Title Water deficit responses of non-nodulated and nodulated *vica faba* (broad bean) when supplied with various forms on concentrations of medium nitrogen nutrition

Name Victoria B McCabe

WATER DEFICIT RESPONSES OF NON-NODULATED AND NODULATED *Vicia faba* (BROAD BEAN) WHEN SUPPLIED WITH VARIOUS FORMS AND CONCENTRATIONS OF MEDIUM NITROGEN NUTRITION

BY

Victoria B. McCabe (B.Sc.)

A Thesis submitted to the Faculty of Science, Technology & Design,

University of Luton,

in partial fulfilment of the requirements

for the Degree of

Doctor of Philosophy

March, 2000.

ABSTRACT

V. faba fixes nitrogen effectively (Richards & Soper, 1979), however nitrogen fixation is reportedly energetically expensive and water deficit sensitive. Research was designed to determine whether medium nitrogen applications would result in increased productivities in *V. faba*, particularly during water deficits. Non-nodulated and nodulated *V. faba* were subjected to gradual water deficit imposition, and were supplied with a variety of medium nitrogen nutrition. Nitrogen fixing *V. faba* exhibited greater productivities than *V. faba* which were supplied with low medium nitrate concentrations (0.8 mM N), even during water deficits.

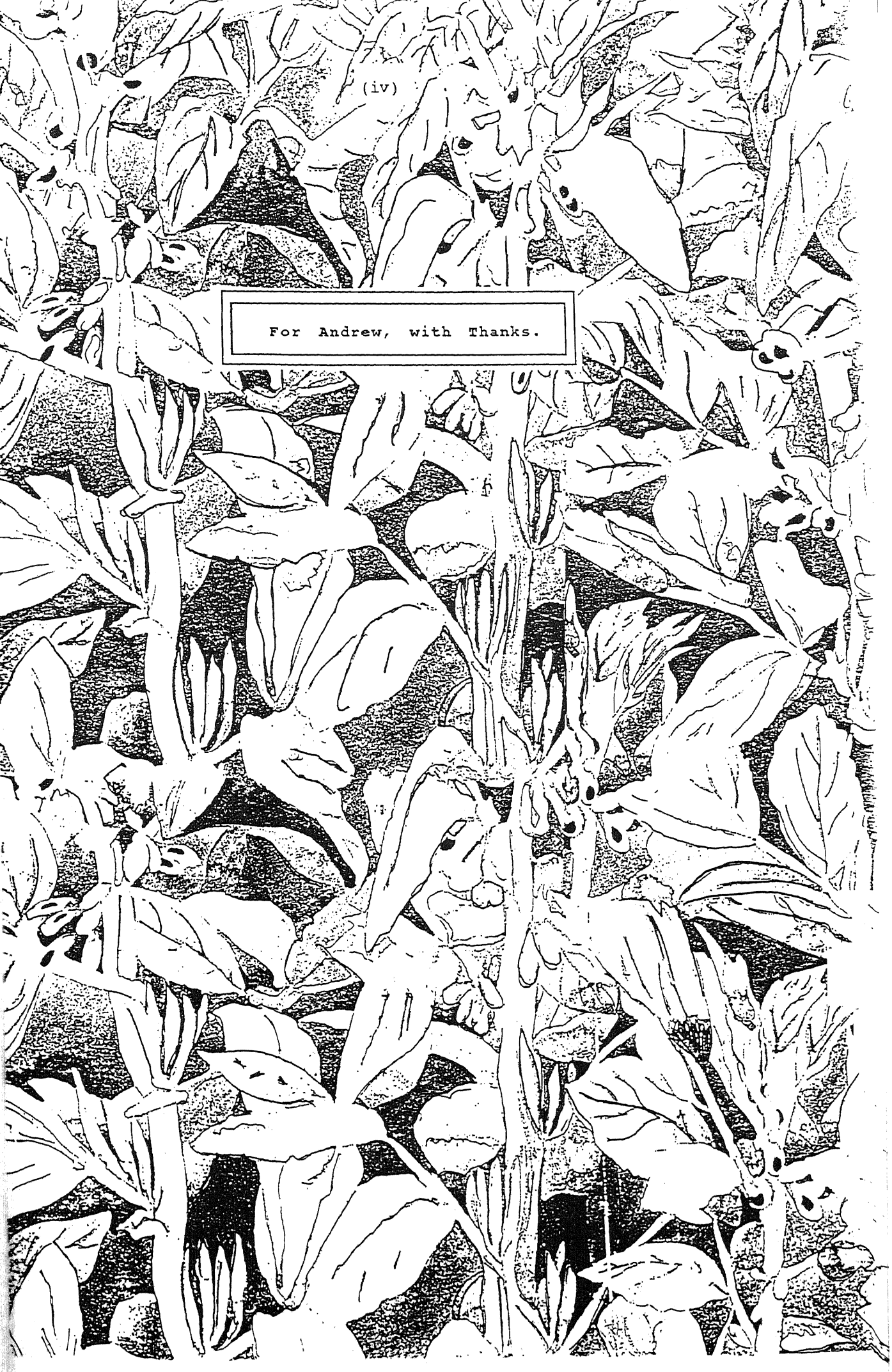
Plant performance parameters (growth; net photosynthesis; nitrogen assimilatory enzyme activities; osmotic adjustment) were greater in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits, inferring water deficit tolerance for nitrogen fixation. However significantly greater plant performance paramaters were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N) than when reliant on nitrogen fixation.

In contrast to the bulk of previous literature, NR activities were maintained in *V. faba* until water deficits became severe, inferring a role for nitrate assimilation in nitrogenous osmotica production.

Medium ammonia additions resulted in the exhibition of significantly increased root biomasses; cumulative leaf areas (important for a green manure crop); heights; and nitrogen assimilation in *V. faba* throughout water deficits, and accordingly in increased osmotic adjustment (including compatible solute accumulation), protein concentrations and vegetative yields. Greater plant productivities in *V. faba* when supplied with medium ammonia additions were attributed in part to lower associated assimilatory

costs for ammonia than nitrate nutrition (Raven, 1992). Results indicated increased metabolism as opposed to storage of medium ammonia, and therefore potentially alleviated 'sink size' feedback inhibition of photosynthesis and nitrogen metabolism in *V. faba* when supplied with medium ammonia additions. Furthermore ammonia supplied *V. faba* may have been predisposed towards water deficit tolerance.

In summary *V. faba* exhibited significantly greater nitrogen assimilation; osmotic adjustment; net photosynthesis; and growth when supplied with increasingly concentrated medium nitrogen nutrition (and particularly with medium ammonia additions) than when reliant on nitrogen fixation, both during periods of adequate irrigation and during water deficits.



For Andrew, with Thanks.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

Acknowledgements are due to Dr. Phil Ladley
for his inspiration and help throughout.

The proof-reading skills of Dr. Graham Steele and Mr. Peter Keay are gratefully acknowledged, as are Dr. Mike Batham's help with GC analyses and Mr. Chris Shoostarian's advice re. statistical analyses. Acknowledgements are also due to Dr. John Pearson.

Thanks also to Mr. David Orwin, particularly for the consideration shown to me upon my arrival in Luton.

Finally, a massive 'Thank-You' to my partner Andrew
for your support throughout,
and acknowledgements to Mr. Jack Hudson.

DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Luton. It has not been submitted before for any degree or examination at any other University.

VICTORIA McCABE

26 th day of March, 2000.

ABBREVIATIONS

ANOVA - Analysis of Variance

CLA(s) - Cumulative Leaf Area(s)

C:N - Carbon : Nitrogen Ratio

cv. - Cultivar

DW(s) - Dry Weight(s)

FW(s) - Fresh Weight(s)

GDH (A) - Glutamate Dehydrogenase (Activity)

GOGAT - Glutamate synthase

GS (A) - Glutamine synthetase (Activity)

HDH - Homoserine Dehydrogenase

I.D. - Internal Diameter

Intro. - Introduction

LA - Long Ashton

LAR(s) - Leaf Area Ratio(s)

LEA(s) - Late Embryogenesis Abundant Proteins

NAR(s) - Net Assimilation Rate(s)

Nase - Nitrogenase

N:C - Nitrogen : Carbon Ratio

N-fixation - Nitrogen Fixation

NR (A) - Nitrate Reductase (Activity)

NiR - Nitrite Reductase

NUE - Nitrogen Use Efficiency

NUR(s) - Net Uptake Rate(s)

OD - Optical Density

O.D. - Outer Diameter

PAR - Photosynthetically Active Radiation

PEPcase - Phosphoenolpyruvate carboxylase

PFK(A) - Phosphofructokinase (Activity)

RGR(s) - Relative Growth Rate(s)

R:S(s) - Root : Shoot ratio(s)

RUBP - Ribulose bisphosphate

RWC(s) - Relative Water Content(s)

ABBREVIATIONS

ANOVA - Analysis of Variance

CLA(s) - Cumulative Leaf Area(s)

C:N - Carbon : Nitrogen Ratio

cv. - Cultivar

DW(s) - Dry Weight(s)

FW(s) - Fresh Weight(s)

GDH (A) - Glutamate Dehydrogenase (Activity)

GOGAT - Glutamate synthase

GS (A) - Glutamine synthetase (Activity)

HDH - Homoserine Dehydrogenase

I.D. - Internal Diameter

Intro. - Introduction

LA - Long Ashton

LAR(s) - Leaf Area Ratio(s)

LEA(s) - Late Embryogenesis Abundant Proteins

NAR(s) - Net Assimilation Rate(s)

Nase - Nitrogenase

N:C - Nitrogen : Carbon Ratio

N-fixation - Nitrogen Fixation

NR (A) - Nitrate Reductase (Activity)

NiR - Nitrite Reductase

NUE - Nitrogen Use Efficiency

NUR(s) - Net Uptake Rate(s)

OD - Optical Density

O.D. - Outer Diameter

PAR - Photosynthetically Active Radiation

PEPcase - Phosphoenolpyruvate carboxylase

PFK(A) - Phosphofructokinase (Activity)

RGR(s) - Relative Growth Rate(s)

R:S(s) - Root : Shoot ratio(s)

RUBP - Ribulose bisphosphate

RWC(s) - Relative Water Content(s)

CHAPTER ONE

INTRODUCTION

> If it is assumed that 10% (or some such value) of the energy in a diet should come from good quality digestible protein, and that because of uneven distribution within the family and community, more protein than that amount should be available, it is clear that protein concentrates are needed. Wheat or potatoes will not suffice - especially if some fat and sugar is being eaten. Special attention must therefore be attached to foods that contain more than 20% protein. That excludes all the cereals at present on the market (Pirie, 1979).

Over 20,000 species of *Leguminosae* are known, the majority of which can fix atmospheric nitrogen, however less than fifty are exploited agriculturally, and only five are regularly utilised to upgrade soil nitrogen status (Postgate, 1987).

Vicia faba is the oldest cultivated bean (Mairs, 1996), and is associated with the Mediterranean, the suspected centre of diversity of the species, however a wild form of *V. faba* has not been identified to date (Zohary, 1977). As the first foods to be easily stored, pulses changed the nature of civilisation; carbonised pulses found in Neolithic villages in the Middle East are dated as 8-9000 years old (Simpson & Ogarzaly, 1995). It has even been claimed that the pyramids were built on faba beans (Darling, 1982). Early cultivation by Egyptians, Greeks, and Romans, led to the world-wide spread of *V. faba* via Asia. Historically important, the second governor of Columbia brought *V. faba* with him from Spain to America in 1543. Theft of *V. faba* from fields once warranted the death penalty, and the historical value of *V. faba* is

highlighted by tradition, such as the belief that broad beans offered in marriage ensure the birth of a son (Mairs, 1996).

V. faba cultivation is often associated with cool climates (Kizirian & Taha, 1997), however *V. faba* is widely grown as a winter crop in sub-tropical countries (Saxena, 1982). China produces around 60% of the total world yield of *V. faba* (Cockbain, 1981), see table 1.1.

Table 1.1 TOP PRODUCERS OF *V. faba*.

Country	1,000 MT (metric tonnes)
China	2500
Egypt	390
Ethiopia	282
Italy	141
Australia	100

(Simpson & Ogarzaly, (1995), after FAO Production Year-book, for 1992, vol. 46, 1993. FAO, Rome).

V. faba is also grown in many European countries, see table 1.2.

Table 1.2 Area grown, average yield, and production of dried *V. faba* in Europe, (1980).

	Area Grown (1000 ha)	Average Yield t/ha	Production 1000t
Czechoslovakia	41	1.8	72
France	20	3.2	64
Germany (reunited)	10	5.4	27
Greece	11	1.4	15
Italy	172	1.3	228
Portugal	35	0.5	16
Spain	84	1.2	104
UK	46	3.1	149
Europe Total	374	1.4	529
World Total	6980	0.9	6709

Source: FAO, 1980 (Hebblethwaite *et al*, 1984).

In marginal and low input areas in the Middle East, Asia, and Africa *V. faba* is grown as a staple food source, providing the main ingredient in the popular dishes foul and falafel, and in salad. 150,000 metric tonnes per year of

(mostly dried) pulses are imported to the Gulf Cooperation Council alone (includes Bahrain, Kuwait, Oman, Qatar, and United Arab Emirates) at a value of $90 million *per annum* (Kizirian & Taha, 1997). In contrast to other legumes (including *Glycine max*) toxin constituents are low (Frauen *et al,* 1981), and lectins in *V. faba* reportedly reduce the progress of cancer (Comber, 1999). *V. faba* is also utilised as a green manure (Corak *et al,* 1992), upgrading soil nitrogen status for subsequent crops in rotation.

In temperate northern areas of Europe and the USA *V. faba* is utilised as animal fodder (sometimes harvested immature allowing biannual silage; Faulkner & Steen, 1984), and also as a food crop.

The versatility and thus the widespread cultivation of *V. faba* reflects the capacity of *V. faba* to yield a high protein product per unit ground area (see table 1.3). The average (whole plant) crude protein content of *V. faba* is sixteen per cent (Falisse *et al,* 1984), (*G. max* has forty per cent protein; Simpson & Ogarzaly, 1995).

Table 1.3 Kg crude protein per unit support energy and fertiliser nitrogen for some UK crops.

	kg crude protein/ kg fertiliser N	kg crude protein/ 103MJ support energy
Wheat	4.8	30
Barley	4.6	26
Grass (UK average)	4.9	40
Rapeseed	2.2	19
V.faba	30.0	74

Data from J.C.O. (1976), (Hebblethwaite *et al*, 1984).

V. faba contains several amino acids at comparable concentrations to steak, the traditional symbol of a complete protein, and is a much more concentrated source of amino acids than whole cows' milk (Simpson & Ogarzaly, 1995). The EEC commission on plant productivity has reported that *V. faba* represents one

of the best indigenous (Western Europe) plant proteins (Griffiths, 1981), with a long term future as a rival to peas.

During and immediately following World War II up to 110 000 hectares of *V. faba* were cultivated in the UK, predominantly for utilisation as a protein-rich livestock feed (Lawes *et al*, 1980). However this dwindled to around 50 000 hectares by the 1980s, perhaps in part because *V. faba* is often characterised by large unpredictable yield variations (Bond, 1977; Dekhuijzen *et al*, 1980). The average UK yield of *V. faba* can reportedly vary from one to six tonnes per hectare in different years on the same farm with the same management (Lawes, 1980).

Nitrogen is an essential plant macro-nutrient; increasingly concentrated medium nitrogen nutrition reportedly results in increases in plant aerial growth; leaf expansion; individual leaf areas; stem elongation; and reductions in root growth, (roots need not 'nitrogen seek', Marschner, 1986). Furthermore nitrogen is an essential constituent of the enzymes which ultimately control plant growth; cell replication; metabolic reactions etc. In addition to 'roles' in an array of metabolic reactions, nitrogen is a constituent of amino acids and thus of protein, the content of which influences the nutritional value of crops (Pirie, 1979).

Fig. 1.1 illustrates that nitrogen is available to *V. faba* as atmospheric nitrogen via nitrogen fixation, or from the medium in the form of nitrate and/or ammonia. The enzymes which assimilate these potential nitrogen sources, and the reported water deficit tolerance of these enzymes are also given in fig. 1.1.

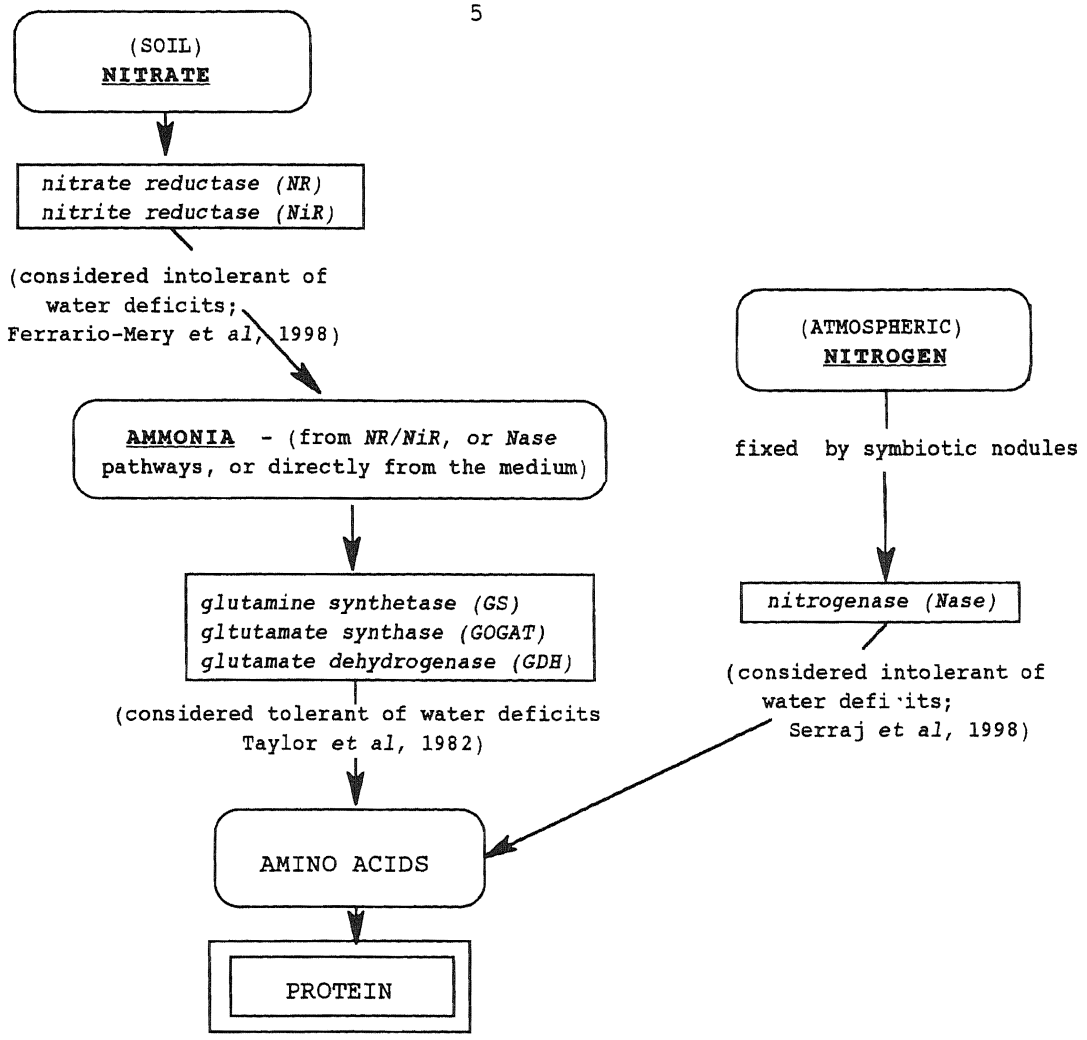


Fig. 1.1 Potential nitrogen sources of *V. faba*, with enzymes of assimilation (given in italics) and accepted water deficit tolerance of these enzymes (given in brackets).

Soil nitrate is reportedly the most common nitrogen source utilized by plants (Marschner, 1986). NR was discovered by Evans & Nason, (1953); is the first enzyme in nitrate assimilation; and is probably rate-limiting due to its small concentration (Campbell, 1988b). The nia gene (which encodes NR) is reportedly expressed early during root meristem formation (Vuylsteker *et al*, 1998), highlighting the importance of reduced nitrogen availability to plant metabolism. Prior to maximum rapid root NR induction some free nitrate escapes to the shoot where NR is quickly induced (Wallace & Pate, 1965).

Increasingly concentrated medium nitrate nutrition may reportedly result in increased plant nitrate uptake (possibly through induced synthesis of nitrate carriers, or because increasingly concentrated nitrogen nutrition reportedly results in increased root and shoot growth; Ourry *et al*, 1995); and in increased nitrate reductase activities (Shaner & Boyer, 1976a, & b). Furthermore *V. faba* reportedly exhibits greater aerial growth (Sprent & Thomas, 1984), and maintains greater cumulative leaf areas (CLAs; Hebblethwaite *et al*, 1984), when supplied with increasingly concentrated medium nitrate nutrition; these parameters being reportedly strongly correlated with dry matter yield and seed matter yield in *V. faba* (Hebblethwaite *et al*, 1984).

Ammonia may also provide plants with a nitrogen source (Raven *et al*, 1992). Although potentially toxic, in the soil ammonia is quickly converted to ammonium (Haynes & Goh, 1978). Ammonium (a soluble ion) may accumulate in abundance in many soils so that the medium pH rises into the alkaline range. Ammonium may then be converted into gaseous ammonia which is lost from the soil. Furthermore medium acidification may reportedly result from plant ammonia assimilation, and may reportedly result in growth rate restrictions; wilting; leaf expansion and photosynthetic rate decreases; marginal necrosis; interveinal chlorosis of terminal leaves; stem and leaf lesions; inhibited water uptake; and decreased leaf water potentials (Pill & Lambeth, 1977; Tolley-Henry & Raper, 1986), and sometimes ultimately in plant death (Maynard & Barker, 1969), in other plant species.

However the media pH was measured throughout the experiments conducted in this research, and remained between 6.5 and 7.5, indicating that neither

increases in media alkalinities (due to ammonium accumulation), nor media acidification (due to ammonia assimilation) were significant. Regulation of the medium pH (around neutral) reportedly results in equal growth in other plant species whether supplied with equimolar nitrate or with ammonia nutrition (Kirkby & Hughes, 1970; Marschner & Romheld, 1983; Rufty *et al*, 1983; Raven, 1985; Trolley-Henry & Raper, 1986; Chaillou *et al*, 1991); thus many of the reported 'toxic' effects associated with ammonia nutrition may result from medium pH perturbations rather than from ammonia assimilation *per se*. Medium acidification is reportedly inhibitory for nitrogen absorption (Ryan & Walker, 1994), indicating that ammonia-associated 'abnormal' growth may result from nitrogen deficiency as opposed to from toxicity *per se*. Indeed symptoms associated with ammonia-toxicity are consistent with those of nitrogen deficiency (Rufty *et al*, 1984).

However other workers have reported that plant nitrate uptake is not suppressed by the inclusion of medium ammonia additions, and that other plant species may exhibit greater plant nitrate concentrations when supplied with ammonia as opposed to with nitrate nutrition (Orebamjo & Stewart, 1975a; Haynes & Goh, 1978). However increased nitrate concentrations could result from decreased NR activities, as reported in other plant species when supplied with medium ammonia nutrition (Breteler & Siegerist, 1984).

Yet other workers have reported that medium ammonia additions may stimulate plant NR activities (Hofstra *et al*, 1985; Bennet *et al*, 1986; Bungard *et al*, 1999), and increasingly concentrated ammonia nutrition reportedly results in increased plant GDH activities (Taylor & Havill, 1981), and GS activities (Ortega *et al*, 1999) in other plant species, and hence potentially in increased nitrogen assimilation.

Unsuitable experimental design may be partly responsible for reported ammonia-

associated toxic affects. A combination of high concentrations of media ammonia, with the low light levels found in many controlled environment cabinets can lead to high rates of ammonia absorption and low rates of ammonia assimilation, encouraging the accumulation of free ammonia (Bloom, 1988). However ammonia was provided as a supplement to nitrate in this research, and the concentrations of medium ammonia; pH; and photosynthetically active radiation (PAR) levels were controlled (see chapter two); accordingly toxicity symptoms were not expected when medium ammonia additions were supplied to *V. faba*. In the field molybdenum may be limiting (particularly in acid soils; Gutschick, 1981), and ammonium assimilation is the only form of nitrogen assimilation without a Mo requirement, an advantage *in vivo*.

V. faba may also utilise atmospheric nitrogen as a nitrogen source. In 1800, Humphry Davy suspected nitrogen fixing systems in plant roots; Boussingault realized that atmospheric N_2 was utilised by legumes in the 1830s; and nodules were described by Hellriegel & Wilfarth in 1886, (Burris, 1974).

V. faba infected with *Rhizobium leguminosarum* may form symbiotic nodules which are extremely effective at fixing atmospheric nitrogen; fixation reportedly accounts for over eighty per cent of the total plant nitrogen content in this species (Richards & Soper, 1979).

However nitrogen fixation is reportedly an energy intensive process (Pate *et al*, 1979). When carbon-limited populations of nitrogen fixing bacteria are compared with similar populations which are utilizing nitrate or ammonium ions as their nitrogen source, yields of the latter are often substantially higher (Pate *et al*, 1979; Schilling, 1983), inferring that ATP may be diverted away from biomass production to nitrogen fixation.

Accordingly host plants dramatically sever their associations with rhizobia when external nitrogen is plentiful, producing fewer curled root hairs and fewer effective nodules, both in *V. faba* (Dean & Clark, 1980), and in other plant species (Bauer, 1981; Caba *et al*, 1998). *Phaseolus vulgaris* reportedly exhibits reduced nitrogenase activities when supplied with medium nitrate; <200 ppm nitrate in the medium reportedly results in fifty per cent decreases in nitrogen fixation (Trinchard & Rigaud, 1981).

As NR activities and nitrogenase activities both rely on phloem translocated substrates (Oghoghorie & Pate, 1971) competition may occur. NR is reportedly activated in the shoots of *V. faba* when supplied with medium nitrate nutrition (Sutherland *et al*, 1985), and may therefore be better positioned to compete for substrates, which may contribute to the reported inhibition of nitrogen fixation in *V. faba* when supplied with medium nitrogen. Nitrogen fixation is also reportedly suppressed in other plant species when supplied with medium ammonia nutrition (Kennedy & Eady, 1979; Serraj *et al*, 1999), and feed-back inhibition mediated by increasing xylem amino acid concentrations has also been implicated as a possible cause of decreasing nitrogen fixation in plants when supplied with medium nitrogen nutrition (Baker *et al*, 1997).

Accordingly methodology was designed so that one group of *V. faba* was germinated in a *Rhizobia*-rich medium (see sections 2.3 & 2.4.2) and supplied with nutrition ranging from 'nitrogen-free' to 4.0 mM nitrogen, in order that the effects of increasingly concentrated medium nitrogen nutrition on nitrogen fixation could be determined.

Other *V. faba* seeds were surface-sterilised and germinated in *Rhizobia*-free media and were thus unable to fix atmospheric nitrogen (see section 2.3), and relied upon medium nitrogen nutrition (supplied to different groups as

increasingly concentrated nitrate solutions, with and without increasingly concentrated ammonia additions) in order that the effects of different forms and concentrations of medium nitrogen nutrition on the physiology of non-nodulated *V. faba* could be determined (see fig. 2.1 for an overview of the methodology).

The efficiency of utilisation of specific nitrogen sources may be species dependant. *T. pseudonana* exhibits the same specific growth rates whether supplied with nitrate or with ammonia nutrition (Thompson *et al*, 1989); however many photolithotrophs reportedly exhibit lower maximum growth rates when supplied with ammonia as opposed to with nitrate nutrition (even when the medium pH is controlled; Allen & Smith, 1986); while species 'preferring' ammonia and supplied with nitrate reportedly develop iron deficiencies (Nelson & Selby, 1974). However species already adapted towards the utilisation of a specific nitrogen source reportedly do not exhibit toxicity or deficiency symptoms when provided with their 'preferred' nitrogen source (Krajina *et al*, 1973), and the metabolic pathway of nitrate utilisation reportedly differs among species, which reportedly exhibit varying degrees of 'nitrophilia'.

Legumes reportedly exhibit lower km values (higher affinities) for ammonia than cereals, and translocate absorbed nitrogen to the shoots more quickly (Hogh-Jensen *et al*, 1997). As a legume *V. faba* is adapted to receive nitrogen from the roots via nitrogen fixation; via ammonia assimilation (which is a root phenomenon); or via nitrate reduction (which is also predominantly a root phenomenon in *V. faba*; Sutherland *et al*, 1985). Indeed similarities are noted when nitrogen fixation and ammonia assimilation are compared, for example xylem sap compositions are similar in ammonia supplied and in nitrogen fixing legumes (Baker *et al*, 1997), and ammonia assimilation and nitrogen fixation

both result in the production of H^+ ions (Raven, 1985). If the entire physiology of *V. faba* is predisposed towards root assimilation, medium ammonia additions may not result in the exhibition of toxicity symptoms in this leguminous species.

In addition to nitrogen, water is also fundamentally important to plant productivity (Sinha & Nicholas, 1981), driving expansive growth; providing support; forming the medium in which metabolic reactions occur; and acting as a reactant in photosynthesis, etc.

Water deficits are often labelled 'drought stress', a vague umbrella as the effects of such 'stress' on *V. faba* may vary as affected by many factors including the life cycle stage during which the water deficits are experienced, and the severity and duration of water deficit imposition (Plies-Balzer *et al*, 1995).

The term 'drought stress' when used in biology, has general connotations and not precise definitions (Osmond *et al*, 1987). Drought is described in the Collins English Dictionary as a 'prolonged period of scanty rainfall', while Katz & Glantz, (1977), suggested that there are meteorological and agricultural definitions of drought; meteorological drought being a time period of less than expected precipitation; agricultural drought referring to unseasonable vegetative development (in which case one day of dry, hot weather may be classed as 'drought'). Furthermore Palmer, (1974), suggested that a dry spell is not drought until the economy is affected, and Morris, (1974), defined drought as an unseasonably rapid rise in agricultural prices.

The definition of stress as an 'overpowering pressure of some adverse force or influence' (Shorter Oxford English Dictionary, 1983) is useful when describing the effects of 'drought stress' on plants (Jones *et al*, 1989). However while

Osmond *et al*, (1987), defined 'stress' as a factor which results in decreases in plant growth and reproduction below the genotype's potential, it is apparent that the term 'drought stress' is an unscientific description. Water deficit is the preferred term in this thesis. Water deficits occur when rates of transpiration exceed those of water uptake (Bray, 1997), and are characterised by alterations in the physiology and metabolism of plants (including *V. faba*) in efforts to maintain water uptake, and to conserve water contents and metabolism.

In Maharashtra (India) rainfall was twenty-five per cent below average in 1971. In 1972 it was forty per cent below average. Average pulse/cereal crop production was 64,000 tonnes per year for ten years prior to 1971, and dropped to 44,000 tonnes in 1971-72, then to 30,000 tonnes in 1972-73 (Subramanian, 1975), indicating the extent to which crop yields may be devastated by water deficits.

Harvest index is defined as the ratio of dry seed yield to the maximum dry matter production of the aerial part of the plant during vegetative growth, and is variable in *V. faba* (Duc & Picard, 1982). A water deficit is possibly the primary factor to which limited and varied *V. faba* yields may be attributed throughout Europe (Thompson & Taylor, 1981; Hebblethwaite *et al*, 1984).

Water deficits at any stage of growth reportedly result in yield reductions in *V. faba*, with little evidence of a particularly sensitive developmental stage (Day & Legg, 1981). *V. faba* can only extract water up to about 0.9 m, which is much more shallow than cereals, sugar beet, and grasslands, and *V. faba* roots predominate in the top 30 cm of the medium (an adaptation to regular limited

water supplies), inferring an increased susceptibility to root dehydration during water deficits in *V. faba* as compared with cereals (Hebblethwaite, 1982).

Water deficits provide an interesting platform for research as a variety of water deficit tolerance adaptations are invoked in nodulated and in non-nodulated *V. faba* (Sau & Ines-Minguez, 1990). Plant responses to water deficits may be categorised as structural; physiological; stomatal; or metabolic.

Physiological adaptations to water deficits often involve altered growth patterns. Root : shoot ratios (R:Ss) reportedly alter from 1:3 in well watered plants, to 10:1 in plants experiencing severe water deficits (Etherington, 1962), during which more branched root profiles are reportedly exhibited, deeper in the soil (Sharp & Davies, 1979). Increasing R:Ss have the consequence of increasing the root area available for water (and nitrogen) uptake while simultaneously reducing the above ground biomass, and hence the potential for transpiration (McDonald & Davies, 1996). However cumulative leaf areas, photosynthetic potential and yields may simultaneously be reduced as a consequence (Yoshida, 1972).

The effects of nitrogen applications on growth parameters during water deficits are of interest, as nitrate nutrition reportedly stimulates aerial growth in *V. faba* (Sprent & Thomas, 1984), and sixty kg N ha^{-1} urea applications to *Vigna unguiculata* during late water deficits reportedly results in the exhibition of growth which is comparable to that of adequately irrigated plants (Elowad & Hall, 1987). Accordingly growth parameters were quantified in nodulated and in non-nodulated *V. faba* when supplied with

different forms and concentrations of medium nitrogen nutrition, throughout water deficits (fig. 2.1).

A second adaptation to water deficits involves stomatal closure, which results in reduced transpirational water losses, however CO_2 uptake and hence net photosynthesis are simultaneously reduced (Lawlor, 1995). Stomatal closure may therefore incur decreased plant survival prospects during water deficits as photosynthates and reductants are required to 'fuel' plant water deficit tolerance adaptations. Within the physiology of any plant, water use, nitrogen metabolism and carbon metabolism are intertwined; for example adequate nitrogen, carbon and water supplies are required for the maintenance of growth, photosynthesis, nitrogen assimilation etc. (Martinez-Carrasco *et al*, 1998).

Some plant species reportedly increase the capacity of the light reactions of photosynthesis in response to increasingly concentrated medium nitrate nutrition (Bjorkman, 1981; Marques *et al*, 1983). Furthermore lower stomatal resistances; greater photosynthetic rates; and greater water use efficiencies are reportedly exhibited in some plant species when supplied with increasingly concentrated medium nitrate nutrition (Radin & Ackerson, 1981; Radin *et al*, 1985; Ghashghaie & Saugier, 1989).

However 2 mM ammonia reportedly results in an uncoupling of photophosphorylation in isolated chloroplasts (Good, 1960; Chaparro *et al*, 1976), and ammonia nutrition reportedly results in lowered photosynthetic rates and thylakoid malformations (Takacs & Tecsi, 1992). Furthermore 1-3 mM ammonia reportedly inhibits respiration in isolated mitochondria (Vines & Wedding, 1960), however Wakiuchi *et al*, (1971), reported that respiration was maintained with ammonia nutrition. Wann & Raper, (1979), postulated that if

photosynthetic reserves fall below a critical level which allows maintenance respiration, then leaf organic nitrogen compound degradation may be required as an energy source with the release of free ammonia, and an exasperation of toxicity symptoms (Barker, 1966). However at concentrations below those required to uncouple photophosphorylation the ammonia ion reportedly has a stimulative effect on photosynthesis in intact chloroplasts (de-Beneditti *et al*, 1976), through an activation of ribulose bisphosphate (RUBP) carboxylase reactions, and ammonia nutrition may reportedly result in increased CO_2 fixation (Michael *et al*, 1970).

It is thus proposed that medium nitrogen applications prior to and during mild water deficit development may result in the exhibition of increased photosynthetic rates and improved water use efficiencies in nodulated and in non-nodulated *V. faba* (as reported for other plant species) during this key time (Radin *et al*, 1985). Accordingly stomatal conductances and net photosynthesis were quantified in nodulated and in non-nodulated *V. faba* when supplied with different forms and concentrations of medium nitrogen nutrition, throughout water deficits (see fig. 2.1).

Increased root growth and increased stomatal resistances reportedly occur during mild water deficits, while at lower water potentials osmotic adjustment is initiated, both in *V. faba* (Sau & Ines-Minguez, 1990), and in other plant species (Turner & Stewart, 1986).

Osmotic adjustment refers to the regulation of cellular osmotic pressure and therefore water potentials during water deficits as mediated by increases in cellular solute concentrations as opposed to decreases in cellular volumes (Hanson & Hitz, 1982; Morgan; 1984; Rhodes & Samaras, 1994; Zhang *et al*, 1999), and has been observed in *V. faba* (Van der Wal, 1981; Sharma & Rai,

1989), and in other plant species (Waldren & Teare, 1974; Wignarajah *et al*, 1975; Sharp & Davies, 1979; Stewart & Lahrer, 1980; Stewart, 1981; Borowitzka, 1981; Hanson & Hitz, 1982; Singh & Gupta, 1983; Wyn Jones, 1983; Morgan, 1984; Morgan & Condon, 1986; Whittington & Smith, 1992; Wang *et al*, 1995; Bussis & Heineke, 1998; Cellier *et al*, 1998; Clifford *et al*, 1998; Zhang *et al*, 1999). Plants which accumulate greater concentrations of osmotica reportedly maintain greater solute potentials; greater relative water contents (Singh & Gupta, 1983); greater stomatal conductances (and by inference carbon acquisition); extract more water (Kumar & Singh, 1998); and exhibit greater yields (Van der Wal, 1981; Rodriguez-Maribona *et al*, 1992) during water deficits than those which accumulate lower concentrations of osmotica.

Sugars are reportedly major contributors to osmotic adjustment, while amino acids and quaternary ammonium compounds reportedly contribute smaller concentrations of osmotically active solutes (Hanson & Hitz, 1982; Turner & Stewart, 1986). Nitrate may also act as an osmoticum (Cram, 1974; Martinoia *et al*, 1981; Pate, 1983).

Water deficits can have deleterious consequences for proteins which are easily denatured (Stryer, 1988). Brown & Simpson, (1972), introduced the term 'compatible solute' which describes solutes which are non-inhibitory to metabolism and which accumulate in the cytoplasm of cells subjected to low external water potentials. Potential compatible solutes include polyols; sucrose; fructose; glucose; proline; alanine; beta-alanine; taurine; and glycine betaine, and appear to reduce water deficit associated protein degradation at almost every stage of various 'stresses' (Paleg *et al*, 1985; Samaras *et al*, 1995; Smirnoff, 1995). Compatible solute accumulation may contribute significantly to overall osmotic adjustment

(Wood, 1998).

Z. mays supplied with increasingly concentrated medium nitrogen nutrition reportedly exhibited lower leaf osmotic potentials (Bennet *et al*, 1986). As osmotic adjustment and therefore RWCs may be maintained at greater levels throughout water deficits in plants when supplied with increasingly concentrated medium nitrogen nutrition, stomatal conductances may be maintained at lower water potentials, allowing maintained carbon acquisition (Bennet *et al*, 1986; Hawkins & Lewis, 1993). Accordingly osmotic adjustment (including compatible solute accumulation) was quantified in *V. faba* when supplied with different forms and concentrations of medium nitrogen, throughout water deficits.

V. faba which are reliant on nitrogen fixation reportedly exhibit statistically similar biomasses as *V. faba* which are supplied with medium nitrogen nutrition during periods of adequate irrigation (Dekhuijzen *et al*, 1981; Simon & Skrdleta, 1983). Indeed Richards & Soper, (1979), reported that nitrogen fixation rendered medium nitrogen applications superfluous during periods of adequate irrigation, as very concentrated medium nitrogen nutrition was required before yield increases were exhibited in nodulated *V. faba* when supplied with sufficient water.

However nodule numbers and nitrogen fixation reportedly decrease during water deficits, both in *V. faba* (Hamdi, 1982; Guerin *et al*, 1990; Sangakkara *et al*, 1996), and in other plant species (Serraj *et al*, 1998). The survival of *Rhizobium* in the medium during water deficits is probably not limiting as rhizobial strains are reportedly resistant to soil desiccation and may survive in water films surrounding soil particles (Serraj *et al*, 1999). However rhizobial motility and the infection process

(mucigel production, root hair curling and infection thread formation) are reportedly seriously inhibited during water deficits (Sprent, 1971; Greaves & Derbyshire, 1972). Water deficit resistance in nodulated plants is reportedly proportional to the position of the nodule in the cortex (Sprent, 1971). *V. faba* has a large root diameter, however nodules form close to the epidermis and are often positioned close to the substrate surface and may therefore be susceptible to dehydration during water deficits (Sprent, 1971). Up to half of the water required by nodules may be supplied via the phloem along with carbohydrates making nitrogen fixation expensive to *V. faba* in terms of water economy (Sprent, 1971). Furthermore nitrogen fixation is reportedly energetically expensive (Schilling, 1983) and water deficits result in decreases in carbon acquisition (Epron, 1997; Clifford *et al*, 1998); and in reductions in the phloem transportation of carbohydrates and water to nodules (Walsh, 1995).

Indeed the water deficit sensitivity of nitrogen fixation has been attributed to many factors, including increasing nodular asparagine concentrations (Serraj *et al*, 1999); increasing glutamate : glutamine ratios (Curioni *et al*, 1999); decreasing leghaemoglobin contents (Oghoghorie & Pate, 1971; Arrese-Igor, 1998); decreasing bacteroid respiration rates (Guerin *et al*, 1990); and more recently to decreasing nodular sucrose synthase activities during water deficits (Gordon & James, 1997; Gonzalez *et al*, 1998; Serraj *et al*, 1998). However the meristematic nodules of *V. faba* reportedly recover well upon re-watering (Sprent, 1972). Plies-Balzer *et al*, (1995), reported that growth in *V. faba* during water deficits was not limited by decreases in nitrogen fixation, and that water deficits during pod-filling resulted in maintained nitrogenase activities and biomass production. Furthermore a *G. max* cultivar, ('Jackson'), has been

identified as water deficit tolerant for nitrogen fixation (Serraj & Sinclair, 1997). Thus controversy surrounds the sensitivity of nitrogen fixation to water deficits. Accordingly the effects of water deficits on parameters of growth; stomatal conductance and net photosynthesis; osmotic adjustment; and nitrogen assimilatory enzyme activities were quantified in nodulated *V. faba* grown without medium nitrogen, in order that the effects of increasing water deficits on nitrogen fixing *V. faba* could be determined.

Plies-Balser *et al*, (1995) also reported that medium 'combined nitrogen' applications did not result in the exhibition of greater aerial biomasses, total nitrogen concentrations, or yields by *V. faba* (cv. 'Alfred') than were exhibited by nitrogen fixing 'Alfred' during water deficits. However Purcell & King (1996) reported greater nitrogen and biomass accumulation rates and seed yields in *G. max* when supplied with 'combined nitrogen' nutrition (336 kg/ha NH_4NO_3) than when reliant on nitrogen fixation during water deficits. A major aim of this research was to determine whether medium nitrogen applications (in increasing concentrations, and particularly with ammonia additions) would result in the exhibition of increased productivities by *V. faba*, particularly during water deficits.

NR is also classically considered to be sensitive to water deficits (Mattas & Pauli, 1965; Ferrario-Mery *et al*, 1998), however a small number of studies have reported that NR activities are maintained during water deficits in other plant species (Smirnoff *et al*, 1985; Ladley, 1990).

V. faba supplied with increasingly concentrated medium nitrate nutrition reportedly exhibits increasing NR activities (Sutherland *et al*, 1985; Hocking & Meyer, 1991). Furthermore while ammonia nutrition has previously been reported as inhibitory for NR activities in some plant species (Orebamjo &

Stewart, 1975a; Raper *et al*, 1991; Muller & Janiesch, 1993), contrasting earlier literature describes increases in NR protein, and in NR activities in *G. max* and in other plant species when supplied with 'combined nitrogen' nutrition as opposed to with nitrate or with ammonia nutrition (Lillo & Henriksen, 1984; Guerrier, 1991). A proposal is that increasingly concentrated nitrate or 'combined nitrogen' plant nutrition during water deficits may result in the production of increasing concentrations of nitrogenous osmotica should NR prove water deficit tolerant.

In contrast to NR, the enzymes of ammonia assimilation (GDH and GS GOGAT; enzymes which also assimilate the ammonia produced via nitrate reduction; see fig. 1.1), are classically considered insensitive to periods of water deficit in other plant species (Sinha & Nicholas, 1981; Taylor *et al*, 1982). As such plant medium ammonia additions may incur 'benefits' during water deficits, as nitrogen is central to water deficit tolerance adaptations, being a component of many of the compatible solutes previously described. Furthermore increasingly concentrated medium ammonia nutrition may reportedly result in increased plant NR (Hofstra *et al*, 1985; Bennet *et al*, 1986; Bungard *et al*, 1999); GDH (Taylor & Havill, 1981); and GS (Ortega *et al*, 1999) activities in other plant species, and hence in increased nitrogen assimilation, which in turn may result in increased growth maintenance, net photosynthesis, and osmotic adjustment as previously discussed. Indeed continuing nitrogen metabolism (as opposed to nitrate storage) during water deficits may result in an alleviation of 'sink size' feedback inhibition of photosynthesis and of nitrogen assimilation (and potentially in reduced photoinhibition during water deficits Smirnoff, 1985). If ammonia proves a 'beneficial' supplicant, economic benefits may be incurred, as ammonia is cheaply available in the form

of bird droppings.

Accordingly methodology was designed so that groups of nodulated and non-nodulated *V. faba* were supplied with increasingly concentrated medium ammonia additions in order that the effects of medium ammonia additions could be determined on growth; stomatal conductances and net photosynthesis; osmotic adjustment; and nitrogen assimilatory enzyme activities (fig. 2.1).

The discussion so far has concentrated on the reported effects of single nitrogen sources (either nitrate or ammonium alone) on plant physiology during water deficits. However the optimum nitrogen source is often 'combined'. 'Combined nitrogen' nutrition refers to simultaneous nitrate and ammonium applications. A 'combined nitrogen' source may offer benefits as compared with nitrate or with ammonia nutrition, for example earlier literature which described greater NR activities in other plant species when supplied with 'combined nitrogen' as opposed to with nitrate nutrition has already been detailed (Guerrier, 1991). Furthermore although ammonia uptake reportedly competes with magnesium, calcium, and potassium uptake, medium nitrate additions may reportedly aid cation uptake (Fagena, 1974).

