9 ± 0.8	4.2 ± 0.9	27.3 ± 0.2
13	W	1.2 ± 0.5	1.3 ± 0.4	13.7 ± 0.2	2.3 ± 0.1	1.9 ± 0.1
	D	0	2.8 ± 0.1	12.7 ± 0.3	0.6 ± 0.1	46.4 ± 3.3
14	W	7.9 ± 0.5	8.6 ± 0.3	23.8 ± 2.8	6.4 ± 0.8	3.0 ± 0.8
	D	3.0 ± 0	6.2 ± 0.5	32.7 ± 2.1	2.2 ± 0.1	42.8 ± 0.9

*Pinitol, except where noted otherwise.
†Ononitol;
‡*O*-Methyl-scyllo-inositol.
W, Wet; D, dry.

Table 4. Effect of water-stress on concentrations of organic acids in leaves of tropical legumes (mg/g dry wt)

Species		Malonic	Malic	Citric	'γ-Lactone'*
1	W	5.7 ± 0.4	44.5 ± 5.0	4.6 ± 0	0
	D	0	0	0	105 ± 7
2	W	1.9 ± 0.2	5.2 ± 0.4	4.2 ± 0.8	0
	D	0.4 ± 0	2.0 ± 0.4	0	39 ± 4
3	W	4.0 ± 1.5	32.2 ± 7.5	30.6 ± 6.4	0
	D	3.3 ± 1.1	4.7 ± 0.7	4.2 ± 0.5	7 ± 3
4	W	7.3 ± 0.3	36.1 ± 1.8	4.6 ± 0.9	4 ± 0
	D	8.1 ± 0.6	15.5 ± 3.2	9.3 ± 0.2	35 ± 0
5	W	2.5 ± 0.7	22.2 ± 6.2	30.5 ± 4.3	6 ± 2
	D	4.0 ± 0.3	14.3 ± 2.6	13.2 ± 2.0	26 ± 6
6	W	7.1 ± 3.0	15.1 ± 2.4	30.1 ± 1.7	0
	D	16.0 ± 3.6	8.5 ± 0.9	6.6 ± 0.9	0
7	W	12.9 ± 1.4	12.9 ± 2.9	5.6 ± 0	0
	D	6.9 ± 0.1	19.9 ± 2.2	17.0 ± 0.6	65 ± 5
8	W	0.5 ± 0.2	6.1 ± 1.1	0	0
	D	1.0 ± 0.1	15.7 ± 1.5	6.2 ± 0.2	0
9	W	0.6 ± 0	6.6 ± 0.6	1.7 ± 0.2	0
	D	1.0 ± 0.1	23.9 ± 2.2	3.2 ± 0.3	0
10	W	2.1 ± 0.3	33.4 ± 0.4	59.0 ± 2.6	0
	D	2.3 ± 0.1	71.2 ± 9.4	24.4 ± 3.0	48 ± 5
11	W	4.5 ± 0	19.4 ± 0.8	14.2 ± 1.8	0
	D	3.7 ± 0.3	27.2 ± 14.8	10.3 ± 2.9	0
12	W	9.2 ± 1.1	13.6 ± 4.9	16.0 ± 10.0	0
	D	22.6 ± 2.0	13.1 ± 2.3	2.6 ± 0.1	13 ± 1
13	W	8.8 ± 2.1	4.6 ± 0.4	10.2 ± 0.1	0
	D	4.8 ± 0.3	7.0 ± 1.3	0	49 ± 7
14	W	0.4 ± 0.1	9.1 ± 1.0	14.0 ± 6.1	17 ± 1
	D	1.1 ± 0.2	19.7 ± 1.2	5.0 ± 2.9	30 ± 3

*2-Methyl-2,3,4-trihydroxybutanoic acid-1,4-lactone [6].
W, Wet; D, dry; 0, not detected.

and lower in five, while citrate levels decreased in the dry tissue of nine species and increased in four species. Some notable changes in malate levels were measured. In species 10, 71.2 mg/g was found in the dry tissue, corresponding to an increase of 37.8 mg/g (282 μmol/g) of the acid. Some large decreases with water-stress were also found, particularly in species 1 where malate levels apparently dropped from a value of 44.5 mg/g in the wet tissue, to zero in the dry tissue. The maximum decrease for citrate values (34.6 mg/g, 180 μmol/g), was found for species 10.

