FREE AMINO ACID COMPOSITION OF FLATPEA (LATHYRUS SYLVESTRIS L.) AS INFLUENCED BY WATER-DEFICIT STRESS, NITROGEN FERTILIZATION, DEVELOPMENTAL STAGE, AND RHIZOBIUM INOCULATION

by

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(ABSTRACT)

A_2bu composed 20-40% of total free amino acids in flatpea tissues and constituted 2-4% of the tissue dry weight. Higher concentrations of A_2bu were found in the leaves than in the roots. A_2bu concentration in leaves and stems increased slightly with plant age. Higher nitrogen availability increased the content of A_2bu in flatpea, a response accompanied by an increase in the contents of soluble protein and other nitrogenous compounds. When exogenous nitrogen supply was decreased, A_2bu levels decreased significantly. *Rhizobium* infection had no effect on the A_2bu production by flatpea. Ammonium was toxic to flatpea growth. Together with typical toxic symptoms, A_2bu elevation was observed in flatpea plants fed with ammonium. Water-deficit stress also elevated A_2bu content of flatpea. The elevation of A_2bu concentration in aerial tissues of flatpea under stress may not be high enough to decrease the value of flatpea as a forage.

4-Aminobutyric acid (Abu), homoserine (Hse), and asparagine (Asn) were the other major free amino acids in flatpea. As with A_2bu, levels of Hse were higher in the leaves than in the roots. The opposite was true for Abu and Asn. The concentration of Abu in the stems increased consistently with plant age. In response to stress conditions, Abu accumulated in flatpea, especially in stems and roots. Asn was the most prevalent free amino acid in the roots of flatpea. Asn levels in roots increased with plant age and accounted for the greatest portion of the increase in the free amino acid pool in the roots of plants subjected to the water stress or supplied with nitrogen in the form of ammonium ions. Levels of Hse in flatpea were changed little in response to the experimental treatments. Relative amounts of major amino acids in flatpea changed with respect to plant organs and experimental factors. If expressed as ratios, the resulting values suggest metabolic relationships.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abu, 4-aminobutyric acid;

A_2bu, 2,4-diaminobutyric acid;

Ala, alanine;

Arg, arginine;

Asn, asparagine;

Asp, aspartate;

Gly, glycine;

Gln, glutamine;

Glu, glutamate;

His, histidine;

HPLC, high performance liquid chromatography;

Hse, homoserine;

Ile, isoleucine;

Leu, leucine;

Lys, lysine;

Met, methionine;

OPA, o-phthalaldehyde;

Phe, phenylalanine;

Pro, proline;

Thr, threonine;

Tyr, tyrosine;

Val, valine.

CHAPTER I. INTRODUCTION

Flatpea (Lathyrus sylvestris L.) is a perennial legume with a deep root system and narrow, paired leaves. The species has some characteristics which are essential for a forage crop. Early feeding and grazing trials indicated that flatpea was palatable and easily digested by animals. Livestock such as cattle, sheep, horses and hogs have been reported to grow and gain weight when fed with flatpea forage. Some animals were initially reluctant to graze flatpea, but after a period of adaptation, they eagerly ate the plant (Daniel and Ensminger 1945; Daniel et al. 1946; Grunder and Dickson 1948). Nutrient levels of flatpea are comparable to those of common forage crops such as alfalfa and clover (Grunder and Dickson 1948; Long et al. 1977). Flatpea has higher digestible protein percentage than alfalfa (Hodgson and Knott 1936; Daniel et al. 1946). It is highly productive (Piper 1916), even in stands known to be more than 50 years old (Slayback and Dronen 1974). Unlike many other forage crops, flatpea plants are resistant to abiotic stresses including low soil fertility, low soil pH, extremes in temperature, and drought stress (Daniel and Ensminger 1945; Robocker and Kerr 1964; and Slayback and Dronen 1974).

Flatpea is toxic to some domestic animals (Grunder and Dickson 1948 and Long et al. 1977). Death and neurological abnormalities of

sheep have been reported after feeding these animals flatpea forage although other sheep in the same experiment were not poisoned by flatpea (Daniel et al. 1946).

The toxic effects of flatpea have been attributed to the high content of a neurotoxin, 2,4-diaminobutyric acid (A_2bu) (Ressler 1962). The concentration of this nonprotein amino acid in flatpea seedlings has been reported to be as high as 2% of the dry weight (Nigam and Ressler 1964). Because of the toxic effect of A_2bu on animals, control or regulation of the levels of A_2bu in flatpea is essential for the utilization of flatpea as a forage legume. What physiological or biochemical functions A_2bu may have in plants is unknown; however, the high level of A_2bu may be related to the tolerance of the species to environmental stresses.

Tissue composition and metabolism of free amino acids are controlled by many developmental and environmental factors. The free amino acid concentration in some plants changes during transitions between developmental stages (Matsumoto et al. 1979; Culianez et al. 1981; Madhusubanan and Nandakumar 1982; and Watnabe and Ishigaki 1982). Concentrations of some storage amino acids usually increase with plant age (Noguchi et al. 1964; Lahdesmaki 1968; and Lekhak and Sen 1981). Nitrogen availability and water status of plants are other factors which have been reported to cause a significant change in the concentration of free amino acids and in the ratios of individual amino acids (Saunier et

al. 1968; Hanover and Brozozowska 1975; Court et al. 1982; Jensen 1982; Mazur et al. 1982; and Yamada and Fukutoku 1982). Change in free amino acids levels in response to environmental stress have been proposed to be either simply a result of stress or related to some kinds of resistance mechanisms (Barnett and Naylor 1966; Stewart et al. 1966; Richter et al. 1975; and Levitt 1980).

The overall objectives of this study were to determine how drought stress, nitrogen source and concentration, plant age, and rhizobium inoculation affect the composition of free amino acids, particularly A_2bu and other nonprotein amino acids, in flatpea tissues. Results of such studies could be beneficial in determining biochemical and physiological functions of nonprotein amino acids and potential control measures that could be used to regulate the synthesis of toxic components such as A_2bu.

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CHAPTER II. LITERATURE REVIEW

POTENTIALLY VALUABLE FORAGE SPECIES

Flatpea (Lathyrus sylvestris L.), a perennial, rhizomatous legume, was first introduced into the United States from Germany in the late nineteenth century. It was considered a potential forage crop because the species possesses many good morphological, physiological, and biochemical features which make it particularly suitable for growth in the hilly, infertile regions of Appalachia, where environmental stresses abound.

Flatpea is characterized by slender, weak, and reclining stems; long, narrow, paired leaflets; and a deep root system. It is an indeterminate perennial and continuously forms flower buds after seeds have matured, if environmental conditions remain suitable (Daniel et al. 1946; Slayback and Dronen 1974).

Early analysis showed that the nutrient content of flatpea was comparable to that of alfalfa (Medicago sativa). Percentages of crude protein, crude fiber, and crude fat of the two species are similar (Hodgson and Knott, 1936; Daniel et al. 1946). Furthermore, flatpea is nutritious and highly digestible as shown by feeding experiments with sheep (Hodgson and Knott 1936; Daniel et al. 1946). Sheep and cattle seemed reluctant to graze flatpea if suddenly changed from another feed source. However, after animals became accustomed to flatpea, it was very palatable to both species.

Cows, horses, sheep, and hogs were reported to eat flatpea readily with a resulting increase in the richness of milk and a more compact meat texture (Virginia Agricultural and Mechanic College, 1892). Grazing and feeding tests by Daniel and Ensminger (1945) and Grunder and Dickson (1948) also showed that flatpea could be used successfully as a nutritious forage.

Flatpea is highly adaptable to unfavorable environments and is tolerant to various environmental stresses. It performs well on a wide variety of soils and may be a valuable crop for some dry and infertile soils (Slayback and Dronen 1974; Allen and Allen 1981). In moderately dry areas and during periods of intermittent drought, flatpea exhibits drought tolerance (Daniel et al. 1946; Robocker and Kerr 1964). Flatpea also exhibits good winter hardiness (Daniel and Ensminger 1945), and competes well with other plant species for water, nutrients, and solar radiation (Slayback and Dronen 1974). In the northeast, flatpea was tested as a plant for maintaining road and power line rights-of-way and forest access roads. It suppressed invasion of native trees and shrubs, developed an excellent cover, and demonstrated good erosion control (Slayback and Dronen 1974). These results confirmed earlier findings by Daniel and Ensminger (1945) that flatpea could compete with the native bracken fern (Pteridium aquilinum) and brush on logged-off and cutover land. However, in soil with poor drainage or a pH below 5, flatpea growth was suppressed (Slayback and Dronen 1974).

Earlier experiments revealed a high productivity potential for

flatpea. Hay yields, in tons per hectare, have been reported in Vermont, (1.55-1.70), Pennsylvania (4.15), and Michigan (4.50) (Piper, 1916). As a consequence of such promising results, Piper (1916) predicted that flatpea would be a choice species for use in areas of the northern states where a long-lived, perennial legume was needed.

However, there are some disadvantages to using flatpea. The seed coat is hard, leathery, and impervious to water. Therefore, it germinates slowly under natural conditions (Slayback and Dronen 1974). Chemical scarification with sulfuric acid has proved to be a effective way to improve the germination rate of flatpea (Perry and Wright 1985; Perry et al. 1985). In addition, establishment of the species is slow (Slayback and Dronen 1974). Experiments during the 1930's met with difficulties in establishing stands of flatpea (Hodgson and Knott 1936). Lime and fertilizer are needed for early establishment (Slayback and Dronen 1974). However, once stands are established, no further maintenance is required for excellent growth and vigor for several decades (Daniel et al. 1946 and Slayback and Dronen 1974).

TOXICITY OF FLATPEA AND RELATED PLANTS

A problem associated with the utilization of flatpea as a forage crop for livestock is the toxic component(s) in the plant. Some early feeding experiments did not show any harmful effects of flatpea on livestock. Flatpea hay showed no toxic effect on sheep (Hodgson and Knott 1936). When seven-year-old sheep were fed solely a diet of fresh, green, flatpea forage, there were no detrimental effects on the animals (Daniel et al. 1946). Young growing flatpea was readily eaten by cattle and sheep during field grazing trials conducted by Daniel et al. (1946) with no adverse effects reported. Grazing studies involving cows, calves, steers, and heifers showed no ill effects due to grazing flatpea. Furthermore, animals in these studies gained weight quickly, especially in the early season (Grunder and Dickson 1948).

However, other reports indicated that flatpea was poisonous to animals. Daniel et al. (1946) reported that three out of 21 sheep died after eating flatpea. The remainder survived six months on the diet with no ill effects. Grunder and Dickson (1948) reported that sheep and rabbits were poisoned by eating immature flatpea plants. Flatpea was also found to be neurotoxic to rats and mice (Ressler, 1975; Long et al. 1977).

Other species of *Lathyrus* have also been found to contain substances toxic to animals and man. For example, it has been known for centuries that the consumption of *L. sativus* seeds could result

in severe, nonreversible paralysis in human beings and in some animals (Barrow et al. 1974; Ramanjuam et al. 1980). In India, such a problem has been especially severe because few crops successfully survive the environmental stresses typical of the area and Lathyrus pulses are frequently the only ones available (Nagarajan and Gopalan, 1968; Roy and Bhat 1975; Ramanjuam et al. 1980). Annual sweet pea (L. odoratus) is another Lathyrus species in which the toxic effects are well known. Rats fed sweet pea developed disorders in conjunctive and bone tissue and were slow to develop (Ressler 1964; Barrow et al. 1974).

The physiological responses of animals that have consumed Lathyrus tissues are referred to as lathyrism, and are categorized as neurolathyrism, the disease of the nervous system, and osteolathyrism, the curvature of long bones and kyphoscoliosis of the thoracic spine. Neurolathyrism is caused by the consumption of L. sativus, flatpea, perennial sweet pea (L. latifolius), and other Lathyrus species, and osteolathyrism is caused by the ingestion of annual sweet pea (Selye 1957; Barrow et al. 1974).

Studies on lathrogens, the toxic compounds of the genus Lathyrus, have resulted in the isolation and identification of several chemicals known to be toxic to animals. Most of the neurotoxins identified were nonprotein amino acids or related compounds. Ressler (1962) demonstrated that 3-cyano-L-alanine (BCLA) and 3-amino-propionitrile (BAPN) had neurotoxic effects on animals, and the symptoms observed were similar to those characteristic of

lathyrism. Later, 4-glutamyl-3-aminoproprionitrile isolated from seeds of annual sweet pea was found to produce changes in mesenchymal tissue and bone of chickens (Ressler 1964). L-2,4-diaminobutyric acid (A_2bu) was identified as the major toxic substance in flatpea and perennial sweet pea (Ressler 1962). Concentrations of A_2bu in the seeds of the two species were found to be as high as 0.5-1% of the dry weight (Ressler 1962). Bell (1962) isolated A_2bu from ten other Lathyrus species. 3-N-oxalyl-2,3-diaminoproponic acid (ODPA), also a neurotoxin, has been isolated from the seeds of L. sativus (Rao et al. 1964; Murti et al. 1964). ODPA was shown to be prevalent in flatpea, perennial sweet pea, and L. clymenum, species known to cause human neurolathyrism. Bell (1962,1964) reported L-homoarginine to be present in seeds of toxic Lathyrus species. In 1964, Murti et al. showed that homoarginine from the seeds of L. sativus resulted in skeletal changes in animals.

Of the five poisonous compounds identified in Lathyrus species A_2bu and homoarginine are exclusively accumulated by some species. Bell (1964) categorized the genus Lathyrus into five subgroups based on the nonprotein amino acid composition. This grouping closely resembled the classification of Senn (1938), which was based on traditional cyto-taxonomic methodology.

Biochemical studies have suggested some possible pathways of lathrogen biosynthesis. BCLA was found to be an intermediate in the synthesis of Asn from cyanide and serine (or cysteine) in flatpea

and annual sweet pea. Radioactively labeled potassium cyanide [$K^{14}CN$] administered to seedlings of these species resulted in significant labeling of Asn and BCLA. BCLA was found to replace the Asn requirement by some plants (Giza et al. 1963; Nigam and Ressler 1964). Asparagine and BCLA are interconvertible in several Lathyrus species (Murti et al. 1964; Barrow et al. 1974). BAPN is the product of decarboxylation of BCLA in sweet pea (Ressler et al. 1961; Murti et al. 1964; Barrow et al. 1974). In flatpea, BCLA may be reduced to A_2bu, the possible major toxic compound in the species (Ressler et al. 1961; Murti et al. 1964). Nigam and Ressler (1964) found only 0.27% of labelled A_2bu after administering ^{14}C-BCLA and proposed an alternative pathway of A_2bu biosynthesis. When L-[^{3}H]-homoserine and D,L-[1-^{14}C]-aspartic acid were administered to flatpea seedlings, a significant labeling of A_2bu resulted. It was concluded that both homoserine and aspartic acid might be precursors for A_2bu (Ressler 1962). Aspartic acid may be converted into aspartic semialdehyde, and the latter can be converted to A_2bu via a transamination reaction and to homoserine by a dehydrogenation reaction (Ressler 1962). As with BCLA and A_2bu, ODPA may also be synthesized through Asn in Lathyrus. It was suggested that the $-CONH_2$ group of Asn is replaced by an $-NH_2$ group by way of the Hofmann reaction. A subsequent acylation with oxalic acid results in the formation of ODPA (Murti et al. 1964; Barrow et al. 1974). Oxalylation of lathrogen is a common reaction in Lathyrus species. In addition to ODPA, both BAPN and A_2bu have been found in

oxalylated form (Murti et al. 1964; Bell and O'Donovan 1966). Oxalylation might increase the toxicity of these lathrogens (Bell and O'Donovan 1966). Bell (1964) suggested that L-homoarginine is synthesized from lysine in a manner analogous to the synthesis of Arg from ornithine.

Physiological functions of nonprotein amino acids in Lathyrus species are not known. Accumulation of nonprotein amino acids may merely be the result of alternation of nitrogen metabolism and reduction of protein biosynthesis in some plants under stressed conditions (Misra and Barat 1981). Roots of Lathyrus plants exude amino acids into the rhizosphere. It is possible that the nonprotein amino acids exuded may inhibit the germination of seeds of other plants or may be toxic to neighboring plants and microflora (Kuo et al. 1982). Production of root exudates by flatpea may account for its success in competing with other plants. Nonprotein amino acids may also increase the resistance of plants to attack by seed-eating beetles and leaf-eating caterpillars (Navon and Bernays 1978).

PLANT DEVELOPMENTAL AND AMINO ACID COMPOSITION

Some plants show a relatively stable amino acid concentration throughout the growing season. Plant age was not a factor in affecting the amino acid composition of alfalfa (Trevino and Hernandez 1977). Taylor et al. (1983) reported a constant amino acid composition in two Cucurbitaceae species during a 14-week growth period. However, in most cases, the amino acid composition of plants varies dramatically with plant age, organ, and specific species (Kumar and Gupta 1976).

In Acanthus ilicifolius and Aegialitis annulata, Pro composes more than 20% of the total plant nitrogen (Popp et al. 1984). A significant decline in Pro concentration with age was found in the two species. In old plants, the Pro concentration was only 50% of the concentration found in young plants (Popp et al. 1984). Similar results have been reported for Lepidium crassifolium, a typical halophyte (Popp and Albert 1981). In Chara brachypus, free amino acid concentration of was higher in the early vegetative stage than during reproductive stages (Venkataramaiah et al. 1983). Kandasamy and Prasad (1973) measured the amino acid concentration of root exudates of green gram (Phaseolus aureus), black gram (Phaseolus mungo), and sunhemp (Crotalaria juncea) at vegetative, flowering, and pod-bearing stage. Amino acid concentrations decreased as plants of the three species matured. If the plants were sprayed with indole acetic acid before flowering, the amino acid

concentration was highest at the flowering stage.

High amino acid concentrations in young plants have been attributed to high rates of carbon and nitrogen metabolism during periods of active growth. As C. brachypus plants aged, the transformation of soluble nitrogen into insoluble structural or storage forms resulted in a decrease in amino acid concentrations (Venkataramaiah et al. 1983). In addition, increases in the C to N ratios in older plants could also have an effect on the amino acid concentration (Hu et al. 1985).

Other species were reported to accumulate amino acids with age. In Medicago leaves, accumulation of both Gln and 4-aminobutyric acid (Abu) were correlated with aging. Abu concentrations in Datura and Salvinia (Lahdesmaki 1968) and tobacco (Nicotiana tabacum) (Noguchi et al. 1964) also tended to increase with age. Total free amino acids in corn (Zea mays) were found to be positively correlated with the number of leaves on the plants (Pinter et al. 1977). The concentration of amino acids, especially Pro, was positively correlated with plant age in rice (Oryza sativa) (Biswas et al. 1982). In the study of 18 arid-zone species, Lekhak and Sen (1981) found that Phe, Leu, Try, Cys, Ser, and ornithine accumulated mostly at maturity.

Fluctuation in the concentration of amino acids during plant development reflects specific characteristics of nitrogen and carbon metabolism controlled by genetic composition and the growth environment. Asparagine is the major nitrogen fixation product in

faba bean (Vicia faba). It accounts, therefore, for a great portion of the free amino acids in roots, stem, and leaves of the species. Another amino acid, Arg, was the form of nitrogen thought to be transported into the faba bean seeds for protein synthesis. An increase in free Arg in stems and pods coincided with the period of most rapid growth of faba bean seeds (Hill-Cottingham and Purves 1983).

Khlyastikov (1984) related the free amino acid concentration in plants to photosynthetic carbon metabolism. Adequately illuminated young bean (Vicia faba) plants contained high amounts of urea, Pro, and Gln. In old plants grown under low illumination Asn, aspartic acid, and Arg accumulated in the leaves. Durzan (1967) reported that, in Picea glauca, concentrations of major components of soluble nitrogen (Arg, Gln, and Pro) showed changing patterns which were correlated to each other. These changes represented the storage during the onset of dormancy and mobilization of nitrogenous compounds during the regrowth of shoot. In many cases, amino acids, which are major storage forms, are accumulated in the tissues. Pro and Abu were the storage form of nitrogen in the leaves of Citrus sinensis and were present at a higher concentration during winter dormancy (Culianez et al. 1981). Glu was the predominant storage form of nitrogen in tomato (Lycopersicum esculentum) fruits and gradually increased in quantity with advancing ripeness (Inaba et al. 1980).

The accumulation of amino acids in old plants was also

influenced by other environmental factors. Increasing nitrogen supply enhanced the Abu accumulation in tomato leaves (Margolis 1960). Infection by tomato mosaic virus (TMV) (Cooper and Selman 1974) and deficiencies in Mo, Fe, and Zn (Selman and Cooper 1978), which limit growth and protein synthesis, also promoted the Abu accumulation in tomato leaves.

In some circumstances, accumulation of amino acids, as a character evolved through systematic development, could have certain ecological significance. For example, as brussels sprouts (Brassica oleracea) plants aged, a reduction in amide content and an increased Abu content rendered the plant an increased resistance to aphids (Van Emden et al. 1971).