The capacities to absorb nitrate or ammonia as sole nitrogen sources are reportedly similar, but *Z. mays* reportedly assimilates twice as much ammonia as nitrate when supplied with 'combined nitrogen' nutrition, inferring that ammonia may satisfy nitrogen requirements a more energy efficient way than nitrate (Taylor & Bloom, 1998; Colmer & Bloom, 1998; Martinez-Carrasco *et al*, 1998). Indeed ammonia is theoretically easier to utilise (its assimilation requires less photosynthetically derived energy; water; and metal ions per unit yield, than nitrate; Bloom, 1988; Raven, 1985; Atwell 1992; Raven *et al*, 1992), but provides the same function as a nitrogen source. During water

deficits when photosynthate and reductant availabilities may become limiting due to stomatal closure, 'energy' may theoretically be 'saved' by assimilating some ammonia (Raven, 1985; Bloom, 1988; Atwell 1992; Raven *et al*, 1992). Indeed Cox & Reisenauer, (1973), reported greater growth rates and yields when *Triticum aestivum* was supplied with 'combined nitrogen', as opposed to with nitrate or with ammonia nutrition, which may have been attributable to reduced energy requirements in plants when supplied with 'combined nitrogen' nutrition. Improvements in the quality and quantity of leaf protein (an important consideration for a 'green manure' crop; Corak *et al*, 1992) have also been reported in other plant species when supplied with 'combined nitrogen' as opposed to with nitrate or with ammonia nutrition, as nitrate applications reportedly result in the exhibition of increased plant methionine contents and ammonia applications reportedly result in increased aspartate contents (Weissman, 1964; Domska, 1974).

Furthermore increased organic acid concentrations (which are reportedly required to enable maintained nitrate uptake (Davies, 1973), and maintained nitrogen assimilation (Bourgeais-Chaillou *et al*, 1992); see chapter five) are reportedly exhibited in *G. max* when supplied with 'combined nitrogen' as opposed to with nitrate or with ammonia nutrition (Bourgeais-Chaillou *et al*, 1992). Net nitrogen acquisition and translocation to the shoot are also reportedly substantially greater in other plant species when supplied with 'combined nitrogen' as opposed to with nitrate or with ammonia nutrition, indicating a potential reduction in root nitrogen assimilation feedback inhibition in plants when supplied with 'combined nitrogen' nutrition (Ourry *et al*, 1995; Kronzucker *et al*, 1999).

'Combined nitrogen' applications may be especially 'beneficial' during water deficits as 'combined nitrogen' nutrition reportedly results in the exhibition

of greater plant maximum specific growth rates and dry weight gains per unit water transpired than nitrate or ammonia nutrition (Delwiche, 1951; Orebamjo & Stewart, 1975a; Haynes & Goh, 1978; Raven *et al*, 1992). Furthermore water deficits often involve warm temperatures which favour ammonia (as opposed to nitrate) uptake (Bloom *et al*, 1998). An optimum ratio of nitrate to ammonia may exist which differs as dependant on plant species, age etc. (Michael *et al*, 1970).

Accordingly nitrate and 'combined nitrogen' were supplied to *V. faba* at increasing concentrations so that the effects of increasingly concentrated nitrogen nutrition could be determined on growth; stomatal conductances and net photosynthesis; osmotic adjustment; and nitrogen assimilatory enzyme activities in *V. faba*, both when supplied with adequate irrigation, and during increasing water deficits (see fig. 2.1).

The preceding pages detailed earlier contradictory reports regarding the effects of varying nitrogen nutrition and of water deficits on plant physiology. Chapter two highlights the major aims of this research, and details the methodology designed to meet these aims.

CHAPTER TWO.

GENERAL METHODOLOGY & PLANT GROWTH CONDITIONS

A method is a specific application of a technique to solve an analytical problem (Willard *et al*, 1988).

Methodology is the science of method (Concise Oxford Dictionary, 8th Ed).

2.1 GENERAL GROWTH CONDITIONS

This chapter details the aims and objectives of the research, and the rationale behind the methodology (see fig. 2.1). The specifics of the nitrogen regimes (section 2.4) and water deficit regimes (fig. 2.2) are explained; and general growth conditions and sample preparation techniques are outlined.

2.2 AIMS & OBJECTIVES

The preceding chapter reviewed the contradictory conclusions of earlier workers as to the effects of medium nitrogen applications on the productivities of leguminous species; the effects of water deficits on the activities of various enzymes associated with nitrogen metabolism; and the effects of ammonia nutrition on plant growth.

2.2.1 Plies-Balser *et al*, 1995 reported that medium 'combined nitrogen' applications did not result in increased aerial biomasses, total nitrogen concentrations, or yields in *V. faba* (cv. 'Alfred') during water deficits. This is unexpected, as although *V. faba* is reportedly an effective nodulator (Richards & Soper, 1979; Sau & Ines-Minguez, 1989), earlier workers have described greater root growth (Giordano & Bowes, 1997); greater heights (Quebedeaux & Osbun, 1973); greater net photosynthesis (de-Benedetti *et al*, 1976): greater NR, GDH, and GS activities (Bungard *et al*, 1999; Taylor & Havill, 1981; Ortega *et al*, 1999); greater osmotic adjustment (Bennet *et al*,

Fig. 2.1 Rationale of Methodology.

SEEDS WERE RANDOMLY ASSIGNED TO ONE OF THREE SCHEMES, (a; b; or c)

a

SCHEME (a) NON-NODULATED *V. faba*

NITROGEN REGIME

72 beans within scheme (a) were surface sterilized to prevent nodulation, (see section 2.3).
Scheme (a) was then sub-divided into 4 groups. Each group was supplied with one of the following nitrogen treatments;

(ai) 'No Nitrate'
(aii) 1.6 mM Nitrate
(a iii) 4.0 mM Nitrate
(a iv) 4.0 mM 'Combined Nitrogen' (see table 2.4).

WATER DEFICIT REGIME

Six water deficit levels were imposed gradually, by adding increasing amounts of Polyethylene Glycol 6000 to the hydroponic medium, (see section 2.7; table 2.5).

EXPERIMENTS

Growth data, stomatal conductance data, osmotic adjustment, amino transferase activities, and nitrogen assimilatory enzyme activities were quantified in response to water deficits, (in all four nitrogen source groups). Comparisons were made as to which nitrogen source(s) produced the greatest water - deficit - tolerance response(s) in non-nodulated *V. faba*, and hence maintained the greatest productivities, and would be best able to survive water deficits.

RATIONALE

Non-nodulated *V. faba* is often found in the field, as although medium nitrogen (i.e. fertilization) is expected to increase yields with water deficits, it depresses N-fixation, (Kennedy & Eady, 1979; Caba *et al*, 1998). Furthermore nitrogenase is considered water deficit sensitive (Wilson, 1942; Serraj *et al*, 1998).
Scheme (a) plants allowed research into the effects of nitrogen applications on plant physiology during increasing water deficits, without the added variable of N-fixation.

b

SCHEME (b) NODULATED *V. faba*

NITROGEN REGIME

72 beans within scheme (b) were surface sterilized and then germinated with *Rhizobium leguminosarum* (see section 2.5). Initially all seedlings within this group were supplied with 'No Nitrate', as medium nitrogen reportedly inhibits nodulation (Kennedy & Eady, 1979).
Scheme (b) was then sub-divided into 4 groups on day 12, following which each group was supplied with one of the following nitrogen sources;

(bi) 'No Nitrate'
(bii) 1.6 mM Nitrate
(b iii) 4.0 mM Nitrate
(b iv) 4.0 'Combined Nitrogen' (see table 2.4).

WATER DEFICIT REGIME

Six water deficit levels were imposed gradually, by progressively witholding water, (see section 2.7; table 2.6).

EXPERIMENTS

Growth data, stomatal conductance data, osmotic adjustment, amino transferase activities, and nitrogen assimilatory enzyme activities were quantified in response to water deficits, (in all four nitrogen source groups). Comparisons were made as to which nitrogen source(s) produced the greatest water - deficit - tolerance response(s) in nodulated *V. faba*, and hence maintained the greatest productivities, and would be best able to survive water deficits.

RATIONALE

'No Nitrate' scheme (bi) *V. faba* relied soley on nitrogen fixation for a supply of nitrogen. Comparisons of the water deficit tolerance responses of scheme (ai) non-nodulated 'No Nitrate' plants, with scheme (bi) nodulated 'No Nitrate' plants, allowed an evaluation of the effects of water deficits on N-fixation.
Evaluations of the effects of various nitrogen sources on nodulation were also made, during increasing water deficits, (table 2.2).

PRELIMINARY DATA SUGGESTED THAT 'COMBINED NITROGEN' NUTRITION RESULTED IN THE EXHIBITION OF THE GREATEST PRODUCTIVITIES & WATER DEFICIT TOLERANCE ADAPTIVITY. HENCE A THIRD SCHEME (c) WAS DESIGNED

c

SCHEME (c) 'SPIKED' *V. faba*

NITROGEN REGIME

72 beans within scheme (c) were surface sterilized (see section 2.3). Group (c) was then sub-divided into 4 groups. Each group was supplied with one of the following nitrogen sources;

(ci) 'No Nitrate'
(cii) 1.6mM Nitrate
(c iii) 2.8mM Nitrate
(c iv) 'Combined Nitrogen' (see table 2.4).

However from day twelve onwards, each plant in this scheme received a 2mM ammonium sulphate 'spike', in addition to it's original allocated nitrogen source (see table 2.4).

WATER DEFICIT REGIME

Six water deficit levels were imposed gradually, by adding increasing amounts of Polyethylene Glycol 6000 to the hydroponic medium (see section 2.7; table 2.5).

EXPERIMENTS

Growth data, stomatal conductance data, osmotic adjustment, amino transferase activities, and nitrogen assimilatory enzyme activities were quantified in response to water deficits (in all four 'spiked' nitrogen source groups).

RATIONALE

Comparing differences in the physiological responses to water deficits between plants from the 'spiked' group (c), and plants from the 'non-spiked' group (a) allowed insight into the hypothesis that ammonium additions result in the exhibition of increasing productivities and increase the ability to survive water deficits in this species.

1986): and greater growth rates and yields (Cox & Reisenhauer, 1973) in other plant species when supplied with increasingly concentrated medium nitrogen. Increases in such parameters may increase the water deficit tolerance of a plant, for example by increasing substrate availabilities for osmotica production. Indeed Purcell & King (1996) reported greater nitrogen and biomass accumulation rates and seed yields in *G. max* when supplied with 'combined nitrogen' nutrition (336 kg/ha NH_4NO_3) than when reliant on nitrogen fixation during water deficits. A major aim of this research was to determine whether medium nitrogen applications (in increasing concentrations, and particularly with ammonia additions) would result in the exhibition of increased productivities by *V. faba* during water deficits.

2.2.2 The introduction highlighted controversies surrounding the water deficit tolerance of NR. This research evaluated NR activities during gradually imposed water deficits. The potential contribution of primary nitrogen assimilation to metabolism maintenance throughout water deficits was thus determined. The activities of key transaminases (which influence which amino acids accumulate) were also determined during water deficits, and related to the metabolic derivation of each amino acid and to the potential 'roles' of accumulating amino acids within plant metabolism.

2.2.3 The introduction detailed contradictory reports of the effects of medium ammonia on plant growth (and NR activities). A research aim was to determine the effects of medium ammonia additions on the physiology of this leguminous species (in the presence and absence of increasing water deficits).

2.2.4 A *G.max* cultivar 'Jackson' has been described as water deficit tolerant for nitrogen fixation (Serraj & Sinclair, 1997). Furthermore it has been

reported that the growth of *V. faba* (cv. 'Alfred') is not limited by decreasing nitrogenase activities during water deficits (Plies-Balser *et al*, 1995). Physiological parameters were quantified in nodulated *V. faba* during water deficits, allowing some inference into the water deficit sensitivity of nitrogenase in 'Bunyards Exhibition'.

The potential ability to understand factors which encourage reliable yields of high protein *V. faba* by optimising water and nitrogen metabolism (these two parameters being major yield-limitation factors; Sinha & Nicholas, 1981), justifies research into the physiology of this species. While crop breeding is important, protein content is so affected by environment that it cannot be selected for as easily as other characters, and more physiological information is required to aid selection.

2.3 SEED TREATMENT

V. faba (cultivar 'Bunyards Exhibition') were purchased from Unwins throughout. Similar sized seeds were randomly allocated to nitrogen and water deficit groups. Prior to germination all seeds were soaked in 95% ethanol for 90 minutes, and then full strength commercial bleach (5.25% sodium hypochlorite) for 20 minutes to ensure surface sterilisation (Yang *et al*, 1992). Inoculation with *R. leguminosarum* (when required) was thus controlled. *V. faba* synchronises the exhaustion of seed nitrogen reserves with the availability of fixed nitrogen well; as a hypogeal species *V. faba* begins photosynthesis when nitrogen fixation has already commenced, which is prior to cotyledon nitrogen depletion. Indeed cotyledons of *V. faba* are large enough to supply sufficient substrates for seedling production that elevated CO_2 has no effect on seedling development (Radoglou & Jarvis, 1993). However seed-shed was at approximately four weeks, after which the only available nitrogen until

harvest was from a specified Long Ashton (LA) source (or potentially from nitrogen fixation when *V. faba* was germinated in *Rhizobia*-rich media, see section 3.4).

V. faba were cultivated in Town & Country walk-in controlled environment cabinets (model no. TC001) which allow controlled PAR (photosynthetically active radiation) levels; % humidity levels (humidity changes affect stomatal apertures; Mansfield & Davies, 1981); and day and night temperatures, and were operated as outlined in table 2.1.

Table 2.1 Controlled environment conditions (after Saxena, 1982).

		Light Period (06.00 - 18.00)	Dark Period (18.00 - 0600)
Temperature		20 oC	15 oC
Relative Humidity		60 %	60 %
PAR	Plant Apex	295 µmol/sec/sq. m	-
	Plant mid-zone	155 µmol/sec/sq. m	-
	Soil Level	78 µmol/sec/sq. m	-

Thus environmental parameters (except nitrogen source/scheme; water deficit imposition; medium physical state) were standardized.

Fig. 2.1 outlines the rationale behind the methodology and details three overall nitrogen schemes. Within each nitrogen scheme (non-nodulated; nodulated; 'spiked') seventy-two *V. faba* were grown per experiment. These seventy-two *V. faba* per scheme were sub-divided into four nitrogen groups (tables 2.3 & 2.4). Within each of the four nitrogen groups *V. faba* were further sub-divided into six pots (each containing three plants) each of which was subjected to one of six levels of water deficit imposition (tables 2.6 & 2.7).

2.4 NITROGEN REGIMES - NON-NODULATED, NODULATED, & 'SPIKED' *V. faba*.

Three major nitrogen schemes (see fig. 2.1) allowed:

(a) Research into the proposal that increasingly concentrated medium

nitrogen nutrition (supplied either as nitrate or as 'combined nitrogen') may result in improved water deficit tolerance in non-nodulated and in nodulated *V. faba*;

(b) Comparisons between water deficit associated responses of *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition as opposed to when reliant on nitrogen fixation;

(c) Comparisons of water deficit associated responses of *V. faba* when supplied with medium nitrate or 'combined nitrogen' nutrition equivalent to 4 mM nitrogen, as opposed to when supplied with an additional 4 mM ammonia 'spike', to allow investigation into the hypothesis that medium ammonia additions may result in increased water deficit tolerance (see introduction).

Nitrogen was supplied to *V. faba* as one of four LA solutions (recipes are given in appendix I; pg. 280), which contained equal concentrations of all macro-nutrients except nitrogen (see table 2.2).

Table 2.2 Nitrogen sources; nodulated and non-nodulated *V. faba*

NITROGEN SOURCE	NITROGEN SUPPLIED	NITROGEN CONCENTRATION
'NO NITRATE'	NO NITROGEN	0.0 mM N
'1/10 NITRATE'	SUPPLIED EQUALLY AS KNO3 & Ca(NO3)2	0.8 mM N
'1/2 NITRATE'	SUPPLIED EQUALLY AS KNO3 & Ca(NO3)2	4.0 mM N
'COMBINED NITROGEN'	SUPPLIED AS 4 mM NH4NO3	4.0 mM N

Micronutrients are reportedly required for nitrogen fixation (Mengel *et al*, 1974; Othman *et al*, 1991), and deficiencies reportedly result in reduced yields (Hafner *et al*, 1992). Accordingly micronutrients were included at equal concentrations in all LA solutions. LA solutions have advantages over solid media in that they contain all essential nutrients and are completely defined

and controllable, eliminating problems of batch variation.

A 'no nitrate' nitrogen source allowed research into the effects of nitrogen deprivation on non-nodulated *V. faba* (as reviewed by Ghashghaie & Saugier, 1989), and provided a 'baseline' nitrogen level against which the water deficit tolerance adaptations of *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition could be compared. Although the LA solutions were made up in distilled water, it is possible that low levels of nitrogen might have been present in the 'no nitrate' solution' (see section 6.3.1.1; pg. 180). Nitrogen fixation potentially provided nitrogen to 'no nitrate' supplied *V. faba* within the nodulated scheme (fig. 2.1; scheme b). The introduction detailed earlier reports of the effects of increasingly concentrated medium nitrate nutrition and of 'combined nitrogen' nutrition on plant physiology during water deficits. Accordingly nitrate solutions were provided at two concentrations; and a fourth group of *V. faba* was supplied with 'combined nitrogen' nutrition (see table 2.2).

2.4.1 NON-NODULATED *V. faba* NITROGEN REGIMES

Non-nodulated *V. faba* were grown hydroponically in LA media. Hydroponic growth results in minimal root damage upon harvesting, and is not conducive to the growth of mycorrhizal fungi (Min *et al*, 1999), thus this variable on nitrogen nutrition was eliminated. Blackened pots complete with blackened lids (10.5 cm deep; 10.5 cm diameter) were utilised for growth. Lids were perforated allowing plants to sit with their roots in the media. The media were aerated by means of an air-pump attached to a series of tubes which bubbled air into each pot. Solutions were changed every three days to remove exudates; to maintain nutrient levels; and to prevent pH changes. pH was monitored throughout and remained between 6.5 and 7.5. A disadvantage with liquid growth is that it is unnatural and does not mimic field growth, for example root

mechanical impedance during water deficits is reduced when hydroponic growth is utilised. However systems of drying soil are difficult to control, standardise and reproduce. As nodulated plants were grown on a (solid) medium of mixed vermiculite, bark and sand (see 2.3.2), the water deficit responses of *V. faba* when grown in solid medium and when grown hydroponically were compared.

2.4.2 NODULATED *V. faba* NITROGEN REGIMES

The rhizobia which infect *V. faba* (*R. leguminosarum*) are fast growing, requiring high water contents to maintain their relatively high enzyme activities (Sprent, 1976). The first steps in infection are especially sensitive to water deficits (Sprent, 1971a). Accordingly excessive numbers of *R. leguminosarum* (1 l of solution containing 10^9 viable cells/ml per seventy-two seeds; Ligero *et al*, 1991) were added (when required; i.e. to scheme b plants; fig. 2.1) to a vermiculite, sand, and bark germination mixture (1:1:1), and germinating seeds were adequately irrigated with 'no nitrate' LA solution. Such seed inoculation reportedly results in the production of maximum numbers of healthy, discretely positioned nodules (Worrall & Roughly, 1976). Furthermore inoculation at the germination stage allows the maximum time for nitrogen fixation, and increasing the length of time of infection may reportedly result in a doubling of the amount of nitrogen fixed (Hardy & Havelka, 1976).

Nodulation is reportedly inhibited in liquid media unless a fixed nitrogen source e.g. glutamine is supplied (Postgate, 1974). However glutamine supplies were incompatible with this study where specific nitrogen sources were supplied. Accordingly nodulated *V. faba* were grown in the vermiculite, sand, and bark mixture throughout water deficit imposition (Caba *et al*, 1998), the nitrogen content of which was negligible (< 0.01 mM g^{-1}).

Initially all inoculated *V. faba* plants were supplied with 'no nitrate' nutrition, as medium nitrogen nutrition reportedly results in inhibited nodulation (Kennedy & Eady, 1979; Caba *et al*, 1998). However from day twelve onwards nodulated *V. faba* were sub-divided into four nitrogen groups each of which was supplied with one of the four pre-specified LA solutions (table 2.2). Pots measured 9.5 cm in depth, with a 10.5 cm diameter. Despite reports that nitrate absorption may be reduced in nodulated plants (perhaps as root growth itself is limited by increased cytokinin levels as produced by both bacteria and roots; Rigaud, 1981), nodulation was reduced in inoculated *V. faba* when supplied with medium nitrogen nutrition.

Table 2.3 Numbers of healthy nodules observed in *V. faba* when supplied with various nitrogen sources during water deficits, (average of three experiments).

Volume Long Ashton's per pot (% control)	Average Number of Nodules			
	Nitrogen Treatment			
	No Nitrate	1/10 Nitrate	1/2 Nitrate	Combined N
100	>12	9	7	0
85	11	9	7	0
70	7	5	5	0
60	5	3	3	0
45	4	3	0	0
30	4	0	0	0

Table 2.3 records that inoculated 'no nitrate' supplied *V. faba* were the only plants which maintained significant nodulation throughout water deficits.

2.4.3 'SPIKED' *V. faba* NITROGEN REGIMES

Some non-nodulated *V. faba* were allocated to a 'spiked' nitrogen scheme (fig. 2.1; scheme c). Such plants were initially supplied with the four nitrogen

sources outlined in table 2.2. However from day twelve onwards an additional ammonia 'spike' was provided to these *V. faba* (according to table 2.4), to allow investigation into the hypothesis than medium ammonia additions may result in increased water deficit tolerance.

Table 2.4 Nitrogen concentration of the media supplied to 'spiked' *V. faba*

NITROGEN SOURCE	NITROGEN CONCENTRATIONS (UNTIL DAY 12)	ADDITIONAL NITROGEN (FROM DAY 12)	TOTAL NITR AVAILABLE (FROM DAY
NO NITRATE (& AMMONIA)	NO NITROGEN	(+ 4.0 mM N SUPPLIED AS (NH4)2SO4)	4 mM
1/10 NITRATE (& AMMONIA)	0.8 mM N SUPPLIED EQUALLY AS KNO3 & Ca(NO3)2	(+ 4.0 mM N SUPPLIED AS (NH4)2SO4)	4.8 mM
1/2 NITRATE (& AMMONIA)	4.0 mM N SUPPLIED EQUALLY AS KNO3 & Ca(NO3)2	(+ 4.0 mM N SUPPLIED AS (NH4)2SO4)	8 mM
COMBINED NITROGEN (& AMMONIA)	4.0 mM N SUPPLIED AS NH4NO3	(+ 4.0 mM N SUPPLIED AS (NH4)2SO4)	8 mM

2.5 WATER DEFICIT IMPOSITION

Plant age may reportedly affect plant water deficit responses (Etherington, 1962), and was therefore standardised by germinating *V. faba* (for around four weeks) until the fifth leaf stage was achieved before water deficits were imposed.

Sudden water deficit imposition may reportedly result in altered plant responses during water deficits. Increased cell wall elasticities rather than active solute accumulation reportedly becomes the dominant cause of increased solute concentrations during rapid water deficit imposition (Grossnickle & Russell, 1996; Clifford *et al*, 1998). Subsequently lower levels of osmotic adjustment and hence earlier declines in stomatal conductance and photosynthetic activities have been reported in other plant species when subjected to rapid water deficit imposition (Jones & Rawson, 1979; Richardson & McCree, 1985), along with decreased growth parameters such as relative water contents and organ weights, and decreased yields

Fig. 2.2 Water Deficit Regimes

RWCs > 90% = slight water deficits; 90 - 80% = moderate water deficits; <80% = severe water deficits (Hsaio, 1973)

NON-NODULATED & 'SPIKED' *V. faba* WATER DEFICIT IMPOSITION

	POT NUMBER*, with percentage PEG 6000 supplied per pot.					
	1*	2*	3*	4*	5*	6*
day 3	0%	0%	0%	0%	0%	0%
day 6	0%	5%	5%	5%	5%	5%
day 9	0%	5%	10%	10%	10%	10%
day12	0%	5%	10%	15%	15%	15%
day 15	0%	5%	10%	15%	20%	20%
day 18	0%	5%	10%	15%	20%	25%
day 21	0%	5%	10%	15%	20%	25%

Final degree of water deficit per pot as indicated by Leaf RWCs (fig. 3.17), and also by plant growth and osmotic responses in *V. faba* when supplied with 4 mM N.

- pot 1* ADEQUATE WATER- CONTROL
- pot2* SLIGHT WATER DEFICIT
- pot3* SLIGHT / MOD. WATER DEFICIT
- pot4* MODERATE WATER DEFICIT
- pot5* SEVERE WATER DEFICIT
- pot6* SEVERE WATER DEFICIT

NODULATED *V. faba* WATER DEFICIT IMPOSITION

	POT NUMBER*, % Control Water supplied per pot.					
	1*	2*	3*	4*	5*	6*
day 3	100%	100%	100%	100%	100%	100%
day 6	100%	85%	85%	85%	85%	85%
day 9	100%	85%	70%	70%	70%	70%
day12	100%	85%	70%	60%	60%	60%
day 15	100%	85%	70%	60%	45%	45%
day 18	100%	85%	70%	60%	45%	30%
day 21	100%	85%	70%	60%	45%	30%

Final water deficit levels were maintained for one week prior to harvest to allow equilibration , (Clifford *et al*, 1998).

and post-water-deficit recovery rates (Pearson & Stewart, 1987). Furthermore the rate of water deficit development reportedly affects the relationships between cell division and cell elongation, and therefore stomatal density and carbon acquisition (Heckenberger *et al*, 1998). Accordingly non-nodulated, nodulated, and 'spiked' *V. faba* were subjected to gradual water deficit imposition as outlined below.

2.5.1 WATER DEFICIT IMPOSITION IN NON-NODULATED & IN SPIKED *V. faba*

Polyethylene glycol 6000 (PEG) has a high molecular weight and PEG additions to aqueous solutions effectively limit water uptake by stereochemically occupying solvent space (Paleg *et al*, 1985). LA solutions containing 0 % PEG formed the media of control plants. Prior to use a 50% PEG solution was prepared in distilled water, and then passed through a Duolite MB50 indicator mixed bed resin column to remove aluminium and phosphate contaminants. The hydroponic media of non-nodulated *V. faba* were changed every three days, and replaced with solutions which contained more concentrated PEG (PEG concentrations increased in 5 % increments according to fig. 2.2) when increased water deficit imposition was required. Thus water deficits were imposed in a gradual and finely controlled manner.

PEG additions may result in increased viscosities and hence decreased oxygen availabilities in hydroponic media, however hydroponic media were aerated throughout as previously discussed.

2.5.2 WATER DEFICIT IMPOSITION IN NODULATED *V. faba*

Nodulated control plants were supplied with 100% water (>80 mls LA per pot thrice weekly; with adequate drainage) throughout the water deficit regime. Media osmotica (such as PEG 6000) reportedly result in decreased nitrogen fixation (Sprent, 1976; Caba *et al*, 1998), furthermore nodulated *V. faba* were grown in solid media which plasticizes (becomes less gas permeable) with PEG

additions. Thus water deficits were imposed in nodulated *V. faba* by supplying decreasing quantities of water.

Grajeda, (1990), reported that late water deficits do not affect the yields of nodulated plants, however water deficits were sustained throughout the vegetative and reproductive phases of *V. faba* using the water deficit imposition regime outlined in fig. 2.2.

Drying vermiculite may reportedly result in root growth which is more sensitive to water deficits than oxygenated PEG (Verslues & Sharp, 1998), perhaps attributable to increased root mechanical impedance. However the similar responses recorded by *V. faba* whether exposed to water deficits using the nodulated (vermiculite/bark/sand/LA) regime, or the non-nodulated (LA/PEG 6000) regime, indicate that PEG applications do mimic soil based systems, and are thus a viable technique for gradual controlled water deficit imposition (as previously reported by Taylor *et al*, 1982).

2.6 GENERAL EXTRACTION PROCEDURES - FRESH & DRY WEIGHTS

If the medium sized leaves of plants subjected to water deficits are compared against the larger leaves of adequately irrigated (control) plants, then the ratio between these tissues which have different biochemical stocks may differ and may potentially bias results as affected by changes in biochemical composition (Heckenberger *et al*, 1998). Organs chosen for analyses from different water deficit treatments were of similar cytological status, as reflected in the similar leaf sizes exhibited by newest fully expanded leaves within all treatments. For all experiments, organs analysed were of equivalent physiological age and were harvested from same-age plants (from leaves 4-8; and from young roots; within 5cm of the root tip) as the highest enzyme activities (Wallace & Pate, 1965; Dudel & Kohl, 1974; Rigaud, 1981; Durzan & Stewart, 1983), and the highest concentrations of osmotic adjustment (Aspinall

& Paleg, 1981; Wyn Jones & Storey, 1981; Wang *et al*, 1995) are reportedly exhibited in young tissue.

Organ fresh weights (FWs) were recorded following harvest. Dry weight (DW) values were obtained by wrapping the organs (of known fresh weight) in labelled perforated foil, and then oven drying at 80°C for one hour, and then at 60°C until constant weights were achieved.

2.6.1 SPECTROPHOTOMETRIC ASSAY SAMPLE PREPARATION

Dried samples were finely ground using a pestle, mortar, and liquid nitrogen (which disrupts the cell membranes, allowing detection of subcellular solutes). For spectrophotometric analyses 0.2 - 0.5 g dried crushed organs were placed in boiling tubes containing 10 ml methanol (95 %). Samples were stored at 4°C, and agitated three times daily using whirlli-mixers. After three days samples were filtered through Whatman's No. 1 general purpose filter paper. Solutions were made up to constant volumes (10 ml), covered and stored at 4°C. In the case of starch analyses, the remaining plant material was retained for final analysis (see section 5.2.4, pg. 137, for starch analysis method).

2.6.2 GC ANALYSES SAMPLE PREPARATION

Samples for GC analyses were weighed, dried, and crushed as described in section 2.6.1. Crushed samples were suspended in a methanol: methylene chloride: water mixture, (12:5:3), refrigerated (at 4°C), and agitated three times daily. After three days 1 ml methylene chloride was added to each sample, followed by small water additions (using a pasteur pipette) until phase separation was achieved. Samples were stored overnight at 4°C to ensure complete phase extraction. The lower (non-aqueous) layers were discarded. The

(a) indicates that differences in the measured parameter were significantly affected by the increasingly concentrated medium nitrogen nutrition and were not likely to be attributable to chance. Significant 'F calc' values in the water deficit column in appendix II indicates that the mean values of those parameters were significantly different as affected by water deficits (as opposed to error).

Appendix II (b) contains 'F calc' and 'F crit' values following ANOVA analysis of the data derived from non-nodulated 'no nitrate' supplied *V. faba* when compared against the data derived from nodulated 'no nitrate' supplied *V. faba*. A significant 'F calc' value in the 'nitrogen treatment' column of appendix II (b) indicates that the means of the measured parameters differed significantly in the nodulated as compared against the non-nodulated 'no nitrate' supplied *V. faba*, and that these differences were not likely to be attributable to chance. As the only treatment difference between 'no nitrate' supplied plants from these two nitrogen schemes was potential nodulation, a significant 'F calc' in the 'nitrogen treatment' column of appendix II (b) was attributed to the effects of nitrogen fixation.

Appendix II (c) contains 'F calc' and 'F crit' values following ANOVA analysis of the data derived from non-nodulated 'non spiked' *V. faba* compared against the data derived from non-nodulated 'spiked' *V. faba*. A significant 'F calc' value in the 'nitrogen treatment' column of appendix II (c) indicates that the means of the measured parameters differed significantly in 'spiked' as compared against 'non spiked' *V. faba*, and were not likely to be attributable to chance. It was therefore possible to determine whether the observed differences in the growth parameters and the enzyme and water deficit responses between *V. faba* when supplied with 'spiked' as opposed to with 'non-

spiked' nutrition were statistically significant.

In each section of appendix II significant 'F calc' values are given in **bold.**

CHAPTER THREE

GROWTH DISTRIBUTION, GROWTH RATES, STOMATAL CONDUCTANCES & NET PHOTOSYNTHESIS IN NON-NODULATED; NODULATED; AND AMMONIA 'SPIKED' *V. faba*, WHEN SUPPLIED WITH VARIOUS FORMS AND CONCENTRATIONS OF MEDIUM NITROGEN NUTRITION DURING INCREASING WATER DEFICITS

Photosynthesis is the driving force of plant productivity and the ability to maintain the rate of photosynthetic carbon dioxide and nitrate assimilation under environmental stresses is fundamental to the maintenance of plant growth and production (Lawlor, 1995).

Stomata have been delegated the task of providing food while preventing thirst (Raschke, 1976).

3.1 INTRODUCTION

Growth parameters must be quantified prior to assessing the responses of *V. faba* when supplied with different nitrogen sources during water deficits. Diminishing relative water contents (RWCs), organ weights, and growth measurements are all reportedly indicative of, correlated with, and attributable to water deficits (Hsaio *et al*, 1976), and were therefore quantified in *V. faba* under the nitrogen and water deficit regimes outlined in chapter two.

Nitrogen and water supplies have been highlighted as major limitors of crop yield (see introduction). With adequate nitrogen supplies and irrigation crop growth is reportedly largely determined by the capacity to intercept solar radiation (Sinha & Nicholas, 1981; Hebblethwaite, 1982), as plant dry matter

accumulation is a function of the expansion of photosynthetic surfaces, and of the intensity of photosynthesis per unit area (Raab & Terry, 1994). Maintained high cumulative leaf areas have been strongly correlated with dry matter yield and seed matter yield in *V. faba* (Hebblethwaite, 1982). Large leaf areas are desirable, as they represent a large potential capacity for photosynthesis (Yoshida, 1972). Optimum cumulative leaf areas (CLAs) exist however, as photosynthetic rates per unit leaf area may decrease with increasing leaf size (Evans & Dunstone, 1970; Hageman, 1979), due to increased mutual leaf shading, and canopy photosynthesis rapidly approaches an asymptote when leaf area indices exceed three (Yoshida, 1972).

R:Ss reportedly increase during water deficits (Etherington, 1962; Sharp & Davies, 1979). Increased root growth potentially allows increased water (and nitrogen) uptake (McDonald & Davies, 1996), and has previously been associated with the postponement of dehydration in *V. faba* (Sau & Ines-Minguez, 1990). Reduced above ground biomasses result in reduced potentials for transpiration, which are important for water conservation, particularly as stomatal densities are reportedly higher in water deficit treated plants due to the developmental pattern of the formation of stomatal complexes (Heckenberger *et al*, 1998), accentuating potential water losses. However CLAs and hence photosynthetic potentials and yields may simultaneously be reduced (Yoshida, 1972). Average leaf thicknesses are very low compared to the rate at which water may be lost by transpiration (Mansfield & Davies, 1981), and therefore little leaf water is held in reserve, and while large leaf areas may result in increased photosynthetic capacities, potential water losses via transpiration are simultaneously increased. Accordingly water deficits reportedly induce stomatal closure when leaf RWCs approach eighty per cent (Lawlor, 1995), and stomatal closure simultaneously results in decreased CO_2 assimilation

(Wellburn *et al*, 1996). Below eighty per cent leaf RWCs changes in metabolism reportedly become marked, with cessation of photosynthesis (Epron, 1997; Clifford *et al*, 1998), at a time when photosynthates and reductants are required not only for growth maintenance, but also for the maintenance of nitrogen assimilation and for water deficit tolerance adaptations such as osmotic adjustment (which reportedly results in maintained water uptake and water contents (Kumar & Singh, 1998), and has previously been positively correlated with yield in *V. faba* (Van der Wal, 1981), and in other plant species (Rodriguez-Maribona *et al*, 1992)). Thus a 'stomatal compromise' must be negotiated, whereby stomata close sufficiently to avoid unsurmountable water losses during water deficits, but remain sufficiently open that the carbon demands of water deficit tolerance adaptations may be met.
It has previously been reported that severe water deficits result in a near complete inhibition of photosynthesis and structural damage to chloroplasts (Poljakoff-Mayber, 1981), decreased chlorophyll and chloroplastic enzyme levels (Quartacci *et al*, 1997; Bussis *et al*, 1998; Iturbe-Ormaetxe *et al*, 1998), and increasing conversions of violaxanthin to zeaxanthin, indicating photosynthetic apparatus damage (Iturbe-Ormaetxe *et al*, 1998) in other plant species. In the field the high light intensities associated with water deficits may accentuate water deficit effects on photosynthesis, as UV-B radiation decreases adaxial stomatal conductance in *P. sativum* by approximately 65%, reducing CO_2 uptake by 10-15% (Nogues *et al*, 1998).

RWCS were quantified, as evidence suggests that water deficit tolerance adaptations may be evoked over a narrow range of plant RWCs (as opposed to water potentials, Sinclair & Ludlow, 1985).

Relative growth rates (RGRs) were determined throughout water deficits in

V. faba when supplied with the pre-specified forms and concentrations of medium nitrogen nutrition. RGRs represent the amount by which a plant has grown in proportion to its original weight, and therefore represent an approximation of the efficiency of the plant as a producer of new material (Blackman, 1919; Hunt, 1978). However RGRs do not account for whether the measured plant material is structural or productive (e.g. photosynthetic), but instead provide a convenient integration of the growth of combined plant parts over time, and are thus useful when comparing treatment differences (Hunt, 1978).

While RGRs imply that all of the weight of a plant is equally productive to further weight it is known that larger plants contain proportionally larger amounts of structural material (Hunt, 1978). Accordingly plant net assimilation rates (NARs) refer to the net gain in weight per unit photosynthetic area (Gregory, 1926), and attempt to index growth independently of plant size. However NARs may alter as environments change (Thorne, 1961), for example as affected by stomatal closure, and are thus useful plant growth indicators during water deficits.

While NARs estimate the carbon-assimilatory capacities of plants per unit leaf area, leaf area ratios (LARs) represent the ratio of the total plant leaf area to the whole plant dry weight (Briggs *et al*, 1920b), and attempt to describe plant growth rates in terms of both the efficiency of the leaves as producers of new material, and in terms of plant leafiness (Hunt, 1978).

3.2 MATERIALS & METHODS

3.2.1 WEIGHT & GROWTH DATA

Organ fresh (touch dried), and dry (oven dried @ 80°C for 1hr, then 60°C until

constant weight was achieved) weights were obtained using standard laboratory balances. Plant heights and leaf areas were repeatedly measured, using standard measuring equipment.

3.2.2 RELATIVE WATER CONTENT (RWC) (after Kemp, 1960)

RWC measurements were made on the fourth plant leaf, which reportedly provides a good indication of whole plant water status in *V. faba* (Kassam, 1971). Fresh plant sections (1cm X 1cm) were weighed (FW1), and then incubated on wetted filter paper in petri dishes for 24 hours. They were touch-dried and re-weighed (FW2). After drying (as in 3.2.1), sections were re-weighed (DW). RWCs were then determined using the following equation:

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

3.2.3 RELATIVE GROWTH RATE (RGR) (after Thorne, 1960)

$$RGR = \frac{\text{Log e FW2} - \text{Log e FW1}}{\text{sample time} - \text{start time}}$$

3.2.4 NET ASSIMILATION RATE (NAR) (after Thorne, 1960)

$$NAR = \frac{w2 - w1}{t2 - t1} \times \frac{\text{logeL2} - \text{LogeL1}}{L2 - L1}$$

Where,

t1 = start time, t2 = sample time, w1 = dry weight start, w2 = dry weight sample, L1 = leaf area start, L2 = leaf area sample.

3.2.5 LEAF AREA RATIO (LAR) (after Thorne, 1960)

$$LAR = RGR / NAR$$

3.2.6 PHOTOSYNTHETIC & STOMATAL CONDUCTANCE MEASUREMENTS

Stomatal conductances and net photosynthetic rates were determined using a portable Infra Red Gas Analyser (IRGA - ADC LCAZ) on newly fully expanded, photosynthetically competent, non-senescing leaves. The IRGA measures CO_2 partial pressures entering and leaving a cuvette (containing the attached leaf), cuvette air temperature, relative humidity, and PAR levels, from which stomatal conductances and net photosynthetic rates can be calculated (von Cammerer & Farquhar, 1981).

3.3 RESULTS & DISCUSSION

3.3.1 FRESH AND DRY MATTER ACCUMULATION

Figs. 3.1 - 3.10 and anova analyses reveal that plant organ biomasses increased significantly in non-nodulated *V. faba* as the concentration of the supplied nitrogen source increased, and were maintained in the following order with respect to medium nitrogen nutrition: '1/2 nitrate' = 'combined nitrogen' > '1/10 nitrate' > 'no nitrate'. Indeed increasing nitrogen applications have previously been associated with the production of increased aerial growth in *V. faba* (Sprent & Thomas, 1984), and in other plant species (Breteler & Smit, 1974; Marschner, 1986; Ines-Minguez & Sau, 1989).