Accumulation of the 'γ-lactone' in dry tissue was found in 10 of the legumes, including species 3, which had comparatively low levels (Table 4). The exceptions were species 6, 8, 9 and 11. Generally, this compound could not be detected in the wet tissue, the exceptions being species 4, 5 and 14. A high value of 105 mg/g (795 μmol/g) was measured in species 1, but most values were much less than half that amount.

Proline was found to accumulate in the dry tissue of nine species, only traces being measured in species 3, 4 and 12 (Table 5). A high value of 41.3 mg/g (359 μmol/g) was measured in the dry tissue of species 8. Generally, wet tissue had only trace quantities of free proline.

The betaines, glycinebetaine, trigonelline and stachydrine (prolinebetaine) did not accumulate to any great degree in wet or dry tissue (Table 5). Glycinebetaine was detected in five species (max. 2.2 mg/g, species 10), trigonelline was present in all species (max. 1.7 mg/g, species 11) and stachydrine was detected in nine species (max. 2.1 mg/g, species 5). Lack of sufficient plant material precluded the analysis of some samples.

Free amino acid levels (excluding proline) were typically low (Table 6), and the maximum value found for an individual acid was 10.8 μmol/g (arginine, species 3). Of the seven species analysed, consistently increased levels in dry tissue were found for glycine (five species) and valine (six species), and decreased levels for serine (six species) and alanine (seven species).

Further studies using pigeon pea showed that during the drying period leaf water potential fell consistently until rewatering, when there was rapid recovery to near the beginning value (Fig. 1). Pinitol levels, on the other hand, increased rapidly up to 20 days stress and then tended to a maximum after about 40 days, before dropping at the end of the drying cycle to a value less than that found after 20 days stress. On rewatering, pinitol levels fluctuated greatly and five out of six values measured were much greater than any found during the stress period. Pinitol levels had a linear relationship with leaf water potential down to *ca* −4.0 MPa (Fig. 2). Regression analysis of data from the first drying cycle gave the equation $y = -1.40x + 4.26$ ($r = 0.9816$), where y = pinitol (mg/g) and x = leaf water potential (MPa). As water potential values decreased below −4.0 MPa,

Table 5. Effect of water-stress on the levels of proline and betaines in leaves of tropical legumes (mg/g dry wt)

Species		Proline	Glycine betaine	Trigonelline	Stachydrine
2	W	tr*	0	0.2	0
	D	32.5†	0.9	0.5	1.3
3	W	tr	0	0.5 ± 0.1	0.2 ± 0.1
	D	tr	tr	1.3 ± 0.1	0.4 ± 0.1
4	W	tr	0	0.5 ± 0	tr
	D	tr	0	0.6	tr
5	W	tr	1.1 ± 0.1	0.3 ± 0	0
	D	5.4 ± 2.0	tr	0.4 ± 0.1	2.1 ± 0.7
6	W	tr	tr	0.7 ± 0.3	0
	D	n.d.	n.d.	n.d.	n.d.
7	W	tr	0	0.2 ± 0.1	tr
	D	22.0 ± 0.8†	0	0.3 ± 0.1	0.4 ± 0.1
8	W	tr	0	0	0
	D	41.3 ± 1.5	0	0.5 ± 0	tr
9	W	0	0	0.3 ± 0	tr
	D	30.2 ± 5.9	0	0.3 ± 0.1	0.7 ± 0
10	W	tr	2.2	0.4 ± 0	0
	D	14.8 ± 4.3	1.6	0.8 ± 0.2	0.5 ± 0.2
11	W	n.d.	n.d.	n.d.	n.d.
	D	10.5	0.9	1.7	0
12	W	0	tr	0.1 ± 0	0
	D	tr	0	0.1 ± 0	1.0 ± 0.1
13	W	n.d.	n.d.	n.d.	n.d.
	D	29.1 ± 0.7	0.6 ± 0.4	0.2 ± 0	1.7 ± 0
14	W	tr	0	1.0 ± 0.1	0
	D	28.9 ± 2.3†	0	1.4 ± 0.1	0.2 ± 0

*tr = trace, < 2 mg/g (proline), < 0.2 mg/g (glycine betaine and stachydrine).
†Values supported independently on an automatic amino acid analyser.
n.d., Not determined; 0, not detected; W, wet; D, dry.