Numerous results indicated a close relationship between amino acid fluctuation and the transition of developmental stage. Development of new organs can cause a depletion of or decrease in free amino acids in the old organs. Concentrations of free amino acids and caffeine declined at the stage of bud-opening in tea (Camillia sinensis) plants (Watanabe and Ishigaki 1982). Glutamic acid was the major amino acid excreted from corn roots and showed the highest concentration in the roots before tassel emergence and lower values during seed development (Matsumoto et al. 1979). Fruit development in orange (Citrus sinensis) was accompanied by an increase in free amino acids in the fruits and a reduction in the adjacent leaves (Culianez et al. 1981). In some plants, onset of the reproductive growth may produce a "signal" which promotes the

degradation of insoluble nitrogen and the accumulation of free amino acids in the leaves. Nitrogen in such soluble forms can be easily transported to the new growth center for the formation of reproductive organs. It was reported that plant reproduction was sometimes preceded by or associated with an increase in free amino acids in vegetative tissues (Madhusudanan and Nandakumar 1985). Lichi chinensis leaves exhibited a wide array of amino acids at flower bloom and fruit onset. Afterwards amino acids declined due to rapid translocation to the fruits (Prasad et al. 1979). In tobacco, the concentration of free amino acids, including Asp, Glu, Gly, Ala, Pro, Ser, and Abu, peaked when the plants switched from vegetative to reproductive growth (Hu et al. 1985). In pineapple (Ananas comosus) plants, a three-fold increase in the concentration of total amino acids was observed in the leaves at the preflowering stage (Madhusudanan and Nandakumar 1985). In response to a change in the growth pattern, each individual free amino acid may react specifically. For example, in timothy (Phleum pratense), meadow fescue (Festuca elatior), and ryegrass (Lolium perenne), leaf concentrations of His, Thr, Pro, Gly, Ala, Met, and Ile were found to increase at the stage when the first panicles were visible. At the same time, the concentration of Arg and leu, which may be the forms of nitrogen temporarily stored in the leaves, decreased (Mela and Rand 1979).

WATER DEFICIT STRESS AND AMINO ACID CONTENT

Water stress has profound effects on nitrogen metabolism as well as other aspects of plant growth. The influence of water stress varies with species, plant organ, stage of plant development, and other environmental conditions under which plants have developed and are growing (Hsiao 1973). Sbaaban et al. (1979) studied the effect of soil moisture content on the absorption of nitrogen by bean and barley (Hordeum vulgare) plants. Higher percentages of nitrogen and higher concentrations of ammonium, nitrate, and total nitrogen were observed in plants under low soil moisture. Such effects were attributed to the ability of the plant to absorb nitrate at soil-moisture levels below the wilting point and the effects of soil-moisture content on redox processes which favor mineralization of nitrogen in soil.

Effects of water stress on the nitrogen content of plants differ with organ and developmental stage. Nitrogen content of vegetative parts may remain constant (Sanchez et al. 1983; Whitehead 1983) or decrease (Whitehead 1983). A decrease might result from mobilization of nitrogen in old parts and redistribution to growth sinks (Whitehead 1983). Water shortage speeds up reproductive development and maturing of plants and increases the nitrogen content in seeds. Cowpea (Vigna cylindrica), subjected to water-deficit stress during the flowering and pod-filling stages, produced seeds with high nitrogen concentrations (Labanauskas et al.

1981).

Plant protein concentrations decrease significantly when plants are subjected to moisture stress (Saini and Srivastava 1981), a result of inhibition of protein synthesis and increased protein degradation. Protein synthesis in the mesocotyl of corn seedlings ceased and a marked reduction in total polyribosomes occurred when seedlings were subjected to a mild water-deficit stress (Bewley and Larsen 1980,1982). Protein synthesis may stop without a decline in polyribosome content as shown in the nongrowing region of the mesocotyl of stressed corn seedlings (Bewley et al. 1983). Bewley and Larson (1982) reported that two classes of polyribosomes, membrane bound and free, declined in the cells of the growing region of water-stressed corn mesocotyl. No protein was missing or newly synthesized when the plants were grown under water stress. Therefore, it has been concluded that water stress induces quantitative rather than qualitative changes in the pattern of protein synthesis (Bewley et al. 1983).

Significant changes in the concentration of free amino acids were found in water stressed plants (Levitt 1980; Yamada and Fukutoku 1982; Jensen 1982). When ryegrass was subjected to water-deficit stress, the concentration of all amino acids, except Pro, decreased, in several instances significantly, while Pro concentration increased dramatically. The essential amino acids decreased more than the nonessential amino acids in Jensen's (1982) experiment so that the ratio of essential to nonessential amino

acids decreased in response to increasing soil water deficit.

Total free amino acids often increase in plants subjected to water-deficit stress over extended periods (Barnett and Naylor 1966). When soil moisture for soybean (Glycine max) was maintained below -2 MPa (Yamada and Fukutoku 1982), changes in the concentration of free amino acids were different from the results of Jensen (1982). Concentrations of free forms of some protein amino acids, which were usually not present in the control plants, increased during the stress period while other amino acids, such as Glu, Ala, Asp, Ser, and Gly which are normally present as free amino acids in the control plants, remained unchanged. Nonprotein amino acids, 2-aminoadipic acid and 2-aminobutyric acid also increased. The level of free Pro was relatively constant until the stress became severe (below -1.5 MPa). At -2.6 MPa, Pro was 30% higher than that in the control plants. Moderate water stress caused an increase in Asp, Glu, and Gln in cotton (Gossipium hirsutum) (Paulin 1972); Gly, Ser, and Gln in sunflower (Helianthus annuus) (Hanover and Brozozowska 1975); and Phe and Glu in creosote bush (Larrea divaricata) (Saunier et al. 1968). Pro has also been reported to increase in the four species as a result of water-deficit stress.

Accumulation of Pro and other amino acids was attributed to the degradation of protein and the inhibition of protein synthesis (Barlow et al. 1976; Levitt 1980; Yamada and Fukutoku 1982). Barnett and Naylor (1966) and Boggess et al. (1976) suggested that de novo synthesis of Pro from glutamic acid was a major source of Pro

accumulation under water stress. Promotion of synthesis and inhibition of oxidation of free amino acids, such as Pro, in water-stressed plants were reported by Stewart (1978) and Stewart and Boggess (1976).

The physiological function of amino acid accumulation in response to water-deficit stress is still unclear. Amino acids may be more innocuous than other forms of nitrogen and may, therefore, accumulate without causing injury. These compounds have been proposed to serve as storage forms of reduced carbon and nitrogen for future use under less stressed conditions (Barnett and Naylor 1966). A function of Pro in osmo-adjustment has been proposed by several researchers (Levitt 1980). However, there are no further data to confirm the hypothesis, and there may be no cause and effect relationships between Pro accumulation and osmo-regulation in plants under water-deficit stress (Stewart et al. 1966).

EFFECT OF NITROGEN FORMS ON PLANT GROWTH

Application of ammonium nitrogen and nitrate nitrogen has been reported to have different effects on the growth and nitrogen metabolism in many species of plants examined. Pure ammonium nitrogen is toxic to plants and inhibits the growth of plants (Bennett et al. 1964; Warncke and Barber 1973; Gaffaney et al. 1982; Ganmore-Neumann and Kafkafi 1983). In poinsettia (Euphorbia pulcherrima), height, number of nodes, and shoot dry weight were reduced as the ammonium concentration in the soil solution was increased (Gaffaney et al 1982). Severe stunting, leaf chlorosis, leaf abscission and stubby brown roots were observed (Gaffaney et al. 1982). Kristic and Saric (1982) reported a decrease in root dry weight, shoot dry weight, leaf area, and total root nitrogen in sugar beet (Beta vulgaris) plants grown with ammonium. Leaves of tomato plants treated with ammonium were rolled and wilted (Magalhaes and Wilcox 1983). Compared to plants grown in nitrate medium, tomato plants fed with ammonium showed an increased leaf and root resistances to water flux and a decreased water use efficiency (Pill and Lambeth 1977). Rate of uptake and levels per unit dry weight of Ca, Mg, K, and Na of sunflower, Dupontia fisheri, and tomato were depressed in ammonium-treated plants (Magalhaes and Wilcox 1983; Ulrich and Gersper 1985). Uptake and accumulation of P, on the other hand, were increased in ammonium treatments.

Another characteristic of plants supplied with ammonium as the

sole nitrogen source was the inhibition of lateral growth. Limited lateral growth and reduced cytokinin levels were found for several species (Darrall and Wareing 1981). Within the leaves, accumulation of ammonium ions was found to interfere with electron transport in chloroplasts, resulting in the uncoupling of noncyclic photophosphorylation and an increase in the concentration of NADPH and ADP in the chloroplasts (Chaparro et al. 1970; Losada et al. 1973). A blockage of electron transport and oxidation of NADH in the mitochondra was also observed (Vines ans Wedding 1960; Haynes and Goh 1978). Increased respiration in ammonium-fed plants was reported by Berner (1971) and Ikeda et al. (1974).

Ammonium toxicity is exacerbated by other factors. A foliar spray of ammonium ions showed more severe damage than did root feeding. Increased light levels reduced ammonium toxicity in foliarly sprayed plants due to increased production of carbon precursor used for ammonium detoxification. Higher light levels enhanced transport of free ammonium up to the leaves and, thus, enhanced ammonium toxicity when ammonium was provided through roots (Magalhaes and Wilcox 1983). Although many plants are susceptible to ammonium toxicity, some plants, such as onion (Allium cepa) and Dupontia fisheri, are able to tolerate the toxic effects of high ammonium levels (Maynard and Barker 1969; Ulrich and Gersper 1985).

Nitrate is a preferred form of nitrogen for plants such as corn (Bennett el al. 1964; Schrader et al, 1972), strawberry (Fragaria ananassa) (Ganmore-Neumann and Kafkafi 1983), tomato (Pill and

Lambeth 1977), bean (Barker et al. 1966), and poinsettia (Gaffaney et al. 1982). In some instances, nitrate could cause some negative effects on the utilization of plant products. For example, in sugarbeet, higher levels of Na, K, NO_3^-, and oxalate occurred in nitrate-fed plants, thus decreasing the nutritional value of the crop (Breteler 1973).

Experiments using several different species have shown that the best growth and yield were obtained when a mixture of nitrate and ammonium fertilizers was used (Haynes and Goh 1978). Fresh and dry weight of corn increased more rapidly when plants were provided with nitrogen as a combination of nitrate and ammonium than when either of the two forms was applied alone (Schrader et al. 1973). Wheat (Triticum aestivum) yield was higher when the ratio of ammonium to nitrate was 1 to 5 (Cox and Reisenauer 1973). The maximum nitrogen uptake by corn occurred when an ammonium/nitrate ratio was 2.46 (Warncke and Barber 1973). The optimum ratio varies with species, plant age, temperature, and pH of the growth medium (Haynes and Goh 1978).

When hydroponically grown plants were supplied with different forms of nitrogen, the uptake of nitrogen ions by plants caused a dramatic change in the ambient acidity. Rapid absorption of ammonium in ammonium-treated plants resulted in a substantial increase in solution acidity (Barker et al. 1966; Raven and Smith 1976). Absorption of nitrate ions, on the other hand, caused an alkaline drift in the growth medium (Smiley 1974). The change in

the environmental acidity, in turn, decreased the further uptake of nitrogen by plants (Marcus-Wyner 1983). Some researchers studying the toxicity of ammonium showed that the toxic effect of ammonium was actually due to the non-optimal pH conditions following unbalanced uptake of anions and cations. Even if plants were provided solely with ammonium nitrogen, a normal growth was obtained as long as the ambient pH was well controlled (Barker et al. 1966; Barker 1967; Sander and Barker 1978). Rice, bean, tomato, and Dupontia fisheri have been reported to be able to use either ammonium or nitrate as an effective nitrogen source when the pH in the hydroponic solutions was controlled by buffering the system with calcium carbonate (Karim and Vlamis 1962; Barker et al. 1966), frequent solution renewals, or automatic titration with acid or base (Peet et al. 1985; Ulrich and Gersper 1985). Detoxification through pH maintenance was thought to be due to an improvement in nitrogen uptake (Peet et al. 1985) and increases in the incorporation of ammonium into other soluble or insoluble forms (Barker et al. 1966). However, in experiments conducted by Karim and Vlamis (1962), rice plants fed with ammonium in a calcium-carbonate-buffered solution still produced less biomass than did plants provided with nitrate. Other studies showed that ammonium toxicity could not be diminished by means of environmental pH control (Kirkby 1968; Pill and Lambeth 1977; Breteler 1973). Mustard (Brassica rapa) plants grown hydroponically with continuous circulation and maintenance of pH at 5.5 produced 50% higher dry matter in nitrate solutions than in

ammonium treatments (Kirkby 1968). For tomato plants fed with ammonium, pH control did not improve plant production or mineral composition of roots and shoot (Pill and Lambeth 1977). Lack of detoxification by pH control was attributed to the ineffective incorporation of ammonium into nontoxic forms of nitrogen, such as some protein amino acids, in the roots. Therefore, toxic, free ammonium ions were transported up to the shoot where the biochemistry and physiology of the plant were greatly disturbed (Barker and Mills 1980). Even if ammonium is effectively incorporated into other organic forms, lower productivity in ammonium-fed plants of some species will still be expected, since a large portion of energy is used for the incorporation and detoxification of ammonium.

NITROGEN FERTILIZATION AND AMINO ACID COMPOSITION

The amount and form of nitrogen available in the environment influences nitrogen metabolism and the levels of nitrogenous compounds in plants. In general, a high nitrogen level in the soil promotes the accumulation of free amino acids (Court et al. 1982; Jensen 1982; Mazur et al. 1982). Addition of 67.2 kg/ha nitrogen to the soil caused a 28% increase in the total free amino acids of tobacco compared to the control plants to which no additional nitrogen was added. The greatest increase was in the level of Asp (61%) while the lowest increase was the level of Met (28%) (Court et al. 1981). Increased nitrogen application caused significant increases in amino acid concentrations in ryegrass. The relative concentrations of Glu and Asp increased while Lys, Ala, Val, Ile, Leu, Tyr, and Phe decreased. Thus, the ratio of nitrogen in essential and non-essential amino acids declined in response to an increase in nitrogen fertilization (Jensen 1982). Effects of different forms of nitrogen on the amino acid content of plants most likely reflect differences in plant species and the environmental conditions. Plants supplied with ammonium had higher levels of free ammonium, free amide and free basic amino acids in comparison with nitrate-treated plants (Haynes and Goh 1973; Kristic and Saric 1982). The concentrations of Asp and Asn were higher in rice, corn, and barley when grown with ammonium nitrogen (Schrader et al. 1972; Oji and Izawa 1974; Richter et al. 1975). In tomato, serine

accumulated in the leaves as a result of feeding with ammonium (Ikeda et al. 1974).

Plants accumulate Gln as a result of excess ammonium fertilization. Lorenz (1976) observed that large amounts of Gln accumulated in the roots, shoots, and the sap of tomato when nitrate was replaced with ammonium. Schrader et al. (1973) found high concentrations of glycine in nitrate-fed corn plants. Nitrate was reported to decrease the Met content of corn plants (Schrader et al. 1973; Domska 1974).

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CHAPTER III. CHANGES IN THE DISTRIBUTION AND COMPOSITION OF FREE AMINO ACIDS IN FLATPEA (LATHYRUS SYLVESTRIS L.) PLANTS SUBJECTED TO DROUGHT STRESS

ABSTRACT

2,4-Diaminobutryic acid (A_2bu) in the drought-tolerant perennial legume Lathyrus sylvestris L. may be responsible for the apparent toxicity of flatpea forage to some livestock. To obtain information relative to the regulation of A_2bu, approximately 3-month old flatpea (cv. 'Lathco') plants were subjected to water-deficit stress for 1, 2, and 4 weeks. A_2bu, the most abundant free amino acid in roots, stems, and leaves, increased nearly 100% in roots of stressed plants. Increases in the concentrations of asparagine (Asn), proline (Pro), and arginine (Arg) in roots; Asn, Pro, and 4-aminobutyric acid (Abu) in stems; and Pro, Abu, A_2bu, and homoserine (Hse) in leaves also occurred in response to drought stress. Pro was a minor constituent of the free amino acid pool, even under water-deficit stress. The distribution of A_2bu and Pro in the stressed plants (roots > stems > leaves) was the reverse of that in plants supplied with adequate water (roots < stems < leaves). As concentrations of Asn and Abu decreased from roots to leaves in control tissues, concentrations of Hse and A_2bu increased in roughly the same proportions. This relationship suggests that Abu and Asn may be precursors of A_2bu and Hse, respectively, the former two amino acids

being synthesized in the roots and transported to the stems and leaves where A_2bu and Hse are synthesized. Elevated levels of A_2bu in root tissue of drought-stressed plants could result from transport of this amino acid from the leaves. The increase in A_2bu levels in aerial parts of drought-stressed flatpea plants is probably not sufficient to lower the feed value of the forage.

INTRODUCTION

Flatpea (Lathyrus sylvestris L.) is a long-lived, deep-rooted, perennial legume that is tolerant to environmental stresses such as drought (Slayback and Dronen 1974), low soil fertility (Allen and Allen 1981), temperature extremes (Robocker and Kerr 1964), and low soil pH (McWilliams 1970). Because of these characteristics, flatpea is currently under study as a potential forage or cover crop for use in the Appalachian region of the United States, where severe environmental stresses limit the growth and productivity of other legumes (Slayback and Dronen 1974).

Flatpea forage is palatable and easily digested by animals as shown in early grazing trials (Daniel and Ensminger 1945), and its nutritive value is comparable to that of alfalfa, clover, and crownvetch (Daniel et al. 1946, Long et al. 1977). However, 2 to 3% of the dry weight of flatpea forage is composed of 2,4-diaminobutyric acid (A_2bu) (Ressler 1964, Przybylska and Rymowicz 1965), a nonprotein amino acid that has neurotoxic activity in rabbits and rats (Ressler 1964). A_2bu may have been responsible for the death of some sheep observed during feeding studies involving flatpea (Grunder and Dickson 1948).

Drought stress affects plant nitrogen metabolism. Major changes include enhanced proteolysis, increased free amino acid concentrations, and inhibition of protein synthesis (Petrie and Wood 1938, Kemble and MacPherson 1954, Barnett and Naylor 1966, Rosinger

et al. 1984, Drossopoulos et al. 1985). Associated with the these various responses is the accumulation and/or depletion of protein-and nonprotein-amino acids. The amino acid proline (Pro) has been of particular interest to plant scientists because it appears to respond more to drought stress than do most other amino acids (Palfi et al. 1974, McMichael and Elmore 1977, Jensen 1982). Although the Pro stress response has bee much studied, the physiological function of free Pro in plants has not been clearly defined. Pro accumulation has been proposed as a drought stress indicator, but all plants apparently do not respond to drought stress by increasing Pro concentrations (Palfi et al. 1974). Little attention has been given to other nitrogenous compounds which also may accumulate in plants and contribute to drought tolerance (Stewart and Larher 1980). Asparagine (Asn) and 4-aminobutyric acid (Abu) have been reported to increase in water-stressed wheat plants (Drossopoulos et al. 1985), citrus seedlings (Chen et al. 1964), cotton leaves (McMichael and Elmore 1977), and bermudagrass shoots (Barnett and Naylor 1966). In wheat the response differed with cultivar, organ, and stage of development.

The purpose of the present study was to quantify A_2bu and other protein- and nonprotein-amino acids in the leaves, stems, and roots of flatpea grown under different levels of drought stress in an effort to obtain information relative to the control of potentially toxic, nitrogenous compounds and the physiological function of such compounds in the plants. Results suggest that biosynthetic

relationships exist among several of the predominant amino acids in flatpea and that water-deficit stress does not increase the A_2bu-associated toxicity of flatpea forage.

MATERIALS AND METHODS

A typical Appalachian top-soil, Lily (fine-loamy, siliceous, mesic Typic Hapludult), was used to grow experimental plants. Soil pH was 5.5 and was supplemented with phosphorus and potassium in the form of K_2HPO_4 at a rate of 0.5 g per 1 kg of dry soil. The soil was covered with a heavy plastic sheet and sterilized with methylbromide (50 g per 1 kg of soil, Dow Chemical Co., Midland, MI) for 2 days. Moisture retention curves for the soil were determined using a pressure plate extractor (Soil Moisture Equipment Co., Santa Barbara, CA).

Flatpea (Lathyrus sylvestris L. cv. 'Lathco') seeds were scarified with concentrated H_2SO_4 for 10 min and imbibed in continuously aerated water for 2 days at 20°C in the dark. After imbibition, each seed was inoculated with about 50 µl of a late-log phase Rhizobium leguminosarum culture (10^9 cells/ml), strain 92F2 (Nitragin Co., Milwaukee, WI). Inoculated seeds were sown in 20-cm plastic pots (10 seeds per pot) containing 3.5 kg of dry soil. Experiments were conducted in environmentally controlled growth chambers with a photoperiod of 16 h. An average photosynthetic photon flux density of 400 µmol m^{-2} s^{-1} (400-700 nm) at plant height was provided by a combination of fluorescent (215 W cool white/very high output 96T12, Philips Lighting Corporation, Bloomfield, NJ) and incandescent (Sylvania 60 W, inside frosted, GTE Corp., Springfield, VA) lamps. Light/dark temperature were 27/20°C and the relative

humidity was 75%. Soil water potential was maintained as close to -0.1 MPa as possible by weighing and adding an appropriate amount of water to each pot daily. One week after emergence, plants were thinned to four per pot. At 40 days post emergence, shoots were pruned to the soil surface. After 40 days of regrowth, the pots were divided into two groups. Soil water potential of the control group was maintained at approximately -0.1 MPa. Moisture was withheld from plants in the second group until the soil water potential reached -1.2 MPa (approximately 2 to 3 days), after which water was added daily, as needed, to maintain this potential. One, 2 and 4 weeks after initiation of the stress three pots each of water-stressed and control plants were harvested. Water potentials of leaves from the two treatments were measured with a pressure bomb (Soil Moisture Equipment Corp., Santa Barbara, CA). Harvested plants were separated into leaves, stems, and roots. Tissues were then crushed in liquid nitrogen with a mortar and pestle. A portion of the fresh, frozen tissue was used for soluble protein analysis. Five ml of 0.1 M HEPES-NaOH (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5; Calbiochem-Behring Corp., LaJolla, CA), containing 0.01 M isoascorbate (Sigma Chemical Co., St. Louis, MO), and 0.05% (v/v) antifoam C emulsion (Sigma Chemical Co.) were added to 0.5 g of insoluble polyvinylpyrrolidone (Sigma Chemical Co.) and 1 g of fresh, frozen tissue. The mixture, held in an ice bath, was homogenized using a Polytron homogenizer (Brinkman Model PT 10-35, Westbury, NY) with a Model PT 10-ST probe generator at

maximum speed for 20 s. Extracts were centrifuged at 10,000 g for 20 min in a Beckman (Palo Alto, CA) model JA-20 rotor. The resulting pellet was resuspended in 5 ml of the initial homogenizing medium and centrifuged as before. Supernatants from the two centrifugations were combined and soluble protein was assayed using the Bradford (1976) procedure.

