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STRESS EFFECTS
IN ALFALFA (*Medicago sativa*)
SEEDLINGS IN RELATION TO PROLINE AND BETAINES

by

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To my parents whose inspiration made this possible.

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SUMMARY

1. Betaines from alfalfa plant samples were separated successfully on thin layer chromatography with an initial purification on a strongly acid cation exchange resin column. The individual Betaines in alfalfa plant samples were identified on ^{1}H and ^{13}C NMR spectroscopy. The presence of stachydrine, homostachydrine and trigonelline was confirmed with these studies.

2. Water stress increased the proline content in all the organs of alfalfa. Stachydrine and trigonelline were also increased in all the organs. When the stress was relieved proline and stachydrine decreased by 14 hours, but trigonelline did not decrease. The level of stress was monitored by measuring the plant water potential.

3. NaCl stress increased the proline content in all the organs in alfalfa. It also increased stachydrine and trigonelline contents in all the organs. The increase of these compatible solutes was proportional to the concentration of the NaCl applied. The levels of stress was indicated by the plant water potential. As in water stress, the proline and stachydrine content decreased after the relief of stress. Trigonelline, however, did not decrease within 24 hours.

4. Alfalfa seedlings when subjected to cold stress ($5^{o}C$) increased in proline, stachydrine and trigonelline contents in all the organs tested. This increase was not related to water status of the plant as there was no change in the tissue water potential. However, when the plants were relieved of cold stress the proline content decreased within 24 hours. Stachydrine and trigonelline did not decrease after stress relief.

5. Under PEG stress proline, stachydrine and trigonelline increased in all the organs of the alfalfa seedlings. This was proportional to the level of stress indicated by water potential. Like in other stress treatments when the stress was relieved, proline and stachydrine content decreased. Trigonelline, however, continued to increase even after the stress relief.

6. In alfalfa shoots in addition to proline, stachydrine and trigonelline there was an increase in abscisic acid under water, NaCl and cold stress. This may probably be the factor which triggers the accumulation of all these compatible solutes. This was shown by the increase of proline, stachydrine and trigonelline in plants when abscisic acid was applied exogenously through roots.

7. Nodules proved to be one of the important factors responsible for the accumulation of compatible solutes in alfalfa. Nodulated alfalfa plants accumulated more proline, stachydrine and trigonelline under water and NaCl stress as against the non-nodulated plants. This was also supported by the presence of large amounts of proline, stachydrine and trigonelline in alfalfa root nodules.

8. It was evident that stachydrine was synthesised from proline under both non-stress and stress in alfalfa seedlings. This may be mediated by the co-factors like methionine, pyridoxine and folic acid. The synthesis of trigonelline, however, is either, on a different pathway possibly with both ornithine and proline.

9. The pre-treatment of proline, stachydrine, and glycine betaine protected the photofunction of alfalfa leaves when subjected to stress under strong light. This was showed with the retention of variable fluorescence (Fv) and quench level (Ql) under stress with these pre-treatments.

DECLARATION

The investigation described in this thesis were performed in the Department of Plant Physiology, Waite Agricultural Research Institute, University of Adelaide.

To the best of my knowledge and belief, this thesis contain no material previously submitted for a degree or diploma in any university, except where due reference is made in the text.

G. PARAMESHWARA

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CHAPTER I

GENERAL INTRODUCTION

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CHAPTER I

GENERAL INTRODUCTION

Environmental effects and the resistance to them offered by plants is a complex phenomenon, in that the physiology and biochemistry of plants are quickly and extensively altered by these stresses. Accumulation of compatible solutes such as proline and betaines has been studied in several crop species in response to different environmental conditions. Proline accumulation, particularly, has occupied a special position in plant physiology research during recent times. It accumulates in response to water stress (Barnett and Naylor, 1966; Singh et al, 1973b; Stewart and Lee, 1974; Quarrie, 1980; Jan and Halloram, 1982; Singh and Gupta, 1983; Karmanos et al, 1983); salinity stress (Palfi and Jahasz, 1970; Buhl and Stewart, 1983, Dreier, 1983a); and cold stress (Pauli and Mitchell, 1960; Wilding et al, 1960; Draper, 1972; Thompson and Zalic, 1974; Stefl et al, 1978; Chu et al, 1978; Vegina and Paquin, 1982). Inspite of this abundant evidence and the correlation between proline accumulation and the water status of the tissue, the mechanism of accumulation is still not conclusively understood. Experiments suggests that proline accumulates as a result of loss of feedback inhibition under stress (Noguchi et al, 1968; Boggess et al, 1976a); inhibition of proline oxidation (Stewart et al, 1977; Stewart and Boggess, 1978); enhanced synthesis from glutamate (Stewart, 1981). Abscisic acid, which also accumulates under stress in plants, has been suggested as the 'factor' initiating these metabolic inhibitions (Chrispeels and Varner, 1967: Aspinall et al, 1973; Stewart, 1981; McDonnell et al, 1983) and, finally, increasing the proline levels.

Accumulation of betaines also is now realized as being physiologically important in assisting plants in withstanding adverse conditions and among these, glycine betaine is probably the only compound which has drawn much attention (Storey, 1976; Storey et al, 1977, 1978a, b; Wyn Jones and Storey, 1981; Hanson et al, 1978; Hanson and Scott, 1980). Inspite of the interest shown in the physiology and biochemistry of proline accumulation, the betaines of proline, and other betaines, like trigonelline, have received little attention. Stachydrine (proline-betaine) is known to occur in several plant species (Steenbock, 1918; Vickery, 1924; Weihler and Marion, 1958; Robertson and Marion, 1959a,b,c, 1960; Conner et al, 1973; Sethi and Carew, 1974), but changes in its levels, or it's biosynthesis under stress is not known. Similarly, trigonelline (nicotinic acid-betaine) is widely distributed in many species (Klein et al, 1931; Blake, 1954; WynJones and Storey, 1981) and also has not been studied much in relation to stressful environments.

Accumulation of either proline or betaines (glycine betaine) has been related to adaptive mechanisms. They have been reported to be the osmoregulators (Storey and WynJones, 1977, 1978a, b; Jagels, 1983; Jolivet et al, 1983); enzyme protectors (Paleg et al, 1981; Nash et al, 1981); membrane protectors (Jolivet et al, 1982, 1983); and as nitrogen sources following alleviation of stress (Barnett and Naylor 1966; Larher et al, 1982). However, the concept that the accumulation of these solutes is an adaptive response has been disputed (Hanson et al, 1977 and Stewart and Hanson, 1980; Hanson and Nelson, 1978).

Alfalfa, (*Medicago sativa*) is one of the most widely grown legumes in the world. It provides an economical source of protein and other

nutrients to all classes of livestock. It is also known to contain proline (Wilding et al, 1960; Vezina and Paquim, 1982), stachydrine (Vickery, 1924; Robertson and Marion, 1959a,b,c, Weihler and Marion, 1958), trigonelline (Robertson and Marion, 1959a) and homostachydrine (Robertson and Marion, 1959c). The mechanisms of accumulation and biosynthesis, and the physiological and biochemical significance of these compounds, especially under stressful conditions, is not known. A better understanding of the physiology and biochemistry of resistance would not only aid in the improvement and maintenance of this crop but also be of importance to other economically important crop species. This thesis is an attempt to explore the issues of accumulation, biosynthesis and significance of proline, stachydrine, homostachydrine and trigonelline in alfalfa under different environmental conditions.

CHAPTER II

LITERATURE REVIEW

CHAPTER II

LITERATURE REVIEW

1. Plant-water relations under a stress environments

When plants of most species are stressed, a common response is the reduction in the water status of the tissue. This decrease in water status represented by a decline in water potential, may be due primarily to a decrease in the osmotic potential of the cell or to a decrease in the turgor pressure or, more commonly, to a combination of both. This has been well established by Chu et al, (1974).

The state of water in a plant leaf cell under equilibrium conditions may be written in terms of the various components of the potential energy.

$$\psi = \psi\Pi + \psi p + \psi m$$

where ψ is the total water potential, $\psi\Pi$ is the osmotic potential component, ψp is the pressure (Turgor) potential component and ψm is the component due to absorption forces. Partitioning of the energy between osmotic and absorption components is somewhat arbitrary since a proportion of the water in the leaf tissue will be subjected to both osmotic and absorption forces, particularly at low-leaf water contents (Gardner and Ehlig, 1965). In the vacuole the osmotic component will predominate and ψm is usually neglected as a consequence: ψ of the

leaf tissue is expressed as simply the sum of osmotic and pressure potentials:

$$\psi = \psi\Pi + \psi p$$

With initially fully turgid tissue, a decrease in tissue water content causes a large decrease in ψ. In this phase of dehydration, decreases in ψp are usually much more marked than decreases in $\psi\Pi$ and account for the major part of the dimunition in ψ. After more water is lost, and ψp falls to a negligible level, decreases in $\psi\Pi$ alone account for most of the further decreases in ψ (Gardner and Ehlig, 1965). It has been generally assumed (Slatyer, 1967) that the wilting point corresponds to zero turgor potential in leaves, when $\psi = \psi\Pi$. The first value of water potential at which $\psi = \psi\Pi$ (when $\psi p = 0$) varies from species to species, -32 bars for barley (Miller et al, 1970), -19 bars for cotton and -22.4 bars for sunflower (Gardner and Ehlig, 1965). However, it has been reported that the value of ψp is always greater than zero at the first sign of wilting; for dicotyledons, visible wilting symptoms have been reported to occur at +2 or +3 bars ψp (Gardner and Ehler, 1965) and for rice plant it was found to be approximately +3 bars (Tomor and Ghildayal, 1973). In this case it was associated with a marked change in the elastic property of leaf tissue. Because of the methodological difficulties, matric effects are least well understood. While absolute values may be questionable it appears reasonable to conclude that matric potential becomes increasingly important as water potential decreases (Boyer, 1967; Roberts and Knoerr, 1977; Shepherd, 1975).

Under a saline environment the deleterious effects of salinity are due to the decreased $\psi\Pi$ of the surrounding medium and to the fact that

the plants are exposed to a physiologicl drought (Bernstein and Hayward, 1958). However, it is now well established that when the osmotic potential of the growing medium around plant roots decreases, the ψ_π of the plant tissue also decreases proportionately (Slatyer, 1961; Cooper and Dumbroff, 1972, Storey and Wyn Jones, 1975, 1977) thereby causing the decrease in the total water potential.

Under low temperatures, the loss of water from the leaves may be the indirect result of root cooling or the direct outcome of the chilling of the leaves. Kleinendorst and Brouwer, (1972) reported that cooling the root medium reduced the water potential of maize plants by a primary effect on the water permeability of the root tissue. Such a possibility of reduction in total water potential during cold stress was ruled out in barley and raddish (Chu et al, 1974). However, the effects of chilling on membrane permeability (Lyones, 1973) have been measured in response to chilling and increased permeability has been related to greater water loss from the cells which may lead to a situation in which symptoms of drought would occur (Wright and Simon, 1973).

Correlation between water potential and the relative water content in examining the tissue water relations has provided considerable evidence (Boyer, 1969; Richter, 1978; Tyree and Hammel, 1972). Various authors have determined that as tissue matures the relative water content becomes higher at a given leaf water potential or at zero turgor (Kassam and Elston, 1974, 1976; Knipling, 1967; Robersts and Knoerr, 1977).

The methodologies of measuring the plant water potentials and its components have been modified in recent times and corrected for possible

errors (Turner, 1980, 1981a, 1981b). This may help in better understanding of the water relations of the plants. In brief it can be said that the changes in the water potential of the tissue may be brought about by different environments and this has a pronounced effect on the metabolic process, some of which have been studied in this work.

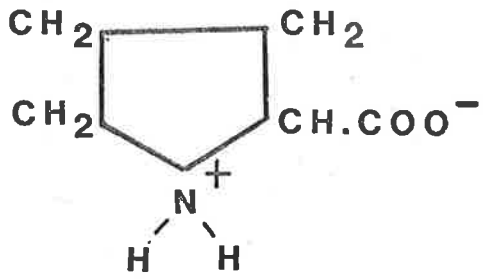
2. Betaines: Distribution, synthesis and response to environmental stress

Betaines form a natural group of simple nitrogenous compounds in which the nitrogen is fully methylated. They are widely distributed in plants and occur most abundantly in those parts of the plant in which the vegetative processes are most active. Of the wide variety of betaines found in plants, glycinebetaine was the first to be isolated and has been subjected to the closest scrutiny. The distribution and response to environmental stress of glycinebetaine in several species have been reviewed recently (Wyn Jones and Storey, 1981). In relevance to the present study the literature review is concentrated on stachydrine, homostachydrine and trigonelline.

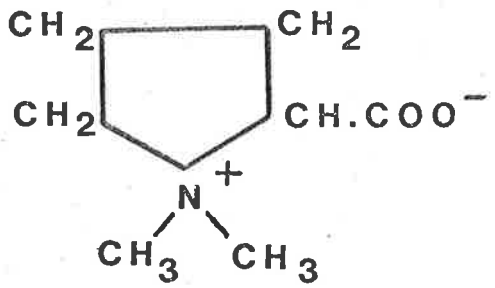
Distribution

Stachydrine, (Figure - 1B) an anhydride of methylated proline, occurs in several plant species. It has been reported to be present in chrysanthemum (Yoshimura, 1913), various citrus species (Guggenheim, 1958), and the fruits of *Courbonia virgata* and *Capparis tomentosa* (Cornforth and Henry, 1952). Some of the plants with pharmaceutical importance have also been reported to contain stachydrine. It was found

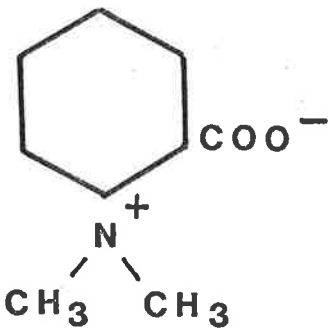
Fig. 1 Structure of Proline, Stachydrine, Homostachydrine and Trigonelline.



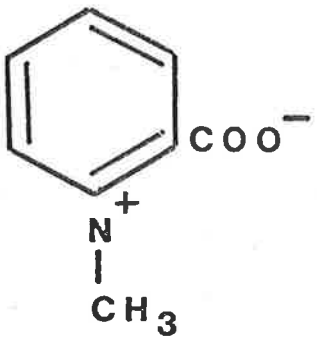
A . PROLINE



B . STACHYDRINE



C . HOMOSTACHYDRINE



D . TRIGONELLINE

in the bark of *Erythrina species* (Singh et al, 1975); *Teucrium polium* (Wassel and Ahmad, 1974); *Onopordon alexambinum* (Wassel, 1975), and in the leaves of *Cadaba fructocosa* (Ahmad and Basha, 1971). Stachydrine was also been reported in marine algae (Boit, 1961; Abe and Kaneda, 1967; Hori, 1979; Blunden, 1983) and in oysters (Yasumoto et al, 1978).

In alfalfa, stachydrine was first reported (Steenbock 1918) as the hydrochloride from the phosphotungstic acid fraction of water soluble constituents of alfalfa hay. Vickery (1924) and Leavenworth et al, (1924) confirmed this finding by reporting large amounts of stachydrine in the juice extracted from the alfalfa plants and, Leete et al, (1955); Robertson and Marion, (1958, 1959); Wheiler and Marion, (1958); Essery et al, (1962) all studied it's biogenesis in alfalfa seedlings. Connor et al, (1973) identified stachydrine in alfalfa as the major quaternary ammonium nitrogen base and correlated it to the protein content. Sethi and Carew (1974) found stachydrine in static and suspension tissue cultures of alfalfa. Recently stchydrine has been reported in alfalfa shoots (Wyn Jones and Storey, 1981), in roots, and in nodules (Le Rudulier et al, 1982).

Homostachydrine (Figure - 1C), a methyl betaine of pipecolic acid has also been reported in alfalfa seeds and seedlings, (Weihler and Marion, 1958; Robertson and Marion, 1959). There is not much evidence of the biological importance of homostachydrine in plants.

Trigonelline (Figure - 1D), a betaine derived from nicotinic acid, was first discovered in Fenugreek. Klein et al, (1931) found trigonelline to be widely distributed in many plant families. *Pisum sativum* has been reported to contain large amounts of trigonelline.

(Arnoff, 1956; Evans et al, 1979; Evans and Tramontano, 1981; Tramontano et al,1982, 1983). It was found in several pharmaceutically important plant species (Ogata and Kamoda, 1943; Goshal et al, 1972). Some marine animal species also contain trigonelline (Ackerman and List, 1958; Beers, 1967; Hayashi and Konosu, 1977). Seeds of several plant species have been reported to contain trigonelline. (Blaim, 1962; Pollock and Stevans, 1965). Recently, Wyn Jones and Storey (1981) found trigonelline in several plant species under different growing conditions. In alfalfa, however, trigonelline was reported in seeds and plants at various ages (Robertson and Marion, 1959; Wyn Jones and Storey, 1981).

Biosynthesis

The presence of these betaines both in plant and animal species encouraged scientists to study their synthesis.

Although several studies have been reported, the biosynthesis of stachydrine in plants is still not clearly understood. Klein and Linser (1932) showed a possible synthetic route of stachydrine in *Trigonella foenum graceum* seedlings by injecting proline. Since then Weihler and Marion (1958); Morgan and Marion, 1956; Robertson and Marion (1959a,b,c, 1960); and Essery et al, (1962) have reported several studies of stachydrine synthesis in alfalfa. They proposed a pathway involving a sequential methylation of proline. Later, Robertson and Marion (1960) showed the conversion of N-Methyl proline (Hygric acid) to stachydrine but were unable to show the conversion of proline itself to stachydrine unless there were cofactors present such as methionine, pyridoxine and folic acid. This has led to the still unsolved question of whether the

synthesis is a simple sequential methylation, or whether there are any other enzymatic reactions involved.

There is no direct report on the biosynthetic pathway of homostachydrine in plants. However, it has been suggested (Weihler and Marion, 1958), that homostachydrine is formed by ring closure of lysine to form pipecolic acid, which is then methylated. Lysine is known to be the precursor of pipecolic acid (Fujii and Miyoshi, 1975; Norbest and Leistener, 1981). The presence of pipecolic acid in alfalfa has yet to be demonstrated, but in view of it's ocurrence in other legumes (Lowey, 1953; Mester, et al, 1979), and in maize (Palfi and Pinter, 1980), it would not be surprising if it were found as a non-protein nitrogen constituent of alfalfa.

Joshi and Handler (1960) showed trigonelline synthesis in peas preparations by methyl transfer from S-adenosylmethionine to nicotinic acid. They also reported the metabolism of trigonelline in *Torula cremoris* and young pea plants (Joshi and Handler, 1962). In Fenugreek, Klein and Linser (1932) found a large increase of trigonelline after feeding proline through the stem. In corn seedlings feeding nicotinic acid increased trigonelline content (Blake, 1954). In alfalfa, though trigonelline has been reported (Robertson and Marion, 1959, 1960), it's biosynthesis is not known.

Response to stress

Recent reviews by Wyn Jones (1980) and Wyn Jones and Storey (1981) provide considerable information about glycinebetaine in response to environmental stress. However, very little information is available for

other betaines in relation to stress. Stachydrine has been reported to increase as a result of NaCl stress (Wyn Jones and Storey, 1981). The presence of high amounts of stachydrine in species grown in dry habitats (Walter, 1971) and coastal regions (Le Rudulier, 1982) may also suggest an adaptive response to stress environments. Although trigonelline has been tested for it's response to NaCl (Wyn Jones and Storey, 1981) no significant accumulation was found. This dearth of information suggests the need for further investigations regarding the Betaines which accumulate under environmental stress.

3. Proline: accumulation, location, and metabolism during environmental stress

Water Stress

Accumulation of proline in various organisms is initiated by a reduction in the water status of the tissue or cells. Proline has been shown to accumulate to a concentration as high as 10% of the dry weight of the tissue (Stewart and Lee, 1974) under stress conditions. Proline accumulation occurs not only in mesophytes but also in halophytes (Stewart and Lee, 1974; Storey et al, 1975, 1977), resurrection plants (Tymms and Gaff, 1979), and lower plants (Schobert, 1977; Brown and Hellebust, 1978; Setter and Greenway, 1979). In higher plants proline accumulation has been found in a number of different species. Routley (1966) reported accumulation of proline in Ladino clover leaves when they lost 15% of the water content. A 10-100 fold increase in proline content was demonstrated in stressed *Cynodon dactylon* shoots by Barnett and Naylor (1966). Similar proline accumulation following reduced moisture content in intact plants has been reported for; tissue include

wheat (Rajagopal et al, 1977; Bengston et al, 1978; Munns et al, 1979; Quarie, 1980; Jan and Halloram, 1982, Karmanos et al, 1983), maize (Carcellar and Fraschina, 1980). Sorghum (Blum and Ebercon, 1976; Parameshwara and Sastry, 1980), barley (Saviskaya, 1967; Singh et al, 1973a; Chu et al, 1974; Ayer, 1981), rice (Mali and Mehta, 1976b), raddish (Chu, 1974), and soyabean (Waldren et al, 1974; Singh and Gupta, 1983). A recent review by Aspinall and Paleg (1981) has catalogued more than fifty species of plants from fourteen families in which proline accumulated as a result of water deficit.

The addition of polyethylene glycol (PEG) to the root medium simulates a water stress condition by lowering the water potential, and also produces an increase in free proline in intact plants. Singh et al, (1973a) have shown a linear increase, over several days, in proline concentration up to 15 mg g^{-1} D.W. in intact barley plants in response to -20 bars PEG; and experiments with intact *Pennystum typhoides* plants showed a five fold increase in proline concentration when stressed with -20 bars PEG for 24 hours (Huber and Schmidt, 1978). Similar results have been observed with PEG (Lawlor, 1970; Aiyer, 1981; Sanchez-Diaz et al, 1982; Spyropoulos, 1983). Water stressing excised tissue also results in proline accumulation, in turnip (Thompson et al, 1966) bermuda grass (Barnett and Naylor, 1966), wheat (Tyankova, 1967), capsicum (Palfi et al, 1974).

Salinity stress

Accumulation of proline in plants subjected to saline environment has been reported by Palfi and Jahasz (1970). Similar increases in proline, as a result of salt, has been found in barley (Chu et al,

1976a; Storey and Wyn Jones, 1978a,b; Buhl and Stewart, 1983), in wheat (Dreier, 1983a,b). A substantial increase was also found in several halophytes (Storey and Wyn Jones, 1978a,b). Aspinall and Paleg (1981), in their recent review, reported several plant species which accumulate proline as a result of salt treatment. A parallel has been found between the decrease in leaf water potential and proline accumulation in intact plants subjected to an increasing salinity in the root environment (Chu et al, 1976a).

Low temperature or cold stress

Increases in amino acids as a response to low temperature has been reported in several crop species; wheat (Pauli and Mitchell, 1960), alfalfa (Wilding et al, 1960; Peoples and Koch, 1978), rye (Thompson and Zalic, 1974) and lolimum (Draper, 1972). Proline was invariably the main contributor to this increased amino acid pool. In alfalfa crowns and roots a proline increase was found when exposed to 1.5^{o}C for two weeks (Vezina and Paquin, 1982). Accumulation of proline in citrus leaves was reported during cold hardening (Yelenosky, 1979). In wheat, proline increased 10 fold in a winter and 4 fold in a spring variety (Trione et al, 1967). Several authors have reported proline accumulation as a result of low temperature or cold stress (Palfi and Juhasz, 1970; Gates et al, 1971; Chu et al, 1978; Ozturk and Szaniwsk, 1981; Vezina and Paquin, 1982). In contrast to the situations in water stress or saline stress, the proline accumulation under cold stress cannot be ascribed to a change in the water potential of the tissue (Chu, 1974).

The source of accumulated proline may be the increased proteolytic activity or the decreased rate of protein synthesis from the amino acid

pool. Such a decreased rate of protein synthesis at chilling temperatures (10°C) has been reported (Rodchenko and Maricheva, 1973). A close correlation has been demonstrated between the accumulation of free proline and tissue resistance to chilling temperatures (Bokarev and Ivanova, 1971) which has also been suggested to depend upon the degree of hydration of the protoplast.

Location and the metabolism of proline

The subcellular location of proline in leaf cells has still not been defined. However, the available evidence indicates a cytoplasmic localisation. Goring et al, (1977), analysing proline distribution in salt stressed maize roots, found that proline accumulation occurred only in the root and tip region where there is little or no vacuolation. Recent studies by Leigh et al, (1981) showed the compartmentation of proline in both vacuoles and cytoplasm, however, the amount of proline was higher in the cytoplasm. Ths may be due to leakage from the cytoplasm to vacuole, but the authors discounted this as there was no relationship between the size of the vacuolar and cytoplasmic pools. They suggested the active and reversible movement across the tonoplast in response to the osmotic stress experienced by the cell. Work by Wyn Jones et al, (1977a) clearly excluded the vacuole as a possible site of proline accumulation.

Since the early studies of proline accumulation in plants (eg. Barnett and Naylor, 1966; Stewart et al, 1966; Thompson et al, 1966), it has been known that most of the proline accumulated under stress is synthesised de novo. The majority of the proline accumulated is believed to be derived from glutamate via the intermediate Δ^1-Pyrroline-5-

Carboxylic acid. However, arginine and ornithine can also be converted to proline in plant tissue (Mazelis and Fowden, 1969; Splittstoesser, 1969; Stewart, 1974). Arginine can be converted to ornithinine by hydrolysis catalysed by arginase, producing urea as the end product. Ornithine can be converted to proline by two possible routes, both involving transamination of ornithine, followed by Cyclization and reduction. The pathways from arginine and ornithine may be as important as the glutamate pathway in some species under stress. It has been suggested that proline synthesis occurs in the cytoplasm and chloroplasts. This view is supported by the distribution of Δ^1-Pyrroline-5-Carboxylate reductase in the cytoplasm and chloroplasts, but not in mitochondria (Noguchi et al, 1966, 1968; Stewart and Lee, 1974). Under non-stressed conditions proline synthesis appears to be under the control of feedback inhibition (Noguchi et al, 1968; Boggess et al, 1976b). This inhibition is believed to involve the formation of Δ^1-Pyrroline-5-carboxylate rather than it's conversion to proline. However, this control is believed to be lost in stressed tissue, resulting in high levels of proline.

Plants have the capability of oxidising proline (Barnard and Oaks, 1970; Oaks et al, 1970; Stewart, 1972a; Wang, 1968; Rena and Splittstoesser, 1974c). This oxidation results in carbon being fed into the Kreb's cycle and eventually respired to CO_2 (Stewart, 1972c). In stressed plants this oxidation is believed to be inhibited (Stewart et al, 1977; Stewart and Boggess, 1978) thereby contributing to the increase in the proline pool.

It has been well established that the incorporation of proline into protein is inhibited by stress (Stewart 1972c; Stewart et al, 1977). The

possibility that proline accumulation is a consequence of impaired protein synthesis has been examined by Boggess and Stewart (1980). Based on experiments with inhibitors of protein synthesis, they concluded that inhibition of protein synthesis was not sufficient to cause proline to accumulate. However, since protein synthesis accounts for the major part of proline utilisation in turgid tissue, it's impairment contributes to proline accumulation by slowing down the rate of proline utilisation (Stewart, 1972c).

Proline accumulation under stress can also be mimicked by the application of ABA. Aspinall et al, (1973) induced proline accumulation in both intact and excised leves of turgid barley by supplying ABA exogenously. More evidence of this nature is reported in Section 4. The mechanism of ABA action on proline accumulation has been suggested; inhibition of protein synthesis (Chrispeels and Varner, 1967; Aspinall et al, 1973), inhibition of the oxidation of proline (Stewart, 1980) and stimulation of the enzyme P-5-C reductase (Huber, 1974). As ABA did not increase proline levels in tobacco (Aspinall, 1980) or sunflower (Wample and Bewley, 1975), the ABA effect on proline cannot be considered as universal.

On alleviation of stress accumulated proline disappears rapidly from the tissue. This has been related to the oxidation of proline to glutamic acid (Stewart, 1972c; Singh et al, 1973c); Blum and Ebercon, 1976). The time taken for proline levels to drop to non-stressed levels after removal of stress varies with the plant and the severity of the stress (Chu et al, 1974; Greenway and Setter, 1979; Bengston et al, 1978; McMichael and Elmore, 1978; Jager and Mayer, 1977).

4. Accumulation of ABA under environmental stress and it's relationship to proline and betaine

Changes in the levels of endogenous hormones and ABA in particular, have been recorded as a result of environmental stress. Increases in the endogenous levels of ABA as a result of water stress, has been reported in several species. Beardsell and Cohen (1975) observed an increase in ABA in sorghum. Similarly, in barley and lolium (Aspinall et al, 1973), Dougbus-fir (Newville and Ferrell, 1980); maize (Ilahi and Dorffling, 1982); wheat (Quarrie and Jones, 1979; Quarrie, 1980, 1983; Quarie and Lister, 1983), rice (Henson, 1983) and in tomato (Bradford, 1983). ABA has also been known to increase as a result of salinity. Mizhrahi et al, (1971) found increased levels of ABA in tobacco as a result of salinity and relative humidity. Downton and Loveys (1978) found an ABA increase in grape berries as a result of salinity. Excised leaves of various plant species have also been reported to increase ABA levels under salinity (Hartung et al, 1983). Other environmental conditions are known to cause an increase in ABA levels in plants; low temperature or cold stress (Rifkin et al, 1976; Daie and Campbell, 1981; Chen et al, 1983; Eamus and Wilson, 1983), high temperature or heat stress (Itai and Ben-Zioni, 1974), and flooding (Hiron and Wright, 1973). The pathway of ABA synthesis, it's accumulation under stress and some of the possible implications of it's presence have recently been reviewed by Milborrow (1981).

ABA has also been reported to induce the accumulation of glycinebetaine. Huber and Sankla (1980) found increased amounts of glycinebetaine in four and fourteen day old Pennisetum seedlings as a result of ABA and salt treatments. This was further confirmed by the

results of McDonnel et al, (1983) where they found increased amounts of both proline and glycinebetaine in *Pennysteum typhoides*.

5. Plant nodules and their significance under environmental stress

A large portion of total plant nitrogen in legumes is derived from symbiotic N_2 fixation (Heichel, et al, 1981). Sizeable amounts of ammonia are generated in bacteriods as the initial product of nitrogen fixation (Bergerson, 1965). Most of this ammonia is exported from bacteriods into the surrounding nodule plant cell cytoplasm (O'Gara and Shanmugen, 1976), assimilated into organic compounds and transported to the shoots in several forms. In a recent review, Minchin et al, (1981) emphasised the importance of nodules in leguminous plants for nitrogen fixation. They concluded that nodules provided part of the carbon and nitrogen nutrition for the growth of the plant. Most of the biochemical processes concerned with nitrogen metabolism are more active in root nodules than in other parts of the leguminous plants (Henson et al 1982; Patterson et al, 1982).

Drought causes significant changes in nodules and their physiochemical aspects. Sprent (1976) reported that root nodules of soyabean subjected to water stress showed lower nitrogenase activity due to the restriction in the supply of intermediates from aerobic pathways. Other reports on the effects of water stress include the decreased total nodule activity in soyabean (Patterson et al, 1979) and the decreased nitrate reductase activity in alfalfa (Tezo and Sanchez-Diaz, 1982). Accumulation of ABA in the nodulated root system (Watts et al, 1983), the presence of Betenocine in Canavalia ensiforms root nodules, the presence of trigonelline in Pisum sativum root nodules (Mears and Mabry,

1971) and the accumulation of proline and 'onium compounds' in root nodules of alfalfa (Le Rudulier et al, 1982) have also been reported.

A systematic study of the effect of stress on the nodules and nodulated plants, in comparison with non-nodulated plants of the same species is still lacking. A portion of this thesis briefly explores this question and the significance of the nodules to stressed alfalfa seedlings.

6. Physiological significance of Compatible Solutes

The significance of compatible solutes in plants under stress is an important issue. Based on experimental results suggestions have been made that the accumulation of proline and glycine betaine as a result of stress may be an adaptive response to help alleviate the detrimental effects of stress. These suggestions have been questioned by Hanson and co-workers. On the basis of series of experiments with two barley cultivators, they concluded that neither proline acumulation (Hanson et al, 1977; Stewart and Hanson, 1980) nor glycinebetaine accumulation (Hanson and Nelson, 1978) is an adaptive response to stress. The other school, which believes in the adaptive response (eg. Aspinall and Paleg, 1981; Dix and Pearce, 1981; Wyn Jones and Storey, 1981), have disagreed with these conclusions of Hanson and co-workers. They suggested that such solute accumulation may be related to survival, if not growth, of the plants under stress conditions and, therefore, may confer some advantage on the plants. In this, one should not forget that complete protection is not possible and the solutes can only extend the limits of tolerance. This is also clear with the death of plants which accumulate these compatible solutes under severe stress.

Several adaptive roles have been postulated for proline and glycine betaine. The role of osmoregulation has drawn much attention (eg. Hellebust, 1976; Jefferies, 1980; Wyn Jones 1980; Boyer and Meyer, 1980; Wyn Jones and Storey, 1981; Larher et al, 1982; Jolivet et al, 1983; Jagels, 1983). The mechanism of osmoregulation in plants, marine species etc. is extensively discussed in "Genetic Engineering of Osmoregulation" edited by Rains et al, (1980). Proline has also been suggested to maintain cellular integrity by protecting the cell constituents from damage. Heber et al, (1971) reported that freezing chloroplasts from spinach for 4 hours at -25°C inactivated the photophosphorylation of thylakoids by irreversibly altering the essential membrane properties. However, thylakoids frozen in the presence of proline were protected against the damage. Further increases in the concentration of proline in the medium increased photophosphorylation until the thawed samples had the same activity as the unfrozen samples. This increased stability was suggested to be due to the protection of the membranes by proline against the toxic organic and inorganic cellular substances concentrated during stress. Glycine betaine has also been reported to protect the membrane against, heat and oxalate destabilisation (Jolivet et al, 1982, 1983) in beetroot cells. The post-thaw viability and growth of maize cells was increased in the presence of 10% proline (Withers and King, 1979). Not only can proline protect organelle integrity during stress but it has also been reported to protect severalcytoplasmic, mitochondrial and chloroplastic enzymes. Paleg et al, (1981); Nash et al, (1981) found proline induced protection of α-amylase, fumarase, and isocitrate dehydrogenase pyruvate carboxylase (both chloroplastic and mitochondrial) of peas against heat inactivation. With the addition of 0.4M proline solution to the enzyme prior to heating at 50°C. Similar protection was shown against NaCl

inhibition of the enzyme malate dehydrogenase (Pollard and Wyn Jones, 1979).

Other roles have been suggested for proline and betaines. They may be sources of carbon, nitrogen and reducing power for use after the stress is relieved (Barnett and Naylor 1966; Stewart et al, 1966; Singh et al, 1973b). Both proline and glycine betaine may act as sinks for surplus reducing power generated during stress (Hanson and Nelson, 1978). It was found that proline solutions were able to increase the solubility of sparingly soluble proteins (Schobert and Tschesche, 1978), and it was postulated that a hydrophobic interaction of proline with the hydrophobic surface residues of protein, increased the hydrophyllic areas of the complex. The presence of protein-bound-proline in plants (Singh, 1970; Stefl, 1978) may influence the configuration of proteins (Blandts et al, 1977), thus influencing the rate of folding and unfolding of the secondary structure of proteins.

The physiological significance of the other nitrogenous compounds which accumulate under stress, such as stachydrine and trigonelline is also not known.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III : MATERIALS AND METHODS

1. MATERIALS

Alfalfa (*Medicago sativa*) seeds of varieties Hunter River and Paravivo, used throughout the experimental programme, were obtained from the South Australian Department of Agriculture. The chemicals, stachydrine and homostachydrine, were synthesised in the laboratory with standard methods (Cornforth and Henry, 1952a,b). The source of other chemicals and reagents are given where they are first mentioned.

2. GENERAL METHODS

2.1 PLANT CULTURE

2.1.1 POT CULTURE

Seeds were planted in 10 cm. plastic pots and, depending on the nature of the experiment, 5-10 seedlings were retained after an initial thinning soon after emergence. The plants were grown either in fertile soil (Urbrrae loam: Plympton sand (4:1) by volume), or well-washed river sand depending on the nature of the study. The pots were irrigated daily with half strength Hoagland solution (Hoagland and Arnon, 1938) till the treatments were imposed. No nutrient solution was applied to plants grown in soil; they were watered daily until the treatments were imposed.

2.1.2 WATER CULTURE

The plants, after growing in sand and in some cases oil-dry (a non-absorbent support medium) up to a certain period, were transferred into

50 ml glass tubes containing half strength Hoagland's solution. Depending on the nature of the experiment, 3-5 plants were grown in each tube. Aeration was provided to each tube by thin tubing connected to a manifold. In order to hold the plants upright a small amount of soft sponge was placed at the top of each tube.

2.2 ENVIRONMENTAL CONDITIONS

Growth cabinets with controlled environmental conditions were used for growing plants. In all the experiments the photon flux density at the canopy of the plants was maintained at approximately 600 $\mu Em^{-2}s^{-1}$. The light source was a bank of 32/80 watt 'cool white' fluorescent tubes (Philips TLE 80/33) and in some cases sodium lights supplemented with 8 incandescent bulbs (60 watts). In all the experiments plants were grown in a 12 hour photoperiod. The temperature of the cabinet was maintained at 24 $\pm$ 1^{o}C during the day and 21 $\pm$ 1^{o}C during the night.

2.3 HARVESTING THE TISSUE

Plant tissue in all the experiments was harvested immediately at the end of each treatment. Leaves, stem, roots and, in some experiments, nodules were separated with a scalpel. They were quickly frozen with liquid nitrogen and the frozen samples were freeze dried for 24 or 48 hours. The dried samples were then weighed and used for chemical analysis.

2.4 MEASUREMENT OF WATER POTENTIAL

Leaf water potential was measured with a Spanner thermocouple psychrometer (Barrs, 1968). Freshly harvested plant parts were placed in the chamber enclosing the thermocouple and equilibrated for 2-3 hours before recording the thermocouple output. The water potential was calculated by comparing the readings with those obtained from a graded series of NaCl solutions.

Water potential was also measured with a pressure bomb. The plant stem was cut with a scalpel and was then inserted invertedly into the pressure chamber. After ensuring the chamber was airtight, dry nitrogen gas was released into it. The water droplets which appeared on the cut end of the stem were observed with a magnifying lens. The point of exudate appearance was taken as the measure of water potential of the tissue. The values were read in bars or MPa's from a meter attached to the chamber.

2.5 DETERMINATION OF RELATIVE WATER CONTENT (RWC)

Relative water content was determined according to the method of Barrs and Weatherly (1962). Leaf samples were selected from the treated plants and weighed exactly with a single pan analytical balance (Initial Weight). The weighed leaves were then floated in 20 ml distilled water in 9 cm. petridishes and allowed to take up water for three hours in room temperature. After three hours the leaves were taken out and surface blotted and weighed (Turgid Weight). After taking the turgid weight, the leaf samples were dried in an oven at $80^{o}C$ for 24 hours.

The dry weights were taken, and RWC was calculated using the formula:

$$RWC(\%) = \frac{\text{Initial Weight - Dry Weight}}{\text{Turgid Weight - Dry Weight}} \times 100$$

2.5.1 EXTRACTION AND MEASUREMENT OF PROLINE

A rapid method for estimating free proline, developed by Singh et al (1972), was used. 250 mg Zerolit SF Decalso resin was placed in a Kantes Dual glass homogeniser. To this was added 10-100 mg of freeze dried alfalfa tissue and 5-10 ml of methanol:chloroform:water (12:5:3 v/v) solution. The mixture was homogenized and decanted into a 50 ml centrifuge tube and 5 ml water was added to break the emulsion. The mixture was then shaken and centrifuged and the volume of the supernatant was noted. An aliquot was transferred to a boiling tube containing glass beads (to reduce bumping) and a glass marble covered the mouth of the tube (to prevent evaporation). 5 ml fresh ninhydrin reagent (3 ml of glacial acetic acid: 2 ml of 6M orthophosphoric acid and 125 mg. ninhydrin) and 5 ml glacial acetic acid was added to the sample extract. The mixture was heated in a boiling water bath for 45 minutes, then cooled to room temperature and shaken with a known volume of toluene (5-20 ml depending on the concentration of proline). The optical density of the ninhydrin product dissolved in toluene was measured at 520 nm on a Unicam Colorimeter. Proline content was then calculated from a standard linear regression curve obtained from optical densities of standard proline solutions.

2.5.2 EXTRACTION AND MEASUREMENT OF QUATERNARY AMMONIUM COMPOUNDS

Described in the chapter IV Section (1 and 2)

2.5.3 EXTRACTION, PURIFICATION AND MEASUREMENT OF ABSCISIC ACID (ABA)

Free ABA was extracted and purified by a method modified from that of Coombe and Hale (1973) as indicated below.

2.5.3.1 PREPARATION OF CHROMATOGRAPHY PAPERS

Whatman no. 3 mm papers were washed by sequential elution for 24 hours with each of 0.1M EDTA, distilled water, 2N acetic acid, distilled water and distilled methanol. The papers were air dried before use.

2.5.3.2 COLUMN PACKING PREPARATION

ABA was measured using a glass column packed with 3% Ov-17 on Gaschrom Q (mesh size 100-120). In order to prepare 3% Ov-17 column packing, 30 g of Gaschrom Q (Applied Science Lab, USA) was silinized by treatment with distilled benzene containing 0.5% hexamethyl disilazene (HMDS; Applied Science Lab, USA) to just cover the Gaschrom Q in a round bottom flask. The benzene and excess HMDS were removed under reduced pressure at 55-60^{o}C on a rotory evaporator. The Gaschrom Q was then heated to 60^{o}C for about 30 minutes and the whole procedure was repeated twice. The silinised Gaschrom Q was then suspended in methylene chloride until the liquid was 6 mm above the solid. To this, 30 ml of anhydrous ethylene chloride containing 1 g of Ov-17 (Applied Science Lab, USA) was added. The slurry was thoroughly mixed by swirling and the methylene chloride was removed under reduced pressure at 55-60^{o}C on a rotary evaporator. The Ov-17 coated support was then oven dried at 60^{o}C.