Nitrate nutrition reportedly results in the greatest biomass production in *T. aestivum* while abnormal foliar symptoms have been observed in *T. aestivum* when supplied solely with ammonia nutrition (Hawkins & Lewis, 1993). Several other plant species are reportedly intermediate in growth and appearance when supplied with 'combined nitrogen' nutrition (Bennet *et al*, 1964; Yin & Raven, 1997; Giordano & Bowes, 1997). However the data collected illustrates that ammonia additions did not result in reduced biomasses in *V. faba*. Indeed 'combined nitrogen' nutrition of non-nodulated *V. faba* resulted in the exhibition of total aerial DWs which were greater than those recorded by

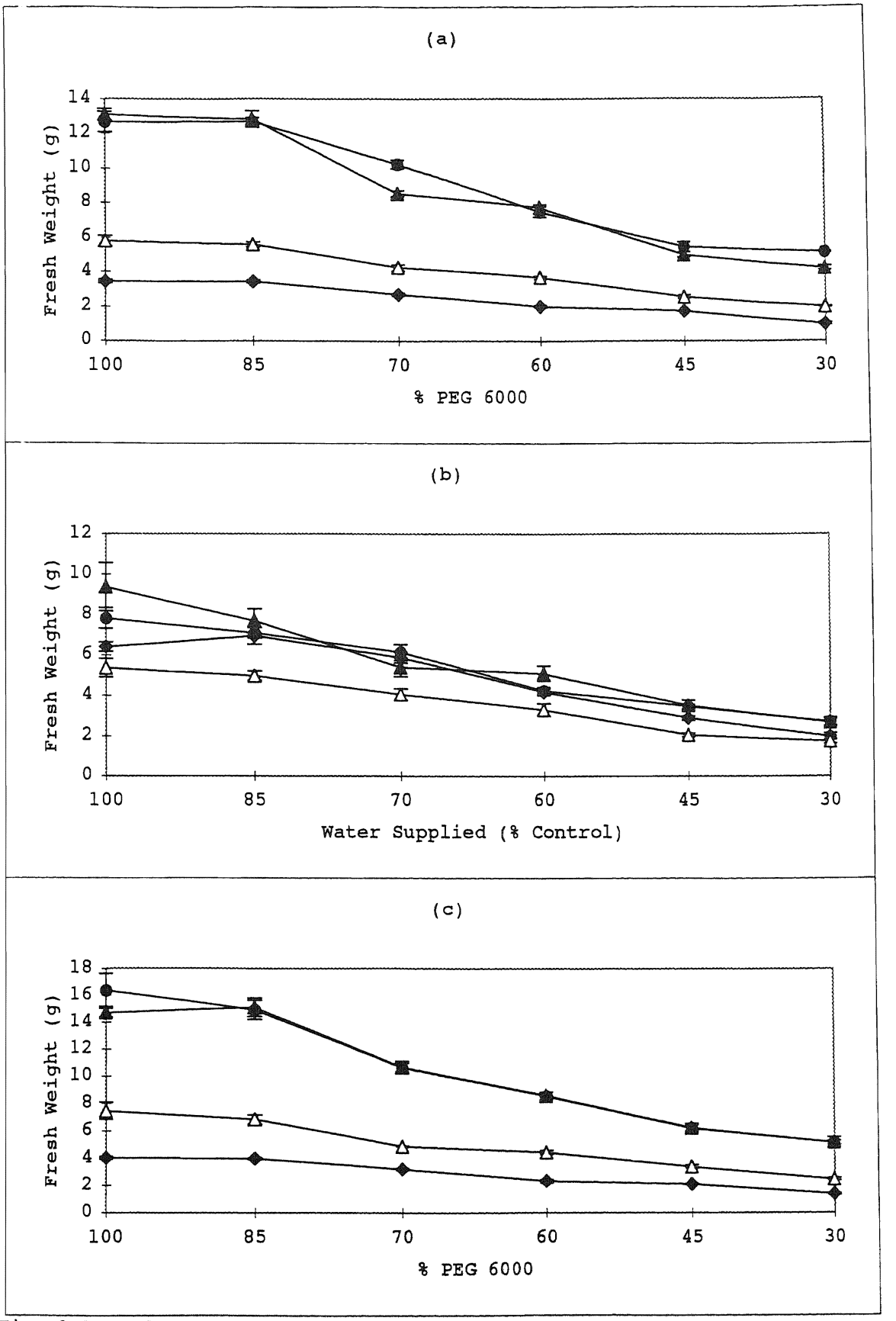


Fig. 3.1 Leaf FWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

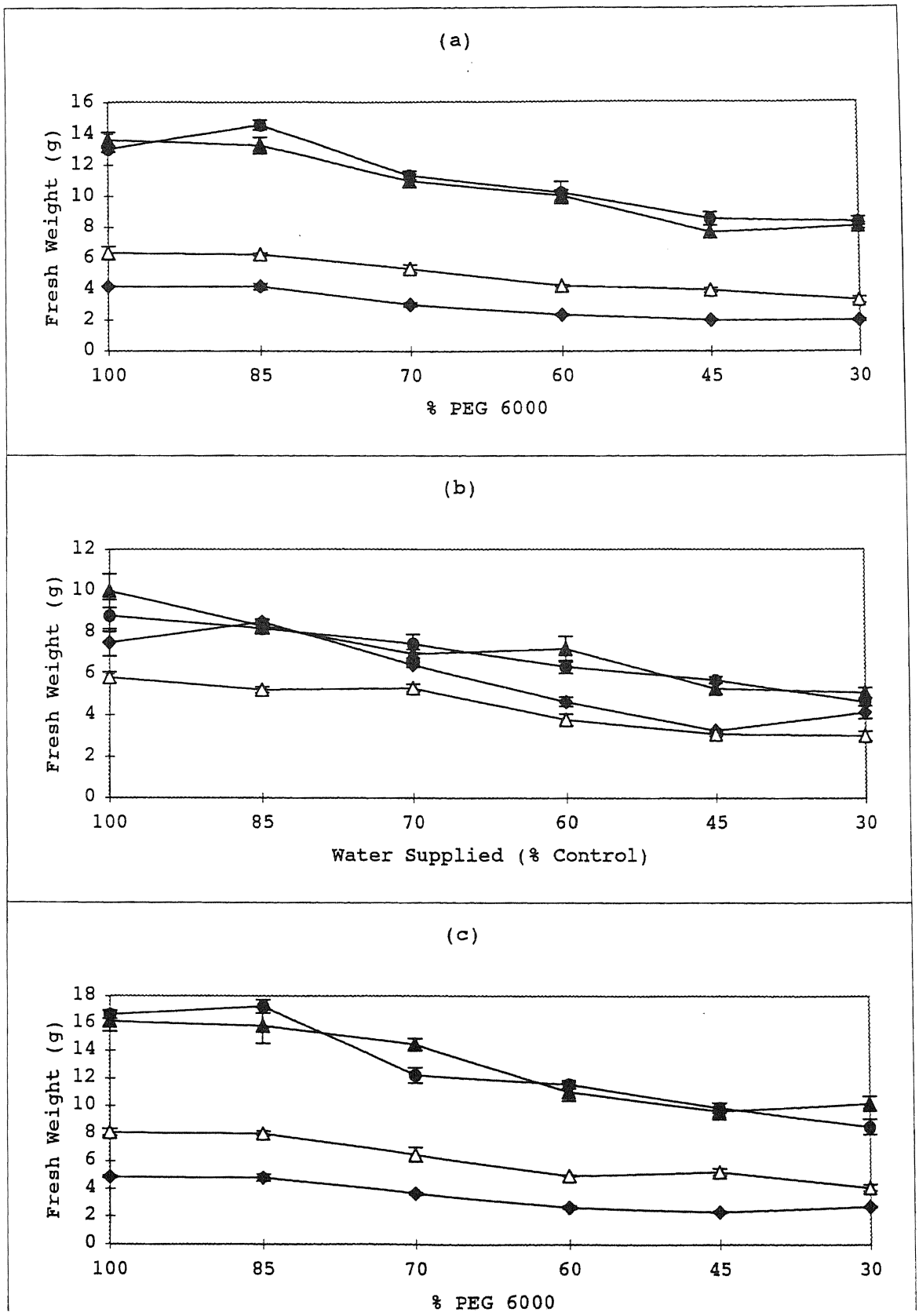


Fig. 3.2 Stem FWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

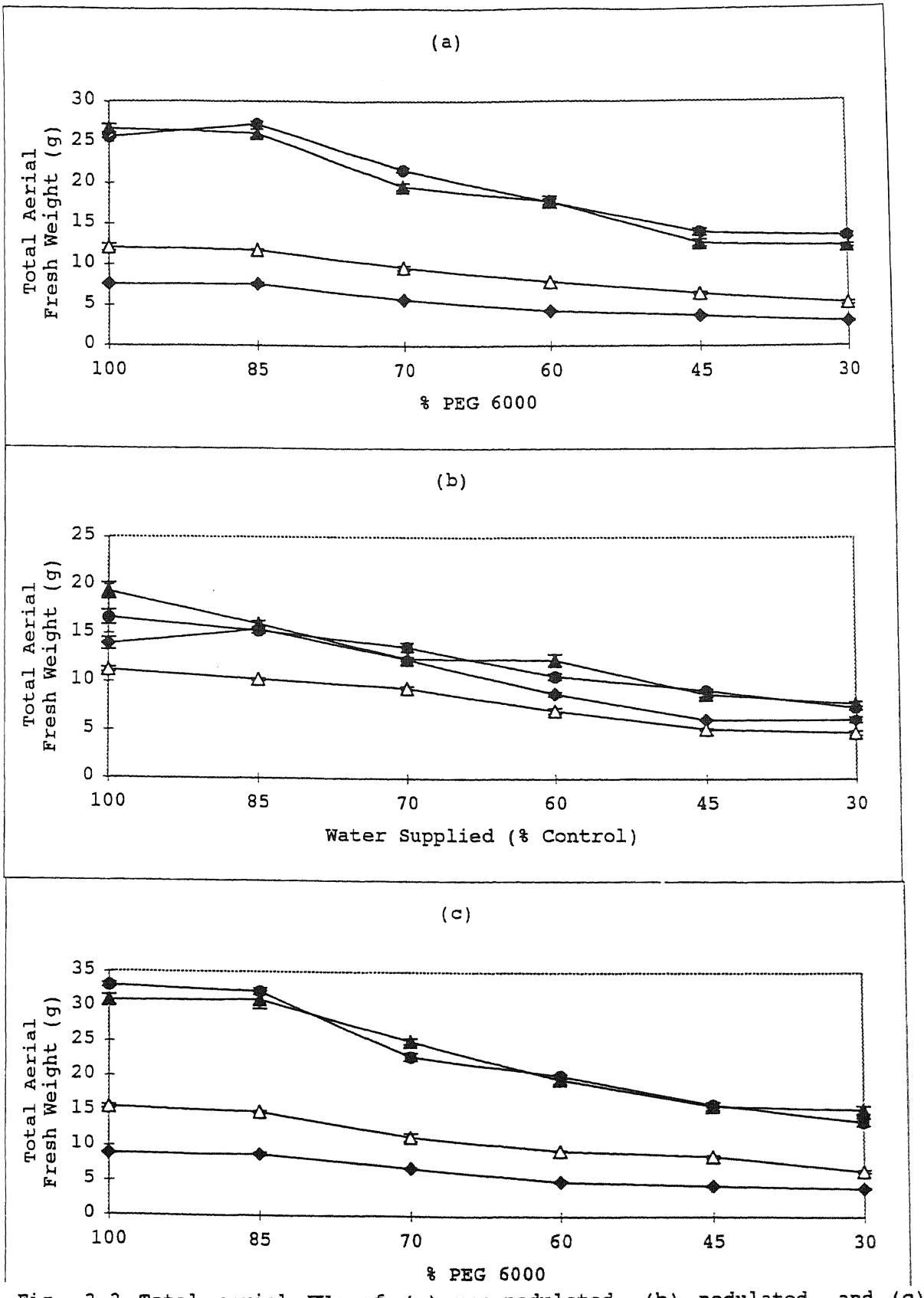


Fig. 3.3 Total aerial FWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

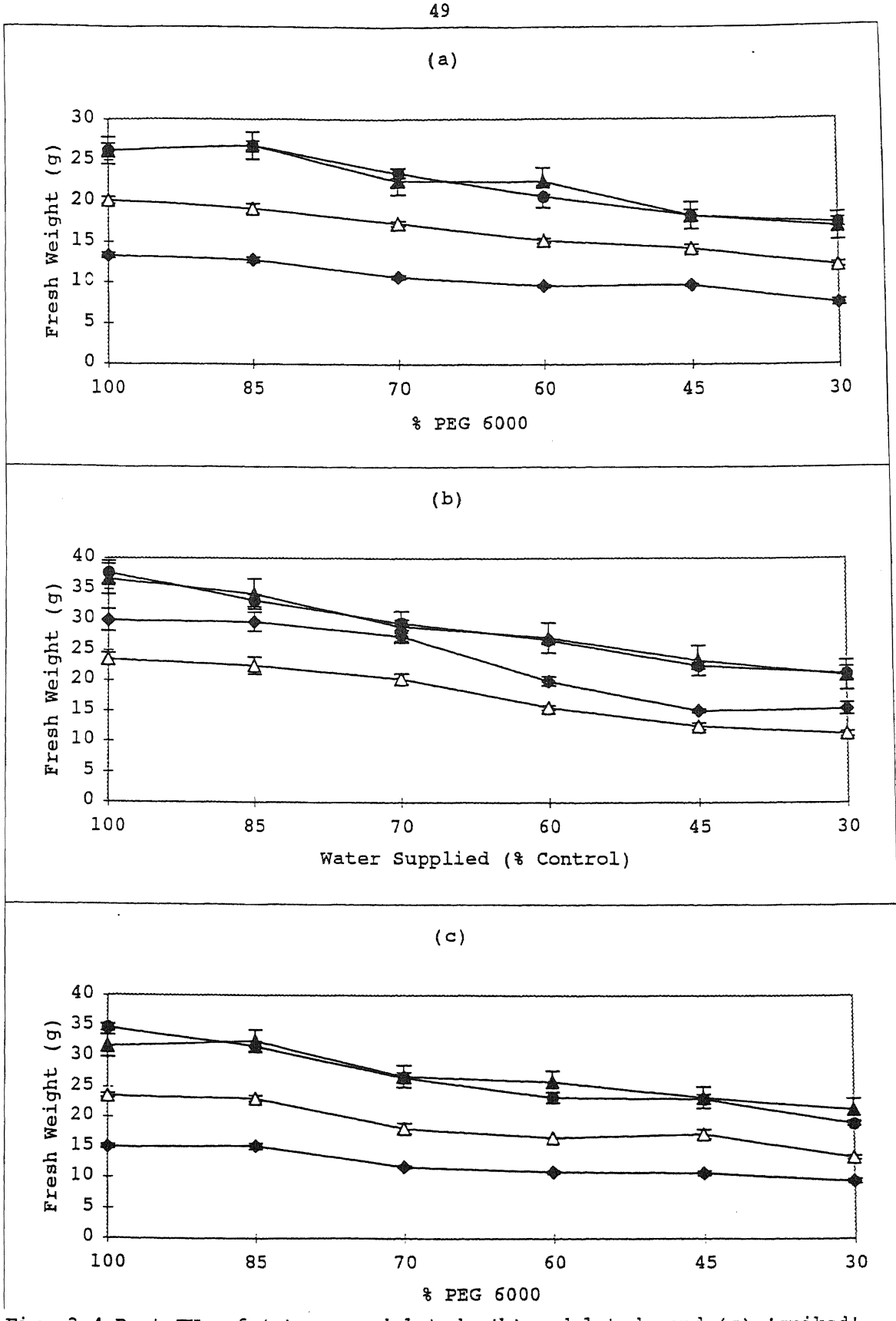


Fig. 3.4 Root FWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

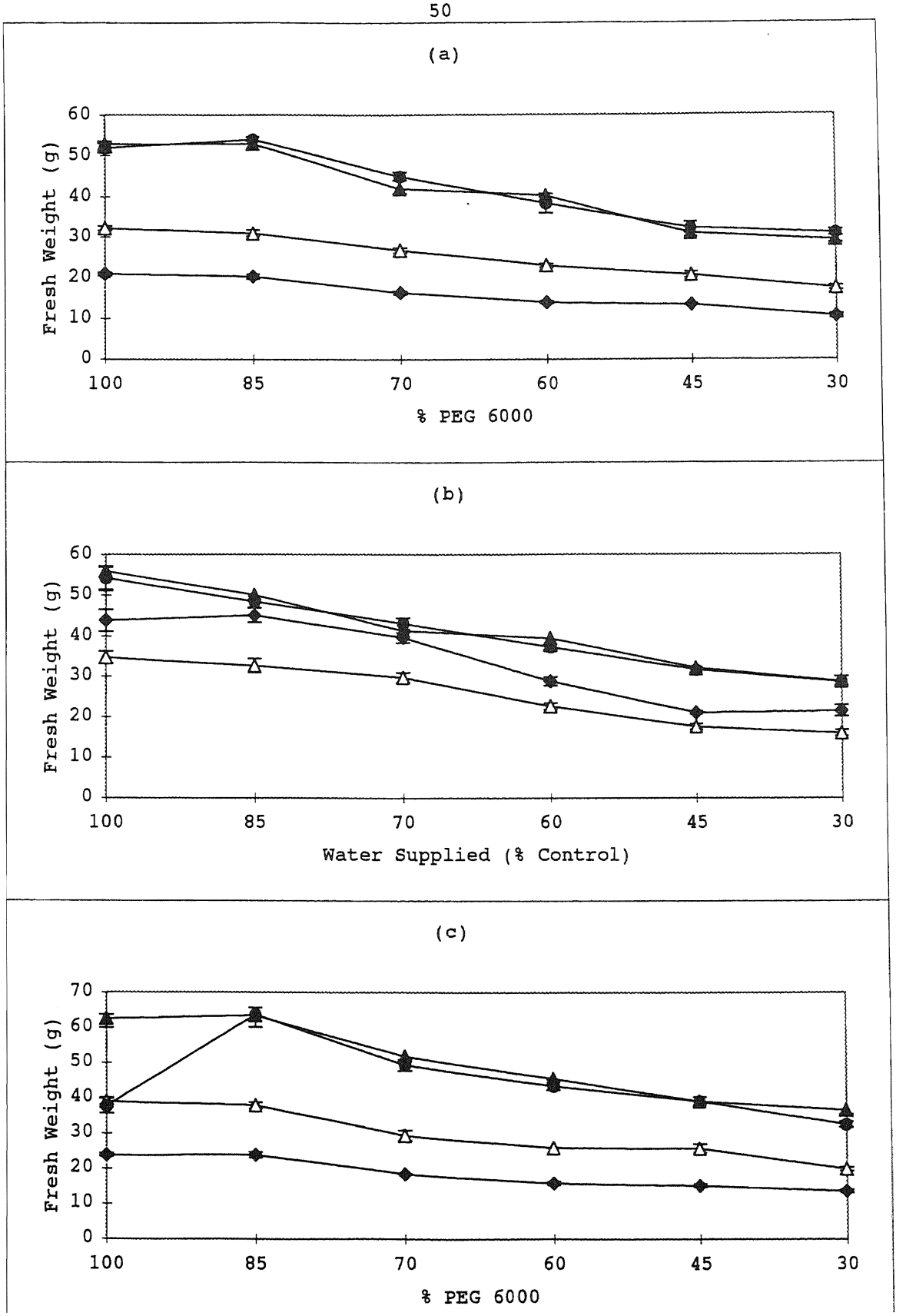


Fig. 3.5 Total FWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

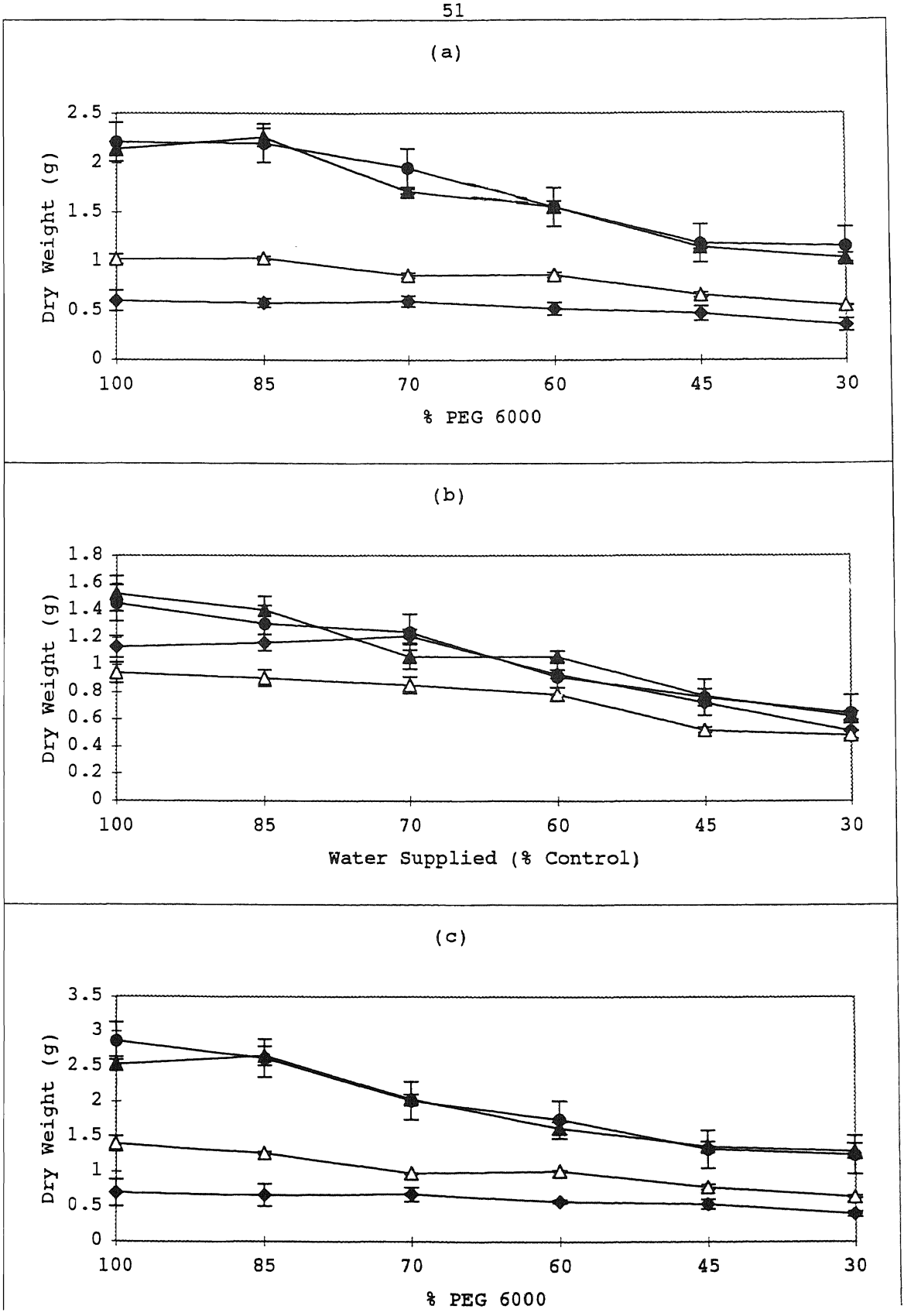


Fig. 3.6 Leaf DWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

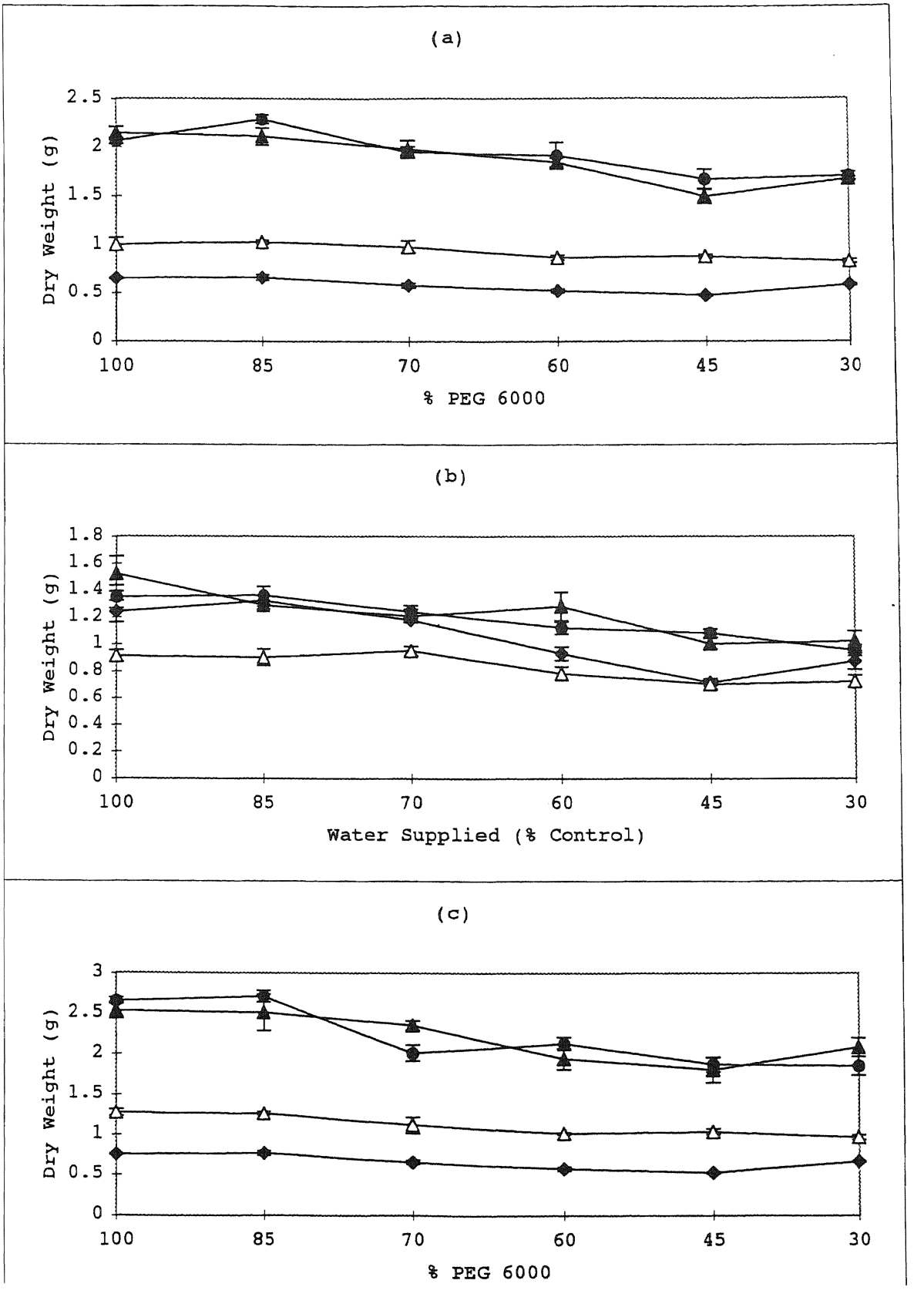


Fig. 3.7 Stem DWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

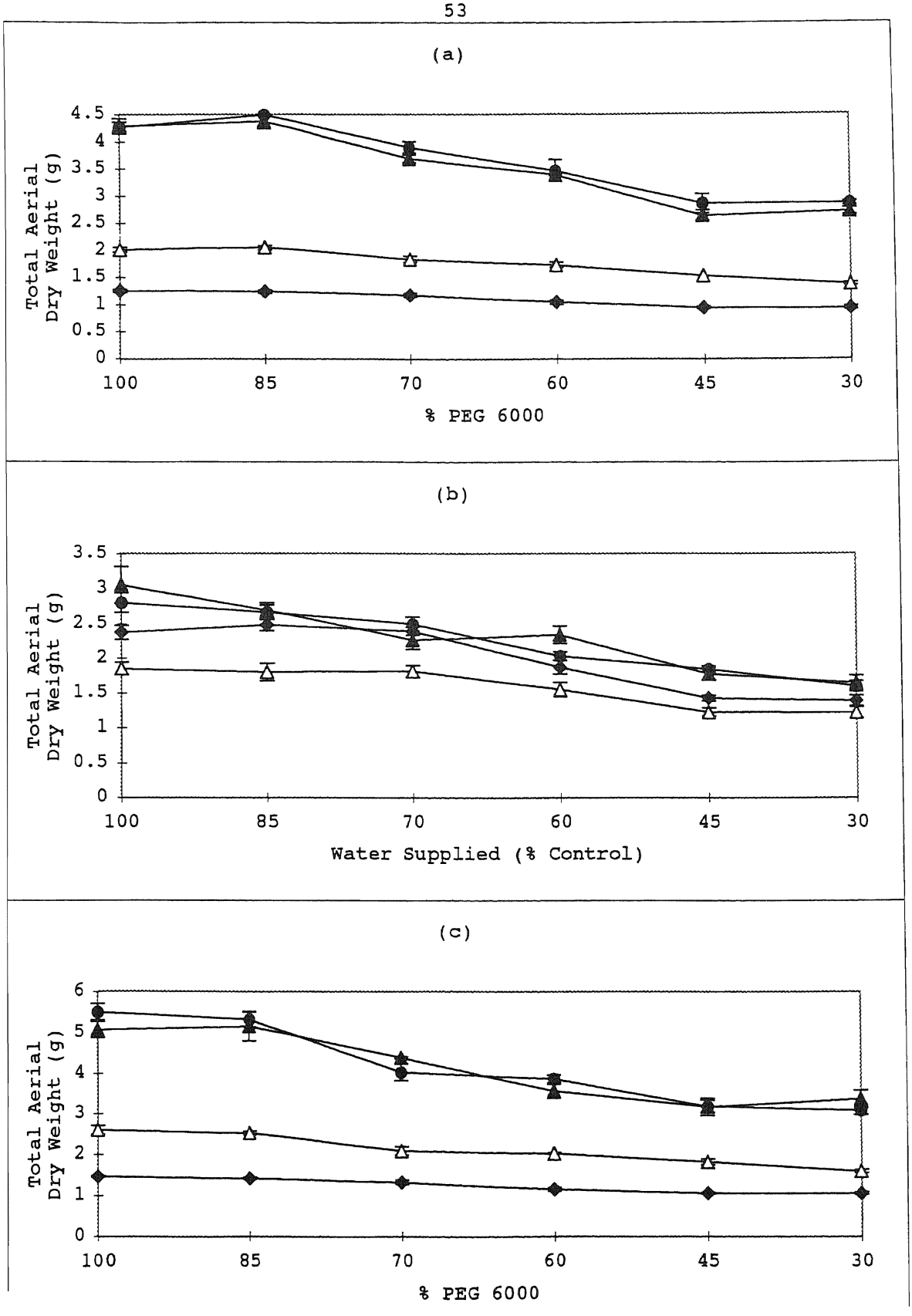


Fig. 3.8 Total aerial DWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

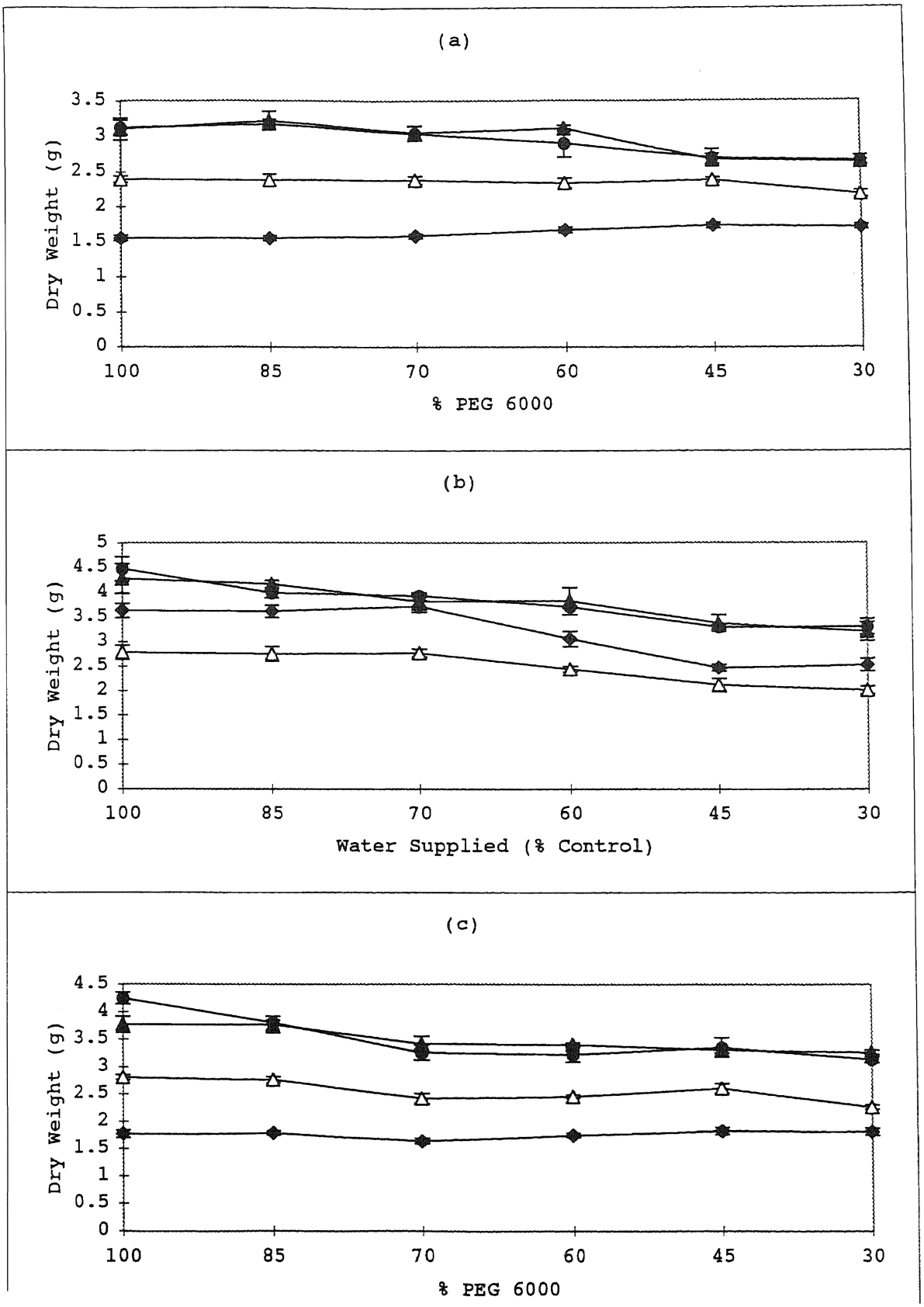


Fig. 3.9 Root DWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

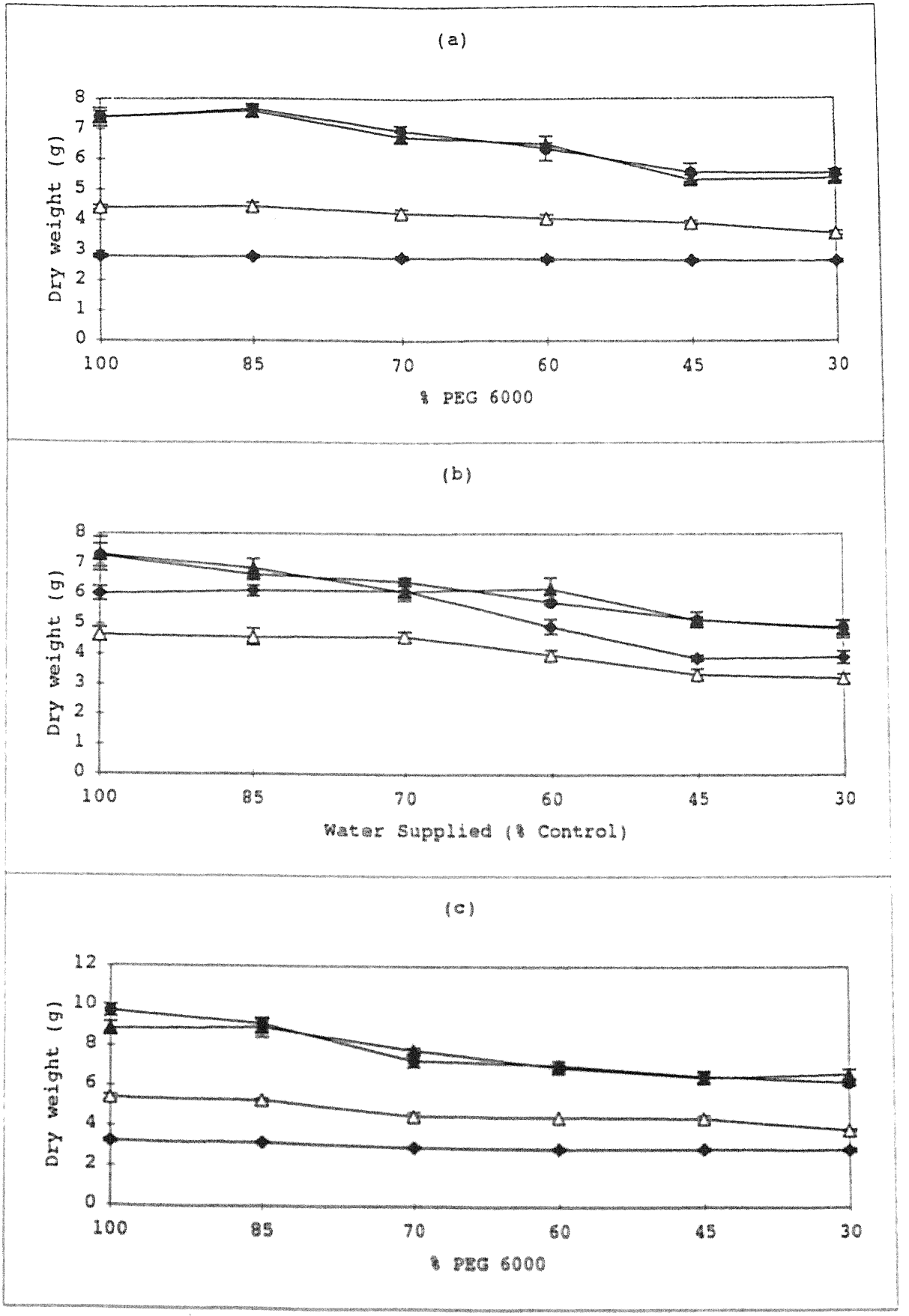


Fig. 3.10 Total DWs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

V. faba when supplied with '1/2 nitrate' nutrition (fig. 3.8); and root and total DWs which were comparable with those recorded by *V. faba* when supplied with equimolar '1/2 nitrate' nutrition (figs. 3.6; 3.7; 3.9 (a graphs)). Some previous workers have reported greater growth in other plant species when supplied with ammonia than with nitrate nutrition (Layzell *et al*, 1985; Troelstra *et al*, 1992), which Raven *et al*, (1985 & 1992) attributed to the lower associated water and photon costs of ammonia as opposed to nitrate assimilation. Previous workers have also reported a preferential uptake of ammonia in other plant species when supplied with 'combined nitrogen' nutrition, which indicates that ammonia may satisfy plant nitrogen requirements in the most energy efficient way (Taylor & Bloom, 1998; Colmer & Bloom, 1998). Increased uptake rates and reduced assimilation costs associated with ammonia nutrition may have contributed to the greater aerial growth recorded in *V. faba* when supplied with 'combined nitrogen' as opposed to with equimolar '1/2 nitrate' nutrition.

Figs. 3.1 - 3.10 and anova analyses reveal that ammonia 'spike' nutrition resulted in the exhibition of significantly greater root FWs and DWs than 'non-spiked' nutrition in *V. faba*, both when supplied with adequate irrigation and during water deficits. Increased root growth has previously been reported in other plant species when supplied with ammonia as opposed to with nitrate nutrition (Lewis *et al*, 1989; Dighton, 1991; Raven *et al*, 1992; Raab & Terry, 1994). Indeed assuming identical leaf morphology, and identical uptake characteristics for ammonia and for nitrate, and equal steady state concentrations of ammonium and nitrate in the medium, when the rate of uptake is limited by the solute concentration in the bulk phase the rate of ammonium uptake is reportedly much lower (ten times) than that of nitrate (Robinson, 1986). The lower diffusion coefficient for ammonia in soil (Gutschick, 1981)

indicates that plants supplied solely with ammonia nutrition may require more extensive root/hair/mycorrhiza systems than those supplied with nitrate (Dighton 1991; Fitter 1991; Pankow *et al*, 1991; Read 1991; Raven *et al* 1992). Additional energy is required to build additional roots, and energy may be at a premium during water deficits as 'fuel' is required for water deficit tolerance adaptations. However root increases occur independently during water deficits (potentially resulting in increased water uptake; Sharp & Davies, 1985; McDonald & Davies, 1996), thus such costs are not 'additional' when ammonia is supplied during water deficits.

Aerial biomasses were not significantly greater in *V. faba* when supplied with ammonia 'spiked' as opposed to with 'non-spiked' nutrition. Plants supplied with ammonia may have lower requirements for large aerial biomasses than those supplied with nitrate, as ammonia applications have previously been reported as stimulative for photosynthesis in intact chloroplasts (de-Beneditti *et al*, 1976). Furthermore the quantified 'aerial biomasses' did not discriminate between structural and photosynthetic plant material and it is possible that medium ammonia additions may have resulted in the exhibition of increased photosynthetic growth at the expense of structural aerial growth (see sections 3.3.5 & 3.3.9).

Figs. 3.1 - 3.10 and anova analyses reveal that nodulated 'no nitrate' supplied *V. faba* exhibited significantly greater organ weights than non-nodulated 'no nitrate' supplied *V. faba*. This was expected as increasing nitrogen supplies have previously been shown to result in increased growth in *V. faba* (Sprent & Thomas, 1984), and nitrogen fixation potentially provided a nitrogen source to the nodulated 'no nitrate' supplied *V. faba* (see table 2.3). Greater organ weights were recorded in nodulated 'no nitrate' supplied *V. faba* than in ('non spiked') '1/10 nitrate' supplied *V. faba*; an indication

of the effectiveness of nitrogen fixation in this species (as previously reported by Richards & Soper, 1979). Previous workers have reported that *V. faba* exhibits greater biomasses when supplied with medium nitrogen than when reliant on nitrogen fixation during periods of adequate irrigation (Ryle *et al*, 1978; Ines-Minguez & Sau, 1989), however these differences are reportedly less marked for *V. faba* than for any other legume grown under a controlled environment; a further indication of the effectiveness of nitrogen fixation in this species.

Dry matter accumulation reportedly decreases as water deficits increase, both in *V. faba* (Hebblethwaite, 1982), and in other plant species (Gallacher & Sprent, 1978; Nonami & Boyer, 1990) as leaves, flowers and pods may be shed, lodging may increase, and ultimately seed yields may decrease (Heatherly & Elmore, 1986). Expansive growth loss is reportedly a sensitive indicator of 'stress' (Hsaio *et al*, 1976). Accordingly figs. 3.1 - 3.10 (and anova analyses) illustrate that leaf fresh and dry biomasses, and root fresh biomasses (and root dry biomasses in *V. faba* when supplied with nodulated and with 'spiked' nutrition) decreased significantly in *V. faba* (when supplied with all nitrogen sources) as water deficits increased. That root dry weights were better maintained than aerial weights reflects the R:S ratio increases which are reportedly characteristic during water deficits (see 3.3.4). The highest organ weights were maintained in non-nodulated *V. faba* when supplied with the most concentrated medium nitrogen nutrition; organ weights were maintained in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' = '1/2 nitrate' > '1/10 nitrate' > 'no nitrate' nutrition, throughout water deficits.

That significantly greater weights were maintained in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits, infers that

nitrogen fixation may not be as susceptible to water deficits as is classically reported (e.g. by Sprent, 1971; Serraj et al, 1998). It has been reported that growth in *V. faba* is not limited by decreases in nitrogen fixation during water deficits (Plies-Balzer *et al*, 1995), and a *G. max* cultivar, 'Jackson', has been identified as water deficit tolerant for nitrogen fixation (Serraj & Sinclair, 1997), inferring that nitrogenase activities may be maintained during water deficits in some plant cultivars.

Figs. 3.4 & 3.9 and anova analyses reveal that ammonia 'spiked' *V. faba* maintained significantly greater root biomasses than 'non-spiked' *V. faba* throughout water deficits (the preferential stimulation of root growth as opposed to shoot growth in plants when supplied with medium ammonia nutrition has previously been discussed; pg. 56). Increased root growth is reportedly associated with increased water uptake in *V. faba* (Sau & Ines-Minguez, 1990), and is therefore desirable during water deficits.

It is thus apparent that while water deficits do result in reduced biomass accumulation, *V. faba* maintained significantly greater biomasses throughout water deficits when supplied with increasingly concentrated medium nitrogen nutrition.

