

THE ASSESSMENT OF PROLINE ACCUMULATION AS A MECHANISM OF DROUGHT RESISTANCE IN SUGARCANE

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Abstract

Shortage of water is the main factor which limits the yield of sugarcane grown under rainfed conditions in South Africa. However, local knowledge of drought resistance mechanisms in sugarcane is lacking. The strategies of drought avoidance, as found in N12; and of drought tolerance, as found in N11; are discussed. Possible selection criteria are identified.

Introduction

Proline is commonly accumulated by stressed plants as an osmoregulant (Aspinall and Paleg¹, Levy¹⁴). Osmoregulation involves the active accumulation of solutes in response to water loss and results in a decline in leaf water potential. This in turn leads to an increase in the ability of the plant to extract water from a drying soil because water potential gradient is maintained.

Osmoregulation typifies the drought tolerance strategy whilst that of avoidance is characterized by the maintenance of a relatively high tissue water potential through the stomatal restriction of water loss. The water potential of a leaf can be conveniently described as:

$$-\psi_l = -\psi_o + \psi_t$$

(-MPa) (-MPa) (+MPa) (Passioura¹⁸)

where ψ_o is the potential for water uptake due to dissolved solutes (osmotic potential) and ψ_t is the positive turgor pressure exerted on the confining cell wall.

In the avoidance strategy any decline in leaf water potential occurs through loss of turgor and passive concentration of solutes due to water loss. The tolerance strategy allows the maintenance of turgor due to an active accumulation of solutes.

Maintenance of turgor pressure is necessary for cell expansion as well as for many other physiological processes, most notably the fixation of carbon dioxide (Hsiao⁸).

Ludlow¹⁶ has argued that crops originating from favourable climates, as does sugarcane, tend to have less ability to osmoregulate and therefore would be expected to show avoidance characteristics in any drought resistant genotypes. However Rao and Asokan¹⁹ found that some drought resistant sugarcane genotypes accumulated up to one hundred times more of the amino acid proline, when stressed, than susceptible genotypes. Ho *et al*⁷ report similar results.

Nevertheless some controversy exists over whether proline accumulation is an adaptive response to water stress or a symptom of that stress (Hanson *et al*⁵; Ibarra-Caballero *et al*¹⁰). Ilahi and Dorffling¹¹ have even shown that genotypes susceptible to drought produce more proline. A possible reason for this lies in the release of proline during senescence-related protein breakdown and also the conversion of similarly released arginine and glutamate to proline (Stewart²³). The drought resistant sugarcane variety N11 is known for its reduction in leaf area by senescence during water stress (Inman-Bamber¹²).

Proline accumulation has been suggested to be beneficial for reasons other than osmoregulation. It may act as a reservoir of nitrogen and energy for recovery after alleviation of stress (Singh *et al*²¹; Stewart *et al*²⁴); and it may protect proteins essential for cell activity from denaturation (Schobert and Tschesche²⁰). These functions could be fulfilled simply by an increase in the proportion of total free nitrogenous solutes in the form of proline. This may or may not constitute osmoregulation.

The variety N11 has been shown to continue leaf extension and to close stomata at lower leaf water potentials than the more drought-susceptible NCo376. On the other hand N12, another drought resistant variety, responds to declining water potential by rapidly rolling its leaves and closing stomata earlier than does NCo376 (Inman-Bamber¹²). The implication is that N11 maintains greater turgor at lower leaf water potentials than the other two varieties. It should therefore be expressing the osmoregulatory characteristics of drought tolerance whilst N12 appears to exemplify drought avoidance.

In order to test for proline accumulation in varieties of sugarcane a system using polyethylene glycol MW4000 (PEG 4000) was devised. Excised leaf material was floated on aqueous solutions of this material. The water potential of the leaf segments then adjusted, either actively or passively, to match the potential of the exterior solution.

Materials and Methods

Experiment 1

Leaf blade segments, each 40 mm long, were obtained from the third fully expanded leaves of six month old N11 and N12 plants (30 March 1988). These were fully hydrated by floating on distilled water for 4 hours and were then floated on solutions of different concentrations of PEG4000 for 48 hours at 25°C under constant illumination. Proline concentrations were determined on a $\mu\text{g/g}$ fresh weight basis using the method of Bates *et al*². Figure 1 shows both PEG4000 concentration and the water potential of the solution derived using the graph of Bressan *et al*⁴.