2.5.3.3 SILINISATION AND PACKING THE COLUMN

The method for silinisation of the column was developed by Firn (1968). The glass column was cleaned by passing acetone and anhydrous benzene through it with the aid of a suction pump. Following in an oven, the column was rinsed thrice with 3% HMDS in benzene and dried at 60^{o}C for 15 minutes after each rinsing.

The column was packed by plugging one end with glass wool and sucking in the packing material from the other end with a suction pump. The coiled portion of the column was packed with 3% Ov-17 on Gaschrom Q and the straight portions with Gaschrom Q alone. The column was then installed in the GLC and conditioned at 270^{o}C for 24 hours.

2.5.3.4 EXTRACTION AND PURIFICATION OF THE SAMPLE

The procedure for the extraction of ABA is shown in (Fig. 2) as a flow diagram. The quantity of all reagents and solvents used was determined by the amount of plant tissue. When it was necessary to interrupt the extraction procedure, the sample extract was stored for no more than 24 hours at 4^{o}C at alkaline pH.

These partially purified extracts were applied at 40 mm streaks to acid-washed Whatman no. 3 mm paper, using about 5 µg of ABA as the marker on each side of the paper. The paper was developed for 4 hours in a descending mixture of isopropanol: water: NH_4OH(10:1:1v/v) and dried for 30 minutes. The marker ABA spots were located as fluorescent areas under UV light and were outlined while the rest of the chromatogram was shielded with aluminium foil. The zones of the extracts at equivalent Rf

Fig. 2 FLOW DIAGRAM FOR THE EXTRACTION OF ABA

Grind 1.5 g matured alfalfa tissue with acetone and water (1:1 volume/volume) using the Ultra Turrax under nitrogen.

Titrate with 3N NH_4OH to pH 9.0

Centrifuge at 2000 rpm for 10 min.

Residue — Supernatent

Resuspend in 5 ml of 2% NH_4HCO_3. Centrifuge as before.

Residue (Discard) — Supernatent

Shake vigorously with 7 ml Chloroform and centrifuge as before.

Chloroform (lower) layer — Aqueous (upper) layer

Rewash by shaking with 2 ml of 2% NH_4HCO_3 and centrifuge as before.

Chloroform layer (Discard) — Aqueous layer

Acidefy with 10% H_3PO_4 to pH3 and partition with 10 ml ethyl acetate. Repeat.

Aqueous layer (lower) (Discard) — Pooled Ethyl Acetate (upper) layer

Partition with 10 ml 2% NH_4HCO_3 centrifuge. Repeat.

Ethyl acetate (upper) layer (Discard) — Pooled Alkali (lower) layer.

Acidify with 10% H_3PO_4 to pH 3.0. Partition twice with Ethyl acetate (10 ml : 5 ml).

Aqueous (lower) layer (Discard) — Ethyl Acetate (upper) layer

Concentrate to = 0.5 - 1.0 ml by drying under stream of dry nitrogen.

were excised and eluted overnight with 70% methanol. The eluates were dried under a warm stream of N_2 and washed with anhydrous petroleum spirit and evaporated to dryness.

2.5.3.5 METHYLATION OF THE SAMPLES

Before injecting the samples into the GLC they were dissolved in a mixture of 0.5 ml ethyl acetate and 0.5 ml methanol and methylated with diazomethane. The gas was prepared by reacting 2 ml carbitol (diethylene glycol monoethyl ether), 2 ml 60% (W/V)KOH and about 1 g p-tolylsulphonylmethyl nitrosoamide (TONY). The resulting diazomethane was carried in a stream of N_2, saturated with methanol, through a small bore Teflon tubing with a glass nozzle and bubbled through the extract. Bubbling was stopped after the solution had turned yellow, indicating the presence of excess diazomethane. The extract was then dried under N_2 and stored at $0^{o}C$ in the dark until injected on to a GLC column.

2.5.3.6 DETERMINATION OF ABA ON GLC

Methyl-Cistrans-Abscisic acid was determined by electron capture detection on a Varian 2700 GLC. Extracts were dissolved in a known volume of ethyl acetate and 0.5-2 ml of the dissolved sample was injected on a 3% Ov-17 packed column fitted in the GLC with a ^{3}H-foil electron capture detector. The operating conditions of the GLC were:

Injector - $210^{o}C$

Column - $180^{o}C$

Detector - $245^{o}C$

Flow rates of Carrier gas (N_2) was 26 ml min^{-1}.

Methyl-cistrans-abscisic acid peaks were identified by the coincidence of retention times with those of authentic ABA and confirmed by the decrease in the height of the peak coupled with the increase in the methyl-transtrans-abscisic acid peak following UV irradiation. The amounts of ABA in an extract injected into GLC was measured by comparison of peak area with the standard curve obtained from a series of known ABA (Sigma Chemical Co., USA) concentrations between 0.1 and 0.4 ng.

2.5.4 NITROGEN DETERMINATIONS IN PLANT MATERIAL AND ORGANIC COMPOUNDS

The total nitrogen content of the plant material was measured by a modified micro-kjeldahl method. In the case of organic compounds, nitrogen was measured by digesting the purified fractions of each compound by the micro-kjeldahl method. Nitrogen in ^{15}N labelled samples was measured with the same procedure.

2.5.5 BIOSYNTHESIS OF STACHYDRINE

2.5.5.1 FEEDING PROLINE, GLUTAMATE, ORNITHINE, METHIONINE, PYRIDOXINE AND FOLIC ACID

Proline, Glutamate, Ornithine, Pyridoxine (all from Sigma Chemical Co., USA), Methionine (Merck, USA), and Folic acid (Nutritional Biochemical Corporation, USA) were fed to plants grown in glass tubes containing half strength Hoagland solutions. The compounds were fed for 48 hours under the growing conditions described in section 2.2. The tubes were aerated throughout the experiment.

2.5.5.2 FEEDING ^{15}N PROLINE

Plants were grown in glass tubes as described in section (2.1.2). ^{15}N proline (KOR, USA) was dissolved in water to the required concentration and applied with a syringe into the glass tubes containing nutrient solutions.

2.5.5.3 DETERMINATION OF ^{15}N INCORPORATION ON MASS SPECTROMETER

A ^{15}N-enriched sample was transferred to a micro-kjeldahl flask and digested in 5 ml, 36 N H_2SO_4 containing 0.2 g HgO. The ammonia produced was distilled under alkaline conditions into 1% (W/V) basic acid and it was concentrated to 2 ml after adding one drop of 1N H_2SO_4. The sample was then transferred into one of the limbs of a Rittenberg tube and an excess of alkaline hypobromite added to the other. The tube was then affixed to a mass spectrometer vaccum system and was rigorously evacuated to 10^{-7} mm Hg N_2 gas was generated by mixing the contents of the Rittenberg tube. The tube was immersed in a liquid nitrogen trap to freeze water vapour and the oxides of nitrogen. The gas was then introduced into an evacuated expansion flask and passed into the Micromass 602C mass spectrometer (AEI, Manchester). The ^{15}N enrichment was determined by measuring mass 28, 29 and 30, representing ^{14}N - ^{14}N, ^{14}N - ^{15}N and ^{15}N - ^{15}N respectively.

2.6 CHLOROPHYLL a FLUORESCENCE EXPERIMENT

2.6.1 INSTRUMENTS

The chlorophyll a fluorescence was measured with a Branker model SF-20 plant productivity fluorometer fitted to a data capturing device

and further modified to enable simultaneous activation of energising beam and data aquisition. The measurements were made at a constant room temperature ($23 \pm 1^{o}C$).

CHAPTER IV

RESULTS AND DISCUSSION

SECTION 1 QUANTITATIVE ESTIMATION OF BETAINES BY COLUMN AND THIN LAYER CHROMATOGRAPHY

1.1 INTRODUCTION

The quantitative determination of betaines (N-methylated compounds) has become important in recent times in the study of plants under adverse environmental conditions. Most of the presently available methods for estimating these compounds are unsatisfactory in terms of specificity, sensitivity or convenience. The periodide method, as described by Storey (1976), is not specific or sensitive and requires much care and proper separation of individual betaines. Several authors have used other techniques to assay betaines:-

(a) isotape dilution/micro-kjeldahl method (Hanson and Nelson, 1978),

(b) low-temperature pyrolysis gas chromatography of the OH^- form, and estimation of the trimethylamine fragment (Hitz and Hanson, 1978),

(c) esterification before or after applying the sample on the column (Dubois and Simand, 1976),

(d) gas chromatography of an uncharacterised silylation product (Ranfft and Gerstl, 1975),

(e) and nuclear magnetic resonance spectrometry (Chastellian and Hirsbrunner, 1976).

Martin and Finkelstein (1981) have described an isotape dilution assay for glycine betaine which relies on it's ability to act as a methyl donor in a reaction catalysed by betaines: homocysteine methyl transferase. Gorham et al, (1982) have recently developed a method to measure betaines as u.v - abosrbing esters.

None of the above mentioned methods can be used easily with plant extracts, as a regular, routine analysis with high specificity and convenience.

Since there were many quaternary ammonium compounds in alfalfa it was very important to isolate them separately for this study. This lead to an investigation of available methods in order to devise a technique to estimate these compounds specifically and quantitatively.

This section describes the method used to measure betaines throughout the project.

1.2 METHODS

1.2.1 EXTRACTION

Betaines were extracted from alfalfa tissue with methanol: chloroform: water (12:5:3) by using a mechanical glass homogenisor. The extract was centrifuged for 10 minutes at 5000 rpm and the supernatant was taken to dryness on a rotary evaporator. It was then redissolved in a known amount of distilled water and used for column-TLC analysis.

1.2.2 PURIFICATION

Alfalfa plant extracts contained various contaminants which interferred with the separation of betaines on thin layer chromatography. In order to obtain a specific betaine on TLC it was necessary to purify the extract. Purification necessitated the retention of betaines on a strongly acid cation exchange resin at a mildly acid pH and their subsequent elution by a strong acid.

Dowex 50 H^+, 2% cross linked (50-100 mesh) resin was washed with distilled water and a slurry poured into 300 x 12 mm size glass column which was plugged with fibre glass at the tapered end. The resin was washed with distilled water till the pH of the elutent was 6.0 - 6.5.

The pH-adjusted (6.0-6.5) sample was applied slowly to the column and washed with distilled water (approx. 250 ml). The betaines were eluted from the column with 3N HCl (100-125 ml). Eluted samples were then evaporated to dryness on a rotary evaporator at 50^{o}C and preserved at -20^{o}C for further analysis.

1.2.3 SEPARATION OF BETAINES ON THIN LAYER CHROMATOGRAPHY (TLC)

The separation of quaternary ammonium compounds was achieved successfully be developing the samples on thin layers of silica gel. A combination of solvent mixtures was developed to separate glycine betaine, stachydrine, homostachydrine and trigonelline.

1.2.4 PREPARATION OF TLC PLATES

Thin layer plates (20 x 20 cms) were made using Kieselgel 60G, Art 7731 (Merk, USA) powder. 38 g of Kieselgel 60G was mixed with 68 ml of water and de-gassed before applying on plates. Using a Desaga spreader, five 20 x 20 cms plates were coated with the silicagel slurry to a thickness of 0.20 mm. The plates were left to dry overnight at room temperature. Two hours before applying samples, the plates were activated for 30 minutes at 110^{o}C.

1.2.5 APPLICATION OF SAMPLES

Samples were applied both as spots and bands of known length at a position 2 cms above the lower edge of the layer. The samples were applied with the Camag Linomat III mechanical spotter/streaker. The solvent front was marked at 15 cms from the origin. A maximum of four samples each ranging from 50-100 µl were applied to a full sized plate. Standards were applied on either side of the plates. The thin layer was scored in the direction of the flow.

1.2.6 DEVELOPMENT OF THE PLATES

After the samples were applied the plates were developed in chloroform, 60; methanol, 30; ammonia, 10 ml) for about 1.5 hours in an airtight glass tank. After the first run, i.e. when the solvent reached the marked solvent front, the plates were taken out and air dried. They were re-developed in the same direction with the same solvent mixture for another 1.5 hours. The resolution of the betaines was most satisfactory when the plates were developed twice in the same direction.

All the solvents used were A.R. grade and fresh mixtures were left in the tank overnight to equilibrate.

1.2.7 DETECTION OF BETAINES

Several reagents have been used to detect the betaines (Stahl, 1969). Dragendorff's reagent (Radeka et al, 1971) was found to be most satisfactory.

1.2.7.1 DRAGENDORFF'S REAGENT:- (according to Radeka et al, 1971)

Stock solution: Bismuth carbonate (2.5 g) and Sodium Iodide ~~(8.7 g)~~ 7.8 in glacial acetic acid (25 ml) were boiled for a few minutes until dissolved and after cooling to room temperature, ethyl acetate (10 ml) was added.

1.2.7.2 SPRAY REAGENT

10 ml stock solution was diluted with a mixture of conc. HCl (3 ml), H_2O (11 ml). To this diluted solution 10 ml ethanol was added.

1.2.7.3 SPRAYING PROCEDURE

After drying the TLC plates the reagent was sprayed gently from a distance of 6 - 8" to prevent wetting of the surface area. A second spray, if necessary, was applied 15 - 30 minutes later.

1.2.8 ELUTION OF BETAINES FROM THE PLATES

After identifying the individual betaines, they were marked with reference to the standards. The marked area of the silica gel was carefully removed from the plate with a razor blade and the silica gel was then dissolved in distilled water. The water fraction contained individual betaines and was used for qualitative measurement with periodide method.

1.2.9 QUANTITATIVE MEASUREMENT OF BETAINES BY A MODIFIED PERIODIDE METHOD

The quantitative measurements of betaine was made with the periodide method which was later modified by Storey, (1976) and Wall et al, (1960). This method is based on the precipitation of the periodide quaternary ammonium complex in water. The complex was then redissolved in 1,2-dichloroethane and quantitatively determined from absorption measurements with a spectrophotometer at 365 nm.

REAGENT

Potassium tri-iodide solution:- Iodine (7.5 g) and potassium iodide (10 g) were dissolved in 1N HCl (100 ml) by continuous stirring for 30 minutes and filtered.

Samples containing purified individual betaines in water were placed in 25 ml test tubes and 0.1 ml of potassium tri-iodide was added accurately. The mixture was shaken and kept in an ice bath for a minimum period of 90 minutes. Intermitent shaking was done to enhance the

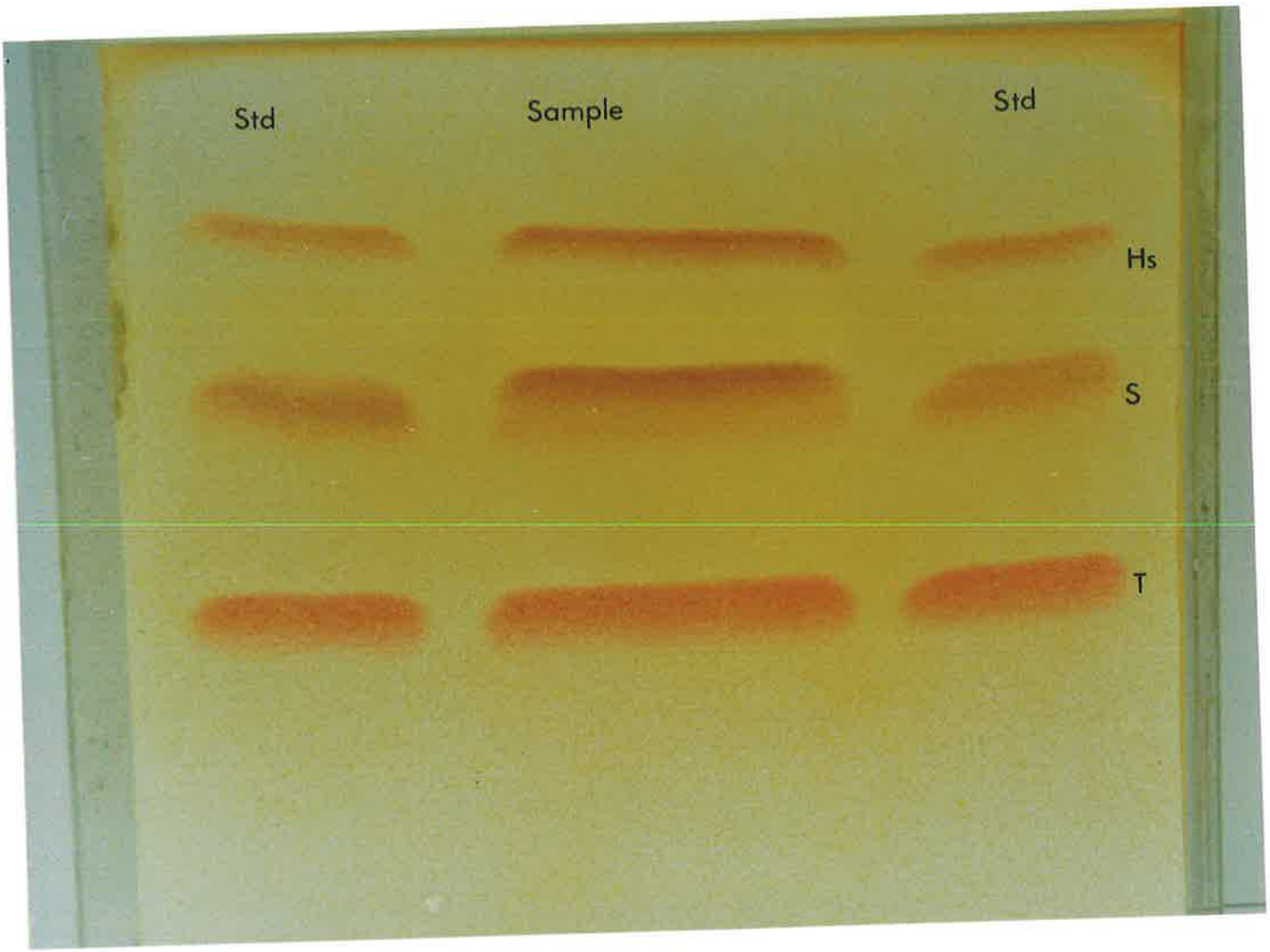
precipitation. After 90 minutes, 1 ml of ice-cooled water was added to the mixture and after shaking, 10 ml of 1,2-dichloroethane was added. The mixture was shaken well and left to settle at $4^{o}C$ for 10 minutes. The upper aqueous phase was discarded and the optical density of the organic layer was measured at 365 nm with a Unicam SP800 spectrophotometer against a blank.

1.3 RESULTS

1.3.1 SEPARATION OF BETAINES WITH THINLAYER CHROMATOGRAPHY (TLC)

Separation of betaines was carried out by applying samples to silica gel-coated thinlayer chomatography plates. When known amounts of standard glycinebetaine, stachydrine, homostachydrine and trigonelline solutions were applied to TLC plate and developed with the solvent mixture (chloroform : methanol : ammonia), the separation was very poor (results are not shown here). When the plates were developed two times in the same direction with an in between drying, the resolution of betaines was better, as shown in Fig. 3. When all alfalfa extract, after initial purification on a Dowex 50 H^{+} column, was applied, same results were achieved. The chromatographic position of these separated betaines was located by spraying with Dragendorff's reagent. The compounds were then eluted from the plates and taken into water for quantitative estimation by periodide assay as described in the methodology in this section (1.2.9).

Fig. 3 Thin layer chromatogram showing the separation of betaines.


Std
Sample
Std
Hs
S
T

1.4 DISCUSSION

Separation of quaternary ammonium compounds from plant samples has been done on column (Christianson et al, 1960) and on TLC (Bazer, 1964; Storey, 1976). The present exercise shows the separations of QACs on thin layer chromatography (Plate 1). It is essential to purify the sample to achieve such a separation. The samples without the initial purification, due to the presence of impurities, posed several problems (Storey, 1976).

In these experiments also, such problems were encountered. However, unlike the method used by Storey (1976), the present separation, inspite of the complexity in alfalfa sample, was sufficient for quantitative measurements of individual betaines. The separated compounds were measured quantitatively with the periodide method. This method, also used by Storey (1976), has been criticised for it's less sensitivity and non-specificity (Gorham et al, 1982). Although new methods of estimating betaines have been reported (Gorham et al, 1982) the esterifiction procedures used are time consuming and are non-specific and were not useful in the experiments in this project where periodical and quick specific measurements were necessary. However, if one can purify the sample and separate it on TLC and obtain the pure compound of interest as was achieved in this exercise, the periodide method will be more specific and more sensitive. For determinations of stachydrine, homostachydrine and trigonelline throughout this project, the same method was followed with further confirmational studies on NMR which is discussed in the next section.

SECTION 2. STANDARDISATION AND IDENTIFICATION OF BETAINES WITH NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

2.1 INTRODUCTION

Nuclear magnetic resonance spectroscopy has been a powerful biochemical tool in recent times due to its ability to provide information about biologically important molecules in solution. Several authors have used NMR for identifying betaines in plant and animal tissue (Chastellian and Hirsbrunner, 1976; Norton and de Rome, 1980; Ahman and Basha, 1975).

In the present study, NMR was used to identify betaines in alfalfa and also to confirm some of the critical steps in the purification procedure.

2.2 METHODS

2.2.1 IDENTIFICATION OF STACHYDRINE, HOMOSTACHYDRINE AND TRIGONELLINE IN ALFALFA EXTRACTS

Several Dragendorff's positive compounds were noticed following TLC of the alfalfa extracts. It was essential to identify these compounds to study their behaviour during these experiments. Three TLC-separated compounds were eluted from the silica gel and were labelled as probable stachydrine (ST), homostachydrine (HS) and trigonelline (TR) and were taken in 0.5 - 1.0 ml distilled water and centrifuged at 5000 rpm for 10 minutes. The supermatants were then taken to dryness on a rotary evaporator and were redissolved in Deuterium oxide (0.6 ml) for NMR analysis.

2.2.2 STANDARDISATION OF THE ANALYTICAL METHOD (PURIFICATION PROCEDURE)

The plant extract, after adjusting the pH to 6-6.5, was applied to a Dowex 50 H^+ cation exchange column and washed with distilled water (25 ml). Each 50 ml of the water washings was collected and taken to dryness on a rotary evaporator. The residue was washed with ethanol (5 ml) and redried with dry nitrogen. The dried sample was then redissolved in Deuterium oxide (0.6 ml) and analysed on a JEOL FX 90Q NMR in order to check

(a) the loss of betaines, if any, in the water washings, and

(b) the removal of the contominants.

After the water washings, the betaines were entered from the column with 3N HCl. The elution fractions of 100 ml (50 ml at a time) were collected and dried as described in the previous paragraph. The Deuterium oxide-dissolved samples were used for NMR analysis.

2.3 RESULTS

2.3.1 IDENTIFICATION OF STACHYDRINE, HOMOSTACHYDRINE AND TRIGONELLINE IN ALFALFA EXTRACT

^{1}H and ^{13}C NMR Spectra of the three silica gel-eluted fractions, were obtained at 89.55 MHz and 22.49 MHz respectively using a JEOL FX-90Q Fourier transform NMR spectrometer at an ambient probe temperature of 24^{o}C. An internal reference of deuterated TSP (sodium 3-trimethyl silyl propionate -2, 2, 3, 3-d4) was used. Proton spectra were

acquired into 8K memory address using a spectral width of 1000 Hz and a pulse width of 15 μS (45^{o}). Sixteen aquisitions with a recycle time of 5.3 seconds were routinely made.

Proton-decoupled ^{13}C spectra were acquired into 8K memory address using a pulse width of 11 μS (45^{o}). 20,000 scans using 1 kHz wide broad band ^{1}H decoupling with a recycle time of 2.8 seconds were usually required to obtain adequate signal to noise. After acquisition a line broadening of 0.5 Hz was applied to the data before fourier transformation.

^{1}H NMR spectra of the ST fraction (Fig. 4A) consists of peaks at 4.31 δ (t, J 9.0Hz, 1H), 3.71 S (m, 2H), 3.34 δ (S, 3H), 3.15 δ (S, 3H) and broad multiplets in the region 2.01 - 2.72 δ (4.H). The above signals have been assigned to C-2, C-5, $N\text{-}CH_3$, $N\text{-}CH_3$, and C-3,4 proton respectively. A proton decoupled ^{13}C-spectrum of this fraction (Fig. 5A) contains six sharp peaks (again the carbonyl resonance was not observed) at 21.45, 28.01, 48.81, 54.82, 69.99 and 79.31 ppm with respect to an internal TSP reference. These resonances have been assigned to C-4, C-3, $N\text{-}CH_3$, $N\text{-}CH_3$, C-5 and C-2 carbons respectively and were consistent with authentic stachydrine (Figs. 4B and 5B).

^{1}H NMR spectra (Fig. 6A) of the HS fraction in D_2O gave complex multiplets in the region 3.34 - 4.09 ppm (4H) and 1.55 - 2.32 ppm (4H) and sharp singlets at 3.27 ppm (3H) and 3.27 ppm (3H). These have been assigned to C-3, C-4,5, $N\text{-}CH_3$, $N\text{-}CH_3$ protons respectively.

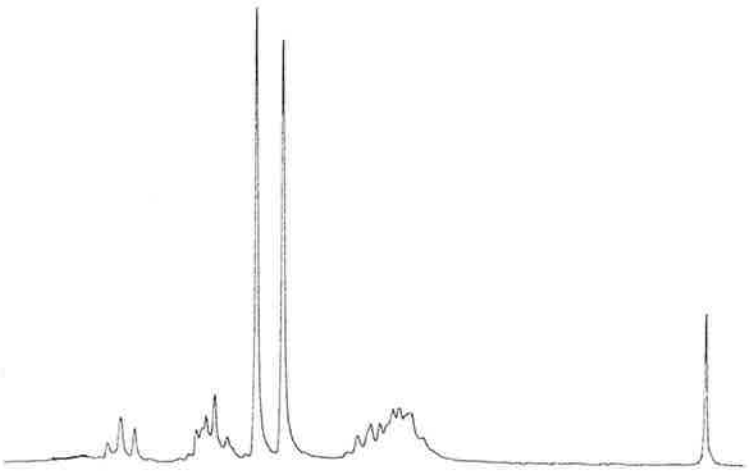
The ^{13}C proton decoupled spectrum of this compound (Fig. 7A) consists of seven sharp singlets (the carbonyl carbon was not observed)

Fig. 4 ^{1}H NMR spectrum of stachydrine.

A. Isolated from alfalfa plants.

B. Authentic stachydrine preparation.

A



B

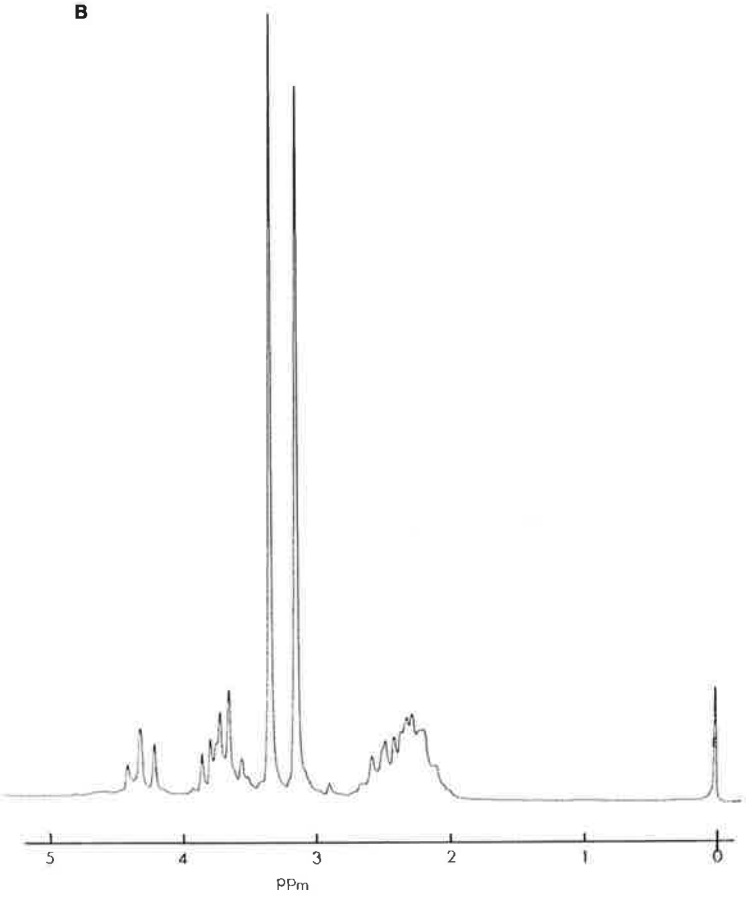
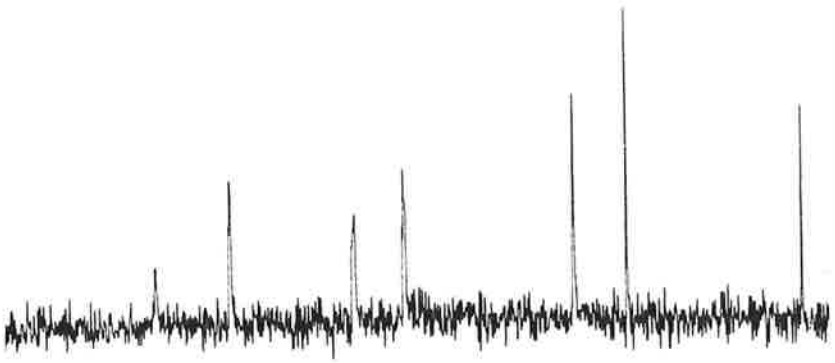


Fig. 5 ^{13}C NMR spectrum of stachydrine.

A. Isolated from alfalfa plants.

B. Authentic stachydrine preparation.

A



B

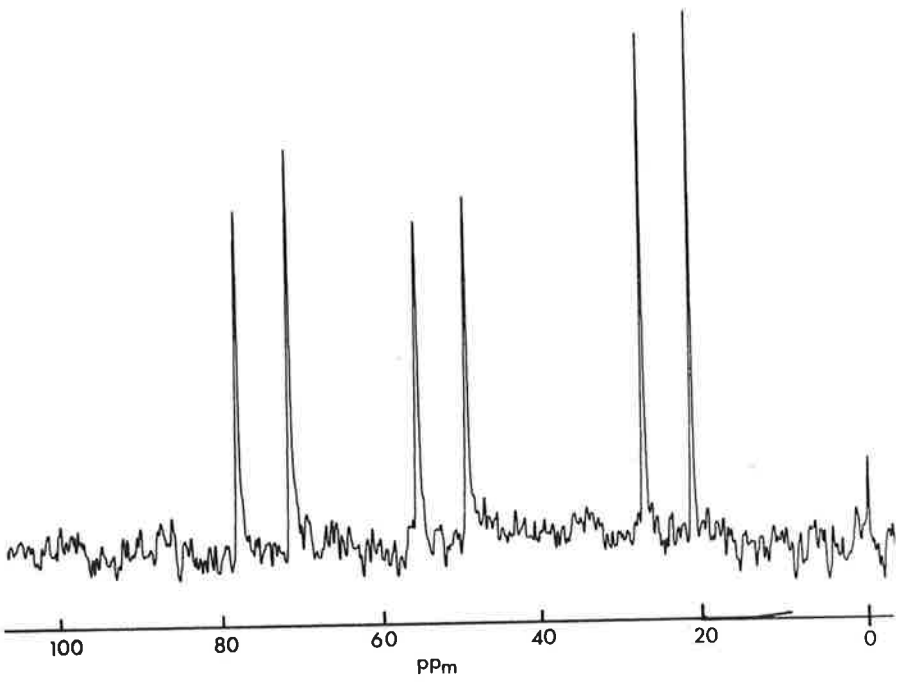
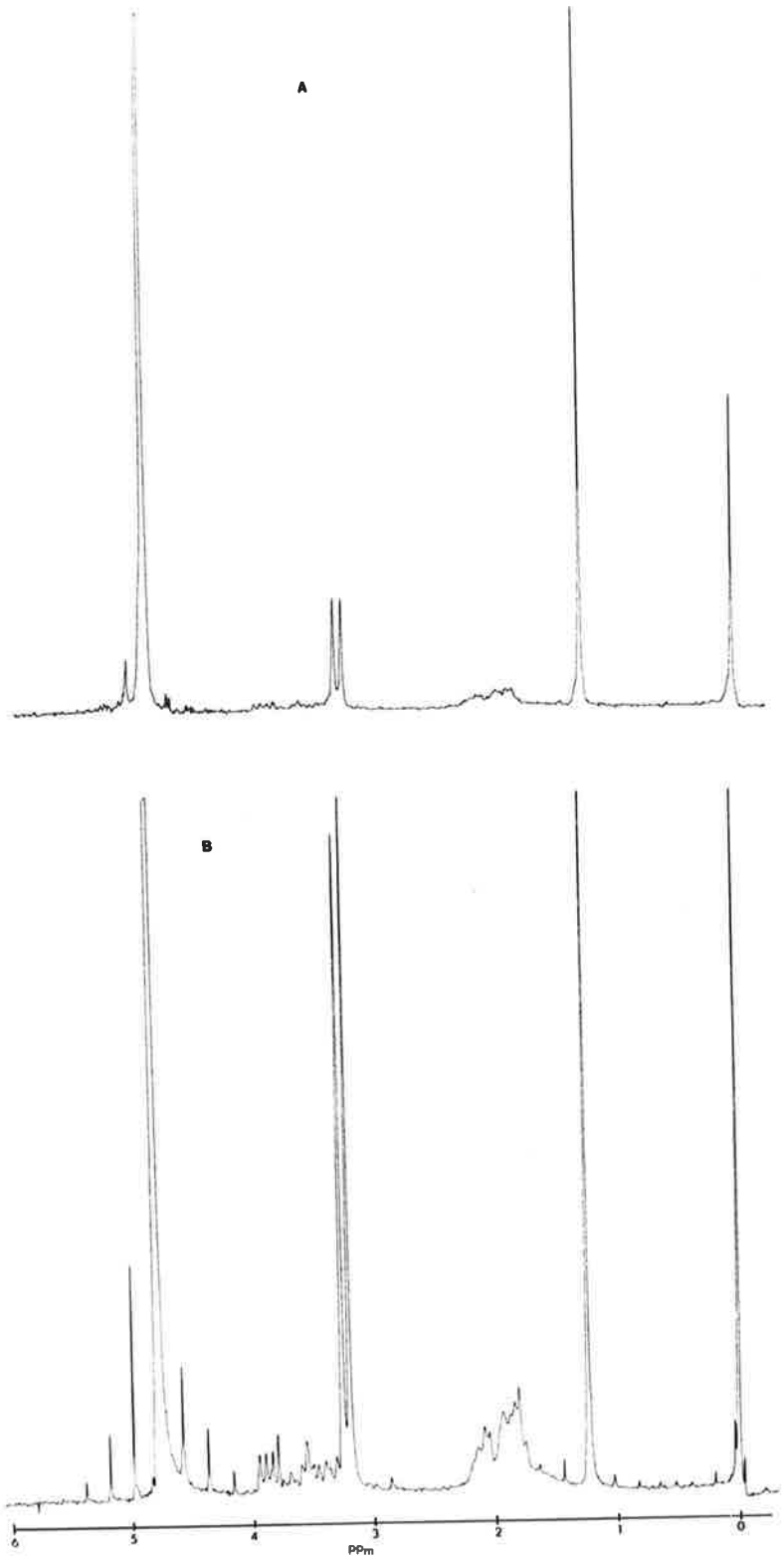


Fig. 6 ^{1}H NMR spectrum of Homostachydrine.

A. Isolated from alfalfa plants.

B. Authentic homostachydrine preparation.


A
B
6
5
4
3
2
1
0
PPm

at 22.59, 23.40, 27.20, 47.57, 56.61, 67.83 and 77.25 ppm. These have been assigned to C-4, C-5, C-3, N-CH_3, N-CH_3, C-6, and C-2 carbons respectively. Both the ^1H and ^{13}C spectra were consistent with the authentic sample of homostachydrine (Figs. 6B and 7B).

The ^1H NMR spectrum (Fig. 8A) of the TR fraction in D_2O gave peaks at 9.33 δ (S, 1H), two overlapping doublets centred at 8.96 δ (2H), 8.16 δ (t, 1H) and 4.47 δ (S, 3H). The overlapping doublets at 8.96 δ collapsed to a broad singlet on homonuclear spin decoupling when irradiated at 8.16 δ. The above signals were assigned to the C-2, C-4, C-6, C-5 and N-CH_3 protons respectively. The fully decoupled ^{13}C spectrum (Fig. 9A) consists of seven sharp singlets at 51.30, 130.67, 133.72, 147.84, 148.87, 149.30 and 170.54 ppm, relative to TSP. These have been tentatively assigned to N-CH_3, C-5, C-3, C-6, C-4, C-2, and COO^- carbons respectively. Both the proton and ^{13}C spectra were consistent with an authentic sample of trigonelline (Figs. 8B and 9B).

2.3.2 STANDARDISATION OF THE ANALYTICAL METHOD (PURIFICATION PROCEDURE)

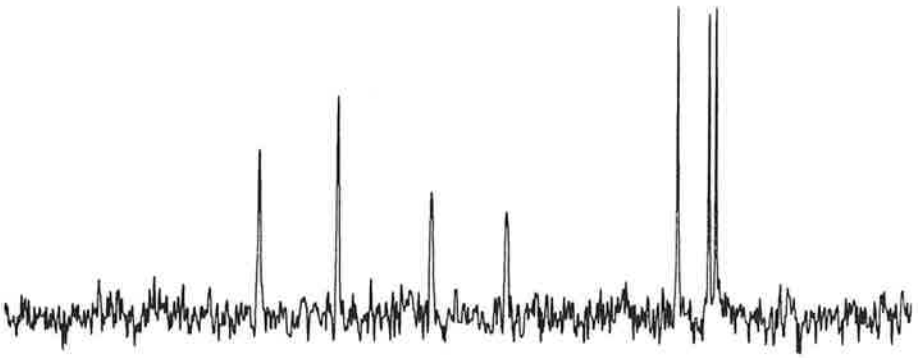
The purification and elution procedures were developed with a mixture of standard betaines (glycine betaine, stachydrine, trigonelline, homostachydrine). The 50 ml washing fractions did not show any of the betaines even after 5 washings or 250 ml water elution. When 3N HCl was applied on to column, the first 50 ml fraction (Fig. 10A) and in the second 50 mls showed all the betaines (Fig. 10B). This suggested that with 100 ml of 3N HCl, all the betaines were eluted from the column.

Fig. 7 ^{13}C NMR spectrum of Homostachydrine.

A. Isolated from alfalfa plants.

B. Authentic stachydrine preparation.

A



B

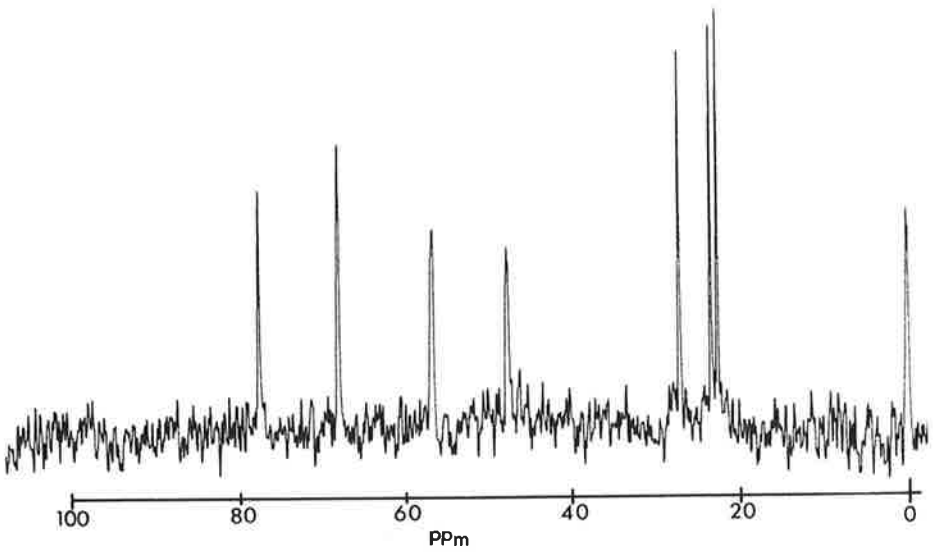


Fig. 8 ^{1}H NMR spectrum of trigonelline.