The remaining fresh, frozen tissue was lyophilized (Virtis 10-100-V, Gardinar, NY) to constant weight and stored at -20°C for other analyses. Nitrogen concentration of lyophilized tissue was measured using a Leco (St. Joseph, MI) model CHN-600 carbon-hydrogen-nitrogen analyzer. For analysis of free amino acids, approximately 0.5 g of lyophilized tissue was extracted with 90 ml of 50% (v/v) aqueous ethanol for 90 min using Soxhlet extractors. Two ml of 15 mM S-(4-pyridylethyl)-DL-penicillamine (Pierce Chemical Co., Rockford, IL) was added as an internal standard. Sample extracts were concentrated under nitrogen at 40°C. The resulting residue was resuspended in 10 ml of extraction medium. A 2.5 ml aliquot of this suspension was centrifuged at 3,000 g for 10 min. The pellet was resuspended in 2.5 ml of extraction medium and recentrifuged two times. The three supernatants were combined and brought to a total volume of 7.5 ml with extraction medium. An extract volume of 0.25 ml was loaded onto a Sep-Pak C_{18} column (Waters Associates, Milford, MA) and eluted successively with 0.5 ml of water and 1.0 ml of methanol. The combined eluate was brought to 2.0 ml with water.

Free amino acids in extracts were derivatized with o-phthalaldehyde (OPA, Pierce Chemical Co.) prepared by dissolving 50 mg of OPA in 1 ml of high performance liquid chromatography (HPLC)-grade methanol, adding 50 ul of 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA) and bringing the solution to a final volume of 10 ml with 0.40 M sodium borate-KOH (pH 9.5) containing 0.1% (v/v) Brij 35 (polyoxyethylene lauryl ether, Fisher Scientific, Pittsburgh, PA). Freshly prepared OPA stock solution was stored overnight at 0 to 5°C before use and was used for 2 days. A 0.1 ml aliquot of the solution was mixed with 0.02 ml of extract 90 s before injection onto the HPLC column. Derivatized samples were analyzed using a Beckman (Berkeley, CA) model 344 binary gradient HPLC system equipped with an Altex (San Ramon, CA) 4.6 x 45 mm, 5μm Ultrasphere-ODS precolumn and an Altex 4.6 x 250 mm, 5 μm Ultrasphere-ODS analytical column maintained at 45°C following the protocol of Jones et al. (1981). Amino acid derivatives were detected using a Gilson (Middleton, WI) model 121 fluorescence detector equipped with a 9 ul flow cell and filters for excitation at 305 to 395 nm and emission at 430 to 470 nm. Detector range and time constant settings were 0.02 relative fluorescence units and 0.5 s, respectively. Amino acids were identified by comparing their retention times to those of pure amino acid standards (Sigma Chemical Co.) and by coinjection of known amino acids. Peak areas were determined using a Nelson Analytical (Cupertino, CA) model 4416 X chromatography data system. Amino acids were quantified using

standard curves generated for each amino acid over the concentration range found to occur in the tissues examined. Yields of individual amino acids in extracts were adjusted, based on recoveries of the internal standard. Pro concentrations were determined using the acid-ninhydrin method of Bates et al. (1973).

RESULTS

Leaf water potentials of control and water-stressed plants ranged from -0.6 to -0.8 MPa and -1.5 to -1.8 MPa, respectively, over the duration of the experiment. A water potential difference ranging from -0.7 to -0.9 was observed between the controls and stressed plants throughout the 4-week stress period, and the maximum standard error observed for three replications was 0.19 MPa.

Relative to controls, total plant biomass of stressed plants was less than that of control over the 4-week period (Fig. 3-1). Leaf growth was inhibited more than stem or root growth during the course of the study, and the longer the duration of the stress, the greater the growth inhibition of the leaves and stems. Root dry matter increased slightly during the first week of stress, but this effect on plant biomass was offset by the major decrease in leaf dry weight. After 2 weeks of exposure of plants to a soil water potential of -1.2 MPa, root dry weight was significantly ($P \leq 0.05$) less than that of controls.

Percent nitrogen was highest in the leaves, with similar concentrations occurring in the stems and roots of both control and stressed plants (Fig. 3-2). In control plants, nitrogen concentration did not change significantly from week 1 to week 4, but a trend towards reduction in nitrogen concentration was observed in roots of control plants as the experiment progressed. Water-deficit stress did not affect the nitrogen concentration in

the leaves or stems of flatpea. However, nitrogen concentration was 42% and 46% higher in stressed roots than in nonstressed roots after 2 and 4 weeks of drought stress, respectively.

The highest concentration of soluble protein was observed in the leaves followed by the stems and roots of control flatpea plants (Fig. 3-3). Soluble protein tended to increase in all tissues of stressed and control plants (exception, stem control) with time. Leaf tissues of stressed plants were lower in soluble protein than control plants at all stress periods. In the stems and roots of 1-week-stressed plants, soluble protein was lower than in the controls, but these levels surpassed the controls after 2 weeks. After 4 weeks the soluble protein concentrations were similar in both stems and roots.

The major free amino acids detected in the leaves, stems, and roots of flatpea control tissues were Abu, A_2bu, Asn, Hse, and Pro (Tab. 3-1). Other amino acids, detected in amounts usually less than 1 mg/g dry weight, were alanine, aspartate, glutamine, glutamate, isoleucine, leucine, phenylalanine, serine, and valine. The qualitative composition of the amino acids differed with tissue type (Tab. 3-1). Control leaves were characterized by having high concentrations of the following amino acids in the indicated relative abundance: A_2bu > Hse > Abu > Pro. However, at week 4, Asn was higher than Pro. In the control stems, the relative abundance of the major amino acids was: A_2bu > Hse (except week 1) > Abu > Asn. Relative concentrations in the control roots were: A_2bu

Table 3-1. Free amino acid composition of flatpea tissues subjected to drought stress. Results are given as means ± SE (n = 3). Total includes all free amino acids quantified by HPLC.

Amino Acid	Week 1		Week 2		Week 4	
	Control	Stressed	Control	Stressed	Control	Stressed
			(mg/g. DW)			
			Leaves			
Abu	3.8 ± 0.8	4.6 ± 0.9	2.1 ± 0.2	2.7 ± 0.4	3.4 ± 0.3	5.6 ± 1.3
A_2bu	26.6 ± 4.9	22.9 ± 3.1	19.8 ± 0.8	19.8 ± 4.0	13.0 ± 0.9	16.3 ± 0.3
Arg	0.7 ± 0.1	0.5 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
Asn	0.9 ± 0.2	2.4 ± 0.5	0.3 ± 0.1	0.6 ± 0.1	2.6 ± 0.5	2.9 ± 1.1
Asp	0.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.7 ± 0.2	0.4 ± 0.1
Hse	15.0 ± 2.5	11.1 ± 1.4	8.7 ± 1.1	10.9 ± 1.2	13.3 ± 0.6	16.1 ± 0.5
Pro	1.3 ± 0.1	4.8 ± 0.5	1.0 ± 0.1	2.5 ± 0.6	0.9 ± 0.2	1.4 ± 0.3
Total	52.2	51.2	33.5	38.2	36.5	45.0
			Stems			
Abu	11.6 ± 1.3	13.9 ± 1.3	3.1 ± 0.5	28.9 ± 1.3	3.6 ± 0.1	17.9 ± 3.2
A_2bu	19.6 ± 1.8	13.3 ± 2.5	18.4 ± 1.8	20.1 ± 1.6	18.1 ± 3.4	20.1 ± 3.8
Arg	0.5 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
Asn	1.4 ± 0.4	1.9 ± 0.2	0.8 ± 09.1	1.6 ± 0.4	1.7 ± 0.2	4.0 ± 0.7
Asp	0.5 ± 0.2	0.3 ± 0.0	0.5 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.2 ± 0.1
Hse	9.2 ± 0.6	7.2 ± 1.0	6.3 ± 0.9	8.4 ± 0.1	9.1 ± 0.1	9.3 ± 0.8
Pro	0.9 ± 0.0	5.0 ± 0.6	0.5 ± 0.0	2.0 ± 0.3	0.8 ± 0.1	1.8 ± 0.1
Total	46.2	44.5	30.7	62.5	36.2	55.2
			Roots			
Abu	22.0 ± 1.0	17.2 ± 1.6	12.9 ± 1.6	22.9 ± 1.1	8.0 ± 0.4	19.7 ± 5.1
A_2bu	12.5 ± 0.7	16.3 ± 2.4	13.4 ± 0.9	23.4 ± 4.0	11.5 ± 1.2	23.0 ± 0.9
Arg	0.9 ± 0.1	1.2 ± 0.3	0.3 ± 0.1	3.4 ± 0.3	0.2 ± 0.0	3.5 ± 0.5
Asn	13.0 ± 2.0	20.9 ± 5.1	6.1 ± 1.6	29.2 ± 2.5	7.2 ± 1.4	25.2 ± 3.8
Asp	2.6 ± 0.2	3.1 ± 0.1	1.5 ± 0.2	3.1 ± 0.2	0.9 ± 0.2	1.8 ± 0.2
Hse	2.8 ± 0.1	4.2 ± 0.5	1.4 ± 0.1	2.2 ± 0.1	1.9 ± 0.2	2.4 ± 0.0
Pro	0.5 ± 0.1	8.0 ± 1.0	0.4 ± 0.0	5.8 ± 1.4	0.5 ± 0.1	3.5 ± 0.8
Total	57.3	74.6	37.9	93.2	31.9	81.7

(except week 1) > Abu > Asn (except week 1) > Hse = Asp. It also appears that a quantitative, inverse relationship exists between Asn and Hse and between Abu and A_2bu. As Asn and Abu decreased in concentration from roots to leaves, Hse and A_2bu increased in roughly the same proportions.

Water-deficit stress caused a differential response in the total amino acid composition of leaves, stems, and roots (Tab. 3-1). The largest change was in the roots, where an increase in total free amino acids of 30%, 146%, and 156% was observed after 1, 2, and 4 weeks of stress, respectively. Stems exhibited the next highest increase (week 2, 104%; week 4, 52%). Stress-related changes in free amino acid levels were the smallest (week 2, 14% ; week 4, 23%) in leaves.

Specific accumulation of several amino acids occurred in flatpea plants subjected to drought stress (Tab. 3-1). Such increases were more prevalent in the roots, but were also observed in the stems and leaves. Pro accumulated in all tissues, the greatest change occurring at week 1 and declining thereafter. The pattern of Pro production seemed to change under drought-stress conditions. In the control plants the highest concentrations of Pro were in the order of leaves > stems > roots. When the plants were stressed, the order was reversed. Asparagine was particularly prevalent in the roots and increased with the duration of the stress to a level 3-4 times that in roots of control plants. As the duration of the stress increased Arg was also observed to accumulate in the roots. In stems, both

Abu and Asn increased when plants were subjected to water stress, but the former decreased during the later part of the experimental period (week 4). With the exception of Pro, Asn responded to stress more than any other amino acid in the leaves. Imposition of water stress did not reverse the relative distributions of Asn, Abu, or Hse among the roots, stems, and leaves from those observed for control tissues (Asn, Abu: roots > stems > leaves; Hse: roots < stems < leaves). Levels of the minor amino acid constituents that were quantified in this study remained relatively constant in all tissues throughout the course of the stress period.

Although total amino acid concentration increased under the stress conditions employed, levels of several amino acids in the leaves and stems declined, particularly at week 1 of the stress period. Such decreases were more prevalent in the leaves than in the stems and included the amino acids A_2bu, Arg, Asp and Hse. The only amino acid to decline in the roots was Abu, and only a small decrease was observed.

Of particular interest to us in this study were the high concentrations of A_2bu in the plant tissues and the changes that occurred as a result of water stress. Little difference was observed in this amino acid between stressed and control plants in leaves or stems (Tab. 3-1). A slight tendency for an increase was observed at the 4th week of stress in these plant tissues. However, root levels of A_2bu were higher in stressed plants and nearly a 100% increase in the concentration of A_2bu was observed in the roots at

week 4 of stress. While A_2bu levels in roots were consistently less than those in stems and leaves when plants received adequate water, the relative distribution favored stems with increasing plant age during the 4 week study period. Water stress tended to have an equalizing effect to the extent that, at the conclusion of the study, the distribution of A_2bu among roots, stems, and leaves (roots > stems > leaves) was the opposite of that observed at the outset (roots < stems < leaves), even with the apparent plant age effect (roots < leaves < stems) (Tab. 3-1).

DISCUSSION

It is clear from this study that various aspects of nitrogen metabolism in flatpea were affected by drought stress. Changes observed in soluble protein, free amino acid, and nitrogen levels are typical of several plant studies in which water stress has led to a reduction in protein content and accumulation of free amino acids. Such changes have been attributed to a reduction in the rates of protein synthesis and an increase in proteolytic activity (Chen et al. 1964; Barnett and Naylor 1966; Thompson et al. 1966; Jager and Meyer 1977), both of which tend to cause an increase in total soluble nitrogen. In flatpea, several free protein- and nonprotein-amino acids responded to drought stress by either decreasing or increasing in concentration, but the particular changes involved were amino acid and tissue specific (Tab. 3-1). Also, the duration of the stress had different effects on the concentration of individual amino acids. Differences in amino acid composition between cultivars, organs, and developmental stages of wheat plants subjected to water stress have also been reported (Drossopoulos et al. 1985).

Although Pro increased in drought-stressed flatpea plants (Tab. 3-1), Pro comprised only 10 to 11% of the total concentration of free amino acids in the stressed plants. Pro accumulation in response to water stress in many plants has been reported to occur only when leaf water potentials were in the range of -1.0 to -1.5

MPa (Singh et al. 1973, Waldren et al. 1974, Hanson et al. 1977). In the present study, leaf water potentials of stressed flatpea plants were consistently less than -1.5 MPa, thus conditions were presumably favorable for Pro accumulation. Plants have been classified into species which do or do not accumulate free Pro under water stress (Palfi et al. 1974). For example, in stressed cotton leaves only 10% of the total free amino acids was Pro (Stewart and Larher 1980), while in stressed coastal bermudagrass, Pro comprised 42% of the total (Barnett and Naylor 1966). The drought-tolerant species Artemesia herba-alba had a low content of free Pro in the nonstressed state and exhibited no accumulation of Pro when stressed by water deficits (Pourrat and Hubac 1974). Observations with the drought-tolerant flatpea are therefore in line with results from other studies.

In addition to Pro, several other amino acids, including Asn, Arg, Abu, and A_2bu in the roots, Abu and Asn in the stems, and Abu, A_2bu, and Hse in the leaves, increased in flatpea in response to the water-deficit stress. These amino acids contributed much more to the total plant nitrogen than did Pro. Accumulation of Asn in water-stressed plants has been reported by Chen et al. (1964), Barnett and Naylor (1966), Thompson et al. (1966), McMichael and Elmore (1977) and Drossopoulos et al. (1985). While the role of Asn as an important nitrogen transport compound in plants has been well established (Lea and Miflin 1980), the specific role of Abu is still unclear. Increases in Abu in response to water stress have been

observed in cotton leaves (McMichael and Elmore 1977) and wheat plants (Drossopoulos et al. 1985), and Chen et al. (1964) reported an increase in the Abu content of leaves and roots of citrus seedlings that generally occurred at severe water stress. This response, similar to that reported for Pro and described above, is consistent with our observations with flatpea (Tab. 3-1). How flatpea plants accumulate Abu in stems when stressed is not clear. Labeling studies are needed to determine the possible precursor roles of Asn and Abu as well as their transport functions and these aspects will be the focus of future studies.

The most abundant amino acid in flatpea tissues was A_2bu. This was true for nonstressed and stressed plants with only a few exceptions where Abu was slightly higher in the stems and roots. While nearly a 100% increase in the concentration of A_2bu was observed in the roots of stressed plants, the concentration of A_2bu in the leaves and stems did not respond to water stress as dramatically as several of the other amino acids. The increase in A_2bu concentration cannot be directly related to protein degradation. Moreover, the magnitude of the increase in leaves and stems is not consistent with the concept of osmotic adjustment. Pro in the drought-tolerant creosote bush (Larrea divarticata) has been reported to have a relative abundance of 43% (1.2 mg/g DW) under nonstressed conditions, a value that was elevated to 66% (4.4 mg/g DW) by water stress (Saunier et al. 1968). In flatpea, the relative abundance of A_2bu (38-51%) was decreased 10 to 20% by water stress

(31-33%) (Tab. 3-1). It should be noted, however, that the A_2bu concentration in flatpea plants (43-63 mg/g DW) was five- to ten-fold higher than the Pro concentration in creosote bush, and the free amino acid pool quantified in the present study was two orders of magnitude greater than that reported by Saunier et al. (1968). These data indicate that A_2bu constitutes approximately 1 to 3% of the dry weight of the plant (Tab. 3-1), a range consistent with values reported by Ressler (1964) and Przybylska and Rymowicz (1965). Certainly the age-related decrease in A_2bu concentration in flatpea leaves and the similarities in the altered distributions of Pro and A_2bu among flatpea roots, stems, and leaves in response to drought stress (Tab. 3-1) are interesting. It is conceivable that A_2bu accumulation in flatpea roots provides a means to store nitrogen for subsequent utilization upon removal of the stress. Considering the low toxicity of A_2bu (Ressler 1975) and the quantity of A_2bu in leaf and stem tissues from control plants at week 1 and those in corresponding tissues from all stressed plants (Tab. 3-1), it is doubtful that the increases observed in the concentrations of A_2bu in the leaves and stems would diminish the quality of flatpea forage, but no information is available regarding acceptable levels of A_2bu in forage used for livestock feed.

The apparent inverse relationship between Asn and Hse and between Abu and A_2bu in the leaves, stems, and roots of flatpea suggests biosynthetic relationships. Comparisons of amino acid levels in roots and leaves revealed that the magnitude of the

decrease in concentration from one plant part to the other for one member of each pair of amino acids was generally similar or equal to the amount of increase of the other member of the amino acid pair for the corresponding tissues (Tab. 3-1). It is possible that Abu and Asn are precursors to A_2bu and Hse, respectively, the former two amino acids being synthesized in the roots and transported to the stems and leaves where A_2bu and Hse are synthesized. Intracellular localization studies (Foster et al. 1987) support the hypothesis that A_2bu synthesis occurs in the leaves, and elevated levels of A_2bu in root tissue of drought-stressed plants could result from transport of this amino acid from the leaves. Evidence in the literature for the synthesis of A_2bu from Abu is lacking, but studies with pea shoots grown initially on $[^{15}N]O_3^-$ resulted in 80% of the amide N of Asn being metabolized in 2 h, and the amino acid receiving the labeled N most rapidly was Hse (Bauer et al. 1977). Studies in which cut flatpea seedlings were provided with either L-[^{3}H]homoserine or DL-[1-^{14}C]aspartic acid resulted in incorporation of the radioactive label into free A_2bu (Nigam and Ressler 1966). These results suggested that both Asp and Hse can serve as precursors for A_2bu. Asn and Asp are readily interconverted enzymatically. With the observed elevations in tissue levels of Asn, Abu, and Hse, the potential exists for enhanced yields of A_2bu if the proposed biosynthetic pathways exist in flatpea.

Particular attention needs to be given to developmental stage

and age of plants with respect to amino acid metabolism because that age of tissues and developmental stage can have considerable effects on amino acid composition and response to the environment (Stewart and Larher 1980; Drossopoulos et al. 1985). Flatpea is a perennial legume with the capacity for continuous growth for up to 50 years or longer. If flatpea is to be used as a successful forage crop, a better understanding of the biosythesis of A_2bu, its physiological function in the plant, and the effect of environmental parameters on its synthesis at different developmental stages and ages are important.

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FIGURE LEGENDS

Fig. 3-1. Percent change in dry weight of flatpea leaves, stems and roots over a 4-week drought period.

Fig. 3-2. Nitrogen concentration of control (---) and drought-stressed (---) leaves, stems and roots of flatpea plants over a 4-week stress period. Error bars represent the standard error of three replications.

Fig. 3-3. Soluble protein concentrations of control (---) and drought-stressed (---) flatpea leaves, stems and roots over a 4-week stress period. Error bars represent the standard error for three replications.