Results

Observation showed that although N11 segments began rolling at a higher water potential than did N12, at lower potentials N12 rolled more rapidly. N11 began accumulating proline at a higher water potential than N12 and also accumulated it to a much greater extent.

Experiment 2

In view of the results of Experiment 1 a PEG water potential of -3.7 MPa was chosen. A drought susceptible variety, N14, was also included. Segments of leaf 40 mm long, this time cut to 15 mm wide, were obtained from fully expanded leaves 1, 3, 5 and 7 of eight month old N11, N14 and N12 plants (15 May 1988). Since chlorophyll levels are known to decline with senescence (Lewington *et al*⁵) these were determined by the method described by Harborne⁶. Chlorophyll retention was used as a measure of senescence.

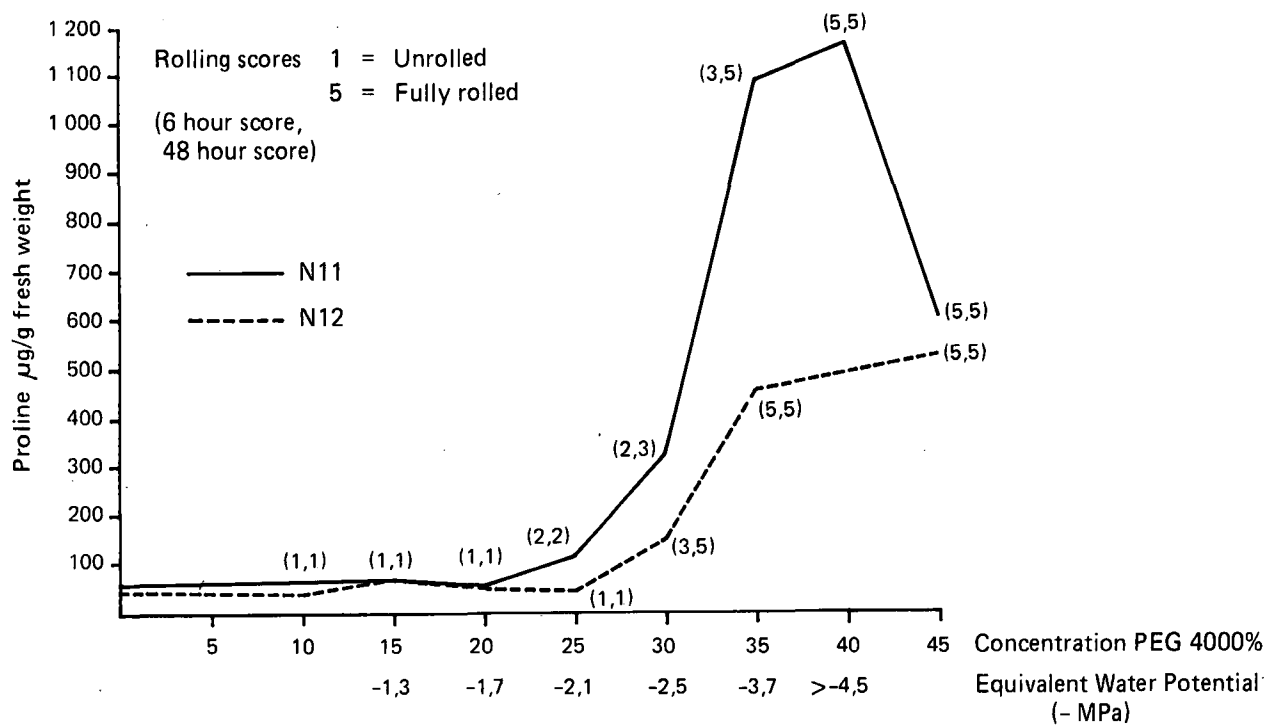


FIGURE 1 (Experiment 1) Proline accumulation by segments of fully expanded leaf 3 from N11 and N12 (30/3/88).