A. Isolated from alfalfa plants.

B. Authentic trigonelline preparation.

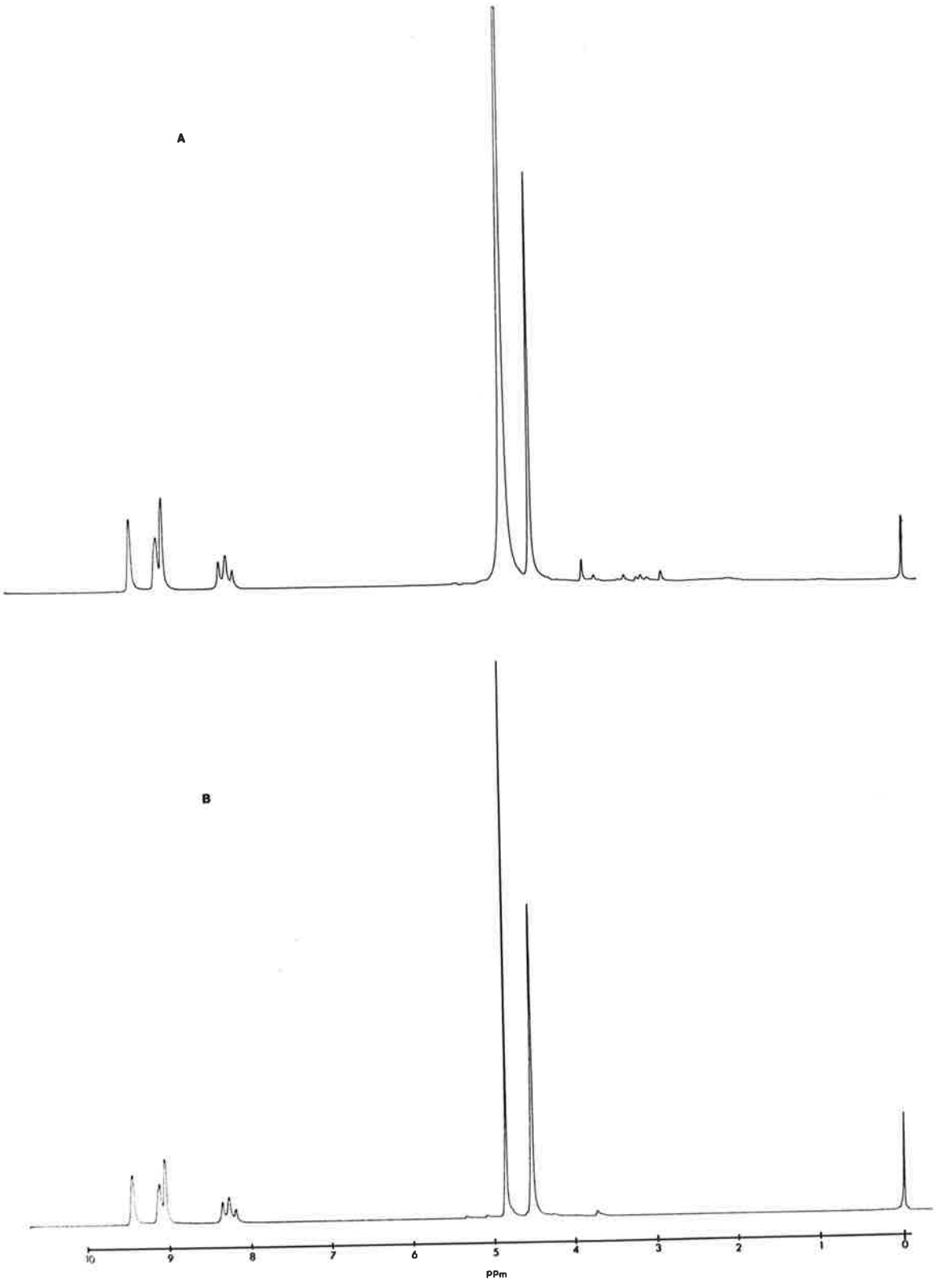

A
B
10
9
8
7
6
5
4
3
2
1
0
PPm

Fig. 9 ^{13}C NMR spectrum of trigonelline.

A. Isolated from alfalfa plants.

B. Authentic trigonelline preparation.

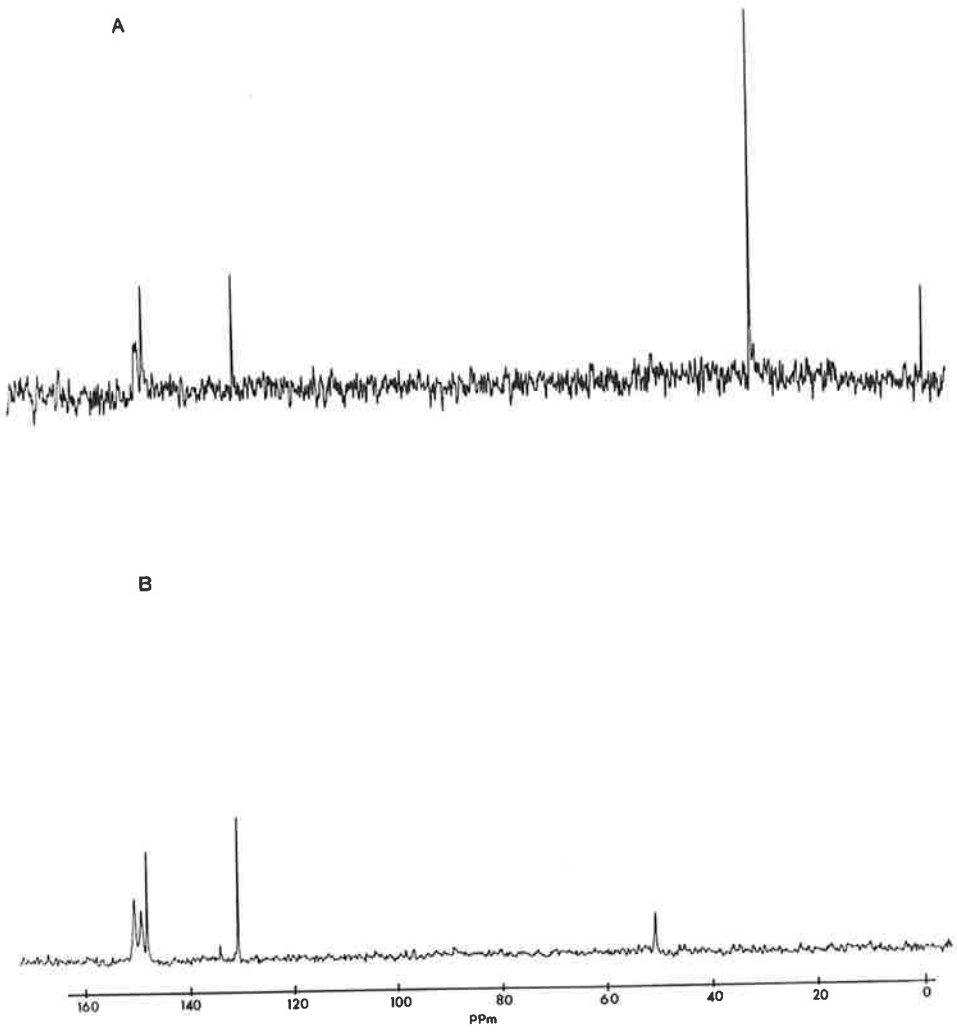

A
B
160
140
120
100
80
60
40
20
0
PPm

Fig. 10 Acid (3N HCl) elution of standard betaines from Dowex 50 H+ column.

A. First 50 ml acid elution.

B. Second 50 ml acid elution.

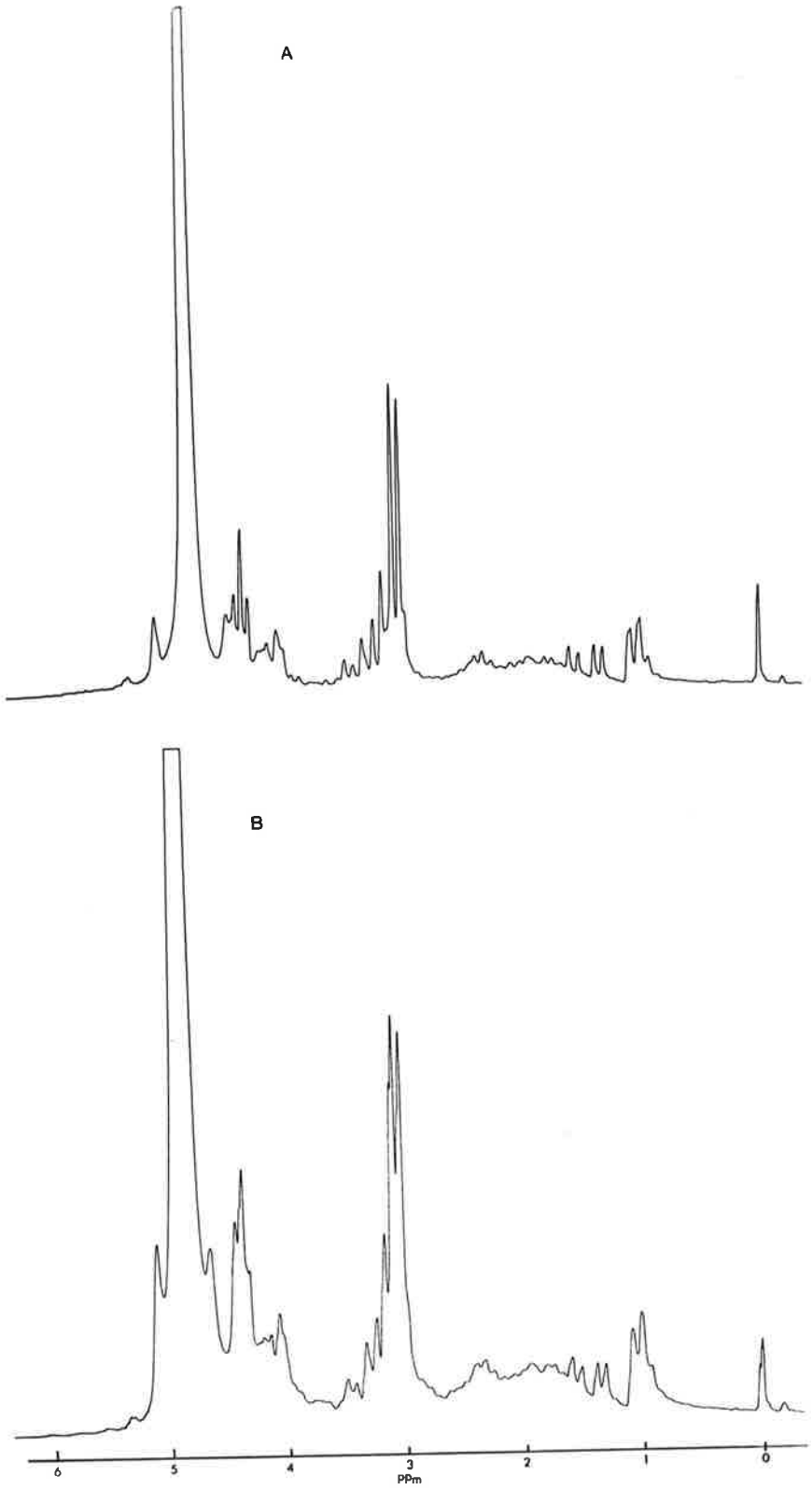
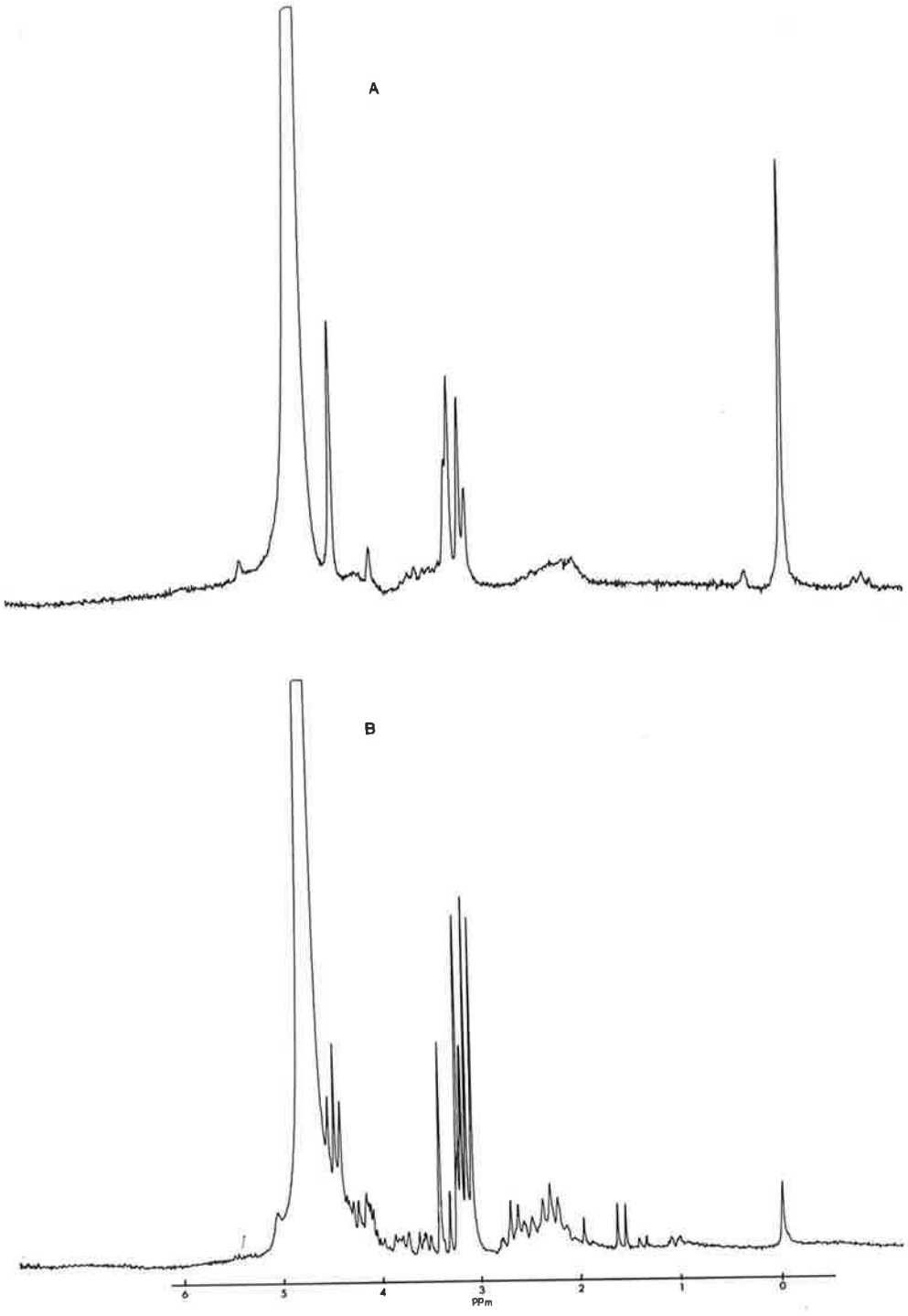

A
B
6
5
4
3
2
1
0
PPm

Fig. 11 Acid (3N HCl) elution of betaines from the alfalfa extracts after initial water washing.

A. First 50 ml acid elution.

B. Second 50 ml acid elution.


A
B
6
5
4
3
2
1
0
PPm

In the second stage, the plant extracts from alfalfa were applied on to column. The first 50 ml water elution showed a broad spectra which did not correspond with any of the authentic betaines. In the second and subsequent water washings all the contominants were washed off, and by the fifth wash it was clear that no contominants remained on the column.

The acid elution (3N-HCl) showed betaines in the first 50 ml (Fig. 11A). The second 50 ml eluted the rest of the betaines from the column (Fig. 11B). This was similar to the elution profile of the standard betaines. The purified samples were collected and used for TLC separation of individual betaines for quantitative study.

2.4 DISCUSSION

NMR spectroscopy provides a convenient means of identifying and quantifying the major organic solutes. Identification of stachydrine (Ahman and Basha, 1975) and glycinebetaine (Norton and Rome, 1980) on NMR have been reported. NMR in this study was used in two ways:

(1) for the standardisation of the column-TLC technique on which the regular assay of betaines was based, and

(2) for the identification of the TLC separated-eluted alfalfa samples, as presented in the results (Figs. 4A - 9B, which established the authenticity of the compounds present in the alfalfa plants.

Purification of the samples on column and subsequent elution of the betaines were also confirmed with the NMR (Figs. 10A - 11B). This

demonstrated that the procedure separates and purifies the sample components without losing any in the washings. The sharp, non-overlapping $N\text{-}CH_3$ proton signals on NMR also provides a method of estimating the levels of these compounds in the tissue. Using t-BuOH as an internal standard and calibration curves of integrated peak interactions against concentrations of the authentic compounds, it has been possible to estimate solute concentrations in the range of 250µg upwards with an accuracy of 5%. However, for the present study this method was not used.

Based on these NMR confirmations the method described in Section 1 was used for measuring stachydrine, homostachydrine and trigonelline throughout this thesis.

SECTION 3: EFFECT OF WATER STRESS ON THE ACCUMULATION PROLINE, STACHYDRINE AND TRIGONELLINE

3.1 INTRODUCTION

For many years information has been accumulating on the effects of water stress on plants. In recent years biologists have been looking for compounds which accumulate in plants under adverse environmental conditions.

The amino acid, L-proline, has occupied a special position in plant physiology research during recent times. Perhaps the major contributing reasons for this interest is that it accumulates in most plants which have been subjected to various environmental stresses. A 10-100 fold increase in proline content was demonstrated in stressed *Cynodon dactylon* shoots by Barnett and Naylor (1966); and proline has been shown to accumulate to levels as high as 10% of the dry weight of the tissue (Stewart and Lea, 1974). Proline accumulation in response to water stress has been reported in many crop species, eg. wheat (Quarie, 1980, Jan and Halloram, 1982; Karmanos et al,1983), maize (Carceller and Fraschina, 1980), sorghum (Blum and Ebercon, 1976; Parameshwara and Sastry, 1980), soyabean (Singh and Gupta, 1983) etc. It has been suggested that this accumulation of proline is advantageous to the plant in coping with drought, and that it can be used as an indicator in selecting for drought resistance in crop breeding programmes (Singh et al, 1973; Blum and Ebercon, 1976).

Quaternary ammonium containing compounds, in particular, glycinebetaine, are another type of compound which accumualtes in plants

in response to salinity or drought (Storey and Wyn Jones 1977). Hanson et al (1978) found increased amounts of glycinebetaine in water-stressed barley leaves. Increased amounts of both proline and glycinebetaine were found in *Euphorbia trigona* leaves and shoots (Huber and Eder, 1982). Stachydrine is present largely in Labiatae and Capparidaceae (Storey et al, 1977) and in alfalfa (Robertson and Marion 1959a,b,c, 1960; Conners et al, 1973) while trigonelline is distributed in alfalfa (Blake, 1954; Robertson and Marion 1959a) and several other families (Wyn Jones and Storey, 1981). Very little or no information is available in the literature in relation to the accumulation of stachydrine and trigonelline in response to water stress.

The experiments reported here were designed to examine the effect of moisture stress on proline accumulation in different parts of alfalfa seedlings. In addition the study was intended to examine the response of stachydrine and trigonelline to water stress.

3.2 METHODS

3.2.1 GROWING PLANTS

Alfalfa seedlings were grown in plastic pots in the glass house under natural conditions using recycled soil as a growing medium. The plants were watered regularly until the treatments were imposed.

3.2.2 WATER STRESS TREATMENTS

When the plants were three weeks old, water stress was imposed by withholding water. Control plants were regularly watered throughout the experimental period.

3.2.3 HARVESTING THE PLANT TISSUE

The plants of both control and stress treatments were harvested at the end of every 48 hours. Leaves, stem and roots were separated immediately and frozen with liquid nitrogen. The frozen samples were then dried in a freeze drier for 48 hours, weighed and analysed for proline, stachydrine and trigonelline. A batch of plants from the same treatments was harvested for the measurement of water potentials as described in materials and methods (Section 2.3).

3.2.4 WATER POTENTIALS MEASUREMENTS

Water potentials were measured on the stem and at the end of each experimental period (48 hours). The methods are described in materials and methods (Section 2.4).

3.2.5 PROLINE MEASUREMENTS

Proline was measured in leaves, stem and roots separately by the Singh et al, (1973) method. (Chapter III, 2.5.1)

3.2.6 STACHYDRINE AND TRIGONELLINE MEASUREMENTS

Stachydrine and trigonelline were measured in the same sample used for proline estimation. The method is described in Chapter IV, Section 1 and 2.

3.3 RESULTS

3.3.1 WATER POTENTIAL

Plants exposed to water stress showed a linear reduction in water potential with time (Fig. 12A). The well-watered control plants maintained water potential around -0.30 to -0.50 MPa throughout the experimental period. Stressed plants, however, showed a significant decrease, from -0.60 MPa to -1.35 MPa, by 240 hours after stress was imposed. When the plants were rewatered the water potential started to increase and by 24 hours after rewatering it had returned to -0.85 MPa, showing a strong alleviation of water stress.

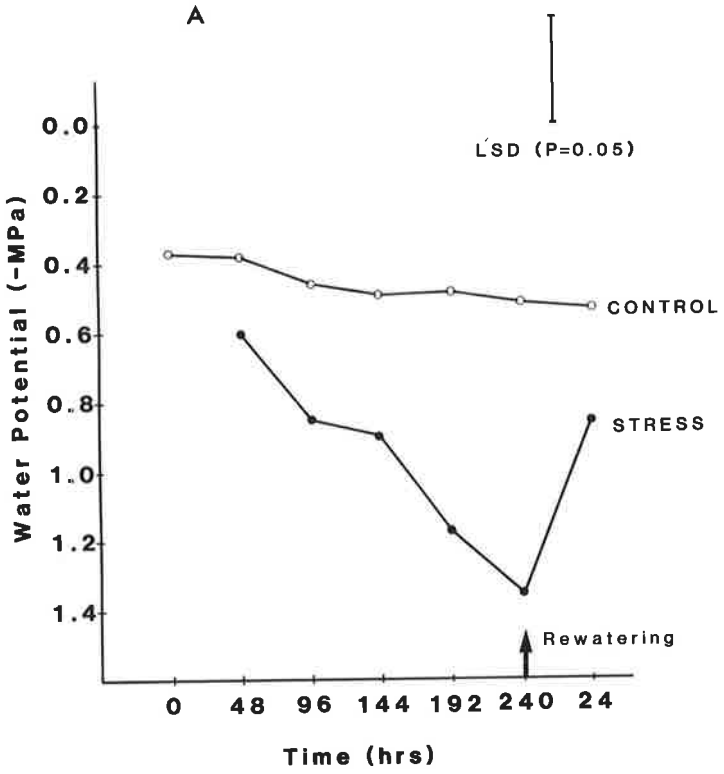
3.3.2 PROLINE CONTENT IN LEAVES, STEM AND ROOTS

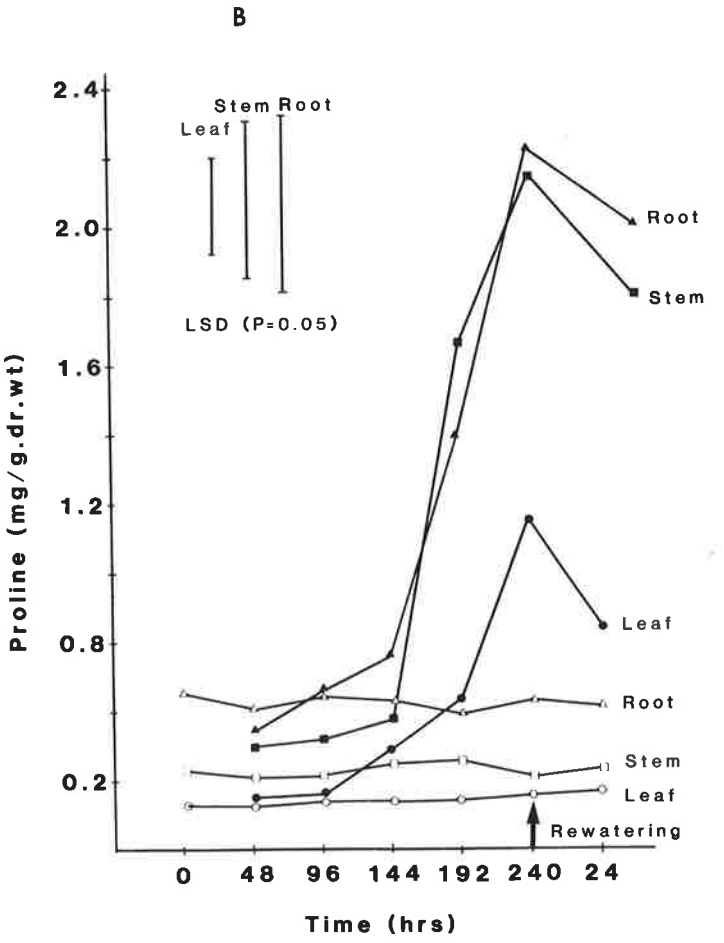
Leaf proline content increased at a slow rate with an increase in stress and was significantly higher than control at the end of 240 hours after stress (Fig. 12B). In stems, proline did not increase significantly until 192 hours; after 144 hours it increased at a faster rate reaching its highest level at 240 hours. Roots followed a similar trend. In all three types of tissue there was no significant change in proline in the first 96 hours which may be due to the lesser reduction in water potential (Fig. 12A) during that period. It was also interesting to observe that the roots and stem showed higher amounts of proline than leaves during the stress.

Proline content determined 24 hours after rewatering showed that in all 3 types of tissue (leaf, stem and roots), the level decreased.

Fig. 12 A. Plant water potential of alfalfa seedlings after exposing to a period of water stress and after alleviation.

B. Accumulation of proline in different organs of alfalfa seedlings after exposing to a period of water stress and after alleviation.


A
LSD (P=0.05)
Water Potential (-MPa)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
CONTROL
STRESS
Rewatering
0
48
96
144
192
240
24
Time (hrs)


B
Stem
Root
Leaf
LSD (P=0.05)
Proline (mg/g.dr.wt)
2.4
2.0
1.6
1.2
0.8
0.2
Root
Stem
Leaf
Root
Stem
Leaf
Rewatering
0
48
96
144
192
240
24
Time (hrs)

3.3.3 STACHYDRINE CONTENT IN LEAVES, STEM AND ROOTS

Stachydrine content in leaves, stem and roots was determined in the same sample used for proline assay. The data is presented in (Fig. 13A). Leaf stachydrine content increased gradually but it was significant only after 192 hours. Stem stachydrine showed similar trend but was significantly higher after 48 hours and continued to increase as the stress progressed. Roots showed a significant increase earlier (48 hours), probably due to a more direct effect of the depletion of moisture. Stachydrine continued to increase till 240 hours.

When the plants were rewatered the stachydrine content in leaves, stem and roots showed a slight decreasing trend after 24 hours. This may indicate that stachydrine, like proline, is re-utilised in metabolic processes during the rejuvination of plant growth.

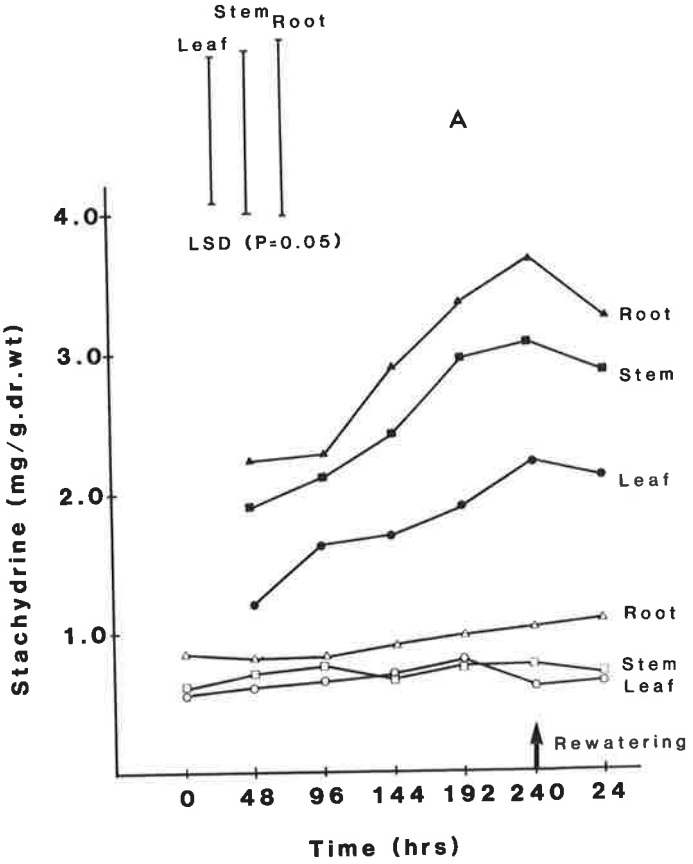
3.3.4 TRIGONELLINE CONTENT IN LEAVES, STEM AND ROOTS

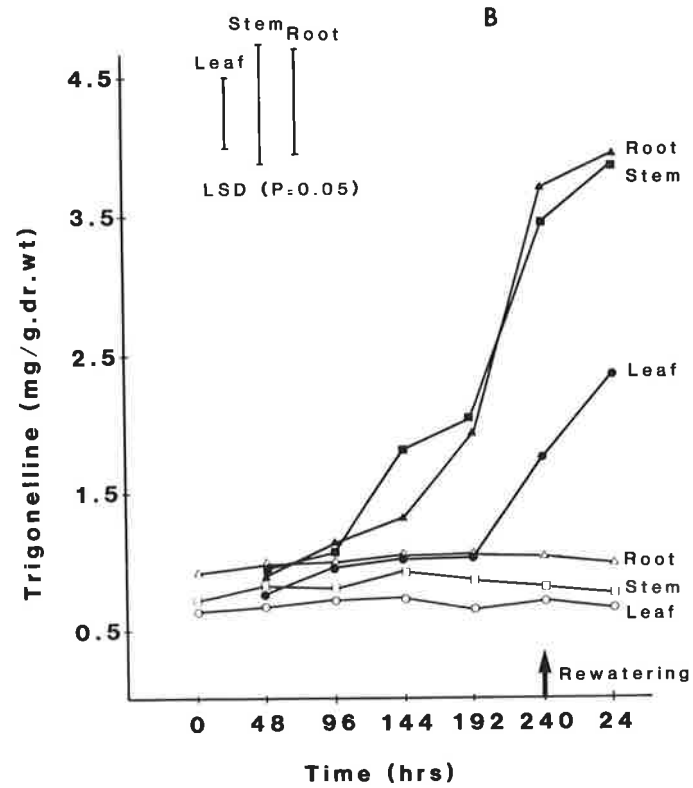
Trigonelline in leaves did not increase significantly until 240 hours after withholding the moisture (Fig. 13B). In stems there was no significant increase in trigonelline until 196 hours, after which it increased sharply. A significant increase was found in roots after 144 hours and trigonelline continued to increase rather sharply after 196 hours. Once again stem and roots showed higher amounts of trigonelline than leaves.

24 hours after rewatering the plants were analysed for trigonelline. It was interesting to find that, unlike proline and stachydrine, trigonelline showed no decrease. The reasons for the

Fig. 13 A. Accumulation of stachydrine in different organs of alfalfa seedlings after exposing to a period of water stress and after alleviation.

B. Accumulation of trigonelline in different organs of alfalfa seedlings after exposing to a period of water stress and alleviation.


A
Stem
Root
Leaf
LSD (P=0.05)
4.0
3.0
2.0
1.0
Stachydrine (mg/g.dr.wt)
Root
Stem
Leaf
Root
Stem
Leaf
Rewatering
0
48
96
144
192
240
24
Time (hrs)


B
Stem
Root
Leaf
LSD (P=0.05)
4.5
3.5
2.5
1.5
0.5
Trigonelline (mg/g.dr.wt)
Root
Stem
Leaf
Root
Stem
Leaf
Rewatering
0
48
96
144
192
240
24
Time (hrs)

continued increase, even after alleviating the stress, are not known at this stage.

3.4 DISCUSSION

The effects of water stress (drought) on plants are extensive. The major physiological changes induced are the reduction in leaf water status and the elevation of endogeneous levels of certain amino acids and quaternary ammonium compounds. The levels of water stress in alfalfa in the present experiments were measured by the reduction of plant water potentials which decreased from -0.30 to -1.35 MPa during the experimental period (Fig. 12A).

Proline accumulation in response to similar water deficits has been reviewed recently by Stewart and Larher (1980), and Aspinall and Paleg (1981). Proline accumulates in all organs of the intact plant during water deficit (Chen et al, 1964; Barnett and Naylor, 1965; Singh et al, 1973b).

In the present experiment, proline content increased significantly in all the organs of alfalfa plants (Fig. 12B). Roots and stem showed higher amounts of proline than leaves. Similar high amounts of proline have been found in roots of several species under stress (Chu, 1974; Goring and Thein, 1979; Carceller and Fraschina, 1980). Apart from the overall reduction in the plant water potential, an alternative explanation may be that leguminous crops synthesise most of their amino acids in their roots and translocate them to the shoot (Wallace and Pate, 1967). Since alfalfa is a legume it falls into that category. The increase in the stem has been reported beofore in (Huber and Eder,

1982). In alfalfa, the accumulation of higher levels of proline in the stem raises the question whether it was synthesised in the stem or translocated there from the roots or leaves. Leaves also accumulated proline but relatively less compared to the roots and stem. In the present experiment in which stress initiation was slow, the effect on leaves may have also been slow. Such differences between slow, gradual stress and shock stress have been considered before (Singh et al, 1973a).

Imposing water stress also brings about the accumulation of nitrogenous compounds group (Storey, 1976; Hanson et al, 1978). Stachydrine and trigonelline are such compounds which are known to occur in alfalfa (Steenbock, 1918; Robertson and Marion, 1959a,b,c, 1960; Conner et al, 1973; Sethi and Carew, 1974). However, there is no evidence of the accumulation of stachydrine under water stress. In the present investigation stachydrine accumulation increased in all the organs when alfalfa plants were exposed to water stress (Fig. 13A). Stachydrine is closely related to proline and the large amounts of proline accumulated in response to the reduction in water potential may have contributed to the accumulation of stachydrine. This may also account for the large amounts of stachydrine in roots and stem in which there were more proline accumulated.

Although trigonelline has been reported in alfalfa (Blake, 1954; Robertson and Marion, 1959a), its behavior under water stress has not been studied before. Water stress in this experiment increased the trigonelline content quite significantly in all the organs (Fig. 13B). The differences between the organs cannot be explained at this stage as there is no evidence of the site of synthesis or metabolism of

trigonelline. However, an hypothetical suggestion has been made by Klein and Linser (1932) to the effect that proline contributes to the synthesis of trigonelline. If so, it is possible that in alfalfa the higher amounts of proline in roots and stem are responsible for the higher trigonelline levels.

Proline accumulated during water stress of alfalfa decreased rapidly 24 hours after rewatering. Such a reduction in proline, after eliminating the water deficit, has been found in many plant species (Stewart, 1972b; Singh et al, 1973b; Blum and Ebercon, 1976; Parameshwana and Sastry, 1980). The decrease in proline has been attributed to the recovery of the water status (Wample and Bewley, 1975) which was also observed in alfalfa (Fig. 12A). A likely mechanism involves the oxidation of proline to glutamate (Stewart, 1972a; Singh et al, 1973b; Sells and Koeppe, 1981). This oxidation results in carbon being fed into the kreb's cycle (Stewart 1972c) and utilised in the metabolic processes.

Stachydrine also decreased, but more slowly, 24 hours after rewatering. The explanation for the difference in rates is not known. However, one would anticipate that a compound structurally related to proline would undergo similar biochemical changes. Trigonelline, on the other hand, did not decrease; instead it continued to accumulate on relief of stress in alfalfa. This suggests that trigonelline may not be involved in similar metabolic processes, at least not within 24 hours. This aspect requires further experimentation.

SECTION 4 : EFFECT OF NaCl STRESS ON PROLINE, STACHYDRINE AND TRIGONELLINE ACCUMULATION IN DIFFERENT PARTS OF ALFALFA

4.1 INTRODUCTION

Salinity creates an ubiquitous problem for crop species which are predominantly sensitive to the presence of high concentrations of salts in the soil. Under these conditions the water potential is lowered due to the lowered osmotic potential. This change in the osmotic potential, and, in turn, water potential, brings about several biochemical changes in plants, such as increases in the concentration of free proline and some quaternary ammonium compounds.

Accumulation of free proline in plants, when subjected to salinity, has been studied by Palfi and Juhasz (1970). A substantial increase in proline concentration under saline conditions was found in halophytes (Storey and Wyn Jones, 1978a,b). Increase in proline were also found in barley (Chu et al, 1976a; Storey and Wyn Jones 1978b; Buhl and Stewart 1983) and in wheat (Dreir, 1983a, 1983b) under saline conditions.

In addition to proline some plant species also accumulate large amounts of quaternary ammonium compounds in response to salinity. Storey and Wyn Jones (1977) found large amounts of glycine betaine in halophytes when they were grown at high NaCl. Storey and Wyn Jones (1975) also found large amounts of glycine betaine in salt tolerant grasses such as barley and *Chloris gayana* at high NaCl. Hanson and Wyse (1982) found an increase in glycine betaine in sugarbeet when subjected to high salinity and *Spartina alterniflora* showed an increase in glycine betaine and proline when subjected to NaCl (Cavalieri and Huang, 1981).

Storey and Wyn Jones (1977) also showed a slight increase in trigonelline in *L.esculentum*, *P.satium*, *P.vulgaris* in response to high salt.

The experiments in this section were designed to study the effect of NaCl on the accumulation of Proline, Stachydrine and Trigonelline in different parts of alfalfa seedlings.

4.2 METHODS

4.2.1 GROWING PLANTS

The alfalfa plants variety Hunter River were grown in fine sand in a growth cabinet with light intensity maintained at 600 μE $m^{-2}s^{-1}$ and with a 12 hour photoperiod. Temperature was 24/21 $\pm 1^{o}C$ day/night. The plants were watered regularly with half strength Hoagland's nutrient solution. When the plants were 3 weeks old, they were used for the experiments.

4.2.2 IMPOSING SALINITY STRESS

Salinity stress was imposed by applying NaCl solutions to the sand medium. The different levels of stress were achieved by applying NaCl ranging from 50 to 500 mM. During the alleviation of stress the sand was washed thoroughly with distilled water to remove the applied NaCl.

4.2.3 HARVESTING THE PLANT TISSUE

After the treatment and at the end of each period of time the plant tissue was taken out of the pots and washed thoroughly. The leaves, stem

and roots were separated quickly by cutting with a scalpel blade and the separated parts were put into glass vials and frozen with liquid nitrogen. The frozen samples were dried in a freeze drier. After taking the dry weights the samples were stored at -20^{o}C for further analysis.

4.2.4 WATER POTENTIAL

Water potential measurements were made on the stem at the end of each 24 hour period after NaCl treatment. The method is described in Chapter III, 2.4.

4.2.5 PROLINE CONTENT

Proline content was estimated by the Singh et al, (1972) method. (Chapter III, 2.5.1).

4.2.6 STACHYDRINE AND TRIGONELLINE CONTENT

Stachydrine and Trigonelline were measured in the same sample used for proline estimation by dividing the extracts into half. The method for the quantitiave estimation is described in Chapter IV, Section 1 and 2.

4.3 RESULTS

4.3.1 WATER POTENTIAL

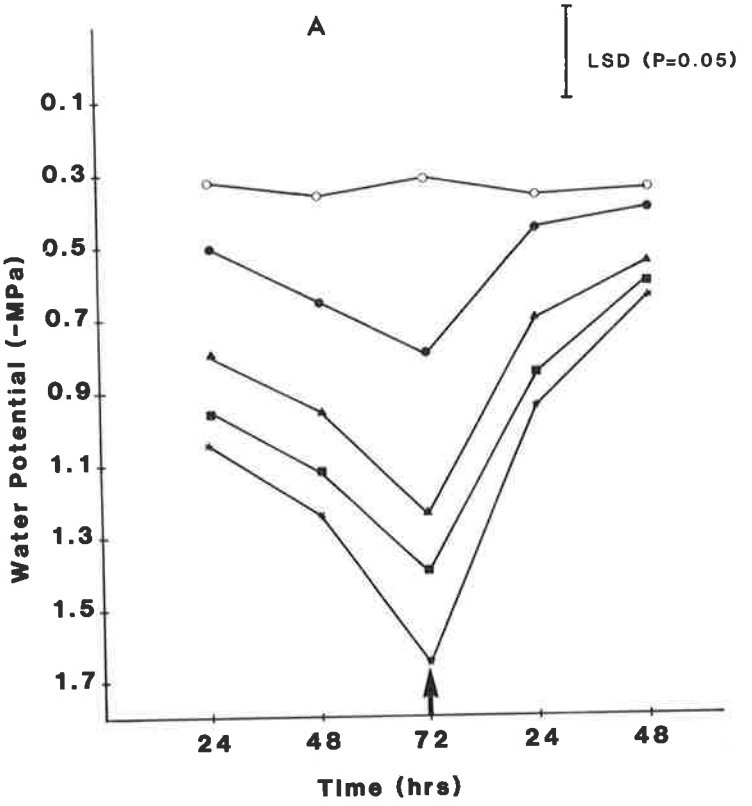
The water potential of the NaCl-stressed plants decreased in relation to the concentration of NaCl applied (Fig. 14A). The plants

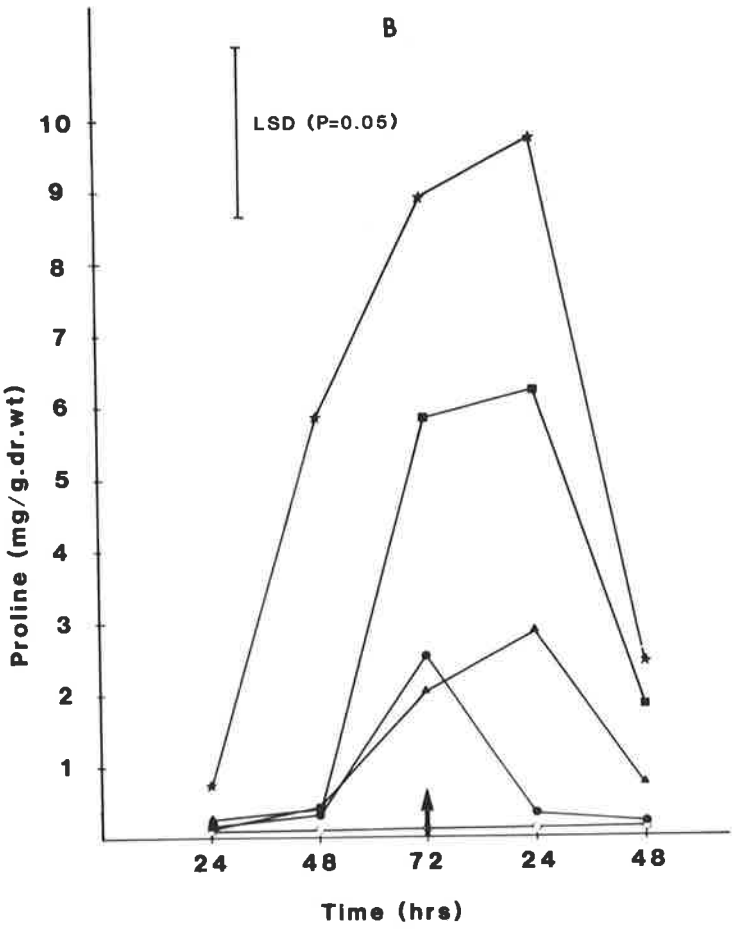
Fig. 14 A. Plant water potential of alfalfa seedlings after exposing them to different levels of NaCl stress.

○- control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★- 500 mM.

B. Proline accumulation in alfalfa leaves after exposing to different levels of NaCl stress.

○- control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★- 500 mM.