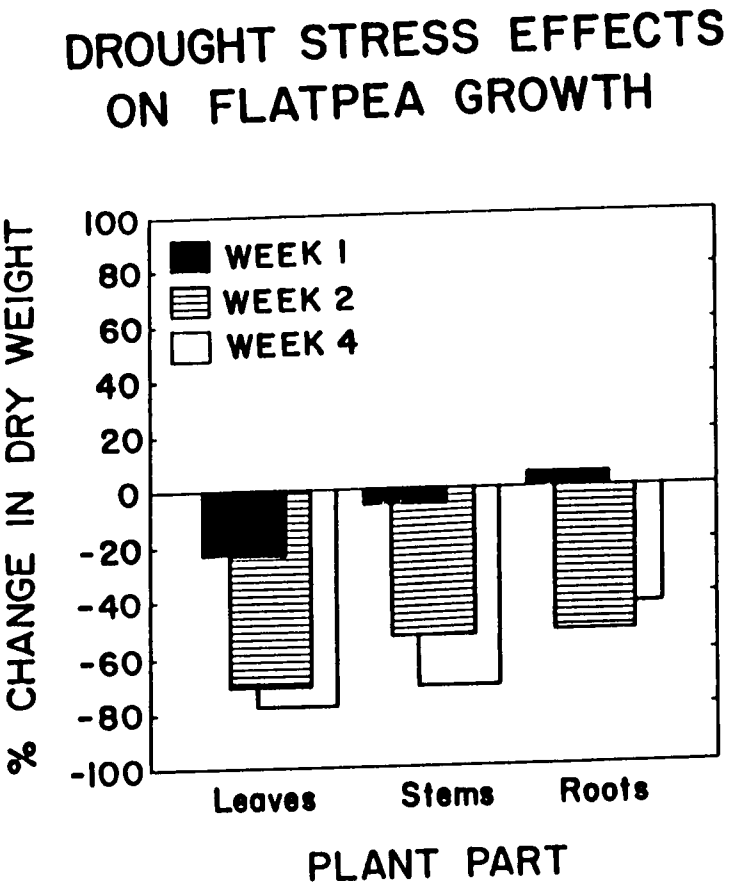

Fig. 3-1. Percent change in dry weight of flatpea leaves, stems and roots over a 4-week drought period.

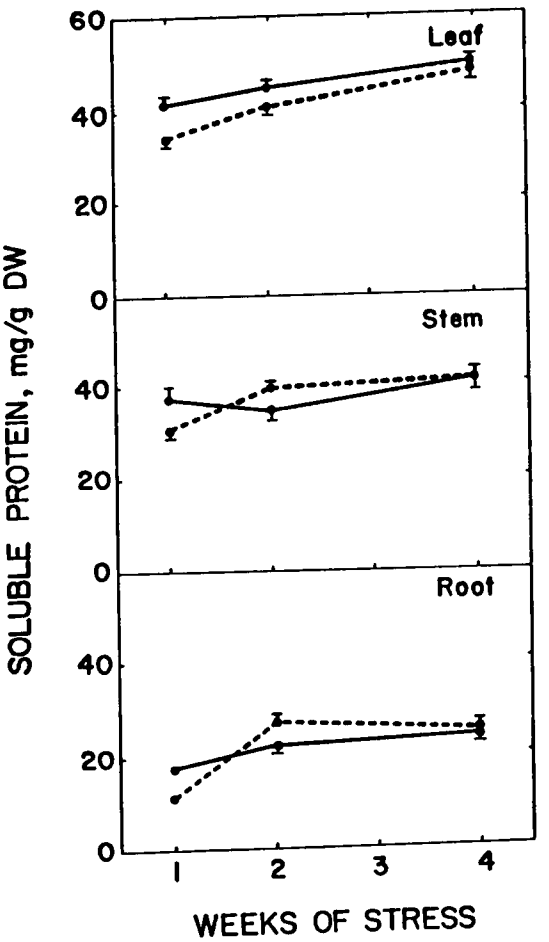

Fig. 3-2. Nitrogen concentration of control (---) and drought-stressed (---) leaves, stems and roots of flatpea plants over a 4-week stress period. Error bars represent the standard error of three replications.

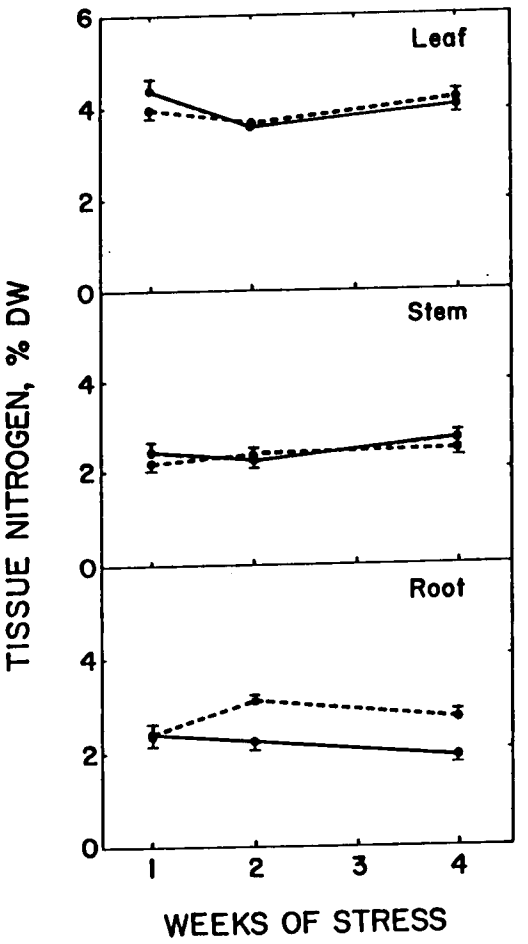

Fig. 3-3. Soluble protein concentrations of control (---) and drought-stressed (---) flatpea leaves, stems and roots over a 4-week stress period. Error bars represent the standard error for three replications.

CHAPTER IV. EFFECTS OF WATER DEFICIT STRESS AND PLANT AGE ON THE COMPOSITION AND DISTRIBUTION OF FREE AMINO ACIDS IN FLATPEA (LATHYRUS SYLVESTRIS L.)

ABSTRACT

Drought-stressed flatpea (Lathyrus sylvestris L.) plants from 8 to 22 weeks of age were analyzed for nitrogen, soluble protein, and free amino acids. A linear increase in nitrogen concentration and a sharp decrease in soluble protein level with age were observed in roots. The level of 2,4-diaminobutyric acid (A_2bu), which represented 1.6 to 2.6% of the dry weight of tissues, was highest in leaves, where it increased with plant age. Concentrations of 4-aminobutyric acid (Abu) is highest in stems and increased dramatically in this tissue with age. Root concentrations of asparagine, arginine, glutamine, and aspartate increased with plant age and reached a peak at the time of flowering (14 to 18 weeks). The cumulative concentration of free amino acids increased with drought stress. The magnitude of change varied directly with plant age. A_2bu levels in stressed leaves increased by 11.9% relative to control. In response to drought stress, Abu in stems increased before week 14 and after week 18. During weeks 14 and 18 the concentrations of valine, isoleucine, leucine, and phenylalanine increased. Proline levels were low in control plants and increased

with plant age, especially under drought stress, but accounted for only 10% of the total free amino acids in drought-stressed plants.

INTRODUCTION

Flatpea (Lathyrus sylvestris L.), a long-lived, perennial legume, is a potential forage species. Early studies showed that flatpea had a nutritive value comparable to other forage crops, such as alfalfa, clover, and crown vetch (Daniel, Wolberg, Miller, Alswager, Ensminger, and Spielman, 1946; Long, Washko, and Palmer, 1977). However, occurrence of a neurotoxic chemical, 2,4-diaminobutyric acid (A_2bu) in the plant (Nigam and Ressler, 1966) and death of some flatpea-fed animals have been reported (Ressler, Redston, and Erenberg, 1961).

Water status of plants has a profound effect on the metabolism of amino acids and other nitrogenous compounds. Plants subjected to water shortage show reduced protein synthesis, enhanced activities of proteases, and an increased concentration of low molecular weight nitrogenous compounds (Mukherjee and Chouduri, 1984).

Tissue composition of nitrogenous compounds also changes during plant development (Culianez, Martin, Guerri, Tadeo, and Millo, 1981). Free amino acids, the concentrations of which change with growth activities, have been considered to be temporary storage form of nitrogen. For example, amino acid levels in leaves of orange (Citrus sinensis L. cv. 'Osbeck') plants decreased during the flowering season, due to active growth and depletion of nitrogen reserves, and increased in the dormancy period (Culianez et al.,

1981). Amino acids have been found to accumulate in storage organs, such as potato tubers and carrot roots, as the plants aged (Selman and Cooper, 1978). In addition, free amino acid concentrations are also related to the growth phase of the plants. An increase in the quantity of free amino acids has been reported to initiate or accompany the reproductive phase of plant growth (Madhusudanan and Nandakumar, 1985). In Camellia sinensis plants, free amino acid levels declined significantly at the stage of bud opening (Watnabe and Ishigaki, 1983).

The purpose of the present study was to investigate the interaction of water deficit stress and plant age on the free amino acids in different tissues of flatpea. Information from this study contributes to our understanding of the regulation of potentially toxic nitrogenous compounds in this species and the physiological functions of such compounds.

MATERIALS AND METHODS

Gilpin soil (fine loamy, mixed, mesic Typic Hapludult) was used to grow experimental plants. Soil pH was 5.5 and initially contained the concentrations of following macroelements: P, 55 ppm; K, 145 ppm; Ca, 420 ppm; and Mg, 67 ppm. Phosphorus and potassium were added in the form of K_2HPO_4 at a rate of 395 mg/kg of dry soil; sulfur was added as $MgSO_4 . 7H_2O$ at a rate of 150 mg/kg of dry soil; and molybdenum was added as $(NH_4)_6Mo_7O_{24} . 4H_2O$ at a rate of 0.17 mg/kg of dry soil. Moisture retention curves for the soil were determined using a pressure plate extractor (Soil Moisture Equipment Co., Santa Barbara, CA).

Flatpea (Lathyrus sylvestris L., cv. 'Lathco') seeds were scarified with concentrated sulfuric acid for 10 min. The scarified seeds were germinated in 9-cm Petri dishes at 20°C in the dark for 5 d until radicles were about 5 mm long. Each seed was then inoculated with 20 µl of a late-log phase Rhizobium leguminosarum culture (10^9 cells/ml), strain 92FZ (Nitragin Co., Milwaukee, WI) and planted in 20-cm plastic pot (10 seeds/pot) containing 3.2 kg (dry weight) of soil. Experiments were conducted in environmentally controlled growth chambers with a 14 h photoperiod and a photon flux density of 400 $\mu E\ m^{-2}\ s^{-1}$ (400-700 nm) provided by a combination of fluorescent and incandescent lamps. Temperatures for the light and dark period were 27°C and 20°C, respectively. Relative humidity was

maintained at 75%. Soil water potential was maintained as close to -0.1 MPa as possible by weighing pots and adding an appropriate amount of water to each daily. One week after emergence, the plants were thinned to four plants per pot. Plants began to flower 12 weeks post emergence.

Plants were harvested 8, 10, 12, 14, 16, 18, 20, and 22 weeks after emergence. Two different water treatments were imposed one week before each harvest: soil water potential of water-stressed plants was maintained at approximately -1.2 MPa; that for control plants was maintained at -0.1 MPa. There were three replications for each combination of age and water treatment. Prior to harvesting, leaf water potentials for the two water treatments were determined using a pressure bomb (Soil Moisture Equipment Co., Santa Barbara, CA). Harvested plants were separated into leaves, stems, and roots. Tissues were lyophilized (Virtis 10-100-v, Gardinar, NY) for 72 h and ground (0.1 mm particle size) with an analytical mill (Model A-10, Tekmar Company, Cincinnati, OH). All ground tissues were stored at -10°C until analyzed. Nitrogen, soluble protein, and free amino acid concentrations were determined as previously described (Chapter 3).

RESULTS

Soil water percentages and the corresponding soil water potentials were approximately 20%/-0.3 MPa and 10%/-1.2 MPa for the control and stressed treatments, respectively. Leaf water potential of control plants ranged from -0.3 to -0.9 MPa, while those of stressed plants varied from -1.5 to -2.7 MPa. The differences in the leaf water potential between the two treatments tended to be large when plants were older.

Drought stressed plants had lower biomass. Differences in the leaf, stem, and root dry weights due to water-deficit stress ranged from 18.1% to 42.3%, 9.5% to 31.4%, and 3.2% to 30.4%, respectively. Shoots showed greater sensitivities than did the roots. Shoots from younger plants were more sensitive to the water shortage than were those from older plants. An opposite trend was observed for the roots.

High concentrations of nitrogen (up to 6% of the dry weight) were found in flatpea leaves and older roots. Stems had the lowest nitrogen concentration (< 4% of the dry weight). Nitrogen concentrations in the leaves and stems did not vary with plant age. In the roots, however, nitrogen levels increased linearly from week 10 to week 14 (R^2 = 0.95) and remained at a constant, high level (> 5% of the dry weight) for the remainder of the study period. No differences in nitrogen concentration between the two water

treatments were observed for all the age treatments.

Soluble protein concentration in the leaves (about 100 mg per g dry weight) was higher than those in roots or stems and tended to increase with plant age. Lower concentrations of soluble protein were detected in the stems (about 60 mg per g dry weight). Root soluble protein levels declined sharply at 8 to 10 weeks of age and remained at a relatively low level (< 50 mg/g dry weight) thereafter. Water deficit did not significantly affect soluble protein concentrations.

Total free amino acid concentrations, calculated as the sum of the concentrations of the 15 individual free amino acids quantified, in the leaves and stems increased slightly with plant age. In roots, total free amino acids increased sharply after week 12, reached a peak (about 100 mg/g dry weight) at week 16, decreased afterwards to about 80 mg/g dry weight at week 18, then remained constant until the end of the experiment (Fig. 4-1). The increase in free amino acid concentrations in roots coincided with an increase in the nitrogen concentration in the roots and the initiation of flowering. Total free amino acid concentration, which was highly correlated with nitrogen concentration ($r = 0.952$) during the 22 weeks of observation, increased significantly under water-deficit stress (Fig. 4-1). In response to drought stress, elevations in the quantity of total free amino acids per gram dry weight of root tissue ranged from 2.4 to 17.1 mg with an average of

7.1 mg. The corresponding values for the stems and leaves were 7.3 mg to 27.8 mg (average of 17.2 mg) and 2.9 mg to 18.2 mg (average of 13.0 mg), respectively. The elevations were higher in the older plants.

The fifteen free amino acids that were quantified in flatpea tissues fall into four categories according to their concentrations and responses to plant age and water supply. Group 1 amino acids, including A_2bu, 4-aminobutyric acid (Abu), and homoserine (Hse), are nonprotein amino acids and the major free amino acids, present at >15 mg/g dry weight. The sum of the three nonprotein amino acids constituted 42.3%, 76.1%, and 80.8% of the total free amino acids in the roots, stems, and leaves, respectively. The highest concentrations of A_2bu and Hse were found in the leaves followed by the stems and the roots (Fig. 4-2). In leaves and stems, A_2bu showed a slight increase with plant age and peaked around week 16 (data not shown). Water deficit caused an 11.9% increase in the concentration of A_2bu in the leaves and little change in the stems and roots (Fig. 4-2). Concentrations of Hse in the tissues were not affected by age (data not shown) or water status (Fig. 4-2) of the plants.

Abu concentration was less than 10 mg per g dry weight in leaves, and the level remained constant during the 22-week experiment (Fig. 4-3). Abu concentrations were higher in the stems of older plants (> 15 mg/g dry weight). Water deficit caused a

decline in Abu in the roots during the entire experimental period and in the stems and leaves around weeks 14 and 16. However, an accumulation of Abu occurred in response to water deficit in the stems and leaves. The accumulation was more prevalent in the stems, before week 14 and after week 18.

Group 2 amino acids consisted of Asn, Arg, Gln, and Asp, which exhibited some common characteristics relative to the experimental treatments. Concentrations of amino acids in this group were not affected by water status. Therefore, only the data from the control treatment are presented in Fig. 4-4. Secondly, amino acids in this group were much more concentrated in the roots than in the shoots. In addition, the concentration of Asn, Arg, Gln, and Asp in the roots were closely correlated with the nitrogen concentration in the roots ($r=0.878$, 0.936, 0.812, and 0.845, respectively). Finally, changes in concentration with plant age were similar for these amino acids. Concentrations of the four compounds in the roots reached peak values between weeks 14 and 18 (Fig. 4-4). A slight increase in the level of these amino acids also occurred in the shoots in the mid-aged plants. Asn differed from the other amino acids in the group in that it was a major component in the roots, composing 41.4% of the total free amino acids. The concentration of Asn in flatpea roots reached as high as 60 mg/g dry weight at week 16.

Group 3 amino acids were Val, Ile, Leu, and Phe (Fig. 4-5). A common characteristic of these amino acids was a constant low level

(< 0.5 mg/g dry weight) in nonstressed plants. Stem concentrations of Val, Ile, and Leu and the leaf concentration of Phe showed a peak value around week 14. Another characteristic of this group was a significant accumulation upon water-deficit treatment. The concentrations of these four amino acids in the roots, stems, and leaves of water-stressed plants were 3.5, 3.5, and 2.3 times higher than those of control plants, respectively. The elevation of these amino acid levels following water-deficit stress was more prominent during weeks 14 to 18.

Proline was the only compound in group 4. Response of Pro to plant age and water treatments was different from the amino acids in the other groups (Fig. 4-6). Proline was approximately evenly distributed among the different tissues of nonstressed plants. Concentrations were low (< 1 mg/g dry weight) in young plants with normal water supply and increased linearly with plant age, from 0.3 mg per gram dry weight at week 8 to 2.69 mg per gram dry weight at week 22 (R^2 = 0.85). Water-stressed plants showed a significant increase in Pro concentration in all of the tissues, a response that was linearly correlated with plant age (R^2 = 0.91).

DISCUSSION

This study shows that nitrogen metabolism of flatpea varies with organ, age, and water status. Distributions of free amino acids among flatpea tissues were consistent with our previous results (Chapter 3). The ratios of A_2bu to Abu and of Hse to Asn tend to be lower in the roots and higher in the leaves. The distribution of the four major amino acids thus seems to imply some substrate/product relationship between these compounds as suggested by Fowden (1965). Both Asn and Hse have been shown to function as precursors for A_2bu. Administration of DL-(1-^{14}C)Asp and L-(^{3}H)Hse resulted in significant labeling of A_2bu (Nigam and Ressler, 1966). No evidence has been found in the literature concerning the synthesis of Abu from Asn, but a close relation between Abu and succinate (Bachrach, 1960) suggested an involvement of Asn in the synthesis of Abu through succinate. It may be postulated that Asn can be transported or converted to Abu in the roots and/or stems and that Asn and Abu are transported to the leaves, where A_2bu and Hse are synthesized. Localization studies (Foster, Cress, Wright, and Hess, 1987) have suggested that A_2bu is synthesized in chloroplasts; however, more detailed study is needed concerning the site of synthesis of those compounds in flatpea.

Relatively constant nitrogen and soluble protein levels were observed in leaves and stems during the 22 week study. On the other

hand, nitrogen concentration increased and soluble protein decreased sharply in the roots with plant age. The increase in nitrogen level with age may be related to increased nitrogen fixation. Decreases in quantities of nitrogenous compounds with age in tobacco (Nicotiana tabacum L.) (Hu, Chen, Tsai, Yu, Chung, Sung, and Su, 1985) and cassava (Manihot esculenta C.) (Gomez, Valdivieso, and Noma, 1985) were thought to be a dilution effect resulting from increased photosynthate in older plants. The decline in soluble protein concentration in roots in the present study may also be a dilution effect as a result of photosynthate transported from the shoots.

The high correlation between the total free amino acid concentration and the nitrogen concentration suggests a relatively constant allocation of nitrogen to free amino acids in flatpea across the entire observation period. The changing pattern of individual amino acids with plant age was distinct. A_2bu concentration tended to increase during the 22 weeks of the experiment. The maximum increase was about 40%, therefore, age seems to be a factor regulating the level of A_2bu in the vegetative tissues of flatpea. The alteration in A_2bu concentration with age should be considered if flatpea is used as a forage crop. Study of field-grown flatpea during growing season is necessary to further understand the change of amino acid composition of flatpea in response to plant age. Of the nonprotein amino acids quantified in

flatpea in this study, Abu levels in the stem showed the greatest change with plant age. Abu accumulation occurs in a wide spectrum of species, including tomato (Lycopersicum esculentum L.) (Selman and Cooper, 1978), tobacco (Hu et al., 1985), and other Lathyrus species (Simola, 1968). In some cases, the accumulation was greatest in the stems and increased with plant age, similar to the response observed in the present study. Although the reason for the accumulation of nonprotein amino acids in flatpea is still unclear, some explanation about Abu accumulation in other plants has been proposed. The compound has been considered to be a temporary storage form of nitrogen and carbon for subsequent biosynthesis of macromolecules. Abu may be an intermediate in the synthetic processes of nitrogen-containing structural compounds and may accumulate when synthesis of those compounds is at a low level, such as during aging, infection by viruses, and deficiency of micronutrients (Selman and Cooper, 1978). Other age-related changes in the concentrations of amino acids in flatpea were elevated levels of Asn, Arg, Gln, and Asp in the roots and Val, Ile, Leu, and Phe in the shoots from weeks 14 to 18 (Figs. 4-4 and 4-5). Such increases, especially in the case of Asn in the roots, may be related to the high nitrogen concentration in roots during the period. Increases in free amino acid levels may also be related to initiation of reproductive growth, since the changes in amino acid concentrations coincided with flowering. Numerous studies with various plant

species have shown a close relationship between the free amino acid concentration and developmental stages (Prasad, Jha, and Mishra 1979; Culianez et al., 1981; Hu et al., 1985; Madhusudanan and Nandakumar, 1985). Concentrations of free amino acids were maximal in four grass species when the panicles were first visible (Mela and Rand, 1979). In Camellia sinensis, free amino acids and caffeine increased five- and two-fold, respectively, at the bud opening stage. Associated with these increases was a 50% reduction in soluble protein (Watnabe and Ishigaki, 1983). Free amino acids in rice (Oryza sativa L.) plants at the boot stage, the milk-ripe stage, and the mature fruiting stage increased 31.1%, 61.6%, and 17.0%, respectively, compared to the concentrations in plants at the vegetative stage (Biswas, Nayek, and Choudhuri, 1982). In our study, the elevated concentrations of free amino acids in the roots during the initiation of flowering may be a result of a higher demand for nitrogen by the reproductive sinks in the shoots. Higher levels of amino acids in the stems may reflect translocation of nitrogenous compounds to the reproductive sinks to support protein synthesis during development of reproductive organs.