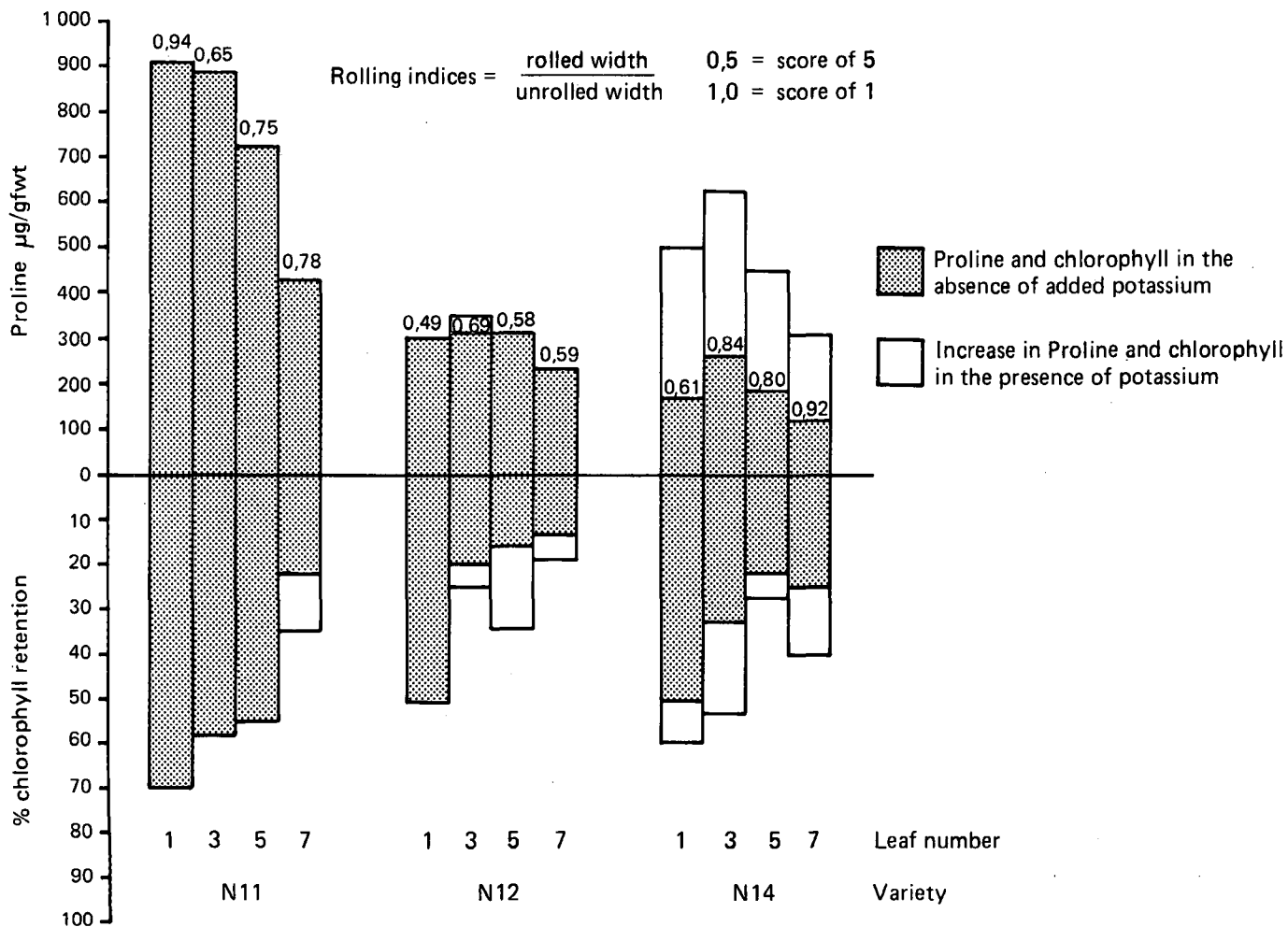


FIGURE 2 (Experiment 2) Proline accumulation and chlorophyll retention by leaf segments of three varieties when stressed by $-3,7$ MPa PEG (15/5/88).

Results

In N11 leaves of increasing age (leaf number) showed a declining ability to accumulate proline. N11 produced more proline than N12 did, whilst N14 produced the least (Figure 2).

Mukherjee¹⁷ showed that maize plants in stressed leaf segments with high potassium contents produced more proline than plants with low potassium contents. The addition of 50 mM potassium chloride to PEG solutions resulted in a substantial increase in proline production by N14 but not by N11 or N12. Production by N14 then exceeded that by N12. In addition increasing senescence with leaf age may have been delayed by the potassium chloride treatments. In the field leaf potassium content fell slightly during the winter whilst calcium content increased in both N11 and N14 (Figure 3).