Table 6. Variations in concentrations of free amino acids with increase in water stress in leaves of tropical legumes (μmol/g dry wt)

Species		Ser	Gly	Thr	Ala	Arg	Val	Trp	Phe	Ile	Leu
3	W	7.1	1.1	0.8	3.4	10.8	2.2	0	0.3	0.5	0.8
	D	0	3.7	0	2.5	1.0	1.2	0	1.2	0.6	0.2
4	W	1.1	0.6	0.6	3.8	10.0	0.8	0.1	0	0.1	0.1
	D	0	2.8	0.4	3.5	5.6	4.0	0.3	2.9	1.3	1.2
5	W	4.6	0.9	0.4	3.4	4.0	0.3	0.6	0	0	0
	D	0.8	5.9	0.4	1.9	3.4	3.3	0.3	2.9	1.5	0.8
7	W	0.4	0.6	0.8	3.8	n.d.	0.5	n.d.	n.d.	n.d.	n.d.
	D	0	7.1	1.0	1.9	0.8	4.8	0.5	0.4	1.9	1.3
10	W	5.6	1.3	1.2	13.5	4.7	0	1.4	0	0	0
	D	0.4	1.1	0.8	6.7	8.7	2.5	1.4	0.9	0.5	0.4
12	W	0.9	0.3	0.3	1.8	n.d.	0	n.d.	0.2	0.2	0.1
	D	0.3	0	0.2	0.7	n.d.	0.5	n.d.	0	0.2	0.1
14	W	8.1	1.0	1.0	4.1	6.3	0.4	0.2	0	0	0
	D	n.d.	3.8	n.d.	1.6	n.d.	1.6	n.d.	n.d.	n.d.	n.d.

n.d., Not determined; 0, not detected; W, wet; D, dry.

pinitol levels tended to a maximum of about 60 mg/g at around −5.0 MPa, before gradually reducing to about 40 mg/g at the end of the drying cycle. Data from a second drying cycle confirmed this general trend in pinitol concentration (Fig. 2).

Levels of free 'γ-lactone' also tended towards a maximum well before the end of the drying cycle, possibly at a slightly higher water potential than pinitol (Fig. 2).

Fructose, glucose and sucrose concentrations (not shown) showed no noticeable trends with decreasing

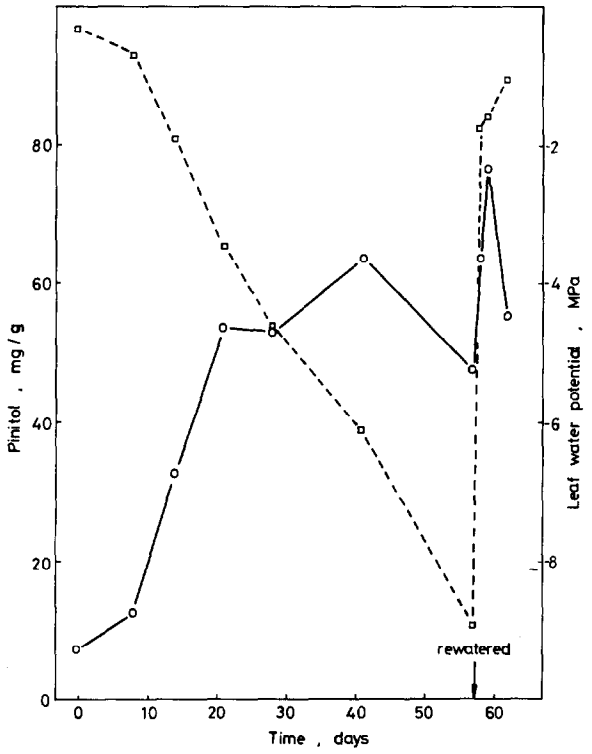

Fig. 1. Changes in leaf water potential (ψ_l) and pinitol levels in leaves of pigeon pea with increasing time without water and after rewatering. (O——O), Pinitol; (□----□), ψ_l.

water potential. Myo-inositol values dropped quickly from an initial high of *ca* 4 mg/g to a constant of *ca* 1 mg/g at −1.9 MPa.