A_2bu accumulation in drought-stressed leaves was consistent with our earlier results (Chapter 3). Accumulation of A_2bu and other amino acids may conserve nitrogen and/or aid in osmoregulation of stressed plants (Morgan, 1984). It is interesting that in the stressed stems Abu accumulated only before week 14 and after week

18. Between weeks 14 and 18, Val, Ile, Leu and Phe accumulated in the stems (Fig. 4-5). A_2bu accumulation in the drought-stressed leaves was also greater during weeks 14 to 18. Results showed that accumulation of amino acids in the stressed plants was closely related to, and possibly also controlled by, plant age. With a few exceptions, water shortage did not significantly affect the nitrogen concentration, soluble protein concentration, or the concentrations of two major free amino acids, Asn and Hse. These results differed from those of our previous study (Chapter 3), in which nitrogen concentration, soluble protein concentration, and concentrations of Asn and Hse were significantly affected by the water-deficit stress. The contradictions may be due to the use of younger plants and longer duration of water deficits in our earlier experiment. As plants mature, their tolerance to water-deficit stress tends to increase (Labanauskas, Shouse, Stolzy, and Handy, 1981). Although the concentrations of Asn, Arg, Gln, and Asp in the roots were closely correlated with nitrogen concentration, they did not change in response to water stress. It seems that these amino acids were more closely related to the general nitrogen status than the water status of flatpea.

Proline concentration in control plants, though at a low level, increased linearly with plant age (Fig. 4-6), similar to the observations recorded for tobacco (Hu et al., 1985) and Lepidium crassifolium (Popp and Albert, 1981). A significant increase in Pro

concentration in water-stressed flatpea plants in this study was consistent with the responses observed with other species, (Mukherjee and Choudhuri, 1984), yet the physiological function of proline accumulation is still unknown. The compound may not be an important osmoregulant in flatpea, because it comprised only 10% of the total free amino acids in the stressed plants. The greater accumulation of Pro in older stressed plants could reflect an increased, Pro-dependent tolerance to water deficit stress when plants were older.

The qualitative and quantitative changes in the free amino acid composition of flatpea observed in this study may reflect specific functions for individual amino acids in growth and developmental processes or in stress tolerance or adaptation. Because neurotoxic activity has been ascribed to A_2bu (Ressler et al., 1961) which normally occurs at relatively high levels in 'Lathco' flatpea (Fig. 4-2), even small increases in the levels of these compound, due to changes in plant age or duration of drought or other environmental stress can have significant physiological impact in animals that consume the plant. Further studies are therefore needed to extend our knowledge of the regulation of A_2bu in flatpea and thereby aid in the development of cultural and management practices to enhance the agronomic value of flatpea.

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FIGURE LEGENDS

Fig. 4-1. Total free amino acid concentration of control (_____) and drought stressed (-----) flatpea plants over a 14 week observation period.

Fig. 4-2. A_2bu and Hse concentration of control and drought stressed flatpea plants.

Fig. 4-3. Abu concentration of control (_____) and drought stressed (-----) flatpea plants over a 14 week observation period. Error bars represent the standard error larger than 10% of means (n = 3).

Fig. 4-4. Several amino acid concentration in the leaves (●), stems (■), and roots (▲) of well-watered flatpea over a 14 week observation period. Error bars represent the standard error larger than 10% of means (n = 3).

Fig. 4-5. Amino acid concentration of leaves (●), stems (■), and roots (▲) of control (_____) and drought stressed (-----) flatpea plants over a 14 week observation period. Error bars represent the standard error larger than 10% of means (n = 3).

Fig. 4-6. Proline concentration in control (_____) and drought stressed (-----) flatpea plants over a 14 week observation. period. Error bars represent the standard error larger than 10% of means (n = 3).

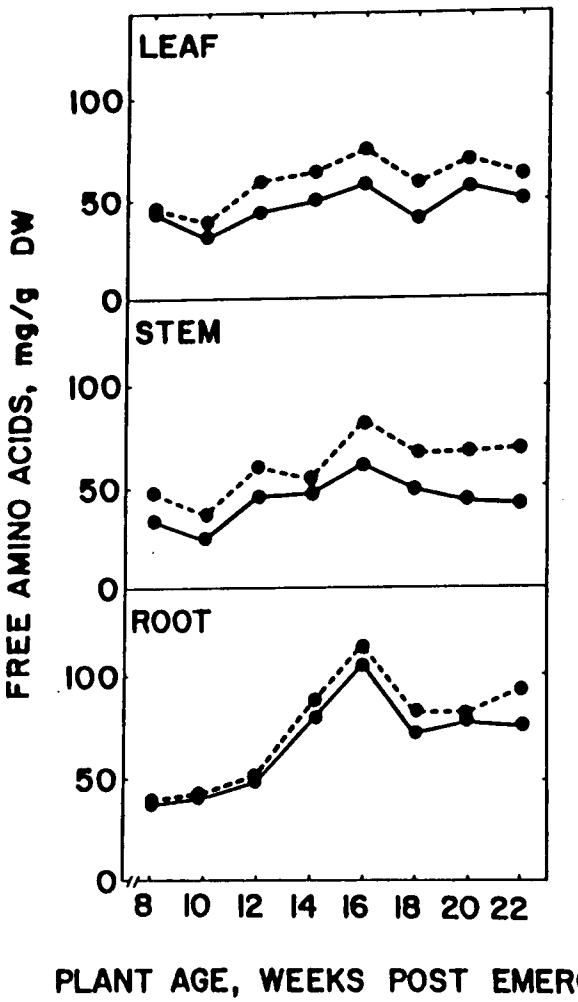

Fig. 4-1. Total free amino acid concentration of control (____) and drought-stressed (----) flatpea plants over a 14 week observation period.

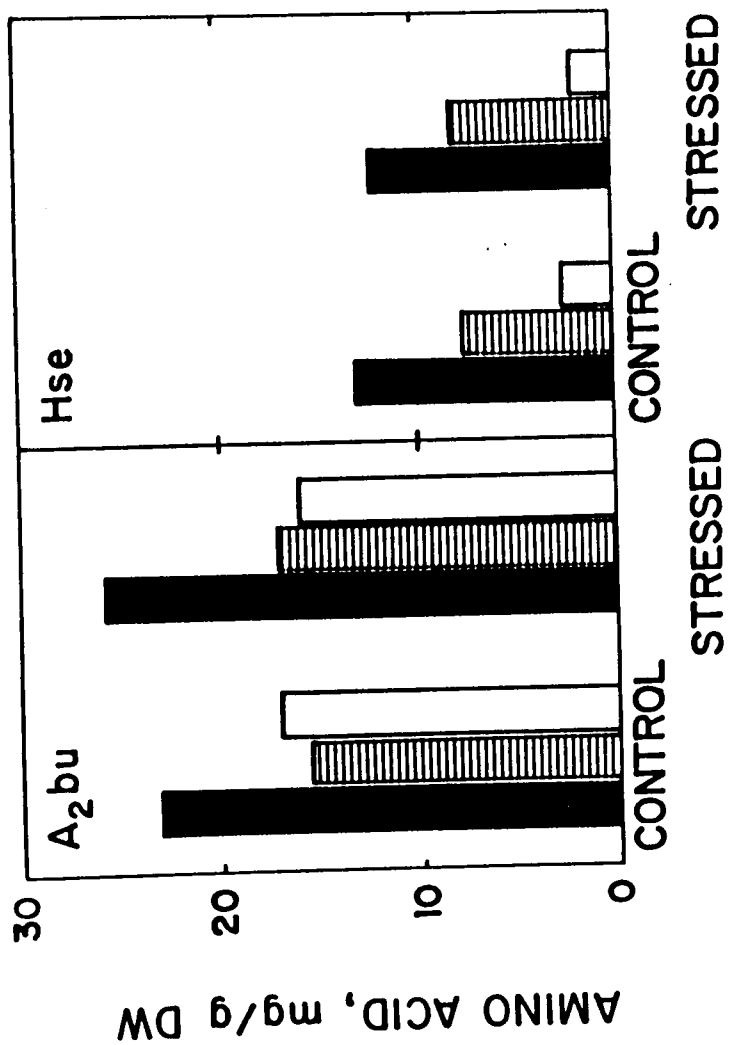

Fig. 4-2. A_2bu and Hse concentration of control and drought-stressed flatpea plants.

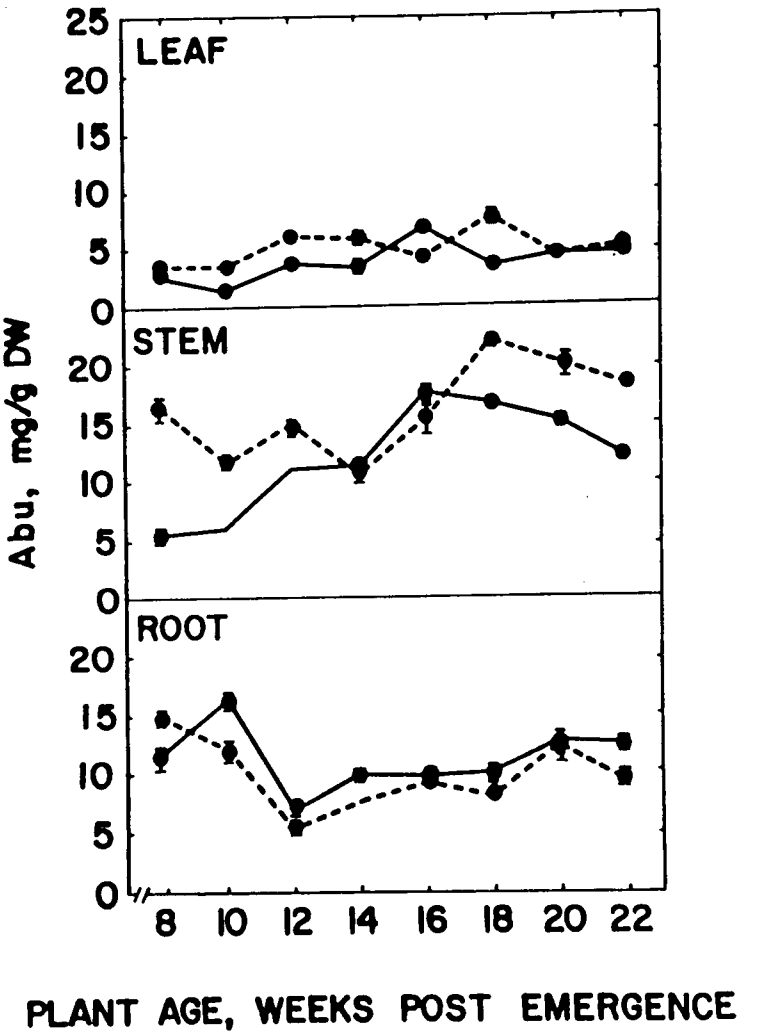

Fig. 4-3. Abu concentration of control (_____) and drought-stressed (-----) flatpea plants over a 14 week observation period. Error bars represent the standard error larger than 10% of means (n = 3).

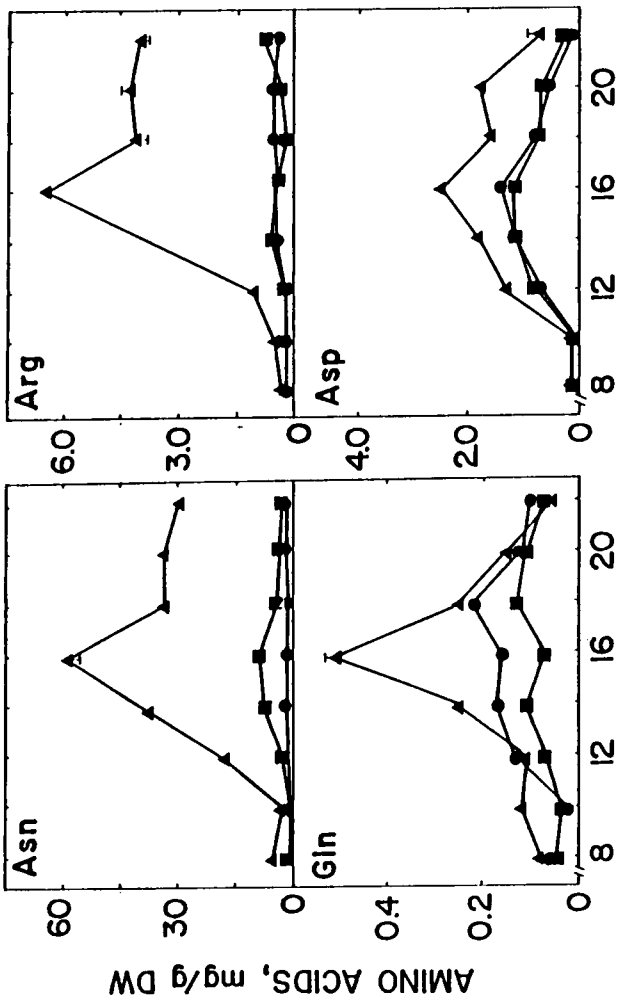

Fig. 4-4. Amino acid concentration of leaves (●), stems (■), and roots (▲) of non-stressed flatpea over a 14 week observation period. Error bars represent the standard error larger and 10% of means (n = 3).

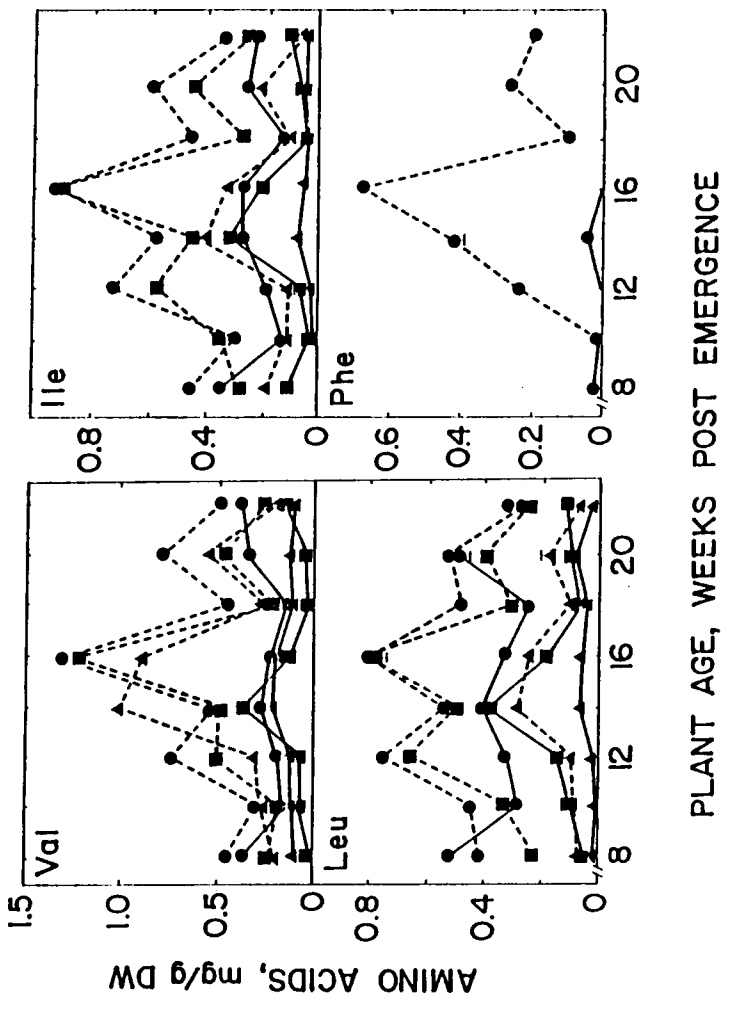

Fig. 4-5. Amino acid concentration of leaves (●), stems (■), and roots (▲) of control (_____) and drought-stressed (-----) flatpea plants over a 14 week observation period. Error bars represent the standard error larger than 10% of means (n = 3).

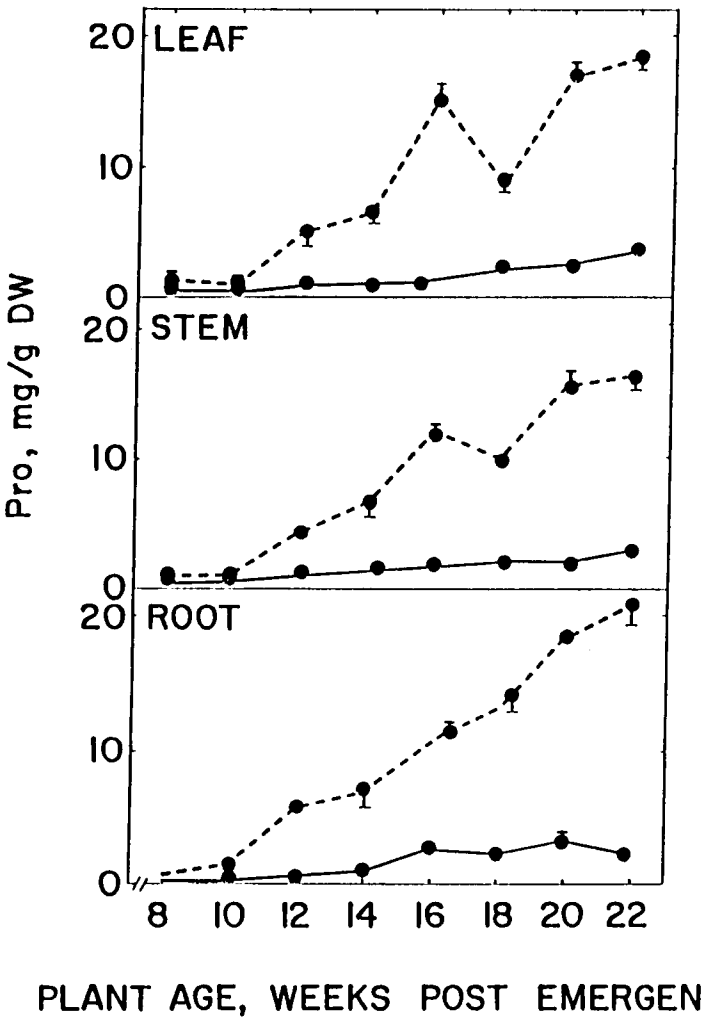

Fig. 4-6. Proline concentration in control (_____) and drought-stressed (-----) flatpea plants over a 14 week observation period. Error bars represent the standard error larger than 10% of means (n = 3).

CHAPTER V. INFLUENCE OF NITRATE AND AMMONIUM SUPPLY ON THE GROWTH AND FREE AMINO ACID COMPOSITION OF FLATPEA (*LATHYRUS SYLVESTRIS* L.)

ABSTRACT

Presence of 2,4-diaminobutyric acid (A_2bu), a neurotoxin, in tissues of flatpea (*Lathyrus sylvestris* L.) necessitates a thorough understanding of the regulation of this nonprotein amino acid before the species can be recommended to livestock producers for use as a forage. To determine how different concentrations and ratios of NO_3^- and NH_4^+ in growth media influence A_2bu levels in flatpea, plants were grown hydroponically under controlled environments. A_2bu concentration increased in tissues when the NO_3^- to NH_4^+ ratio was low. Responses of amides and other nonprotein amino acids, especially in the roots, followed a similar trend. Free protein amino acids in leaves and stems were generally unaffected by changes in NO_3^- to NH_4^+ ratios. In roots, protein amino acids increased as the NO_3^- to NH_4^+ ratio in the rooting medium increased. Ammonium inhibited shoot and root growth and development; nitrate alleviated the toxic effects of ammonium. Soluble protein concentrations were higher in the shoots of NO_3^--fed plants and in the roots of plants supplied with ammonium. Changes in nitrogen metabolism, including free amino acid composition, corresponding to the level of NH_4^+ supplied may relate to the plant adaptation to NH_4^+ toxicity.

INTRODUCTION

Flatpea (Lathyrus sylvestris L.) is a long-lived, perennial legume that has shown promise as a forage crop. It is palatable and easily digested by animals (8), highly productive (20), and tolerant of environmental stresses, such as low fertility (1), drought (27) and temperature extremes (25). However, some animals fed flatpea forage have developed neurotoxic symptoms which sometimes have led to death (23, 24). A nonprotein amino acid, 2,4-diaminobutyric acid (A_2bu), was found to be especially high in flatpea tissues and has been correlated with the toxic effects in some animals (23). If flatpea is to be utilized as a forage, an understanding of how the environment influences its productivity and quality is essential.

Nitrogen is required in high quantity for plant growth and is frequently the most limiting nutrient in soils. Nitrogen form and concentration considerably affect plant growth and nitrogen metabolism (12, 16). Qualitative and quantitative composition of the free amino acid pools in plants (4, 10, 13), including nonnodulated legumes (3), were changed dependent on the concentration and forms of nitrogen supplied: NO_3^- versus NH_4^+. Such changes may reflect physiological adjustments by plants to detoxify toxic nitrogen sources, produce translocatable forms of nitrogen or store nitrogen for future incorporation into protein (12, 16). Studies using different ratios of NO_3^- and NH_4^+ support the concept that most plant species grow optimally when supplied with both forms of nitrogen (12). However,

the more or less favorable effect of one form of N, versus the other, varies with species, pH, and the concentration of available nutrients (12, 16).