A
LSD (P=0.05)
0.1
0.3
0.5
0.7
0.9
1.1
1.3
1.5
1.7
Water Potential (-MPa)
24
48
72
24
48
Time (hrs)


B
LSD (P=0.05)
10
9
8
7
6
5
4
3
2
1
Proline (mg/g.dr.wt)
24
48
72
24
48
Time (hrs)

supplied with 50 mM NaCl showed a decrease in water potential from -0.30 MPa to -0.50 MPa by 24 hours. The water potential decreased significantly to -0.80 MPa by 72 hours. 100 mM NaCl showed a significant decrease in 24 hours (-0.80 MPa) and decreased further to -1.25 MPa by 72 hours stress. Plants supplied with 200 and 500 mM NaCl showed -0.95 and -1.05 MPa after 24 hours and -1.4 and -1.65 MPa after 72 hours of stress respectively. After 72 hours the decrease in water potentials of the plants was significant in all the treatments. The control plants showed no significant change in water potential during this period.

When the stress was relieved (all the NaCl washed out), the plants water potentials returned to almost normal levels within 48 hours depending on the concentration of NaCl applied. This indicates that the plants were capable of returning to their normal water status after the alleviation of stress.

4.3.2 PROLINE ACCUMULATION IN DIFFERENT PARTS

LEAVES

Free proline content was measured in the leaves at 24 hours intervals. In the first 24 hours after treatment, except for a slight increase in 500 mM NaCl treated leaves, there was no significant increase in leaf proline content (Fig. 14B). At the end of 48 hours a similar trend was observed. Proline content increased significantly in all the concentrations of NaCl by 72 hours. The highest level was reached in 500 mM (8.9 mg/g.dr.wt) and the lowest 50 mM (2.5 mg/g.dr.wt). The increase in proline content was related to the decrease in plant water potential.

At the end of 72 hours, when the stress was relieved (washed all the NaCl out of the medium), the proline content of the leaves started to decrease. The plants with 50 mM showed the fastest decrease which may have been due to the mild stress the plants experienced. In the other three NaCl concentrations, the leaf proline content was still increasing even after 24 hours of recovery, suggesting that the more severely stressed plants may require a longer period to recover. However, at the end of 48 hours after stress relief in all the treatments, the proline content decreased to approximately the same level as the control. The control plants showed no significant changes in proline content during this period.

STEM

The proline content in the stem was also measured periodically. No significant increase was observed in the first 24 hours of NaCl treatment at any concentration (Fig. 15A). At the end of 48 hours only 500 mM NaCl showed a significant increase in proline content (2.83 mg/g.dr.wt). At the end of 72 hours all the concentrations showed a significant increase, the highest (5.8 mg/g.dr.wt) of which was observed in 500 mM and the lowest (2.2 mg/g.dr.wt) of which was observed in 50 mM NaCl.

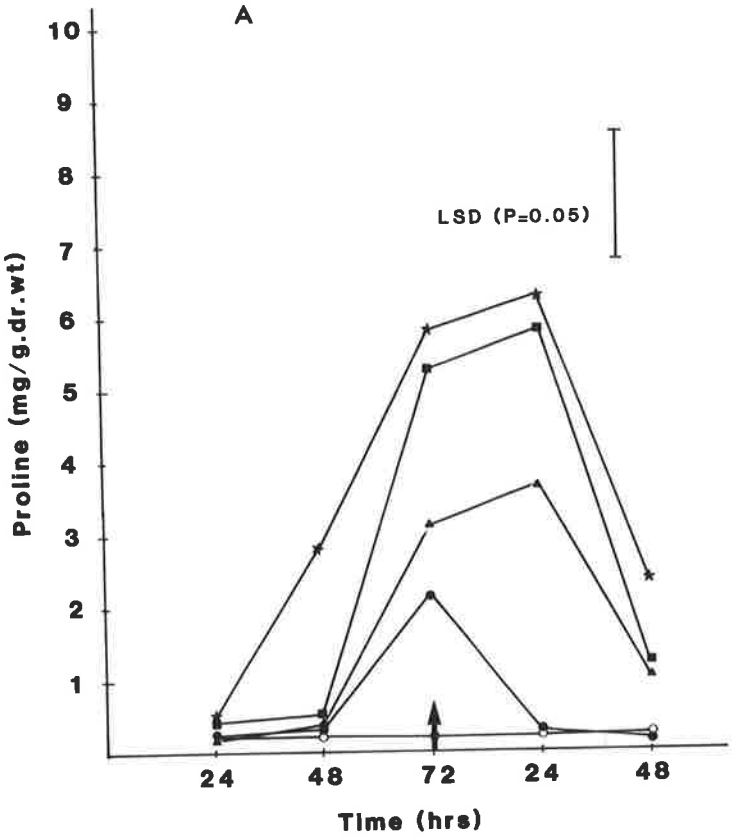
When the stress was relieved the proline content in the stem decreased in the 50 mM NaCl-treated plants to control levels within 24 hours. The plants with higher concentrations of NaCl (100, 200 and 500 mM) showed a further increase in proline content for the next 24 hours. However, 48 hours after the alleviation of stress they showed a rapid

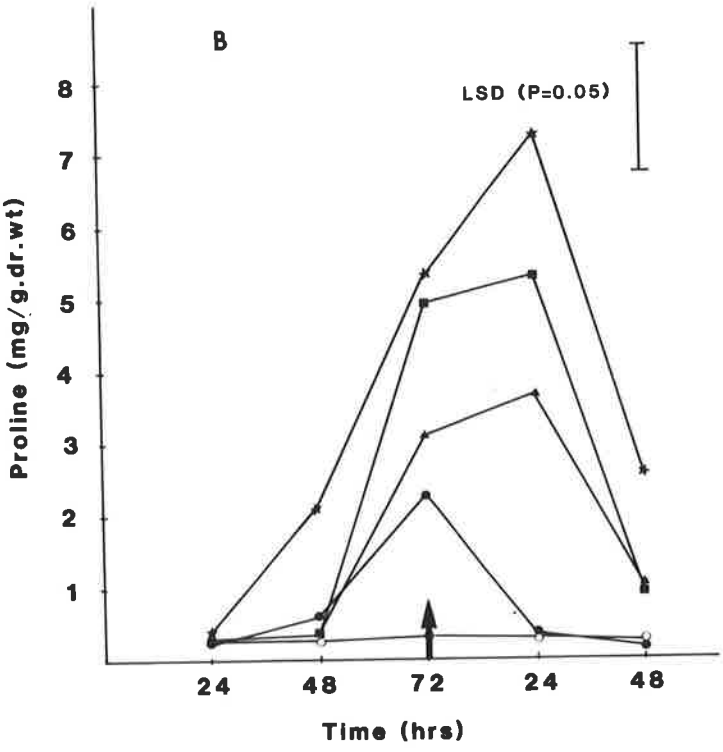
Fig. 15 A. Proline accumulation in alfalfa stem after exposing to different levels of NaCl stress.

○- control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★- 500 mM.

B. Proline accumulation in alfalfa roots after exposing to different levels of NaCl stress.

○- control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★- 500 mM.


A
LSD (P=0.05)
Proline (mg/g.dr.wt)
10
9
8
7
6
5
4
3
2
1
24
48
72
24
48
Time (hrs)


B
LSD (P=0.05)
Proline (mg/g.dr.wt)
8
7
6
5
4
3
2
1
24
48
72
24
48
Time (hrs)

decrease in proline content. The proline content in the control plants showed no significant changes throughout the experimental period.

ROOTS

Roots also accumualted large amounts of proline with the NaCl stress (Fig. 15B). Like leaves and stem, roots also showed no significant increase in proline content in the first 24 hours. The response was significant at 72 hours after stress. The proline content increased signficiantly at all the salt concentrations (50 mM - 2.3, 100 mM - 3.1, 200 mM - 5.0, 500 mM - 5.3 mg/g.dr.wt) respectively.

After the alleviation of stress the proline content in the roots decreased significantly. As in leaves and stem only 50 mM treated plants showed a fast decrease. The other concentrations showed the decrease in proline content after 48 hours. This prolonged time for the decrease may be due to the severity of stress at higher concentrations.

4.3.3 STACHYDRINE ACCUMULATION IN DIFFERENT PARTS

LEAVES

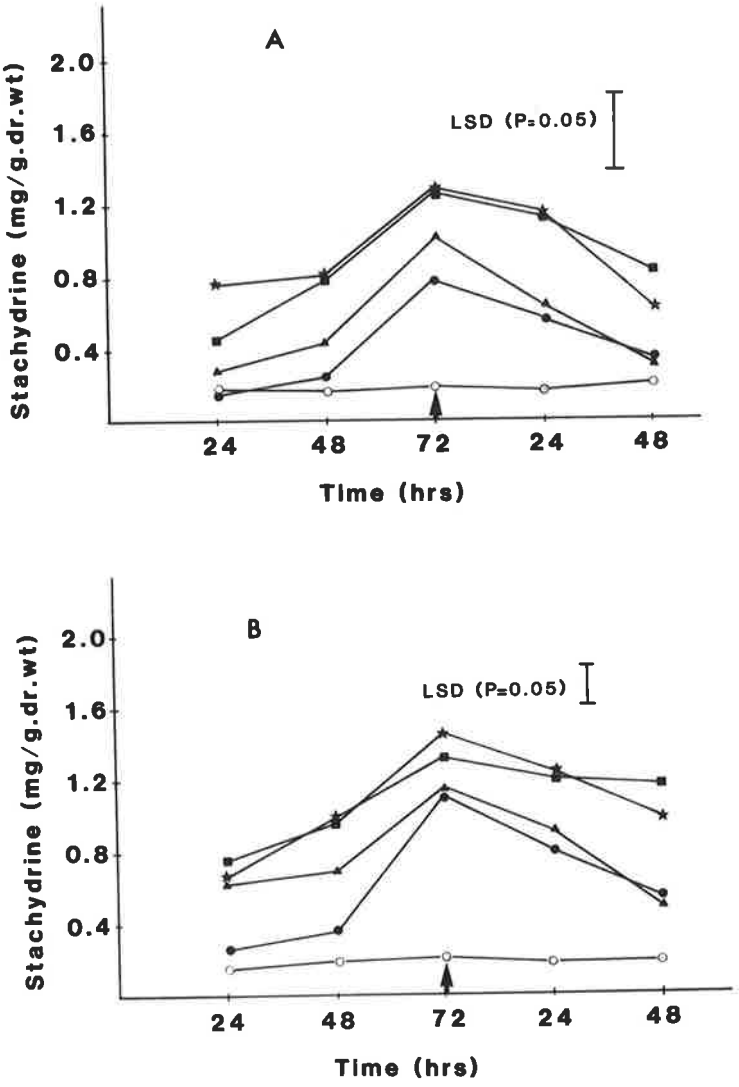
The leaf stachydrine content was measured periodically after subjecting the plants to NaCl stress. The stachydrine content increased after 24 hours of stress to varied degrees depending on the concentration of NaCl applied (Fig. 16A). At the end of 72 hours of stress the stachydrine content had increased significantly in plants at all the concentrations of NaCl. Though the stachydrine content increased significantly when compared to control plants, there was no significant variation in the effects of the different NaCl concentrations.

Fig. 16 A. Stachydrine accumulation in alfalfa leaves after exposing to different levels of NaCl stress. Arrow indicates the relief of stress.

○- control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★- 500 mM.

B. Stachydrine accumulation in alfalfa stem after exposing to different levels of NaCl stress. Arrow indicates the relief of stress.

○- control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★- 500 mM.


A
LSD (P=0.05)
Stachydrine (mg/g.dr.wt)
2.0
1.6
1.2
0.8
0.4
24
48
72
24
48
Time (hrs)
B
LSD (P=0.05)
Stachydrine (mg/g.dr.wt)
2.0
1.6
1.2
0.8
0.4
24
48
72
24
48
Time (hrs)

After rejuvinating the plants by washing away all the NaCl, the stachydrine content, unlike proline, decreased in all the treatment. The decrease of stachydrine was gradual and by 48 hours after recovery it had decreased substantially in all the treatments.

STEM

The stachydrine content in the stem, as shown in Fig. 16B), increased to approximately the same levels as in leaves. Within 24 hours the effect of stress was evident and the stachydrine content in the stem increased dramaticaly in 100, 200 and 500 mM NaCl. At the end of 72 hours of stress the stachydrine content was significantly greater than in control plants.

After the alleviation of stress, unlike in the leaves, the stachydrine content of the stem also decreased but at a slower rate. Even after 48 hours of recovery none of treatments decreased to control levels, suggesting that the stachydrine content in stems may not be metabolized as quickly as in the leaves.

ROOTS

In roots the accumulation of stachydrine was clearer, and more directly a function of the different concentrations of NaCl applied (Fig. 17A). Within 24 hours there was a significant increase with 200 and 500 mM NaCl treatments. By 48 hours of stress even 100 mM treated plants showed a significant increase. At 72 hours after stress all the concentrations showed a large increase in stachydrine content over the

control plants. 500 mM NaCl showed the highest root stachydrine content (3.2 mg/g.dr.wt) and the lowest was with 50 mM NaCl (1.4 mg/g.dr.wt).

After the alleviation of stress the root stachydrine content decreased gradually in all treatments. Due to the direct contact with NaCl, the roots may have been more affected and, perhaps, needed more time to recover and establish normal biosynthetic activities.

4.3.4 TRIGONELLINE ACCUMULATION IN DIFFERENT PARTS

LEAVES

Trigonelline content was measured in the samples which were used for proline and stachydrine measurements. Trigonelline content in the leaves, like proline and stachydrine, also increased (Fig. 17B). By 24 hours of stress 500 mM treatment showed a significant increase. The other concentrations, except 50 mM, showed a significant increase after 48 hours of stress. At the end of 72 hours of stress the increase in trigonelline content was significantly higher in plants treated with 50 mM NaCl showed a lesser amount of trigonelline (1.7 mg/g.dr.wt).

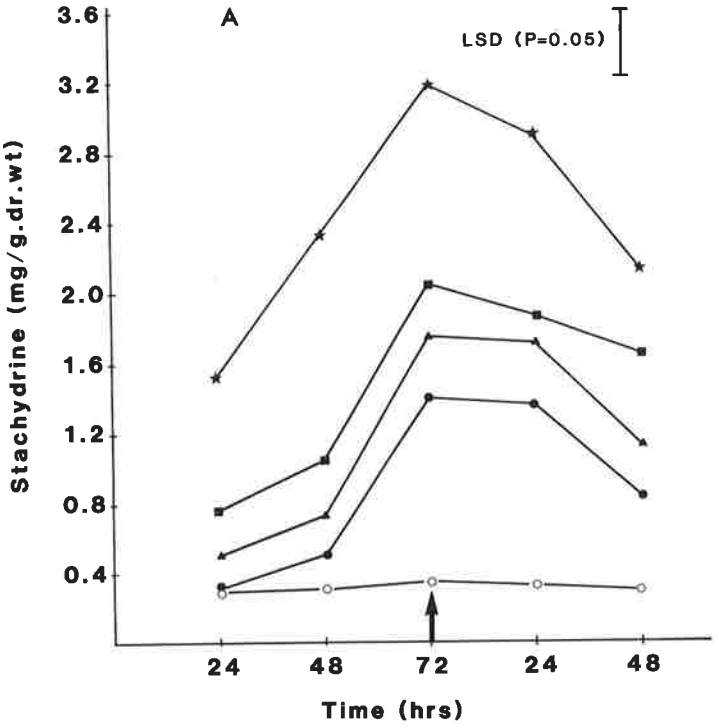
It is interesting to note that when stress was alleviated the trigonelline content continued to increase for a further 24 hours (Fig. 17B) in all treatments. Surprisingly, the trigonelline content did not decrease even afater 48 hours recovery. The reason for the maintenance of the trigonelline level even after the alleviation of stress, is not known.

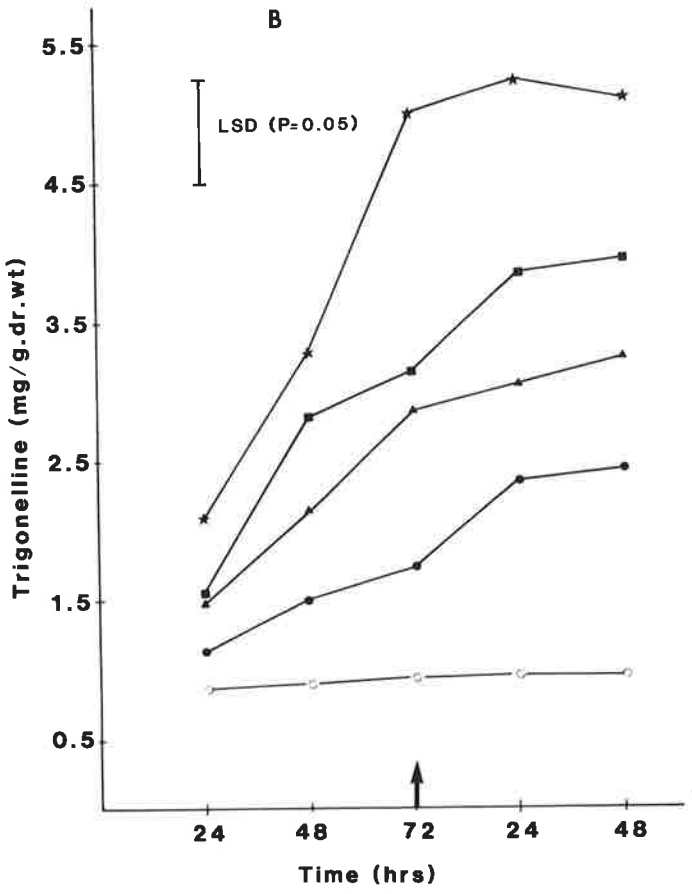
Fig. 17 A. Stachydrine accumulation in alfalfa roots after exposing to different levels of NaCl stress. Arrow indicates the relief of stress.

○- control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★- 500 mM.

B. Trigonelline accumulation in alfalfa leaves after exposing to different levels of NaCl stress. Arrow indicates the

○- control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★- 500 mM.


A
LSD (P=0.05)
Stachydrine (mg/g.dr.wt)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
24
48
72
24
48
Time (hrs)


B
LSD (P=0.05)
Trigonelline (mg/g.dr.wt)
5.5
4.5
3.5
2.5
1.5
0.5
24
48
72
24
48
Time (hrs)

STEM

The trigonelline content in stems did not increase in the first 24 hours of NaCl stress in any of the concentrations tested (Fig. 18A). At the end of 48 hours the plants with 200 and 500 mM NaCl showed a significant increase over control. At the end of 72 hours, all but 50 mM treated plants showed a significant increase in trigonelline.

After the alleviation of stress the trigonelline content in the stem, at all NaCl concentrations, showed a steady increase even after 48 hours of recovery (Fig. 18A). However, the rate of accumulation seemed to have been reduced. Once again, as in leaves, there was no decrease in trigonelline in the stem.

ROOTS

Interestingly, the trigonelline content in the roots increased significantly over the control plant within 24 hours of stress treatment (Fig. 18B). A similar trend was observed at 48 hours after stress. The rapidity of trigonelline accumulation in roots may be due to the direct contact with NaCl which could alter the root water status very quickly. At the end of 72 hours the increase was even more pronounced.

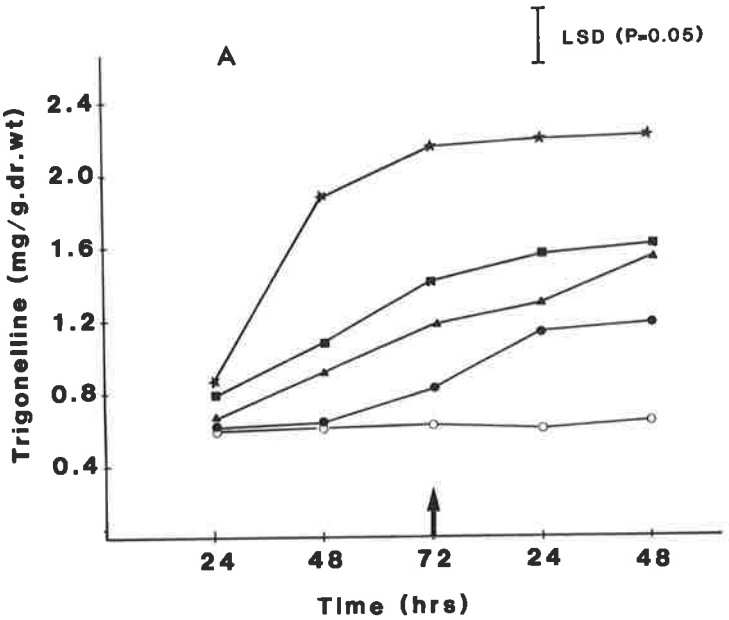
The alleviation of stress did not decrease the trigonelline content in roots (Fig. 18B). Instead, there was a continued increase up-to 48 hours after alleviation of stress. Once again the reasons for this response are not known. The control roots, like in leaves and stems, did not show any significant change in trigonelline content.

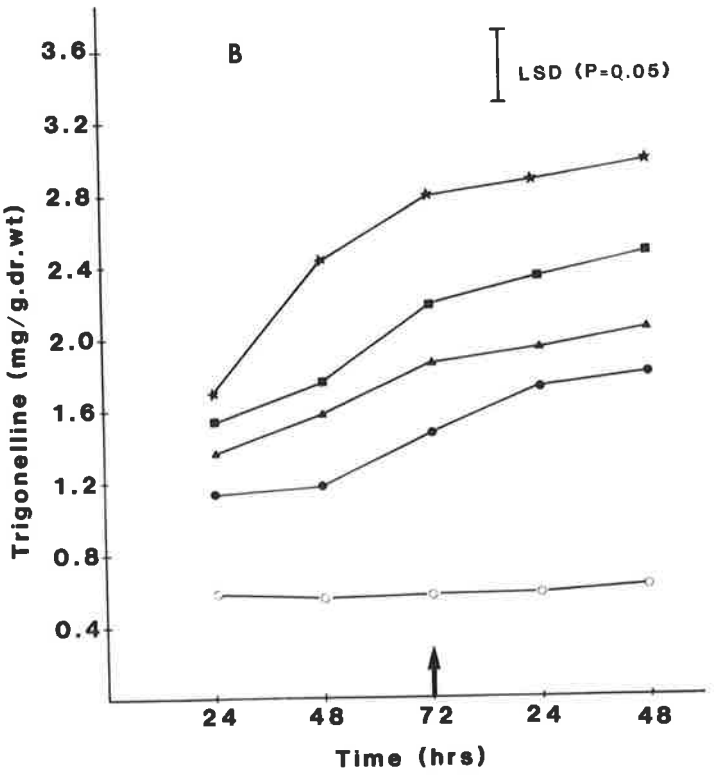
Fig. 18 A. Trigonelline accumulation in alfalfa stem after exposing to different levels of NaCl stress. Arrow indicates the relief stress.

○- control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★- 500 mM.

B. Trigonelline accumulation in alfalfa roots after exposing to different levels of NaCl stress. Arrow indicates the relief stress.

O - control, ●- 50 mM, ▲- 100 mM, ■- 200 mM and ★ - 500 mM.


A
LSD (P=0.05)
Trigonelline (mg/g.dr.wt)
2.4
2.0
1.6
1.2
0.8
0.4
24
48
72
24
48
Time (hrs)


B
LSD (P=0.05)
Trigonelline (mg/g.dr.wt)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
24
48
72
24
48
Time (hrs)

4.4 DISCUSSION

Exposure of plants to increased levels of salt in the medium surrounding the roots results in physiological changes which are potentially inhibitory to plant growth. One such noticable change is the reduction in the water status (Stewart and Lee, 1974; Chu et al, 1976a; Wyn Jones and Storey, 1981; Buhl and Stewart, 1983) as a result of reduction in the potential gradient for water transfer from the medium into the cells of the plants. The present work in alfalfa has demonstrated that there is a striking reduction in the plant water-potential as a result of exposure to different concentrations of NaCl (Fig. 14A). The reduction in plant water-potential was proportional to the concentration of the NaCl.

In the present investigation, it has also been shown that proline accumulation increases in response to salt stress in different parts of the alfalfa plant. Similar accumulation of proline as a result of salt stress have been found in tomato and cotton plants (Strogonov, 1964); paprika, sunflower, peas (Palfi and Juhasz, 1970); barley (Chu, 1974; Buhl and Stewart, 1983) and in wheat (Chauhan et al, 1983; Dreier, 1983a,b). Proline accumulated to different amounts in the different organs measured. At lower concentrations of NaCl, the differences in proline between the organs was not pronounced. However, when the concentration of NaCl increased to 500 mM, the differences in the amounts of proline in the organs were apparent. Although the effects of salinity appeared to be dependent on the rate at which the plant was exposed to the increase in salinity, proline accumulation was also related to the level of salinity imposed in the different treatments. In addition to the reduction of tissue water status, other effects of salt

on proline metabolism have been suggested. Increased synthesis of proline from glutamic acid, and no feedback inhibition of proline synthesis, have been found by Buhl annd Stewart (1983).

A similar control of proline synthesis from glutamate has been demonstrated in non-stressed barley leaves (Boggess et al,. 1976b). The decrease in proline oxidation (Buhl and Stewart, 1983), and the inhibition of incorporation of proline into protein (Stewart and Boggess, 1978; Buhl and Stewart, 1983) have also been suggested to be the major contributors to the accumulation of proline under salt stress. Such metabolic changes due to salt may also have caused the increased levels of proline in alfalfa.

Increased amounts of stachydrine have been found in alfalfa when exposed to salt treatment Wyn Jones and Storey (1981). Increases in stachydrine in the present exercise, as a result of NaCl, is in accordance with their findings. Although there is no definite evidence, one possible explanation may be that the reduction in water-potential due to salt, which increased proline, may also have increased the synthesis of stachydrine. Alternatively, alfalfa plants may accumulate stachydrine as an adaptive mechanism to abrupt environmental conditions. This possibility may be more likely with the presence of higher amounts of stachydrine in alfalfa normally grown in coastal habitats (Le Rudulier, 1982). Stachydrine accumulation seems to be more sensitive to NaCl than proline accumulation. This was demonstrated by the increase in stachydrine within 24 hours (Figs. 16A,B and 17A), whereas, proline increased only after 48 hours. Stachydrine accumulation was proportional to the concentration of NaCl applied. Such concentration dependent accumulation was also found by Wyn Jones and Storey (1981). It is

possible, of course, that no increase in proline was found in the first 24 hours because it was being converted into stachydrine.

Trigonelline in the present experiments also increased significantly in response to salt treatment (Figs. 17B, 18A,B). Such changes (although not to this level), have been reported in *Pisum sativum*, and *Phaseolus vulgaris* as a result of NaCl treatment (Wyn Jones and Storey, 1981). Leaves showed higher levels (Fig. 17B) followed by roots (Fig. 18B) and stem (Fig. 18A). Storey and Wyn Jones (1977) also found such differential accumulation between shoots and roots in *Pisum sativum*. The exact explanation for this differential accumulation is not known.

Relief of stress, by washing the NaCl from the root zone, returned the plant water potential almost to control levels (Fig. 14A). Such changes in water status have been observed in several species (Storey and Wyn Jones, 1977; Stewart, 1972b). Depending on the level of NaCl applied, proline decreased after stress relief. 50 mM NaCl treatment showed a faster reduction in proline than 500 mM, in all the plant parts.

These reductions can be related to the change in tissue water status (Wample and Bewley, 1975). Stachydrine also increased but the metabolic processes involved in the reduction of stachydrine after stress relief is not known. However, a direct relationship between stachydrine and proline cannot be ruled out. Trigonelline continued to accumulate even after stress relief. Similar results were found with water stress experiments in the previous section.

SECTION 5 : EFFECT OF LOW TEMPERATURE (COLD STRESS) ON THE ACCUMULATION OF PROLINE, STACHYDRINE AND TRIGONELLINE IN DIFFERENT PARTS OF ALFALFA

5.1 INTRODUCTION

The effects of low temperature (cold stress) on plants have long been of interest to biologists, both from the practical and the theoretical points of view. Only in recent years, however, have the biologists made an attempt to discover the biochemical and physiological processes of plants under low temperature conditions.

Several authors have reported an increase in total amino acids in response to low temperature. Pauli and Mitchell (1960) found an increase in free amino acids of from 5-12% of the total nitrogen in wheat when exposed to 2°C for two weeks. Wilding et al, (1960) reported a 20% increase in free amino acids in alfalfa roots. Reports of increases in free amino acids in other plants include spring wheat and winter rye seedlings exposed to 4°C for 3 weeks (Thomson and Zalik, 1974) and Lolium plants at 2°C for 2 weeks (Draper, 1972). Proline was invariably the main contributor to this increased free amino acid pool. There are inumerable reports showing the increase in proline content in crop species when exposed to low temperatures. (Trione et al, 1967; Gates et al 1971; Palfi and Juhasz, 1970; Stefl et al, 1978; Chu et al, 1978; Vezina and Paquin, 1982).

The present experiments were carried out to study the effect of cold stress on the accumulation of proline and particularly stachydrine and trigonelline.

5.2 METHODS

5.2.1 GROWING PLANTS

The alfalfa plants, var. Hunter River, were grown in fine sand in 5", 6" plastic pots. The pots were kept in a growth cabinet with temperatures of 25/21^{o}C day/night. The light intensity was maintained at 600 $\mu E\ m^{-2}s^{-1}$ with a 12 hour photoperiod. The plants were watered regularly with half strength Hoagland's nutrient solution, and, when they were three weeks old, they were treated.

5.2.2 LOW TEMPERATURE (COLD STRESS) TREATMENT

In order to impose cold stress the plants were transferred to a 2^{o}C cold room. The temperature at the canopy of the plants was maintained at 5^{o}C. 600 $\mu E\ m^{-2}s^{-1}$ light intensity was maintained with a 12 hours photoperiod and the plants were watered regularly with half strength Hoagland's nutrient solution.

5.2.3 HARVESTING THE PLANT TISSUE

After the treatment and at the end of each experimental period (48 hours interval) the plant tissue was harvested as described in Chapter III, 2.3.

5.2.4 WATER POTENTIAL MEASUREMENT

Water potential measurements were made on the stem at the end of each 48 hour period after the cold treatment. The method is described in Chapter III, 2.4.

5.2.5 PROLINE MEASUREMENT

Proline content was estimated by the Singh et al, (1972) method Chapter III, 2.5.1.

5.2.6 STACHYDRINE AND TRIGONELLINE MEASUREMENTS

Stachydrine and trigonelline were measured in the same sample used for proline estimation by dividing the extracts into half. The method is described in Section 1 and 2 of Chapter IV.

5.3 RESULTS

5.3.1 WATER POTENTIALS

The water potentials of the plants were measured at the end of every 48 hours. No significant change in the plant water potential was observed at any stress period (Fig. 19A). The water potentials were maintained around -3 to -4 MPa in both control and stressed plants. The picture was the same even after alleviating the stress.

5.3.2 PROLINE ACCUMULATION IN LEAVES, STEM AND ROOTS

Proline content was measured in leaves, stem and roots separately every 48 hours after the start of the stress. All the values are compared to the non-stressed control plants. The data is shown in (Fig. 19B).

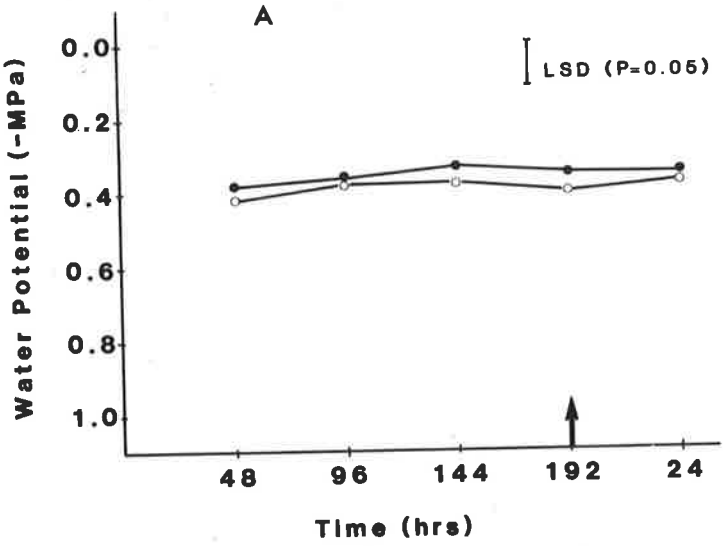
There was no significant increase in proline content, in the leaves after 48 hours of cold stress. Proline increased significantly and the

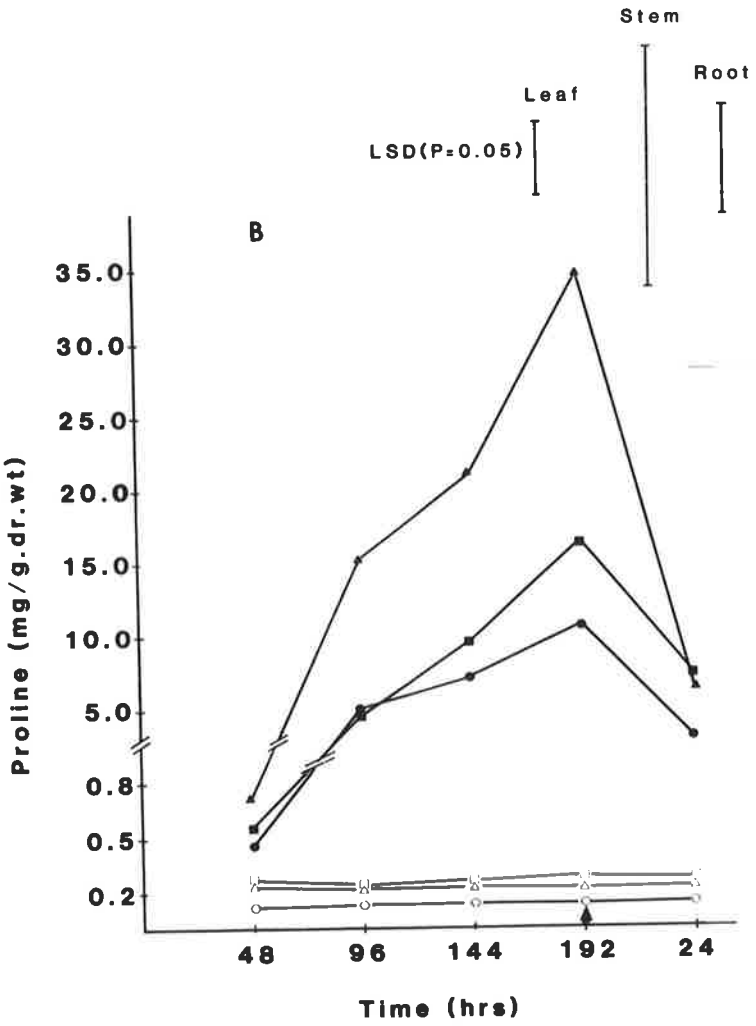
Fig. 19 A. Plant water potential of alfalfa seedlings after exposing to a period of cold stress ($5^{o}C$). Arrow indicates the relief of stress.

○- control, ●- stress.

B. Accumulation of proline in different organs of alfalfa after exposing to a period of cold stress ($5^{o}C$). Arrow indicates the relief of stress ($5^{o}C$). Arrow indicates the relief of stress.

○- control leaf, ●- stress leaf, △- control stem, ▲- stress stem, □- control roots, ■- stress roots.


A
LSD (P=0.05)
Water Potential (-MPa)
0.0
0.2
0.4
0.6
0.8
1.0
48
96
144
192
24
Time (hrs)


Stem
Root
Leaf
LSD(P=0.05)
B
Proline (mg/g.dr.wt)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.8
0.5
0.2
48
96
144
192
24
Time (hrs)

level continued to increase by 96 hours 192 hours after stress. Stems showed no significant changes by 48 hours after stress. However, the proline in stems increased dramatically after 48 hours and continued to increase at a faster rate. The maximum amount of proline (34.72 mg/g.dr.wt) was found at the end of 192 hours after stress. Once again there was a significant change, after 48 hours, in root proline and the level increased till 192 hours.

In order to study the behaviour of proline during recovery, the plants were released from stress by transfering them to their previous growth conditions. Within 24 hours the proline content of the leaves, stem and roots decreased significantly. The stem, which accumulated large amounts of proline, showed the greatest decrease in 24 hours after the alleviation of stress. This, perhaps, shows the capacity of the plants to recover metabolically after exposure to a stressful environment.

5.3.3 STACHYDRINE ACCUMULATION IN LEAVES, STEM AND ROOTS

Stachydrine content was measured periodically in leaves, stem and roots. The data is presented in (Fig. 20A). Leaves showed no significant increase in stachydrine at the end of 48 hours of stress. However, it increased significantly after 96 hours. As the stress progressed the stachydrine content continued to increase till the end of 192 hours (2.34 mg/g.dr.wt).

In stems, there was no significant increase in stachydrine for the first 48 hours. It increased at a faster rate after 48 hours and continued to increase till 192 hours (4.26 mg/g.dr.wt). The roots showed

a similr response in terms of time of accumualtion, and the stachydrine content of the roots was lower as compared to leaves and stem.

When the stachydrine content was measured 24 hours after alleviation of the cold stress there was, unlike proline, no decrease in any three types of tissue. Stems, which showed the highest amount of stachydrine, continued to accumulate it at the same rate. This continued increase in stachydrine 24 hours after alleviation of cold stress is quite different than the response to NaCl stress where it showed a decline.

5.3.4 TRIGONELLINE ACCUMULATION IN LEAVES, STEM AND ROOTS

Trigonelline was measured in the same samples used for proline and stachydrine measurements. In the first 48 hours of cold stress there was no significant increase (Fig. 20B). Though there was a slow increase at 96 and 144 hours, a significant increase was found only after 192 hours (1.79 mg/g.dr.wt). Stems responded differently. There was no change in the first 48 hours, but trigonelline increased drastically after 96 hours and continued to rise till 192 hours, and even after the alleviation of stress (6.46 mg/g.dr.wt). In roots trigonelline did not increase significantly until 144 hours and, as in leaves and stem, the maximum amount induced by stress was observed at 192 hours of stress (3.5 mg/g.dr.wt).

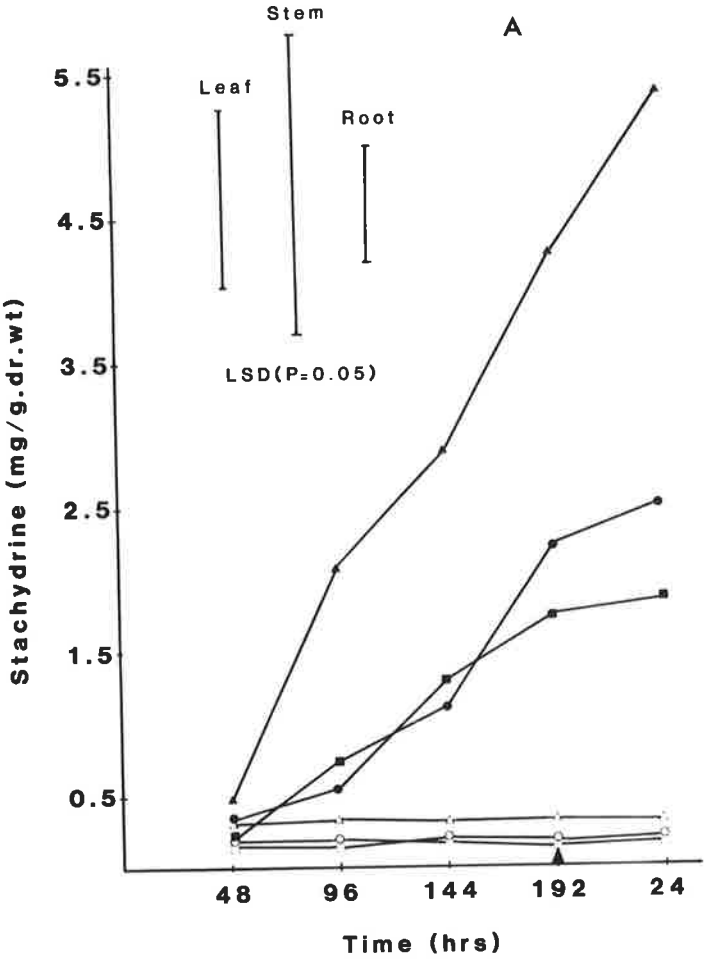
When the stress was alleviated by transferring the plants to normal growing conditions, the trigonelline continued to increase in leaves and stems. The content in the roots, however, showed no change.

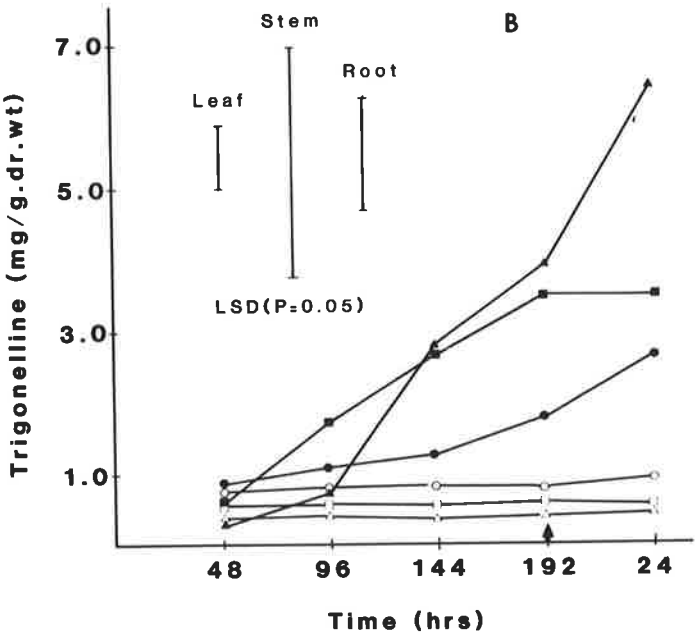
Fig. 20 A. Accumulation of stachydrine in different organs of alfalfa after exposing to a period of cold stress ($5^{o}C$). Arrow indicates the relief of stress.

○- control leaf, ●- stress leaf, △- control stem, ▲- stress stem, □- control roots, ■- stress roots.

B. Accumulation of trigonelline in different organs of alfalfa after exposing to a period of cold stress ($5^{o}C$). Arrow indicates the relief of stress.