3.3.2 FW:DW ratios

Figs. 3.11 - 3.15 and anova analyses reveal that FW:DW ratios were maintained at significantly greater levels in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and that FW:DW ratios were maintained in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate'. By inference the greatest water contents were maintained in *V. faba*

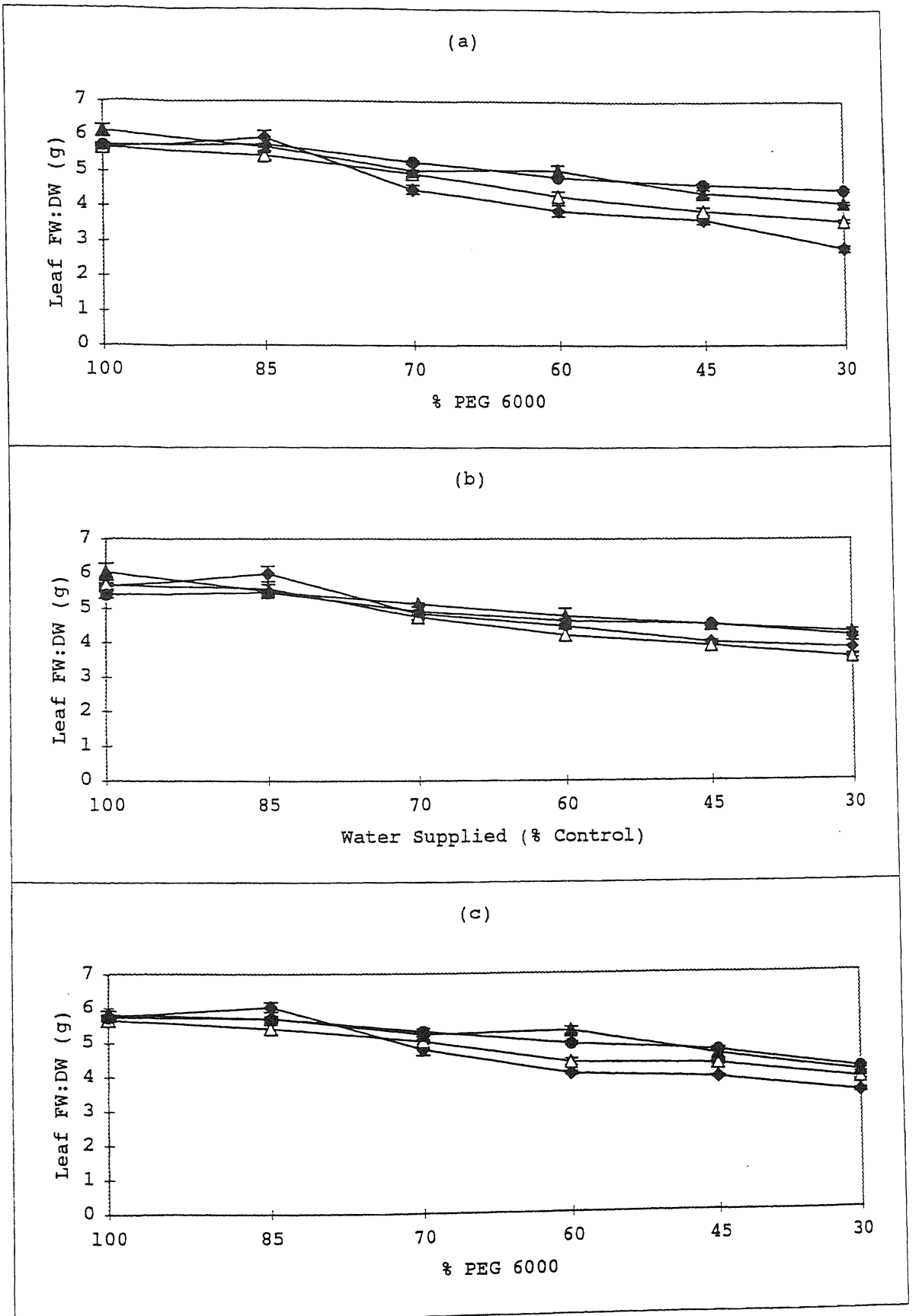


Fig. 3.11 Leaf FW:DWs in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

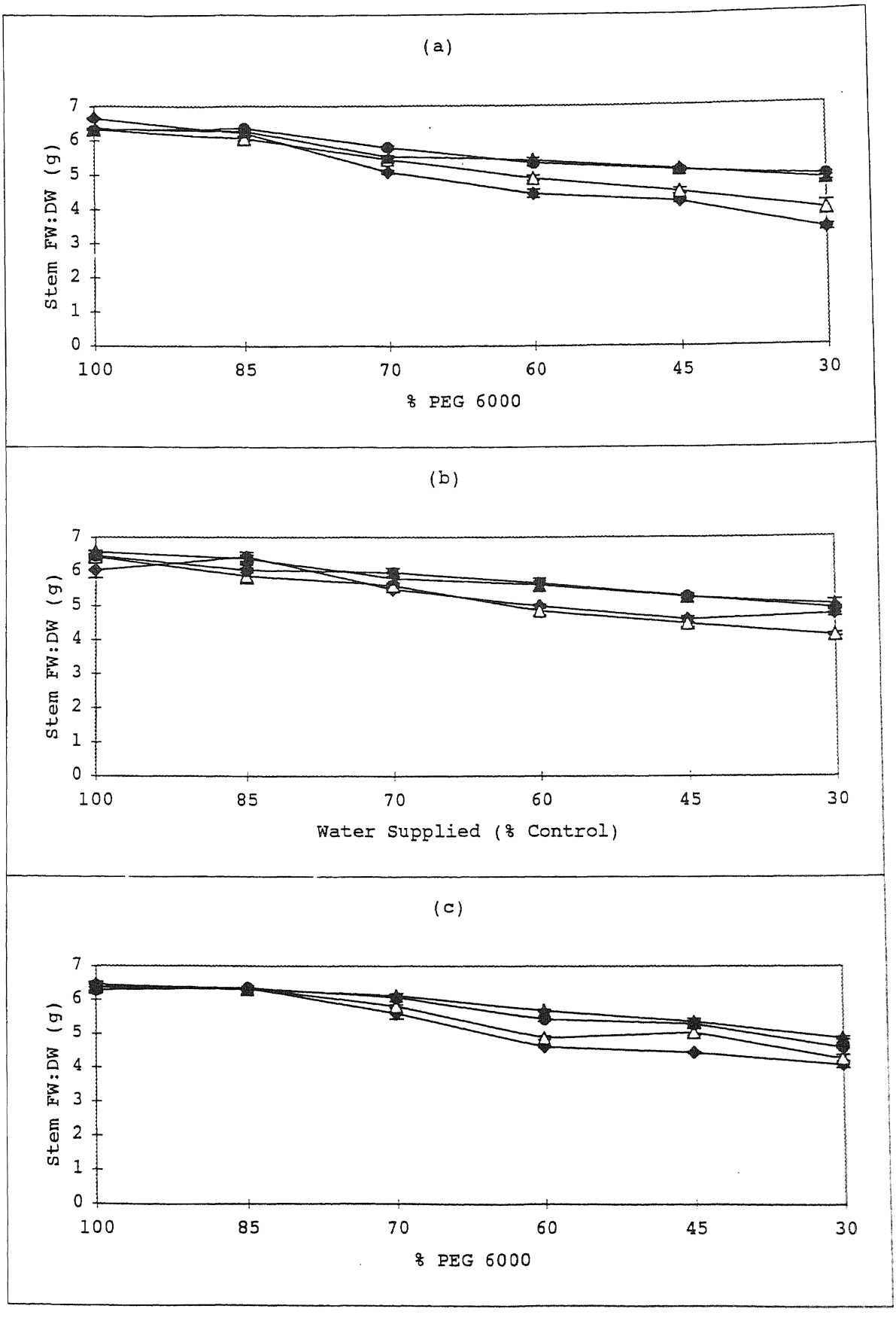


Fig. 3.12 Stem FW:DWs in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

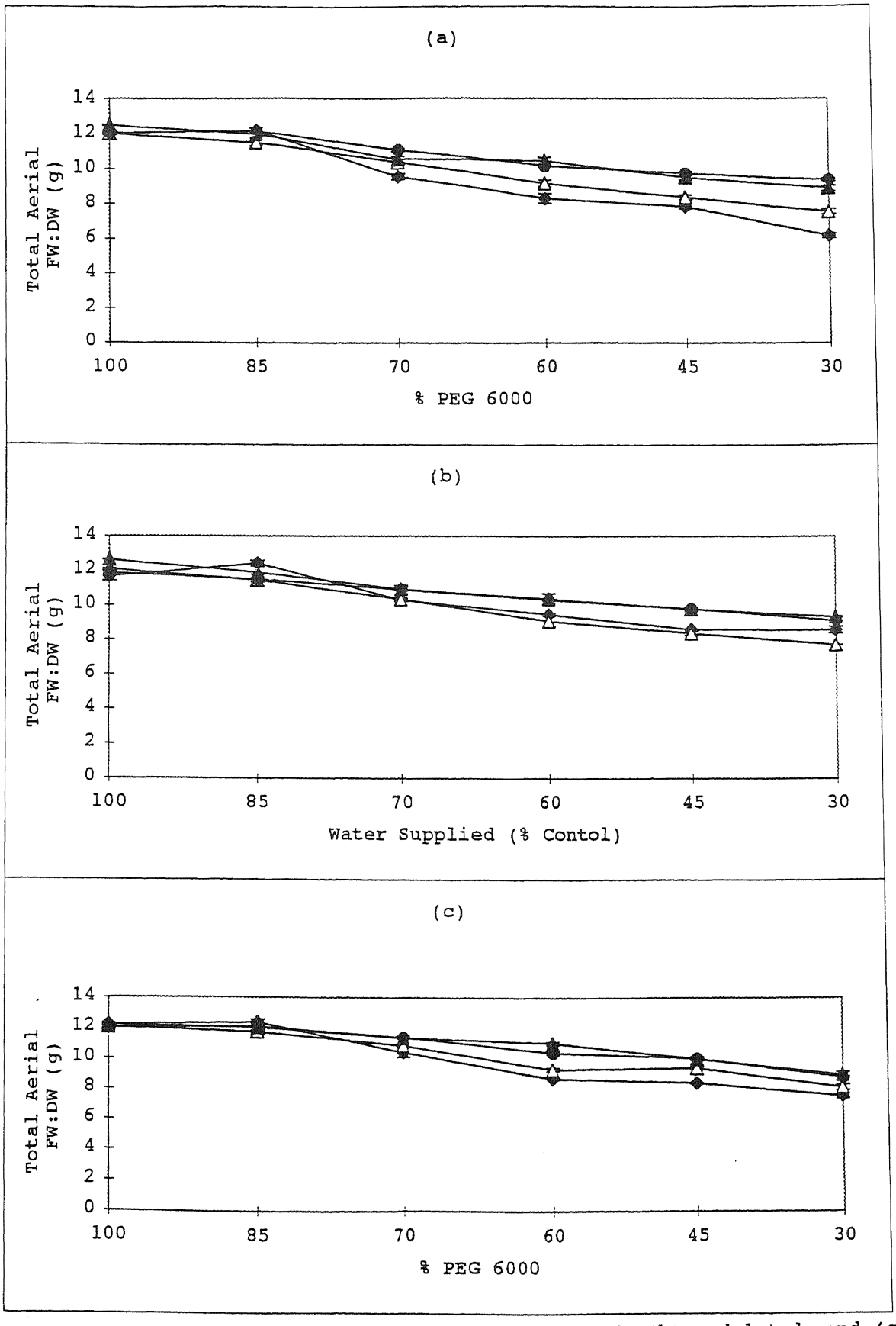


Fig. 3.13 Total aerial FW:DWs in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

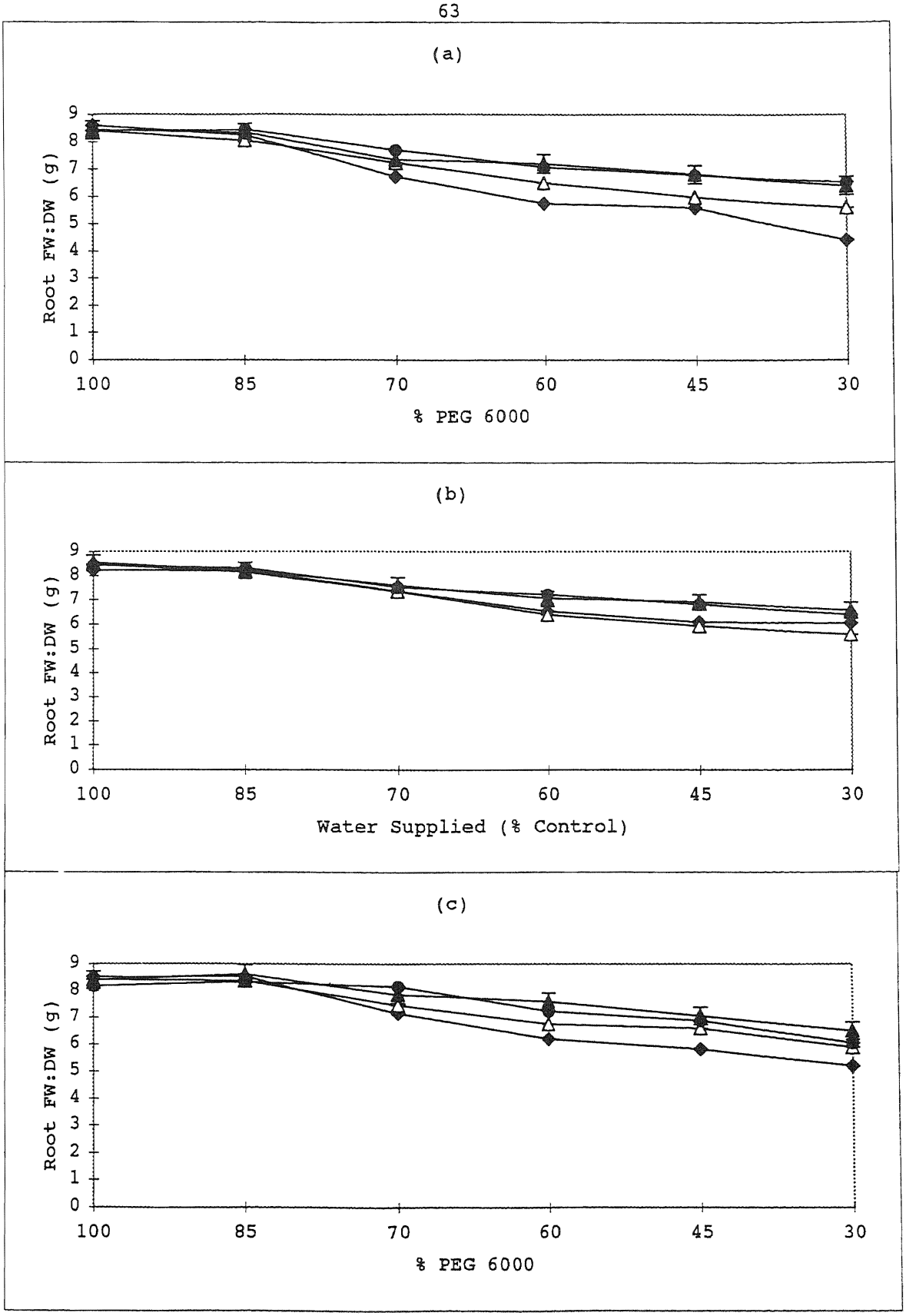


Fig. 3.14 Root FW:DWs in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

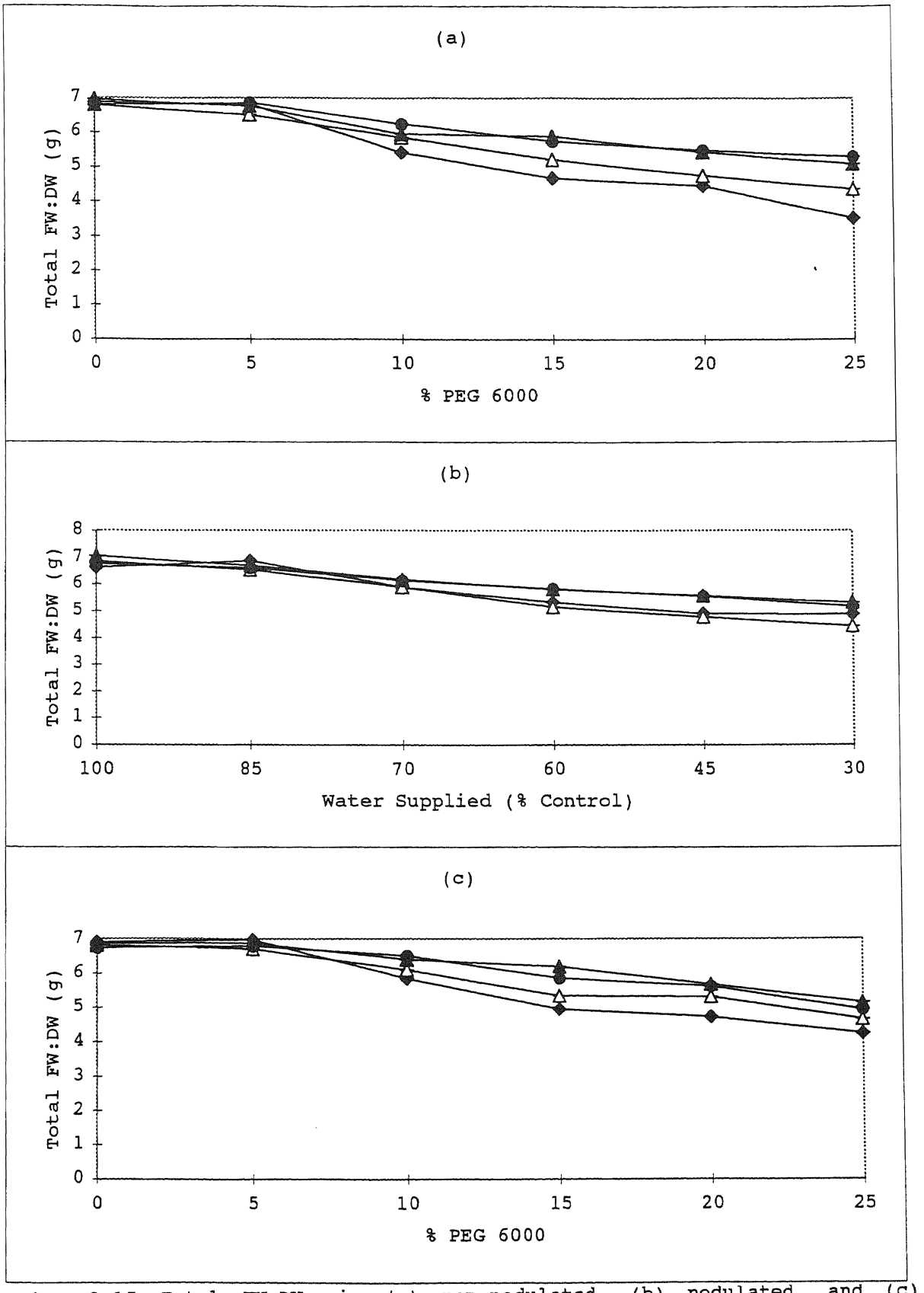


Fig. 3.15 Total FW:DWs in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

when supplied with increasingly concentrated medium nitrogen nutrition. FW:DW ratios decreased significantly (in all organs) during water deficits (as previously reported in other plant species, Ferrario-Mery *et al*, 1998), and indicate that water contents decreased in all organs as water deficits increased.

Non-nodulated *V. faba* supplied with 'no nitrate' nutrition recorded significantly lower FW:DW ratios than nodulated 'no nitrate' supplied *V. faba*, indicating that water contents were better maintained in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*. Again this may reflect the additional nitrogen potentially available to nodulated *V. faba* via nitrogen fixation, which may be utilised in nitrogen dependent water deficit tolerance adaptations (e.g. compatible solute production as described in the introduction; pg. 16).

Comparing FW:DW ratios between *V. faba* when supplied with different nitrogen sources reinforces the observation that (DW) biomasses increased as *V. faba* was supplied with increasingly concentrated medium nitrogen nutrition. Indeed *V. faba* supplied with ammonia 'spike' nutrition recorded significantly greater FW:DW ratios than 'non-spiked' *V. faba*, in the roots only. Increased root growth is reportedly associated with increased water uptake in *V. faba* (Sau & Ines-Minguez, 1990), and is therefore desirable during water deficits. *V. faba* which exhibited the greatest FW:DWs contained more water, however the water contents at full turgor were not considered in the calculation of FW:DW, and as such FW:DW data is not as informative as RWC data.

3.3.3 RELATIVE WATER CONTENTS (RWCs)

Figs. 3.16 & 3.17, and anova analyses reveal that leaf and root RWCs increased significantly in non-nodulated *V. faba* when supplied with increasingly

concentrated medium nitrogen nutrition, and that RWCs were maintained in the following order in the leaves and roots of non-nodulated *V. faba* with respect to medium nitrogen nutrition: 'combined nitrogen' = '1/2 nitrate' > '1/10 nitrate' > 'no nitrate', throughout water deficits. Although ammonia nutrition reportedly results in the exhibition of low leaf water potentials (Quebedeaux & Osbun, 1973), 'combined nitrogen' nutrition resulted in the maintenance of RWCs at similar levels as those recorded in *V. faba* when supplied with equimolar '1/2 nitrate' nutrition. Greater RWCs may reflect the greater root biomasses (3.3.1), and increased osmotic adjustment (see section 4.4), which were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and which have previously been associated with maintained water uptake in *V. faba* (Sau & Ines-Minguez, 1990), and with maintained RWCs in other plant species (Singh & Gupta, 1983; Turner & Stewart, 1986).

Figs. 3.16 & 3.17 and anova analyses reveal that significantly greater leaf and root RWCs were maintained in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*. Again the additional nitrogen potentially available to nodulated 'no nitrate' supplied *V. faba* via nitrogen fixation may have contributed towards greater root growth and higher levels of osmotic adjustment than could be achieved in non-nodulated 'no nitrate' supplied *V. faba* (see section 4.4).

However figs. 3.16 & 3.17, and anova analyses reveal that ammonia 'spiked' *V. faba* did not exhibit significantly greater RWCs than 'non-spiked' *V. faba*. It has previously been reported that ammonia nutrition may result in reduced leaf RWCs (Raab & Terry, 1994), while increasing the amount of dry matter per unit area. It is possible that 'spiked' as opposed to 'non spiked' *V. faba* preferentially exhibited increased growth as opposed to increased RWC

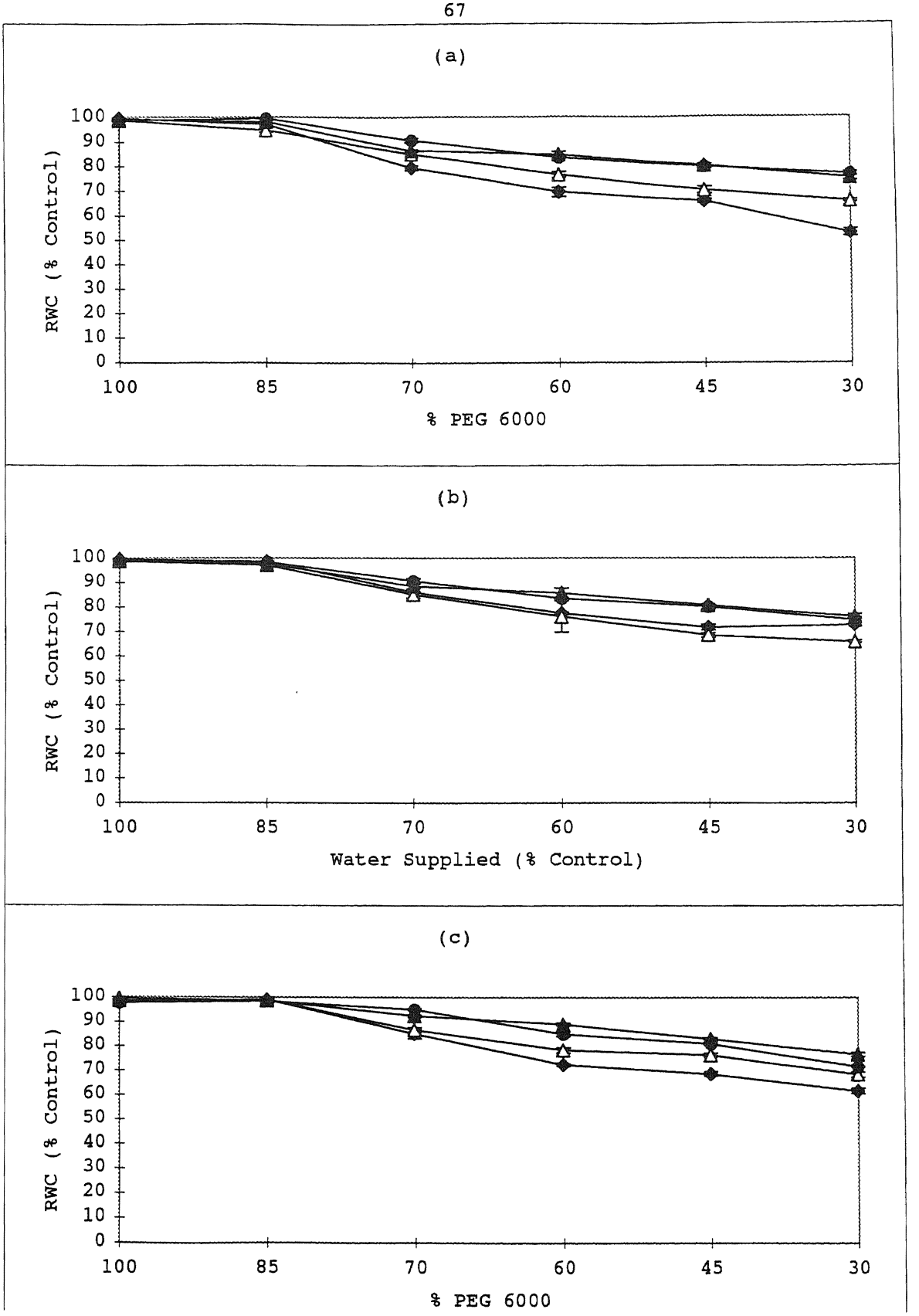


Fig. 3.16 Leaf RWCs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

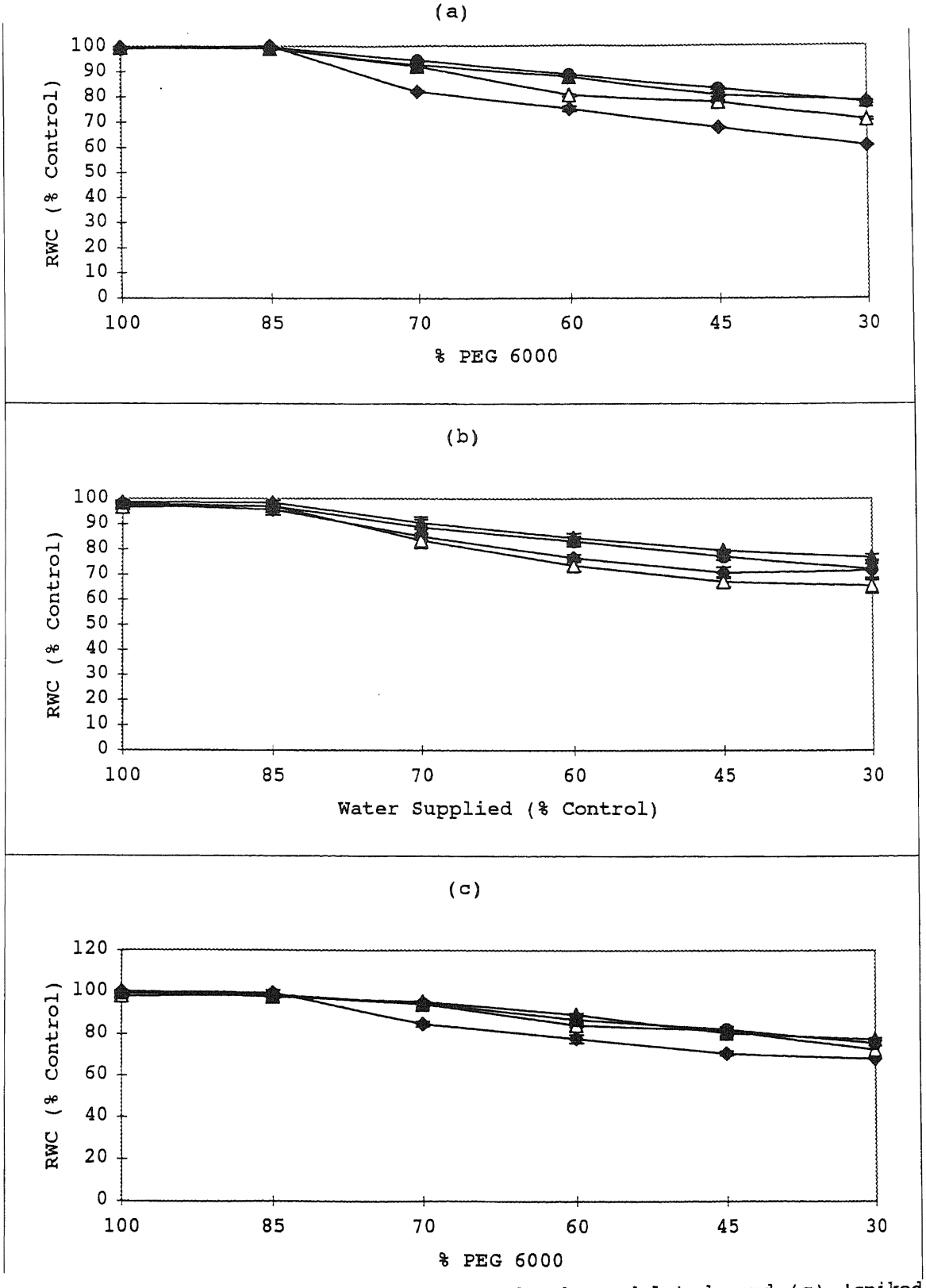


Fig. 3.17 Root RWCs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

maintenance as ammonia requires assimilation, as it is toxic and cannot be stored (Raven, 1985). 'Spiked' *V. faba* did not exhibit significantly different RWCs than 'non-spiked' *V. faba*, however ammonia 'spike' nutrition resulted in the exhibition of increased root growth in *V. faba* during water deficits (figs. 3.4 & 3.9), and also resulted in the exhibition of significantly greater cumulative leaf areas (section 3.3.5; pg. 74) and heights (section 3.3.10; pg. 94), which indicate increased nitrogen assimilation as opposed to nitrogen storage (as further discussed in chapter seven).

Figs. 3.16 & 3.17 and anova analyses demonstrate that leaf and root RWCs decreased significantly during water deficits (as previously reported in other plant species, Collinson *et al*, 1997), and illustrate that RWCs were maintained at increasing levels throughout water deficits in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. Metabolic processes are reportedly more sensitive to turgor and cell volume changes than to absolute water potentials, indicating that maintained RWCs (as opposed to e.g. maintained leaf water potentials due to increased cell wall elasticities, which would simultaneously reduce cell volumes and intermolecular distances - which may be critical for continued metabolic activities, Clifford *et al*, 1998) may represent a prior requirement for the maintenance of metabolism during water deficits. Maintained RWCs may result in continued expansive growth, greater stomatal conductances and net photosynthesis, and hence in continued dry matter accumulation during water deficits (Raab & Terry, 1994). The inference here is that as RWCs were maintained at increasingly great values in *V. faba* when supplied with increasingly concentrated nitrogen nutrition, net photosynthesis and overall metabolism may also have been maintained at increasingly great values in *V. faba* when supplied with the same increasingly concentrated medium nitrogen

nutrition (see section 3.3.7), Sinclair & Ludlow, 1985.

3.3.4 ROOT : SHOOT RATIOS (R:Ss)

Some plant species e.g. *Phaseolus vulgares* reportedly produce new conductive roots roughly in proportion to new leaves during periods of adequate irrigation (Fiscus & Markhart, 1979). However fig. 3.18 and anova analyses reveal that R:Ss in *V. faba* were significantly affected by the supplied nitrogen source, and that R:Ss increased significantly in non-nodulated *V. faba* when supplied with decreasingly concentrated medium nitrogen nutrition during periods of adequate irrigation (i.e. with 0% PEG or 100% control water; fig. 2.2). This may reflect the fact that total nitrogen uptake is reportedly related to root length density (Hodge *et al*, 1999), indicating that plants growing in nitrogen deficient media may require longer roots for adequate nitrogen uptake (McDonald & Davies, 1996).

Sutherland *et al*, (1985), working with *V. faba*, and Sprent & Thomas, (1984) and Vessey *et al*, (1990), working with other leguminous species suggested that roots grow in response to (transported) nitrogen deficiencies, however the growth potential and sink strength for nutrients may decrease in the leaves of nitrogen limited plants, indicating that nitrogen deficiencies may not solely be responsible for the relatively increased root growth exhibited in plants which grow in nitrogen deficient environments (Hocking & Meyer, 1991). A greater R:S (DW) has been observed in *T. aestivum* when grown with elevated CO_2, indicating that carbon may potentially limit root growth (Hocking & Meyer, 1991).

Indeed net photosynthesis decreased significantly in *V. faba* as the concentration of the supplied nitrogen source decreased, even during periods of adequate irrigation (section 3.3.7), inferring that reduced photosynthate availabilities may have contributed to the reduced shoot growth exhibited in

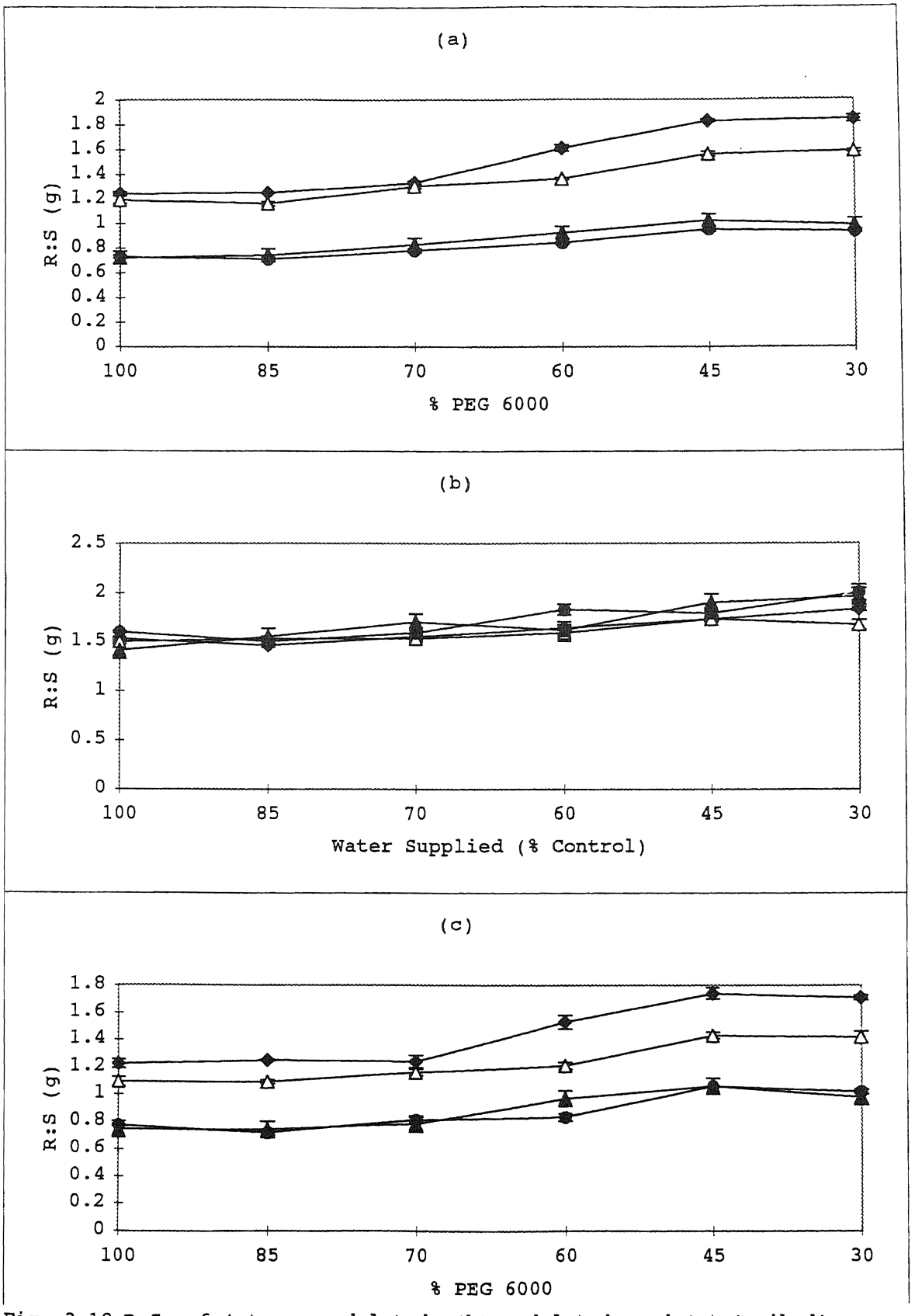


Fig. 3.18 R:Ss of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

V. faba when supplied with decreasingly concentrated medium nitrogen nutrition during periods of adequate irrigation. Furthermore decreasing leaf cell plasticities (Snir & Neumann, 1997) and increasing root cell plasticities (McDonald & Davies, 1996) are reportedly exhibited in other plant species when supplied with decreasingly concentrated medium nitrogen nutrition, and may contribute to relative shoot growth reductions.

Earlier research has indicated that water deficits result in proportionally similar growth reductions in the stems, leaves, and roots of *V. faba*, and that dry weight organ ratios alter little (Grzesiak *et al*, 1989), however fig. 3.18 and anova analysis reveal that R:Ss increased significantly in *V. faba* during water deficits, allowing a larger potential area for water (and nitrogen) uptake (as previously reported in other plant species, Etherington, 1962; Rufty *et al*, 1984; Winzer *et al*, 1992; McDonald & Davies, 1996).

Anova analyses reveal that significantly greater R:Ss were recorded in non-nodulated as opposed to in nodulated 'no nitrate' supplied *V. faba*. Nodulated 'no nitrate' supplied *V. faba* may have utilised nitrogen produced via nitrogen fixation (table 2.3) in order to maintain higher root and shoot biomasses (Marschner, 1986), and did not exhibit the proportionally greater root growth increases which are reportedly characteristic of plants growing in nitrogen deficient environments.

'Spiked' *V. faba* did not exhibit statistically different R:Ss than 'non-spiked' *V. faba*, but rather exhibited relatively increased root (fig. 3.9) and aerial (figs. 3.20 & 3.25) growth.

R:S alterations during water deficits may result in part from a reduced capacity to maintain shoot expansive growth (Boyer 1968; McDonald & Davies, 1996), as shoot cell wall extensibilities (Passioura *et al*, 1993), and RWCs

(Singh & Gupta, 1983) may decrease, and stomatal resistances increase (Sperry *et al*, 1998) during water deficits, inferring that decreasing photosynthate and reductant availabilities may limit shoot growth during water deficits. Indeed RWCs (figs. 3.16 & 3.17) and net photosynthesis (fig. 3.22) were maintained at decreasing values during water deficits in *V. faba* when supplied with decreasingly concentrated medium nitrogen nutrition; the same nitrogen sources which when supplied to *V. faba* resulted in the exhibition of the greatest R:S increases during water deficits.

Masle & Passioura, (1988), rejected the influence of limited carbon and nutrient supplies on reduced relative leaf growth rates in favour of a root induced hormonal message in soils of high mechanical impedance (see also Passioura & Fry, 1992; McDonald & Davies, 1996). While some root growth may be inhibited by drying soil during water deficits, other roots may grow in moister soil and may have access to increased carbohydrates as aerial sink strengths decrease (Hsaio & Jing, 1987; Wardlaw, 1993), which may potentially 'fuel' root biomass increases during water deficits. However R:S increases were similar in *V. faba* whether grown hydroponically or in solid media (fig. 3.18; comparing graphs a & b), indicating that increased root:medium mechanical impedance may not have solely mediated the R:S increases recorded in *V. faba* during water deficits, and that soil water contents were probably significant.

It is apparent that controversial theories abound as to the extent to which nitrogen (Vessey *et al*, 1990) or carbohydrate deficiencies (Hocking & Meyer, 1991) as opposed to altered cell wall extensibilities (Snir & Neuman, 1997) result in relatively increased root growth in plants which grow in nitrogen depleted environments, and the extent to which decreasing shoot growth is attributable to decreasing leaf water potentials (Comstock &

Mencuccini, 1998), to decreasing photosynthate supplies (Sperry *et al*, 1998), or to mediation by root induced hormonal messages (Masle & Passioura, 1988) during water deficits (see McDonald & Davies, 1996).

3.3.5 LEAF AREA RATIOS (LARs) & CUMULATIVE LEAF AREAS (CLAs)

Section 3.1 explained that LARs represent ratios of leaf total areas to plant total weights, and that they aim to describe plant growth rates in terms of both the assimilatory efficiencies of the leaves and in terms of plant leaf areas (Hunt, 1978). Fig. 3.19 illustrates that LARs were similar in non-nodulated, nodulated and in 'spiked' *V. faba*, however anova analyses reveal that the LARs of *V. faba* within the 'non-nodulated' and 'spiked' nitrogen schemes were significantly affected by the form of the supplied nitrogen source (from 'no nitrate' through to 'combined nitrogen' nutrition). Indeed fig. 3.19c illustrates that greater LARs were exhibited in 'spiked' *V. faba* when supplied with decreasingly concentrated medium nitrogen nutrition. This was expected as the equation for LAR (section 3.2.5) dictates that increasing LARs describe plants which require greater leaf areas to maintain equivalent plant biomass increases. As RWCs were maintained at lower values in *V. faba* when supplied with decreasingly concentrated medium nitrogen nutrition during water deficits (figs. 3.16 & 3.17), it is possible that lower stomatal conductances and net photosynthesis (as supported by the data; figs. 3.21 & 3.22) may have contributed to the greater LARs which were exhibited in non-nodulated and 'spiked' *V. faba* when supplied with decreasingly concentrated medium nitrogen nutrition. Difference in LARs were not significant within the nodulated nitrogen scheme. Nodulated 'no nitrate' supplied *V. faba* potentially acquired nitrogen via nitrogen fixation (table 2.3) and may not have been nitrogen deficient (in contrast to non-nodulated 'no nitrate'

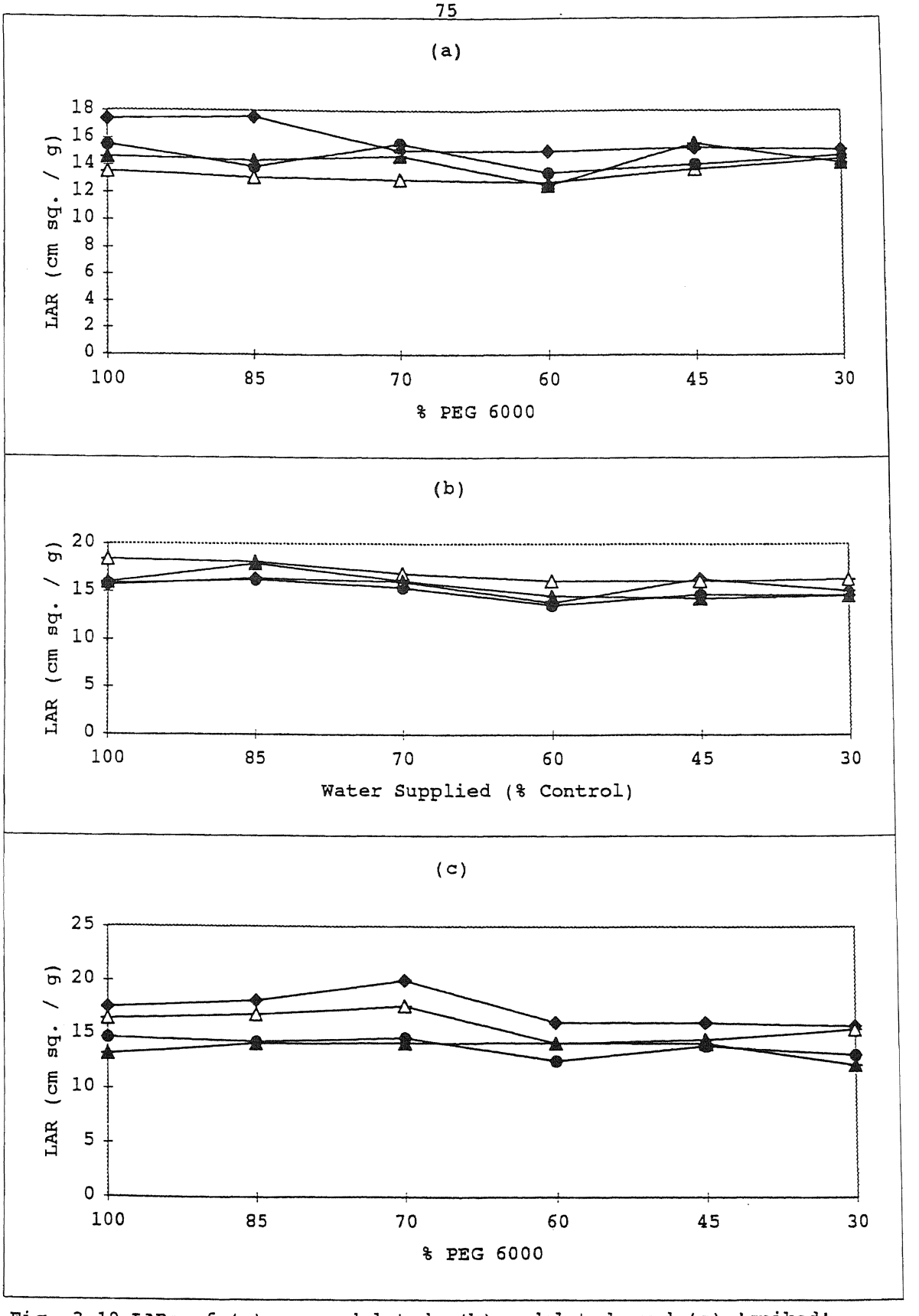


Fig. 3.19 LARs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

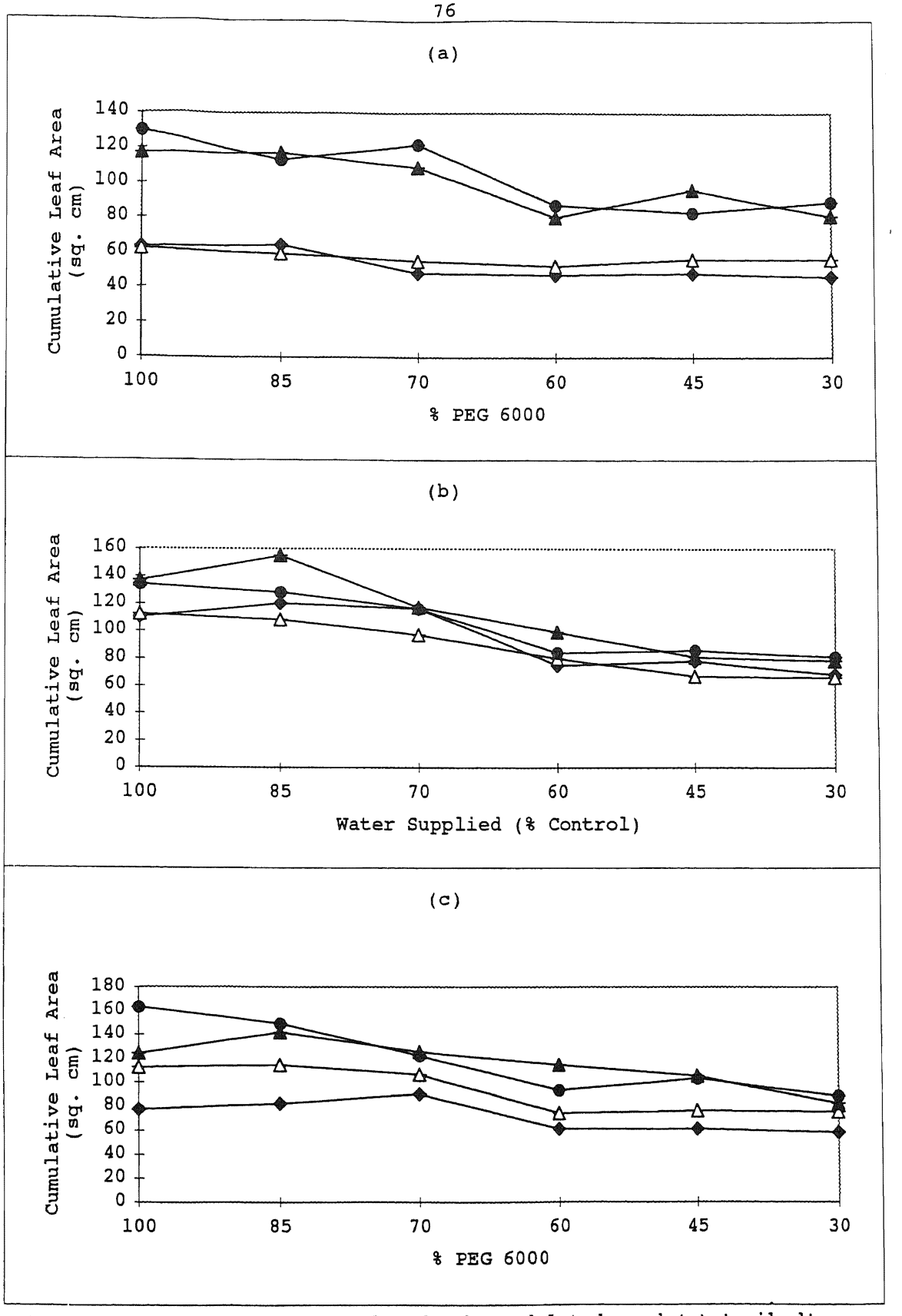


Fig. 3.20 CLAs of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

supplied *V. faba*). Therefore in addition to exhibiting significantly greater RWCs (figs. 3.16 & 3.17), and growth (figs. 3.1 - 3.10), significantly greater net photosynthesis may also have been exhibited in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* (fig. 3.22) which would result in more efficient leaf assimilatory capacities and hence in relatively lower LARs, and in a reduction in the differences between LARs in *V. faba* when supplied with different forms of medium nitrogen nutrition within the nodulated scheme.

Fig. 3.19 illustrates that LARs were maintained in *V. faba* during water deficits (as previously described in other plant species, Martinez-Carrasco *et al*, 1998). The indication is that overall reductions in growth (fig. 3.24) may have been maintained at similar rates to the overall reductions in leaf area (fig. 3.20) and in leaf net photosynthetic rates (fig. 3.22) during water deficits, resulting in the exhibition of maintained LARs (fig. 3.19) during water deficits.

Fig. 3.20a and anova analyses reveal that significantly greater CLAs were exhibited in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and that CLAs were maintained in *V. faba* in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' = '1/2 nitrate' > '1/10 nitrate' > 'no nitrate'. Indeed greater leaf areas and dry weights have previously been reported in *V. faba* (Sutherland *et al*, 1985) and in other plant species (Sprent & Thomas 1984; Hocking & Meyer, 1991) when supplied with increasingly concentrated medium nitrogen nutrition, and with ammonia as opposed to with nitrate (or N_2) nutrition (Hawkins & Lewis, 1993; Martinez-Carrasco *et al*, 1998). Ammonia (as compared with nitrate) nutrition reportedly results in reductions in the area

expansion of individual leaves in other plant species (Pill & Lambeth, 1977; and Tolley-Henry & Raper, 1986; Raab & Terry, 1994), however non-nodulated *V. faba* exhibited similar CLAs whether supplied with 'combined nitrogen' nutrition or with equimolar '1/2 nitrate' nutrition, indicating that medium ammonia additions were not detrimental to leaf growth in this species. Furthermore significantly greater CLAs were exhibited in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* (which potentially fixed atmospheric nitrogen); and in 'spiked' than in 'non-spiked' *V. faba*, further indicating increasing CLAs in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition.