Amino acid pool in flatpea may be also affected by the nitrogen source available to plant. Study about nitrogen source and amino acid composition of flatpea can provide information concerning the biological function of A_2bu and physiological regulation of the potential neurotoxic compound. The purpose of the present study was to determine how different ratios of NO_3^- and NH_4^+ affect the growth of nonnodulated flatpea plants and to determine if the free amino acid composition changes as the NO_3^- to NH_4^+ ratio is adjusted. Results showed that NH_4^+, a potentially toxic form of nitrogen for flatpea, elevated the concentration of A_2bu and other nonprotein amino acids. However, the elevation may not be sufficient to decrease the feeding value of flatpea.

MATERIALS AND METHODS

Sulfuric acid-scarified (Chapter 3) flatpea (Lathyrus sylvestris L., cv 'Lathco') seeds were imbibed on germination paper moistened with deionized, distilled water. When the radicals were about 10 cm long (10 d after germination), the seedlings (four/container), supported in styrofoam lids, were transferred to plastic containers (8 L) filled with quarter-strength Hoaglands's nutrient solution minus nitrogen. Ten nitrogen treatments were applied. For each treatment, nitrogen was supplied as $Ca(NO_3)_2$ or $(NH_4)_2SO_4$ at NO_3^- to NH_4^+ ratios (meq NO_3^--N L^{-1}:meq NH_4^+-N L^{-1}) of 1:0, 2:0, 4:0, 1.5:0.5, 1:1, 0.5:1.5, 0:1, 0:2, 0:4, and 0:0, respectively. All treatments were replicated three times. The experiment was conducted in a controlled environment growth chamber using a 14 h, 27°C light/10 h, 20°C dark cycle. A combination of fluorescent and incandescent lamps provided PAR of 400 $\mu E\ m^{-2}\ s^{-1}$. Relative humidity was maintained at 75%.

Nutrient solutions were aerated with filtered, compressed air and changed weekly for the first four weeks of growth and then twice weekly for another two weeks. For the last two weeks of the experiment, culture solutions were replaced every other day. Solution pH was monitored daily and adjusted to 6.5 with dilute KOH or H_2SO_4. Shoot and root lengths were measured weekly. Eight weeks after initiation of treatments the plants were harvested, and leaves, stems, and roots were frozen, crushed in liquid nitrogen, lyophilized, and stored at -10°C until analyzed. Lyophilized tissue was used for dry

weight determinations. Nitrogen, soluble protein, and free amino acid concentrations were determined as previously described (11, Chapter 3).

RESULTS

Flatpea root and shoot growth varied considerably with different NO_3^- to NH_4^+ ratios in nutrient solutions containing 2 meq N L^{-1} (Figs. 5-1 and 5-2). In the roots of plants grown with NH_4^+ as the sole source of nitrogen, a 3-week growth lag was observed (Fig. 5-1). The parallel nature of the remaining root growth curves suggest that growth rates were similar after week 1. However, differences in cumulative growth among treatments suggest that a lag in growth, related to the NH_4^+ concentrations, occurred in these treatments prior to week 1. A similar trend was observed in the shoots except the lag period was not as pronounced (Fig. 5-2). At harvest time, dry weights of leaves, stems, and roots of flatpea plants grown in different ratios of NO_3^- to NH_4^+ were directly related to the content of NO_3^- in the growth medium (Fig. 5-3). Root growth, as measured by dry matter accumulation, was particularly responsive to changes in the ratio, with highest yields occurring in plants provided with NO_3^- alone.

Differences in plant development were also observed as a result of changes in the NO_3^- to NH_4^+ ratio. Ammonium inhibited the number of lateral shoots produced (Fig. 5-3) and resulted in decreased lateral root development (data not shown). Also, NH_4^+, as sole nitrogen source, resulted in thin and brown roots compared to a healthy, white, thick appearance of roots grown in NO_3^-. A little browning was observed in roots grown in the growth medium containing 25% of nitrogen as NH_4^+. Discoloration increased progressively with

increasing NH_4^+ concentration.

Flatpea growth studies were also conducted using nutrient solutions containing either NO_3^- or NH_4^+ at concentrations of 1, 2 and 4 meq N L^{-1}. No differences were observed in root and shoot lengths, dry weight accumulation, or shoot number over the concentration range tested for either nitrogen source (data not shown).

Nitrogen concentration in leaves and roots was higher than that in stems for all NO_3^- to NH_4^+ ratios evaluated (Fig. 5-4a). However, no statistically significant difference in nitrogen concentration was observed in a given type of tissue with respect to the various NO_3^- to NH_4^+ ratios. When either NO_3^- or NH_4^+ was supplied alone, nitrogen accumulated in all tissues as the concentration of either nitrogen source in growth medium increased. Leaves and stems from plants grown with NO_3^- as the sole source of nitrogen accumulated more nitrogen than did the corresponding tissues of plants supplied with NH_4^+ (Fig. 5-4b). This NO_3^--related nitrogen accumulation was also observed in roots when plants were supplied with 1 meq of N but at higher levels of nitrogen, there was little difference in root nitrogen accumulation between NO_3^-- and NH_4^+-fed plants (Fig. 5-4b).

Soluble protein increased in the stems, but decreased in the roots, as the ratio of NO_3^- to NH_4^+ in the growth media was increased (Fig. 5-5a). No significant change in soluble protein concentration occurred in the leaves except when plants were grown using NO_3^- alone, in which case soluble protein increased. Concentrations of soluble protein were higher in the leaves than in the stems and roots (Fig.

5-5a). The only exception was in plants provided with nutrient solution containing only NH_4^+. In these plants the highest soluble protein concentration was in the roots. When plants were supplied with either NO_3^- or NH_4^+ as the sole source of nitrogen, soluble protein was higher in the leaves and stems of NO_3^--grown plants than in NH_4^+-grown plants receiving a similar amount of nitrogen (Fig. 5-5b). The opposite was true for the roots. Soluble protein increased in the leaves and stems of NO_3^--grown plants and in the roots of NH_4^+-grown plants as nitrogen concentrations in nutrient solutions were increased from 1 to 2 meq L^{-1} and remained at this higher level when even higher levels of nitrogen (4 meq L^{-1}) were supplied. Little change in protein concentration was observed in the leaves and stems of NH_4^+-grown plants while a decrease was observed in root tissues of plants grown with an increasing supply of NO_3^-.

The most abundant free amino acids present in flatpea tissues were Asn and the nonprotein amino acids A_2bu, 4-aminobutyric acid (Abu) and homoserine (Hse). Smaller, quantifiable amounts of Gln, Ala, Glu, Leu, Ile, Asp, Arg, Val and Phe were also present (Figs. 5-6 and 5-7). The free amino acid content of flatpea plants grown using different ratios of NO_3^- to NH_4^+ varied greatly with respect to the specific amino acid and tissues affected (Figs. 5-6 and 5-7). Ammonium inhibited the accumulation of the protein amino acids Ala, Glu, Leu, Ile, Asp, Arg, Val, and Phe, particularly in the roots, while progressive increases in NO_3^- concentrations in the culture solution stimulated the accumulation of these amino acids in the roots. Little

effect of the ratio of NO_3^- to NH_4^+ on the level of these amino acids was observed in leaves or stems with the exception of Glu and Ile, which increased and decreased in the leaves, respectively, as the NO_3^- content of the growth medium increased (Fig. 5-6). Tissue contents of the amides Asn and Gln, known to be storage and transport forms of nitrogen, and the nonprotein amino acids A_2bu, Abu, and Hse were also responsive to changes in the ratios of NO_3^- to NH_4^+ in the growth medium (Fig. 5-7). In roots, A_2bu, Abu, Hse, Asn, and Gln all declined when NO_3^- concentrations in growth media were high. This was also true of A_2bu and Gln in the leaves and stems, Abu in the stems, and Hse and Asn in the leaves. Levels of Abu in the leaves and Hse and Asn in the stems were relatively constant.

Growth of flatpea without exogenous nitrogen was severely inhibited. Dry weights of tissues from nitrogen-starved plants were only about 5% of the dry weights of corresponding tissues from plants supplied with inorganic nitrogen. No discoloration of roots was observed for nitrogen-starved plants. Nitrogen, soluble protein, and free amino acid concentrations in tissues from these plants were consistently less than 40% of their corresponding concentrations in tissues from plants supplied with NO_3^- and/or NH_4^+ at all levels tested.

DISCUSSION

Growth of flatpea was best in the absence of NH_4^+. Inhibitory and toxic affects of NH_4^+ on plant growth has been documented on several occasions (2, 12), and the effect of NH_4^+ on root discoloration and inhibition of lateral root and shoot production has also been reported (2, 9). The mechanism of NH_4^+ toxicity probably involves the uncoupling of photophosphorylation in the chloroplasts (6). The damaged root systems of NH_4^+-grown plants also result in reduced nutrient uptake and, consequently, can result in nutrient deficiencies (5, 17, 20). Magalhaes and Wilcox (17) and Rajasekhar and Mor (22) have reported that plant response to NH_4^+ depends on the amount of light to which the plants are exposed. the apparent toxicity of NH_4^+ observed in this study may reflect the low light level (approximately 25% of full sunlight) available in growth chambers used.

The uptake of NO_3^- or NH_4^+ changes the pH of the rhizosphere dramatically (14). Under controlled pH conditions, NO_3^- and NH_4^+ were comparable sources of nitrogen for tomato (19) and Dupontia fisheri (26) grown hydroponically. However, other reports document NH_4^+ toxicity, even under pH-controlled conditions (5, 14). In our study the pH was maintained at 6.5 with a deviation range of 1 to 1.5 pH units for the different treatments. The maximum deviation from this value was a reduction of 1.5 pH units in the 25% NH_4^+ treatment in which there were no apparent symptoms of NH_4^+ toxicity. Thus, the toxic affect in this study appears to be a direct result of NH_4^+.

In the present study, NH_4^+ supply resulted in the accumulation of the nonprotein amino acids A_2bu, Abu, and Hse and the amides Asn and Gln. Increased amide production was also observed in NH_4^+-treated Lolium multiflorum plants while NO_3^--treated plants contained little amide (18). Accumulation of amides in NH_4^+-treated plants has been related to detoxification and transport functions (12, 16). The specific role of nonprotein amino acids is as yet unclear, but they may also serve to aid in detoxification, storage, and translocation of nitrogen. The low level of protein amino acids in the roots and the low soluble protein concentration in the leaves and stems of flatpea plants grown under high NH_4^+, compared to plants supplemented with NO_3^-, most likely reflect a change in metabolic activity in favor of amino acids involved in detoxification and storage. The high level of soluble protein observed in the roots of plants grown in NH_4^+ (Fig. 5-5) suggests increased production of enzymes which are responsible for the change in the concentrations of nonprotein amino acids or amino acids involved in detoxification and storage. Roots exhibited uniform nitrogen concentration across the NO_3^- to NH_4^+ ratios studied. Leaf and stem nitrogen concentrations were likewise consistent among treatments involving different combinations of NO_3^- and NH_4^+. Such consistency suggests that uptake of nitrogen is closely regulated by the plant, but the mechanisms involved are unclear (7).

When combinations of NO_3^- and NH_4^+ were used, the toxicity of NH_4^+ was reduced. This probably reflects the nontoxic effects of NO_3^- and its ability to accelerate the development of photosynthetic organs

which, in turn, produce photosynthate that can be transported to the roots for use as a carbon and energy source to detoxify the NH_4^+ through the formation of amides and nonprotein amino acids (15). Combinations of NO_3^- and NH_4^+ were no more advantageous to the growth of flatpea than NO_3^- alone. In other plant species combinations of the two nitrogen forms have proved beneficial to growth (2).

It has been suggested that hormonal differences or imbalances may occur as a consequence of inadequate nitrogen levels or source of nitrogen available. Darrall and Wareing (9) reported the lack of cytokinin-like compounds in Betula pendula and Acer pseudplatanus plants supplied with NH_4^+ rather than NH_4NO_3 or NO_3^-, and plants fed with NH_4^+ failed to produce lateral shoots. These observations suggested a relationship between nitrogen nutrition, cytokinin production, and lateral shoot growth since applications of cytokinins to dormant lateral buds could induce growth (9). A good deal of evidence supports the concept that cytokinins may influence nitrogen partitioning in plants and that cytokinins produced in the root tips and exported to the leaves are responsive to nitrogen nutrition and are presumed to affect protein synthesis in leaves (26).

The neurotoxin, A_2bu, was the most abundant free amino acid in flatpea tissues. Accumulation of A_2bu was greater when the ratio of NO_3^- to NH_4^+ was low, but when the NO_3^- to NH_4^+ ratios were increased, A_2bu levels did not decline as drastically as did those of Abu, Hse, Asn, and Gln. Recent studies indicate that A_2bu is also not as responsive to drought stress as Abu, Arg, Asp and Pro in flatpea

(Chapter 3). The physiological role of A_2bu in flatpea is not known. One hypothesis is that it could serve as a storage or translocatable form of nitrogen under stressed conditions such as drought stress (Chapters 3 and 4) and ammonium toxicity (Fig. 5-7). Obviously it could serve as a deterrent to predation. However, in view of the considerable energy expended to produce this amino acid in large quantities it seems that other physiological and biochemical functions may exist. Further studies related to nodulation, plant age and hormonal control are being conducted to further elucidate the importance of this amino acid in flatpea. Knowledge of environmental regulation of A_2bu levels will provide information essential to the development of management strategies for the utilization of flatpea as a forage crop.

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FIGURE LEGENDS

Fig. 5-1. Root growth of flatpea seedlings as influenced by nitrogen source and ratio. Each line represents 2 meq N/L supplied as NO_3^- to NH_4^+ ratios of 0:2.0 (○), 0.5:1.5 (●), 1.0:1.0 (■), 1.5:0.5 (▲), 2.0:0 (◆). Values are means ± SE (n = 3).

Fig. 5-2. Shoot growth of flatpea seedlings as influenced by nitrogen source and ratio. Nitrogen treatments were as described for Fig. 5-1. Values are means ± SE (n = 3).

Fig. 5-3. Influence of NO_3^- to NH_4^+ ratio on dry weight of leaf (____), stem (__ __), root (.....), and shoot number (_ _ _) of flatpea. Values are means ± SE (n = 3).

Fig. 5-4. Tissue nitrogen percentage of flatpea plants supplied with 2 meq N/L as different ratios of NO_3^- and NH_4^+ (A) or with either NO_3^- (____) or NH_4^+ (_ _ _) different concentrations (B). Leaf (●), stem (■), root (▲). Values are means ± SE (n = 3).

Fig. 5-5. Soluble protein concentrations of flatpea plants supplied with 2 meq N/L as different ratios of NO_3^- and NH_4^+ (A) or with either NO_3^- (____) or NH_4^+ (_ _ _) different concentrations (B). Leaf (●), stem (■), root (▲). Values are means ± SE (N=3).

Fig. 5-6. Free protein amino acids in flatpea plants grown with different ratios of NO_3^- to NH_4^+. Symbols are defined as in Fig. 5-3. Values are the means of three replications. Standard errors larger than 5% of means are shown by vertical bars.

Fig. 5-7. Amides and nonprotein amino acids in flatpea plants grown with different ratios of NO_3^- to NH_4^+. Symbols are defined as in Fig. 5-3. Values are the means of three replications. Standard errors larger than 5% of means are shown by vertical bars.

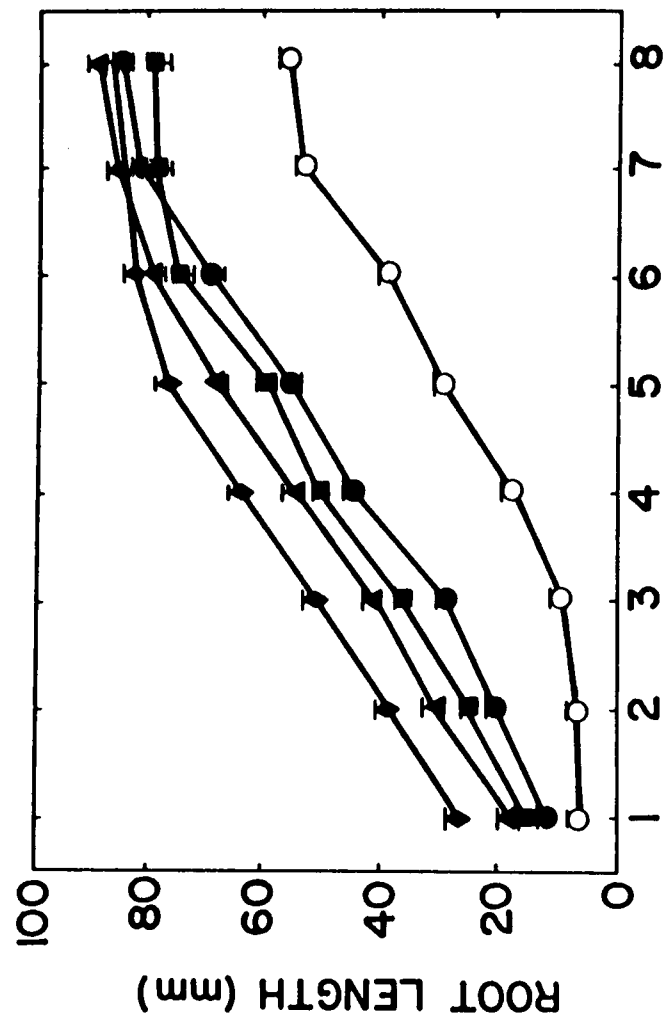

Fig. 5-1. Root growth of flatpea as influenced by nitrogen source and ratio. Each line represents 2 meq N L^{-1} supplied as NO_3^- to NH_4^+ ratios of 0:2.0 (○), 0.5:1.5 (●), 1.0:1.0 (■), 1.5:0.5 (▲), 2.0:0 (◆). Values are means ± SE (n = 3).

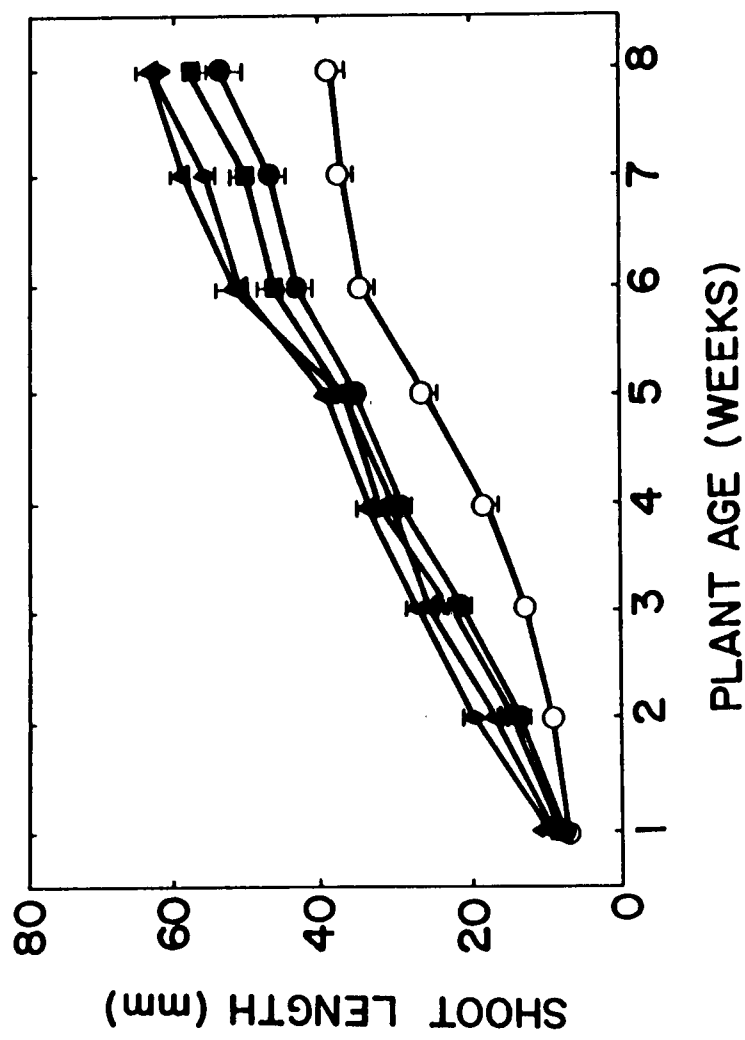

Fig. 5-2. Shoot growth of flatpea plants as influenced by nitrogen source and ratio. Nitrogen treatments were as described for Fig. 1. Values are means ± SE (n = 3).

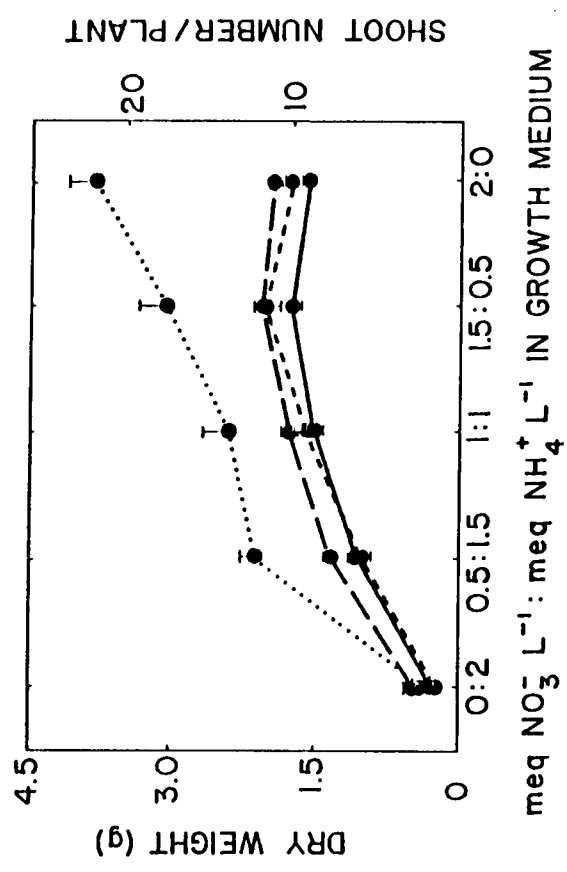

Fig. 5-3. Influence of NO_3^- to NH_4^+ ratio on dry weight of leaf (____), stem (_ _ _), root (.....), and shoot number (_ _ _) of flatpea. Values are means ± SE (n = 3).