○- control leaf, ●- stress leaf, △- control stem, ▲- stress stem, □- control roots, ■- stress roots.


A
Stem
Leaf
Root
LSD(P=0.05)
Stachydrine (mg/g.dr.wt)
5.5
4.5
3.5
2.5
1.5
0.5
48
96
144
192
24
Time (hrs)


B
Stem
Leaf
Root
LSD(P=0.05)
Trigonelline (mg/g.dr.wt)
7.0
5.0
3.0
1.0
48
96
144
192
24
Time (hrs)

5.4 DISCUSSION

The effects of cold stress on metabolic processes seems to be of a different nature than those of water or salt stress. One of the immediate effects observed due to water or salt stress was the change in water status (decrease in the water potential), but this was not the case under cold stress. This observation has been reported earlier in barley and radish (Chu, 1974). In the present experiments no significant changes in the plant's water potential were observed (Fig. 19A) and this is in accordance with the findings of (Chu, 1974).

The initiation of proline as a result of cold stress cannot be ascribed to a change in the water potential of the tissue. It is clear that proline accumulates in barley at temperatures below 12^{o}C without any concomitant decrease in the water potential of the tissue (Chu, 1974, Chu et al, 1978; Vezina and Paquin, 1982). Differences in the light- dependent processes have been suggested to be the cause for this accumulation (Chu, 1974). In the present experiments alfalfa showed a large increase in proline content in all the organs tested. Stem and roots accumulated more proline than the leaves (Fig. 19B). Similar differences in proline accumulation in response to cold stress have been found in wheat (Stefl et al, 1978) in *Zea mays* and *pisum sutivum* (Ozturk Szaniaboski, 1981). Vezina and Paquin (1982) suggested that leaves are necessary for proline synthesis and that the distribution and metabolism of amino acids are influenced by low temperature. They also found higher amounts of proline in alfalfa roots exposed to 1.5^{o}C. These findings have been related to the changes in the protein-proline interactions. One possible explanation could be that since proline accumulation is not due to changes in the water potential of the tissue under cold stress

there must be a specific factor/s which is enhancing the accumulation of proline, which may be more active in roots and stem than in leaves. However, it is difficult to decide whether proline accumulated in roots and stems during cold stress was translocated there form leaves, or synthesised *in situ* in roots and stem. Considering the nitrogen metabolism of alfalfa roots, one can conclude that the majority of proline is synthesised in the roots and translocated to the shoots. This is also supported by the higher amounts of proline in nodulated plants which is discussed in Section 9 of this chapter.

The accumulation of quaternary ammonium compounds under cold stress has not been studied so far. In alfalfa, as in the response to water or salinity stress, cold stress increased stachydrine and trigonelline (Figs. 20A and B). This increase cannot be related to tissue water potential as there was no change in tissue water potential during cold stress (Fig. 19A). Though the distribution of stachydrine and trigonelline in the plant parts is different to proline, there are similarities in the accumulation of all three compounds. The biosynthetic pathways of stachydrine and trigonelline under cold stress are not known. In fact, knowledge of the controls on the biosynthesis of proline under cold stress is also incomplete and scarce. The fact that all three compounds accumulate in response to cold stress, without alteration in the tissue water potential, may indicate a common 'factor/s' which initiates the accumulation. Abscisic acid is thought to be one such factor. Increases in ABA in plants under cold stress (Eamus and Wilson, 1983; Chen et al, 1983) may support such a claim. The findings in this regard are discussed in Section 7 of this chapter.

When plants were returned to normal temperature conditions, proline content dropped within 24 hours. Such a reduction in proline after alleviating the cold stress has been reported (Aspinall et al, 1973). Stachydrine and trigonelline continued to accumulate even after 24 hours. These differences suggest that there may be different mechanisms operating in the metabolising of proline, stachydrine and trigonelline following cold stress. Reduction in stachydrine within 24 hours after relieving water and salt stress may also indicate such a different mechanism.

SECTION 6 : EFFECT OF PEG STRESS ON THE ACCUMULATION OF PROLINE, STACHYDRINE AND TRIGONELLINE IN DIFFERENT PARTS OF ALFALFA

6.1 INTRODUCTION

Iso-osmotic solutions of different salts or non-permeating solutes have been used to assess the relative importantce of osmotic components of stress. Polyethylene glycol of high molecular weight appears to be suitable for this purpose. Several authors have used polyethylene glycol (PEG) as an osmotic agent to attain a uniform stress in their experiments (Lawlor 1970; Singh et al, 1973a; Steuter et al, 1981; Sanzchez-Diaz et al 1982; Spyropoulous, 1983).

Singh et al (1973a), Ayer (1981) and several others have found increased amounts of proline in barley in response to PEG treatment, and increased amounts of glylinebetaine were also found in PEG-treated barley plants (Storey et al 1978b, Hanson 1980).

For a better understanding of the complexities of alfalfa plants under adverse conditions, and possibly to compare the different kinds of environmental effects, the present exercise was designed to study the effect of PEG 4000. The accumulation patterns of proline, stachydrine and trigonelline were studied.

6.2 METHODS

6.2.1 GROWING PLANTS

The plants were grown as described in Chapter III, 2.1.1.

6.2.2 PEG STRESS TREATMENTS

Different concentrations of polyethylene glycol 4000 were prepared to obtain -5, -10 and -20 bars water potential. When plants were 3 weeks old, the PEG solutions were applied to the pots. The stress was relieved by washing out all the PEG with distilled water for the recovery studies.

6.2.3 HARVESTING THE PLANT TISSUE

Harvesting and preserving the tissue was done as described in Chapter III, 2.3 every 24 hours after PEG application.

6.2.4 WATER POTENTIAL MEASUREMENTS

Plant water potential was measured with a pressure bomb (Chapter III, 2.4).

6.2.5 PROLINE MEASUREMENTS

Proline content was measured at the end of each experimental period by the Singh et al (1972) method. (Chapter III, 2.5.1)

6.2.6 STACHYDRINE AND TRIGONELINE MEASUREMENT

Stachydrine and trigonelline were measured by the method described in Section 1 and 2 of this chapter.

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6.3 RESULTS

6.3.1 WATER POTENTIAL

Water potentials were measured on a separate set of plants in each treatment at all experimental periods. The water potentials of plants exposed to different levels of PEG declined in the first 24 hours (Fig. 21A). The decreases were significant 72 hours after stress. At this time, -20 bars PEG showed a rapid decrease in water potential (-2.2 MPa). However, when the osmotic stress was relieved, the water potential in the three different levels of PEG increased in 24 hours and by 48 hours it reached the level of well-watered control plants. This signifies the recovery of plants after a period of osmotic stress.

6.3.2 PROLINE CONTENT IN LEAVES, STEM AND ROOTS

In the leaves of plants exposed to PEG proline accumulation was dependant on the levels of PEG applied (Fig. 21B). There was no significant difference in proline accumulation between -5, -10 and -20 bars PEG in the first 48 hours. However, the increase during this period was significant over the well-watered control plants. The maximum accumulation of proline of all PEG levels was found 24 hours after relieving the stress. This may have been due to the application of PEG to the root zone. During this period maximum proline was found in plants treated with -20 bars PEG and the minimum was in plants treated with -5 bars PEG.

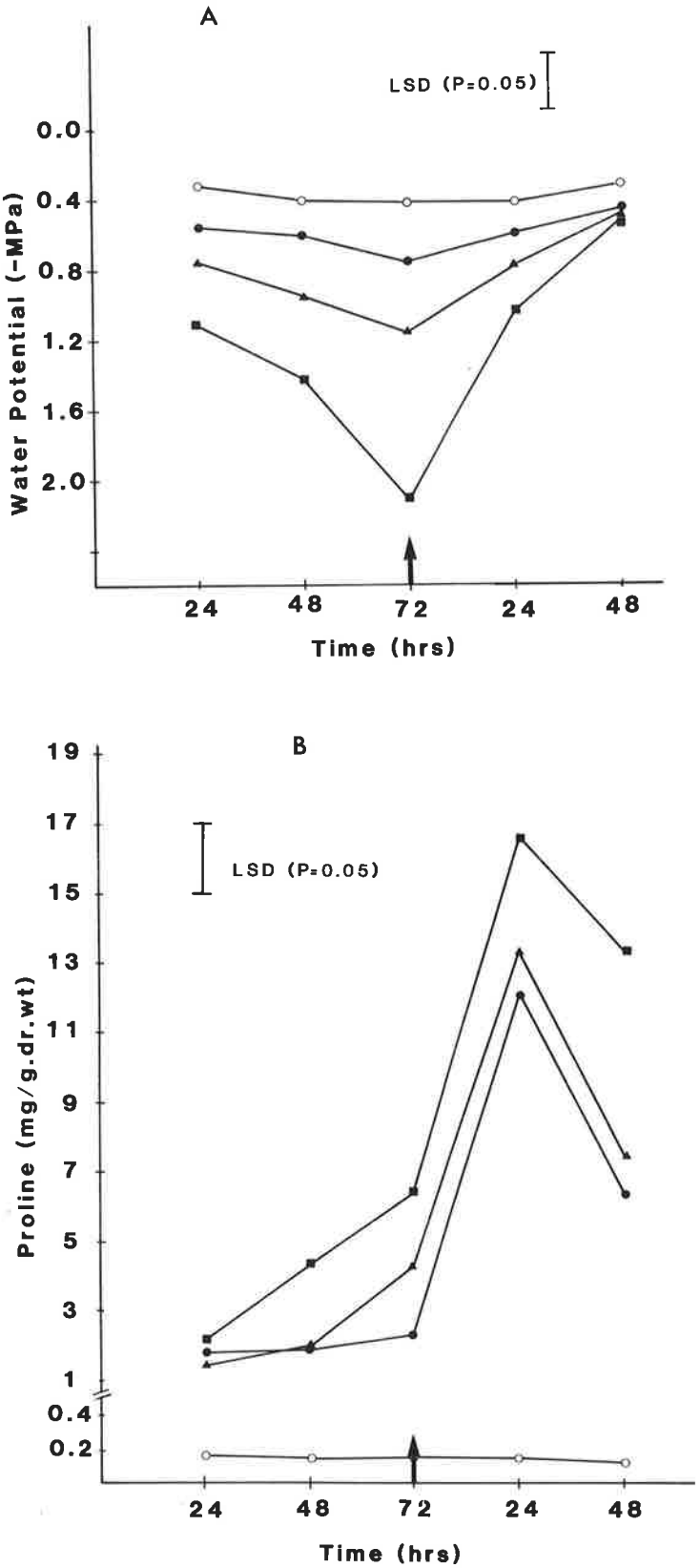
Proline continued to increase during the first 24 hours after relieving the stress and it increased rapidly after this time in all three PEG treatments.

Fig. 21 A. Plant water potential of alfalfa seedlings under PEG stress. Arrow indicates the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.

B. Proline accumulation in alfalfa leaves under different levels of PEG stress. Arrows indicate the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.


A
LSD (P=0.05)
0.0
0.4
0.8
1.2
1.6
2.0
Water Potential (-MPa)
24
48
72
24
48
Time (hrs)
B
LSD (P=0.05)
19
17
15
13
11
9
7
5
3
1
0.4
0.2
Proline (mg/g.dr.wt)
24
48
72
24
48
Time (hrs)

In stems, proline content increased significantly over control plants at all levels of PEG (Fig. 22a). No significant differences were found between -5, -10 and -20 bars PEG up to 72 hours. Significant differences were observed only 24 hours after relieving the stress at which time, as in leaves, proline was still showing an increasing trend. Between 24 and 48 hours after the stress was relieved, proline content decreased. This response was identical to the response of leaves.

Root tissue accumulated more proline than leaves and stems in the first 24 hours. There were no significant differences between the three levels of PEG until the beginning of the stress relief (Fig. 22b). However, as in the other tissues, the increase was significant over control plants. During this period -20 bars PEG was most effective in enhancing the proline levels in roots. The results also suggests that 48 hours after relieving the stress proline content was decreasing in all three levels of PEG.

6.3.3 STACHYDRINE CONTENT IN LEAVES, STEM AND ROOTS

Stachydrine content in leaves increased when the plants were exposed to different levels of PEG (Fig. 23a). No significant increase was found in the first 48 hours when -5 bars PEG was applied. However, stachydrine increased significantly after 48 hours. The plants supplied with -10 bars PEG showed a similar response. When -20 bars PEG was applied, stachydrine increased significantly within 24 bars and continued to increase at a fast rate. When the stress was relieved, stachydrine decreased significantly within 48 hours at all three levels of stress.

Fig. 22 A. Proline accumulation in alfalfa stem under different levels of PEG stress. Arrow indicates the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.

B. Proline accumulation in alfalfa roots under different levels of PEG stress. Arrows indicate the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.

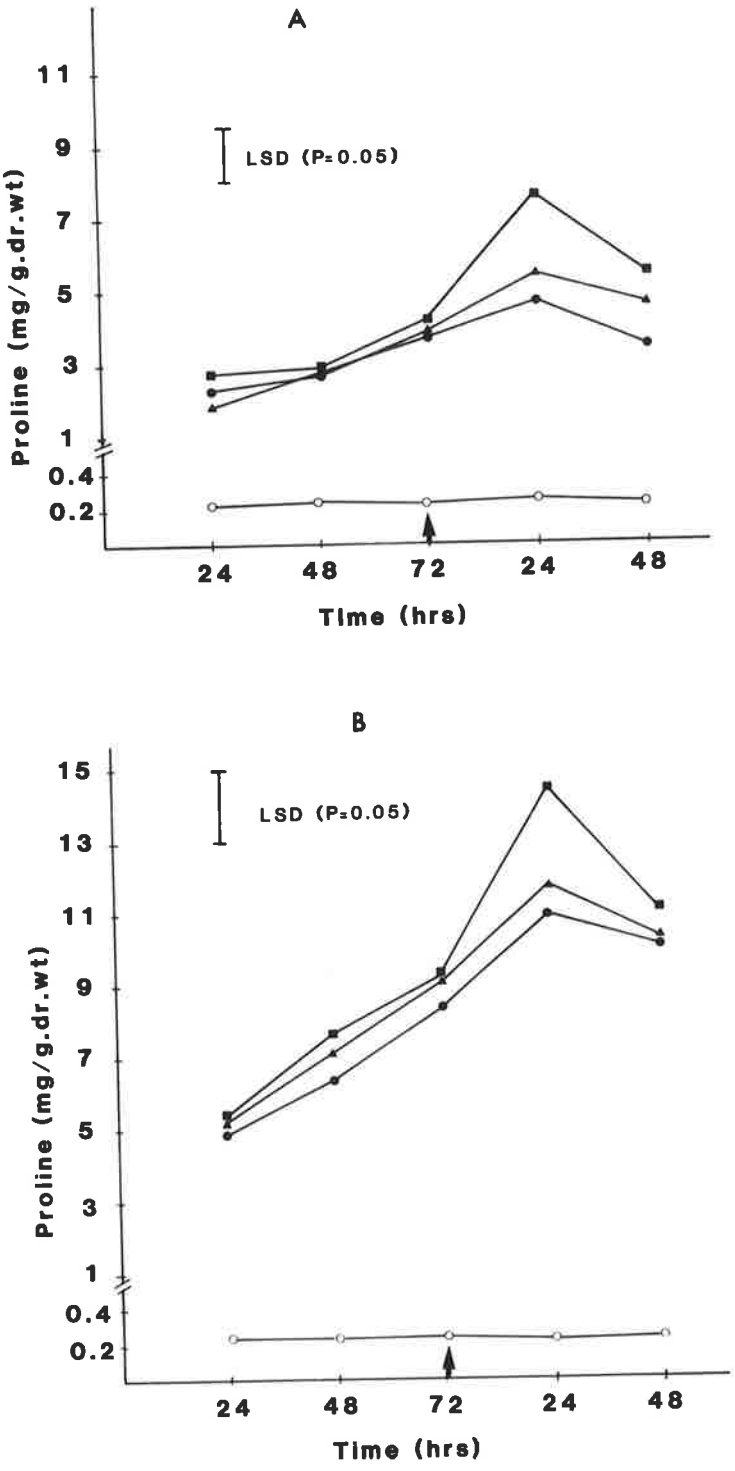
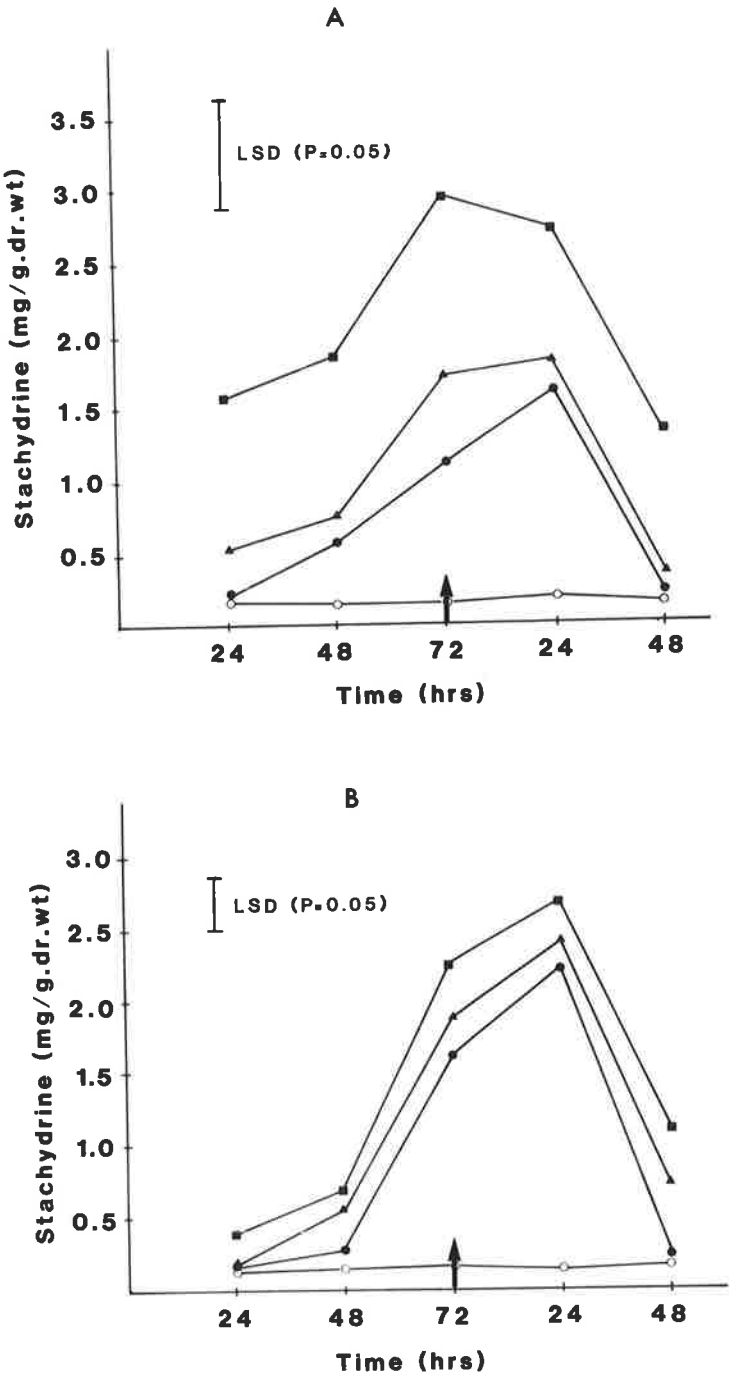

A
11
9
7
5
3
1
0.4
0.2
LSD (P=0.05)
Proline (mg/g.dr.wt)
24
48
72
24
48
Time (hrs)
B
15
13
11
9
7
5
3
1
0.4
0.2
LSD (P=0.05)
Proline (mg/g.dr.wt)
24
48
72
24
48
Time (hrs)

Fig. 23 A. Stachydrine accumulation in alfalfa leaves under different levels of PEG stress. Arrow indicates the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.

B. Stachydrine accumulation in alfalfa stem under different levels PEG stress. Arrow indicates the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.


A
LSD (P=0.05)
Stachydrine (mg/g.dr.wt)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
24
48
72
24
48
Time (hrs)
B
LSD (P=0.05)
Stachydrine (mg/g.dr.wt)
3.0
2.5
2.0
1.5
1.0
0.5
24
48
72
24
48
Time (hrs)

In stems there was an increase in stachydrine at all the three levels of stress, (Fig. 23B). Though the increase was significant over control plants, the differences between the levels of PEG were not always significant. Stachydrine levels at -5, and -10 bars PEG were not significantly different at any experimental period. The reduction in stachydrine after relieving the stress was observed only after 48 hours. In the first 24 hours after stress relief, however, as leaves, the levels continued to increase.

Significant amounts of stachydrine were found in roots in the first 24 hours after application of -10 and -20 bars PEG (Fig. 24a). In -5 bars PEG application it increased after 48 hours. Significant differences in stachydrine between the levels of PEG were found only at 72 hours. When stress was alleviated, the stachydrine content decreased after 48 hours in all three levels of stress.

6.3.4 TRIGONELLINE CONTENT IN LEAVES, STEM AND ROOTS

In leaves, application of PEG at different levels increased trigonelline content significantly when compred to well-watered control plants (Fig. 24B). During the first 24 hours after stress, -10 and -20 bars PEG showed a significant increase in trigonelline, whereas in -5 bars a significant increase was found only after 48 hours. However, at all three levels of stress the icnrease was essentially linear over time. The differences between the levels of PEG may account for the differential effect on plants. When the PEG was washed away, to study the trigonelline levels in recovered plants, a continued increase was observed at all three levels of stress.

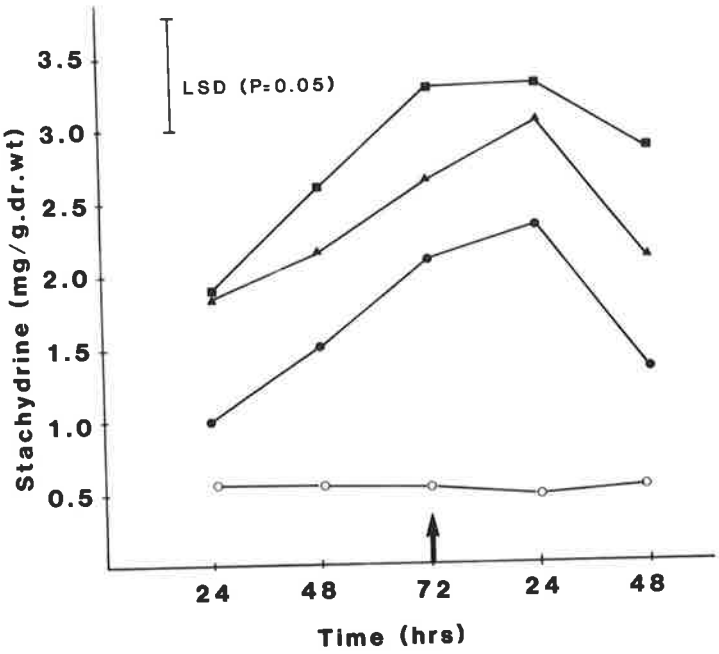
Fig. 24 A. Stachydrine accumulation in alfalfa roots under different levels of PEG stress. Arrow indicates the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.

B. Trigonelline accumulation in alfalfa leaves under different levels of PEG stress. Arrow indicates the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.

A


LSD (P=0.05)
Stachydrine (mg/g.dr.wt)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
24
48
72
24
48
Time (hrs)

B

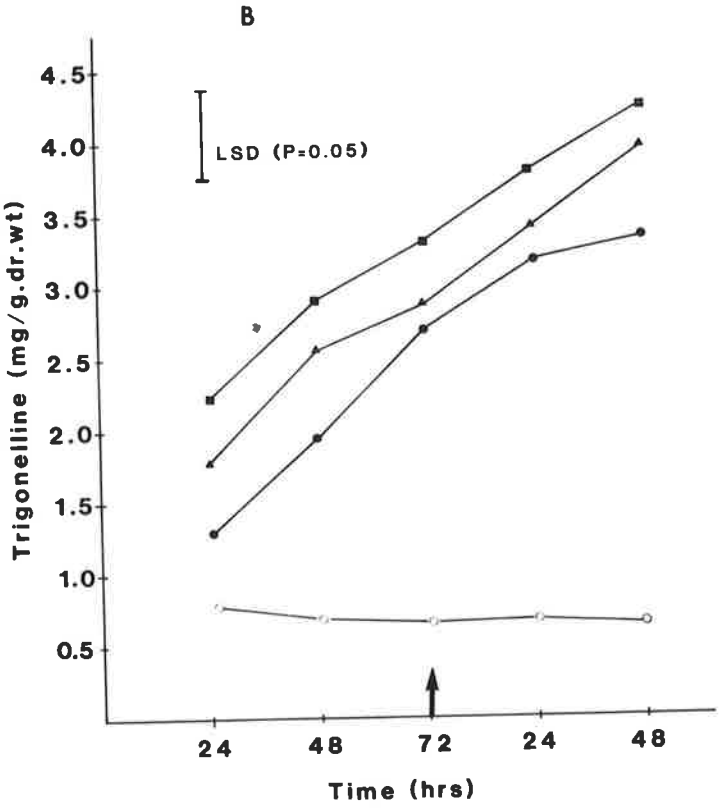
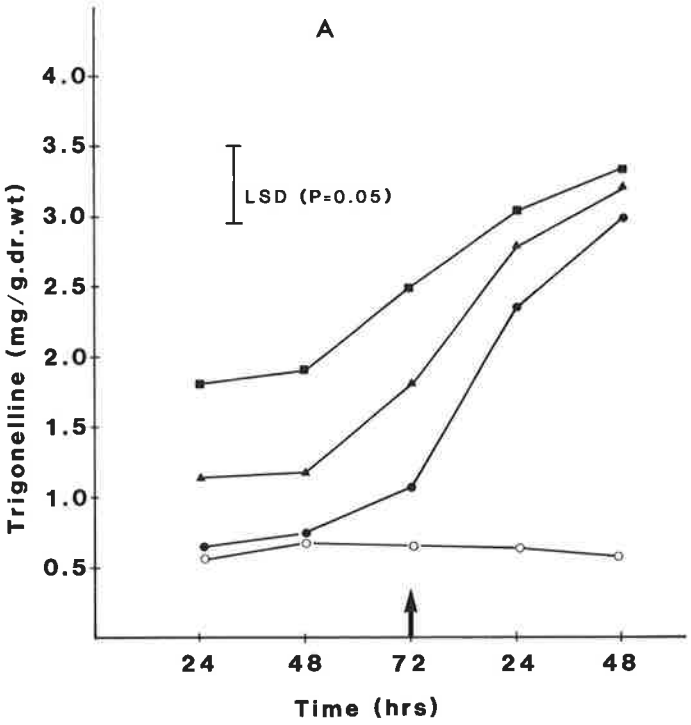

LSD (P=0.05)
Trigonelline (mg/g.dr.wt)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
24
48
72
24
48
Time (hrs)

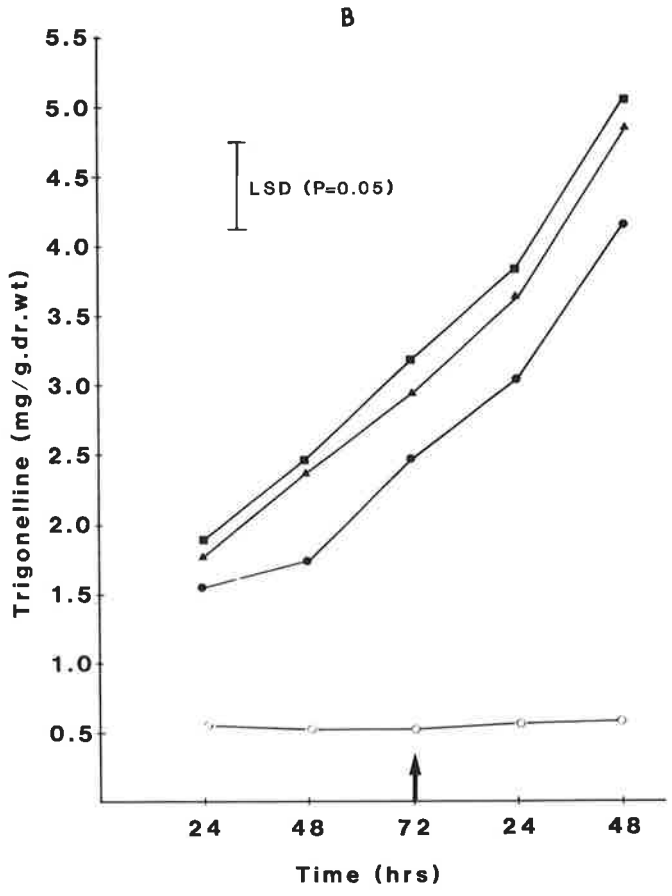
Fig. 25 A. Trigonelline accumulation in alfalfa stem under different levels of PEG stress. Arrow indicates the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.

B. Trigonelline accumulation in alfalfa roots under different levels of PEG stress. Arrow indicates the relief of stress.

○- control, ●- 5 Bars, ▲- 10 Bars, ■- 20 Bars.


A
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
LSD (P=0.05)
Trigonelline (mg/g.dr.wt)
24
48
72
24
48
Time (hrs)


B
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
LSD (P=0.05)
Trigonelline (mg/g.dr.wt)
24
48
72
24
48
Time (hrs)

Trigonelline content increased in stems as it did in the leaves. Again, there was a gradient in response from -5 bars to -20 bars PEG (Fig. 25A). This response also relates to the water potential data (Fig. 21A) measured on stems tissue. As in leaves trigonelline continued to accumulate even after 48 hours recovery.

The roots showed a proportionate increase in trigonelline over time more clearly (Fig. 25B). The PEG which was applied to the root zone may have caused more stress to the roots than to leaves and stem, and there was a greater accumulation of trigonelline in roots. Alleviation of the stress did not alter accumulation at any of the three levels of stress, even after 48 hours.

6.4 DISCUSSION

Polyethylene glycol (PEG), a non-penetrating solute, is known to cause effects in plants similar to those caused by water stress or salinity stress (Singh et al, 1973a; Chu et al, 1974; Steuter et al 1981; Sanchez-Diaz et al, 1982). In the present investigation, when PEG was applied to the root zone of alfalfa plants, there was a concentration-dependent reduction of plant water potential within 24 hours (Fig. 21A). Such reductions in water potential due to PEG have been observed before (Singh et al, 1973a; Chu et al, 1978; Hanson, 1980). This decrease in water potential is believed to be different from that of osmoticum such as NaCl, where there are sequential changes in the components of the water potential (Slatyer, 1961). However, in alfalfa the reduction in water potential due to PEG stress was much faster than that due to water stress or NaCl stress (Fig. 14A). This suggests that PEG brings about stress in plants very quickly.

The consequences of this PEG stress were also reflected in proline accumulation. In alfalfa, proline accumulated in all the organs of the plant (Figs. 21B, 22A and B) and the amounts of proline were in proportion to the levels of PEG applied. Such accumulations of proline have been found in wheat (Protsenko et al, 1968); in barley (Singh et al, 1973a); in radish (Chu, 1974). PEG (-20 bars) induced a high accumulation of proline in all the organs. In leaves a maximum of 17 mg of proline/gm dry weight tissue was accumulated. This is in accordance with the findings of Chu, (1974) in which they found 15 mg of proline accumulated per gram dry weight of tissue at the same level of PEG. This is probably due to the greater reduction in tissue water potential (Fig. 21A). The differences in accumulation between the organs were as apparent as they were in the cases of water, salt and cold stress. The reason for this type of distribution is not clear at this stage. The increase (return to normal level) in water potential, after washing the PEG solution from the root zone, was reflected in the reduction in proline content. In the first 24 hours after washing out the PEG, proline was still increasing, but after 48 hours it decreased sharply. This may be due to a time lag in the return of the tissue to normal metabolic processes. The continued increase in solute after the cessations of stress, was also observed above in the case of alfalfa.

Stachydrine increased in all the organs of alfalfa as a result of PEG stress depending on the applied PEG level (Figs. 23A,B and 24A). Unlike the effects of other stresses, stachydrine under PEG stress increased quickly. Roots, which came into contact with PEG showed relatively high amounts of stachydrine (Fig. 24A). Though there is no evidence in the literature regarding the accumulation of stachydrine under PEG stress, it is probable that the reduction in water potential

caused the increased level of stachydrine. Such an explanation is suggested by the fact that PEG caused an increased level of glycinebetaine, a relative compound (Storey et al, 1978a; Ladyman et al, 1980). The reduction of stachydrine after the relief of PEG stress in all the organs (Figs. 23A,B and 24A) is in accordance with earlier experiments where stachydrine decreased after the release of water stress (Section 1.3.3). The metabolism of stachydrine after the relief of stress is not known and so needs further experimentation.

Similarly, trigonelline increased due to PEG stres (Figs. 24B, 25A and B). Such an increase due to PEG has not been reported before. Water potential or, alternatively, a factor like ABA may be involved in this response also, and further experimentation is necessary to find an exact explanation. Lack of information on trigonelline metabolism also restricts any explanations as to why trigonelline content did not decrease after the relief of stress.

SECTION 7 : EFFECT OF DIFFERENT ENVIRONMENTAL STRESSES ON THE LEVELS OF ABA, PROLINE, STACHYDRINE, HOMOSTACHYDRINE AND TRIGONELLINE IN ALFALFA SHOOTS

7.1 INTRODUCTION

A pronounced increase in the endogenisis level of ABA has been observed in a variety of plants subjected to moisture stress (Aspinall et al, 1973; Newville and Ferrell, 1980; Ilahi and Dorffling, 1982; Quarrie and Jones, 1979; Quarrie, 1980; Henson, 1983; Bradford, 1983; Ackerson and Radin, 1983). Osmotic or salinity stress (Mizrahi et al, 1971; Downton and Loveys, 1978; Hartung et al, 1983) and low temperature or cold stress (Rifkin et al, 1976; Daie and Campbell, 1981; Chen et al, 1983; Eamus and Wilson, 1983). In earlier experiments, as a result of environmental stress, there was a large accumulation of proline, stachydrine and trigonelline. Since ABA also accumulates (literature cited) in other crop species as a result of stress, it was thought to be more than useful to study the ABA behavior in alfalfa. It was also considered important to study proline, stachydrine, homostachydrine and trigonnelline in the same experiments so that it would be easier to establish the relationship, if any, between ABA and the accumulated compounds. It was with this objective in mind that the following experiments with the three stress treatments (water, salinity and cold) were conducted.

7.2 METHOD

7.2.1 PLANT CULTURE

The plants were grown on sand culture for all three stress experiments. (Chapter III, 2.1)

7.2.2 STRESS TREATMENT

7.2.2.1 WATER STRESS

Water stress was imposed by withholding moisture for 48 hours. The alleviation of stress was obtained by re-watering the stressed plants after 48 hours.

7.2.2.2 SALINITY STRESS

Salinity stress was created by applying 200 mM NaCl solution to the pots. The NaCl was washed out thoroughly with distilled water after 48 hours to relieve the stress.

7.2.2.3 COLD STRESS

The plants, after growing for 3 weeks in the standard conditions (Chapter III, 2.2), were transferred to $-2^{o}C$ cold room for 48 hours where the temperature obtained at the leaf canopy was $5^{o}C$.

For relieving the cold stress, the plants were returned to the standard conditions. In all three experiments respective control plants were maintained.

7.2.3 HARVESTING THE TISSUE

In all the experiments the plant tissue (shoot) was harvested as described in Chapter III, 2.3.

7.2.4 WATER POTENTIAL MEASUREMENTS

The water potentials were measured with a pressure bomb (as described in materials and methods) at each experimental period.

7.2.5 ABA MEASUREMENTS

ABA was measured in the harvested tissue by the modified method of Coombe and Hale (1973) as described in Chapter III, 2.5.3.

7.2.6 PROLINE MEASUREMENTS

Proline content was measured by the Singh et al, (1972) method as described in Chapter III, 2.5.1.

7.2.7 STACHYDRINE HOMOSTACHYDRINE, AND TRIGONELLINE MEASUREMENTS

All three compounds were measured by the method described in Section 1 and 2 of this chapter.

7.3 RESULTS

7.3.1 WATER STRESS

The data of the effect of water stress are shown in Table 1. Water potentials measured on stem tissue decreased when the plants were exposed to 48 hours of water stress. The water potential decreased from -0.30 to -2.65 MPa. This decrease was statistically significant over values from control plants. Unlike the previous experiment, the decrease

Table 1 Response of ABA, proline, stachydrine, homostachydrine, and trigonelline in alfalfa shoots to water stress.

μg/g dry wt.

	0 Hours		48 hours		24 hours after stress release		
	No-Stress	Stress	No-Stress	Stress	No-Stress	Stress Recovery	LSD (P=0.05)
WATER POTENTIAL	-0.44	-	-0.3	-2.65	-0.3	-0.64	-0.25
ABSCISIC ACID	47.4	-	42.3	548.1	54.3	84.5	39.4
PROLINE	948.4	-	796.5	18,804.7	1,238.1	11,293.0	3143.7
STACHYDRINE	938.3	-	783.6	3,721.5	670.7	3,064.0	834.8
HOMOSTACHYDRINE	376.7	-	391.7	389.4	228.8	227.5	231.7
TRIGONELLINE	481.3	-	548.8	1,955.4	874.4	2,016.0	652.8

was rapid and this may be due to the changed growth conditions. Water potentials increased sharply 24 hours after relieving the stress. This has been a repeated feature of water stress experiments.

Accumulation of ABA in the alfalfa shoot was measured for the first time after water stress treatments. It was interesting to find the increase in ABA content in alfalfa. The increase of approximately 10 times over control was highly significant. There was also a significant decrease in ABA after the allieviation of stress.

Proline content was measured in a separate set of plants under the same treatments. After 48 hours, proline content in the shoots increased significantly over control. The increase was rapid which may be due to the rapid decrease in water potential or the rapid increase in ABA. Proline content decreased after releasing the stress. These results support the earlier experiments in which proline accumulation was significant.

Stachydrine content was measured in the same samples used for proline estimation. It increased significantly after 48 hours, and decreased after relieving the stress. The response also confirms the earlier findings with water stress.

Homostachydrine, which was newly identified in alflalfa in these experiments, was also measured as it was not studied before. During water stress, homostachydrine did not change significantly throughout the experiment.

Trigonelline content increased significantly after 48 hours of water stress. The accumulation of trigonelline continued even after re-watering the plants, similar to earlier findings.

7.3.2 SALINITY STRESS

The data obtained in the salinity stress experiment is presented in Table 2.

Water potentials measured in alfalfa stems after the application of 200 mM NaCl decreased significantly, from -0.30 to 1.60 MPa, in 48 hours. When the stress was relieved, by washing all the NaCl from the pots, the water potential began to rise after 24 hours. ABA accumulation as a result of salinity stress was measured for the first time in these experiments. ABA increased significantly after 48 hours of stress, and, as in water stress, it decreased when the stress was relieved. Though the increase in ABA was significant the absolute amounts were low when compared to the water stress experiments. This, perhaps, may be due to the smaller reduction in water potential with 200 mM NaCl (-1.60 MPa) as against -2.65 MPa with water stress experiments.

There was a significant increase in proline as a result of NaCl stress. When the stress was released proline showed a significant decrease, a trend which was similar to the earlier salinity experiment.

Stachydrine content showed a significant increase as a result of salinity stress. Again, on releasing the stress, it decreased. The salinity effect on homostachydrine was not significant throughout the experiment.

Table 2 Effect of NaCl Stress on ABA, proline, stachydrine, homostachydrine, and trigonelline in alfalfa shoots.

	0 Hours		48 hours		24 hours after Stress release		
	No-Stress	Stress	No-Stress	Stress	No-Stress	Stress Recovery	LSD (P=0.05)
WATER POTENTIAL	-0.36	-	-0.3	-1.60	-0.37	-0.91	-0.33
ABSCISIC ACID	45.9	-	55.3	157.3	48.7	47.8	23.7
PROLINE	922.0	-	921.3	5,227.0	847.4	3,090.3	508.6
STACHYDRINE	863.0	-	1,018.0	2,260.0	956.4	1,524.2	463.8
HOMOSTACHYDRINE	483.0	-	497.0	822.7	428.4	525.5	318.6
TRIGONELLINE	462.3	-	419.4	1,318.3	544.5	1,679.1	355.8

Trigonelline content increased significantly after 48 hours of NaCl stress. It continued to increase even after releasing the stress.

7.3.3 COLD STRESS

The data pertained to cold stress is shown in Table 3.

Water potentials were measured after exposure to 5^{o}C cold stress treatments. After 48 hours of exposure, no significant change in water potential was observed.

ABA accumulation in alfalfa, as a result of cold stress, was studied with a special interest. It was indeed interesting to find a significant increase in ABA as a result of cold stress. It also showed a decline when the plants were returned to normal growth conditions. This finding perhaps points to a possible relationship between ABA, changes in water potential, and accumulation of organic solutes.

Proline accumulation increased significantly under cold stress after 48 hours. On relieving the stress it decreased significantly. Although no change was observed in the water potentials the increase in proline content was of significant magnitude.

Stachydrine content also increased as a result of cold stress. Like proline, it decreased after relief of stress. Homostachydrine showed no significant changes as a result of cold stress. Trigonelline content, as in water and salinity stress, increased significantly under cold stress. The response, after releasing the stress, was also similar, i.e. it continued to increase even after 24 hours.

Table 3 Effect of Cold Stress (5°C) on ABA, proline, stachydrine, homostachydrine, and trigonelline in alfalfa shoots.