The cessation of leaf expansion and subsequent reductions in leaf areas are reportedly among the most sensitive indices of plant water deficit (Boyer, 1970; Rufty *et al*, 1984; Salama & Sinclair, 1994; Bacon *et al*, 1998; Clark *et al*, 1999; Granier & Tardieu, 1999). Indeed individual leaf areas of *Ziziphus mauritiana* may reportedly decrease during severe water deficits (Clifford *et al*, 1998), and *V. faba* reportedly exhibits increased wilting; decreased leaf expansion; a decreased period of leaf expansion; premature senescence; and lower leaf area indices during increasing water deficits (Hebblethwaite, 1982). Anova analyses reveal that CLAs decreased in nodulated and in non-nodulated 'non-spiked' *V. faba* during water deficits, fig. 3.20 and anova analyses reveal that significantly greater CLAs were maintained throughout water deficits in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. Similarly nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba*, and 'spiked' as opposed to 'non-spiked' *V. faba* maintained significantly greater CLAs throughout water deficits.

Importantly fig. 3.20 illustrates that significantly greater CLAs were

maintained than in 'non-spiked' *V. faba* throughout water deficits (perhaps a reflection of the reduced photosynthate, reductant, water and metal ion requirements associated with ammonia as opposed to with nitrate assimilation, Raven, 1985; Bloom, 1988; Raven *et al*, 1992). The indication is that medium ammonia additions result in the exhibition of greater potentially photosynthetic areas (Yoshida, 1972) in *V. faba* throughout water deficits. The maintenance of high CLAs has previously been strongly correlated with increasing dry matter yields and seed matter yields in *V. faba* (Hebblethwaite, 1982). Furthermore in temperate climates where water is limiting and rain is intermittent, large CLAs may limit the evaporation of rain from the substrate surface throughout the growth period (Passioura, 1981; Passioura *et al*, 1993), and large CLAs may therefore contribute towards a postponement of water deficits in temperate-adapted species such as *V. faba* (Sprent 1973; see also introduction; pg. 13). Upon water deficit alleviation surviving leaves reportedly attain photosynthetic rates which are greater than control leaves of the same chronological age, but are comparable with those of leaves of the same physiological age (Ludlow & Ng, 1974), inferring that a capacity to maintain increasingly great CLAs during water deficits (as was particularly apparent in *V. faba* when supplied with medium ammonia additions) may result in increased yields following water deficit alleviation.

Increasingly concentrated nitrate and ammonia nutrition may potentially result in the exhibition of increased osmotic adjustment in *V. faba* (see section 4.4), and certainly resulted in the exhibition of greater RWCs during water deficits (figs. 3.16 & 3.17). These factors may have contributed towards reduced leaf dehydration and senescence (Granier & Tardieu, 1999) and also towards increased expansive growth (Hsaio & Jing, 1987) in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition.

V. faba has previously been reported as relatively insensitive to wilting, and as having the capacity to maintain high CLAs during gradually increasing water deficits (Van der Wal, 1981).

In addition to the described physiological benefits associated with maintained CLAs, high photosynthetic areas may be economically desirable in this species where high vegetative yields may be utilised as green manure (Corak *et al*, 1992).

3.3.6 STOMATAL CONDUCTANCES

Previous workers have reported that the stomatal conductances of some other plant species were not affected by the form of nitrogen nutrition supplied during periods of adequate irrigation (Raab & Terry, 1994; Hogh-Jensen *et al*, 1997). However fig. 3.21 illustrates that even with adequate irrigation (0% PEG; 100% Control Water) stomatal conductances were maintained at greater values in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (as previously reported in other plant species, Kuiper, 1993; McDonald & Davies, 1996; Meinzer & Zhu, 1998). Although 20-40% lower stomatal conductances and transpirations rates have been reported in *T. aestivum* when supplied with ammonia as opposed to with nitrate nutrition (Hawkins & Lewis, 1993), 'combined nitrogen' nutrition resulted in the exhibition of the greatest stomatal conductances in *V. faba*, indicating that medium ammonia additions do not result in stomatal closure in this species. Indeed ammonia as opposed to nitrate nutrition has previously been reported as resulting in the exhibition of greater plant stomatal conductances (Raven, 1985). As such reductions in the reported greater water efficiency of ammonia as opposed to nitrate assimilation, but increases in potential carbon acquisition may be incurred. Maintained stomatal conductances reportedly result in increased rates of transpiration

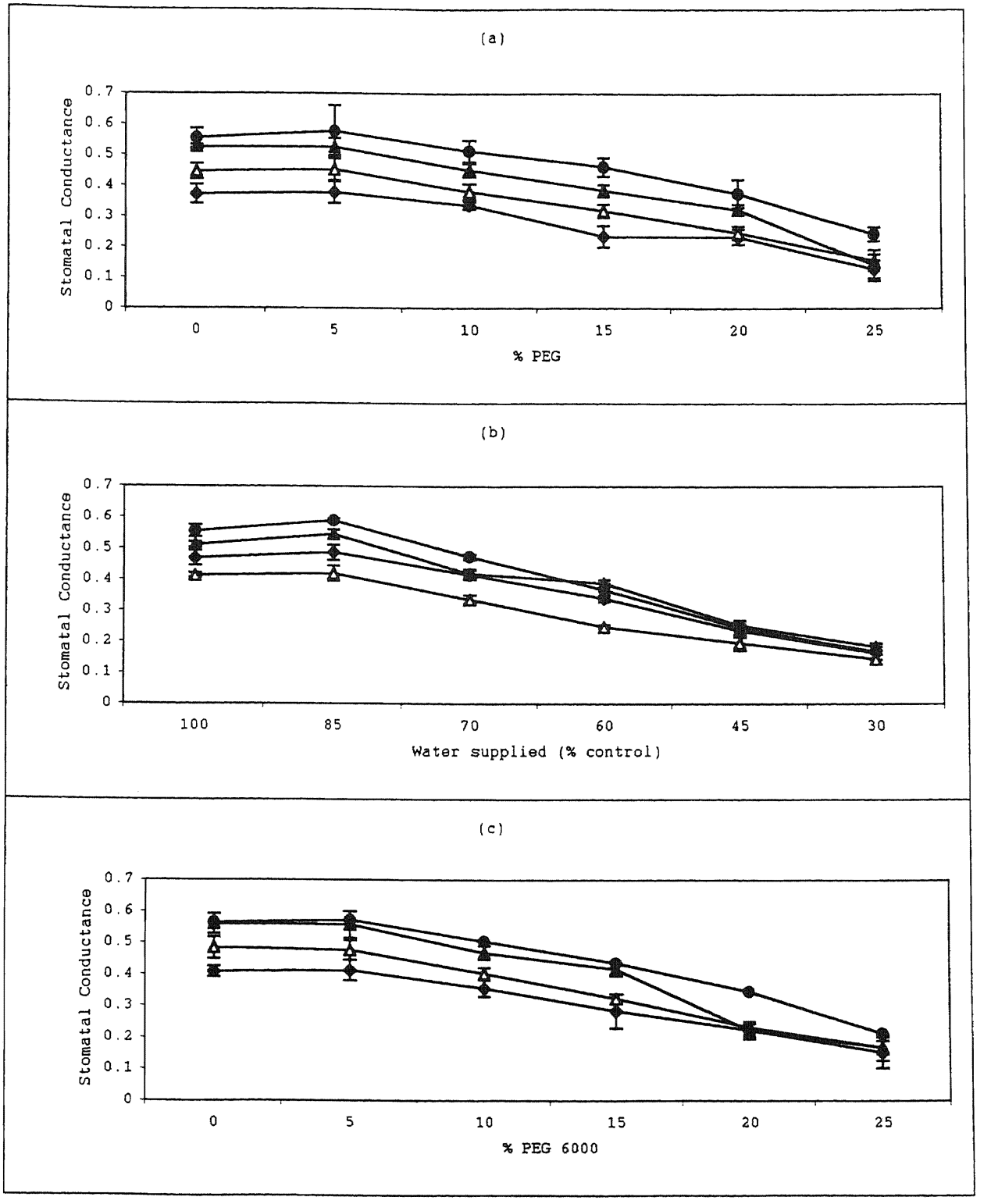


Fig. 3.21 Stomatal Conductance in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

and soil water extraction (Bennet *et al*, 1986), which may be beneficial during water deficits if accompanied by increased root growth (as was exhibited in *V. faba*; fig. 3.9), and by osmotic adjustment (as was exhibited by *V. faba*; see section 4.4), which reportedly enable RWC maintenance (Singh & Gupta, 1983), and further increases in soil water extraction (Sau & Ines-Minguez, 1990; Kumar & Singh, 1998).

Fig 3.21 illustrates that stomatal conductances decreased as water deficits increased (as previously reported in other plant species, Collinson *et al*, 1997; Ferrario-Mery *et al*, 1998; Pankovic *et al*, 1999). This was expected as both nitrogen fixing and medium-nitrogen-supplied *V. faba* reportedly initially rely on increased root growth and on stomatal closure to delay water deficit effects and to maintain internal water contents(Sau & Ines-Minguez, 1990). Fig. 3.21 illustrates that stomatal conductances were maintained in the following order in non-nodulated *V. faba* with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate'> '1/10 nitrate' > 'no nitrate'. This order may reflect the increased RWC maintenance previously recorded in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (figs. 3.17 & 3.18), as maintained RWCs have previously been associated with maintained photosynthetic capacities in other plant species (Hawkins & Lewis, 1993).
Greater stomatal conductances in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition may also reflect decreased ABA production (McDonald & Davies, 1996). Radin *et al*, (1982 & 1985) described increasing stomatal conductance in *Gossypium hirsutum* L. when supplied with increasingly concentrated medium nitrogen nutrition (early during the fruiting season, the points of 50% stomatal closure being separated by 0.5 MPa).

Although stomatal conductances decreased during water deficits, anova analyses reveal that such decreases were only significant in nodulated *V. faba*, which reportedly initiate stomatal closure at higher leaf water potentials than non-nodulated *V. faba* (Sau & Ines Minguez, 1990). Similarly nitrogen fixing *G. max* reportedly exhibit a greater reliance on physiological and stomatal water deficit tolerance adaptations (as opposed to osmotic adjustment) than non-nodulated *G. max* (Ines-Minguez & Sau, 1989). Osmotic adjustment is an energy dependent process which could be detrimental to nitrogen fixation (Sprent, 1971), and conservative behaviour in nodulated *V. faba* at the onset of water deficits might postpone the need to resort to osmotic adjustment.

Fig. 3.21b illustrates that nodulated 'no nitrate' supplied *V. faba* maintained significantly greater stomatal conductances (throughout water deficits) than non-nodulated 'no nitrate' supplied *V. faba*, again perhaps a reflection of the significantly greater RWCs and root growth recorded in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits (3.16; 3.17).

Stomatal conductances were not significantly greater in 'spiked' than in 'non-spiked' *V. faba* (figs. 3.16 & 3.17), which may reflect the previously reported observation that RWCs were not significantly greater in 'spiked' than in 'non-spiked' *V. faba*, throughout water deficits (Comstock & Mencuccini, 1998).

3.3.7 NET PHOTOSYNTHESIS

Net photosynthesis is reportedly closely related to stomatal conductance (Hsaio, 1973), and fig. 3.22 and anova analyses reveal that net photosynthesis was maintained at significantly increasing values in non-nodulated *V. faba* when supplied with increasingly concentrated medium

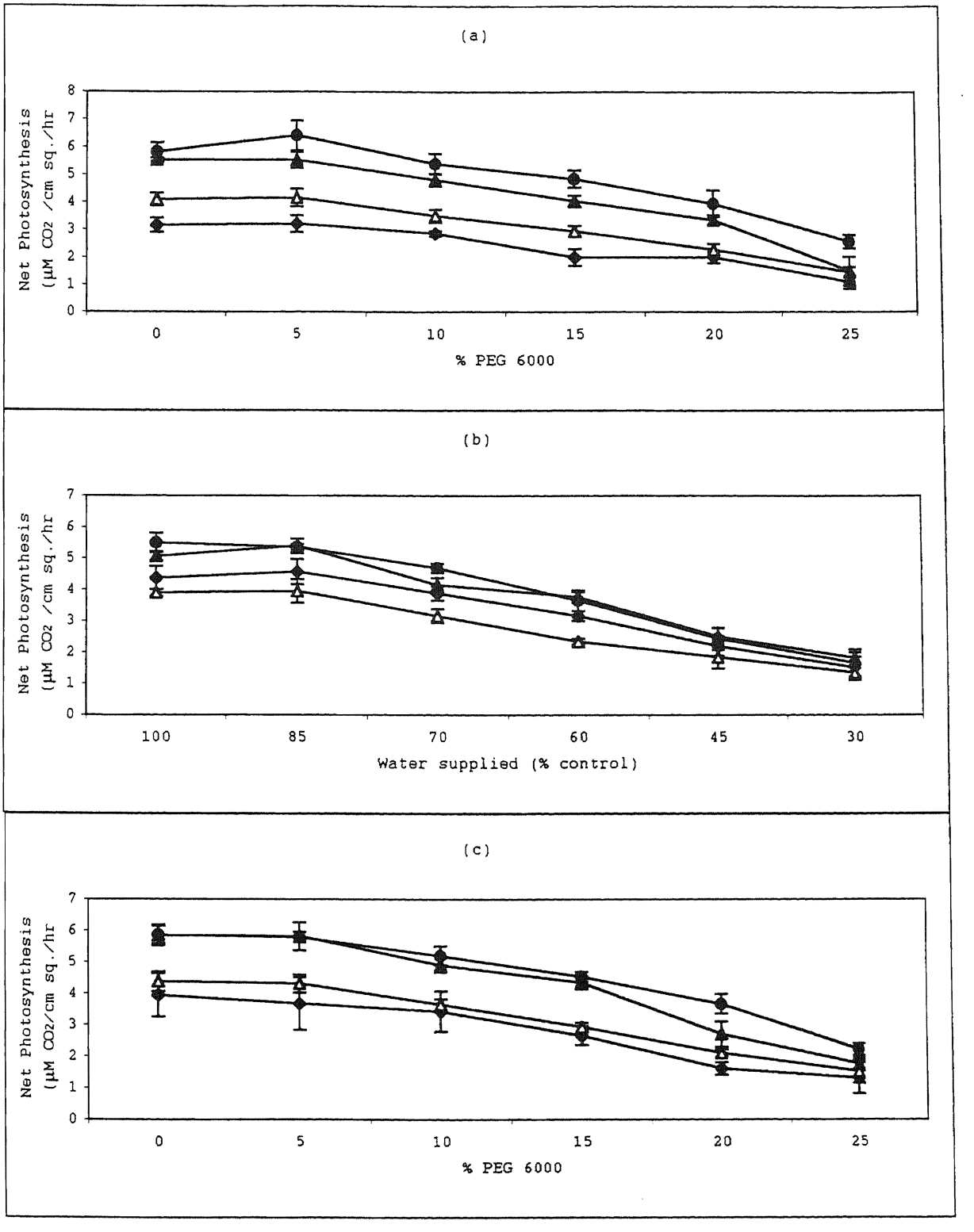


Fig. 3.22 Net Photosynthesis in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

nitrogen nutrition (and particularly with 'combined nitrogen' nutrition). Net photosynthesis was maintained in the following order in non-nodulated *V. faba* with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate'.

Indeed several previous workers have reported that the leaves of other plant species may be able to increase the capacity of the light reactions of photosynthesis in response to increasing nitrate supplies, thus decreasing the competition between nitrate and carbon dioxide assimilation for photochemical energy (Hageman, 1979; Marques *et al*, 1983; Tolley-Henry & Raper, 1986; Doehlert, 1993; Raven & Sprent, 1993).

Some workers have described reduced photosynthesis in other plant species when supplied with medium ammonia nutrition (Losada *et al*, 1973; Rufty *et al*, 1984; Tolley-Henry & Raper, 1986), however non-nodulated *V. faba* exhibited the greatest net photosynthesis when supplied with 'combined nitrogen' nutrition (fig. 3.22), indicating that medium ammonia additions do not result in decreases in net photosynthesis in this species.

Carbohydrate accumulation may reportedly result in the inhibition of photosynthesis via long-term mechanisms involving decreases in the amounts of Rubisco and other Calvin cycle enzymes (Krapp *et al*, 1991; Van Oosten & Besford, 1996). Previous experiments which have involved the feeding of transported analogues have indicated that the metabolism as opposed to the transport of carbohydrates may be required for the maintenance of photosynthesis (Krapp *et al*, 1993). While nitrate may be stored (section 6.3.1.2) ammonia is toxic and cannot be stored (Bourgeais-Chaillou *et al*, 1992; Raven & Sprent, 1993), and ammonia assimilation therefore continually utilises carbon skeletons. The inference is that the end-product inhibition

of photosynthesis may be alleviated in *V. faba* when supplied with medium ammonia additions. Indeed some previous workers have reported increased net photosynthesis in other plant species when supplied with ammonia as opposed to with nitrate nutrition (Krapp *et al*, 1993; Giordano & Bowes, 1997), and *B. vulgaris* reportedly exhibits double the chloroplast volume, and a sixty-two per cent greater chlorophyll concentration when supplied with ammonia as opposed to with nitrate nutrition (Raab & Terry, 1994). Furthermore at concentrations below those required to uncouple photophosphorylation the ammonia ion may have a stimulative effect on photosynthesis in intact chloroplasts (de-Beneditti *et al*, 1976). Such previous observations may partly account for the greater net photosynthesis exhibited in non-nodulated *V. faba* when supplied with 'combined nitrogen' as opposed to with (equimolar) '1/2 nitrate' nutrition.

Fig. 3.22 and anova analysis reveal that net photosynthesis decreased significantly in *V. faba* as water deficits increased. Reduced photosynthetic rates during water deficits, which are fully reversible following mild stress alleviation but persist longer following severe water deficits have previously been reported in *V. faba* (Grzesiak *et al*, 1989), and in other plant species (Salama & Sinclair 1994; Quartacci *et al*, 1997; Jagtap *et al*, 1998; Bussis *et al*, 1998; Iturbe-Ormaetxe *et al*, 1998). That greater levels of net photosynthesis were maintained in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and particularly with 'combined nitrogen' nutrition) throughout water deficits may also reflect the fact that stomatal conductances were maintained at greater values in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with 'combined nitrogen' nutrition (fig. 3.21; Hsaio, 1973), and the fact that ammonia

assimilation reportedly requires less water per unit yield than nitrate assimilation (Raven, 1985; Bloom, 1988; Atwell 1992; Raven *et al,* 1992). Furthermore increased nitrogen supplies may have resulted in increased photosynthetic enzyme production (Evans, 1989; McDonald & Davies, 1996).

RWCs and stomatal conductances reportedly influence net photosynthesis both in *V. faba* (Van der Wal, 1981), and in other plant species (Raab & Terry, 1984; Hawkins & Lewis, 1993). Indeed RWCs (fig. 3.16) and stomatal conductances (3.21) and accordingly net photosynthesis were maintained at significantly greater values in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* (fig. 3.22) throughout water deficits, further inferring that nitrogen fixation may not be as sensitive to water deficits as is classically supposed. Decreasing net photosynthesis reportedly results in decreasing nitrogen fixation in *G. max* which may be attributable to limiting ATP and electron donor supplies, and to the reduced removal of nodular ammonia (Walsh *et al,* 1998). However fig. 3.22 and anova analyses reveal that nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba* recorded significantly greater levels of net photosynthesis, inferring potentially reduced nitrogen fixation inhibition (as mediated by photosynthate limitations) in this species.

Fig. 3.22 illustrates greater net photosynthesis in 'spiked' than in 'non-spiked' 'no nitrate' supplied *V. faba*. However anova analyses reveal that when considering 'spiked' data as a whole (i.e. from *V. faba* supplied with all four of the nitrogen sources within the 'spiked' nitrogen scheme) net photosynthesis was not significantly greater in 'spiked' than in 'non-spiked' *V. faba* during water deficits. Similarly *V. faba* supplied with 'spiked' nutrition did not exhibit significantly greater RWCs and stomatal

conductances (these factors influence photosynthetic rates; Van der Wal, 1981) than 'non-spiked' *V. faba*. However significantly greater CLAs (fig. 3.20) and heights (see section 3.3.10) were exhibited by 'spiked' than by 'non-spiked' *V. faba*, inferring a greater potential overall photosynthetic capacity in *V. faba* when supplied with medium ammonia additions during water deficits.

The effects of water deficits and nitrogen nutrition on carbohydrate and starch concentrations (the ultimate products of photosynthesis) are discussed in chapter five.

3.3.8 RELATIVE GROWTH RATES (RGRs)

Relative growth rates relate to total plant growth over time, and although they are not related to whether the measured plant material is structural or productive (i.e. associated with increased photosynthesis or with increased water uptake) they do provide a convenient integration of the growth of combined plant parts over time (Hunt, 1978). Fig. 3.23 and anova analyses reveal that increasingly concentrated medium nitrogen nutrition resulted in the exhibition of significantly greater RGRs in non-nodulated and in 'spiked' *V. faba* (in agreement with earlier reports which described RGRs in other plant species, Kuiper, 1993). This was expected as increasingly concentrated medium nitrogen nutrition resulted in the exhibition of increased plant biomasses (figs. 3.1-3.10; Marschner, 1986).

Lower plant growth rates have previously been recorded in *Ricinus communis* when supplied with ammonia as opposed to with nitrate nutrition (Allen & Smith, 1986). However fig. 3.23 illustrates that greater RGRs were recorded in *V. faba* when supplied with 'combined nitrogen' nutrition than with equimolar '1/2 nitrate' nutrition, indicating that medium ammonia additions do not

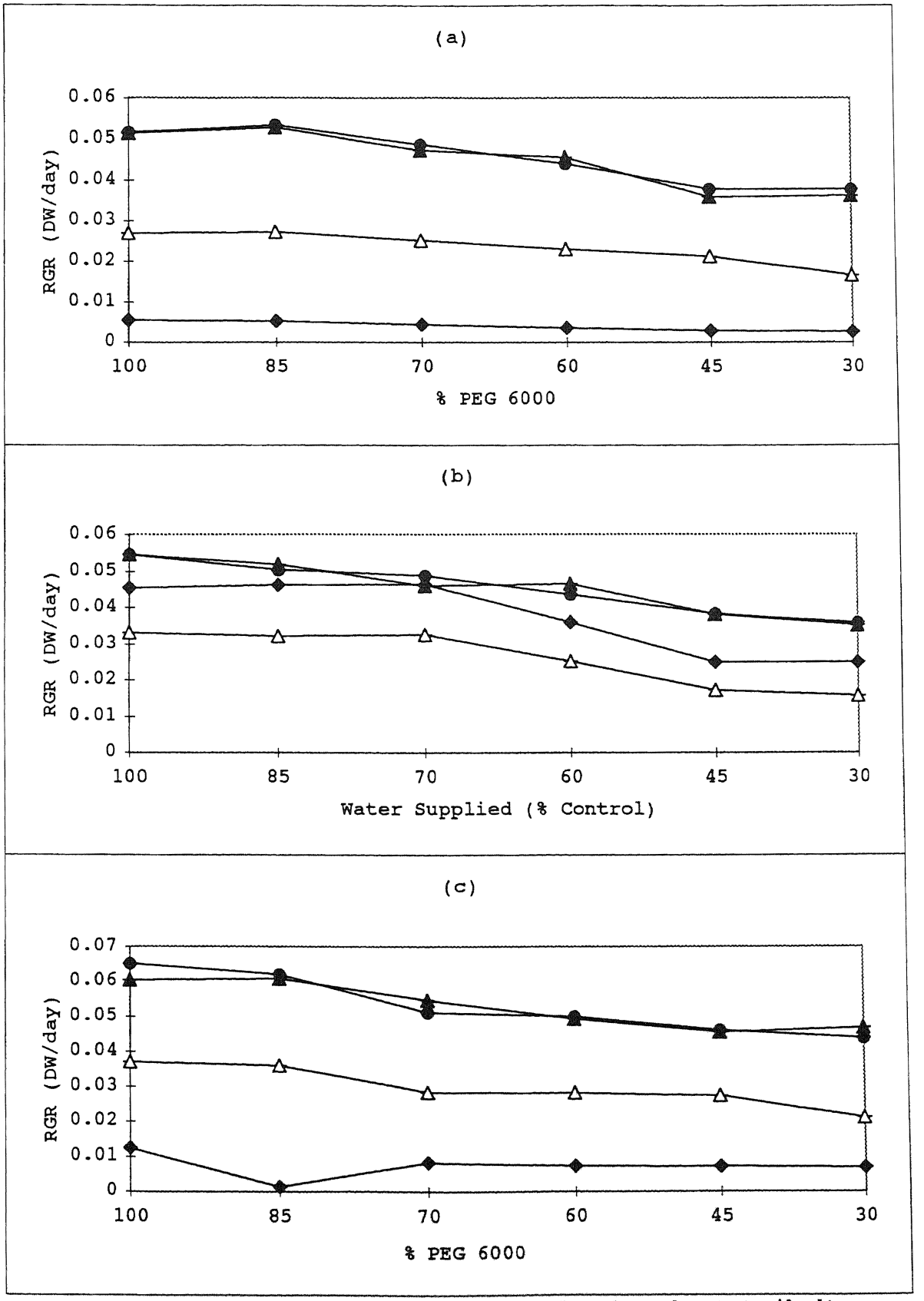


Fig. 3.23 RGRs in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

result in decreased plant growth rates in this species (when medium pH is maintained, see section 2.4.1). The greater RGRs exhibited in *V. faba* when supplied with 'combined nitrogen' nutrition may reflect the previously reported observations that growth may proceed with a higher photon yield (mol carbon assimilated per mol photon absorbed and per unit water), and with faster growth rates in given radiation fields in other plant species when supplied with medium ammonia as opposed to with medium nitrate nutrition (Van Oorschot, 1955; Raven & Smith, 1976; Myers 1980; Raven, 1985; Bloom, 1988; Salac *et al*, 1987; Raven *et al*, 1992). Indeed Troelstra *et al*, (1992), reported that ammonia nutrition, then nitrate nutrition, then nitrogen fixation, resulted in the exhibition of increasing RGRs in other plant species (when media pH was controlled; plants which exhibit high specific growth rates reportedly often exhibit increased pH drift (Gigon & Rorison, 1972), however medium pH drift was not observed during this research (section 2.4.1) under the experimental conditions described in chapter two).

Fig. 3.23 and anova analyses reveal that RGRs were significantly greater in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*, which supports the earlier observation that increasing nitrogen availabilities may result in the exhibition of increasing RGRs. Pate *et al* (1979), compared non-nodulated nitrate supplied *L. albus* L. with nodulated nitrogen fixing plants. The nitrogen regimes promoted closely similar rates of growth. Indeed nodulated 'no nitrate' supplied *V. faba* maintained greater growth rates than '1/10 nitrate' supplied *V. faba* (fig. 3.23b), a further indication of the effectiveness of nodulation in *V. faba* (Richards & Soper, 1979). However the recorded growth rates of nodulated 'no nitrate' supplied *V. faba* were lower than those of *V. faba* when supplied with the most concentrated nitrogen nutrition, i.e. with '1/2 nitrate' or with 'combined nitrogen' nutrition. This

observation supports the work of Purcell & King, (1996), who reported that growth rates and yields of 'combined nitrogen' supplied *G. max* were higher than those of nitrogen fixing *G. max*.

RGRs were not significantly greater in 'spiked' than in 'non-spiked' *V. faba*, indicating that while root growth (fig. 3.9) and CLAs (fig. 3.20) were significantly greater in *V. faba* when supplied with medium ammonia additions, overall growth rates were not. The inference is that rates of 'structural' as opposed to 'productive' growth may have been lower, or that senescence may have been lower in 'spiked' than in 'non-spiked' *V. faba*.

Fig. 3.23 and anova analyses reveal that RGRs decreased significantly during water deficits. The recorded RWC decreases as water deficits increased (figs. 3.16 & 3.17) may have contributed to reductions in expansive growth (McDonald & Davies, 1996). Furthermore net photosynthesis decreased as water deficits increased (fig. 3.22) indicating that reduced photosynthate availabilities may have contributed to reduced RGRs as water deficits increased. In summary RGRs were maintained at significantly increasing levels in non-nodulated and 'spiked' *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition throughout water deficits, which may reflect the increased maintenance of RWCs and net photosynthesis during water deficits in *V. faba* when supplied with the same increasingly concentrated medium nitrogen.

3.3.9 NET ASSIMILATION RATES (NARs)

Differences in crop growth rates in other plant species have previously been attributed to differences in NARs, which are related to photosynthetic rates (Buttery, 1970), and describe the net weight gains per unit photosynthetic area. Increased NARs result in the exhibition of greater biomasses over time, however as leaf areas increase NARs may decrease due to increased mutual

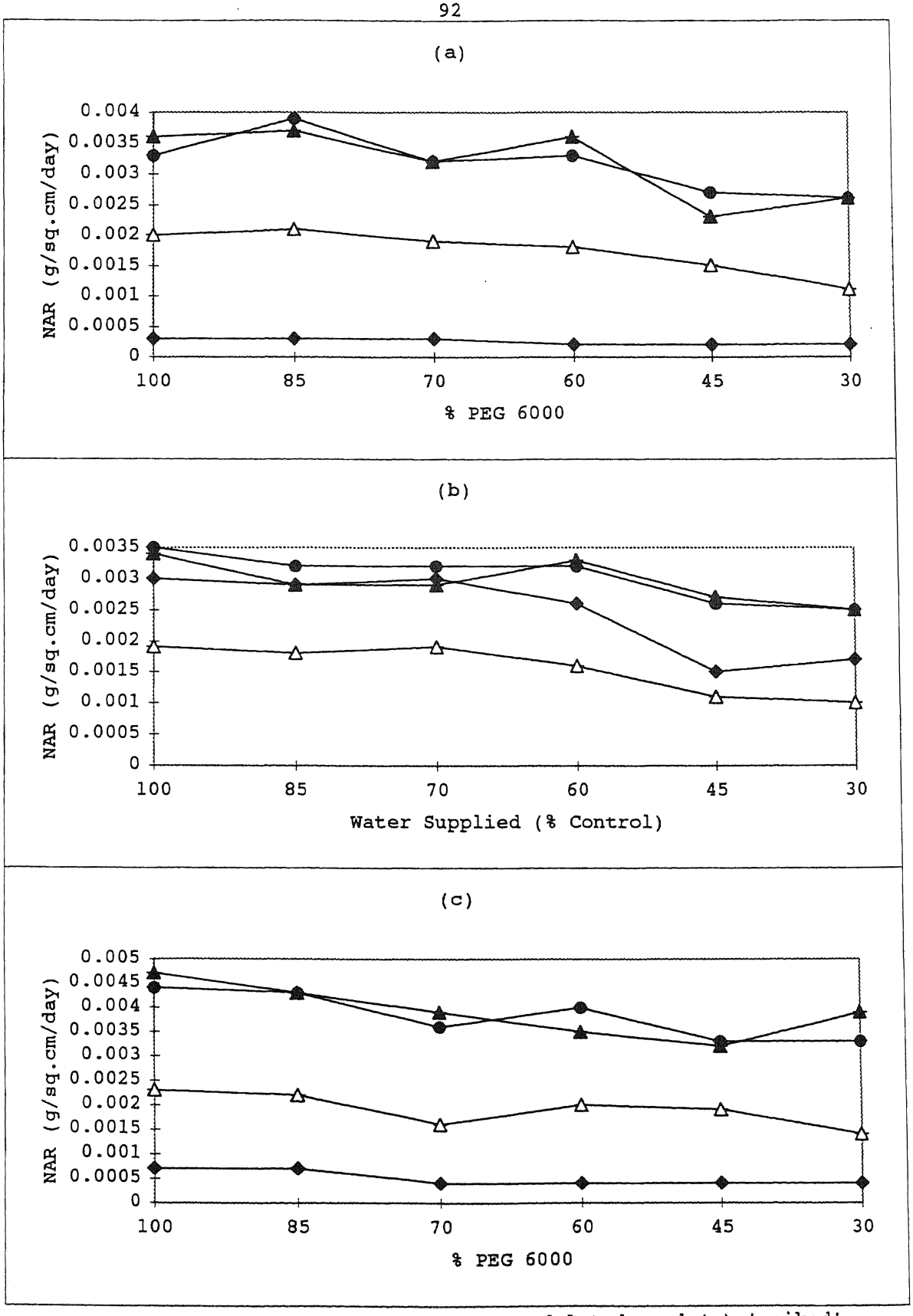


Fig. 3.24 NARs in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

shading (Buttery, 1970).

Fig. 3.24 and anova analyses reveal that NARs increased significantly in non-nodulated *V. faba* as the concentration of the supplied nitrogen source increased. NARs were maintained in the following order in non-nodulated *V. faba* with respect to medium nitrogen nutrition: 'combined nitrogen' = '1/2 nitrate' > '1/10 nitrate' > 'no nitrate', an order which is consistent with the effects of nitrogen nutrition on both net photosynthesis (fig. 3.22) and CLAs (fig. 3.20), and infers that NARs (per unit leaf area) did not decrease due to increased mutual shading in this species under the specified growth conditions (*V. faba* were grown in well-spaced pots; sections 2.4.1 & 2.4.2).

Fig. 3.24 and anova analyses reveal that significantly greater NARs were exhibited in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*, which may have been attributable to the significantly greater net photosynthesis per unit leaf area which was exhibited in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*.

However fig. 3.24 and anova analysis indicate that ammonia 'spiked' *V. faba* did not exhibit significantly greater NARs than non-nodulated 'non-spiked' *V. faba*. It is thus apparent that additional medium ammonia nutrition resulted in the exhibition of proportionally greater CLAs (fig. 3.20) and root biomasses (3.9) in *V. faba*, but not in significantly greater overall biomasses (fig. 3.10), net photosynthesis per unit leaf area (fig. 3.22), or NARs (fig. 3.24). Increased ammonia nutrition has previously been shown to result in the exhibition of increased individual organ weights, but not in increasing NARs in other plant species (Hocking & Meyer, 1991). Indeed results infer that the effects of medium ammonia 'spiking' on biomass maintenance (e.g. root growth; fig. 3.9) in *V. faba* may primarily have been due to the stimulating action of nitrogen on leaf growth, which was significantly greater in 'spiked' than in

'non-spiked' *V. faba* (fig. 3.20), enabling greater total assimilation without changes in net assimilation rates per unit leaf area.

It has been shown that net photosynthesis decreased in *V. faba* during water deficits (fig. 3.22). Similarly anova analyses reveal that NARs decreased significantly during water deficits (as previously reported in other plant species, Clifford *et al*, 1998), and fig. 3.24 reveals that NARs were maintained at increasing values during water deficits in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, an order which reflects the maintenance of net photosynthesis during water deficits.

3.3.10 PLANT HEIGHTS

Optimum crop heights reportedly exist as affected by lodging; shading; proportions of 'non-productive growth' (Yoshida, 1972; Hocking & Meyer, 1991). Fig. 3.25 and anova analyses reveal that significantly greater heights were recorded in *V. faba* as the nitrogen concentration of the supplied medium increased. Greater heights were recorded in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*; and significantly greater heights were recorded in 'spiked' than in 'non-spiked' *V. faba*, supporting the earlier observation that increasing nitrogen supplies result in increased plant growth (Marschner, 1986), and increased growth maintenance during water deficits (figs. 3.1 - 3.10). Indeed increased heights have previously been recorded by other plant species when supplied with increasingly concentrated medium nitrogen nutrition (Quebedeaux & Osbun, 1973).

Anova analyses confirm that plant heights decreased significantly during water deficits, a phenomenon previously reported in *V. faba* (Gallacher & Sprent, 1978; Hebblethwaite, 1982; Plies-Balzer *et al*, 1995), and in other plant species (for example in *G. max*, Salama & Sinclair, 1994; and in *Pinus*

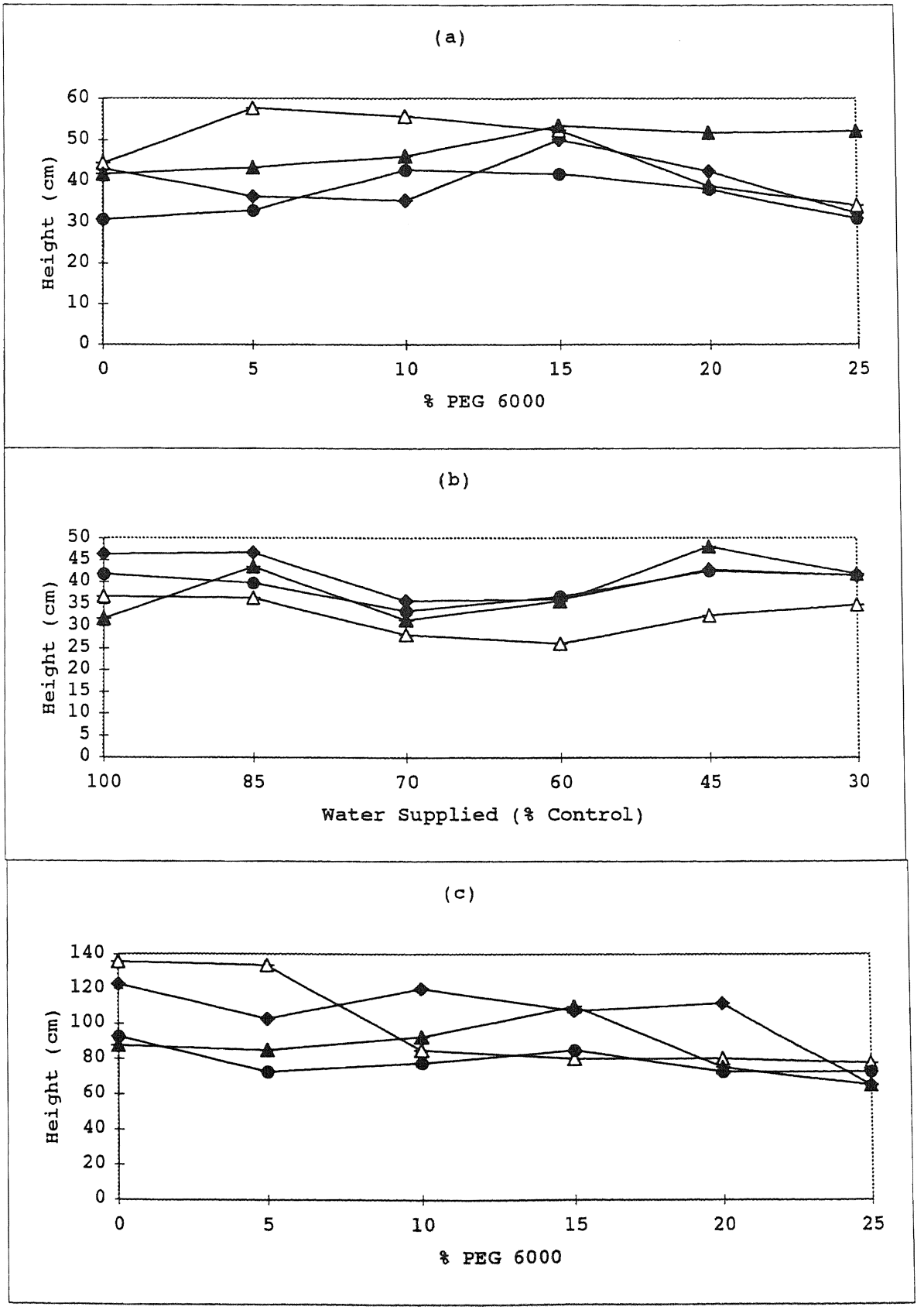


Fig. 3.25 Heights in (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

halepinsus, Wellburn *et al*, 1996).

Reduced aerial growth has briefly been discussed and may have been accentuated by water deficit associated RWC decreases, stomatal conductance decreases, and net photosynthesis decreases (figs. 3.16; 3.17; 3.21; 3.22), which may have resulted in reductions in aerial expansive growth, and in photoassimilate and reductant availabilities (see sections 3.3.1 & 3.3.4; McDonald & Davies, 1996). The significantly greater heights maintained in 'spiked' than in 'non-spiked' *V. faba* throughout water deficits may reflect increased assimilation of (as opposed to storage of) ammonia as compared with nitrate nutrition, and infer potentially increased capacities to intercept solar radiation, and hence potentially increased plant productivities in *V. faba* when supplied with medium ammonia additions (Yoshida, 1972). That heights were greater (yet aerial biomasses similar) in 'spiked' than in 'non-spiked' *V. faba* may infer less branching in 'spiked' *V. faba*.

3.4 CONCLUSION

Organ fresh and dry weights; plant heights; NARs; CLAs, and leaf and root RWCs decreased significantly during increasing water deficits, and were maintained at significantly greater values in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition.

R:Ss increased significantly during water deficits, and were maintained at significantly greater values in *V. faba* when supplied with decreasingly concentrated medium nitrogen nutrition, reflecting the previously reported observation that plants growing in nitrogen deficient media may require longer roots to enable adequate nitrogen uptake (Hodge *et al*, 1999).

RGRs were significantly greater in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition in all but nodulated plants (possibly as LARs and RGRs were significantly greater in nodulated than in non-nodulated

'no nitrate' supplied *V. faba*, thus the gap between the growth rates of *V. faba* when supplied with 'no nitrate' and '1/10 nitrate' nutrition may have been bridged by nitrogen fixation in *V. faba* within the nodulated scheme).

It has been reported that *V. faba* (cultivars 'Alfred', 'Diana', 'Piccolo' and 'Troy') does not exhibit greater aerial growth when supplied with 'combined nitrogen' nutrition as opposed to when reliant on nitrogen fixation, both when supplied with adequate irrigation and during water deficits (Plies-Balzer *et al*, 1995). However fig. 3.8 illustrates that *V. faba* (cultivar 'Bunyards Exhibition') supplied with either 'combined nitrogen' or with '1/2 nitrate' nutrition exhibited significantly greater aerial biomasses than non-nodulated 'no nitrate' supplied (i.e. nitrogen fixing) *V. faba*, both when supplied with adequate irrigation, and during water deficits.

Comparing fig. 3.23 graphs a & c illustrates that RGRs increased in *V. faba* when an ammonia 'spike' was included in the medium, however anova analyses reveal that the greater RGRs in 'spiked' than in 'non-spiked' *V. faba* were not statistically significant. This is of interest as it has been demonstrated that heights (fig. 3.25), CLAs (fig. 3.20) and root biomasses (fig. 3.9) were significantly greater in 'spiked' than in 'non-spiked' *V. faba* throughout water deficits. It is therefore indicated that medium ammonia additions result in proportionally greater root and leaf growth in *V. faba* during water deficits, as opposed to significantly greater overall growth, as supported by the statistically similar total dry weights exhibited in 'spiked' and in 'non-spiked' *V. faba* throughout water deficits (fig. 3.10).

This may be physiologically significant as greater root growth potentially results in deeper substrate penetration, and has been correlated with maintained water uptake in *V. faba* during water deficits (Sau & Ines-Minguez,

1990), while greater heights and CLAs reportedly represent potentially increased overall photosynthetic capacities in *V. faba* (Van der Wal, 1981), and in other plant species (Yoshida, 1972; Clark *et al*, 1999). Accordingly increased capacities for water uptake and photosynthesis are inferred in *V. faba* when supplied with medium ammonia additions (i.e. in 'spiked' *V. faba*) throughout water deficits.

That stomatal conductances and net photosynthesis were maintained at lower external water potentials in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and with 'combined nitrogen' as opposed to with equimolar '1/2 nitrate' nutrition) may reflect the increased RWCs recorded in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, as low leaf RWCs are reportedly associated with stomatal closure during water deficits in *V. faba* (Van der Wal, 1981), and in other plant species (Comstock & Mencuccini, 1998), and stomatal conductances reportedly influence net photosynthesis, particularly during slight to moderate water deficits (McDonald & Davies, 1996).

RWCs of eighty to ninety per cent have previously been reported to correspond with altered relative rates of photosynthesis and respiration (Lawlor, 1995). RWCs were maintained above eighty per cent in *V. faba* (when supplied with all forms of medium nitrogen nutrition) when slight water deficits were imposed (i.e. 5% PEG; 85% contol water; see figs. 3.16 & 3.17), and accordingly fig. 3.22 illustrates that net photosynthesis was maintained in all *V. faba* during slight water deficits. Insensitivities to slight water deficits have previously been described in other plant species (Hsiao, 1973), however changes in metabolism reportedly become marked if RWCs fall below eighty per

cent, and photosynthesis may cease (Hsiao, 1976; Lawlor, 1995). Fig. 3.16 illustrates that leaf RWCs were maintained above eighty per cent until moderate water deficits were experienced by *V. faba* when supplied with 'no nitrate' and with '1/10 nitrate' nutrition; and until severe water deficits were experienced by *V. faba* when supplied with '1/2 nitrate' and with 'combined nitrogen' nutrition. Accordingly fig. 3.22 illustrates that while photosynthetic declines were initiated during moderate water deficits in *V. faba* when supplied with each nitrogen source, net photosynthesis was maintained in the following order in non-nodulated *V. faba* with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate' nutrition throughout water deficits, indicating that medium ammonia additions (as compared against equimolar nitrate nutrition) resulted in the exhibition of an increased capacity for maintained net photosynthesis during water deficits in this species.

However leaf and root RWCs (and accordingly stomatal conductances and net photosynthetic rates) were maintained at similar values in 'spiked' and in 'non-spiked' *V. faba*. This may be explained as ammonia requires rapid assimilation (as opposed to nitrate which may be stored as an osmotically active solute during water deficits, and thus may contribute directly to RWC maintenance) and the assimilation of medium ammonia additions may therefore primarily result not in further RWC increases, but rather in increased growth (as reflected in the significantly greater root growth (fig. 3.9), CLAs (fig. 3.20), and heights (fig. 3.25) exhibited in 'spiked' than in 'non-spiked' *V. faba*), which indicate increased nitrogen and carbon assimilation (as opposed to nitrogen and carbon storage). Although similar (RWCs and accordingly) net photosynthesis was recorded in 'spiked' and in 'non-spiked' *V. faba*, the significantly greater CLAs and heights recorded in 'spiked'

V. faba infer an increased overall photosynthetic capacity in *V. faba* when supplied with medium ammonia additions. Indeed *V. faba* which exhibit increased CLAs reportedly exhibit proportional increases in dry matter and seed matter yield during water deficits (Hebblethwaite, 1982), and increased CLAs may therefore represent increased economic benefits both during water deficits and following water deficit alleviation, particularly as the vegetative yield of *V. faba* is often utilised as green manure (Corak *et al*, 1992).
Fig. 3.16 (pg. 67) illustrates that RWCs fell below eighty per cent during severe water deficits (25% PEG; fig. 2.2; pg. 34), however complete stomatal closure was not recorded by *V. faba* (fig. 3.21; pg. 81). A degree of cuticular transpiration may have contributed towards the recorded stomatal conductance in *V. faba*, particularly as the leaves of this temperate adapted species are not waxy.