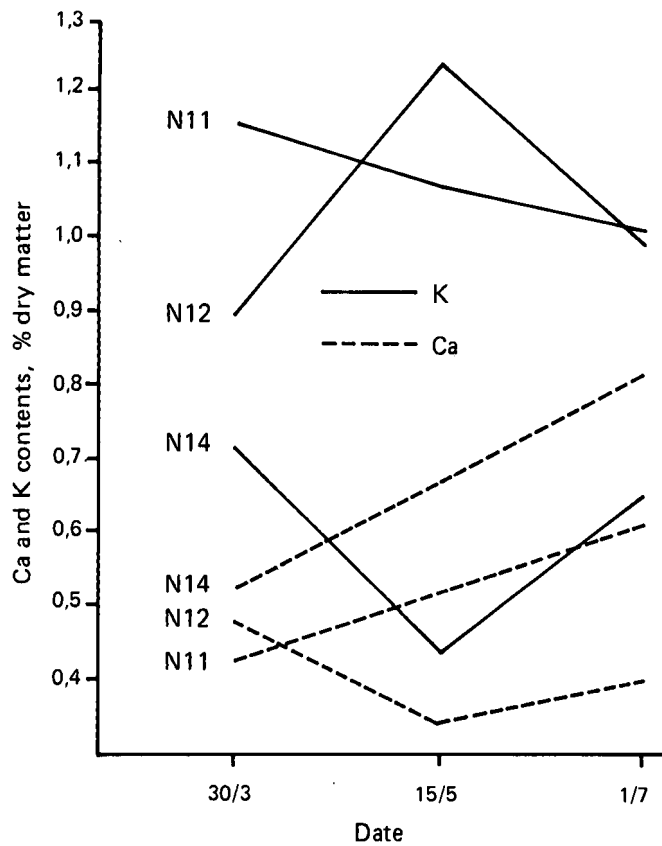


FIGURE 3 Influence of sampling date on leaf 3 calcium (Ca) and potassium (K) contents for N11, N12 and N14.

Experiment 3

Pooled leaf segment extracts of N11 were purified by ion exchange chromatography (Lazarus¹³) for unstressed (distilled water) and stressed (-3,7 MPa PEG) treatments. Leaf 3 was used, and the sampling date was 15 May 1988. The purified amino acids were then derivatized with phenylisothiocyanate to produce phenylthiocarbonyl amino acids. These derivatized amino acids were separated and quantified by reversed phase high performance liquid chromatography with ultraviolet detection at 246 nm (Figure 4). The individual amino acids can be identified by their retention times given in Tables 1(a) and (b) for the chromatograms 1(a) and (b) respectively of Figure 4.

Table 1(a)

Composition of the free amino acid pools of unstressed and stressed N11 leaf segments (15/5/88)

(a) Unstressed (48 hours in distilled water)				
Peak #	Name	Retention	% Area	umoles/gfw
1	Asp/Aspgn	150,8	67,4634	11,75
2	Glu/Glutamn	158,8	8,0416	1,15
3	Serine	226,0	8,2663	1,38
4	Glycine	239,6	2,3247	0,37
5	Histidine	257,6	0,7565	0,18
6	Threonine	313,6	0,6529	0,12
7	Alanine	328,0	7,3137	1,27
8	Proline	338,8	0,4371	0,06
9	Tyrosine	478,0	0,8619	0,16
10	Valine	524,4	0,9543	0,16
11	Isoleucine	634,8	0,7919	0,14
12	Leucine	648,0	0,6880	0,12
13	Phenylalanine	704,4	0,5574	0,09
14	Lysine	752,2	0,8905	0,09
Total				17,04

Table 1(b)