	0 Hours		48 hours		24 hours after stress release		
	No-Stress	Stress	No-Stress	Stress	No-Stress	Stress Recovery	LSD (P=0.05)
WATER POTENTIAL	-0.48	-	-0.43	-0.36	-0.50	-0.46	-0.11
ABSCISIC ACID	56.6	-	50.7	684.5	54.4	434.3	43.7
PROLINE	922.5	-	1,040.6	4,932.1	961.9	1,934.9	456.5
STACHYDRINE	851.0	-	786.7	1,441.1	799.3	1,073.1	208.0
HOMOSTACHYDRINE	438.9	-	511.0	675.8	790.3	707.0	167.0
TRIGONELLINE	673.3	-	480.8	1,601.3	772.1	1,933.1	229.2

7.4 DISCUSSION

Abscisic acid accumulated in alfalfa under different environmental stress. Table 1 indicates the increase of endogenous ABA in alfalfa shoots as a result of water stress. This increase in ABA under water stress, is in agreement with similar ABA increases observed in several species (Aspinall et al, 1973; Anderson and Rajagopal, 1978; Ilahi and Dorffling, 1982; Quarrie, 1983; Bradford, 1983; Henson, 1983; Ackerson and Radin, 1983). Increases in ABA in alfalfa were also found under salinity stress (Table 2) and cold stress (Table 3) as have been found with several crop species under salinity (Mizrahi et al, 1971; Downton and Loveys, 1978; Hartung et al, 1983) and under cold stress (Rifkin et al, 1976; Eamus and Wilson, 1983).

The increase in ABA that occurs in leaves during drought has been suggested as being due to increased biosynthesis rather than release from a pool of bound ABA (Milborrow and Noddle, 1970; Milborrow, 1978). Such a biosynthetic change can also be expected in alfalfa under stress conditions. The increase in ABA in alfalfa under cold stress was not due to the change in tissue water potential as there was no decrease in water potential (Table 3). If there is a common mechanism operating in all three environmental stresses which increases the biosynthesis of ABA, it is difficult to involve water potential in it.

In the experiments above proline accumulated under water, salinity, cold and PEG stresses (Sections 3, 4, 5 and 6). In the present experiments also large differential increases in proline were found in response to water, salinity and cold stresses (Table 1, 2 and 3). This proline accumulation may be due to an ABA-triggered mechanism/s such as

inhibiting the incorporation of proline into protein (Chrispeels and Varner, 1967) or inhibiting proline oxidation (Stewart, 1980) etc. This possiblility is supported by the facts that proline accumulated in plants which also showed ABA accumulation and that when the stress was relieved both ABA and proline levels decreased (Table 1, 2 and 3). Such a possibility is also supported by the fact that exogenous application of ABA usually (but not always) increased proline accumulation (Aspinall et al 1973; Rajogopal and Anderson, 1977; Stewart 1980; McDonell et al, 1983). Inspite of these findings the mechanisms of action of ABA under stress is still not clear.

The relationship of stachydrine to ABA is not known. However, increases in glycinebetaine, another quaternary ammonium compound (Huber and Sankla, 1980) may indicate such a possibility, but needs further experimentation. Levels of homostachydrine did not change in the stressed alfalfa shoots. The metabolic pathways through which homostachydrine is synthesized seems not to have been affected by the stress or the presence of ABA. Trigonelline, however, increased in response to stress (Table 1, 2 and 3). Once again the relationship between ABA and trigonelline is not known. An attempt to find a relationship between ABA and trigonelline is discussed in the next section.

SECTION 8 : EFFECT OF ABSCISIC ACID ON PROLINE, STACHYDRINE AND TRIGONELLINE ACCUMULATION IN ALFALFA SEEDLINGS

8.1 INTRODUCTION

Accumulation of proline, stachydrine and trigonelline in alfalfa in response to different environmental stresses is well documented in Sections 3-6 of Chapter IV. Rapid accumulation of abscisic acid was found in plants during drought stress (Wright and Hiron 1969; Hsiao, 1973; Ilahi and Dorffling, 1982), and in chilled plants (Eamus and Wilson, 1983), and many authors have proposed that abscisic acid may mediate between the imposed stress and the accumulation of organic compounds.

Applied abscisic acid has been shown to induce proline accumulation in both intact plants and exercised leaves of barley (Aspinall et al, 1973; McDonnell et al, 1983), and a similar response to applied ABA has been found intact plants, *Lolium temulentum* (Aspinall et al, 1973), and *Pennisetum typhoides* (Huber, 1974; Eder and Huber, 1977). Applied abscisic acid has also been found to influence the accumulation of glycine betaine in *Pennistem typhoides* (Huber and Sankla, 1980; McDonnell et al, 1983).

In addition to proline, stachydrine and trigonelline, all increase in abscisic acid levels, in response to environmental stresses, was enunciated in the previous section. With this background it was deemed worthwhile investigating whether ABA also influences the accumulation of proline, stachydrine and trigonelline in alfalfa seedlings.

8.2 METHODS

8.2.1 GROWING PLANTS

Plants were grown in sand medium as described in Chapter III, Section 2.1.

8.2.2 ABSCISIC ACID TREATMENT

When plants were three weeks old abscisic acid (Sigma USA) was applied through the roots. Three plants of uniform size were grown in an aerated 50 ml glass tube, containing ABA (either 5 Mg/ml or 50 µg/ml) in half strength Hoagland's solution. A small amount of sponge was placed at the top of the tube to hold the plants together. The plants were grown in ABA solutions for 48 hours under the same conditions they were grown in before (24/21^{o}C day/night, 12 hour potoperiod and 550 µE $m^{-2}s^{-2}$. A set of controlled plants, without ABA, were reared similarly. All the treatments were replicated thrice.

8.2.3 HARVESTING THE TISSUE

The plants were harvested at the beginning of the experiment (0 hours) and subsequently every 24 hours. They were taken out of the tubes and roots were washed thoroughly in distilled water and frozen quickly with liquid nitrogen. The samples were then dried in a freeze dryer for 48 hours. The dry weights of the samples were determined before analysing them for proline, stachydrine and trigonelline.

8.2.4 PROLINE MEASUREMENTS

Proline content of the samples were mesured by the Singh et al (1972) method as described in Chapter III, 2.5.1.

8.2.5 STACHYDRINE AND TRIGONELLINE MEASUREMENT

Stachydrine and trigonelline were measured by the method described in Section 1 and 2 of this chapter.

8.3 RESULTS

Abscisic acid, when fed to alfalfa seedlings, increased the proline content significantly (Fig. 26). In the first 24 hours after feeding, proline increased in both 5 µg/ml and 50 µg/ml ABA. However, there was no significant difference between the two concentrations. After 48 hours of feeding both ABA levels showed a significantly high increase in proline. The increase was also proportional to the concentration of ABA i.e. 50 µg/ml ABA showed a greater increase. The slight change observed in the control plants was statisticaly not significant.

It was interesting to observe the increase in stachydrine as a result of ABA treatment (Fig. 27). The increase in stachydrine was apparent in the first 24 hours but was clearer after 48 hours of feeding. Once again, as with proline, the greatest response was obtained at the higher concentration (50 µg/ml) of ABA.

The trigonelline response to ABA was also studied in the same plants. As shown in (Fig. 28), trigonelline content increased as a

Fig. 26 Effect of Abscisic Acid (when fed through the roots) on the accumulation of proline in alfalfa seedlings under normal growing conditions.

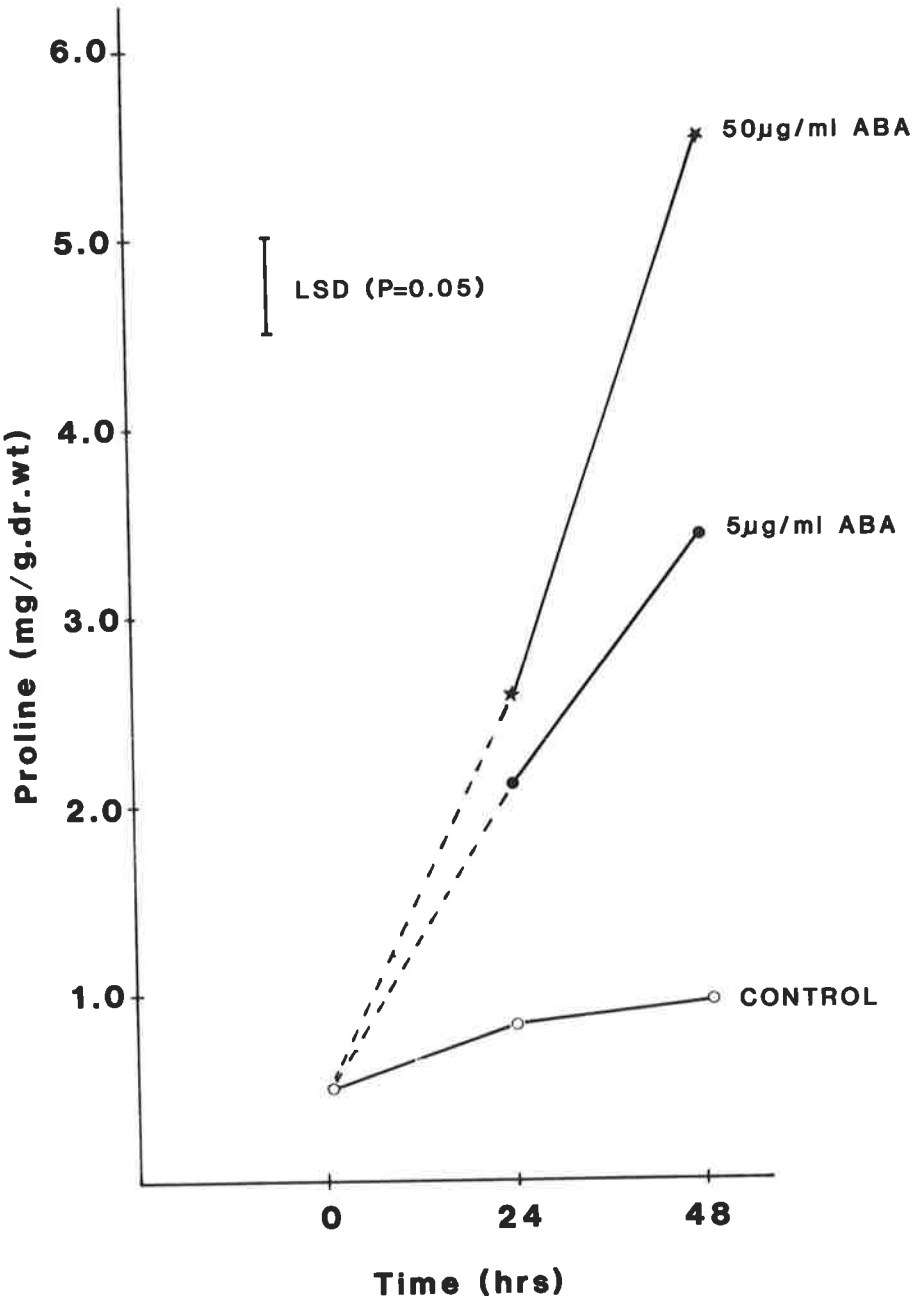

6.0
5.0
4.0
3.0
2.0
1.0
Proline (mg/g.dr.wt)
LSD (P=0.05)
50µg/ml ABA
5µg/ml ABA
CONTROL
0
24
48
Time (hrs)

Fig. 27 Effect of Abscisic Acid (when fed through roots) on the accumulation of stachydrine in alfalfa seedlings under normal growing conditions.

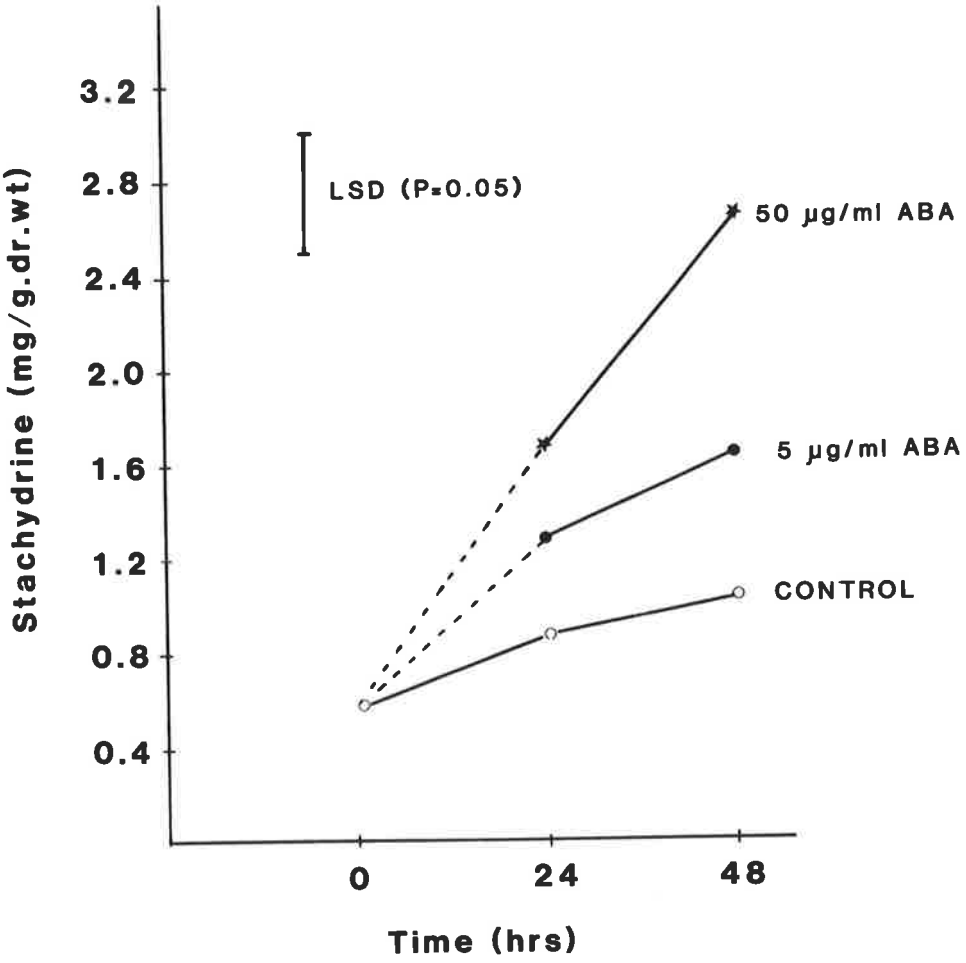
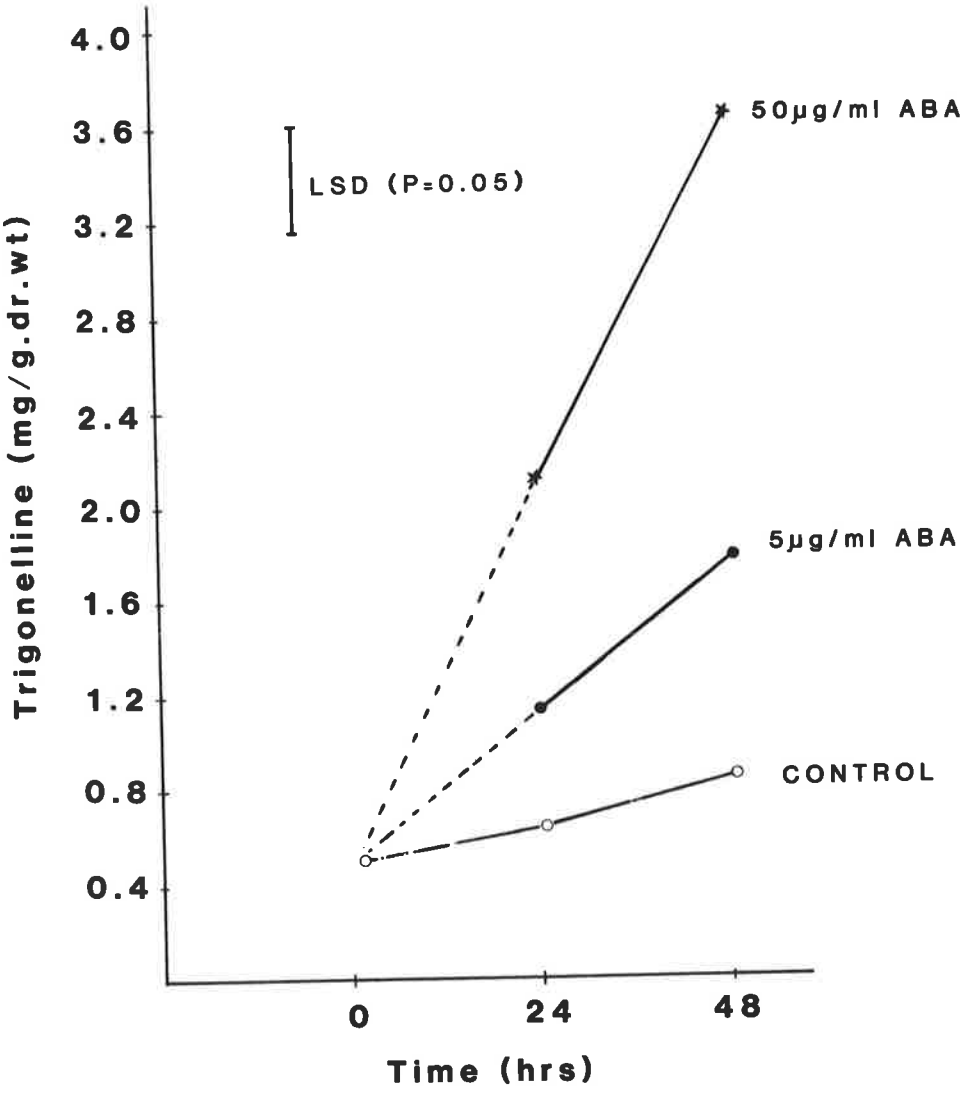

Stachydrine (mg/g.dr.wt)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
LSD (P=0.05)
50 µg/ml ABA
5 µg/ml ABA
CONTROL
0
24
48
Time (hrs)

Fig. 28 Effect of Abscisic Acid (when fed through roots) on the accumulation of trigonelline in alfalfa seedlings under normal growing conditions.


4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
Trigonelline (mg/g.dr.wt)
LSD (P=0.05)
50µg/ml ABA
5µg/ml ABA
CONTROL
0
24
48
Time (hrs)

result of ABA and the increase was highly significant at both 24 hours and 48 hours after application. It is worth noting that though trigonelline is structurally different to proline and stachydrine, and obviously synthesised on a separate metabolic pathway, it responds to abscisic acid in a manner similar to proline and stachydrine.

8.4 DISCUSSION

It is now clear that proline, stachydrine and trigonelline accumulate in alfalfa under different environmental stresses. It was also found in the previous section that ABA accumulated in alfalfa under similar conditions. Such changes have been found in several species (Aspinall et al 1973; Anderson and Rajagopal, 1978; Quarrie, 1980; Ilahi and Dorffling, 1982) and may indicate a relationship between ABA and proline and betaines. Though there is sufficient evidence to conclude that ABA increased proline accumulation, there is no published data available in relation to stachydrine or trigonelline. In the present investigation application of ABA has induced increase in proline (Fig. 26) stachydrine (Fig. 27) and trigonellins (Fig. 28). These increases were also dependent on the concentration of ABA applied as shown with two levels of ABA (5 µg/ml and 50 µg/ml). This strongly suggests that the levels of the three solutes are controlled by similar metabolic processes which probably involve the presence of ABA. Three mechanisms of ABA action have been suggested at least as far as proline accumulation is concerned; inhibiting protein synthesis (Chrispeels and Varner, 1967; Aspinall et al, 1973), inhibition of the oxidation of proline (Stewart, 1980); and Huber's (1974) suggestion that during stress ABA stimulated P5C reduction consequent on increased synthesis of the enzyme, P5C reductase, thereby, increasing proline.

SECTION 9 : NODULES AND NODULATED ALFALFA PLANTS UNDER ENVIRONMENTAL STRESS: ACCUMULATION OF PROLINE, STACHYDRINE, HOMOSTACHYDRINE AND TRIGONELLINE

9.1 INTRODUCTION

The presence of root nodules is an important aspect in legumes in relation to nitrogen fixation. Drought causes significant changes in nodules and their physico-biochemical aspects (Sprent, 1981). Inspite of abundent evidence on nodules in relation to nitrogen fixation, there is litle information available regarding the synthesis or accumulation of amino acids and quaternary ammonium compounds which are of high significance under stress. The occurrence of amino acids and nitrogenous compounds containing a quaternary ammonium group in nodules under normal conditions has been reported. Betonicine was found in nodules of *Canavalia ensiformis* and trigonelline was found in nodules of *Pisum sativum* (Mears and Marby, 1971).

Nodules are very sensitive to stress conditions (Sprent and Gallacher, 1976). Le Rudulier et al (1982) reported for the first time, the occurrence of proline and 'onium compounds' in the root nodules of several nitrogen-fixing plants adapted to dry habitats. They also found proline and 'onium compounds' in alfalfa nodules *Medicago sativa*.

The presence of nitrogen-fixing nodules on leguminous crops makes a significant contribution to the plants in terms of amino acids amides, carbohydrates, etc. synthesis, as was demonstrated by the classic comparisons with cocklebur and field pea (Wallace and Pate, 1967). Their findings suggested that the presence of nodules might also make a

significant difference to the response of plants under stress conditions (Sprent, 1981).

With this background the present experiments were designed to identify the presence of proline, stachydrine, homostachydrine, and trigonelline, if any, in alfalfa, and to determine whether there differences in type of quantity of compatible solutes between nodulated and non-nodulated alfalfa plants.

9.2 METHODS

9.2.1 PLANT CULTURE

Alfalfa plants were grown in plastic pots (6") as described in the materials and methods Chapter III, 2.1.

9.2.2 STRESS TREATMENTS

In both the water stress experiments stress was applied by withholding water for 48 hours. At the end of 48 hours the plants at harvest, divided into nodules, nodulated roots and shoots. In the case of the salinity experiment, 200 mM NaCl was applied to pots and at the end of 48 hours the tissue was harvested. In all the experiments the tissue after harvest was frozen with liquid nitrogen and later freeze dried. The dried samples were then weighed and taken for chemical analysis.

9.2.3 HARVESTING NODULES

Nodules were harvested from the roots with a scalpel and were collected in the glass vials and frozen immediately with liquid

nitrogen. The nodules were freeze dried, weighed and analysed for proline and betaines.

9.2.4 NODULATED AND NON-NODULATED PLANT TYPES

In order to get enough nodules the nodulated plants were treated with Rhizobium culture and were regularly watered with nutrient solution devoid of nitrate. The non-nodulated plants were obtained by growing the plants without Rhizobium but with sufficient amounts of nitrate. The growing medium (sand) and the nutrient solutions were sterilised before use. The plants were grown in a growth cabinet with conditions as described in materials and methods (Section 2.2)

9.2.5 IDENTIFICATION OF PROLINE AND BETAINES IN NODULES ON NMR SPECTROMETER

Proline and betaines in the nodules were extracted with M:C:W and, with the procedure described in Chapter III (Section 2.5.1), were identified with the NMR spectrometer.

9.2.6 PROLINE DETERMINATIONS

The proline content of the tissue was determined by the Singh et al (1972) method. (Chapter III - 2.5.1)

9.2.7 STACHYDRINE, HOMOSTACHYDRINE AND TRIGONELLINE DETERMINATIONS

The stachydrine, homostachydrine and trigonelline contents were determined by the method described in Section 1 and 2 of this chapter.

9.3 RESULTS

9.3.1 IDENTIFICATION OF BETAINES IN THE NODULES

Betaines from the alfalfa nodules extracts were identified with the NMR spectrometer. The NMR spectra were compared with the spectra of authentic betaines, and it was found that nodules contained proline, stachydrine, homostachydrine, trigonelline and choline as the major nitrogenous compounds (Fig. 29).

9.3.2 EFFECT OF WATER AND SALINITY STRESS ON NODULES IN RELATION TO THE ACCUMULATION OF PROLINE AND BETAINES

Nodules obtained from water stressed alfalfa plants showed a large increase in proline, stachydrine, homostachydrine and trigonelline (Table 4). When plants were exposed to salt stress, the solute contents increased but very much less than they did under water stress.

9.3.3 COMPARATIVE EFFECTS OF STRESS ON NODULATED AND NON-NODULATED ALFALFA PLANTS IN THE ACCUMULATION OF PROLINE AND BETAINES

9.3.3.1 WATER STRESS

Under well-watered conditions there was no significant difference in the proline content in shoots between nodulated and non-nodulated plants (Table 5). Roots of well-watered plants, however, showed significantly higher amounts of proline in nodulated roots as compared to non-nodulated roots. When plants were exposed to water stress for 48 hours, both shoots and roots of the two plant types showed a significant

Fig. 29 ^{1}H NMR spectra of an extract from alfalfa nodules indicating the presence of P-Proline, S-Stachydrine, Hs-Homostachydrine, T-Trigonelline, and C-Choline.

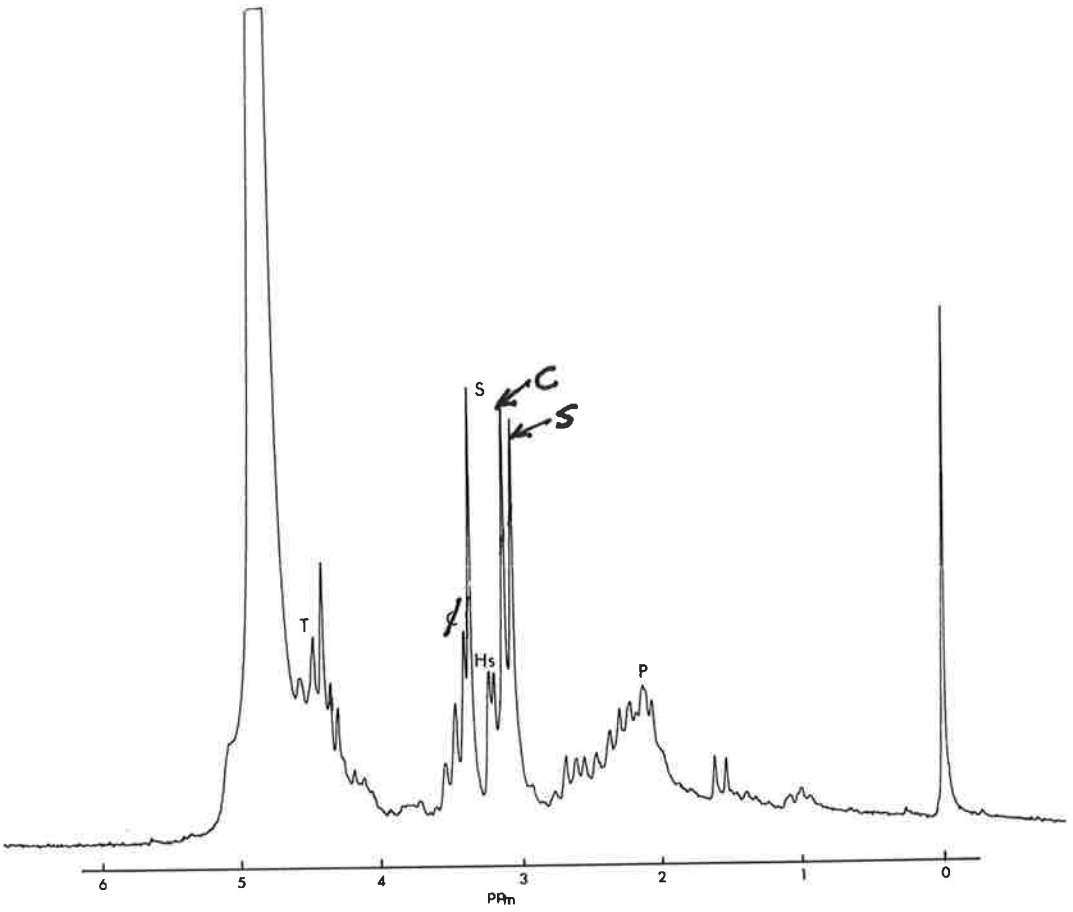

S
C
S
T
Hs
P
6
5
4
3
2
1
0
PPm

Table 4

Effect of water and NaCl stress on proline, stachydrine, homostachydrine and trigonelline in alfalfa nodules.

	mg/g dr. weight*			
	Proline	Stachydrine	Homostachydrine	Trigonelline
At the start of the expt.	0.3656	0.4217	0.0465	0.4969
No Stress (48 hrs)	0.3048	0.547	0.0382	0.4601
Water Stress (48 hrs)	5.9705	3.3296	0.5803	2.1749
NaCl Stress (200 mM) (48 hrs)	1.5805	1.7789	0.1524	1.0626

* Each is a single value obtained from nodules collected from 3 uniform plants from the same treatment.

Table 5

Proline content (mg/g dr. wt) in nodulated and non-nodulated alfalfa plants under water stress.

Treatment		At the start of the expt.	Non-Stress	Stress
NON-NODULATED	SHOOT	0.3692	0.2823	0.8064
	ROOTS	0.4085	0.3976	0.7520
NODULATED	SHOOT	0.5003	0.3854	1.2671
	ROOTS	0.9476	1.0702	2.1030

Overall LSD (P = 0.05) = 348.5 0.3485 ?

increase over their respective non-stressed controls. When comparison was made between the two plant types, nodulated plants showed significantly higher proline in both shoot and roots.

The differences in stachydrine content between nodulated and non-nodulated types was not significant under normal conditions (Table 6). However, under water stress the nodulated plants showed significantly higher stachydrine in both shoot and roots. Trigonelline content was also increased significantly in the nodulated plants under stress (Table 7). The well watered plants showed a marginal, statistically non-significant difference between two types.

9.3.3.2 SALINITY STRESS

Both plant types were exposed to 200 mM NaCl for 48 hours. Proline was not significantly different in shoots between 2 types under normal conditions (Table 8). Proline was marginally higher in the roots of nodulated types under normal conditions. Under NaCl stress the increases in proline in both types were significant. Between the plant types, proline in nodulated plants increased significantly more in both shoot and roots.

Stachydrine levels in both shoot and roots between two types under normal conditions were not statistically different (Table 9). However, under salt stress the differences were significant. The nodulated shoot and roots showed higher amounts of stachydrine. The levels of trigonelline under normal conditions were not statistically different between the two plant types (Table 10). The salt stress increased trigonelline more in nodulated shoot and roots.

Table 6

Stachydrine content (mg/g dr. wt) in nodulated and non-nodulated alfalfa plants under water stress.

Treatment		At the start of the expt.	Non-Stress	Stress
NON-NODULATED	SHOOT	0.3805	0.4032	0.8331
	ROOTS	0.5413	0.4467	1.2662
NODULATED	SHOOT	0.4097	0.4639	1.7932
	ROOTS	0.5954	0.5312	2.2229

Overall LSD (P = 0.05) = 301.6

Table 7

Trigonelline content (mg/g dr. wt) in the nodulated and non-nodulated alfalfa plant under water stress.

Treatment		At the start of the expt.	Non-Stress	Stress
NON-NODULATED	SHOOT	0.4653	0.4007	0.9935
	ROOTS	0.5283	0.5706	1.1822
NODULATED	SHOOT	0.5515	0.6347	1.4982
	ROOTS	0.8040	0.8602	1.9812

Overall LSD (P = 0.05) = 177.5 0.1775

Table 8

Proline content (mg/g dr. wt) in the nodulated and non-nodulated alfalfa plants exposed to NaCl stress.

Treatment		At the start of the expt.	Non-Stress	Stress
NON-NODULATED	SHOOT	0.3697	0.3703	0.7932
	ROOTS	0.5212	0.4865	0.9506
NODULATED	SHOOT	0.5385	0.6449	1.6945
	ROOTS	0.8422	0.8900	2.2107

Overall LSD (P = 0.05) = 293.4 (0.2934?)

Table 9

Stachydrine content (mg/g dr. wt) in the nodulated and non-nodulated alfalfa plants exposed to NaCl stress.

Treatment		At the start of the expt.	Non-Stress	Stress
NON-NODULATED	SHOOT	0.2980	0.3749	0.8909
	ROOTS	0.4439	0.3610	1.0303
NODULATED	SHOOT	0.5728	0.5061	1.5121
	ROOTS	0.5612	0.5105	2.0733

Overall LSD (P = 0.05) = 200.7 0.2007?

Table 10

Trigonelline content (mg/g dr. wt) in the nodulated and non-nodulated alfalfa plants exposed to NaCl stress.

Treatment		At the start of the expt.	Non-Stress	Stress
NON-NODULATED	SHOOT	0.4109	0.4065	0.8607
	ROOTS	0.6305	0.5237	1.1710
NODULATED	SHOOT	0.4112	0.5172	1.1263
	ROOTS	0.5133	0.8375	1.5129

Overall LSD (P = 0.05) = 339.0 0.3390?

9.4 DISCUSSION

The emphasis on the significance of nodules in synthesising or accumulating nitrogenous compounds like proline and quaternary ammonium compounds in alfalfa was brought about by the following facts. A large portion of the total plant nitrogen in alfalfa is derived from symbiotic nitrogen fixation (Heichel et al, 1981). Sizeable amounts of ammonia are generated in bacteroids as the initial product of nitrogen fixation (Bergerson, 1965). Most of the ammonia (90-95%) is exported from bacteroids into the surrounding nodule-plant cell cytoplasm (O'Gara and Shanmugam, 1976), assimilated into organic compounds and transported to the shoots (Rawsthrone et al, 1980). In the present exercise the nodules were tested for the presence of proline and quaternary ammonium compounds. It is evident from the study that high amount of proline and quaternary ammonium compounds are present in the alfalfa root nodules even in unstressed plants. This obervation is in accordance with the report by Le Rudulier et al (1982). The quaternary ammonium compounds found in the nodules in this study were identified on NMR as stachydrine, homostachydrine, trigonelline and choline (Fig. 29). This finding was extended to a study of the effects of stress, if any, on these compatible solutes in the nodules. Indeed, proline, stachydrine, homostachydrine and trigonelline increased as a result of both water and NaCl stress (Table 4). There is no other evidence of similar data although the findings of Le Rudulier et al (1982), from alfalfa nodules grown in coastal environment, can be compared. An adapative mechanism was suggested by them for the presence of high amounts of proline and 'onium compounds'. This suggestion of adaptive mechanism was also put forward by Storey and WynJones (1975, 1977) for coastal halophytes which were found to accumulate large amounts of proline and glycinebetaine.

The mechanism of accumulation of proline or quaternary ammonium compounds in alfalfa nodules is not known, nor it is known whether the accumulated compatible solutes are synthesised in the nodules or translocated there from other parts.

It is evident from the present investigation that alfalfa nodules accumulate compatible solutes and are important in physiological or biochemical terms. It was also found in the present experiments that alfalfa plants with nodules accumulated more proline both under water (Table 5) and NaCl stress (Table 8) when compared to non-nodulated plants. More stachydrine and trigonelline were also found in nodulated plants both under water and NaCl stress (Table 6, 7 and Table 7, 10 respectively). From this study it is clear that the nodules are one of the major contributing factors for the higher amounts of proline, stachydrine and trigonelline accumulated in nodulated plants under stress. If these compounds are of adaptive significance in protecting plant structure or function against the effects of environmental stresses, nodulated plants will have a distinct advantage over non-nodulated plants. It is also important for a species like alfalfa to obtain enough carbon and nitrogen sources for the metabolic processes. Such a requirement is at least partially provided by the presence of nodules in the roots.

An explanation can be suggested for the higher amounts of compatible solutes in the nodulated plants. It is possible that abscisic acid which has been reported to enhance nitrogen fixation (Bano et al, 1983) is involved. Such a possibility is supported (although not from this study) by the presence of ABA in the root nodules (Watts et al

1983) and the increase in proline, stachydrine and trigonelline in alfalfa plants as a result of ABA treatments through roots (Section 8).

However, more experiments are necessary for conclusive explanations of these findings.

SECTION 10 : BIOSYNTHESIS OF STACHYDRINE AND TRIGONELLINE IN ALFALFA UNDER NON-STRESS AND STRESS CONDITIONS

10.1 INTRODUCTION

Stachydrine (Proline-betaine) is a characteristic product of Capparaceae and Labiatae (Wyn Jones, 1980). It has also been reported to be present in chrysanthemum, citrus and alfalfa (Guggenheim 1958). It is difficult to ascribe any physiological or biochemical significance to stachydrine with this little available information.

In the course of these experiments, it was observed that stachydrine increased significantly in response to different environmental stresses. The biosynthesis of stachydrine is not well understood. Robertson and Marion (1959a,b,c) , Leete et al (1955), Morghan and Marion (1956) Essery et al (1962) have made several studies of alfalfa *Medicago sativa* in relation to stachydrine biosynthesis and in their findings radioactive proline yeilded radioactive stachydrine, in the presence of methionine, pyridoxal phosphate and folic acid. These studies however were not related to any environmental variations.

The interest in the present experiments was two-fold: a) The structural similarities between proline (which accumulates in alfalfa in large amounts) and stachydrine, and, b) The increase in the endogenous level of stachydrine when plants were exposed to stressful environments. With these reasons the decision was made to determine whether stachydrine is in fact synthesised from proline, whether it increases during a given stress, and whether other factors may be involved in the synthesis.

The presence of trigonelline in alfalfa was reported first by Robertson and Marion (1959a). Earlier experiments in the present study showed a signifcant increase in trigonelline content in response to environmental stresses. Since proline, stachydrine and trigonelline all responded similarly to stresses, it was anticipated that these three compounds might have some biochemical relationship. With this in mind the study was extended to trigonelline synthesis.

In the present work, although proline was expected to be the most direct precursor of stachydrine, glutamate and ornithine, which are proline precursors were also studied. Methionine, pyridoxine and folic acid were used as co-factors which might enhance the synthesis of stachydrine or trigonelline through biological transmethylation, both under normal and stress conditions.

10.2 METHODS

10.2.1 PLANT CULTURE

2-3 week old plants grown in sand culture were selected. The plants were grown in nutrient solution culture (Chapter III, 2.1.2) for 2-3 days before beginning the experiments. They were grown in a growth cabinet under standard conditions (Chapter III, 2.2).

10.2.2 FEEDING PROLINE, GLUTAMATE, ORNITHINE, METHIONINE, PYRIDOXINE AND FOLIC ACID

The feeding procedure is described in materials and methods (Chapter III section 2.5.5.1) 48 hours after treatment plants were exposed to NaCl (200mM), PEG (-15 bars) to induce stress.

10.2.3 STRESS TREATMENTS

NaCl stress was induced after pre-treatment by simply replacing the nutrient solution with 200mM NaCl for 48 hours. The same procedure was followed with PEG stress for which PEG solutions at -15 bars water potential were applied to the tubes.

10.2.4 HARVESTING THE TISSUE

The tissue was harvested as described in Chapter III, 2.3. Wherever necessary the tissue was rinsed with distilled water before freezing with liquid nitrogen.

10.2.5 PROLINE MEASUREMENTS

The proline content in the harvested samples was measured by the Singh et al (1972) method (Chapter III, 2.5.1).

10.2.6 STACHYDRINE AND TRIGONELLINE MEASUREMENTS

Stachydrine and trigonelline were measured as described in section 1 and 2 of this chapter.

10.2.7. FEEDING ^{15}N-PROLINE AND DETERMINATION OF ^{15}N INCORPORATION

Refer to section (2.5.5.2) and (2.5.5.3) 3 in the materials and methods.

10.3 RESULTS

10.3.1 EFFECT OF PROLINE AND GLUTAMATE PRETREATMENT ON STACHYDRINE AND TRIGONELLINE SYNTHESIS UNDER NON-STRESS AND NACL STRESS CONDITIONS

The data of the first experiment with NaCl stress is shown in (Table 11). The proline content of the plants increased significantly after 48 hours of feeding with proline in the nutrient solution. Glutamate, when fed similarly, also increased the proline content significantly. After 48 hours of pre-treatment, when the plants were stressed with 200 mM NaCl the proline content increased further in both proline and glutamate pre-treated plants when compared to plant stressed without any pre-treatments. In the absence of stress, stachydrine increased significantly in the proline pre-treated plants. It also increased significantly though to a lesser extent in the glutamate-treated plants. When these plants were exposed to NaCl stress after the pre-treatments, stachydrine increased significantly only in proline pre-treated plants. Glutamate had no significant influence on stachydrine levels during stress.

Trigonelline did not increase significantly as a result of proline or glutamate treatment under non-stress conditions. However, under NaCl stress both proline and glutamate increased trigonelline content significantly as against non-treated stressed plants.

Table 11 Effect of proline and glutamate pre-treatment on stachydrine and trigonelline synthesis under non-stress and NaCl stress conditions.

Treatment	Proline		Stachydrine		Trigonelline	
	No-Stress	Stress	No-Stress	Stress	No-Stress	Stress
			Mg per g dry weight			
Control at the start of the experiment	360.3	-	650.0	-	746.0	-
Control no pre-treatment	588.9	4,384.0	762.2	2,195.1	904.7	3,262.3
Proline	3,607.3	7,243.5	1,427.6	2,797.3	1,001.2	3,616.6
Glutamate	2,508.8	5,202.1	1,073.2	2,388.9	933.4	3,764.8
LSD (P = 0.05)	250.2	510.2	210.6	494.4	280.0	326.0

10.3.2 EFFECT OF METHIONINE, PYRIDOXINE AND FOLIC ACID PRE-TREATMENT ON PROLINE, STACHYDRINE AND TRIGONELLINE SYNTHESIS UNDER NON-STRESS AND NACL STRESS CONDITIONS

Under non-stress conditions, methionine, pyridoxine and folic acid treatments caused no signifcant changes in proline, stachydrine or trigonelline, except for the pyridoxine treatment which showed a lesser stachydrine and trigonelline content (Table 12). Under stress all three treatments showed significantly lower amounts of proline. Stachydrine levels were unchanged by the co-factors, and pyridoxine and folic acid reduced the trigonelline levels. This indicates that the individual co-factors have no apparent significant promotive effect on proline, stachydrine and trigonelline synthesis in stressed or non-stressed alfalfa seedlings. The decreases could be the result of decreased uptake or reduced conversion to other products.

10.3.3 EFFECT ON STACHYDRINE AND TRIGONELLINE SYNTHESIS WHEN PROLINE AND GLUTAMATE WERE FED WITH METHIONINE, PYRIDOXINE AND FOLIC ACID INDIVIDUALLY

Significant amounts of proline were taken up by plants when proline was fed with methionine, pyridoxine and folic acid separately under non-stress conditions (Table 13). Lower levels of proline were found in the tissue than when proline was fed alone. However, the proline taken up in the presence of any of the three co-factors had no significant effect on either stachydrine or trigonelline levels under non-stress conditions. Under stress the amount of proline was significantly lower in all three treatments. Similar results were observed with stachydrine and trigonelline under stress conditions.

Table 12 Effect of Methionine, Pyridoxine, and Folic Acid pre-treatment on proline, stachydrine and trigonelline synthesis under non-stress and NaCl stress conditions.