Photosynthesis may be reduced during water deficits via feedback caused by reductions in 'sink size' (Krapp *et al*, 1991; Krapp *et al*, 1993). However maintained growth (figs. 3.1 - 3.10; 3.20; 3.25), nitrogen assimilation and osmotic adjustment (which increased in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with medium ammonia additions; sections 4.4 & 6.4) may have provided sinks for photosynthates and reductants during water deficits, and may have resulted in an alleviation of the feedback inhibition of photosynthesis, and hence may have contributed towards the recorded maintenance of net photosynthesis at greater values in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and with 'combined nitrogen' as opposed to with equimolar '1/2 nitrate' nutrition; and as inferred in *V. faba* when supplied with 'spiked' as opposed to with 'non-spiked' nutrition).
Net photosynthesis data may also reflect the previously reported observation that the ammonia ion may have stimulative effect on photosynthesis in intact

chloroplasts (de-Beneditti *et al*, 1976).

Increasingly concentrated medium nitrogen nutrition may also have enabled increased plant photosynthetic enzyme production, which may have alleviated problems of enzyme damage during increasingly severe water deficits (Poljakoff-Mayber, 1981). Furthermore sections 5.4 & 6.4 will demonstrate that compatible solutes were produced at increasing concentrations in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and increased compatible solute concentrations may have contributed to the recorded maintenance of net photosynthesis at increasing values in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition.

Expansive growth may reportedly decrease during water deficits before photosynthesis is inhibited, and accumulating carbohydrates may therefore reportedly 'fuel' osmotic adjustment (Hsaio, 1973; Wardlaw, 1993), as the metabolic cost of storing photosynthate and using it for osmotic adjustment is reportedly less than the cost of converting it into new biomass (McCree, 1986). However figs. 3.8 & 3.25 illustrate that aerial growth reductions and decreasing net photosynthesis coincided during water deficits in this species. Thus if osmotic adjustment is shown to occur in *V. faba* during water deficits the inference is that excess photosynthate accumulation prior to growth reductions is not the sole (or primary) source of substrates for osmotic adjustment. However substrates for osmotic adjustment may have been provided via starch degradation (see sections 5.3.1.3 & 4) and via medium nitrogen assimilation (see section 6.4).

In summary the collected data illustrates that growth, stomatal conductances and net photosynthesis were maintained at greater values during water deficits in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition.

CHAPTER FOUR

OSMOTIC ADJUSTMENT IN NON-NODULATED, NODULATED, AND AMMONIA 'SPIKED' *V. faba* WHEN SUPPLIED WITH VARIOUS FORMS AND CONCENTRATIONS OF MEDIUM NITROGEN NUTRITION DURING INCREASING WATER DEFICITS

As water is being removed from the cell, osmotic potential is reduced due to the simple effect of solute concentration. However, if during the course of cellular water loss solutes are actively accumulated, osmotic potential would be reduced beyond the rate dictated by the mere effects of concentration. Such accumulation of solutes during the development of water deficit is termed 'osmotic adjustment' or 'osmoregulation' (Blum, 1983; Rhodes & Samaras, 1994).

4.1 INTRODUCTION

It is imperative for growth that plants maintain (a threshold level of) turgor during water deficits (Brownlee *et al*, 1999). Plants which accumulate greater concentrations of osmotic solutes reportedly maintain greater RWCs (Singh & Gupta, 1983); greater stomatal conductances and extract more water during water deficits (Kumar & Singh, 1998; Collinson *et al*, 1997); and exhibit increased yields (Van der Wal, 1981; Rodriguez-Maribona *et al*, 1992); than those which accumulate lower concentrations of osmotic solutes.

Osmotic adjustment has been observed during water deficits in *V. faba* (Van der Wal, 1981; Sharma & Rai, 1989), and in other plant species (Hanson & Hitz, 1982; Morgan, 1984 & 1986; Bussis & Heineke, 1998; Cellier *et al*, 1998; Clifford *et al*, 1998; Zhang *et al*, 1999).

Most accumulating osmotica are not components of major metabolic pathways,

but are synthesized at the end of off-shoots of major pathways (Borowitzka, 1981), in order that sudden solute requirements may be met and that rapid removal may be achieved upon water deficit alleviation. Osmotic solutes must be soluble and uncharged at physiological pH, so that osmotic adjustment does not result in cellular ionic and pH perturbations. Carbohydrates (sucrose; glucose; fructose; starch; and sugar alcohols) are reportedly the most abundant osmotica during water deficits (Foyer *et al*, 1998; Clifford *et al*, 1998; Li *et al*, 1998; Ferrario-Mery *et al*, 1998), representing up to eighty per cent of the total osmotica pool (Ferrario-Mery *et al*, 1998), and 40 mM sucrose reportedly accounts for approximately 0.1 MPa (Nobel, 1991). Sugars reportedly accumulate prior to other osmotica in a wide range of plant species (Hanson & Hitz, 1982; Morgan, 1984; Morgan & Condon, 1986; Bussis & Heinke, 1998).

Infrared spectroscopy has revealed that sucrose may act as a compatible solute during water deficits (Ingram & Bartels, 1996). Hydrogen bonds reportedly form between the hydroxyl groups of sucrose and the polar residues in proteins to produce 'glass', which fills space preventing cellular collapse, and restricts the molecular diffusion required by chemical reactions providing a quiescent state, which is associated with viability (Ingram & Bartels, 1996). Sucrose, maltose, and trehalose reportedly stabilize the activity of phosphofructokinase *in vivo*, which otherwise may dissociate irreversibly during dehydration (Ingram & Bartels, 1996). Furthermore hexose sugars (and proline) reportedly stabilize DNA and membranes and ameliorate the deleterious effects of free radicals which may occur during water deficits (Clifford *et al*, 1998).

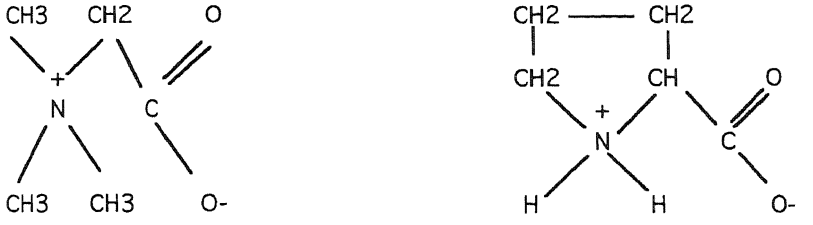
Carbohydrates may also form substrates for organic acid synthesis in the TCA cycle and (eventually) for compatible solute (e.g. proline) production

(Lawlor, 1995). Furthermore accumulated carbohydrates reportedly remain beyond the time of water deficit relief, and may be consumed by respiration, both during water deficits and following water deficit alleviation (Clifford *et al*, 1998). Thus carbohydrates may fulfil many 'roles' both during and following water deficits, and as such may represent particularly 'useful' solutes.

Amino acids also reportedly contribute significantly to osmotic adjustment (particularly the imino acid proline; and glutamate and glutamine; asparagine and aspartate) accumulating during more severe water deficits than are required for carbohydrate accumulation in many plant species (Singh *et al*, 1973b; Jones *et al*, 1980; Foyer *et al*, 1998).
Proline is reportedly rapidly metabolized in irrigated plants (Aspinall & Paleg, 1981), but may represent over eighty per cent of the total amino acid pool during water deficits (Samaras *et al*, 1995; Wood, 1998; Clifford *et al*, 1998).
Proline reportedly exhibits compatible solute 'behaviour', diminishing the PEG induced precipitation of GS in a concentration dependant manner (while some amino acids; alanine; serine; glycine; and threonine reportedly have additive effects; Nash *et al*, 1981; Paleg *et al*, 1985). Proline also reportedly protects albumin from denaturation by ethanol, urea, or ammonium sulphate (Aspinall & Paleg, 1981).

Glycine betaine concentrations may also reportedly increase (up to twenty-six-fold) during water deficits (Hanson & Nelsen, 1978; Wood, 1998), and glycine betaine may also exhibit compatible solute 'behaviour', reportedly protecting enzyme integrity during 'saline stress' (Borowitzka, 1981).
Proline and glycine betaine contain charged nitrogen, which is indicative

of protein stabilising properties (Clifford *et al*, 1998). The hydrophilic carboxyl groups in proline and glycine betaine may bind with cytosolic water, and the hydrophobic ring may attract proteins, resulting in an alleviation of protein dehydration (Borowitzka, 1981; Samaras *et al*, 1985).



Glycine Betaine Proline

Fig. 4.1 Chemical Structures of Glycine Betaine and Proline

Inorganic ions may also accumulate during water deficits (Bougeais-Chaillou *et al*, 1992).

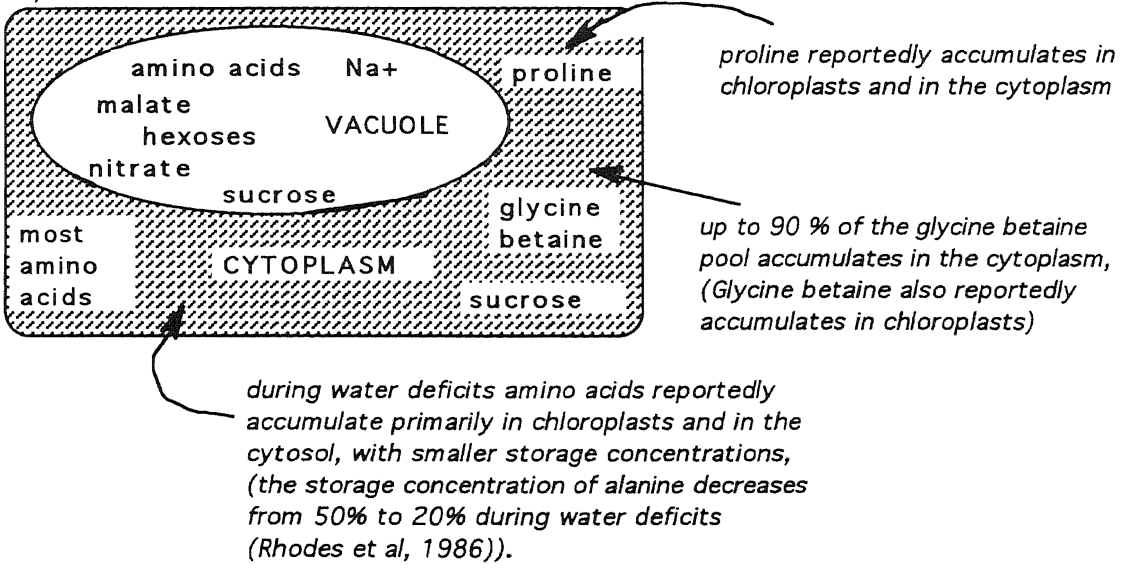


Fig. 4.2 Compartmentation of osmotic solutes (Data from Waldren & Treare, 1974; Wyn Jones, 1983; Deemig & Winter, 1985; Rhodes *et al*, 1986; Bussis & Heinke, 1998).

Decreasing amino acid storage pools were exhibited in suspended *Lycopersicon esculentum* cells when PEG was applied, inferring increasing cytoplasmic pools (Rhodes *et al*, 1986). Indeed there is much evidence for a marked degree of

compartmentation of osmotic solutes in other plant species during water deficits (Wyn Jones & Storey, 1981; Leigh *et al*, 1981; Jeschke 1992), with compatible solutes accumulating to proportionally greater concentrations in the cytoplasm during water deficits than during periods of adequate irrigation (see fig. 4.2), consistent with enzyme stabilization 'roles' (Wyn Jones, 1983; Jeschke, 1992).

4.2 MATERIALS & METHODS

4.2.1 TOTAL SOLUBLE CARBOHYDRATES (after Plummer, 1978; Jermyn, 1975)

100 µl plant extract (see section 2.6.1) was added to 4 ml anthrone (2% anthrone in concentrated $H_2 SO_4$), and 900 µl water. The resultant solution was boiled for 10 minutes then rapidly cooled. OD was determined at 720nm, and concentrations determined using a (glucose) standard curve.

4.2.2 TOTAL AMINO ACIDS (after Pearson & Stewart, 1987)

100 µl plant extract (section 2.6.1) was added to 1.4 ml citrate buffer and 1.2 ml ninhydrin (Citrate buffer: 42g citric acid & 16g NaOH in 250 ml water; Ninhydrin: 1 g ninhydrin/100 ml methoxyethanol & 40 mg ascorbic acid in 40 ml water). The solution was boiled for 20 minutes, and then rapidly cooled, and then diluted with 3ml 60% ethanol. OD was determined at 570nm, and concentrations determined using a (leucine) standard curve.

4.2.3 PROLINE (after Singh *et al*, 1973)

1 ml plant extract (section 2.6.1) was added to 1 ml acid ninhydrin (400 mg ninhydrin per 10 ml glacial acetic acid). The solution was boiled for 45 minutes with 0.5 ml 6 M orthophosphoric acid. The solution was extracted in 5ml toluene. OD was determined at 515nm using glass cuvettes, and concentrations determined using a (proline) standard curve.

4.2.4 GLYCINE BETAINE ANALYSIS (after Stumpf, 1984)

1 ml plant extract (section 2.6.1) was pipetted into a 1.5 ml eppendorf along with 100 µl modified dragondorff reagent, (equal volumes 0.35M $Bi(NO_3)_3$ in 20% acetic acid and 2.45M KI in distilled water). After centrifugation (3000 g for 10 minutes), the pellet was re-dissolved in 1 ml 2.45M KI. 10 µl re-dissolved pellet was added to 1 µl 0.49M KI. OD was determined at 467 nm, and concentrations determined using a standard curve.

4.2.5 TOTAL OSMOLARITIES

When calibrated with the standards provided, the Herman Roebling (Type 5B) micro-osmometer provides direct readings of osmotic concentrations (osmolarities) in mOsm, working on the principle that the temperatures at which solutions freeze relate to the osmotic pressures of the solutions. 0.25 ml plant samples (section 2.6.1) were analysed in the micro-osmometer according to the manufacturers instructions.

4.3 RESULTS & DISCUSSION

4.3.1 TOTAL SOLUBLE CARBOHYDRATES

Figs. 4.3 & 4.4 and anova analyses reveal that total soluble carbohydrates accumulated to significantly greater concentrations in the leaves and roots of non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; and accumulated in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate'.

Figs. 4.3 & 4.4 and anova analyses reveal that nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba* accumulated significantly greater concentrations of total soluble carbohydrates. Indeed nodulated 'no nitrate' supplied *V. faba* accumulated greater concentrations of total

soluble carbohydrates than non-nodulated '1/10 nitrate' supplied *V. faba*; an indication of the effectiveness of nitrogen fixation in *V. faba* (as previously reported, Richards & Soper, 1979).
Furthermore figs. 4.3 & 4.4 and anova analysis reveal that total soluble carbohydrate concentrations were significantly greater in *V. faba* when supplied with 'spiked' as opposed to with 'non-spiked' nutrition. Increasing concentrations of osmotica have previously been reported in other plant species when supplied with increasingly concentrated medium nitrogen nutrition (Bennet *et al*, 1986).

Figs. 4.3 & 4.4 and anova analyses reveal that total soluble carbohydrate concentrations increased significantly during water deficits (as previously reported in other plant species, Foyer *et al*, 1998; Clifford *et al*, 1998; Li *et al*, 1998; Ferrario-mery *et al*, 1998), and were maintained in the following order in the leaves and roots of non-nodulated *V. faba* with respect to medium nitrogen nutrition: 'combined nitrogen > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate' nutrition, throughout water deficits. Furthermore significantly greater carbohydrate concentrations were exhibited in the leaves and roots of nodulated than of non-nodulated 'no nitrate' supplied *V. faba*; and of 'spiked' than of 'non-spiked' *V. faba*, throughout water deficits.

Specific carbohydrates reportedly exhibit compatible solute 'behaviour' during water deficits (Ingram & Bartels, 1996; Clifford *et al*, 1998), indicating that both osmotic and protein stabilization 'benefits' may be incurred in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with medium ammonia additions, during water deficits (see section 4.1, pg. 103).

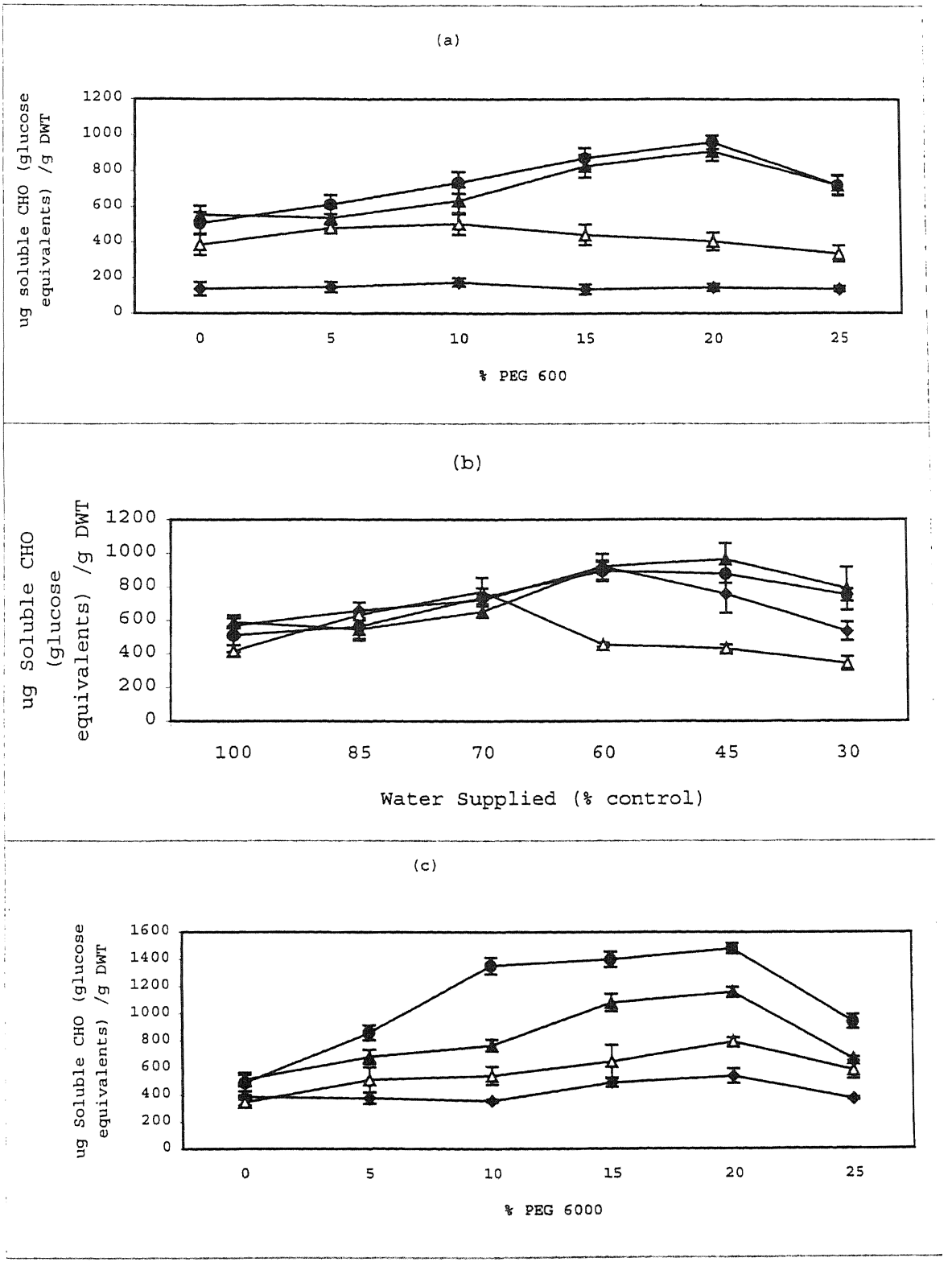


Fig. 4.3 Total soluble carbohydrate concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

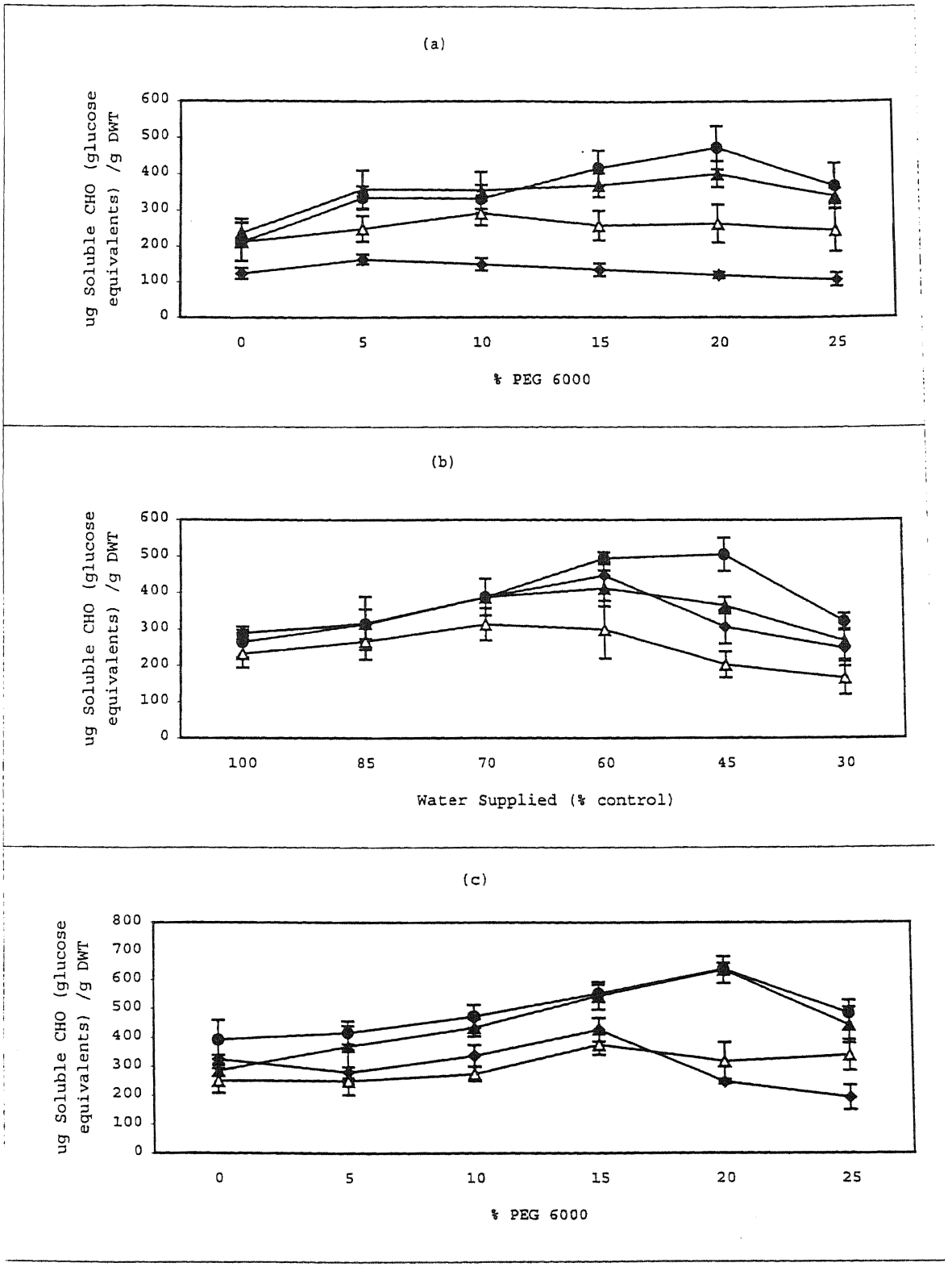


Fig. 4.4 Total soluble carbohydrate concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

4.3.2 TOTAL AMINO ACIDS

Leaf total amino acid concentrations are reportedly similar irrespective of the form of nitrogen supplied to *G. max* under 'control' conditions (Bougeais-Chaillou *et al,* 1992). However figs. 4.5 & 4.6 and anova analyses reveal that total amino acid concentrations were exhibited in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate' in the leaves and roots of non-nodulated *V. faba,* both when supplied with adequate irrigation (i.e. 0% PEG / 100% control water; fig. 2.2), and during water deficits.

Furthermore significantly greater amino acid concentrations were exhibited in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*; and in 'spiked' than in 'non-spiked' *V. faba* both when supplied with adequate irrigation and during water deficits, (in agreement with the data of Chaillou *et al,* 1991).

It is thus apparent that amino acid concentrations increased in *V. faba* as nitrogen availabilities increased. This was expected as nitrogen is a component of the enzymes which catalyse amino acid (and other osmotica) production, and is a component of amino acids (see introduction). Furthermore increased net photosynthesis (fig. 3.22) and total soluble carbohydrate concentrations (figs. 4.3 & 4.4), inferring increased carbohydrate and reductant availabilities for nitrogen assimilation, were recorded by *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. NR (Shaner & Boyer, 1976 a&b; Ourry *et al,* 1995); GDH (Taylor & Havill, 1981); and GS (Ortega *et al,* 1999) activities reportedly increase in other plant species in response to increasingly concentrated medium nitrogen nutrition, indicating that increased nitrogen assimilatory enzyme activities may also have contributed to the increased total amino

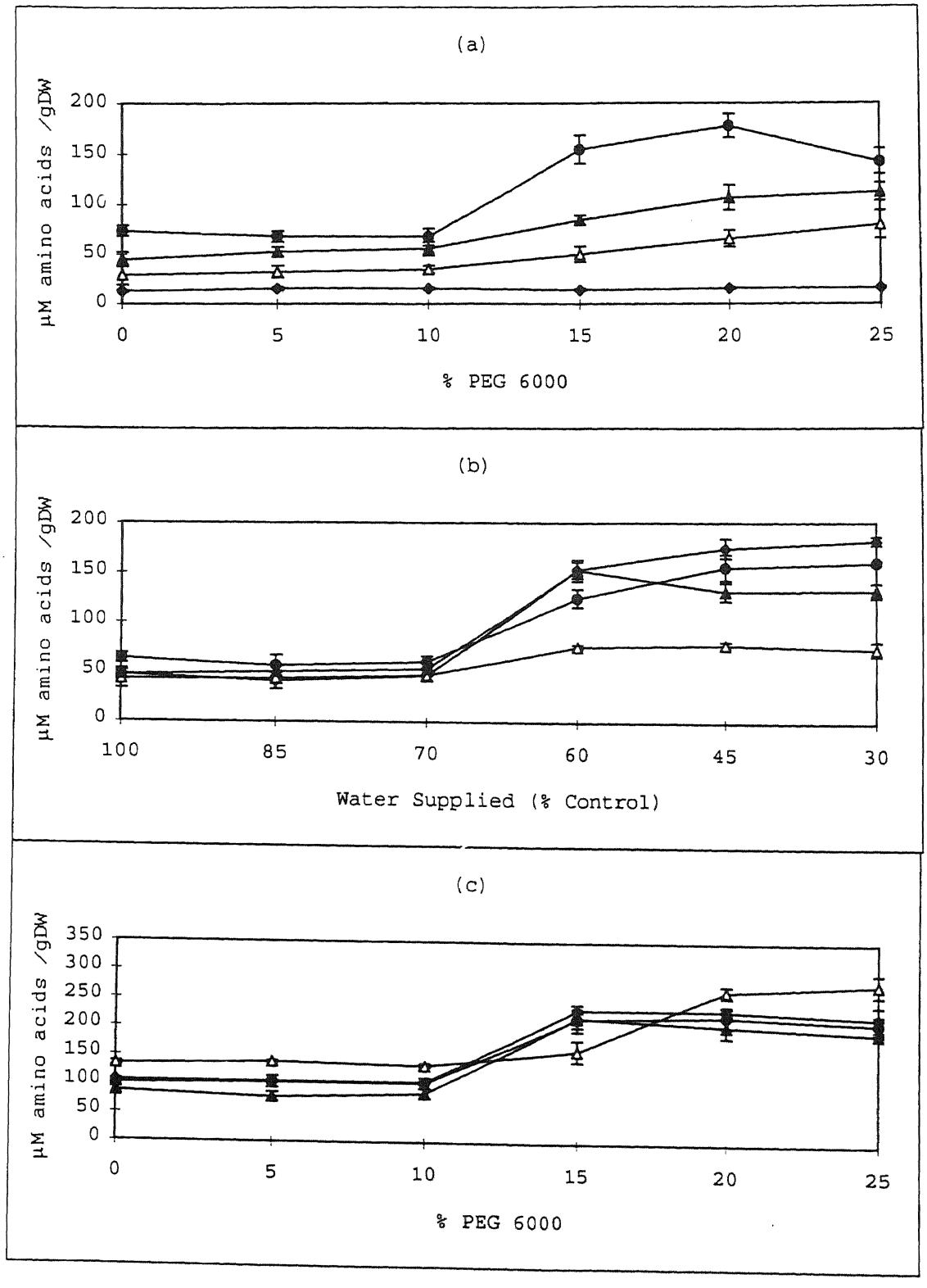


Fig. 4.5 Total amino acid concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

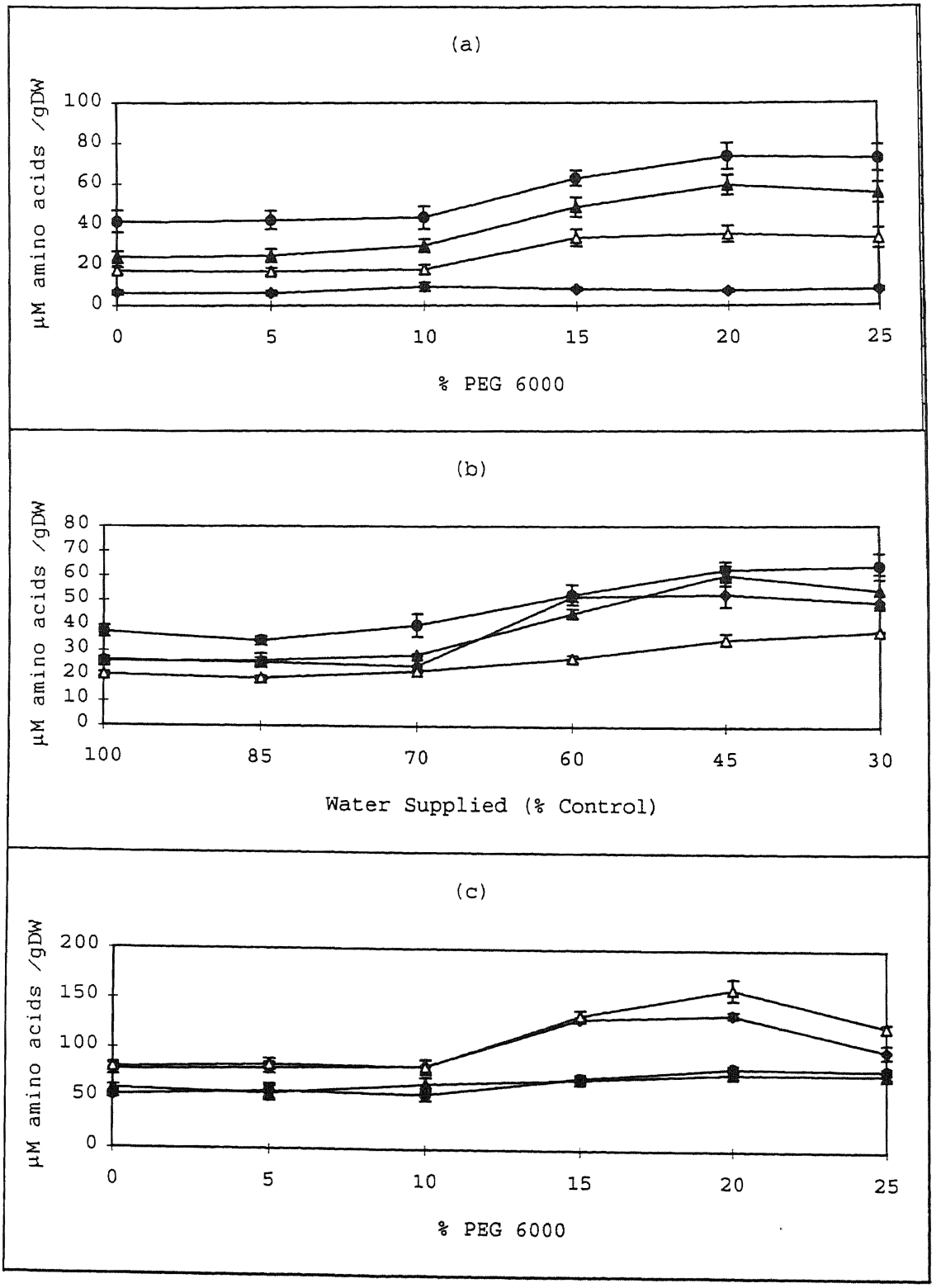


Fig. 4.6 Total amino acid concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

acid concentrations recorded in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (see section 6.4).

Foliar NR activities and accordingly total foliar amino acid concentrations may reportedly decrease rapidly during water deficits (Mattas & Pauli, 1965; Rajagopal *et al*, 1977; Hanson & Hitz, 1982; Wellburn *et al*, 1996; Ferrario-Mery *et al*, 1998; Foyer *et al*, 1998). However figs. 4.5 & 4.6 and anova analyses reveal that total amino acids accumulated significantly in the leaves and roots of *V. faba* during water deficits (as previously reported in other plant species, Singh, 1973b; Jones *et al*, 1980; Foyer *et al*, 1998). Significantly greater total amino acid concentrations were maintained in *V. faba* when supplied with increasingly concentrated medium nitrate nutrition; inferring that nitrate reductase activities may have been maintained in *V. faba* during water deficits (see 4.1, pg. 104).

Specific amino acids reportedly exhibit compatible solute effects during water deficits (Nash *et al*, 1981; Paleg *et al*, 1985; see pgs. 104 & 246) further inferring that osmotic and protein stabilization 'benefits' may be incurred in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with medium ammonia additions during water deficits.

4.3.3 PROLINE

Figs 4.7 & 4.8 and anova analyses reveal that proline concentrations were significantly greater in the leaves and roots of non-nodulated *V. faba* when supplied with 'combined nitrogen' as opposed to with nitrate nutrition, and that proline accumulated in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' >

'no nitrate'.

Furthermore leaf and root proline concentrations were significantly greater in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*. Evidence has emerged that some nodular bacteroids (infected with *Bradyrhizobium japonicum*) may catabolize proline as an energy source (Straub *et al*, 1997), which reportedly results in increased yield maintenance during moderate water deficits, inferring that proline accumulation in nodulated roots (as recorded in *V. faba*; fig. 4.8) may incur metabolic 'benefits' in addition to osmotic 'benefits'.

Proline may be produced from the arginine-ornithine pathway (Barnett & Naylor, 1966), however this pathway is reportedly primarily catabolic (Kueh *et al*, 1984; Gilbert *et al*, 1998), and proline is reportedly primarily produced from glutamate (Boggess 1970; Kato, 1980; Stewart, 1981; Rhodes *et al*, 1986; Samaras *et al*, 1995; see fig. 6.1). Proline may thus represent an ammonia de-toxification product (Shobert, 1977), and a carbon and nitrogen storage compound (Barnett & Naylor, 1966; Aspinall & Paleg, 1981), as reflected in the significantly greater concentrations of proline which accumulated in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (see section 6.4).

'Spiked' and 'non-spiked' *V. faba* accumulated similar concentrations of proline, however chapter six will demonstrate that several other amino acids accumulated to significantly greater concentrations in *V. faba* when supplied with 'spiked' as opposed to with 'non spiked' nutrition.

Anova analyses reveal that proline accumulated significantly during water deficits, as previously reported in *V. faba* (Aspinall & Paleg, 1981), and in other plant species (Barnett & Naylor, 1966; Stewart & Larher, 1980;

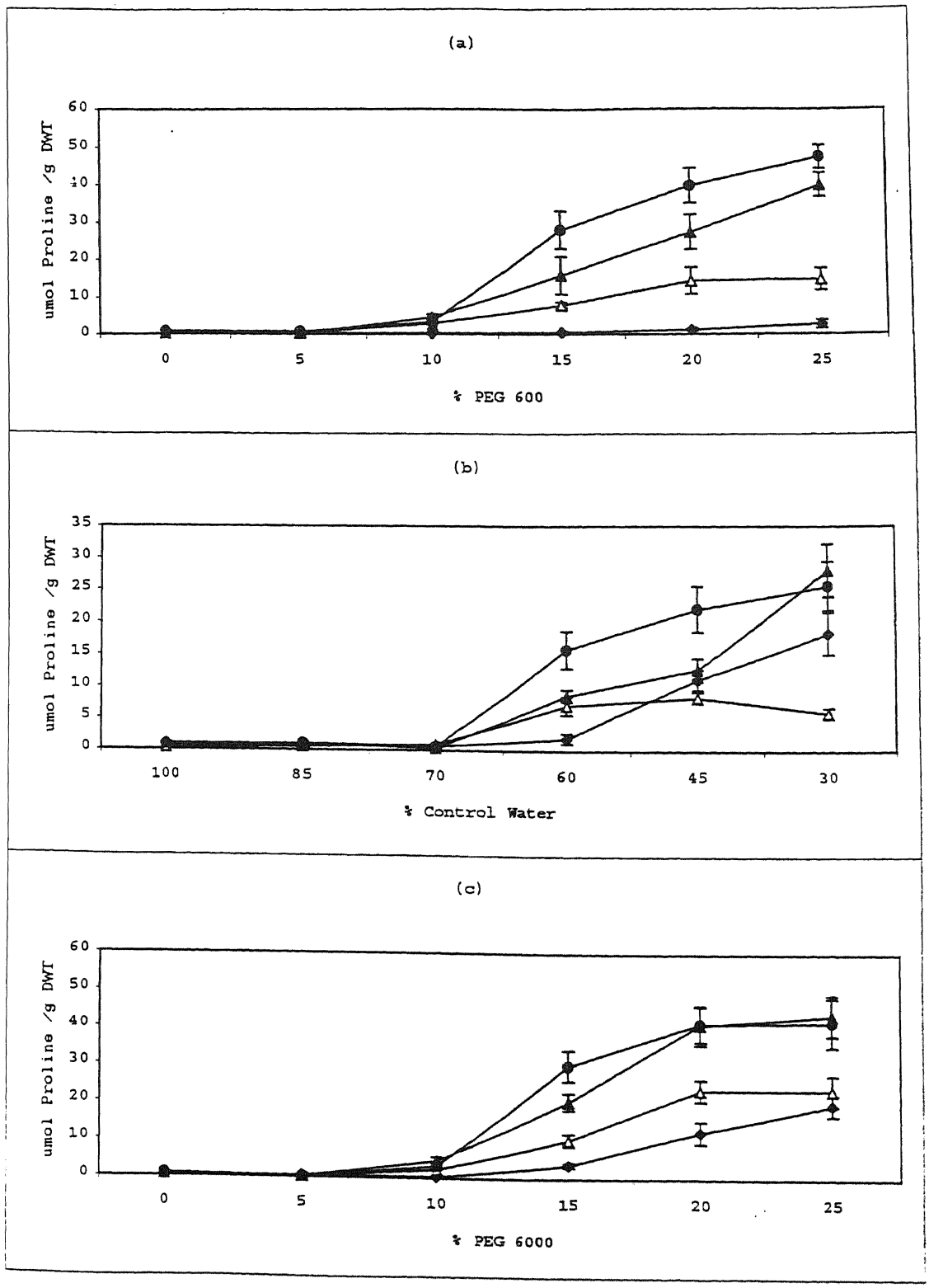


Fig. 4.7 Proline concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

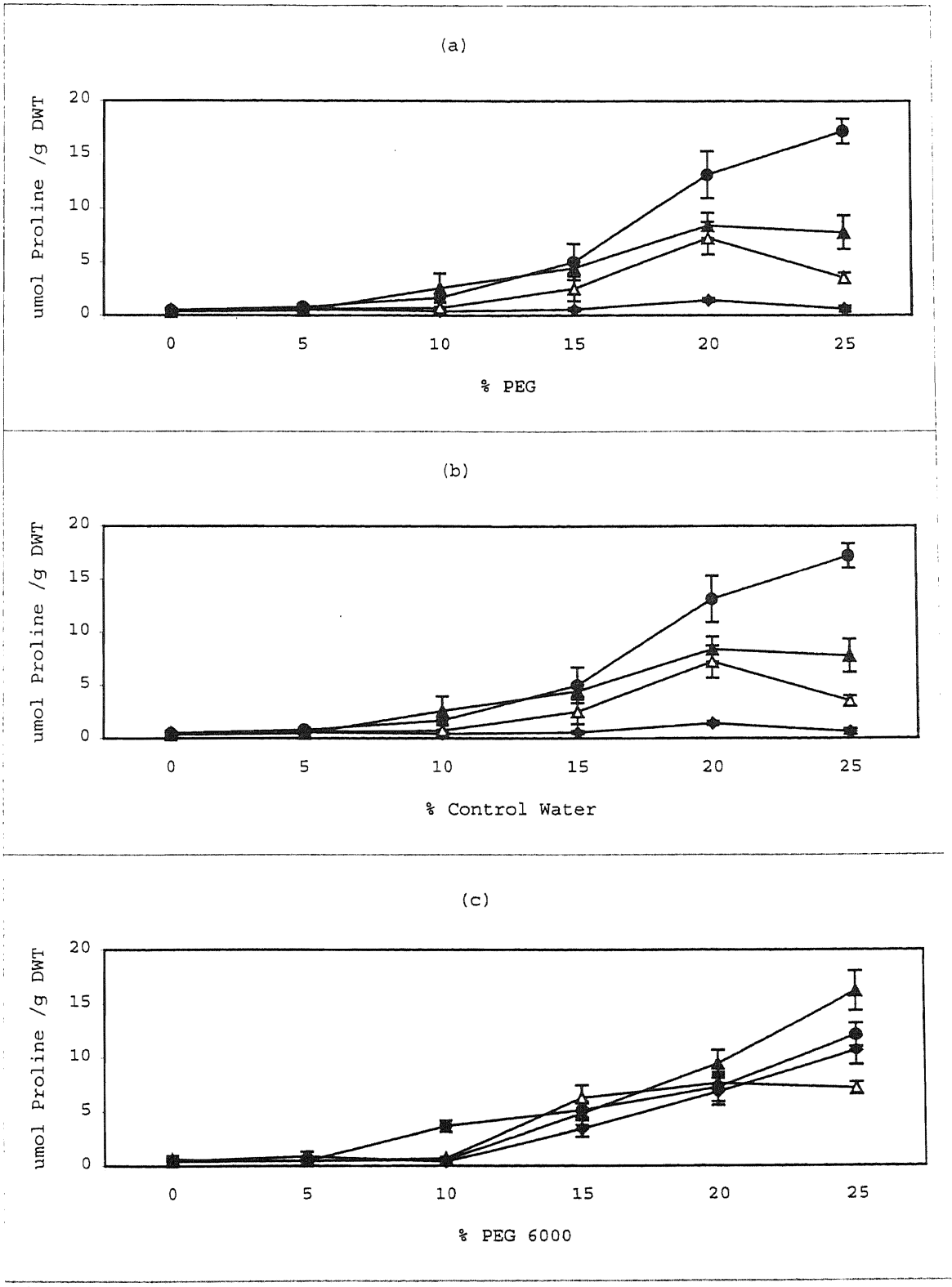


Fig. 4.8 Proline concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

Bray, 1997). Proline is an efficient osmotic solute, the reported slow turnover of which results in proline accumulation at a minimal cost of synthesis (Rhodes *et al*, 1986). Proline accumulation is reportedly positively correlated with leaf tissue survival and water deficit tolerance (Singh *et al*, 1973a), and also with post water deficit growth (Aspinall & Paleg, 1981) in other plant species. Proline may also reportedly fulfil compatible solute 'roles' in other plant species (Paleg & Aspinall, 1981; Nash *et al*, 1981; Paleg *et al*, 1985).

The inference is that when *V. faba* is supplied with increasingly concentrated medium nitrogen nutrition (and particularly with 'combined nitrogen' as opposed to with equimolar nitrate nutrition) greater proline concentrations may contribute towards greater protein stabilization (enzyme activity data is summarised in sections 5.4 & 6.4), and increased growth and leaf tissue survival (as previously described in section 3.4).

4.3.4 GLYCINE BETAINE

Figs. 4.9 & 4.10 and anova analyses reveal that glycine betaine concentrations were significantly greater in the leaves and roots of *V. faba* when supplied with 'combined nitrogen' as opposed to with equimolar nitrate nutrition, and that glycine betaine concentrations were exhibited in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate' nutrition. Furthermore nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba*; and 'spiked' as opposed to 'non-spiked' *V. faba* accumulated significantly greater concentrations of glycine betaine throughout water deficits.