In leaves of well-watered pigeon pea plants, pinitol levels varied with leaf position on the stem. Pinitol levels tended to increase progressively from young, still expanding leaves at the top of the plant, towards a maximum in the most recently expanded leaves (Fig. 3). Thereafter, a steady decline in pinitol concentrations was found. The variation among individual leaflets, as shown by analysis from selected trifoliate leaves of the regrowth plant, although appreciable, suggested that the observed trends from single leaf analysis were genuine. There was no relationship between leaf water potentials and pinitol levels in the well-watered leaf tissue.

Sugar values (not shown) fluctuated greatly. There was a suggestion of a trend towards lower sucrose values in the older plant tissue of the 1400 hr harvest from *ca* 25 mg/g initially to *ca* 15 mg/g. However, in both harvests, changes in sucrose levels tended to be accompanied by an inverse change in glucose and fructose levels. Myo-inositol levels remained relatively constant, e.g. 3.2 ± 0.5 mg/g for the 25 leaves of the 1400 hr harvest, with no apparent trend. Small amounts of ononitol were measured in the unfolding leaves (max. 6 mg/g), which declined rapidly from about leaf position 0 to a relatively constant value of *ca* 0.5–1 mg/g from leaf position 5 downwards.

DISCUSSION

Previous field studies on the effect of water stress on the tropical legume Siratro found that a small amount of osmotic ajustment occurred which could be largely accounted for by changes in the water-holding capacity of the leaf [2]. Except for a small amount of pinitol (15 mg/g), there was no detectable accumulation of a wide range of solutes [4]. However, most of the species in the present study were found to have some degree of osmotic adjustment, after adjustment for changes in tissue hydration [Ludlow, M. M., unpublished results], which was taken to indicate accumulation of solutes [1].

The present work, however, indicates that with particular respect to inorganic ions and sucrose, the effect of water stress on legumes differs greatly from the effect on tropical grasses in which these solutes accumulated to much higher concentrations in water-stressed leaves [4]. For example, potassium, which is considered to be a major osmotic component of glycophytes [7], tended to de-

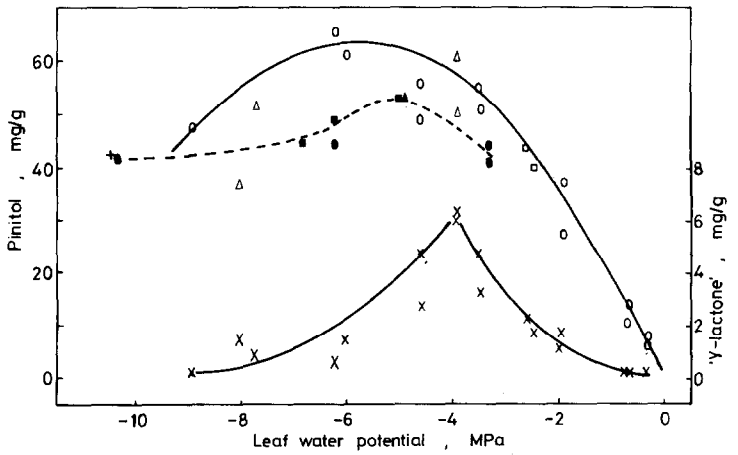

Fig. 2. Relationship between leaf water potential and pinitol and free 'γ-lactone' contents in leaves of pigeon pea during two drying cycles. Pinitol, 1st drying cycle: O, Pot 1; △, Pot 2; □, Pot 3; Pinitol, 2nd drying cycle (dotted line): ●, Pot 1; ▲, Pot 2; ■, Pot 3; +, Pot 4; 'γ-lactone' (×): 1st drying cycle only. Lines are hand drawn to illustrate trends and do not represent any mathematical relationship.