Treatment	Proline		Stachydrine		Trigonelline	
	No-Stress	Stress	No-Stress	Stress	No-Stress	Stress
			mg per g dry weight			
Control at the start of the experiment	360.3	-	650.03	-	746.40	-
Control no pre-treatment	588.9	4,384.0	762.2	2,195.1	904.7	3,262.3
Methionine	657.6	2,711.8	755.4	1,953.5	928.9	2,958.8
Pyridoxine	508.6	2,952.4	480.4	2,003.2	667.2	2,708.9
Folic acid	453.2	2,815.6	853.2	2,554.7	1,009.6	2,603.9
LSD (P = 0.05)	250.2	510.2	210.6	494.4	280.0	326.0

Table 13 Effect on stachydrine and trigonelline synthesis when proline and glutamate were fed with methionine, pyridoxine and folic acid individually.

Treatment	Proline		Stachydrine		Trigonelline	
	No-Stress	Stress	No-Stress	Stress	No-Stress	Stress
			ug per g dry weight			
Control at the start of the experiment	360.2	-	650.0	-	746.40	-
Control no pre-treatment	588.9	4,384.0	762.2	2,195.1	904.7	3,262.3
Proline	3,607.3	7,243.5	1,427.6	2,797.3	1,001.2	3,616.6
Proline + Methionine	1,936.1	3,623.3	805.1	1,961.0	910.7	3,126.7
Proline + Pyridoxine	1,669.5	3,448.6	763.1	1,517.0	1,008.0	2,708.1
Proline + Folic acid	1,515.6	3,371.7	740.0	1,883.4	1,025.1	2,509.8
Glutamate	2,508.8	5,202.1	1,073.2	2,388.9	933.4	3,764.8
Glutamate + Methionine	1,994.4	4,062.4	902.3	2,675.3	1,244.1	3,047.0
Glutamate + Pyridoxine	2,273.1	3,617.9	777.3	2,067.9	1,120.4	2,742.5
Glutamate + Folic acid	1,996.5	4,175.1	658.0	2,206.3	772.4	2,625.5
LSD (P = 0.05)	250.2	510.2	210.6	494.4	280.0	326.0

When glutamate was fed with methionine, pyridoxine and folic acid separately, the proline content increased. Although not as much as when glutamate was fed alone. The increase was significantly higher in the presence of pyridoxine than with methionine or folic acid. No significant changes were observed in stachydrine and trigonelline except when glutamate and methionine were fed together causing a small increase in trigonelline. Under stress glutamate showed lower amounts of proline in the presence of methionine, pyridoxine and folic acid when compared to nontreated stress. It was more clear in the presence of pyridoxine. No significant changes were observed in stachydrine or trigonelline under stress.

10.3.4 EFFECT OF PROLINE AND GLUTAMATE ON STACHYDRINE AND TRIGONELLINE SYNTHESIS IN THE PRESENCE OF DIFFERENT COMBINATIONS OF CO-FACTORS

The data is presented in (Table 14). Significant amounts of proline were found in the plants when it was fed under non-stress conditions along with methionine:pyridoxine, methionine:folic acid and pyridoxine: folic acid. In most of the cases the treatment comparison is made to non pretreated control plants. When proline was fed with methionine, pyridoxine and folic acid together, though it was lower compared to other treatment combinations, it was significantly higher than non-treated control plants. Under stress, in all treatment combinations of proline and co-factors, the proline level found was not significantly higher than in non-treated stress plants.

Stachydrine increased significantly when proline was fed with methionine:pyridoxine and methionine:folic acid. It did not change

Table 14 Effect of proline and glutamate on stachydrine and trigonelline synthesis in the presence of different combination of co-factors.

Treatment	Proline		Stachydrine		Trigonelline	
	No-Stress	Stress	No-Stress	Stress	No-Stress	Stress
			µg per g dry weight			
Control at the start of the experiment	360.3	-	650.0	-	746.40	-
Control no pre-treatment	588.9	4,384.0	762.2	2,195.1	904.7	3,262.3
Proline	3,607.3	7,243.5	1,427.6	2,797.3	1,001.2	3,616.6
Proline + Methionine + Pyridoxine	1,575.1	3,578.5	1,056.7	2,817.1	896.5	3,167.4
Proline + Methionine + Folic acid	1,852.8	3,000.8	1,451.1	2,458.0	1,005.4	3,194.7
Proline + Pyridoxine + Folic acid	1,710.9	3,287.4	734.4	1,929.3	785.8	3,084.7

Table 14 (continued)

Proline + Methionine + Pyridoxine + Folic acid	1,023.1	4,260.4	2,850.1	4,799.3	1,191.4	3,497.7
Glutamate	2,508.8	5,202.1	1,073.2	2,388.9	933.4	3,764.8
Glutamate + Methionine + Pyridoxine	2,086.2	4,189.9	1,016.3	2,873.1	997.3	3,099.5
Glutamate + Methionine + Folic acid	2,064.8	4,191.7	987.6	2,457.6	950.6	3,096.2
Glutamate + Pyridoxine + Folic acid	2,054.9	3,446.0	995.7	2,675.2	1,151.4	3,564.2
Glutamate + Methionine + Pyridoxine + Folic acid	1,546.5	3,430.8	1,166.3	3,277.9	1,009.8	3,712.5
LSD (P = 0.05)	250.2	510.2	210.6	494.4	280.0	326.0

when proline was applied with pyridoxine:folic acid treatment. However, when proline was applied with all three co-factors the increase in stachydrine (3.7 times over control) was highly significant. Under stress this increase was even more clear. The level of stachydrine found with all three co-factors and proline even greatly exceeds that found when proline was fed by itself. This indicates a requirement for all three co-factors for the conversion of proline to stachydrine. No significant changes in trigonelline were observed when proline was fed with different combinations of co-factors, with under non-stress and stress conditons.

When glutamate was applied under non-stress conditons with co-factors in different combinations, the proline content increased signifantly. However, this was not the case under stress with an exception of methionine:pyridoxine. In the presence of pyridoxine: folic acid, and methionine:pyridoxine:folic acid combinations proline appeared in significantly lower amounts compared to non-treated stress. Stachydrine did not change with the glutamate treatment under non-stress, except for a small increase when all co-factors were also fed. Under stress, however, stachydrine increased when glutamate was fed with methionine:pyridoxine and methionine:pyridoxine:folic acid combinations. Once again higher amounts of stachydrine were found in the presence of all the three co-factors, though not as high as when proline, rather than glutamate, was fed with methionine:pyridoxine and methionine:pyridoxine:folic acid combinations. Once again higher amounts of stachydrine were found in the presence of all the three co-factors, though not as high as when proline, rather than glutamate, was fed.

Glutamate did not alter the trigonelline level significantly in the presence of any combination of co-factors under non-stress. Under stress, only when all the three co-factors were provided was there a significant increase in trigonelline as against non-treated stress plant.

10.3.5 EFFECT OF ORNITHINE AND CO-FACTORS ON THE BIOGENESIS OF PROLINE, STACHYDRINE AND TRIGONELLINE UNDER NON-STRESS AND STRESS CONDITIONS

When ornithine was provided under non-stress conditions to alfalfa plants there was a significant increase in the amino acid proline (Table 15). No significant changes were observed with stachydrine under these treatment conditions. There was a significant increase in trigonelline as a result of ornithine treatment. When plants were stressed after ornithine pretreatment proline, stachydrine and trigonelline increased significantly.

When ornithine was provided in the presence of methionine, there was significant increase in proline under non-stress conditions, but it was not as high as when only ornithine was fed. Stachydrine did not change but trigonelline decreased significantly with methionine and ornithine. Under stress only trigonelline showed a significant increase in response to the ornithine-methonine combination compared to ornithine alone. When pyridoxine was fed with ornithine only proline showed an increase under non-stress. Under stress however, both proline and trigonelline increased but not stachydrine. Folic acid with ornithine did not change stachydrine or trigonelline under stress or non-stress but increased proline under non-stress, while decreasing it under stress.

Table 15 Effect of ornithine and co-factors on the biogenesis of proline, stachydrine and trigonelline under non-stress and stress conditions.

Treatment	Proline		Stachydrine		Trigonelline	
	No-Stress	Stress	No-Stress	Stress	No-Stress	Stress
			mg per g dry weight			
Control at the start of the experiment	578.3	-	649.7	-	878.40	-
Control no pre-treatment	563.7	4,788.5	850.2	2,010.4	885.3	2,611.1
Ornithine	2,177.0	5,621.8	830.5	2,569.4	1,195.3	3,126.7
Ornithine + Methionine	1,515.1	5,361.4	865.9	1,849.7	749.3	3,098.2
Ornithine + Pyridoxine	2,872.3	6,541.9	754.6	2,149.6	797.5	2,877.4
Ornithine + Folic acid	1,529.1	4,069.6	822.5	2,032.5	922.0	2,751.7
Orthinine + Pyridoxine + Folic acid	1,182.6	4,424.7	1,047.8	3,090.5	1,114.9	2,828.4
Orthinine + Methionine + Pyridoxine + Folic acid	1,631.7	6,582.1	1,517.1	3,281.2	1,012.6	3,032.5
LSD (P = 0.05)	338.2	321.0	160.5	192.0	100.1	199.7

When only the co-factors were fed without ornithine there was a significant increase in proline, stachydrine and trigonelline under non-stress conditions. With these three co-factors and ornithine, proline and stachydrine increased significantly under non-stress conditions. The picture was different under stress, where all three, i.e.proline, stachydrine and trigonelline, increased significantly.

10.3.6 EFFECT OF PROLINE, GLUTAMATE AND ORNITHINE ON THE BIOGENESIS OF STACHYDRINE AND TRIGONELLINE IN THE PRESENCE OF CO-FACTORS UNDER NON-STRESS AND STRESS (PEG) CONDITIONS

When proline was fed to alfalfa plants under normal conditions, it was expected to increase tissue proline content. Indeed, there was a significant increase in proline in the tissue after 48 hours of feeding (Table 16). Glutamate and ornithine also increased the proline content, but to a lesser extent. Stachydrine content also increased in proline and glutamate treated plants but no significant changes were found in trigonelline in any of the three treatments.

After the pre-treatments with proline, glutamate and ornithine When the plants were fed with proline, and then exposed to PEG stress (-15 bars), there was a significant increase in stachydrine and trigonelline. This was in addition to the presence of higher amounts of proline under these treatments. When methionine, pyridoxine and folic acid were fed, except for proline, no significant changes were observed under non-stress. Under stress, only stachydrine increased significantly.

When proline, glutamate and ornithine were fed with the three co-factors under non-stress conditions, only stachydrine showed a

Table 16 Effect of proline, glutamate and ornithine on the biogenesis stachydrine and trigonelline in the presence of co-factors under non-stress and stress (PEG).

Treatment	Proline		Stachydrine		Trigonelline	
	No-Stress	Stress	No-Stress	Stress	No-Stress	Stress
			mg per g dry weight			
Control at the start of the experiment	723.9	-	722.6	-	915.0	-
Control no pre-treatment	682.6	3,438.1	954.3	1,955.5	994.6	2,526.2
Proline	3,861.1	5,301.7	1,330.1	2,532.7	972.6	3,549.4
Glutamate	2,127.4	4,211.3	1,156.2	2,429.3	1,050.1	3,624.6
Ornithine	2,034.1	3,891.7	962.4	2,332.1	995.0	3,017.1
Methionine + Pyridoxine + Folic acid	1,026.3	3,499.2	904.5	2,239.6	1,033.0	2,614.8

Proline + Methionine + Pyridoxine + Folic acid	1,868.6	3,733.6	2,577.3	3,628.6	1,078.2	2,980.6
Glutamate + Methionine + Pyridoxine + Folic acid	1,571.6	3,406.5	1,668.1	2,889.7	966.8	3,394.7
Orthine + Methionine + Pyridoxine + Folic acid	1,122.3	4,176.8	1,331.5	3,003.5	974.6	3,013.1
LSD (P = 0.05)	242.6	300.1	184.8	215.0	162.9	314.3

Table 17

^{15}N-proline conversion to stachydrine with and without co-factors (methionine, pyridoxine and folic acid) in the presence of stress and non-stress in alfalfa.

Treatment	Tissue type	mg of ^{15}N microportion			
		Proline		Stachydrine	
		Control	Stress	Control	Stress
N^{15} proline					
	Leaves	3.684	2.917	0.381	2.71
	Stem	2.955	2.370	0.361	2.51
	Roots	3.364	3.472	0.415	2.63
N^{15} proline + co-factors					
	Leaves	3.458	1.251	0.551	3.43
	Stem	3.008	1.620	0.971	3.24
	Roots	3.301	2.013	0.665	3.52

significant increase. Under stress, however, in the presence of all three co-factors, proline and glutamate feeding resulted in low proline content in the plants, but all of the other solutes increased in all of the other treatments. Ornithine, however, increased the proline significantly under stress. Stachydrine increased significantly with all the three amino acids under stress and the order of increase being proline, ornithine and glutamate.

10.3.7 ^{15}N-PROLINE CONVERSION TO STACHYDRINE WITH AND WITHOUT CO-FACTORS (METHIONINE, PYRIDOXINE AND FOLIC ACID) IN THE PRESENCE OF STRESS AND NON-STRESS IN ALFALFA ORGANS

^{15}N-proline fed to alfalfa plants through the roots was distributed in leaves, stem and roots (Table 17). The presence of co-factors at the time of ^{15}N-proline feeding did not seem to have any effect on ^{15}N-proline uptake. When the plants were stressed after the feeding there was a reduction in the ^{15}N-proline level compared to the level in non-stressed plants. This reduction was even more pronounced in the presence of co-factors. ^{15}N-enrichment was found in stachydrine after the ^{15}N-proline application to the control tissue, indicating some of the ^{15}N-proline was converted to stachydrine. This conversion was further enhanced by the co-factors. Under stress the conversion was even more pronounced in all the organs both with and without co-factors. This was similar to the earlier findings where proline in the presence and absence of co-factors caused an increase in stachydrine content.

10.4 DISCUSSION

Betaines form a natural group of simple nitrogenous compounds which is widely distributed in plants. Most of them are derived from amino

acids in which the nitrogen atom is fully methylated. In the present experiments the relationship between proline, glutamate and ornithine on the one hand, to stachydrine and trigonelline, on the other, in alfalfa was examined.

It is evident that when proline was fed through the roots to alfalfa seedlings, not only were the endogenous levels of proline increased but the stachydrine levels under non-stress conditions were also increased (Table 11). This suggests that proline may be a close precursor of stachydrine in alfalfa. Similar results were also obtained by Robertson and Marion (1959a,b,c). The increase in stachydrine as a result of proline feeding was further enhanced by NaCl stress. This could be due to faster transmethylation reactions under stress conditions.

Though the presence of trigonelline in alfalfa (Blake, 1954; Robertson and Marion, 1959a), has been known for some time and its increase in response to different environmental stresses (Chapter IV, Section 3-6) seems clear, it's path of synthesis in alfalfa is not known. In these experiments proline did not increse the trigonelline levels under non-stres conditions, but it did increase them significantly under NaCl stres (Table 11). One explanation for this increase is the possible conversion under stress of proline through α - amino veleric acid to the six-membered ringed compound, nicotinic acid (Leete, et al, 1964), from which trigonelline is formed (Klein and Linser, 1932; Joshi and Handler, 1962; Willeke et al, 1979; Tramontano et al, 1983). Alternatively, trigonelline may have been synthesised, under stress, through other pathways, possibly from glutamate or ornithine. Glutamate in the present investigations increased proline and

stachydrine under non-stress but not trigonelline under stress, however, glutamate increased trigonelline as much as proline did. Ornithine, on the other hand, did not enhance trigonelline as much, even though it too is a precursor of proline.

One of the probable requirements for the increase in stachydrine and trigonelline is the biological methylation of amino acids which requires several cofactors and coenzymes. Methionine is known to be the best source of methyl groups. (Brown and Byrrum, 1952; Matchett et al, 1953; Dubek and Kirkwood, 1952; Lehringer, 1972). Pyridoxine and folic acid are also involved in several transmethylation reactions (Sakami and Welch, 1950; Metzler and Snell, 1952; Walsh, 1979; Breslow and Czarnik, 1983). In these experiments, when the cofactors were fed in the same manner as proline, glutamate and ornithine, there was no significant change observed in either stachydrine or trigonelline levels under non-stress or stress conditions (Table 12). It is clear that though these co-factors may contribute to the conversion of proline and glutamate to stachydrine and trigonelline, they are not sufficient by themselves to enhance the betaine levels. It is also likely that when fed separately, not all the necessary factors were present to complete the transmethylation reactions. This was also supported by the fact that when proline and glutamate were fed together with individual cofactors there was no significant increase in stachydrine and trigonelline either under non-stress or stress conditions (Table 13). These results are in agreement with Robertson and Marion (1959a,b); Weihler and Marion (1958) where methionine, pyridoxine and folic acid individually had no effect on stachydrine synthesis under non-stress conditions. However, when all these co-factors were fed at the same time, together with either proline or glutamate, there was significant changes in stachydrine and

trigonelline (Table 14). Stachydrine increased in the presence of proline and all co-factors 3-7 times more than when proline was present alone. The reduction of proline, under both non-stress and stress supports the conclusion that proline is a close precursor of stachydrine in alfalfa.

In contrast to stachydrine, trigonelline increased under stress in the presence of proline and co-factors but there was no increase under normal conditions. This suggests that the level of trigonelline may be under control in non-stress conditions in the same way that proline levels are. The increase under stress may also be comparable to that of proline, for which feed-back control is lost under stress (Boggess et al, 1976b). On the other hand, stress may activate a different pathway to that operating under non-stress conditions.

One of the other sources for trigonelline in this tissue could be ornithine from which proline is also synthesised (Essery et al 1962; Robertson and Marion, 1959a). This was supported by the increase in trigonelline when ornithine was provided under stress (Table 15 and 16). Ornithine has been shown to increase nicotine content in tobacco plants (Klein and Linser, 1932) by serving as a specific precursor of the pyrrolidine ring of nicotine (Dewey et al, 1955). The carbon skeleton of nicotine is formed by a condensation between reduced diphosphopyridine nucleotide and Δ'-pyrroline. The Δ'-pyrroline can arise from glutamic acid, proline or onithnine. One of the routes whereby glutamic acid and ornithine could yield Δ'-pyrroline is via glutamic-γ-aldehyde and Δ-pyrroline-5-carboxylic acid. Decarboxylation of Δ'-pyrroline-5-carboxylic acid yield Δ'-pyrroline (Leete, 1958). Proline, also, has been reported to serve as a precursor for the pyrroline ring but to a

lesser extent than ornithine and glutamic acid (Leete, et al, 1964). The enhancement of trigonelline under stress may be related to a higher rate of conversion to nicotine and, further, to trigonelline.

Ornithine increased proline but not stachydrine under non-stress. This finding is in accordance with Robertson and Marion (1959a,b,c). However, when co-factors were applied together with ornithine, stachydrine increased under both non-stress and stress. Such a co-factor related conversion in alfalfa under non-stress has been reported previously (Essery, et al, 1962). Considering these experimental results it is likely that ornithine serves as a precursor for both stachydrine and trigonelline. Such a possibility has also been suggested before (Karrer and Widmer, 1925).

The effect of stress on these metabolic reactions was further confirmed with PEG stress where similar results were obtained (Table 16). Once again stachydrine and trigonelline were increased as a result of proline, glutamate and ornithine with all three co-factors (Methionine, Pyridoxine and folic acid). The increase in stachydrine, however, was highest in the proline and co-factors treatment suggesting that proline is a closer precursor for stachydrine under stress or non-stres than glutamate or ornithine. Although ornithine and glutamate increased the stachydrine level, it was to a lesser extent. This may be due to the fact that both ornithine and glutamate have to go through several steps before converting to proline, whereas formation of stachydrine from proline is just a stepwise methylation.

The close relationship between stachydrine and proline was confirmed by feeding ^{15}N-proline. Incorporation of the ^{15}N of proline

into stachydrine was evident in the presence and absence of co-factors (Table 17). ^{15}N incorporation into stachydrine was also enhanced under stress suggesting a stress-induced channelling of proline into stachydrine. Similar results, under non-stress, were obtained with ^{14}C-proline in alfalfa by Robertson and Marion (1959b).

To sum up, it is evident that stachydrine is synthesised from proline in alfalfa under both non-stress and stress. This may be mediated by co-factors like methionine, pyridoxine and folic acid. The synthesis of trigonelline, however, is either on a different pathway possibly with both ornithine and proline, or subject to very different controls.

SECTION 11 : PHYSIOLOGICAL SIGNIFICANCE OF COMPATIBLE SOLUTES (PROLINE AND BETAINES) IN MAINTAINING THE PHOTOFUNCTION OF ALFALFA LEAVES UNDER STRESS

11.1 INTRODUCTION

In alfalfa the accumulation of proline, stachydrine and trigonelline has been found under different stress conditions (Chapter IV, Section 3-6). The significance of these accumulated compounds in enhancing the resistance of plants to stress has been related to -

(a) exerting a general osmoregulatory role which favours turgor maintenance despite a reduction in bulk leaf water potential (Storey and Wyn Jones, 1975, Jagels, 1983, Jolivet et al, 1983);

(b) influence on the retention of catalytic effectiveness of enzyme proteins despite high salt concentration or reduced water potential (Wyn Jones and Storey, 1981), or elevated temperature (Paleg et al, 1981, Nash et al, 1981);

(c) maintenance of differential permeability despite elevated temperatures (Jolivet et al, 1982, 1983).

Inspite of these findings, a definite *in vivo* protective role is yet to be established for these compatible solutes. Thus, it is unclear whether the accumulation of proline, stachydrine and and trigonelline in alfalfa has any protective role *in vivo* under stress. This was tested in the present experiments on one of the most important and sensitive plant processes, photosynthesis.

Reduction of chlorophyll a variable fluorescene characteristics, as a result of reduced water status, has been related to injury of the thylakoid structure, thus, affecting the photosynthetic electron transport (Havaux and Lannaye, 1983). The present experiments were designed to assess the protection, if any, of these variable fluorescence characteristics by proline, stachydrine and glycinebetaine in alfalfa. Variable fluorescence (Fv) and quench elvel (Q1) were used as the tools to measure the damage or protection under PEG stress.

11.2 METHODS

11.2.1 PLANT CULTURE

Alfalfa plants, varieties Hunter River and Paravivo were grown in plastic pots (6") on a non-absorbent support medium (oil dry). They were grown in a growth cabinet with conditions as described in materials and methods (Section 2.2). The plants were watered twice a day with half strength Hoagland's nutrient solution.

11.2.2 FEEDING COMPATIBLE SOLUTES

After 3 weeks selected uniform plants from pots were transferred into 50 ml glass tubes containing half strength Hoagland's solution. In each tube 1-3 plants were grown depending on the nature of the experiment. When plants were established in the nutrient solution culture (2-3 days after transfer from pots), 10 mg of one of the following, L-proline, glycinebetaine, trigonelline or choline chloride, were applied to each tube in the form of a solution. The glass tubes were aerated continuously, and the growth conditions in the cabinet were

maintained as described in the materials and methods (Section 2.2). The solutions in the tubes were checked regularly for any bacterial growth throughout the experimental period. After 48 hours of feeding the plants were taken for fluorescence measurements. In the case of stress treatments, the plants were treated as described in the next section.

11.2.3 STRESS TREATMENT

Plants, after 48 hours of feeding compatible solutes, were stressed with PEG 4000 (-15 bars). PEG solution was applied to the glass tubes by replacing the nutrient solution. The plants, after PEG treatment, were kept in the dark overnight after which they were exposed to high light intensity (1000 $uEm^{-2}s^{-1}$) for a period of one to one and a half hours. The result of the conditions were maintained without alterations.

11.2.4 DARK ADAPTION

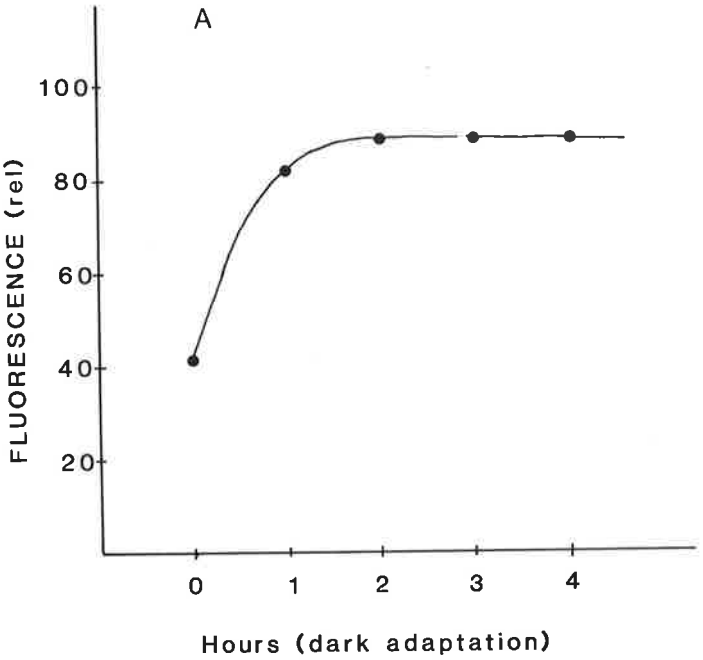
Plants were dark adapted (held in darkness) for a certain length of time in order to obtain a maximum fluorescence signal. A maximum fluorescence peak was obtained after dark adapting the alfalfa plants for 60-90 minutes (Fig. 30A). Before measuring the fluorescence the plants were transferred from the dark chamber to an enclosure flushed with CO_2 free air to achieve full expression of variable fluorescence.

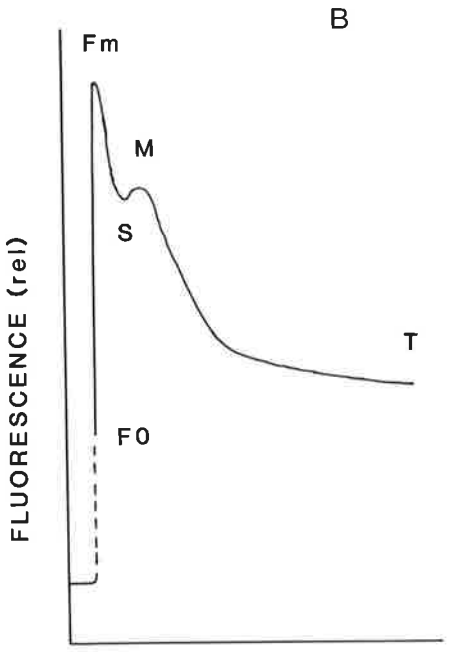
11.2.5 CHLOROPHYLL a FLUORESCENCE INDUCTION KINETICS

Chlorophyll, a fluorescence induction kinetics were measured at room temperature ($25^{o}C$) with a Branker model SF-20 plant productivity fluorometer. The peak signals Fm, Fo, SMT transition on the slow

Fig. 30 A. Dark adaptation period for alfalfa plant to measure the fluorescence characteristics.

B. Example of a fluorescent transient obtained from a maize leaf (Havaux and Lannoye, 1983).
Fo - fluorescence yield from an initial level (0).
Fm - fluorescence yield to a maximum level (m).
S - decrease of fluorescence to a quasi steady state (s).
M - second peak.
T - terminal steady state.


A
100
80
60
40
20
FLUORESCENCE (rel)
0
1
2
3
4
Hours (dark adaptation)


B
Fm
M
S
T
F0
FLUORESCENCE (rel)

kinetics were assigned as indicated in (Fig. 30B), are according to Papageorgiou (1975).

11.2.6 CALCULATIONS OF VARIABLE FLUORESCENCE AND QUENCH LEVEL

Variable fluorescence was calculated as -

$$\underset{(Fv)}{\text{Variable fluorescence}} = \underset{(Fm)}{\text{Maximum Peak}} - \underset{(Fo)}{\text{Constant Yield Fluorescence}}$$

$$Fv = Fm - Fo$$

The nature of the reduction of fluorescence (slope) was taken as quench level (Ql) and calculated as:-

$$\text{Quench level (Ql)} = \frac{\text{Full scale deflection of the recorder}}{\text{distance of the slope}} = \frac{2000}{\text{sec}} \text{ mv/sec}$$

(obtained from the tangent drawn to the slope)

11.2.7 WATER POTENTIAL AND RELATIVE WATER CONTENT (%) MEASUREMENTS

Plant water potential was mesured on the stem with a pressure bomb as described in Section 2.4 of materials and methods. The relative water content (RWC) was measured by the method of Barrs and Weatherly (1962) as described in materials and methods (Section 2.5)

11.2.8 PROLINE, STACHYDRINE AND GLYCINEBETAINE MEASUREMENTS

Proline content of the leaf on which fluorescence measurements were made, was estimated by the Singh et al (1972) method (materials and methods, Section 2.5.1). Stachydrine and glycinebetaine were measured by the method described in the Section 1 and 2 of Chapter IV.

11.3 RESULTS

11.3.1 EFFECT OF PROLINE PRE-TREATMENT ON VARIABLE FLUORESCENCE (Fv) AND QUENCH LEVEL (Q1) IN ALFALFA LEAVES UNDER NORMAL CONDITIONS

Variable fluorescence (Fv) was measured after proline pre-treatment on one leaflet of the selected trifoliate leaflet. There was no significant change in (Fv) due to proline pre-treatment (Fig. 31A). This indicated that proline did not cause any deleterious effect on photofunction when fed exogenously.

The quench level (Q1) showed no significant changes after proline pre-treatment (Fig. 31B) under normal conditions, also indicating no significant effect of exogenous proline.

11.3.2 VARIABLE FLUORESCENCE (Fv) AND QUENCH (Q1) AFTER PROLINE PRE-REATMENT AND SUBSEQUENT EXPOSURE TO PEG STRESS.

Plants, when stressed with PEG and exposed to high light, showed a significant reduction in Fv (Fig. 32A). The quench level (Q1) was also reduced significantly due to stress. However, in proline pre-treated plants the Q1 was maintained at a significantly higher level. (Fig. 32B). This indicates the protection of Fv and Q1 by proline treatment before the onset of stress.

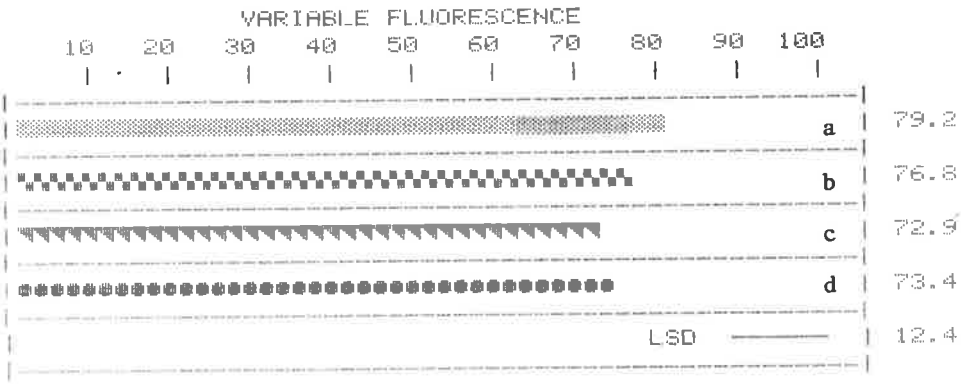
Fig. 31 Effect of proline pre-treatment on fluorescence characteristics under normal conditions.

A - variable fluorescence. (Fv).

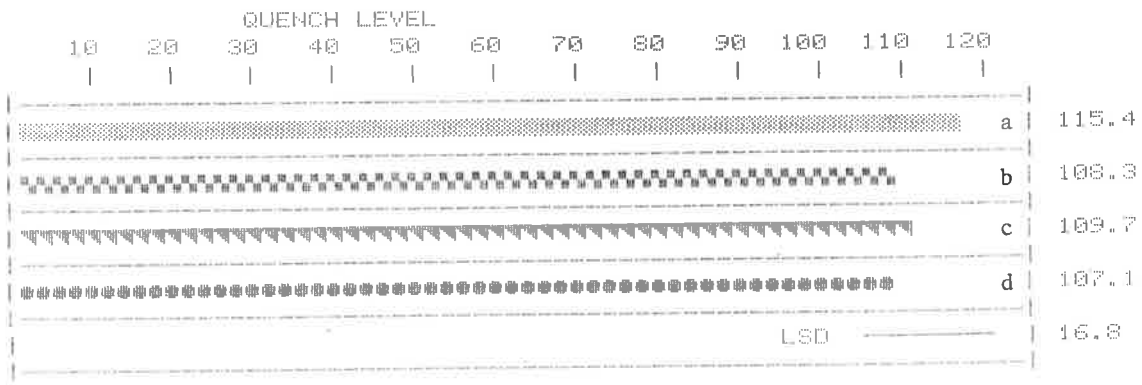
B - quench level. (Ql).

a = non pre-treated control.
b = non pre-treated control.
c = proline pre-treated.
d = proline pre-treated.

A


VARIABLE FLUORESCENCE
10 20 30 40 50 60 70 80 90 100
a
b
c
d
LSD
79.2
76.8
72.9
73.4
12.4

B


QUENCH LEVEL
10 20 30 40 50 60 70 80 90 100 110 120
a
b
c
d
LSD
115.4
109.3
109.7
107.1
16.8

11.3.3 PLANT WATER POTENTIAL AND LEAF PROLINE CONTENT AFTER PROLINE PRE-TREATMENT AND PEG STRESS

Plant water potential of non-stressed plants ~~Change due to proline~~ did not change due to proline pre-treatment. Under PEG stress there was a significant reduction in plant water potential in both proline-treated and non-treated plants (Fig. 33A). Proline content of the leaves measured at the end of the experiment increased significantly in the non-treated stressed plants. The proline-pretreated plants showed much higher proline levels under stress (Fig. 33B), an indication of the uptake of exogenously applied proline.

11.3.4 EFFECT OF PROLINE, GLYCINE BETAINE AND STACHYDRINE ON VARIABLE FLUORESCENCE (Fv) AND QUENCH LEVEL (Q1) UNDER STRESS CONDITIONS

Fv, as in the previous experiment, decreased significantly in the non-pretreated plants (Fig. 34A) as a result of stress. Proline pre-treated plants retained Fv under stress confirming the results of the previous experiment. Interestingly, plants treated with stachydrine, a compound also found to increase in alfalfa under stress, also retained Fv under stress. The retention of Fv was well apparent when plants were fed with glycine betaine, a compound which does not occur in alfalfa.

The protective effects of proline, stachydrine and glycine betaine on quench level (Q1) was also highly significant. It was quite significantly maintained under stress by all three solutes (Fig. 34B). Similar results were observed with the next younger leaves which are more sensitive to stress (Figs. 35A and B).

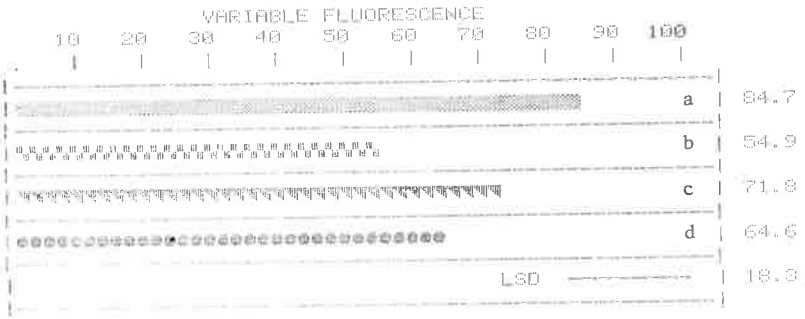
Fig. 32 Effect of proline pre-treatment on fluorescence characteristics under normal stress.

A - variable fluorescence. (Fv).

B - quench level. (Ql).

a = non pre-treated control.
b = non pre-treated control.
c = proline pre-treated.
d = proline pre-treated.

A


VARIABLE FLUORESCENCE
10 20 30 40 50 60 70 80 90 100
a 84.7
b 54.9
c 71.8
d 64.6
LSD 18.3

B

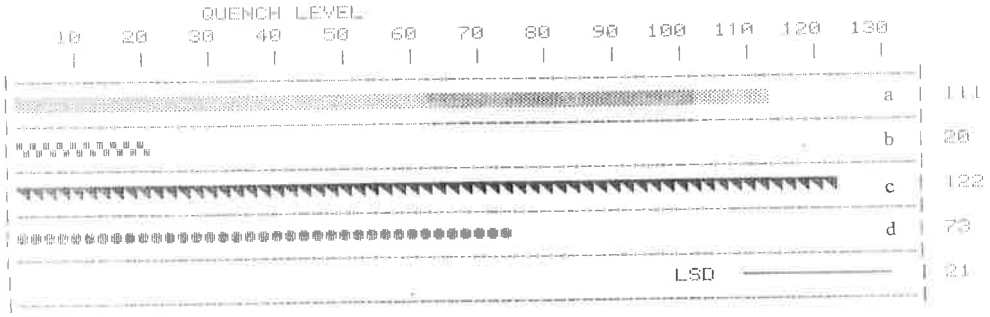

QUENCH LEVEL
10 20 30 40 50 60 70 80 90 100 110 120 130
a 111
b 20
c 122
d 73
LSD 21

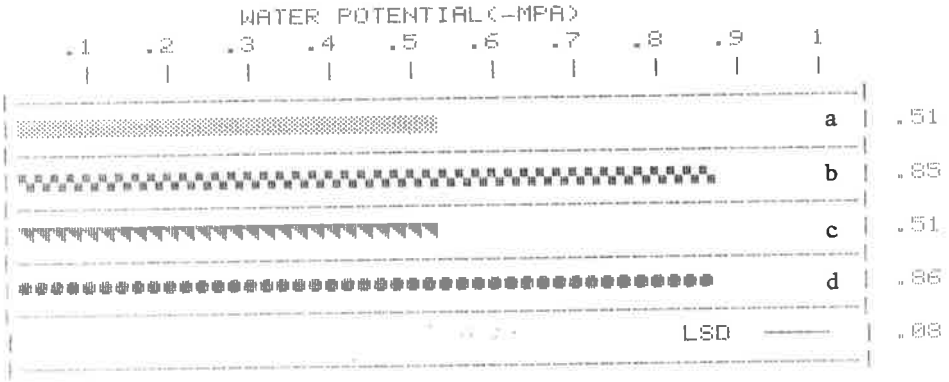
Fig. 33 A. Plant water potential of seedlings exposed to pre-treatment with proline and PEG stress.

a = non pre-treated control.
b = non pre-treated ~~control~~ stressed
c = proline pre-treated.
d = proline pre-treated and stressed.

B. Leaf proline content in alfalfa plants after pre-treatment with proline and PEG stress.

a = non pre-treated control.
b = non pre-treated ~~control.~~ stressed.
c = proline pre-treated.
d = proline pre-treated and stressed.

A


WATER POTENTIAL(-MPA)
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
a
.51
b
.85
c
.51
d
.86
LSD
.08

B

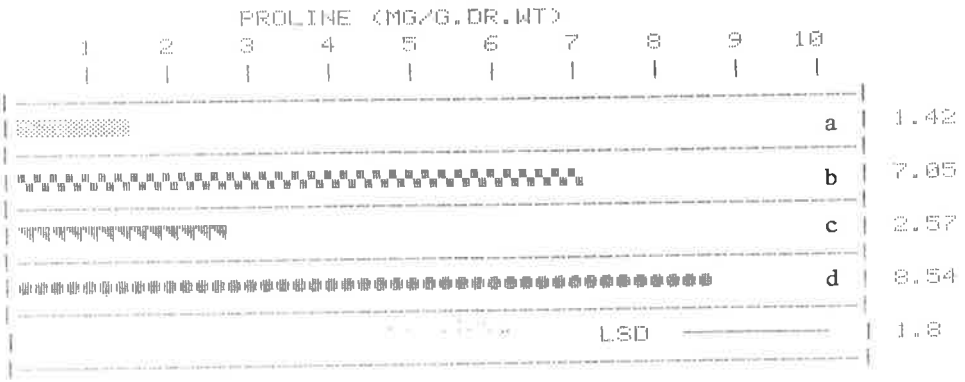

PROLINE (MG/G.DR.WT)
1
2
3
4
5
6
7
8
9
10
a
1.42
b
7.05
c
2.57
d
8.54
LSD
1.8

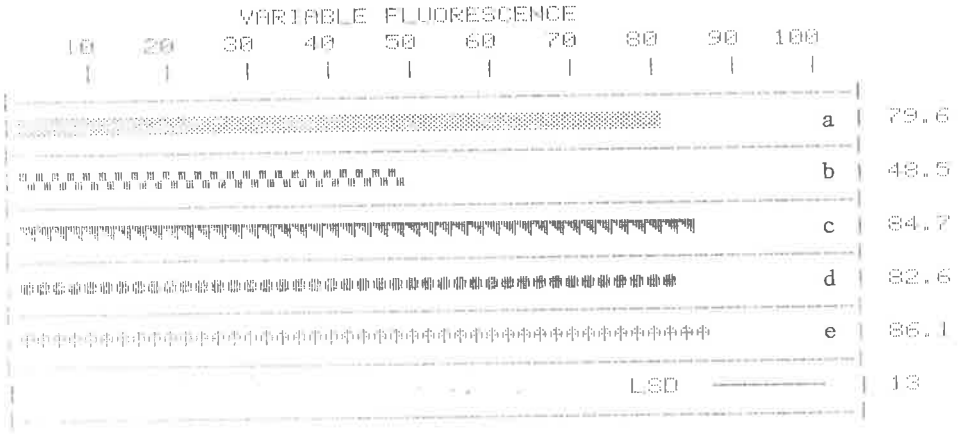
Fig. 34 A. Effect of pre-treatment with compatible solutes on variable fluorescence (Fv) under normal and stressed alfalfa leaves.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed.
c = proline pre-treated.
d = proline pre-treated and stressed.
e = stachydrine pre-treatment and stress.

B. Effect of pre-treatment with compatible solutes on quench level (Ql) under normal and stressed alfalfa leaves.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed.
c = proline pre-treated.
d = proline pre-treated and stressed.
e = stachydrine pre-treatment and stress.