Greater (actual and inferred) net photosynthesis was exhibited in *V. faba* when supplied with medium ammonia additions, and may have resulted in

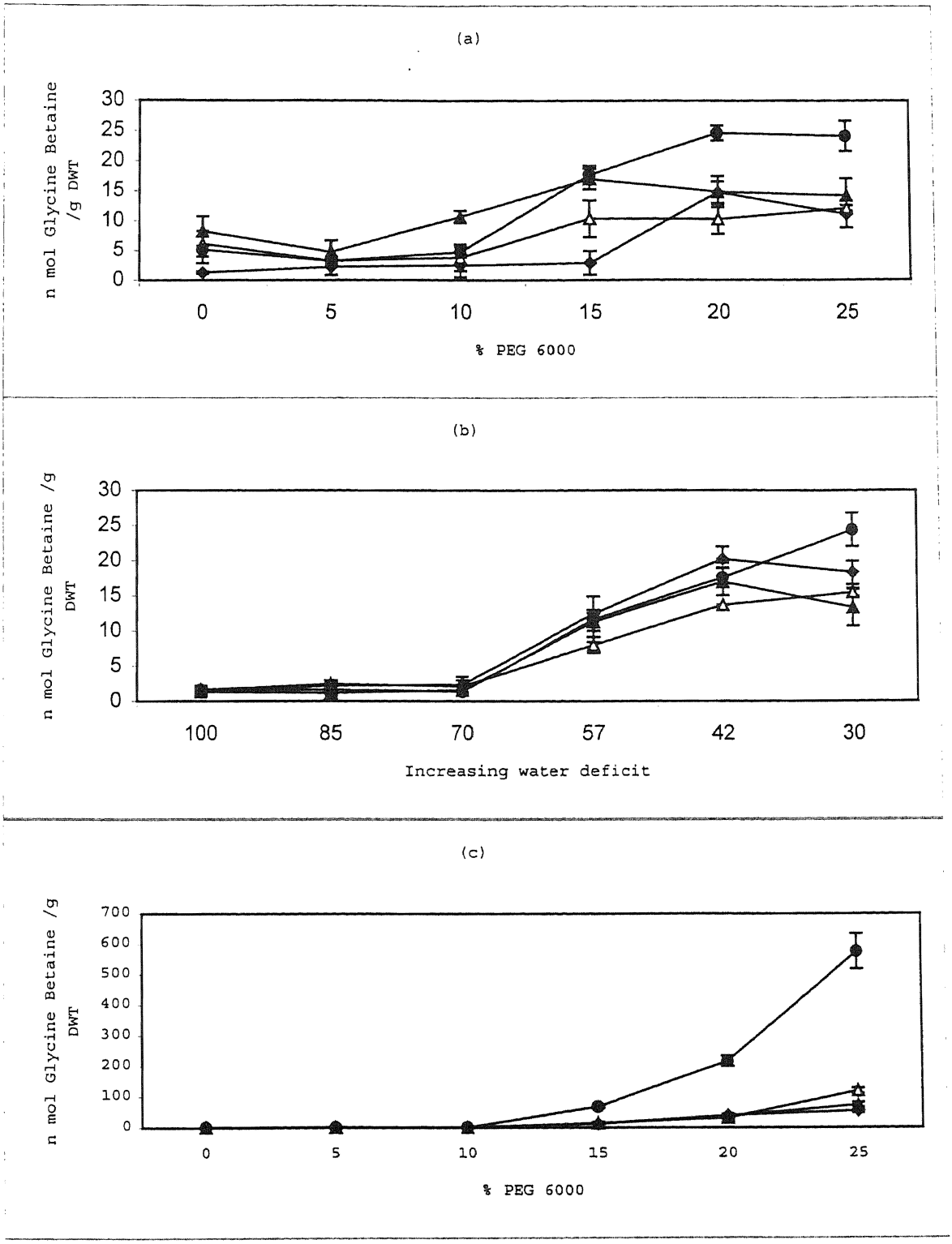


Fig. 4.9 Glycine Betaine concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

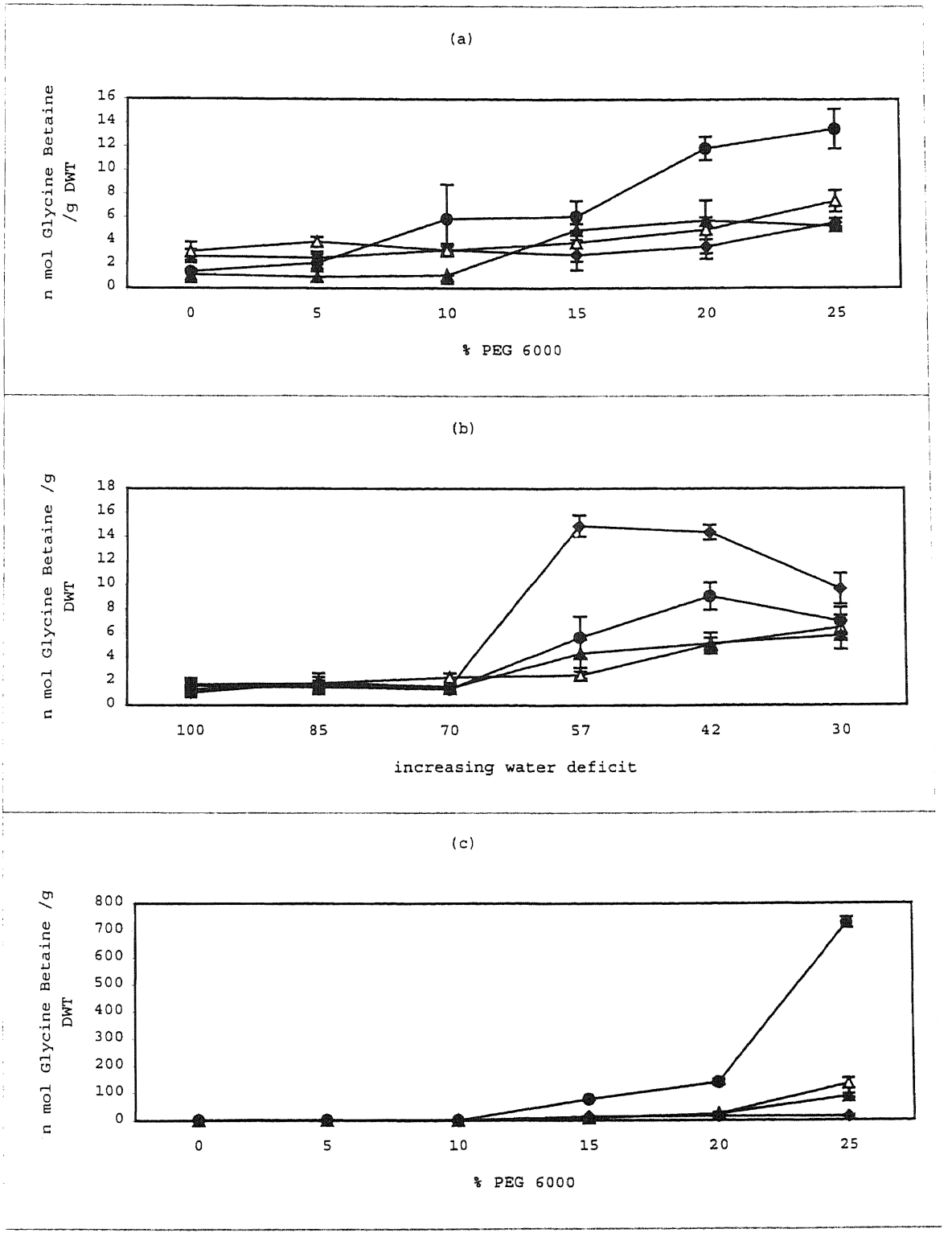


Fig. 4.10 Glycine Betaine concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

greater photosynthate and reductant availabilities (fig. 3.22). Furthermore increased nitrogen assimilatory enzyme activities have been reported in other plant species when supplied with increasingly concentrated medium nitrogen nutrition (Shaner & Boyer, 1976 a&b; Taylor & Havill, 1981; Ortega *et al*, 1997; see section 6.4). These two factors may have resulted in increasing substrate and energy availabilities which may have been partly utilised in the increased synthesis of glycine betaine in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with medium ammonia additions.

Figs. 4.9 & 4.10 and anova analyses reveal that glycine betaine accumulated significantly in *V. faba* during water deficits (particularly when supplied with medium ammonia additions). However while glycine betaine accumulation was statistically significant, the actual concentrations of glycine betaine which were recorded contributed small concentrations to the total amino acid pools (figs. 4.5 & 4.6), and therefore may not have been physiologically significant during water deficits.
However glycine betaine reportedly exhibits compatible solute 'behaviour' during water deficits (Borowitzka, 1981; Clifford *et al*, 1998), inferring the potential exhibition of an increased capacity for metabolism maintenance in *V. faba* when supplied with medium ammonia 'spike' additions during water deficits.

4.3.5 TOTAL OSMOLARITIES

Figs. 4.11 & 4.12 and anova analyses reveal that total osmolarities were maintained in the leaves and roots of *V. faba* in the following order as significantly affected by medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate'. Significantly greater total

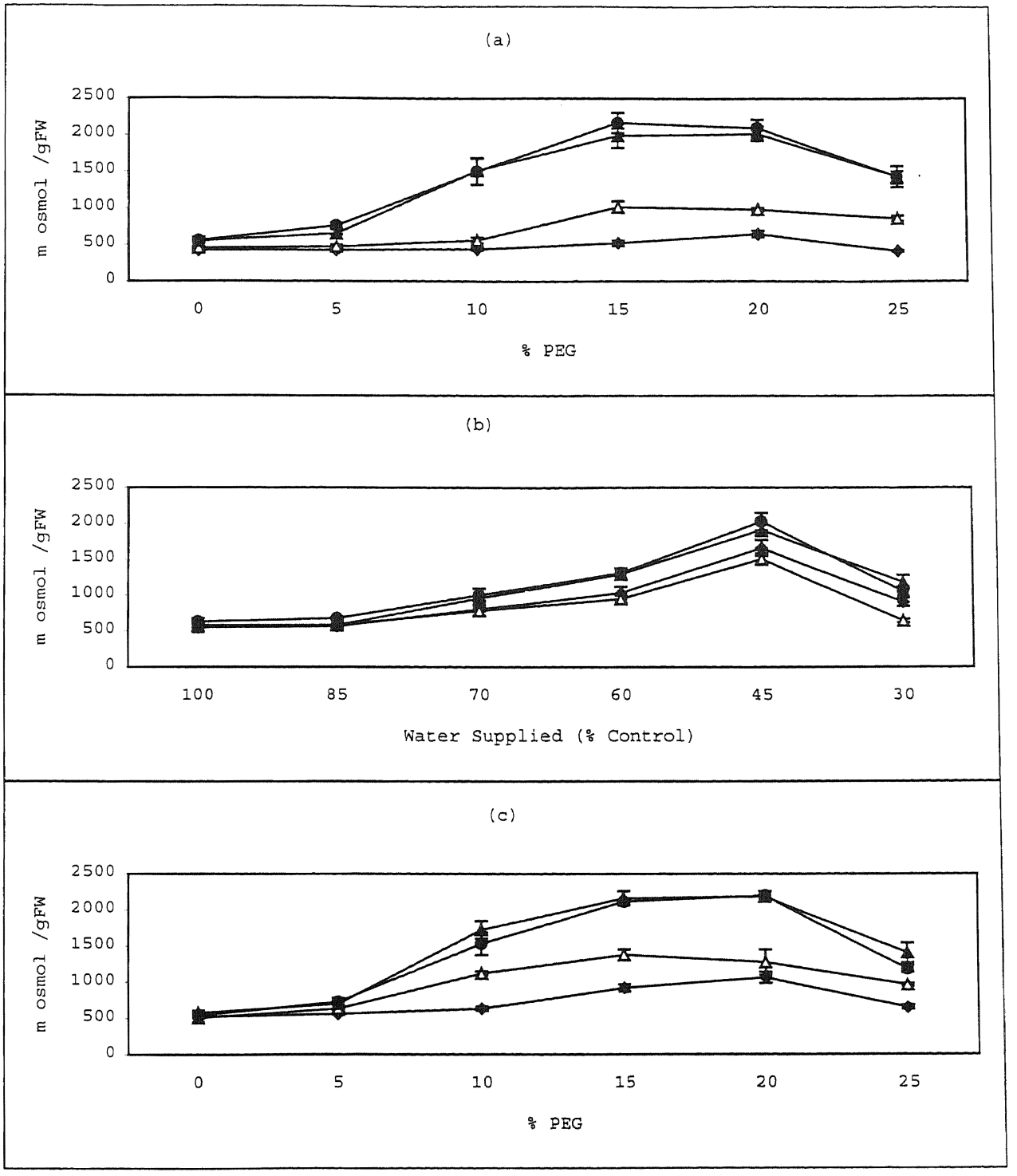


Fig. 4.11 Total osmolarities in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

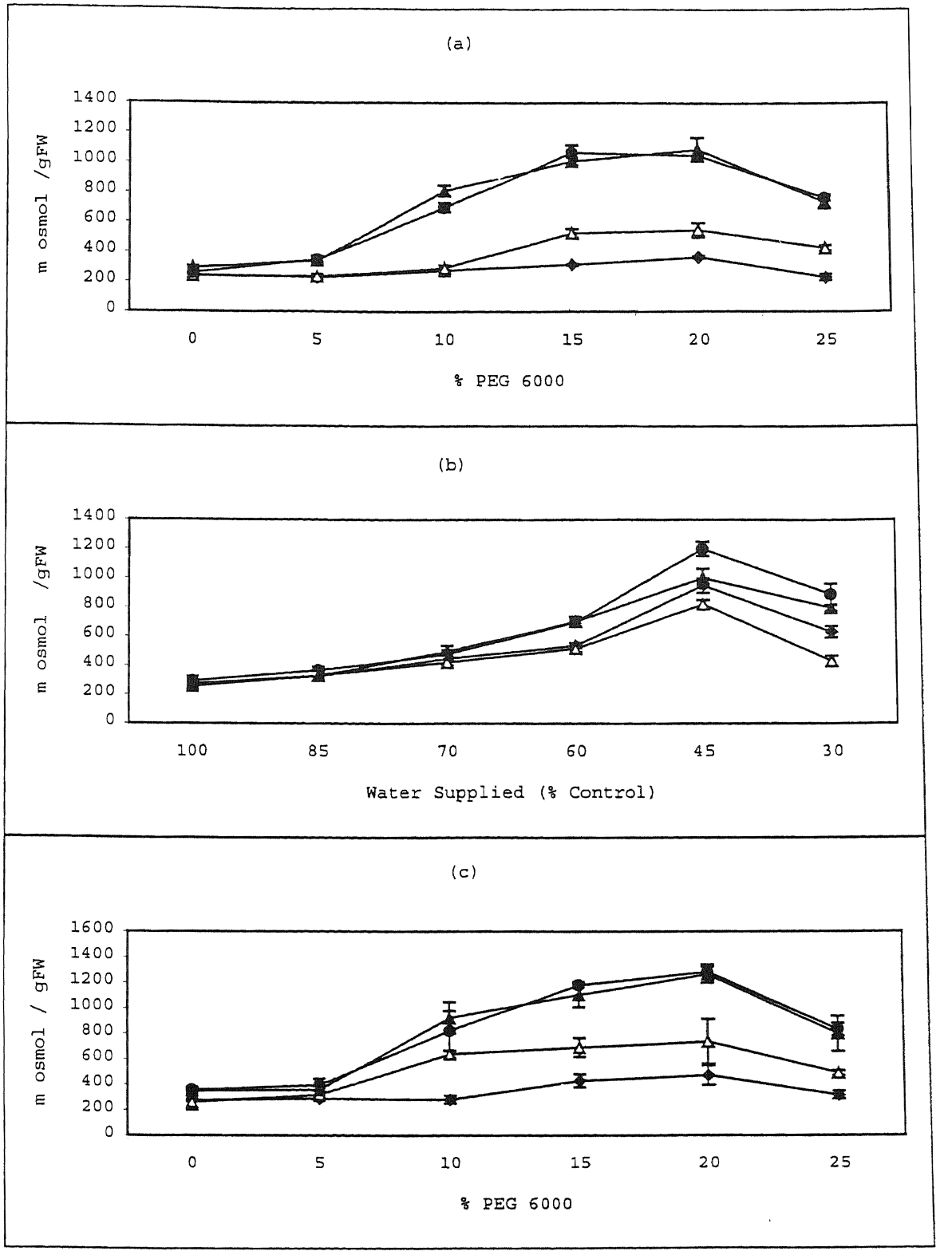


Fig. 4.12 Total osmolarities in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

osmolarities were recorded in the leaves and roots of nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba*; and in 'spiked' as opposed to 'non-spiked' *V. faba*. Total osmolarity data supports earlier data which demonstrated that increasingly great concentrations of specific osmotica were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with medium ammonia additions (figs. 4.3 - 4.10).

Figs. 4.11 & 4.12 and anova analyses reveal that total osmolarities increased significantly in the leaves and roots of *V. faba* during increasing water deficits, which may reflect the significant increases in total soluble carbohydrates; total amino acids; proline; and glycine betaine which were recorded during increasing water deficits (figs. 4.3 - 4.10).

4.3.6 ROOT OSMOTIC ADJUSTMENT

Anova analyses reveal that total soluble carbohydrate concentrations; total amino acid concentrations; proline concentrations; glycine betaine concentrations; and total osmolarities increased significantly in the roots of *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and particularly with 'combined nitrogen'; and with ammonia 'spike' nutrition), and that root osmotic adjustment also increased significantly during water deficits.

Root osmotic adjustment has previously been reported. Choline mono-oxidase (which synthesizes glycine betaine) is reportedly induced in the roots during water deficits (Russell *et al*, 1998), and proline is reportedly transported to plants roots (Verslues & Sharp, 1999), where it accumulates during water deficits in other plant species (albeit less extensively than in the leaves; Aspinall & Paleg, 1981).

Earlier work has indicated that root osmotic adjustment might result from reductions in radial expansion (Wilson *et al*, 1977). However root dry weights were maintained in non-nodulated *V. faba* during water deficits (fig. 3.9), and root RWCs decreased during moderate water deficits (10% PEG / 70% control water supplied; fig. 3.18), by which time substantial root carbohydrate accumulation was exhibited. RWCs were maintained at increasingly great concentrations in *V. faba* which exhibited the greatest osmotic adjustment (i.e. in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition). Furthermore root FWs (fig. 3.4) and root osmotic adjustment (figs. 4.4; 4.6; 4.8; 4.10 & 4.12) were both significantly greater in 'spiked' than in 'non-spiked' *V. faba*. The inference from these two observations is that actual osmotic adjustment as opposed to cell volume decreases occurred in the roots (and in the leaves) of *V. faba* during water deficits.

Furthermore root osmotic solutes accumulated to significant concentrations; root total soluble carbohydrate concentrations doubled, root total amino acid concentrations almost doubled, and root proline concentrations increased twenty-fold in non-nodulated *V. faba* during water deficits (when supplied with medium 'combined nitrogen' nutrition). The indication is that medium ammonia additions may result in the greatest exhibition of root (and leaf) osmotic adjustment in *V. faba*, and the inference is that as the increases in actual concentrations (per gramme dry weight) of the quantified root solutes were substantial during water deficits they may not have been solely attributable to root cell volume decreases. Indeed glycine betaine concentrations increased from zero to over 700 nmoles/g DW during water deficits in the roots of *V. faba* when supplied with ammonia 'spike' nutrition (fig. 4.10); fifty-fold greater glycine betaine concentrations

were exhibited in the roots of 'spiked' than of 'non-spiked' *V. faba*, and while the glycine betaine concentration increases represented a small fraction of the overall quantified osmotica pool, glycine betaine reportedly exhibits compatible solute 'behaviour' (Wood, 1999). The inference is that medium ammonia additions may result in an increased capacity for metabolism maintenance in the roots (and leaves) of *V. faba* during water deficits.

The inter-cellular space apoplast of roots reportedly often contains solutions with high ion concentrations, as water can leave the symplast faster than some solutes which may then accumulate (Canny, 1995), and with low transpiration (as inferred by the stomatal conductance decreases recorded in *V. faba* during water deficits; fig. 3.21) xylem tensions may disappear, and xylem osmotic potential (generated by root solute concentrations, with possible root positive pressure), may be the sole cause of root water uptake (Boyer, 1985). The inference is that *V. faba* which accumulated the greatest concentrations of root osmotica (i.e. *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; and particularly when supplied with an additional ammonia 'spike') may have maintained greater capacities for water uptake during water deficits than *V. faba* which accumulated lower concentrations of root osmotica. Indeed plants which accumulate greater concentrations of osmotic solutes reportedly extract more water during water deficits (Kumar & Singh, 1998; Collinson *et al*, 1997), as supported by the significantly greater osmotic adjustment and RWCs exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (figs. 4.3 - 4.10; 3.16; 3.17).

4.4 CONCLUSION

Total soluble carbohydrates accumulated significantly during water deficits, and to significantly greater concentrations in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; with 'combined nitrogen' as opposed to with equimolar nitrate nutrition; with 'spiked' as opposed to with 'non-spiked' nutrition; and with nodulated as opposed to with non-nodulated 'no nitrate' nutrition. Carbohydrates accumulated earlier during water deficits than other osmotica (as previously described by Bussis & Heinke, 1998). Indeed sucrose accumulation may be a prior requirement for proline accumulation, as sucrose reportedly inhibits proline oxidation (Boggess *et al*, 1975; Stewart, 1981), and the collected data illustrates that sucrose accumulation was initiated prior to proline accumulation in *V. faba* (figs. 4.7; 4.8; 5.2; 5.3). Increased total soluble carbohydrate accumulation in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition was expected, as nitrogen is a component of the enzymes which catalyse metabolic reactions, including photosynthesis. Total soluble carbohydrate data reflects the data described in chapter three which demonstrated that RWCs; stomatal conductances; and hence net photosynthesis (and by inference carbon acquisition; see section 3.4) were maintained at significantly greater levels in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*; and in 'spiked' than in 'non-spiked' *V. faba*.

Total amino acids also accumulated significantly during water deficits, and to significantly increasing concentrations in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition.

This was expected as in other plant species NR (Shaner & Boyer, 1976 a&b; Ourry *et al*, 1995); GDH (Taylor & Havill, 1981); and GS (Ortega *et al*, 1997) activities reportedly increase in response to increasingly concentrated medium nitrogen nutrition (as occurred in *V. faba*; see section 6.4). The reported enhancement of amino acid synthesis relative to sucrose synthesis in other plant species when supplied with concentrated medium nitrogen nutrition (Foyer *et al*, 1991), the reported protein concentration decreases in other plant species during water deficits (Riccardi *et al*, 1998), and alterations in transaminase activities during water deficits (Thompson *et al*, 1966) may also have contributed towards the accumulation of individual amino acids (as discussed in section 6.4).

Proline and glycine betaine concentrations, and total osmolarities also increased significantly in *V. faba* during water deficits; again to increasingly great concentrations in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition.
Specific carbohydrates and amino acids (as discussed in sections 4.1; pgs. 103 & 104), and proline and glycine betaine may reportedly act as compatible solutes during water deficits (Paleg *et al*, 1985; Ingram & Bartels, 1996; Clifford *et al*, 1998). The significant accumulation of these solutes infers an increased capacity for metabolism maintenance in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and particularly with 'combined nitrogen' as opposed to with equimolar nitrate nutrition; and with medium ammonia 'spike' additions) during water deficits. Furthermore specific amino acids, proline, and glycine betaine may represent ammonia de-toxification products in *V. faba* (as previously suggested for other plant species, Barnett & Naylor, 1966; Shobert, 1977; Aspinall & Paleg, 1981; Pulich, 1986), as discussed in section 6.4. Proline

has previously been described as a nitrogen storage compound, as it is reportedly found in relatively large concentrations in storage organs (e.g. seeds; Miflin & Lea, 1977).

Furthermore greater total amino acid and total soluble carbohydrate concentrations were exhibited in *V. faba* when supplied with medium ammonia additions, even when supplied with adequate water (0 % PEG / 100 % Control Water; figs. 4.3 - 4.6). The inference is that *V. faba* may exhibit increased water deficit tolerance when supplied with medium ammonia additions, both prior to and during water deficits, as amino acids and soluble carbohydrates represent potential substrates for osmotic adjustment and for growth maintenance.

Osmotic adjustment increased significantly in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and particularly with ammonia 'spike' additions) during water deficits, which may infer an increased capacity for water uptake in *V. faba* when medium ammonia additions are included (Boyer, 1985), particularly as significantly greater root biomasses were also exhibited in 'spiked' than in 'non-spiked' *V. faba*, and root growth is reportedly correlated with water uptake in this species (Sau & Ines-Minguez, 1990).

Other leguminous species reportedly exhibit lower concentrations of osmotica during water deficits when reliant on nitrogen fixation than when supplied with medium nitrogen nutrition (Sprent, 1971). Previous authors have attributed this to the high ATP and photosynthate demands of nitrogen fixation (Sprent, 1971; Pate *et al*, 1979; Dekhuijzen *et al*, 1981; Schilling, 1983; Caba *et al*, 1998). However significantly greater total

soluble carbohydrate; total amino acid; proline; and glycine betaine concentrations; and leaf and root total osmolarities were maintained in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits. This may reflect the previous observation that significantly greater stomatal conductances and net photosynthesis (and hence potentially photosynthate, reductant, and carbon skeleton concentrations) were maintained in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* (figs. 3.21 & 3.22), and that nitrogen fixation potentially provided nitrogen to nodulated 'no nitrate' supplied *V. faba* (table 2.3). Thus the carbon and nitrogen substrates which are required for osmotic adjustment were potentially available in greater concentrations in nodulated than in non-nodulated 'no nitrate' supplied *V. faba.* The maintenance of greater osmotic adjustment in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits infers that nitrogenase activities may have been maintained in *V. faba* during water deficits (which supports the work of Serraj & Sinclair, 1997, who highlighted that nitrogen fixation may be water deficit tolerant in some *G. max* cultivars; see introduction).

It has previously been reported that during the early stages of water deficits nodulated *G. max* (var. 'Williams') predominantly rely on increased root growth and on stomatal closure to delay water deficit effects, and that osmotic adjustment is initiated later during water deficits in nodulated than in non-nodulated *G. max*, again perhaps as nitrogen fixation is reportedly an energy intensive process (see introduction, pg. 8), to which osmotic adjustment may be detrimental (Ines-Minguez & Sau, 1989). Ines-Minguez & Sau (1989) concluded that the different water deficit tolerance 'strategies' (delayed osmotic adjustment in nodulated as opposed

to in non-nodulated *G. max*) nevertheless resulted in the exhibition of similar leaf areas and inferred turgor maintenance during water deficits. However osmotic adjustment was initiated at similar levels of water deficit imposition in both nodulated and non-nodulated *V. faba* (var. 'Bunyards Exhibition'), perhaps as significantly greater net photosynthesis (fig. 3.22), starch concentrations and amylase activities (section 5.4), and significantly greater nitrogen assimilatory enzyme activities (section 6.4) were exhibited in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits, which may have contributed substrates towards osmotic adjustment in this cultivar.

Increased turgor during early water deficits has been described as resulting from both osmotic and elastic components (Grossnickle & Russell, 1996). Increased cell wall elasticities (as reported in *V. faba* during water deficits, Elston *et al*, 1976) may facilitate osmotic adjustment via cell volume reductions (Meier *et al*, 1992; Clifford *et al*, 1998). However reduced inter-molecular space during water deficits is reportedly correlated with reduced enzyme activities, inferring that turgor and volume maintenance may be more important than maintained water potentials during water deficits (Meier *et al*, 1992). Solutes accumulated to such significant concentrations in *V. faba* that it was unlikely that they were solely attributable to cell volume decreases (figs 4.3 - 4.10), particularly as growth (figs. 3.1 - 3.10) and RWCs (figs. 3.16 & 3.17) were also maintained at significantly greater levels in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (see section 3.4), the same nitrogen nutrition which resulted in the exhibition of the greatest concentrations of osmotica when supplied to *V. faba*.

Furthermore *V. faba* exhibited significantly greater concentrations of root osmotica when supplied with increasingly concentrated medium nitrogen nutrition, and with 'spiked' than with 'non-spiked' nutrition. It has been inferred that plants which accumulate root osmotic solutes may exhibit increased capacities for water uptake during water deficits than non-root-accumulators (Boyer, 1985). Thus increased water uptake capacities are inferred for *V. faba* when supplied with medium ammonia additions, particularly as 'spiked' *V. faba* also exhibited significantly greater root biomasses than 'non-spiked' *V. faba*, and root growth is also reportedly correlated with water uptake in *V. faba* (Sau & Ines-Minguez, 1990). Greater water uptake in 'spiked' than in 'non-spiked' *V. faba* is also inferred by the significantly greater heights and cumulative leaf areas (figs. 3.20 & 3.25) which were exhibited in 'spiked' than in 'non-spiked' *V. faba*, and which infer greater (threshold water content dependant) expansive growth (McDonald & Davies, 1996).

The significantly greater RWCs (figs. 3.16 & 3.16) and vegetative yields (figs. 3.1 - 3.10; 3.20; 3.25) exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and with medium ammonia additions) may reflect the increased osmotic adjustment exhibited in *V. faba* when supplied with such nutrition, as greater RWCs (Singh & Gupta, 1983), increased medium water extraction (Kumar & Singh, 1998), and greater yields (Rodriguez-Maribona *et al*, 1992) have previously been reported in other plant species which have exhibited increasing concentrations of osmotica during water deficits. Indeed *V. faba* exhibited sufficient osmotic adjustment during water deficits to allow the maintenance of RWCs until severe water deficits were imposed (figs. 3.16 & 3.17). A positive correlation between osmotica concentration and yield has

previously been reported in *V. faba* (Van der Wal, 1981).

Osmotic adjustment may reportedly provide an alternative sink for photosynthates and energy during growth reductions in some plant species (Wardlaw, 1993), and may thus reduce the likelihood of photoinhibition (Smirnoff & Stewart, 1985). However it is unlikely that excess photosynthates accumulated prior to growth reductions in *V. faba*, as growth and photosynthetic declines coincided during water deficits in this species (see section 3.4). The inference is that substrates for osmotic adjustment in *V. faba* were not solely derived from accumulating photosynthates, and may potentially have resulted from alterations in carbon metabolism (as described in chapter five), or from alterations in nitrogen metabolism (as described in chapter six), during water deficits.

In summary the data presented in this chapter illustrates that total soluble carbohydrate concentrations, total amino acid concentrations, proline concentrations, glycine betaine concentrations and total osmolarities all increased significantly in the leaves and roots of *V. faba* during water deficits.

Increasing osmotic adjustment was exhibited by the leaves and roots of *V. faba* when supplied with increasingly concentrated medium nitrogen. Furthermore significantly greater total osmolarities, and significantly greater total soluble carbohydrate, total amino acid and glycine betaine concentrations were exhibited by *V. faba* when supplied with medium ammonia additions, inferring an increased capacity for metabolism maintenance in *V. faba* when supplied with some medium ammonia, due to increased compatible solute accumulation.

CHAPTER FIVE

ASPECTS OF CARBON METABOLISM IN NON-NODULATED, NODULATED, AND AMMONIA 'SPIKED' *V. faba* WHEN SUPPLIED WITH VARIOUS FORMS AND CONCENTRATIONS OF MEDIUM NITROGEN NUTRITION DURING INCREASING WATER DEFICITS

Carbohydrates serve as energy stores, fuels, and metabolic intermediates (Stryer, 1988).

5.1 INTRODUCTION

This chapter examines aspects of carbon metabolism which may influence nitrogen assimilation and water deficit responses in *V. faba*.

Net photosynthesis is maintained at greater levels in *V. faba* (fig. 3.22; and reportedly in other plant species, Hageman, 1979; Marques *et al*, 1983; Tolley-Henry & Raper, 1986; Doehlert, 1993; Raven & Sprent, 1993) when supplied with increasingly concentrated medium nitrogen nutrition, inferring potentially increased capacities for the production of carbohydrates as osmotica, and therefore potentially greater plant productivities during and following water deficits in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (Van der Wal, 1981; Sau & Ines-Minguez, 1990).

Decreasing starch concentrations may be correlated with carbohydrate accumulation (Stewart, 1972a; Clifford *et al*, 1998), and reportedly coincide with increasing osmotic adjustment during water deficits (Bussis & Heinke, 1998), inferring that starch degradation and / or a decreased conversion of photosynthates into starch may also contribute substrates towards osmotic adjustment during water deficits.

Decreased respiration may potentially result in increased carbohydrate accumulation during water deficits, however respiration rates reportedly

only decrease during severe water deficits (Nogues *et al*, 1998, working with *P. sativum*) by which stage carbohydrate accumulation is maximal, inferring that reduced respiration may not contribute significantly towards osmotic adjustment.

Nitrate reduction results in the production of OH- ions, which (in the shoots) are biochemically converted via a 'pH stat' into organic (malic/oxalic) acids utilising sugars, starch, and CO_2 (Davies, 1973). The organic acids produced are osmotically active and may be stored in the vacuole, or may be phloem translocated to the roots (as K^+ salts) and then decarboxylated to pyruvate and HCO_3. OH^- is root excreted, while K^+ is recirculated with NO_3^- and other anions back to the shoots via the xylem (Raven & Smith, 1976). The inference is that decreasing carbohydrate and hence organic acid concentrations during water deficits (potentially attributable to decreasing net photosynthesis, fig. 3.22) may represent a decreased capacity for 'pH stat' maintenance, and hence for shoot nitrate reduction.

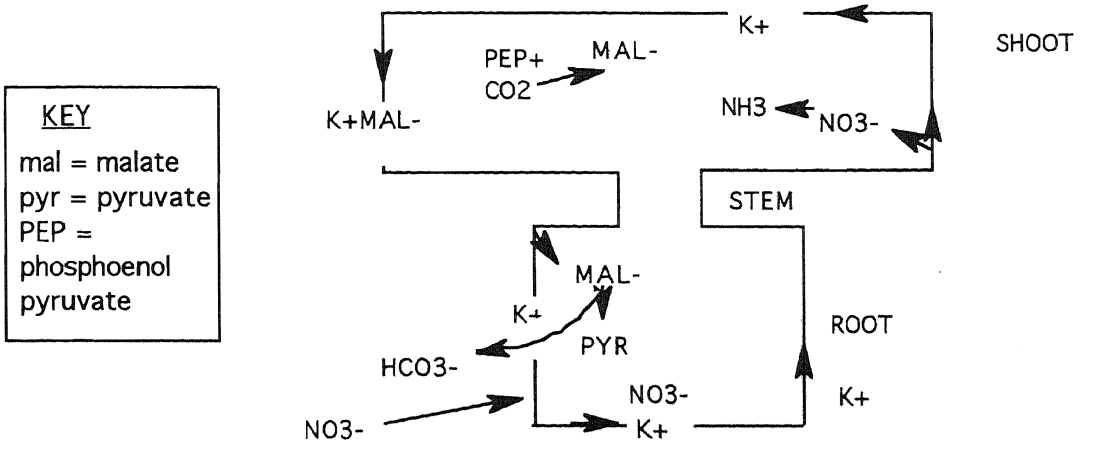


Fig. 5.1 Role of the nitrate-reductase-dependant malate shuttle on potassium and nitrate transport systems (after Lips, 1979).

The carbon skeletons of organic acids are also utilised during ammonia assimilation (Harada *et al*, 1968; Dijkshoorn 1973; Ikeda *et al*, 1974; Bourgeais-Chaillou *et al*, 1992; Raab & Terry, 1994), inferring that decreasing organic acid concentrations would result in decreased capacities for both nitrate and ammonia assimilation in plants. Indeed poor plant performance was reported when *G. max* which exhibited low organic acid concentrations was supplied with medium ammonia nutrition (Bourgeais-Chaillou *et al*, 1992).

5.2 MATERIALS & METHODS

5.2.1 TRINDER'S GLUCOSE OXIDASE : PEROXIDASE METHOD (G.O.D.P.O.D.)

5 mg glucose oxidase was added to 100 ml 0.5 M phosphate buffer (pH 7.0) along with 5 mg peroxidase; 100 mg sodium azide; and 35 mg 4-aminophenazone. 1.5 ml of this reagent was added to 1 ml plant extract (see section 2.6.1), along with 0.5 ml phenol (0.1%). Samples were incubated at 37°C for 20 minutes. OD was determined at 515nm, and concentrations calculated using a (glucose) standard curve.

5.2.2 INVERTASE ASSAY

0.5 ml methanoic extract (section 2.6.1) was incubated at 37°C (20 minutes) with 0.5 ml invertase (3000Eu/ml), which hydrolyses sucrose. Additional glucose was determined using a G.O.D.P.O.D. assay (5.2.1).

5.2.3 REDUCING SUGARS (Benedict's test)

17.3 g $CuSO_4$ was dissolved in 150 ml distilled water forming solution A. 173 g sodium citrate and 90 g anhydrous sodium carbonate were dissolved in 850 ml distilled water, which was then filtered (Whatman's No. 1 paper), forming solution B. Solution A was added to solution B slowly, with constant

agitation. This mixture formed the Benedict's reagent. 100 µl plant sample (section 2.6.1) was added to 2 ml Benedict's reagent. This mixture was boiled for 10 minutes. OD was determined at 515nm. Concentrations were determined using a (fructose) standard curve.

5.2.4 STARCH ANALYSIS (after McCready *et al*, 1950)

The extracted tissue from a methanoic extraction was re-washed twice (or until no soluble carbohydrates were detected using the anthrone detection technique; section 4.2.1) with the methanoic mix (see section 2.6.1), and then washed with 80% ethyl alcohol, then water, and then placed in a solution of 2.5 ml water and 3.2 ml 52% perchloric acid at 0°C for 20 minutes. This liquid was stored and the plant material was again placed in 2.5 ml water and 3.2 ml perchloric acid for a further 20 minutes at 0°C. The liquid from this second extraction was combined with that from the first, and this solution was diluted 1:10, filtered, then further diluted 1:50. 5 ml of this final dilution was added to 10 ml cold anthrone (0.2g anthrone in 100 ml 95% H_2SO_4), and then heated in a boiling water bath for 15 minutes, and then rapidly cooled. OD was determined at 630nm, and glucose equivalents were calculated for starch using a (glucose) standard curve.

5.2.5 AMYLASE ASSAY (after Chrispeels & Varner, 1967)

A starch solution was prepared from the spun down supernatant (8000 g for 25 minutes) of a 250 ml solution which contained 375 g soluble potato starch; 1.5 g KH_2PO_4; 200 µM $CaCl_2$ and had been boiled for 1 minute and then cooled. 5 g plant samples were pulverised in liquid nitrogen in 15 ml 10 mM calcium chloride with a mortar and pestle. Extracts were centrifuged at 8000 g for 25 minutes at 2 °C. 0.2 ml plant extract was added to 0.08 ml distilled water

with 1 ml starch solution. After 10 minutes 1 ml KI (6 g of KI with 600 mg iodine; diluted 1:100 with 0.05M HCl) was added. OD was determined at 620nm.

5.2.6 L-MALATE DETERMINATION (after Mollering, 1985a)

0.1 ml plant extract (prepared as in section 2.6.1) was added to a volume of 1.89 ml (containing 153 µM glycine, 100 µM L-glutamate, 4 µM Beta-NAD, and 61 µg glutamic-oxalacetic transaminase). Optical density, (OD), was determined, (340nm). 30 µg malic dehydrogenase (MDH) was added to start the reaction, creating a final volume of 2 ml. The rise in OD was followed at 340nm until a plateau was reached. The change in OD was used to determine the extract malate concentration.

5.2.7 CITRATE (after Mollering, 1985b).

0.1 ml plant extract was added to a volume of 1.89 ml (containing 352 µM tris buffer, (pH 7.6), 3.0 µg MDH, 74 µg lactic dehydrogenase, (LDH), and 1 µM beta-NADH). OD (340nm) was determined. 2.0 mg of citrate lyase was added to the solution to start the reaction. The change in OD was used to determined the extract citrate concentration.

5.2.8 2-OXOGLUTERATE (after Burlina, 1985)

0.1 ml plant extract was added to a volume of 1.89ml (containing 370 µM Tris-Cl buffer (pH 7.6) and 0.4 µM beta-NADH). The OD was determined (340nm), then L-glutamic dehydrogenase (GDH) was added to start the reaction, creating a final volume of 2.0 ml. The change in OD was used to determine the extract 2-oxogluterate concentration.

5.2.9 PYRUVATE (after Lamprecht & Heinz, 1984)

0.1 ml plant extract was added to a volume of 1.89 ml (containing 370 µM Tris-

Cl buffer (pH 7.6), and 0.4 μM beta-NADH). The OD (340 nm) was determined, and then 124 μM LDH was added to start the reaction creating a final volume of 2.0 ml. The change in OD was used to determine the extract pyruvate concentration.

5.3 RESULTS & DISCUSSION

5.3.1.1 SUCROSE, GLUCOSE AND REDUCING SUGARS

Figs. 5.2 to 5.7 and anova analyses reveal that sucrose; glucose; and reducing sugars accumulated to significantly increasing concentrations in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and accumulated in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate'.

Significantly greater concentrations of sucrose; glucose; and reducing sugars accumulated in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*; and in *V. faba* when supplied with 'spiked' as opposed to with 'non-spiked' nutrition.

Earlier work has indicated that sucrose; glucose; and fructose concentrations may decrease during water deficits in other plant species (Sanchez-Rodriguez *et al*, 1999). However figs. 5.2 - 5.7 and anova analyses reveal that sucrose; glucose; and reducing sugars accumulated significantly in the leaves and roots of *V. faba* during water deficits (as previously reported in other plant species, Wilson *et al*, 1980; Hanson & Hitz, 1982), and were maintained in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate' nutrition, throughout water deficits.