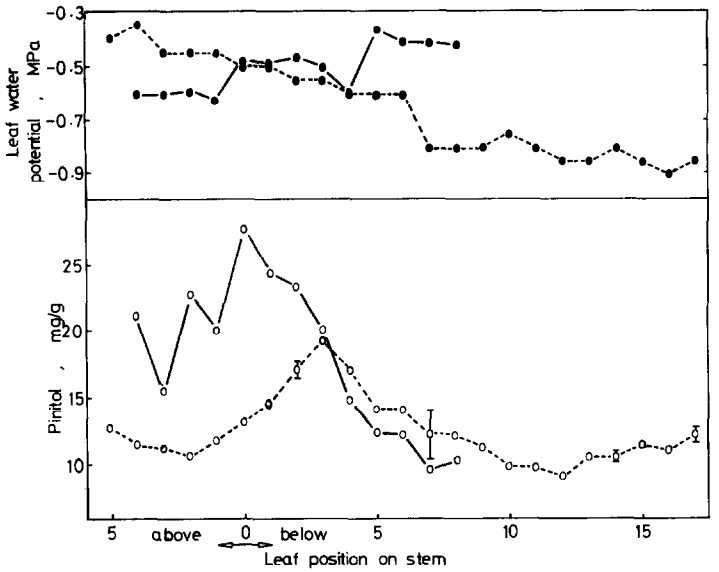

Fig. 3. Variation of leaf water potential and pinitol levels with position of leaf on the stem of well-watered pigeon pea at two times of harvest. Postiion 0 = youngest fully expanded leaf. Vertical bars indicate mean values ± s.d. for individual analysis of the three leaflets on one trifoliate leaf. (——), Dawn harvested. (– – – –), 1400 hr harvested leaves.

crease with water-stress in the legumes. Nor was there any positive relationship between potassium and malate levels as might have been expected [8], particularly in the three species which had increased potassium values. Similarly sucrose, a major accumulation product with water-stress in tropical grasses [4], tended to decrease or remain constant in the legumes. Recent studies on two cultivars of soybean [9] found little change with water stress in soluble sugar or potassium levels, in agreement with the present work, but also reported no osmotic adjustment. In the present study, reasonable osmotic adjustment was measured for soybean [Ludlow, M. M., unpublished results]. Organic acids have also been mentioned as osmotically active components [7, 8], and increased values of 300 μmol/g for malate in water-stressed cotton have been reported [10]. Only *Psoralea*, of the six legumes which accumulated malate with water stress, had increased malate levels approaching that of cotton (280 μmol/g).

The levels of 'γ-lactone' (Table 4) were determined using as reference a redissolved, weighed residue of a chromatographically pure syrup isolated from water-stressed chickpea [6]. The values quoted are considered tentative at this stage, but do indicate that this product could be of major importance in the osmotic regulation of many of the legumes. Much of the compound appears to occur in a combined form whose structure is at present unknown, but from which it is liberated readily with methanolic HCl. Since the 'γ-lactone' has only recently been found in plants [6], little is known about its distribution, other than its occurrence in the species reported here, and that it does not accumulate in some tropical grasses [4].