A


VARIABLE FLUORESCENCE
10
20
30
40
50
60
70
80
90
100
a
79.6
b
48.5
c
84.7
d
82.6
e
86.1
LSD
13

B

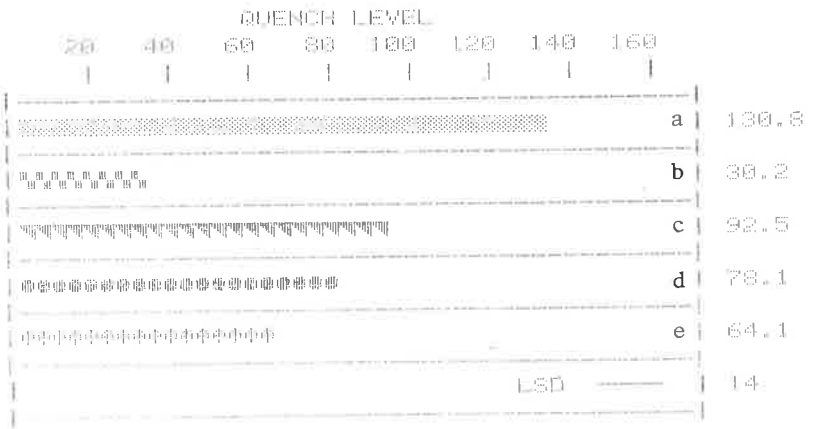

QUENCH LEVEL
20
40
60
80
100
120
140
160
a
130.8
b
30.2
c
92.5
d
78.1
e
64.1
LSD
14

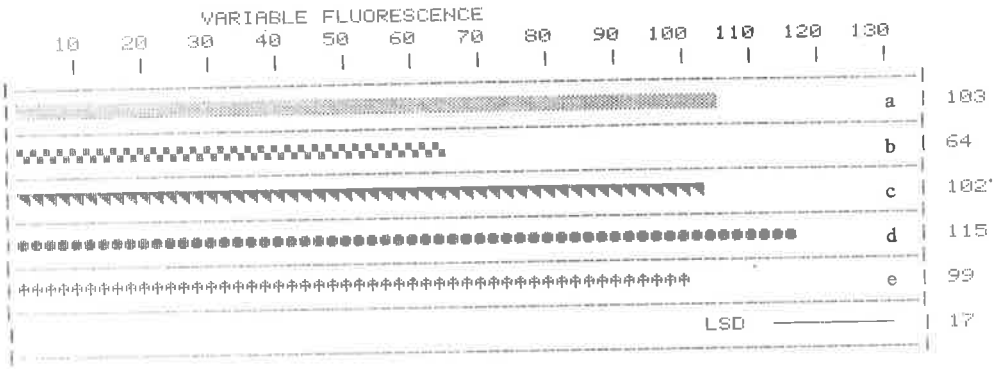
Fig. 35 A. Effect of pre-treatment with compatible solutes on variable fluorescene (Fv) under normal and stressed youngest leaves in alfalfa.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed
c = proline pre-treated.
d = proline pre-treated and stressed.
e = stachydrine pre-treatment and stress.

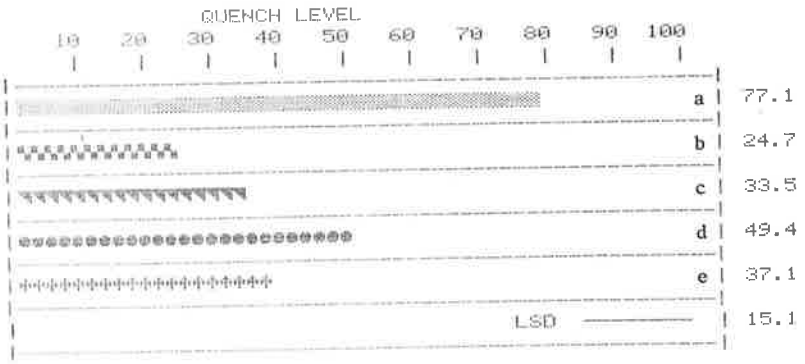
B. Effect of pre-treatment with compatible solutes on quench level (Q1) under normal and stressed youngest leaves in alfalfa.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed
c = proline pre-treated.
d = proline pre-treated and stressed.
e = stachydrine pre-treatment and stress.

A


VARIABLE FLUORESCENCE
10 20 30 40 50 60 70 80 90 100 110 120 130
a 103
b 64
c 102
d 115
e 99
LSD 17

B


QUENCH LEVEL
10 20 30 40 50 60 70 80 90 100
a 77.1
b 24.7
c 33.5
d 49.4
e 37.1
LSD 15.1

Plants were transferred from PEG solutions to fresh nutrient solution in order to study the recovery from stress. After 4 hours in the normal growing conditions Fv of plants in all the treatments returned to almost normal levels (Fig. 36A). With quench levels, however, all pre-treatments except glycine betaine showed a high level of recovery although the control did not recover well (Fig. 36B).

11.3.5 RELATIVE WATER CONTENT (RWC)

Relative water content was measured with one of the leaflets from the same trifoliate leaf used for fluorescence measurements. Relative water content decreased significantly after stress (Fig. 37A) in all the treatments. Interestingly, there was no significant different between the pre-treated plants and non-treated plants suggesting that the compatible solutes did not effect the relative water content under stress.

11.3.6 LEAF LEVELS OF PROLINE, STACHYDRINE AND GLYCINE BETAINE AFTER PRE-TREATMENT AND STRESS

Measurements were made on one of the leaflets on the trifoliate leaves used for fluorescence measurements. Proline content was significantly higher in the proline-pretreated plants compared to the non-treated stressed plants (Fig. 37B). This indicates the uptake of exogenously applied proline from the nutrient solution. It is evident, however, that neither stachydrine nor glycine betaine influenced the proline level of stressed plants. Large amounts of glycine betaine were found in the glycine betaine pre-treated plants (Fig. 38A). This confirms the uptake as alfalfa doesn't synthesise glycine betaine.

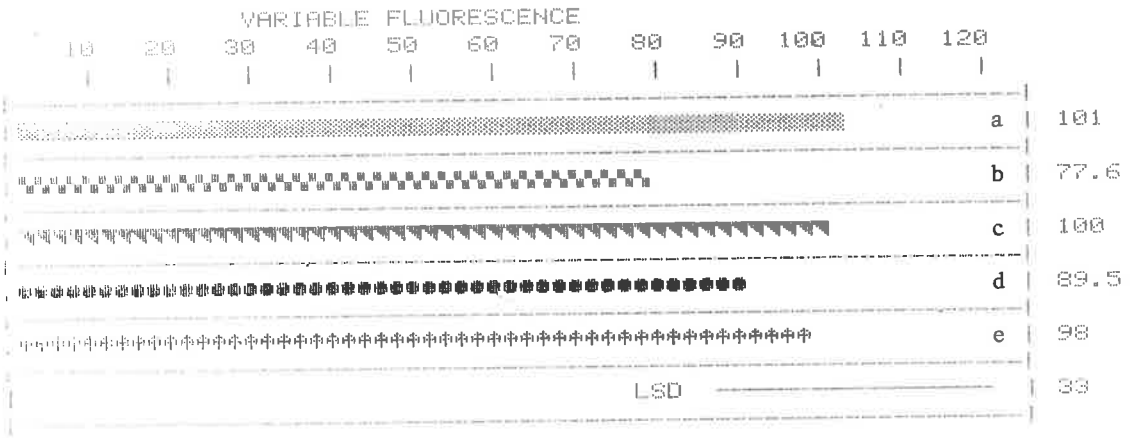
Fig. 36 Recovery of fluorescence characteristics after pre-treatment with compatible solutes and stress.

A = variable fluorescence (Fv)

B = quench level (Ql)

a = non pre-treated control.
b = non pre-treated ~~controls~~ and stressed
c = proline pre-treated.
d = proline pre-treated and stressed.
e = stachydrine pre-treatment and stress.

A


VARIABLE FLUORESCENCE
10 20 30 40 50 60 70 80 90 100 110 120
a 101
b 77.6
c 100
d 89.5
e 98
LSD 33

B

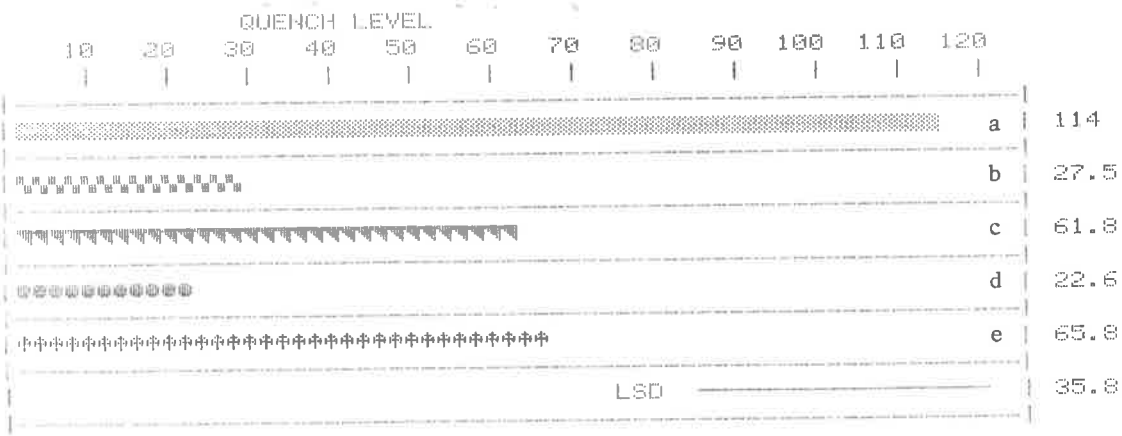

QUENCH LEVEL
10 20 30 40 50 60 70 80 90 100 110 120
a 114
b 27.5
c 61.8
d 22.6
e 65.8
LSD 35.8

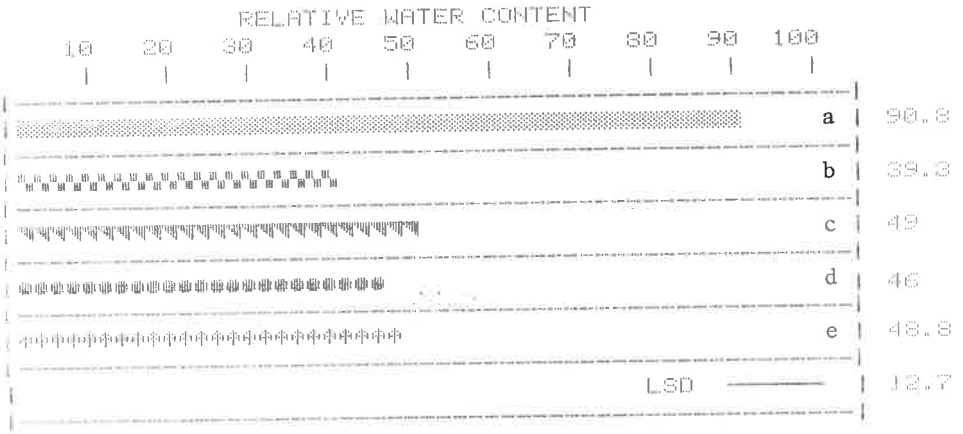
Fig. 37 A. Relative water content of alfalfa plants after pre-treatment with compatible solutes and stress.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed .
c = proline pre-treated.
d = proline pre-treated and stressed.
e = stachydrine pre-treatment and stress.

B. Proline content of alfalfa plants after pre-treatment with compatible solutes and stress.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed.
c = proline pre-treated.
d = proline pre-treated and stressed.
e = stachydrine pre-treatment and stress.

A


RELATIVE WATER CONTENT
10 20 30 40 50 60 70 80 90 100
a 90.8
b 39.2
c 49
d 46
e 48.8
LSD 12.7

B

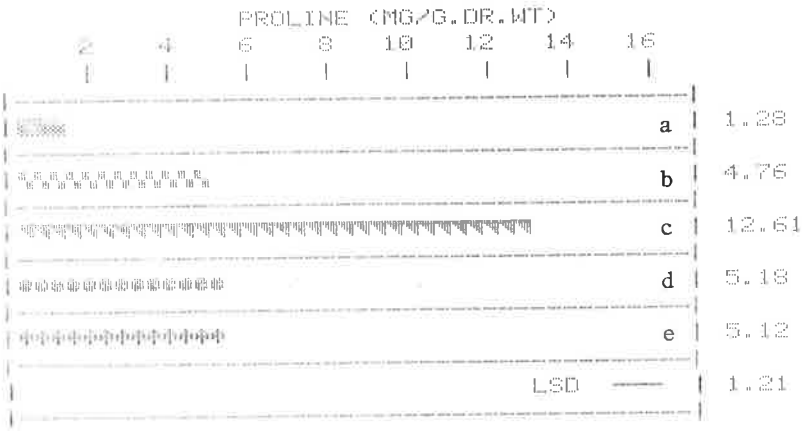

PROLINE (MG/G.DR.WT)
2 4 6 8 10 12 14 16
a 1.28
b 4.76
c 12.61
d 5.18
e 5.12
LSD 1.21

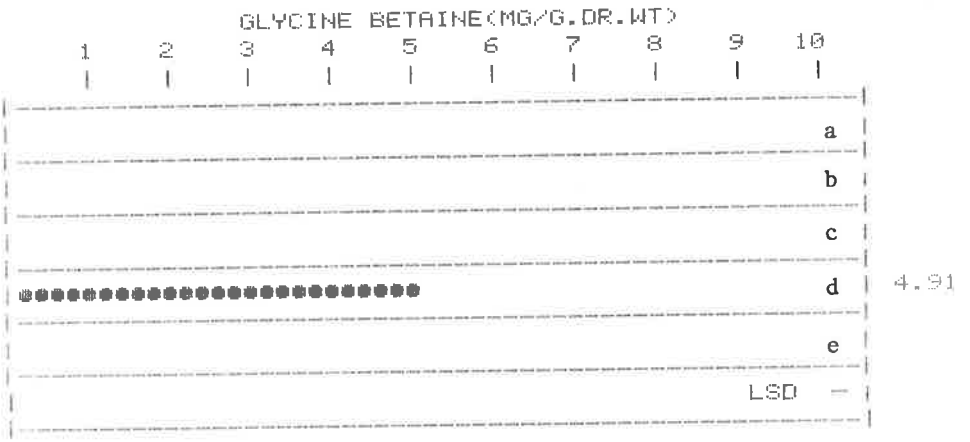
Fig. 38 A. Glycine betaine content of alfalfa plants after pre-treatment with compatible solutes and stress.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed
c = proline pre-treated.
d = proline pre-treated and stressed.
e = stachydrine pre-treatment and stress.

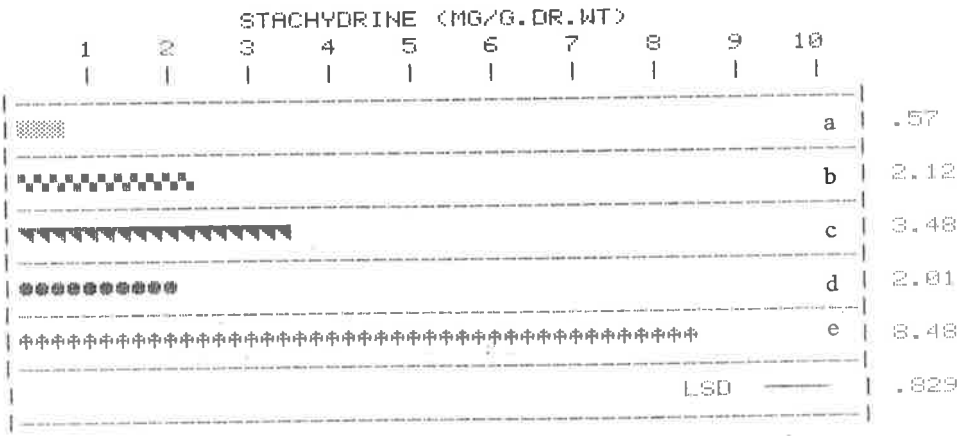
B. Stachydrine content of alfalfa plants after pre-treatment with compatible solutes and stress.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed.
c = proline pre-treated.
d = proline pre-treated and stressed.
e = stachydrine pre-treatment and stress.

A


GLYCINE BETAINE(MG/G.DR.WT)
1 2 3 4 5 6 7 8 9 10
a
b
c
d
e
LSD
4.91

B


STACHYDRINE (MG/G.DR.WT)
1 2 3 4 5 6 7 8 9 10
a
b
c
d
e
LSD
.57
2.12
3.48
2.01
8.48
.829

Stachydrine also increased significantly due to both feeding and stress (Fig. 38B). The endogenous level of stachydrine in the proline-pretreated plants was also increased reflecting the conversion of proline to stachydrine.

11.3.7 GENOTYPIC DIFFERENCES IN VARIABLE FLUORESCENCE (Fv) AND QUENCH LEVEL (Q1) IN RESPONSE TO PROLINE AND STACHYDRINE PRE-TREATMENT AND PEG STRESS

A comparison of two varieties was made of variable fluorescence, and the quench level with and without pre-treatment of compatible solutes under stress. Hunter River was reported to be a drought-resistant genotype and Paravivo a susceptible one (according to Department of Agriculture field observations). Although the report was not based on quantitative parameters, it was felt that a comparative study of these two genotypes might reveal differences in responsiveness to the pre-treatments under stress conditions.

As in (Fig. 39), without pre-treatment the geontype Hunter River showed less percentage decrease of the variable fluorescence compared to Paravivo under stress. However, when proline or stachydrine was fed before stress, the variable fluorescence of both genotypes was preserved to a greater extent when under stress. The effect of stachydrine was significant with Hunter River and the proline effect was significant with Paravivo.

The quench level (Q1) decreased significantly in both the genotypes when stressed (Fig. 40), and proline and stachydrine pre-treatment enhanced retention of the Q1 under stress. In spite of the great

Fig. 39 Effect of pre-treatment with compatible solutes prior to stress on variable fluorescence (Fv) in two alfalfa varieties, Hunter River and Paravivo.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed.
c = proline pre-treated.
d = proline pre-treated and stressed.

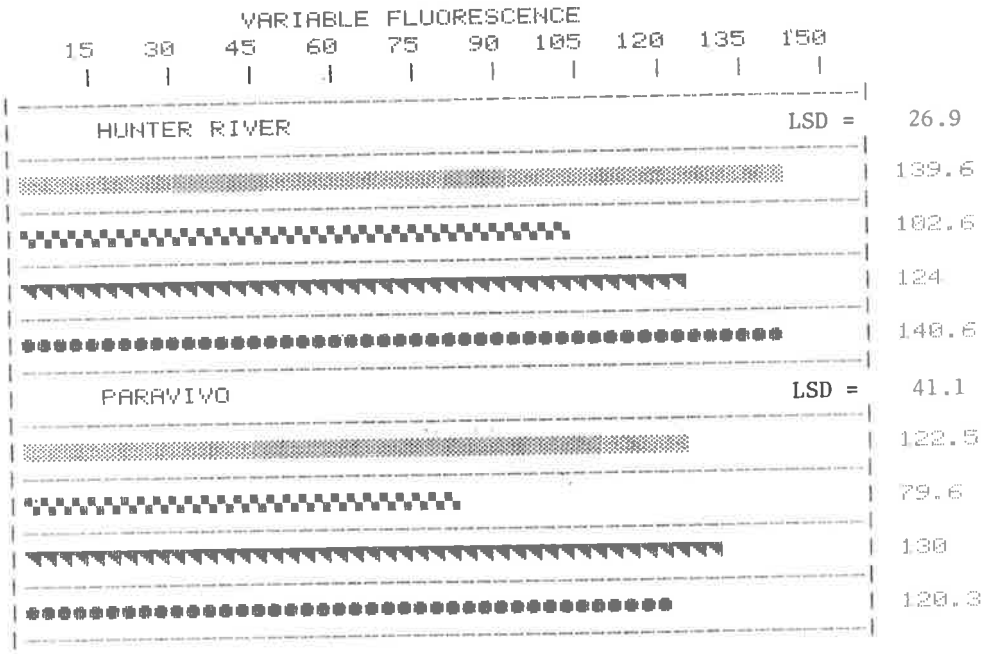

VARIABLE FLUORESCENCE
15
30
45
60
75
90
105
120
135
150
HUNTER RIVER
LSD = 26.9
139.6
102.6
124
140.6
PARAVIVO
LSD = 41.1
122.5
79.6
130
120.3

Fig. 40 Effect of pre-treatment with compatible solutes prior to stress on quench level (Q1) in two alfalfa varieties, Hunter River and Paravivo.

a = non pre-treated control.
b = non pre-treated ~~control~~. and stressed.
c = proline pre-treated.
d = proline pre-treated and stressed.

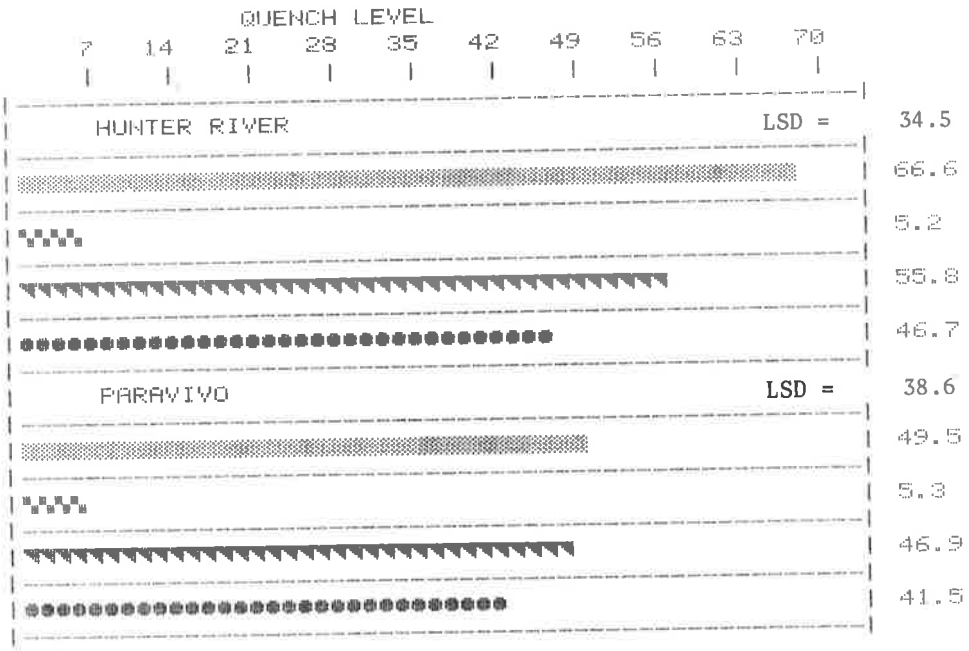
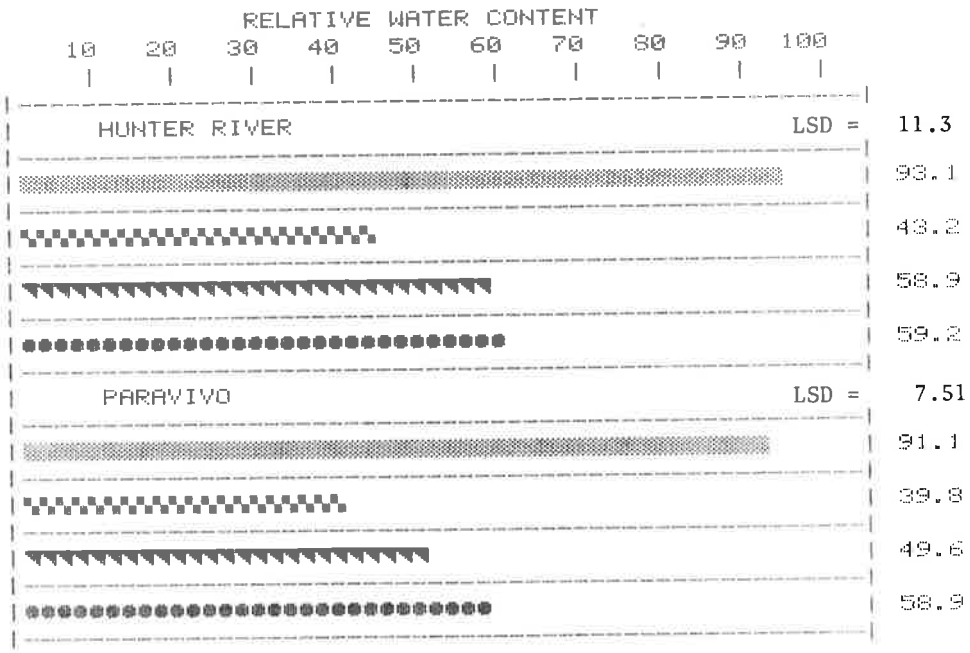

QUENCH LEVEL
7
14
21
28
35
42
49
56
63
70
HUNTER RIVER
LSD =
34.5
66.6
5.2
55.8
46.7
PARAVIVO
LSD =
38.6
49.5
5.3
46.9
41.5

Fig. 41 Effect of pre-treatment with compatible solutes prior to stress on Relative Water Content in two alfalfa varieties, Hunter River and Paravivo.

a = non pre-treated control.
b = non pre-treated control.
c = proline pre-treated.
d = proline pre-treated and stressed.


RELATIVE WATER CONTENT
10
20
30
40
50
60
70
80
90
100
HUNTER RIVER
LSD = 11.3
93.1
43.2
58.9
59.2
PARAVIVO
LSD = 7.51
91.1
39.8
49.6
58.9

variability observed in this experiment, the effects of proline and stachydrine were significant on Hunter River, while only proline was effective with Paravivo. Hunter River showed a relatively higher reduction in Q1 under stress. However, there was a relative difference between genotypes in the Q1 under normal conditions.

11.3.8 RELATIVE WATER CONTENT (RWC) OF GENOTYPES HUNTER RIVER AND PARAVIVO AFTER PRE-TREATMENT AND UNDER PEG STRESS

Relative water content decreased significantly in both the genotypes (Fig. 41). Proline and stachydrine pre-treated plants also showed a decrease in the RWC under stress, but plants with pre-treatment of both genotypes showed significantly higher RWC levels when compared to plants without proline and stachydrine pre-treatment. There was no difference between proline and stachydrine in the genotype Hunter River, but Paravivo showed higher RWC with stachydrine compared to proline under stress. Inspite of this difference between the compounds, the overall stress effects on RWC in both genotypes are the same (statistically non-significant).

11.4 DISCUSSION

Variable fluorescence characteristics calculated from a number of S-M-T transients (Fig. 30B) indicates the relative strength of initial electron flow through PS II, while signal quenching after the initial peak (Fm) provides an additional comparative index of subsequent photosynthetic electron flow and related events on the reduced side of PS II. In the present experiment, when proline was fed through the roots, no significant changes in the variable fluoresence

characteristics were observed (Figs. 31A and B) meaning there were no deleterious effects of proline on the photofunction of the leaf. When plants were stressed overnight with PEG (-1.5 MPa) to a certain level of water potential (Fig. 33A) and subsequently exposed to high irradiance, both variable fluorescence (Fv) and quench level (Q1) were decreased significantly. Similar reductions in variable fluorescence as a result of water stress (Havaux and Linnoye, 1983), salt stress (Downton and Milhouse, 1983) chilling and heat (Smillie and Hetherington, 1983) have been reported. However, when plants were pre-treated with proline before stress, both Fv and Q1 were significantly lighter (Figures 4a, 4b) following exposure to stress (-0.85 MPa). This suggests a possible protective role for proline.

Stachydrine, which accumulates in alfalfa under stress, was also tested for a protective role. In addition, glycinebetaine, a compound which does not occur in alfalfa, was included in the test. Pre-treatment with these compatible solutes before stress significantly maintained the Fv and Q1 (Figs. 34A and B). This was even more evident in the next younger leaf tested (Figs. 35A and B) which is probably more active and sensitive to stress effects. When the stress was alleviated, Fv was restored, but not the Q1, confirming the two different processess suggested by Havaux and Lannoye (1983). Measurement of endogeneous levels of proline, stachydrine and glycinebetaine after the pre-treatment and at the end of the experiment (Figs. 33B, 37B, 38A and B) gave an idea of how much of these compatible solutes were present in the tissue when it was stressed. One of the aspects that may differ between genotypes was observed with Hunter River, a relatively drought-resistant genotype, and Paravivo, a susceptible genotype. In both, water stress caused a reduction in Fv and Q1. The reduction was greater in Paravivo

than in Hunter River, suggesting a link between susceptibility and photofunction. However, pre-treatment with proline maintained Fv and Q1 to the same extent in both genotypes. These differences in response may eventually be of help in screening genotypes for stress resistance.

The reduction in variable fluorescence is attributed to the reduction of Q, the primary electron acceptor of PS II (Papageorgiou, 1975; Barber, 1976; Govindjee et al, 1981). The quenching of fluorescence emission from Fm to T is believed to involve redox changes in Q due to the initiation of CO_2 fixation and, also, an increase in excitation energy transfer from the strongly fluorescent PSII to the weakly fluorescent PSI (Papageorgiou, 1975; Barber, 1976). This change in energy transfer efficiency between the two photosystems is controlled by conformational changes in the thylakoids and is associated with bioenergetic processes such as the generation of electrochemical gradients across the thylakoid membrane and changes in ATP concentration in the stroma (Krause, 1974; Barber, 1976; Briantais et al, 1979; Bennet et al, 1980; Horton and Black, 1980).

If one accept these explanations then the loss or decrease of fluorescence characteristics can easily be attributed to the changes induced in any of the above mentioned factors by stress. However, whatever the mechanism of stress action, it is certain that compatible solutes protect the photofunction as measured by the retaining of variable fluorescence and quench levels under stress.

CHAPTER V

GENERAL DISCUSSION

CHAPTER V

GENERAL DISCUSSION

Development of methods for the ready separation of quaternary ammonium compounds (QACs) is very important in order to study their precise role in plants and animals. Werle (1955), and more recently Gorham et al, (1982), summarised the numerous procedures employed for the analysis of individual QACs. However, none of these methods directly fulfilled the requirements of speed, simplicity and general application necessary for use in this thesis work. These problems were disentangled by purifying the extracts on column chromatography based on the method of Christianson et al, (1960). Purification was carried out on a strong cation exchange resin (Dowex 50 H^+, 50-100 mesh) column, and subsequently eluted with strong acid. This is where most of the previous methods failed; because of the incomplete separation, other compounds intereferred with analytical methods for determining QACs. In the present study, separation of individual QACs in alfalfa extracts was successfully done by the combination of column and thin layer chromatography.

As there were many QACs which reacted with Dragendorff's reagent, identification of individual QACs was very important. After the initial separation and purification, precise identification with the NMR confirmed the earlier reports that alfalfa contains stachydrine, homostachydrine and trigonelline (Steenbock, 1918; Blake, 1954; Robertson and Marion, 1958, 1959; Weihler and Marion, 1959, 1960; Conners et al, 1973; Wyn Jones and Storey, 1981). There was also a mention of the presence of glycine betaine (Sauvage et al, 1983) in

alfalfa but the present study does not support such claims. The NMR analysis not only provided confirmation of separation techniques and identification of individual betaines specifically but it also provided a possibility, if required to measure these compounds quantitatively based on their $N\text{-}CH_3$ proton signals. However, for the present study, only the 'column-TLC' method was used for quantitative estimation. The identification and quantitative estimations of stachydrine, homostachydrine and trigonelline, along with proline, in alfalfa, point to the need for further physiological and biochemical studies under stress conditions.

Apart from a simple water deficiency, similar metabolic changes are brought about by several other unfavourable environmental factors. For example, increasing the NaCl concentration in the medium around the roots of a plant will lower the Gibbs free energy of that medium and hence, decrease the potential gradient along which water flows into the plant resulting in a limitation in the rate of water uptake. Low temperature can also lower the movement of water to the plant resulting in a metabolic change. In all these conditions, the inter-relationships between the direct effects of the environmental factors, and the indirect effects on tissue water deficit in influencing the growth and metabolism of the plant, are difficult to separate. It is particularly difficult to separate the direct effects of these environmental factors on metabolism from those mediated through the concomittant changes in water potential. The effects of leaf water potential on plant growth and metabolism have been studied for a long time and it can be concluded that water potential does not have a uniform effect on the various aspects of plant growth and functioning.

However, the most noticeable metabolic consequence of lowered water potential is the modification of amino acid metabolism leading to a rapid and extensive accumulation of the amino acid proline (Barnett and Naylor, 1966; Singh et al, 1973c; Aspinall and Paleg, 1981 and many others). Such an increase in proline in alfalfa organs, as a consequence of water stress which also caused a reduction in water potential, was observed (Chapter IV, Section 3). Similarly, a water deficit results from salinity stress, but it's effect is more complicated, mainly depending on the salt concentration and the rate of salinisation. Despite the fact that the changes in the water status are different than those caused by a water deficit, there is large accumulation of proline in plants as a result of salt treatment (Palfi and Juhasz, 1970; Chu et al, 1976; Storey and Wyn Jones, 1978, Buhl and Stewart, 1983; Dreier, 1983). In alfalfa, proline accumulated in all the organs as a result of a salt treatment which also caused a reduction in water potential (Chapter IV, Section 4). Again, with salinity, proline accumulation appeared to be mediated through a change in the water status. Another form of reduction in the water status is by lowering the water potential by immersing roots in a solution of PEG, and this also resulted in proline accumulation (Singh et al, 1973a; Ayer, 1981). Similar results were obtained with alfalfa (Chapter IV, Section 6). Chu et al (1976) correlated the leaf proline content and the leaf water potential resulting from PEG stress. Generally proline accumulation has been related to a reduction in water status of the tissue. This may not be universal as there is also a rapid increase in proline accumulation under cold or low temperature stress (Trione et al, 1967; Gates et al, 1971; Chu et al, 1978; Vezina and Paquin, 1982). Alfalfa plants when exposed to $5^{o}C$, accumulated very high amounts of proline in all the organs without any change in water potential (Chapter IV, Section 5). It

is evident, therefore, that proline accumulation is not solely dependent on the water status of the plant or the tissue. This raises the possibility of an alternative factor involved in proline accumulation, which probably respond to stress treatments independent of any changes in water status.

In all the stress experiments proline accumulated differently in roots, stem and leaves of alfalfa than it did in barley. A possible explanation for this differential accumulation may be the stress induced distribution of proline precursors. Alternatively, alfalfa being a leguminous plant in which synthesis and translocation of nitrogenous compounds are different than in non-leguminous plants (Wallace and Pate, 1967) such a difference in organ behaviour may not be surprising. However, it is hard to come to a definite conclusion without understanding the site of synthesis of proline.

One of the very important aspects of proline accumulation in plants is it's disappearance after releasing the stress. It is often suggested that proline is being utilised for metabolic process (Blum and Ebercon, 1976; Aspinall and Paleg, 1981), but the exact involvement of proline in any of the metabolic process is not known. However, it has been reported to be lost principally by oxidation to glutamate (Stewart, 1972b; Singh et al, 1973b, 1973d; Blum and Ebercon, 1976). In alfalfa in all the stress treatment proline levels were reduced after releasing the stress in some cases more quickly than others.

Although there has been a large amount of interest shown in the accumulation of glycine betaine in plants, little attempt has been made to study other betaines. The presence of stachydrine (Steenbock, 1918)

and trigonelline (Blake, 1954) in alfalfa has been known for many years, and was further confirmed by the NMR studies (Chapter IV, Section 1 and 2). Like proline, stachydrine and trigonelline also accumulated to a large extent as a result of water, salt, PEG and cold stress treatments. (Chapter IV, Section 3-6). The accumulation was found in all the organs tested (leaves, stem and roots). Whether this response is due primarily to a reduction in water status in the case of water, salt and PEG stress, or some other factor particularly in cold stress, is not clear at this stage. Whatever the cause, it is certain that stachydrine and trigonelline respond to stress in a manner similar to proline and glycine betaine in the species. This behaviour of stachydrine and trigonelline in alfalfa in response to environmental adversities may be related to the resistance of the plant to stresses. As in the case of proline and glycine betaine (Singh et al, 1973a; Stewart and Lee, 1974; Aspinall and Paleg, 1981; Wyn Jones and Storey, 1981), both stachydrine and trigonelline accumulation may be adaptive responses. If so, species which accumulate these compounds may be of importance for their capacity to resist stress, particularly in screening programmes in selecting and breeding resistant genotypes.

When the stress was relieved, stachydrine levels decreased. This is similar to the response of proline and probably best demonstrates the closeness of the proline-stachydrine relationship. Trigonelline on the otherhand did not decrease as a result of relieving the stress illustrating a case in which the amounts of the solute is independent of the water status of the tissue. However, utilisation of trigonelline as the precursor of pyridine nucleotides in plants have been suggested (Joshi and Handler, 1962), and emphasized the potential metabolic importance of the compound.

ABA concentrations in plants increase markedly during drought (Wright, 1977; Quarrie, 1980; Bradford, 1983; Henson, 1983), salinity (Mizrahi et al, 1971; Hartung et al, 1983) and cold stress (Rifken et al, 1976; Chen et al, 1983; Eamus and Wilson, 1983). Increases in ABA under these situations, particularly during drought, has been suggested to be due to increased biosynthesis rather than release from a pool of bound ABA (Milborrow and Noddle, 1970; Milborrow, 1978). The increase in ABA under different stresses in alfalfa observed in this work may also be due to the increased synthesis, but no conclusions can be made as to why or how it is accumulated.

An interesting aspect of the accumulation of ABA in alfalfa is the simultaneous increase in the levels of proline, stachydrine and trigonelline (Chapter IV, Section 7). Proline accumulation is known to be influenced by ABA (Aspinall et al, 1973; Eder and Huber, 1977; McDonnell et al, 1983). The action of ABA is believed to be due to the ABA-triggered inhibition of the incorporation of proline into protein (Chrispeels and Varner, 1967), or the inhibition of the oxidation of proline (Stewart, 1980). In alfalfa, this inter-relationship was well supported by the fact that proline and ABA both increased during stress. This was further confirmed by the application of exogenous ABA where proline increase was found similar to that under stress conditions. The relationship of stachydrine and trigonelline to ABA is not known and it is impossible at this stage to extrapolate from the proline-ABA relationship with any degree of surity. However, it can be concluded that, under a given stress, ABA probably accumulates first and then triggers other metabolic proceses causing proline, stachydrine and trigonelline accumulation. If this is true, then the changes in the

tissue water status are not the direct causes of solute accumulation which is already clear in the case of cold stress.

One of the possible reasons why alfalfa plants accumulate such large amount of the compatible solutes, is the presence of root nodules. The significance of root nodules in relation to stress has been well documented by Sprent (1976, 1981). In the present study the nodules contained large amounts of proline, stachydrine and trigonelline (Chapter IV, Section 9). In addition, there was a higher rate of accumulation of these organic solutes, under stress, in nodulated alfalfa plants compared to non-nodulated one's. Nodules or nodulated roots are very active enzymatically probably providing the necessary precursors to synthesize these organic solutes more efficiently under a situation like stress. It seems likely that nodulated plants can adapt to a stress more effectively and early nodulation of leguminous plants may be of importance.

As mentioned earlier in the discussion the structural similarities and differences between proline and stachydrine, makes these two compounds of interest with respect to their behaviour under stress. Both compounds have a five membered ring containing nitrogen, as well as a carboxyl group. The major difference between them, of course, is that in stachydrine the nitrogen is a methylated quaternary nitrogen, with a permanent positive charge. Thus, stachydrine contains some element of proline, with some elements of glycine-betaine.

It was of interest to find that both compounds accumulate under stress. The relationship between the compounds was revealed in the biosynthetic studies of stachydrine (Chapter IV, Section 10). Although

there was some information available about the synthesis of stachydrine from proline under normal situations in alfalfa (Robertson and Marion, 1958, 1959; Weihler and Marion, 1959, 1960), the source of the increase in stachydrine under stress was not known. In alfalfa, in the present study, it is evident that stachydrine is synthesised from proline both under normal and under stress situations. In normal situation where there was no feeding of co-fctors it seems appropriate to consider that the normal co-factor levels in the plant were sufficient to support the observed synthesis. Trigonelline, however, was increased by both ornithine and proline treatment. Possibly both are directly or indirectly involved in forming the pyrroline ring of trigonelline. In summary, therefore, it seems very likely that there is in alfalfa, a biosynthetic relationship between proline, stachydrine and trigonelline which needs further experimentation to elaborate.

One of the main concerns regarding the accumulation of these organic solutes (compatible solutes) is their role under stress, i.e., are they of adaptive significance in alfalfa or not. Experiments revealed (Chapter IV, Section 11) that proline, stachydrine and glycine betaine, when fed exogenously, do protect the photofunction of the leaves by maintaining fluorescence characteristics under stress. In these experiments glycine betaine, which does not accumulate in alfalfa, showed similar effects to those of proline and stachydrine. These results strongly suggest that the solutes accumulated by the stressed plant are also of significance in protecting metabolic function against the effects of stress. However, it may not be possible to completely protect the plant's metabolic processes against all adverse conditions. Inspite of this, any delay in the onset of deleterious effects on metabolic processes such as photosynthesis, will certainly be of help to

the plant. Several mechanism of actions can be postulated for proline, and stachydrine in maintaining the fluorescence characteristics. One such possibility involves the maintenance of the enegy transfer from photosynstem II to photosynstem I, which is believed to be damaged by stress (Papageorgiou, 1975; Barber, 1976). This change in energy transfer efficiency is controlled by conformational changes in the thylakoids which are associated with bioenergetic processs, such as the generation of electro-chemical gradients across the thylakoid membrane, and changes of ATP concentration in the stroma (Krause, 1974; Barber, 1976; Bennet et al, 1980; Horton and Black, 1980). Proline (Heber et al, 1971) and glycine betaine (Jolivet et al, 1982, 1983) have been reported to maintain membrane integrity. This would not only help energy transfer efficiency from PSII to PSI, but may also maintain the activity of the reaction centres. In addition the protection of enzymes by proline and glycine betaine (Paleg et al, 1981; Nash et al, 1981) provides an alternative mechanism by which these compounds may protect the enzymatic reactions involved in photofunctioning. Although at this stage a definitive explanation cannot be given, it is still pertinent with the available data to conclude that proline, glycine betaine and stachydrine can protect the photofunctioning of leaves under stress to a significant extent.

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