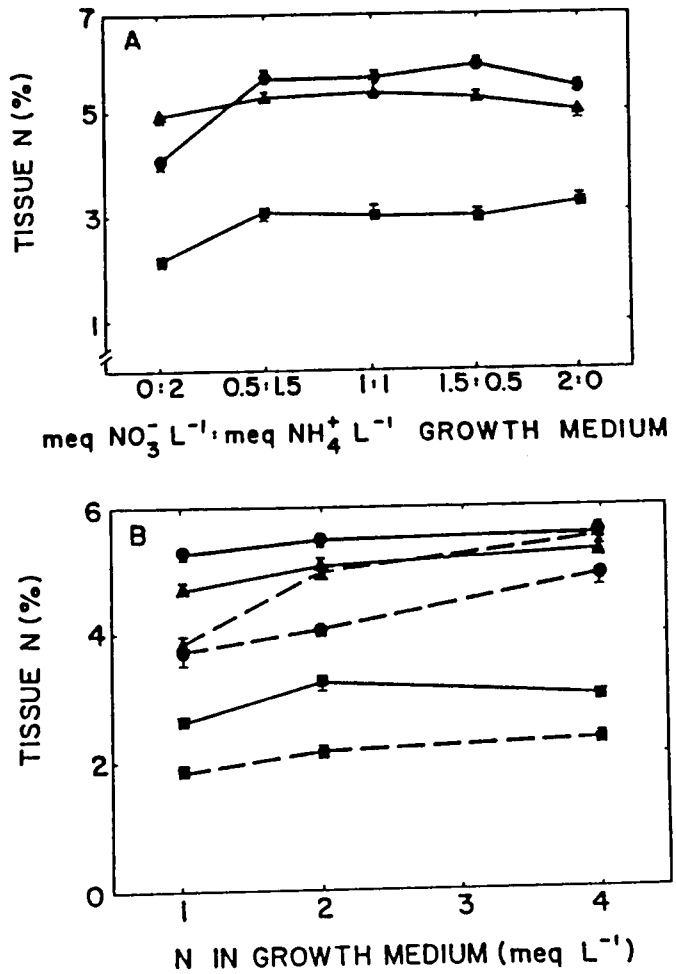

Fig. 5-4. Tissue nitrogen percentage of flatpea plant supplied with 2 mew N L^{-1} as different ratios of NO_3^- to NH_4^+ (A) or with either NO_3^- (_____) or NH_4^+ (_ _ _) at different concentrations (B). Leaf (●), stem (■), root (▲). Values are means ± SE (n = 3).

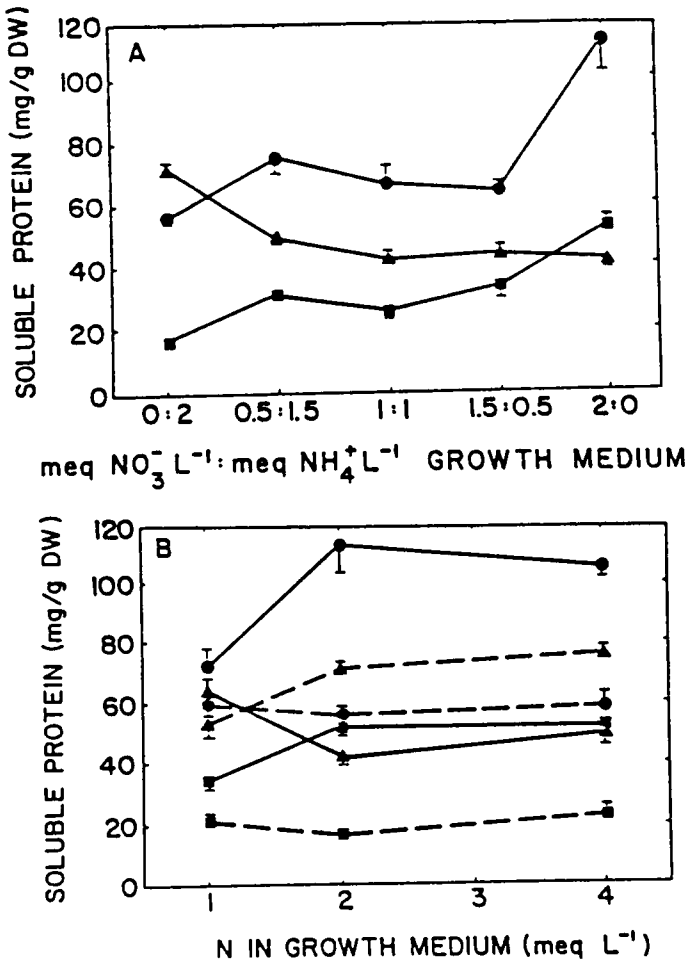

Fig. 5-5. Soluble protein concentrations of flatpea plants supplied with 2 meq N L^{-1} as different ratios of NO_3^- to NH_4^+ (A) or either NO_3^- (_____) or NH_4^+ (_ _ _) at different concentrations (B). Leaf (●), stem (■), root (▲). Values are means + SE (n = 3).

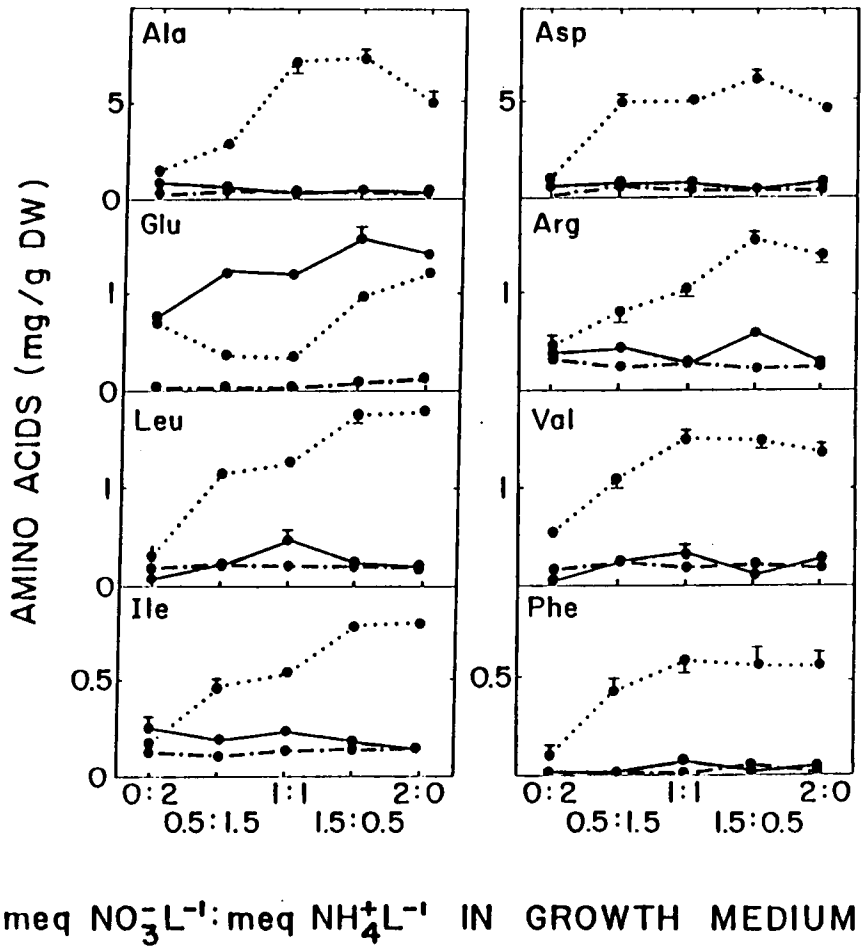

Fig. 5-6. Free protein amino acids in flatpea plants grown with different ratios of NO_3^- to NH_4^+. Symbols are defined as in Fig. 3. Values are the means of three replications. Standard errors larger than 5% of means are shown by vertical bars.

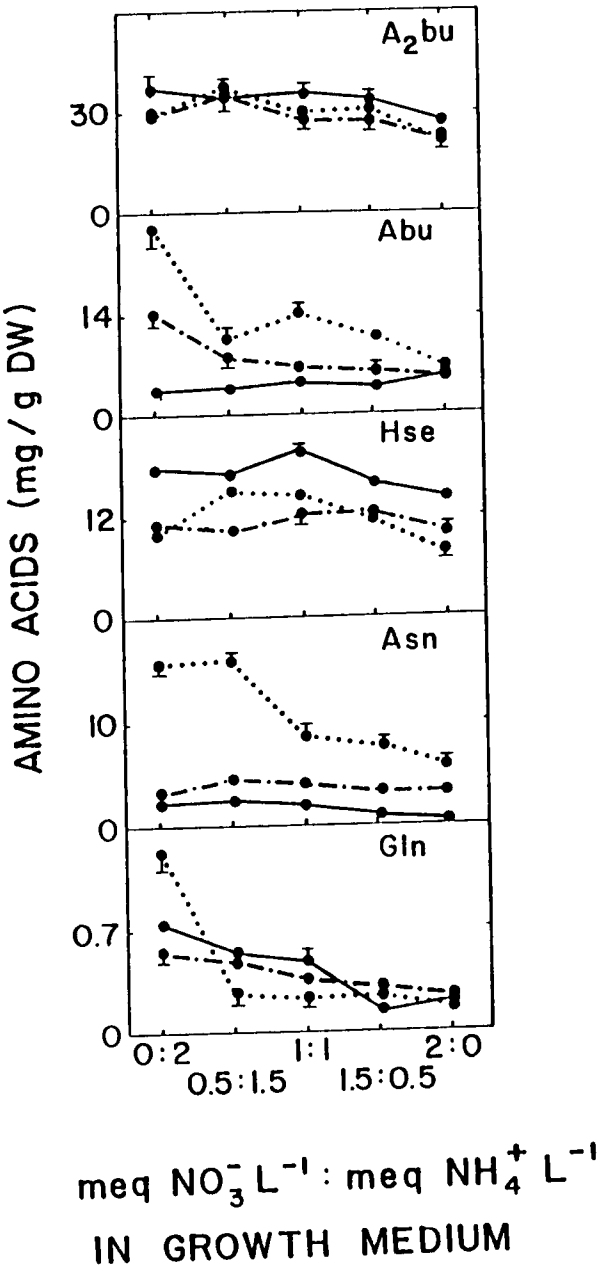

Fig. 5-7. Amides and nonprotein amino acids in flatpea plants grown with different ratios of NO_3^- to NH_4^+. Symbols are defined as in Fig. 3. Values are the means of three replications. Standard errors larger than 5% of means are shown by vertical bars.

CHAPTER VI. EFFECTS OF MINERAL AND SYMBIOTICALLY-FIXED NITROGEN ON THE GROWTH AND FREE AMINO ACID COMPOSITION OF FLATPEA (LATHYRUS SYLVESTRIS L.)

ABSTRACT

Flatpea (Lathyrus sylvestris L.) plants, grown hydroponically, were inoculated with rhizobia, supplied with nitrate nitrogen, or provided with both rhizobia and nitrate. Plants provided with both biologically fixed and inorganic nitrogen had the highest biomass and the highest level of free amino acids in leaves and roots. An 80% decline in nitrogen fixation was observed in inoculated plants supplemented with nitrate. Compared to nodulated or noninoculated plants provided with nitrate, nodulated plants that acquired nitrogen solely through nitrogen fixation had fewer lateral shoot and lower concentrations of nitrogen, soluble protein, and total free amino acids in the shoots, especially in the leaves. Levels of 2,4-diaminobutyric acid (A_2bu) and most of the other free amino acids quantified were significantly lower in the nodulated plants grown in the absence of nitrate than in the nodulated or noninoculated plants supplied with inorganic nitrogen. Arginine and glutamic acid were exceptions in that levels of these two amino acids were higher in the roots with highly effective nodules. Plant responses to the different treatments are attributed to the amount of nitrogen available to the plants instead of methods or forms of

nitrogen supplied. the symbiotic association between plants and rhizobia for nitrogen fixation appeared to be neither essential nor inhibitory to the production and accumulation of A_2bu and other nonprotein amino acids in flatpea.

INTRODUCTION

Flatpea (Lathyrus sylvestris L.) is a deep-rooted, perennial legume that has potential as a forage species. Nutritional value of this plant has been reported to be comparable to such common forage crops as alfalfa, clover, and crownvetch (Daniel et al. 1946, Long et al. 1977). Early feeding studies with sheep and cattle indicated that flatpea was highly palatable and digestible (Hodgson and Knott 1936). However, some sheep died after consuming flatpea (Daniel et al. 1946). High levels of 2,4-diaminobutyric acid (A_2bu) in flatpea has been considered to be a causal factor for toxicity to animals (Ressler et al. 1961). While the toxicity of this nonprotein amino acid is low (Ressler 1975), concentrations in flatpea can reach as high as 2 to 3% of the tissue dry weight (Nigam and Ressler 1966). Therefore, controlling or reducing the levels of A_2bu in flatpea is essential, if the plant is to be used as a forage crop.

Free amino acid concentrations in plants are influenced by the developmental stage, temperature, and water availability (Hu et al. 1985, Mukherjee and Choudhuri 1984). Nitrogen source also has considerable effects on the growth and nitrogen metabolism of plants, including the metabolism of free amino acids (Schrader et al. 1972). For legumes, free amino acid profiles may be altered, depending on the source of nitrogen available to the plants, e.g. nitrogen symbiotically fixed by rhizobia versus inorganic nitrogen absorbed directly by the roots of plants (Vance et al. 1983). The

purpose of this study was to determine whether nitrogen metabolism and amino acid, particularly A_2bu, composition vary when flatpea uses nitrate and/or symbiotically fixed nitrogen to meet its nitrogen requirement.

MATERIALS AND METHODS

Flatpea (Lathyrus sylvestris L., cv. 'Lathco') seeds were scarified by soaking in concentrated sulfuric acid for 10 min followed by rinsing with distilled water. Scarified seeds were imbibed in water in a flask shaken at 100 rpm using a G10 Gyrotory Shaker (New Brunswick Scientific Company Inc., New Brunswick, NJ, U.S.A.)[1] for 1 d. The seeds were then germinated in 9-cm Petri dishes at 20°C in the dark for 5 d. Young seedlings with radicles of approximately 1 cm were cultured further on germination paper moistened with distilled water for another 7 d. Thereafter, four seedlings with radicles approximately 10 cm in length were supported by a piece of polyethane foam and transferred into 8-L plastic containers for hydroponic culture. Plants were grown in nitrogen-free, quarter-strength Hoagland and Arnon (1950) nutrient solution through which nitrogen treatments were imposed. Containers were divided into three groups for nitrogen-source treatments with three replications for each treatment. Plants in one group were provided with 2 meq L^{-1} of nitrate nitrogen in the form of $Ca(NO_3)_2.4H_2O$. Plants in the second group were inoculated with a heavy inoculum of Rhizobium leguminosarum, strain 92FZ (Nitragin Co., Milwaukee, Wisconsin, U.S.A.). The third group of plants was provided with inorganic nitrogen as in group 1 but was also inoculated with rhizobia as in group 2. A non-recycled continuous flow of nutrient solution (5 ml min^{-1}) was provided for each

container. Solutions in reservoirs were prepared every 10 d and were adjusted to a pH of 6.5 with KOH. All containers were aerated with filtered compressed air.

Plants were cultured in an environmentally controlled growth chamber with a 14 h photoperiod. A photon flux density of 400 $\mu E\ m^{-2}\ s^{-1}$ (400-700 nm) was provided by a combination of fluorescent and incandescent lamps. The temperature regime for the light/dark cycle was 27°C/20°C. Relative humidity was maintained at 75%. Nitrogen fixation was measured by the acetylene reduction assay of Hardy et al. (1972). Plants were harvested 80 d post germination and separated into leaves, stems, and roots. Tissues were lyophilized (Virtis 10-100-v, Gardinar, New York, U.S.A.) for 72 h and ground (approximately 0.1 mm particle size) with an analytical mill (Model A-10, Tekmar Company, Cincinati, Ohio, U.S.A.). All tissues were stored at -10°C until analyzed.

Nitrogen, soluble protein, and free amino acid concentrations were determined as previously described (Chapter 3).

RESULTS

No difference in dry matter production was found between flatpea plants supplied with nitrate and those whose nitrogen was acquired through symbiotic fixation. However, plants provided with both nitrate nitrogen and symbiotically fixed nitrogen showed a 31.8% increase in total biomass (Fig. 6-1). Shoot length was not affected by the treatments (Fig. 6-2a). Plants whose nitrogen requirement was met solely through biological nitrogen fixation had significantly fewer lateral shoots than did nodulated or noninoculated plants provided with nitrate (Fig. 6-2b). As expected, nitrogen fixation did not occur in noninoculated plants (Fig. 6-2c). An 80% decline in nitrogen fixation was observed in inoculated plants supplemented with nitrate.

Nitrogen concentration was the highest in the flatpea leaves and the lowest in the stems (Fig. 6-3a). Noninoculated, nitrate-fed plants showed slightly higher nitrogen levels than did nodulated, nitrate-fed plants. Nitrogen concentrations in leaves, stems, and roots of nodulated plants with no inorganic nitrogen supply were only 73.1%, 65.9%, and 82.0%, respectively, of the average of those determined for the nitrate-fed plants whether nodulated or noninoculated, .

The highest soluble protein concentration was found in the leaves of noninoculated plants fed with nitrate (Fig. 6-3b). Plants whose nitrogen was derived solely by symbiotic fixation showed a

significantly lower level of soluble protein in leaves and stems and a slightly higher soluble protein concentration in the roots compared to the corresponding tissues from the other two treatments.

The concentration of total free amino acids (sum of the concentrations of the 14 free amino acids quantified) was similar among tissues of nodulated plants not supplied with inorganic nitrogen (Fig. 6-3c). Amino acid distribution among the tissues of both nodulated and noninoculated plants supplied with nitrate occurred in the following sequence: leaf > stem > root. Free amino acid levels in plants from these two treatments were significantly higher, especially in the shoots, than that in plants whose nitrogen was supplied only by biological fixation. Nodulated plants supplied with nitrate had the highest free amino acid level in the leaves and roots compared to plants from the other two treatments.

Fourteen free amino acids were quantified in flatpea plants. A_2bu, Abu, and Hse were found at very high levels, accounting for 78.9%, 95.1%, and 46.4% of the total free amino acids in the leaves, stems, and roots, respectively. In general, the distribution among the tissues and the response of the three nonprotein amino acids to the nitrogen-source treatments (Fig. 6-4) was similar to the pattern of the total free amino acids (Fig. 6-3c). However, there were differences among the three nonprotein amino acids (Fig. 6-4). A_2bu and Hse accumulated to a greater extent in the leaves, while Abu was higher in the stems than in the leaves and roots. Six protein amino acids, Ala, Ser, Val, Phe, Ile, and Leu, were detected at low

concentration (< 1 mg/g DW) and exhibited similar distributions and responses to the treatments as the total free amino acids and nonprotein amino acids (data not shown). Asn, Asp, Gln, Glu, and Arg were the major free protein amino acids in the roots of nodulated plants grown in the absence of nitrate, but they occurred at low levels in the shoots (Fig. 6-4). With the exception of Arg, these amino acids were also major components in roots of nodulated and inoculated plants supplied with nitrate. Relatively high levels of Asn, Asp, Gln, Glu, and Arg were also detected in the leaves of these plants. In general, concentrations of free protein amino acids were the highest in nodulated plants supplemented with nitrate. Plants whose nitrogen was derived only from nitrate exhibited the next highest concentrations. This trend was especially true for Arg and Asn in the leaves and Asn and Asp in the roots. Although concentrations of free amino acids in nodulated plants not supplied with inorganic nitrogen were, in most cases, lower than those of nodulated and noninoculated plants supplied with nitrate, levels of Arg and Gln were significantly higher in the roots of nodulated, nitrate-deprived plants.

DISCUSSIONS

Compared to flatpea plants whose nitrogen requirement was satisfied either partially or fully by nitrate provided in the nutrient solution, plants which were totally dependent upon biological nitrogen fixation exhibited significantly lower concentrations of soluble protein and free amino acids in their leaves and stems. The decreases were highly correlated with declines in nitrogen concentration (Fig. 6-3a, Table 6-1). The average nitrogen concentration of nodulated plants in the absence of added nitrate (3.49%) was approximately two thirds that of nodulated and noninoculated supplied with nitrate (4.55% and 4.70%, respectively). These results differ from those of Duc et al. (1979) who reported that nodulated mung bean (Phaseolus aureus L.) and vigna (Vigna catjang Walp.) had total nitrogen concentrations that were 23 to 38% higher than those of corresponding plants supplemented with inorganic nitrogen. This nitrogen increase was accompanied by a significant increase in amino acid levels. Van Beusichem (1983) reported that dinitrogen-fixing soybean plants had a nitrogen concentration in the xylem that was 1.7 times higher than that in nitrate-fed plants. These differences in experimental observations suggest that nitrogen fixation in the previous studies may have been sufficient to maintain nitrogen metabolism at a high level. In our study, the amount of nitrogen obtained by nodulated flatpea plants through symbiotic nitrogen fixation alone was less

Table 6-1. Correlation coefficients (r) between nitrogen concentration and the concentrations of nitrogenous compounds in flatpea.