The increased levels of *O*-methyl-inositols in the water-stressed tissue of all the legumes studied here suggests that this class of compound may be of general importance in osmoregulatory processes of the Leguminosae. Although over the years these inositols have been well documented in the Leguminosae [11, 12], it has only recently been observed that their concentrations in some plant tissue could be dramatically increased by water stress [4, 5]. It was demonstrated that, at least for pigeon pea, accumulation of pinitol during water stress was not due to differences in age or position on stem of the leaf sampled (Fig. 3). Older tissue of well-watered plants actually tended to have lower levels of pinitol than the youngest fully expanded leaves. Thus the increase in pinitol levels in water-stressed pigeon pea leaf tissue could be attributed with some certainty to a reduction in the leaf water potential. The magnitude of *O*-methyl-inositol accumulation in the other species suggested that this relationship with water potential may be a general phenomenon in the Leguminosae. However, it was clear there was not a continual increase in pinitol levels as the leaf water potential decreased. A comparison between all species (Fig. 4) indicated that in general the trend was towards a decrease in pinitol levels as the leaf water potential was reduced below about −4 MPa. This trend was confirmed for pigeon pea where additional pinitol values were measured at water potentials between 0 and −4 MPa (Fig. 2). The processes involved in the metabolism of pinitol during water stress conditions, and during the period immediately following rewatering when pinitol levels fluctuated greatly (Fig. 1), have not yet been elucidated.

The legume species studied here could be divided into two groups: those which tolerated low water potentials (< −3 MPa) and those whose leaf water potentials remained greater than that value. The former group was characterized by the accumulation of pinitol in the leaves, while the latter (with the exception of Siratro) contained no pinitol, but accumulated ononitol or *O*-methyl-scyllo-inositol. It may be that pinitol synthesising legumes, in general, are those which can tolerate the lowest water potentials.

Betaines were quantitatively minor components in the

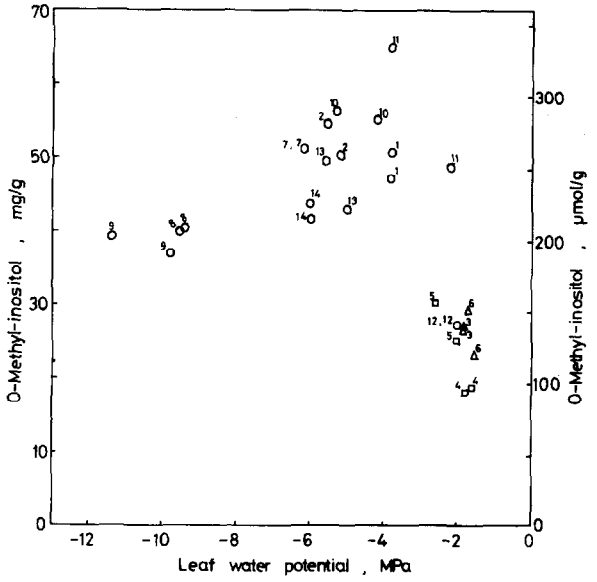

Fig. 4. Variation of *O*-methyl-inositol levels in leaves of water-stressed tropical legumes with changes in leaf water potential. Numbers on graph refer to species identification codes (Table 1). ○, Pinitol. △, Ononitol. □, *O*-Methyl-scyllo-inositol.

legumes studied here (Table 5), and hence are not potential osmoregulators during water stress in these species. This is in agreement with results reported for some temperate legumes [13], but is in contrast to certain species of Gramineae such as barley [14] and the tropical grasses green panic and buffel [4], in which glycinebetaine accumulated during water stress.

Proline accumulation in the dry tissue was related to the water potential (Fig. 5). The regression equation indicated that proline accumulated at water potentials below −0.53 MPa. This is slightly higher than −0.72 MPa estimated for barley [15], and is consistent with previous results with grasses [4] where there was a time lag, while the water potential fell, before proline accumulation commenced. Proline accumulation increased consistently to very low water potentials (Fig. 5), unlike pinitol which tended to a maximum around −4 MPa (Figs 2 and 4). Some of the proline values were much higher than previously reported in water-stressed grasses [4, 16], possibly because of the lower water potentials attained by the legumes in this study. However, similar proline levels have been reported in some water-stressed temperate legumes [17].