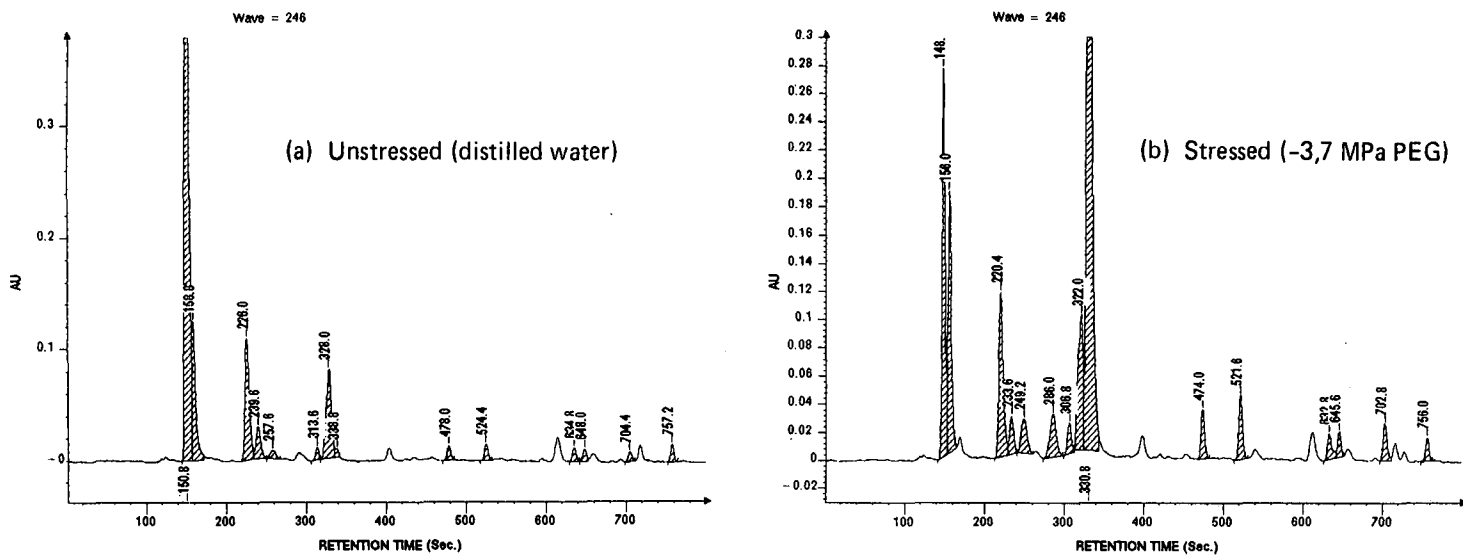
(b) Stressed (48 hours in -3,7 MPa P.E.G.)

Peak #	Name	Retention	% Area	umoles/gfw
1	Asp/Aspgn	148,4	11,8207	3,04
2	Glu/Glutamn	156,0	8,7469	1,84
3	Serine	220,4	7,5486	1,85
4	Glycine	233,6	1,6974	0,40
5	Histidine	249,2	2,2265	0,77
6	Arginine	286,0	2,8943	0,77
7	Threonine	306,8	1,2354	0,35
8	Alanine	322,0	7,0394	1,81
9	Proline	330,8	47,6962	9,86
10	Tyrosine	474,0	1,9297	0,52
11	Valine	521,6	2,5221	0,60
12	Isoleucine	632,8	1,2368	0,32
13	Leucine	645,6	0,9605	0,24
14	Phenylalanine	702,8	1,6222	0,40
15	Lysine	756,0	0,8232	0,12
Total				22,89

In the stressed treatment total free amino acids increased slightly. Asparagine/aspartate decreased greatly whilst proline increased from less than 0,5% of residues in the unstressed treatment to 48% of residues in the stressed treatment.

Experiment 4

Leaf segments of N11 and N14 (leaf 3) were tested for their ability to produce the enzyme nitrate reductase in the presence of -3,7 MPa PEG. Potassium nitrate was added to PEG and distilled water treatments at a concentration of 50 mM. In the case of the potassium nitrate treatments segments were vacuum infiltrated with a 50 mM solution in water immediately before being placed on PEG plus nitrate or distilled water plus nitrate. The PEG only treatment was vacuum infiltrated with distilled water. In vivo nitrate reductase activity was determined at 18, 24, 42 and 48 hours by the method of Stewart *et al*²⁵.



AU scales are in proportion to sample masses.

FIGURES 4a and 4b HPLC chromatograms of the free amino acids obtained by extraction from leaf segments of N11 (15/5/88).

Results

It seems that at least up to 18 hours incubation, PEG does not reduce the level of in vivo nitrate reductase activity that appears in the presence of potassium nitrate (Figure 5). Hsiao *et al*⁹ indicated that protein synthesis and nitrate reductase activity are very sensitive to stress.

Experiment 5

Several commercial varieties were tested for ability to produce proline. Methods were those used in Experiment 2. The sampling date was 1 July 1988 and only leaf 3 was used.