Tissue		Soluble protein	Total amino acids[a]	A_2bu	Abu	Hse
Leaf	r	0.908	0.929	0.848	0.868	0.728
	Significant Probability	0.0007	0.0003	0.0034	0.0024	0.0261
Stem	r	0.901	0.926	0.954	0.725	0.707
	Significant Probability	0.0009	0.0003	0.0001	0.0030	0.0332
Root	r	-0.524	0.344	0.407	0.604	0.405
	Significant Probability	0.1472	0.3624	0.2776	0.0850	0.2800

[a]Total free amino acid concentration is defined as the sum of the concentrations of fourteen free amino acids quantified.

than that assimilated by plants provided with nitrate. This observation is consistent with results reported by McNeil and LaRue (1984). These scientists determined that soybean yields from plants provided with $^{15}N_2$, $^{15}NH_4^+$, and $^{15}NO_3^-$ depended only on the total amount of nitrogen incorporated into the plants and that distribution of nitrogen among different compounds did not depend on the form of nitrogen supplied.

Growth of nodulated flatpea plants with no nitrate supplement, as indicated by similar biomass accumulation and shoot lengthening (Figs. 6-1, 6-2a), was not substantially different from that of noninoculated, nitrate-fed plants. When nitrogen concentrations of nodulated soybean plants declined from 2.48% to 1.68% because of a limited nitrogen supply, the total dry matter production of soybean plants was decreased by 26.4% (Brevedan et al. 1977). These results suggest that flatpea may be more efficient in utilizing nitrogen. The apparent efficiency could be of particular importance if the species is used under nitrogen-limiting conditions.

Decreases in levels of nitrogenous compounds in the roots of nodulated plants grown in the absence of nitrate, relative to noninoculated, nitrate-fed plants, were not as large as in the shoots. In fact, soluble protein concentrations and the concentrations of some amino acids (Arg and Gln) were even higher in the roots of nodulated plants with no nitrate supply than in roots of noninoculated, nitrate-fed plants. This observation presumably reflects the influence of nitrogen status on the distribution of

tissue nitrogen. Roots, as the functional organ involved in nitrogen absorption from the soil, conserve a relatively large portion of nitrogen if the total nitrogen in plants is scarce. Under such conditions less nitrogen would be transported to the shoots.

Another change in free amino acid composition in flatpea due to nitrogen source was the variation in the ratio of A_2bu to Hse, the two major free amino acids in the leaves. Nodulated plants not supplied with nitrate had a much lower A_2bu/Hse ratio (0.44) than noninoculated, nitrate-fed plants (0.99). It seems that in the plant with a limited nitrogen supply, more nitrogen was stored as Hse. However, in the plants with higher levels of nitrogen, more A_2bu was accumulated.

In addition to the effects on dry matter production and nitrogen metabolism, a significant morphological change, relative to nitrogen source, was observed. The lower number of lateral shoot in the nodulated plants (Fig. 6-2b) may reflect the low nitrogen concentration in the plants. Decreased nitrogen concentrations in plants may affect levels of important nitrogen-containing growth hormones (auxins and cytokinins), thus influencing differentiation of lateral meristems with a subsequent reduction in stem number.

Inhibition of nitrogen fixation by nodulated legumes due to nitrate supplementation of the culture solution was consistent with other studies (Dixon, 1969). The growth of flatpea was promoted when both biologically-fixed and inorganic nitrogen sources were available (Fig. 6-1). The combination of the two nitrogen sources

also resulted in greater accumulation of nitrogenous compounds, as shown by high concentrations of free amino acids in the leaves (A_2bu, Asn, Arg) and roots (A_2bu, Asn, Asp). When the data from this study are expressed on a whole plant basis, instead of a dry matter basis, the concentrations of nitrogen, soluble protein, total free amino acids, and individual amino acids are much higher in nodulated plants supplemented with nitrate than in the plants dependent upon either biological nitrogen fixation or inorganic nitrogen in the form of nitrate. Similar effects of combined nitrogen sources on the growth and nitrogen metabolism of soybeans have been reported (Zengbe et al., 1984). Provision of inorganic nitrogen decreased nodulation and nitrogen fixation in soybeans, but increased amino acid concentrations in the xylem sap (Zengbe et al., 1984). In another study, application of nitrogen fertilizer improved not only the yield of nodulated soybeans but also significantly increased the amino acid concentrations in the seeds (Mareckova and Sykora, 1980).

No significant qualitative difference in major free amino acid profiles was observed between the noninoculated, nitrate-fed plants and nodulated plants not supplemented with nitrate. A_2bu and other nonprotein amino acids were consistently the major components. However, since N_2 and NO_3^- are metabolized through different enzymatic pathways, the relative concentrations of some amino acids, which may be either products of early synthetic steps or active transport forms, will change when plants are supplied with different

sources of nitrogen. It has been reported that concentrations of Asn and Met in alfalfa (Medicago sativa L.) increased in urea-fed plants relative to nodulated plants (Strzelec, 1972). Applied nitrogen also changed the distribution of nitrogen among individual amino acids in alfalfa plants (Vance et al., 1983): addition of nitrate appeared to reduce the concentrations of Asn and Ala but increase that of Asp. In our experiments, the high levels of Arg in the roots of nodulated plants not supplied with nitrate and Asn and Arg in the leaves of nodulated plants supplemented with nitrate are particularly interesting. More detailed studies of amino acid metabolism in plants grown on different nitrogen sources are essential to explain these differences.

Comparison between the plants supplied with inorganic nitrogen and those with symbiotically-fixed nitrogen showed that concentrations of nitrogen, total free amino acid, A_2bu and other major free amino acids in flatpea were basically determined by the amount of nitrogen assimilated by the plant but not determined by the nitrogen source (Table 6-1), as long as the source was not toxic to the plants (Chapter 5). Symbiotic association between flatpea and rhizobia for nitrogen fixation appears to be neither essential nor inhibitory to the production and accumulation of nonprotein amino acids.

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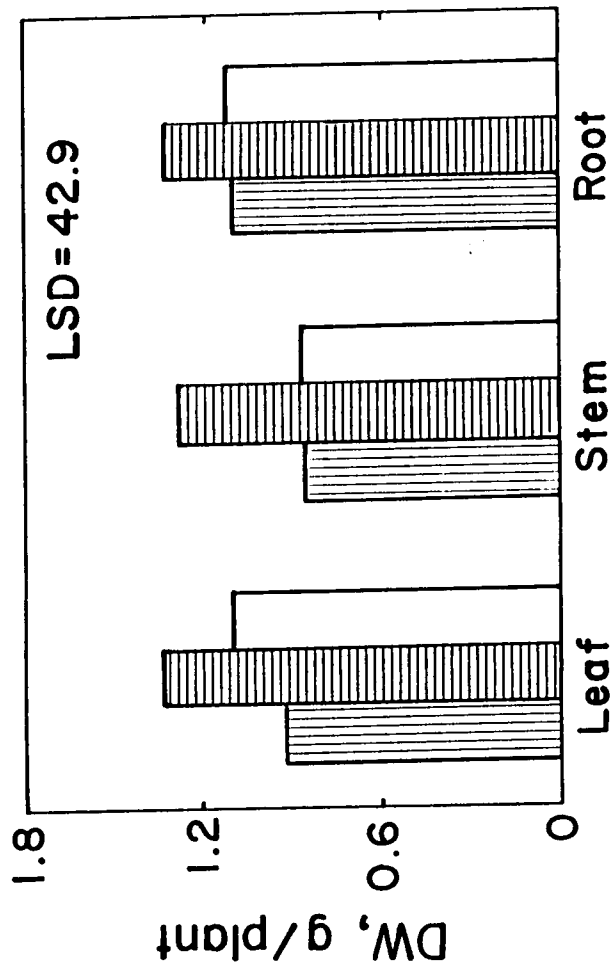
FIGURE LEGENDS

Fig. 6-1. Biomass of nodulated (vertically-lined bars); nodulated, nitrate-fed (horizontally-lined bars); and noninoculated, nitrate-fed (unshaded bars) flatpea plants.

Fig. 6-2. Shoot length (A), stem number (B) and acetylene reduction (C) of flatpea plants inoculated with rhizobia (+R, -N), supplied with 2 meq L^{-1} nitrate (-R, +N) or both (+R, +N).

Fig. 6-3. Nitrogen (A), soluble protein (B), and free amino acids (C) of flatpea tissues. Bar patterns as defined for Fig. 6-1.

Fig. 6-4. Major free amino acids in flatpea tissues. Bar patterns as defined for Fig. 6-1.



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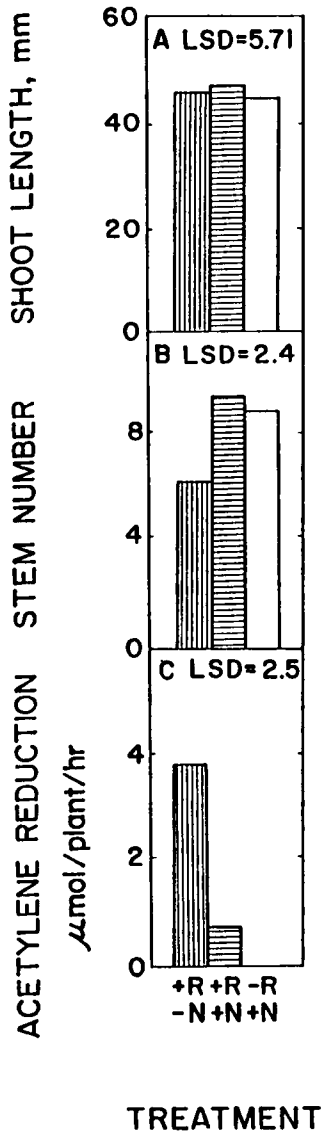

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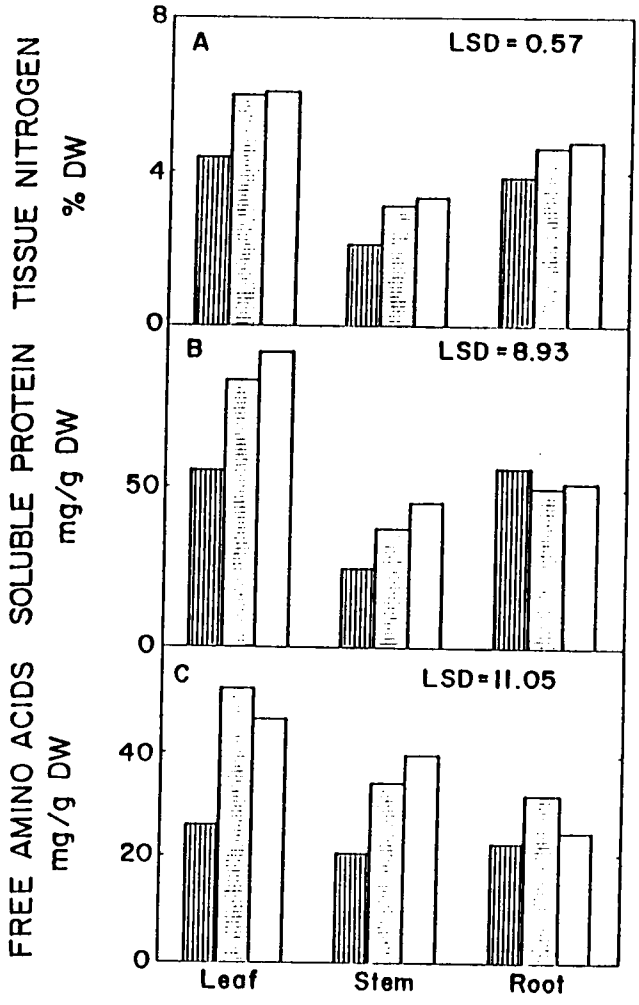

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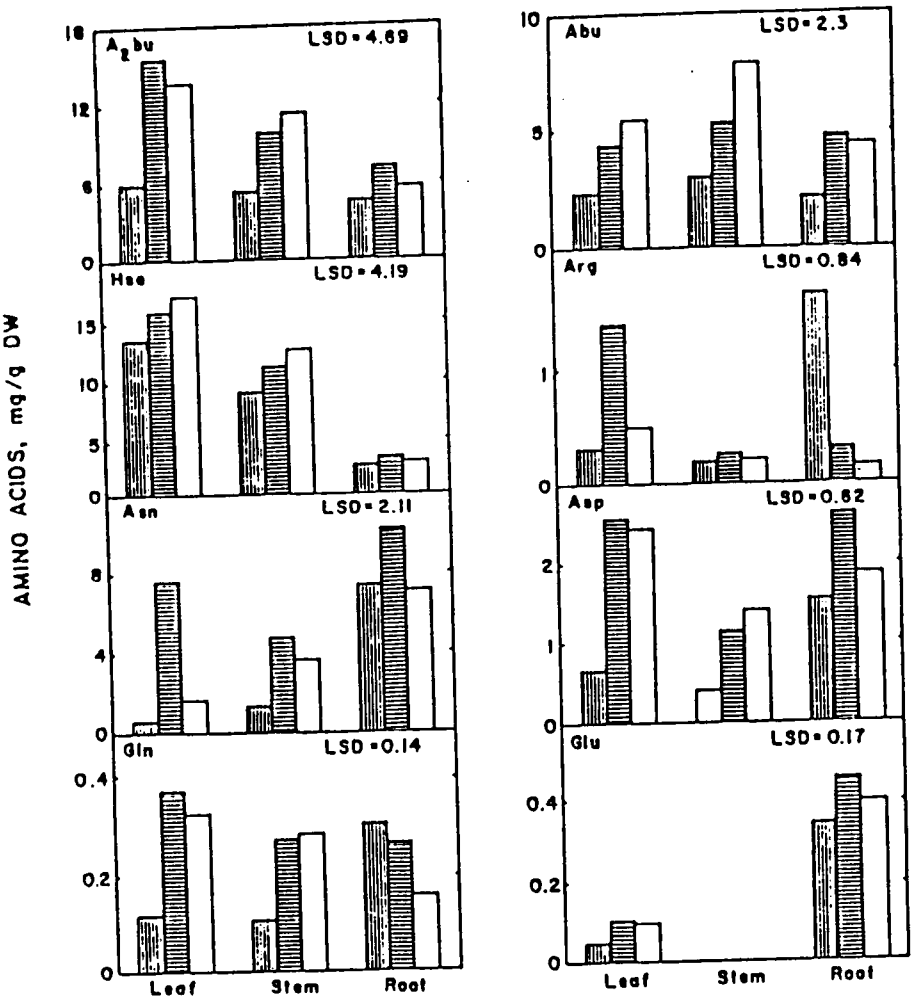

Fig. 6-4. Major free amino acids in flatpea tissues. Bar patterns as defined in Fig. 1.

CHAPTER VII. SUMMARY AND CONCLUSIONS

Influences of developmental stage, nitrogen sources, and water status on growth and nitrogen metabolism, with an emphasis on the composition of free amino acids, were studied in a series of experiments. General growth responses have been presented and discussed in the previous chapters. The discussion here will stress the response of nonprotein amino acids, especially A_2bu, to alterations in developmental status and environmental conditions.

A_2bu generally comprised 20 to 40% of the total free amino acids. In some cases, as much as 70% of the free amino acids was A_2bu (Chapter 5). Concentrations of A_2bu varied with the experimental factors employed.

Water-deficit stress increased A_2bu concentrations in flatpea (Chapters 3 and 4). Levels of A_2bu were dependent on plant age and severity of the stress. In older plants and under shorter duration of stresses, leaves and stems showed a greater accumulation of A_2bu than the roots and no change in nitrogen or soluble protein (Chapter 4). In younger plants subjected to severe drought, more A_2bu accumulated in the roots, coupled with a decrease in soluble protein and an increase in total nitrogen (Chapter 3).

Nitrogen also affected the A_2bu concentrations. High nitrogen availability significantly increased A_2bu concentrations in flatpea, a response that was accompanied by an increase in concentrations of soluble protein and other nitrogenous compounds (Chapter 6).

Application of nitrate nitrogen to flatpea plants ineffectively infected by Rhizobium increased A_2bu concentrations in leaves by 164%. At the same time, leaf concentrations of total nitrogen and soluble protein were increased by 51% and 36%, respectively (Chapter 6). Concentrations of inorganic nitrogen ions in hydroponic media were linearly correlated with the A_2bu concentrations in flatpea roots (Chapter 5). When the nitrogen supply was low, A_2bu was the only amino acid to decline dramatically (Chapter 5). Root nodulation seemed to have no effect on the production of A_2bu (Chapter 6). Ammonium was toxic to flatpea (Chapter 5). As observed with many other species (Bennett et al., 1964; Warncke and Barber, 1973; Gaffaney et al., 1982; and Ganmore-Neumann and Kafkafi, 1983), flatpea apparently could not incorporate ammonium effectively into structural components, even under a pH-controlled condition. Along with the typical symptoms associated with ammonium toxicity, A_2bu increased in flatpea as a result of ammonium fertilization (Chapter 5). Elevation of A_2bu in the leaves and stems of ammonium-fed flatpea was accompanied by a decrease in soluble protein. A_2bu accumulation, therefore, seemed to be connected with protein degradation or an inhibition of protein synthesis in plants repressed by ammonium. In the roots of plants fed with ammonium, increases in A_2bu were associated with an increase in soluble protein. Soluble protein production in response to ammonium fertilization may reflect the synthesis of enzymes related to nonprotein amino acid synthesis or ammonium

detoxification.

Concentrations of A_2bu in flatpea tissues did not change as dramatically as Abu and Asn during a 22-week observation (Chapter 4). However, A_2bu concentration in the leaves and stems tended to increase with plant age, and reached a peak value when plants flowered (Chapter 4). The variation in A_2bu concentrations with age may reflect changes in developmental stages, because the change in A_2bu accumulation coincided with a transition of growth from a vegetative state to a reproductive stage, similar to the observations with other plants (Matsumoto et al. 1979; Culianez et al. 1981; Madhusudanan and Nandakumar 1982; Watnabe and Ishigaki 1982). Increased concentration of A_2bu may be genetically controlled and/or related to the aging process as has been determined for major storage amino acids in other plant species (Noguchi et al. 1964; Lahdesmaki 1968; Lekhak and Sen 1981). Another possible explanation for the increased accumulation of A_2bu in older plants may be related to the greater uptake of nitrogen by older, larger plants, as indicated by the parallel response of nitrogen concentrations and A_2bu concentration with plant age (Chapter 4).

A generalization can be drawn about A_2bu accumulation in flatpea as influenced by the factors examined. The nonprotein amino acid appears to function as a nitrogen storage compound in the legume when the plant is stressed. Stresses such as drought, ammonium toxicity, and aging may promote degradation and/or inhibit synthesis of nitrogen-containing macromolecules. Under those conditions,

nitrogen could not be effectively incorporated into structural nitrogenous components and thus A_2bu could accumulate. On the other hand, when the nitrogen supply was limited, A_2bu level showed a substantial decrease (Chapter 6), a response that reflects a depletion of the storage nitrogen pool. Why A_2bu accumulates to a high level, even in unstressed plants, is still unknown.

A_2bu was not the only major amino acid whose concentration in flatpea changed in response to the developmental and environmental conditions. Nevertheless, most of the amino acids that did change, including A_2bu, were four carbon compounds that may be related to Asn metabolism. Of the three major nonprotein amino acids detected in flatpea (A_2bu, Abu, and Hse), Abu increased consistently in the stem with plant age (Chapter 4). Under water-deficit stress, Abu accumulation accounted for 30 to 50% of the amino acid concentration in the stems (Chapter 4). Abu accumulated to the greatest extent in the roots in response to exogenous ammonium (Chapter 5). However, concentration of Abu was not affected by the nitrogen levels in hydroponic culture solutions. In summary, Abu is the major amino acid that accumulates in flatpea, especially in the stems and roots, in response to water stress or ammonium toxicity. Physiological functions of the accumulation need to be studied. Concentrations of another major free amino acid, Hse, showed a relatively stable level with changes in age, nitrogen supply, and water status.

In flatpea roots, Asn was the most abundant amino acid among 15 quantified (Chapters 4 and 6). Root concentrations of Asn increased

significantly with plant age (Chapter 4) and increased linearly with the concentration of ammonium present in the growth medium (Chapter 5). Asn concentration increased in the roots of drought-stressed plants, especially for the young plants under severe stress (Chapter 3). Asn may be a primary compound in the assimilation and transport of nitrogen in flatpea, as in many other species (Simola, 1968). Asn is a precursor for the biosynthesis of A_2bu and Hse in flatpea (Nigam and Ressler, 1964). Abu and Asn might also be related through succinate which has been shown to be a precursor for Abu synthesis in Pseudomonas (Bachrach, 1960). Therefore, in stressed flatpea plants, Asn may be an intermediate for the production and accumulation of A_2bu and other nonprotein amino acids.

Relative amounts of the four major free amino acids (A_2bu, Abu, Hse, and Asn) in flatpea, changed with respect to plant organs and experimental treatments. If expressed as ratios, the resulting values suggest metabolic relationships. For example, the ratios of Abu/A_2bu and Asn/Hse were higher in the roots than in the shoots and tended to increase in response to water-deficit stress (Chapter 3). These results suggested that Asn and Abu in the roots may be precursors for the biosynthesis of A_2bu and Hse in the leaves.

The other 11 amino acids quantified in flatpea tissues were usually lower in concentration than A_2bu, Abu, Hse, and Asn and changed less relative to differences in the developmental and environmental conditions studied, with the exception that Pro and Arg did show specific responses to the treatment conditions

(Chapters 3-6). The physiological significance of these changes has been discussed in the previous chapters.

Lateral growth of flatpea was suppressed when nitrogen was limiting (Chapter 6) or when toxic levels of ammonium were used (Chapter 5). Growth hormone levels may be involved in such responses since several growth substances (indoleacetic acid, cytokinins, and ethylene) are either derived from amino acids or contain nitrogen in their structures. Further studies are necessary to verify these conclusions.

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