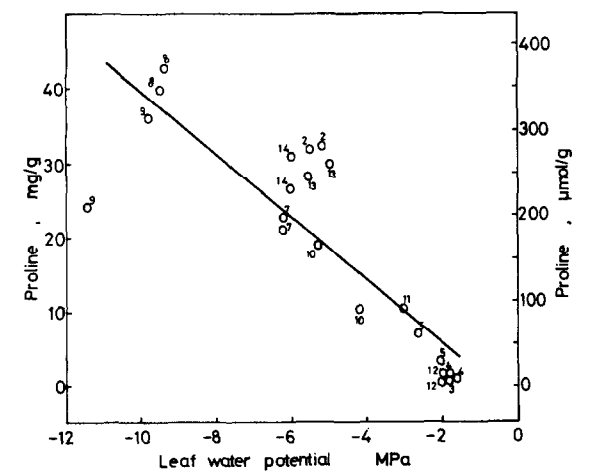

Fig. 5. Variation of proline levels in leaves of water-stressed tropical legumes with changes in leaf water potential. Numbers on graph refer to species identification codes (Table 1). Linear regression equation: $y = -2.24 - 4.19x$ ($r = 0.8602$), where y = proline concn (mg/g) and x = leaf water potential (MPa).

A further point of interest was the valine accumulation in the dry tissue of some of the legumes (Table 6). Previously [6] it had been speculated that the 'γ-lactone' (Table 4) might be involved in the biosynthesis of valine. Of the seven species analysed for amino acids, six species had proportionally large increases in valine, which coincided with accumulation of 'γ-lactone'. The other species accumulated neither valine nor 'γ-lactone' in the dry tissue. Although hardly conclusive, these results do not contradict the proposal.

This study has thus described a reasonably comprehensive analytical investigation of a range of legume species in an attempt to identify, and generalise on, the phytochemical responses of these plants to water stress. The accumulation of *O*-methyl-inositols in all the water-stressed tissue in this work suggests that these compounds may be of importance in osmoregulation in legumes, and identification and quantitation of these products should be included in any water stress studies. Likewise proline, whose levels seem to be directly related to the water potential values, is an important accumulator in legume leaf tissue below −2MPa. Proline is considered to be localized in the cytoplasm [18] which occupies 5–20% of the cell volume [19, 20]. However, many of the proline levels are so high that their contribution to osmotic adjustment would be inordinately large. Hence a modified proline environment must be considered, possibly a degree of association with macromolecules in the cytoplasm [21] or a less specific compartmentation between the vacuole and the cytoplasm. The occurrence of the 'γ-lactone' in most of the legumes, but only in water-stressed tissue, is of interest not only as a possible osmoregulator but also as a possible indicator of a metabolic intermediate which accumulated due to inhibition of amino acid or protein synthesis.

EXPERIMENTAL

Plant material. Plants were grown in large cylinders (1 × 0.25 m i.d.) lined with heavy gauge polythene bags containing 50 kg of an air-dried creek bank loam. Growth took place in a controlled environment room [22] (500 μE/m²/S photosynthetic quantum flux for a 17 hr day and 30°/25° day/night air temp.). The cylinders, two per species, were watered daily to a H_2O content equivalent to pF2. After germination, plants were thinned to three per cylinder. The drying cycle was commenced when plants reached a suitable size and their roots had reached the bottom of the cylinders. This varied from 23 days after planting for *Lablab* and cowpea to 64 days for *Rhyncosia*.

One pot of each species per replicate was kept well watered, while water was withheld from the remaining cylinders. Samples of last fully expanded leaves were harvested at the controlled environment equivalent of dawn from two replicates of both wet and dry plants in each species near the end of the drying cycle. The drying period varied from 20 days for chickpea to 50 days

for *Rhyncosia*, and the harvested water-stressed material had water potentials [23] near those at which the leaves of the respective species were irreversibly damaged. These plants were also used in a study of their water relations characteristics [Ludlow, M. M., unpublished data].

Two further studies were conducted using pigeon pea leaf tissue. In the first, plants were grown in four pots with three plants to each pot. The pots were well watered for 21 days after which time water was withheld for 57 days before watering was recommenced. After a further 5 days some of the plants were cut back, regrown for 43 days with regular watering, and then re-stressed by withholding water for a further 27 days. Samples of youngest fully expanded leaves were harvested at various intervals during each drying cycle and the rewatering period.