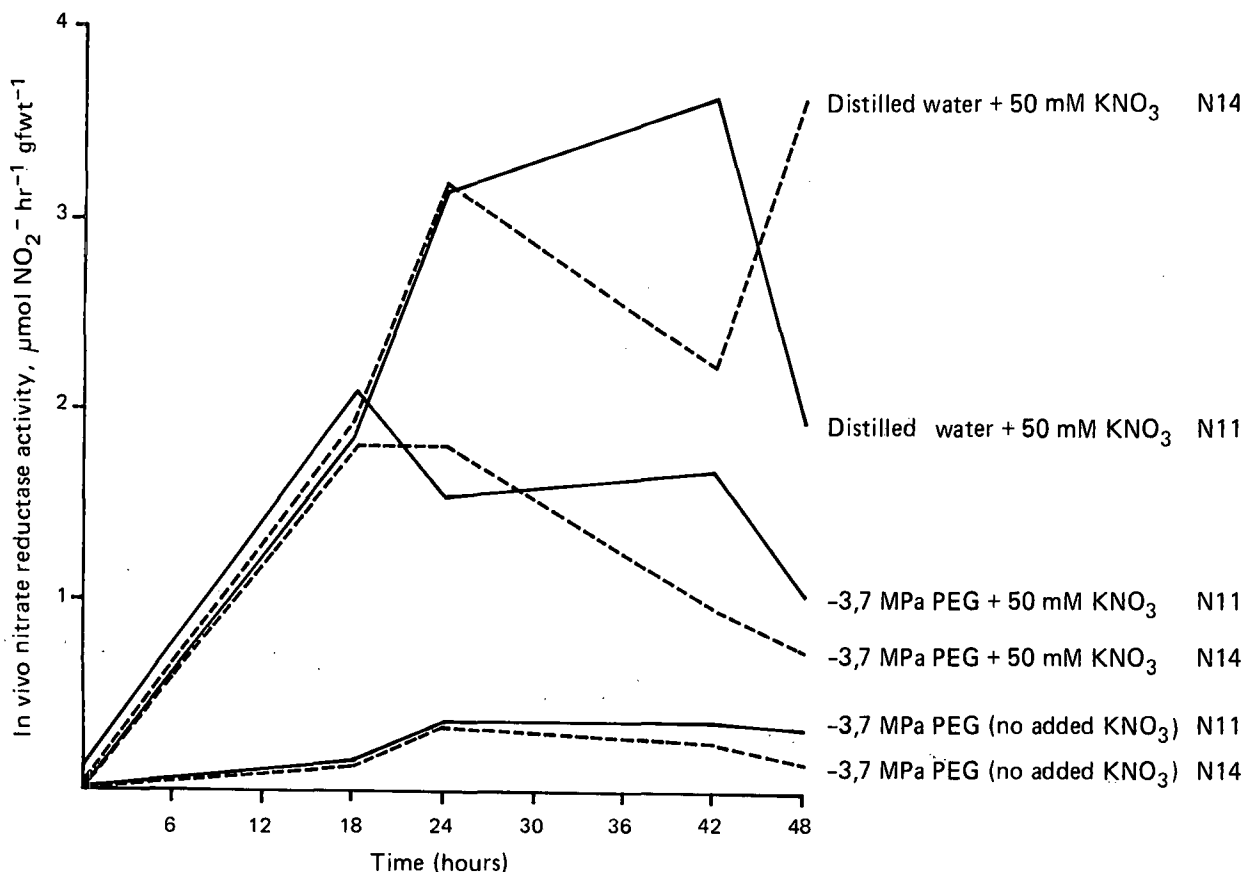


FIGURE 5 (Experiment 4) Effect of duration of floating on -3,7 MPa PEG upon in vivo nitrate reductase activity in N14 and N11 leaf segments (15/5/88).

Results

Table 2

(Experiment 4) Calcium (Ca) and Potassium (K) contents (% of dry matter) of leaf samples and levels of proline accumulation and chlorophyll retention in PEG (-3,7 MPa) stressed leaf segments (1/7).

Variety	% Ca/dm	% K/dm	Rolling Index	Proline ug/gfw	Chlorophyll Retention %
N14	0,82	0,65	0,99	202	55
N13	0,62	0,63	0,94	373	—
N11	0,61	1,10	0,68	429	27
N8	0,60	0,69	0,62	348	—
NCo376	0,58	0,82	0,81	308	—
N18	0,58	0,99	0,89	290	—
N7	0,51	0,94	0,54	220	—
NCo293	0,48	0,73	0,47	287	—
N17	0,46	0,85	0,83	187	—
N12	0,40	1,00	0,41	100	48
N16	0,40	1,06	0,42	184	—

Although N11 still accumulated the most proline the quantity produced was much less than in May and March (Table 2). The leaves of N14 and N11 did not roll as much as they did in May. Lack of leaf rolling was generally associated with high calcium content (N11 and N8 were exceptions). Possibly N13 and N8 would have produced more proline if they had been tested in March.