In the second study, two sources of plant tissue were used. In one case, a plant which had previously been stressed to −8.9 MPa was rewatered and a new branch which subsequently grew was used. The other source was a plant from the regrowth material in the second drying cycle described above in the first study. A single branch from each source was used to provide single leaves (17–27) of varying ages from the top to the bottom of the stem, each leaf to be analysed individually. All harvested material was immediately frozen in dry ice followed by lyophilization, drying in vacuum over P_2O_5 and grinding through a 1 mm sieve. Analytical results are given on a dry wt basis.

General experimental conditions. Evaporations were performed under red. pres. at less than 40° unless stated otherwise. GLC separations were effected on (a) 3% GE-SE 30 on Gas Chrom Q, stainless steel 1800 × 3 mm, or (b) 12% HI-EFF 1BP on Gas Chrom P, nickel 1800 × 3 mm. Trimethylsilyl (TMSi) ethers were prepared using TMSi-imidazole [24]. Injector and detector temps were 220° and 285° respectively. Column temps were initially 140°, programmed to rise at (a) 2°/min to 190°, or (b) 4°/min to 190°. Carrier gas was N_2 at 25 ml/min. HPLC fractionations were performed using a Waters 6000A delivery system and a Radial Compression Module (RCM 100, Waters Associates), with either (a) μ-Bondapak (5μ, C_{18}) or (b) Dextropak 'Radial-PAK' cartridges (10 × 0.8 cm). Mobile phases used were (a) gradient buffer (NaOAc, 0.5 M, pH 7.4) mixture: buffer A (H_2O–NaOAc–THF–MeOH, 16:2:1:1) (1 l) + buffer B (H_2O–NaOAc–MeOH, 1:1:8) (1 l) ('AUTO · TAG' technique, Waters Associates), or (b) H_2O containing 0.02% dibutylamine phosphate (D4, Waters Associates) at pH 3, and a flow rate of 1.5 ml/min. All mobile phases were filtered ('Millipore' FH 0.5 μm) and degassed before use, and samples were clarified ('Millipore' HA 0.45 μm) before injection.

Analytical procedures. (a) *Carbohydrates.* Dried, ground plant tissue (*ca* 50 mg) was sequentially extracted in a Soxhlet apparatus with Et_2O and 95% aq. EtOH. The EtOH-soluble carbohydrates fructose, glucose, sucrose and inositols were determined by GC as their TMSi derivatives on column (a) [24]. (b) *Organic acids.* Plant tissue (*ca* 200 mg) was shaken with H_2O (3 × 15 ml, 0.5 hr each) at 55°. The combined aq. extracts were concd and then adjusted to 10 ml. Aliquots (*ca* 150 mg dry wt equivalent) were evaporated, dried over P_2O_5 and then shaken with 3% MeOH–HCl (1 ml) in a stoppered vial at 55° for 4 hr. After centrifugation, the acid supernatant solns were analysed by directly injecting *ca* 1.5 μl into the GC [column (b)]. Standard solns of organic acids, prepared and derivatized in an identical fashion to that of the tissue H_2O extracts, were used as external standards. (c) *Elemental analysis.* Aliquots from the H_2O extracts described in (b) were used, and Na, K, Mg, Ca (atomic absorption spectroscopy) and Cl [25] required *ca* 30 mg tissue equivalent. (d) *Amino acids.* Dry tissue (0.2–1.2 g) was extracted as in (a). Amino acids were determined in the 95% EtOH extract after partial purification using cation exchange resin [Amberlite IR 120 (H^+)]. Amino acids were eluted from the resin with 2 M NH_3 and then evaporated to dryness. After redissolving in 0.5 ml H_2O, the amino acids were determined either using an automatic amino acid analyser and ninhydrin detection, or by reverse phase HPLC [method (a)], using pre-column derivatization with *o*-phthalaldehyde [26], but measuring the *A* at 336 nm instead of using fluorimetric detection. (e) *Proline and betaines.* These compounds were simultaneously determined by reverse-phase HPLC using method (b) and a differential refractometer and UV absorbance at 264 nm for detection [Ford, C. W., unpublished results].

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