Discussion

It is interesting that a PEG potential of -3,7 MPa is necessary to maximise 48 hour proline accumulation. Inman-Bamber¹² states that full rolling of leaf 1 occurs at around -2,0 MPa. This did not occur in PEG at -3,7 MPa for N11 (Figure 2) which seems to indicate that osmotic adjustment took place. It is possible that leaf segments take some time to equilibrate with PEG solutions and that any osmotic adjustment takes place as leaf water potentials fall towards that of the external solution. In addition, continued photosynthesis with lack of translocation of assimilate out of leaf segments might decrease the leaf osmotic potential, so that a bathing solution of much lower potential than might be expected is necessary to induce full rolling and proline accumulation. It can be seen from the rolling data in Figures 1 and 2 and Table 2 that -3,7 MPa PEG caused less rolling with increasing crop age. This is compatible with the increasing osmotic potential with increasing age found by Inman-Bamber¹². A progressively lower PEG potential would be required to cause the decline in turgor necessary for rolling and probably also for proline accumulation.

From the data of Experiment 4 it appears that -3,7 MPa PEG does not greatly affect protein synthesis or nitrate reductase activity until some time into the incubation period. This supports the above suggestion that time is needed for the water potential of the leaf segments to fall towards that of the PEG solution.

In Experiment 2 it is apparent that proline accumulation in N14 is much increased by the addition of potassium chloride whilst senescence is reduced. This is compatible with a "new assimilate" origin for this proline rather than one derived from senescence. The added potassium probably improved photosynthesis in stressed N14 segments. Berkowitz and Christa³ showed that potassium deficient leaves suffered non-stomatal inhibition of photosynthesis during stress. Low potassium levels in N14 could have consequences for the ripening of this variety during drying off under irrigated conditions.

It appears that in Experiment 2 N11 retained the most chlorophyll and produced the most proline. This is also consistent with a large part of this proline being produced from new assimilate (Figure 2). In Experiment 4 however, N11 leaf 3 showed much reduced proline accumulation and chlorophyll retention when compared with N14 and N12, although it still produced the most proline (Table 2). It is likely that much of this proline was derived from senescence.

Obviously, in the absence of appreciable senescence proline can only accumulate by synthesis from new assimilate or by increasing its proportion of the free amino acid pool. In leaf segments there is no continuous supply of external nitrate or amino acids necessary for an increase in total amino compounds. It therefore follows that only the ability of varieties to repartition nitrogen into proline from a variety of sources (stored nitrate, protein, free amino acids) has been tested. The floating of leaf segments on PEG solutions is equivalent to cutting off the xylem flow of nutrients to the leaves, a process which is supposedly maintained by osmotic adjustment in intact plants. Stress does not develop in this way in the field (Smirnoff *et al*²²). However an increase in proline due to repartitioning of nitrogen undoubtedly is one of the mechanisms leading to proline accumulation.

Results from Experiment 3 suggests that there is considerable repartitioning of nitrogen into proline by N11 (Figure 4), particularly from the amide asparagine. This seems to indicate that stored nitrogen (asparagine and glutamine) was used in the synthesis of proline. Other amino acids, except asparagine/aspartate and glutamine/glutamate, increased on average three times, probably as a consequence of some senescence (Figure 2).

Conclusions

Variation in proline accumulation by leaf segments is present. Whether or not some varieties osmoregulate in the field more than others remains to be determined. In a pot trial Inman-Bamber¹² found no difference in osmoregulation between N11 and NCo376.

Although screening for proline accumulation could be rapid there are several confusing aspects such as the contribution of senescence and declining proline production with plant age. Rao and Asokan¹⁹ also reported declining proline accumulation with age in the field. Further research on proline accumulation and its contribution to osmoregulation, if any, in sugarcane is necessary.

There is lack of evidence that selecting for physiological traits will result in improved yield or adaption to stress. The only conclusive way of doing so is to select for the traits in question and to test the results. Selections could be made from the large number of early stage clones where variation is greatest.

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