Sucrose contributed the greatest concentration to the total quantified

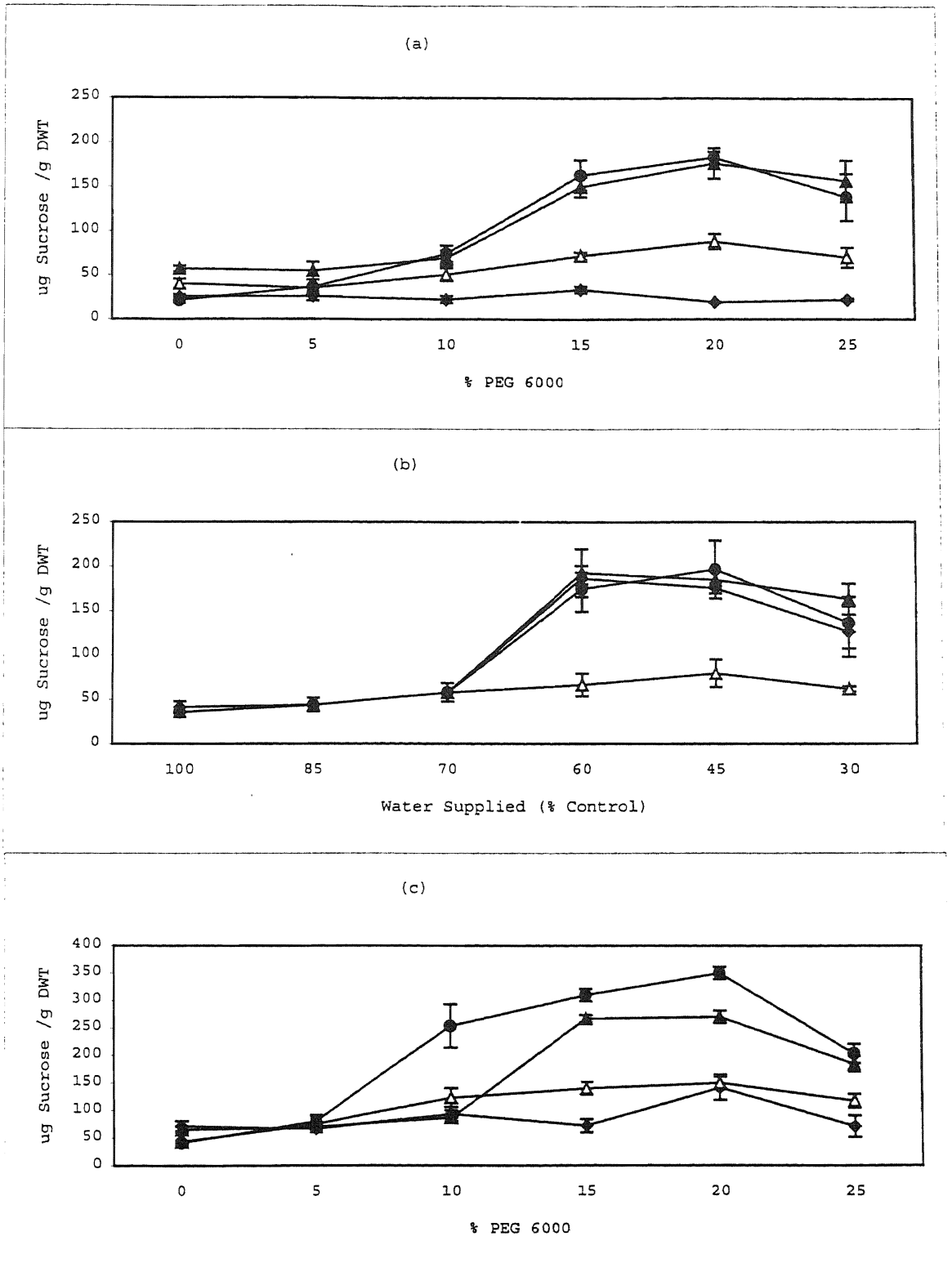


Fig. 5.2 Sucrose concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

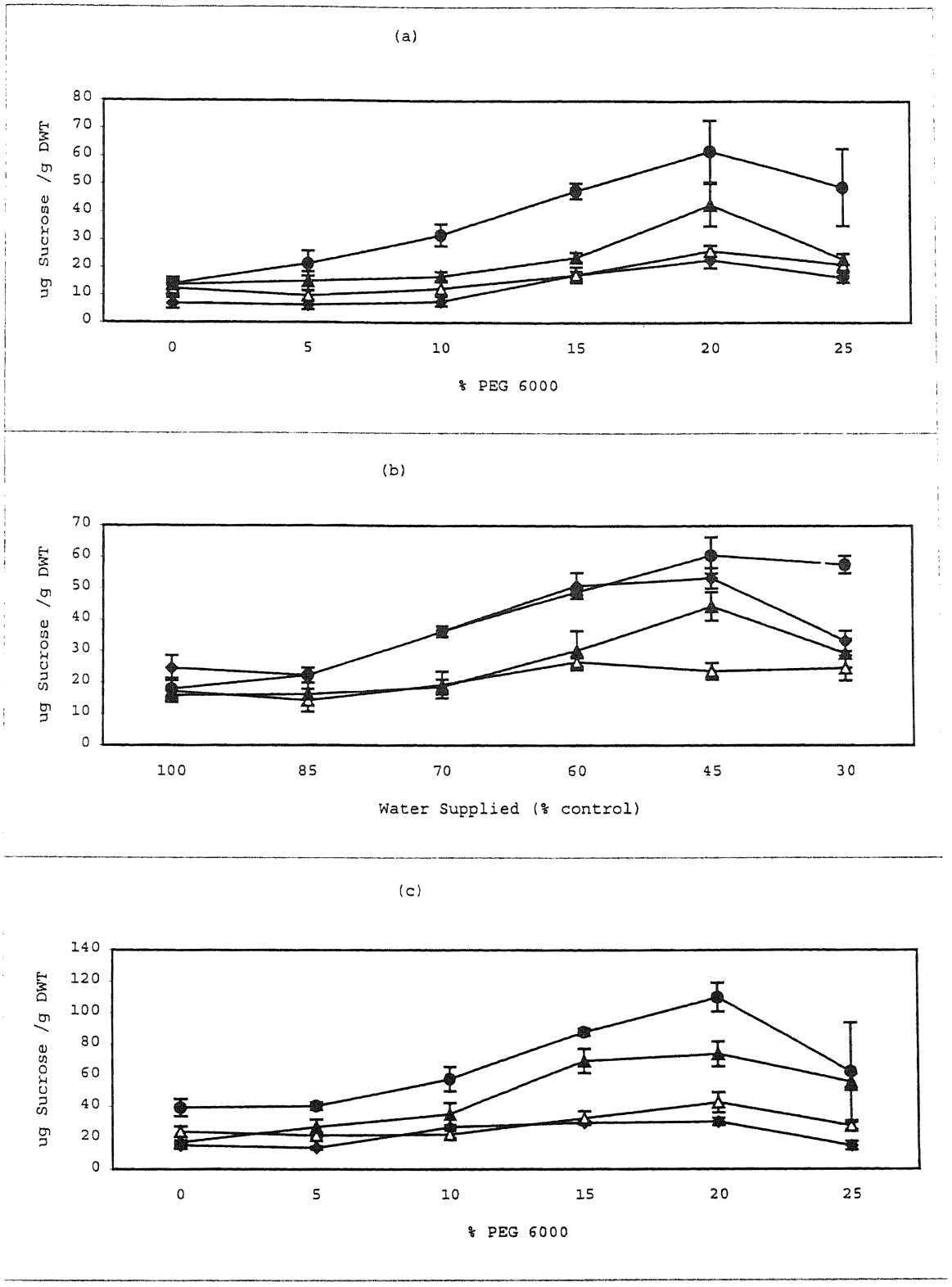


Fig. 5.3 Sucrose concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

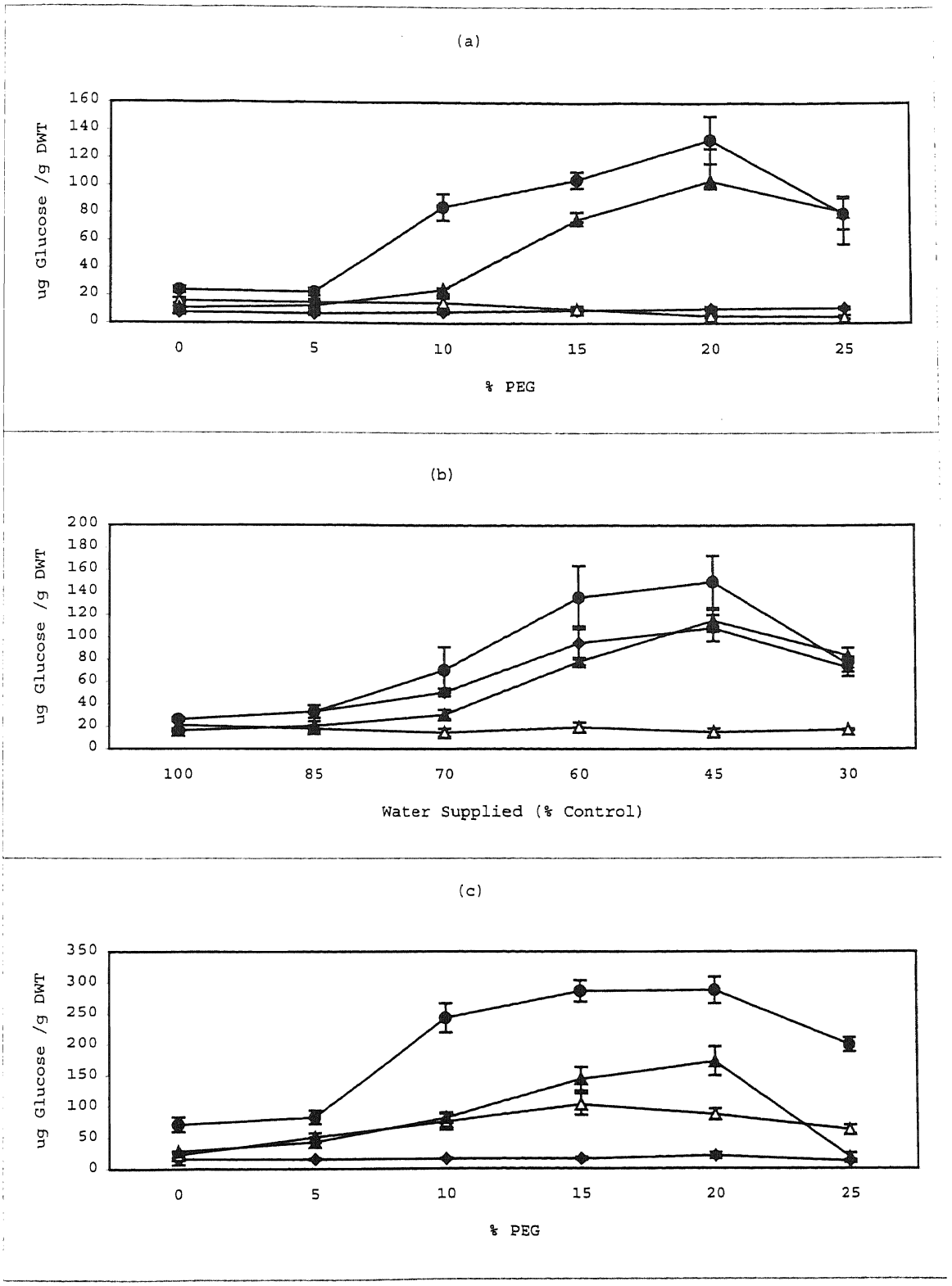


Fig. 5.4 Glucose concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

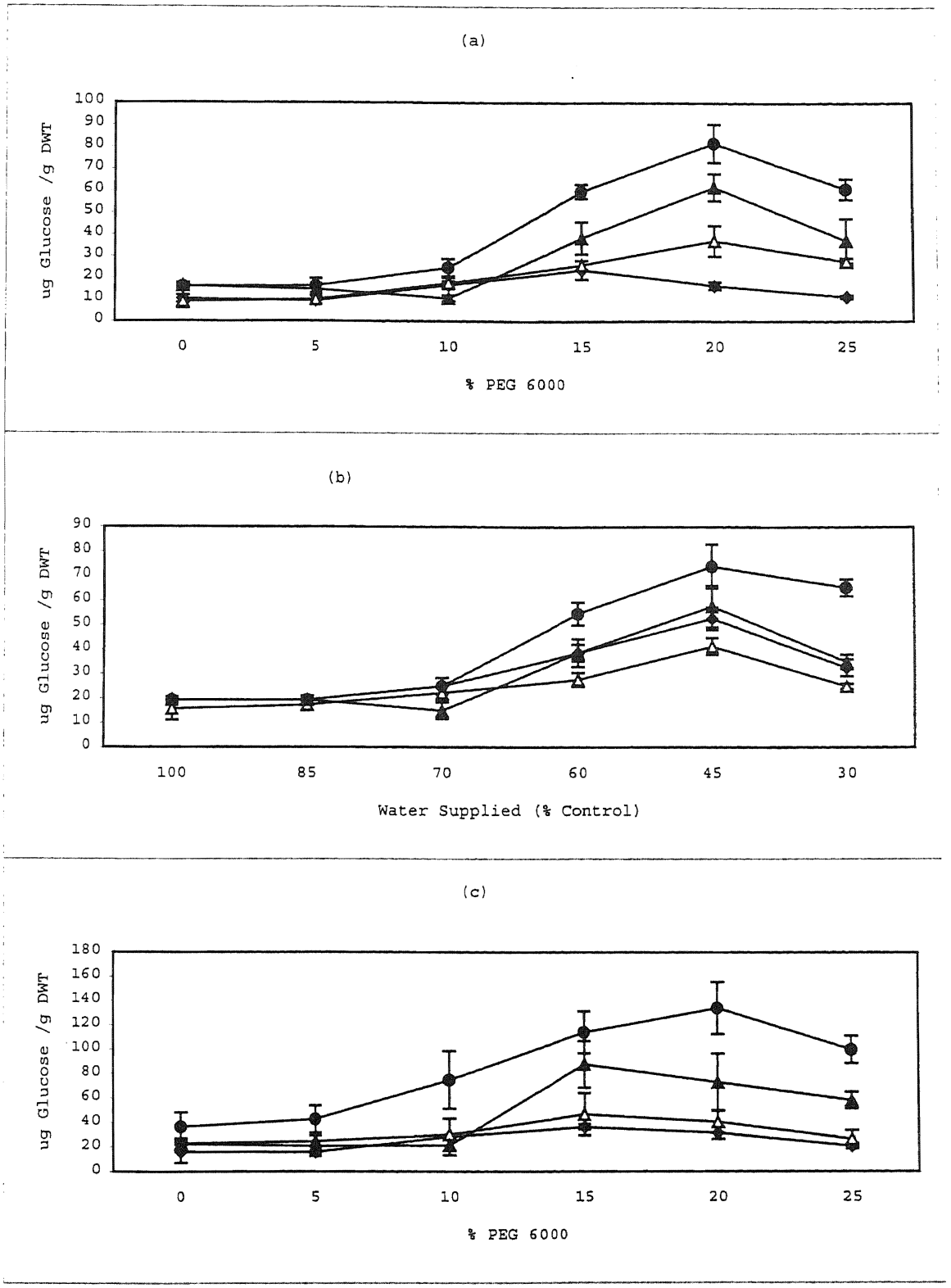


Fig. 5.5 Glucose concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

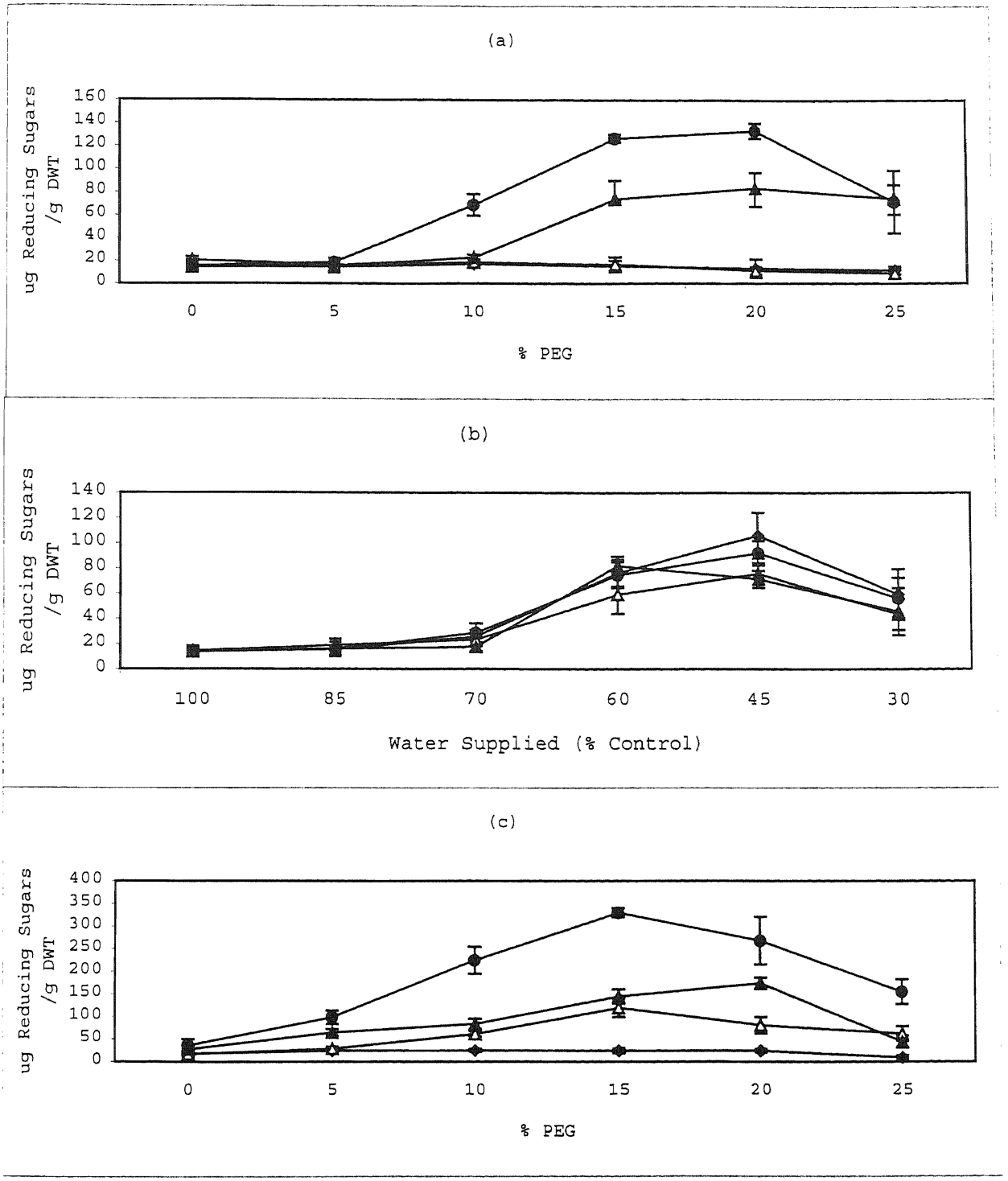


Fig. 5.6 Reducing sugar concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

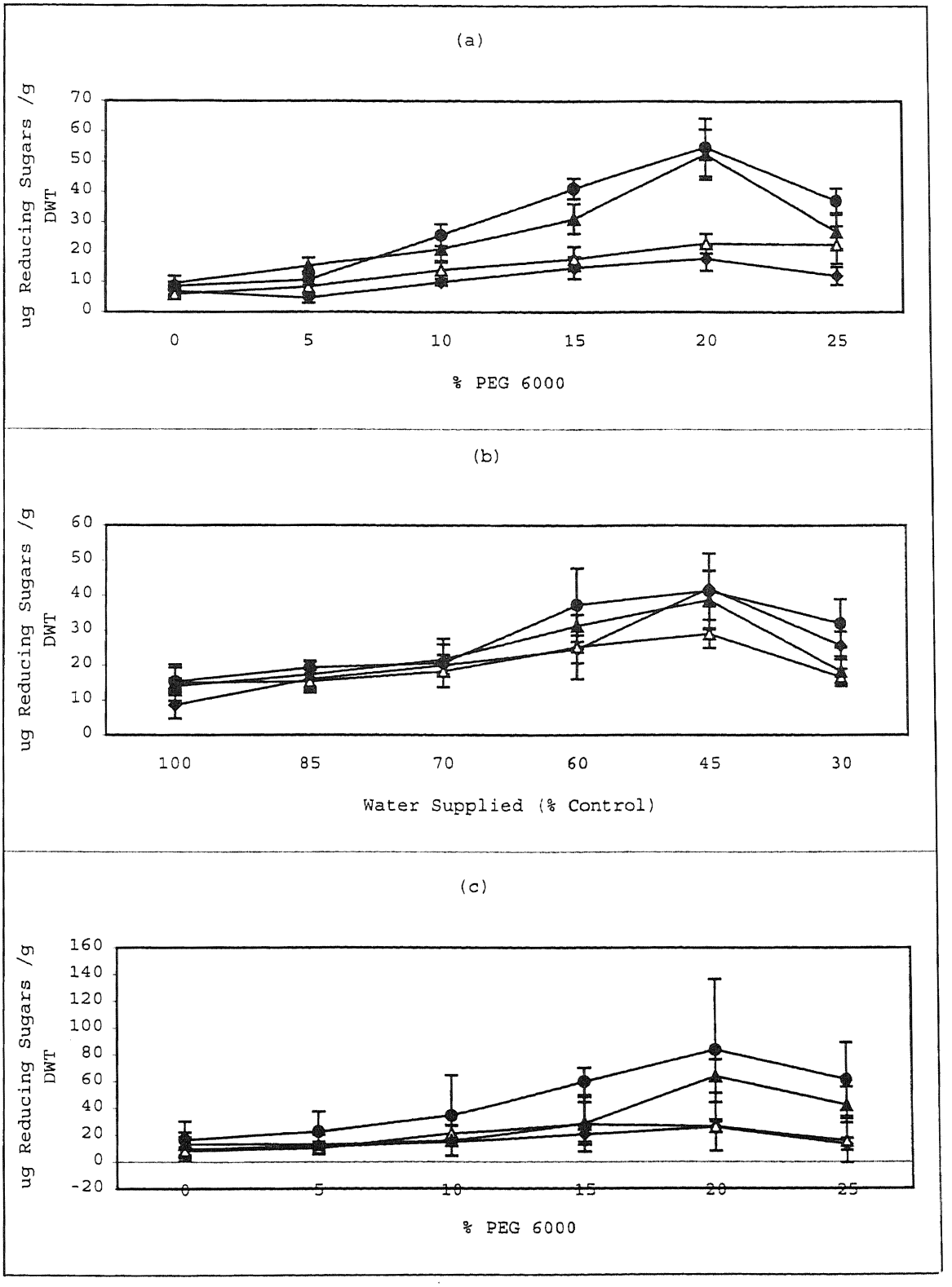


Fig. 5.7 Reducing sugar concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

soluble carbohydrate pool in the leaves and roots of *V. faba* (as previously reported in other plant species, Stewart, 1971; Wilson *et al*, 1980; Ford & Wilson, 1981; Munns & Weir, 1981), while slightly lower and approximately equal concentrations of glucose and reducing sugars accumulated. As osmotic potential is related to particle number increased osmotic adjustment would be expected if sucrose was hydrolysed into fructose and glucose during water deficits (Munns & Weir, 1981; Ladley, 1990), however this did not appear to occur within *V. faba*.

Section 4.1 (pg. 103) highlighted the reported compatible solute 'role' of sucrose during water deficits (Ingram & Bartels, 1996; Clifford *et al*, 1998). Accordingly the significantly greater sucrose concentrations exhibited in *V. faba* when supplied with increasingly concentrated nitrogen nutrition may infer an increased capacity for metabolism maintenance during water deficits.

5.3.1.2 CARBOHYDRATE ACCUMULATION AND NET PHOTOSYNTHESIS

Carbohydrate accumulation commenced prior to net photosynthesis decreases (figs. 3.22; 4.3; 4.4) inferring that net photosynthesis may have contributed substrates towards osmotic adjustment during slight to moderate water deficits (up to 10% PEG / 70% control water).

That significantly greater concentrations of sucrose; glucose; and reducing sugars accumulated in the leaves and roots of *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition may reflect the significantly greater levels of net photosynthesis which were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (fig. 3.22; and as inferred in 'spiked' as opposed to 'non-spiked' *V. faba*).

Indeed although nitrogen fixation is reportedly carbon intensive (Vance *et*

al, 1984; Hardy *et al*, 1968; Pate *et al*, 1979; Sprent, 1971; Minchin & Pate, 1973) nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba* exhibited significantly greater net photosynthesis (fig. 3.22), which may have contributed to the significantly greater carbohydrate concentrations recorded in nodulated as opposed to in non-nodulated 'no nitrate' supplied *V. faba*, (figs. 5.2 - 5.7).

5.3.1.3 STARCH

Starch concentrations are reportedly similar in *G. max* whatever the form of the supplied nitrogen nutrition when adequate irrigation is supplied (Chaillou *et al*, 1991), however figs. 5.8 & 5.9 and anova analyses reveal that starch concentrations were significantly greater in the leaves and roots of non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and with adequate irrigation (i.e. with 0% PEG or with 100% control water). Furthermore significantly greater starch concentrations were recorded in the leaves and roots of nodulated than of non-nodulated 'no nitrate' supplied *V. faba* (which again may reflect the significantly increased net photosynthesis recorded in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, fig. 3.22).

Figs. 5.8 & 5.9 and anova analyses reveal that *V. faba* did not exhibit significantly greater starch concentrations when supplied with 'spiked' as opposed to with 'non-spiked' nutrition, despite the greater inferred net photosynthesis in *V. faba* when supplied with an additional medium ammonia 'spike' (section 3.4). Ammonia cannot be stored and requires rapid assimilation (Raven, 1985); as such ammonia nutrition has a greater (or rather a more immediate) carbon skeleton requirement than nitrate nutrition (Bourgeais-Chaillou *et al*, 1991). The inference is that *V. faba* supplied

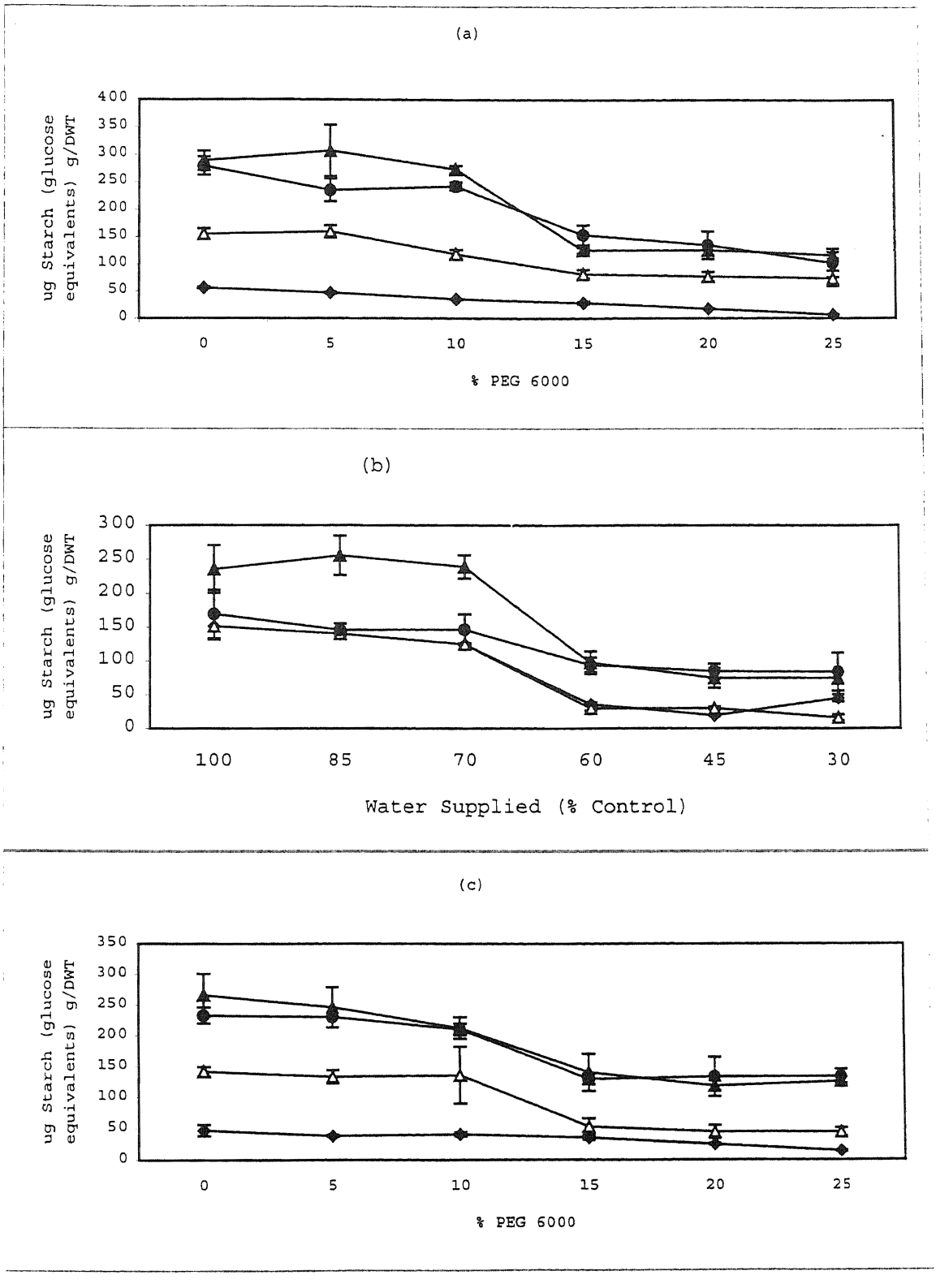


Fig. 5.8 Starch concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

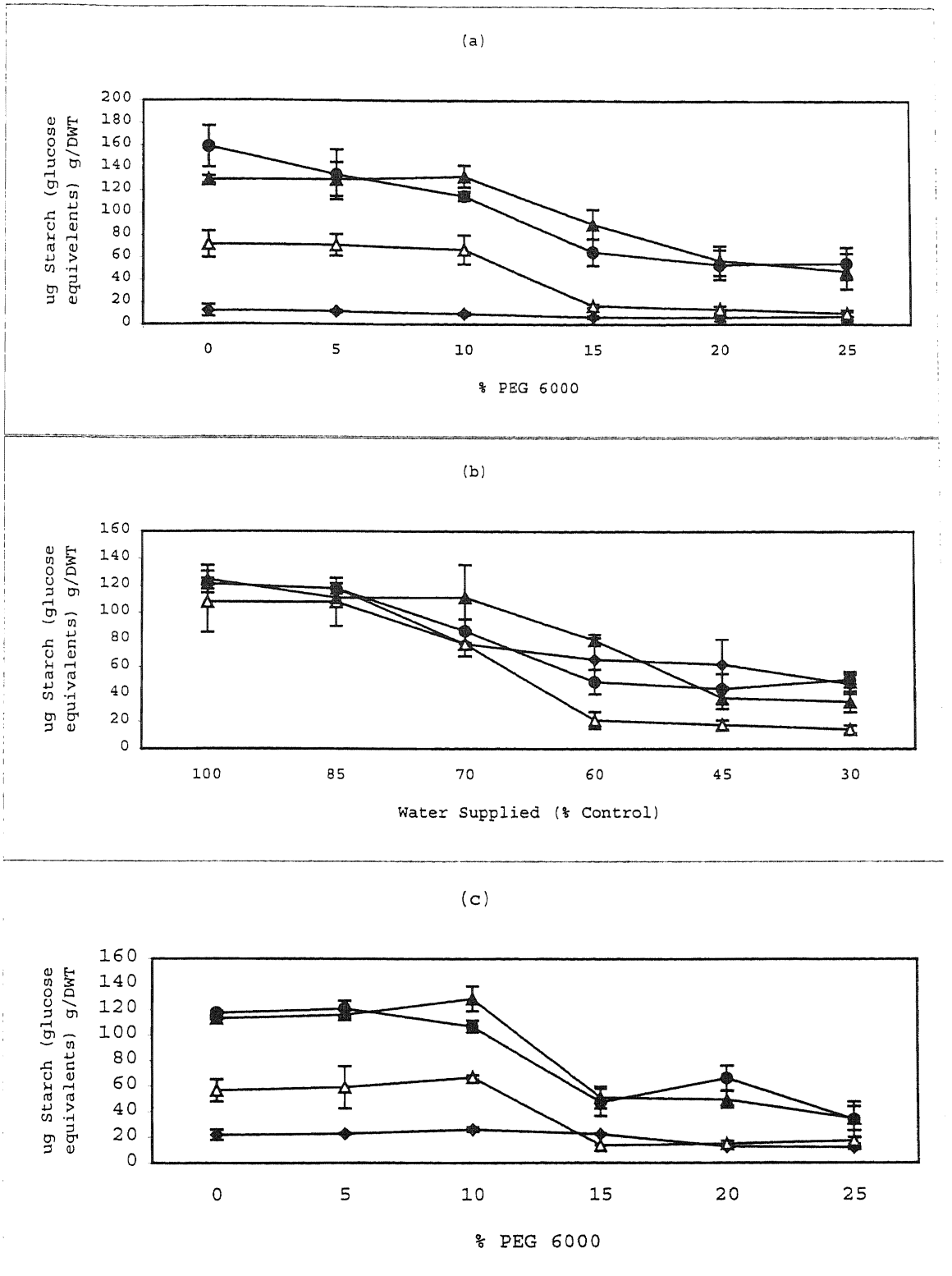


Fig. 5.9 Starch concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

with increasing medium ammonia additions might exhibit greater growth as opposed to greater starch concentrations (as compared with *V. faba* when supplied with less concentrated nitrate nutrition, as previously reported in other plant species, Macduff & Jackson, 1991; Raven *et al*, 1992; Purcell & King, 1996; Giordano & Bowes, 1997), in reflection of the continued utilisation of carbon skeletons in the continued assimilation of ammonia. Indeed while figs. 5.8 & 5.9 reveal that similar starch concentrations were exhibited in 'spiked' and 'non-spiked' *V. faba* despite the exhibition of greater inferred total photosynthesis in *V. faba* when supplied with the former nutrition throughout water deficits, section 3.4 highlighted significantly greater heights, CLAs and root biomasses in 'spiked' than in 'non-spiked' *V. faba*, inferring an increased assimilation of, as opposed to a storage of additional medium ammonia in *V. faba*. In contrast figs. 6.4 & 6.5 illustrate that increasing nitrate concentrations are exhibited in *V. faba* when supplied with increasingly concentrated medium nitrate nutrition (which may represent some nitrate storage as opposed to assimilation; see section 6.3.1.2). Increased growth and therefore metabolism (as opposed to storage) of nitrogen in *V. faba* when supplied with medium ammonia additions rather than solely with nitrate nutrition (as inferred by the greater growth exhibited in *V. faba* when supplied with the former nitrogen nutrition; see section 3.4) may have contributed to an alleviation of 'sink size' feedback inhibition of photosynthesis (Krapp *et al*, 1993; see section 3.4), and nitrogen assimilation (Imsande & Touraine, 1994; see section 6.4), which may have contributed to the greater levels of net photosynthesis (and nitrogen assimilation) and yet the exhibition of maintained as opposed to increased starch concentrations in *V. faba* when supplied with increasingly concentrated medium nitrogen

nutrition, and particularly with medium ammonia additions, throughout water deficits.

Earlier work has indicated that starch concentrations may increase during water deficits in other plant species (Foyer *et al*, 1998; Sanchez-Rodriguez *et al*, 1999). However figs. 5.8 & 5.9 and anova analyses reveal that starch concentrations decreased significantly during water deficits in both the leaves and roots of *V. faba* (when supplied with all utilised forms of nitrogen nutrition), inferring that some carbon skeletons for osmotic adjustment may have been made available from starch degradation (in agreement with earlier work; on different plant species, Stewart, 1972a; Bussis & Heinke, 1998; Clifford *et al*, 1998), or from decreased conversion of photosynthate into starch.

Significantly greater starch concentrations were maintained throughout water deficits in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, which may reflect the significantly increased net photosynthesis exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (fig. 3.22), and infers that less starch degradation may have been required for carbon skeleton production in *V. faba* when supplied with increasingly concentrated nitrogen availabilities, as net photosynthesis (and therefore potentially carbon acquisition) was maintained during increasingly severe water deficits in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (fig. 3.22).

5.3.1.4 AMYLASE ACTIVITIES

Figs. 5.10 & 5.11 and anova analyses reveal that amylase activities were not significantly affected by the form or the concentration of the supplied nitrogen source in either the leaves or the roots of non-nodulated *V. faba*,

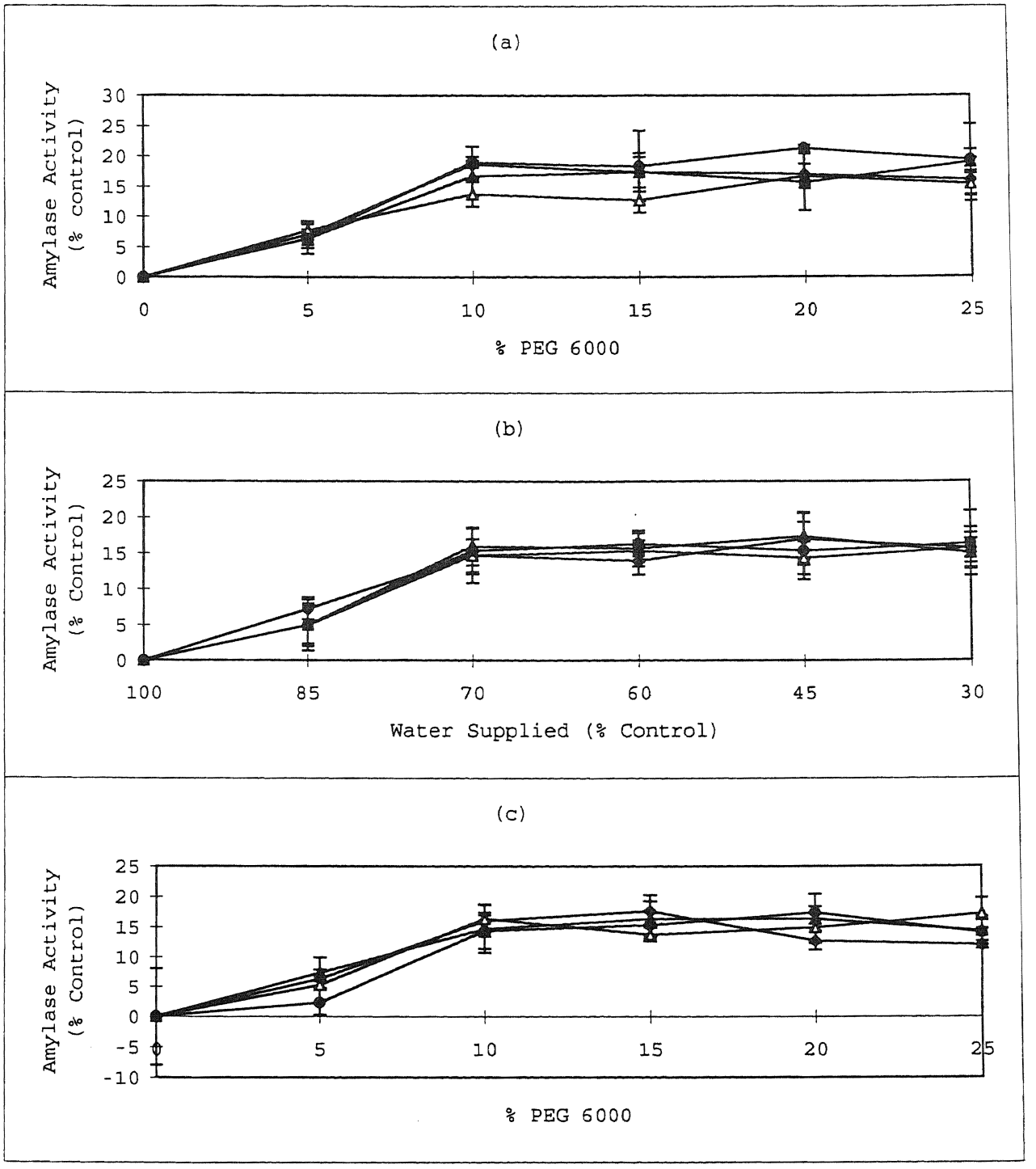


Fig. 5.10 Amylase activities in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

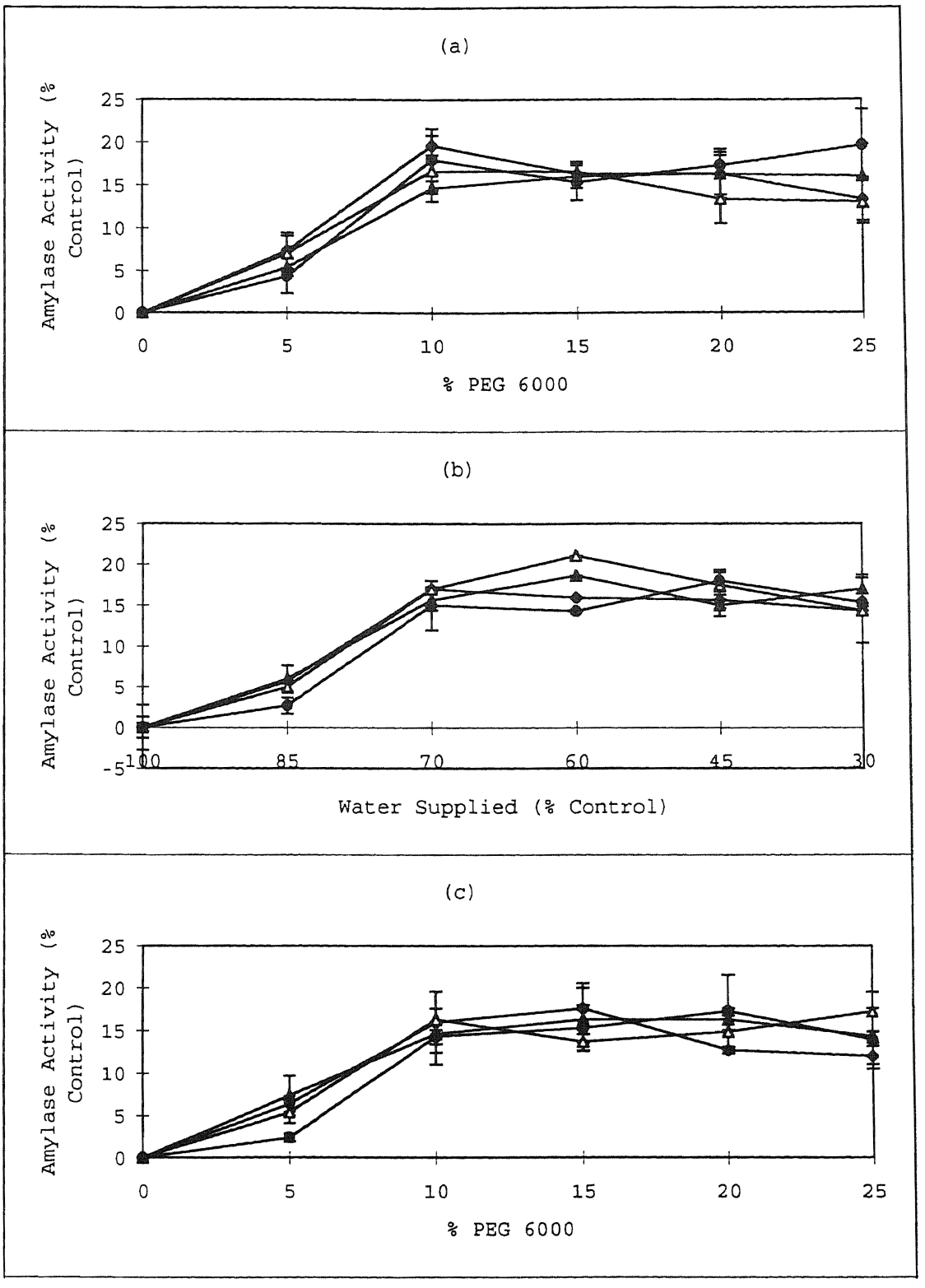


Fig. 5.11 Amylase activities in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

and did not differ significantly in the leaves or roots of nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba*; or of 'spiked' as opposed to 'non-spiked' *V. faba*.

Amylase activities may reportedly increase in some plant species during water deficits (Jones *et al*, 1980), indeed figs. 5.10 & 5.11 and anova analyses reveal that amylase activities increased significantly in *V. faba* during water deficits. However amylase activities reached a plateau level during moderate water deficits (10% PEG / 70% control water) in *V. faba* within all of the pre-specified nitrogen regimes, and amylase activities were maintained at this plateau level throughout severe water deficits. Starch degradation may have accounted for the production of some carbon skeletons for osmotic adjustment, however sufficient carbon skeletons may have been provided for osmotic adjustment at this level of amylase activity, as net photosynthesis was maintained into increasingly severe water deficits in

V. faba when supplied with increasingly concentrated medium nitrogen nutrition, and a potential role for the production of substrates via a reduced incorporation of photosynthates into starch is also inferred (as previously reported in *Lemna minor*, Stewart, 1972a).

5.3.2 ORGANIC ACIDS

It has previously been reported that *G. max* exhibits greater organic acid concentrations when supplied with 'combined nitrogen' nutrition than with nitrate or with ammonia nutrition (Bourgeais-Challou *et al*, 1992), however figs. 5.12 & 5.13 and anova analyses reveal that the leaves and roots of non-nodulated *V. faba* exhibited similar organic acid concentrations irrespective of the form or concentration of the supplied nitrogen source.

Ammonia cannot be stored and the plant assimilation of ammonia reportedly utilises organic acids as a source of carbon skeletons (Michael *et al*, 1970; Bourgeais-Challou *et al*, 1992), while nitrate assimilation also utilises organic acids in the 'pH stat' (Kirkby, 1969), and increasing organic acid concentrations reportedly induce increased nitrate uptake (Imsande & Touraine, 1994).

Tables 5.1 - 5.6 and anova analyses reveal that statistically significantly greater malate and pyruvate concentrations were exhibited in the leaves and roots of nodulated than in non-nodulated 'no nitrate' supplied *V. faba*, which may reflect the significantly greater net photosynthesis exhibited in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* (figs. 3.22; 6.1). Furthermore statistically significantly lower 2-oxogluterate concentrations were exhibited in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* (perhaps attributable to increased organic nitrogen production or to increased root respiration). It is possible that more organic acid skeletons were utilised in amino acid production in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*. However differences in actual concentrations of organic acids between nodulated and non-nodulated 'no nitrate' supplied *V. faba* were small, and while such differences were statistically significant it is unlikely that they were physiologically significant.

Indeed total organic acid concentrations were maintained in *V. faba* during water deficits (figs. 5.12 & 5.13), inferring that 'control' organic acid concentrations may have been above a threshold concentration required for the maintenance of nitrogen uptake and assimilation (via a maintained 'pH stat' and via carbon skeleton donation), and that nitrogen assimilation was unlikely to have decreased due to limitations in organic acids during water

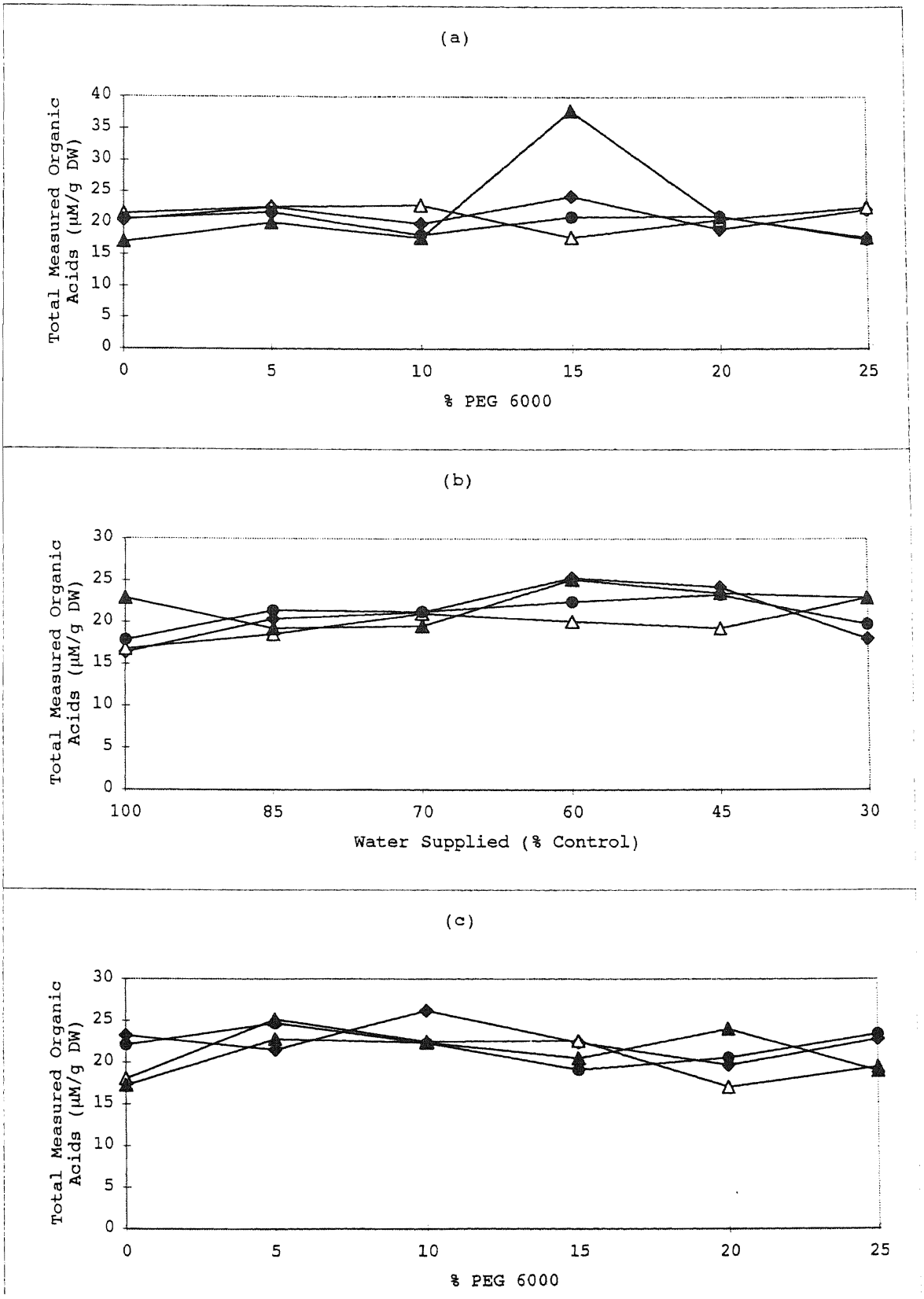


Fig. 5.12 Total measured organic acid concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

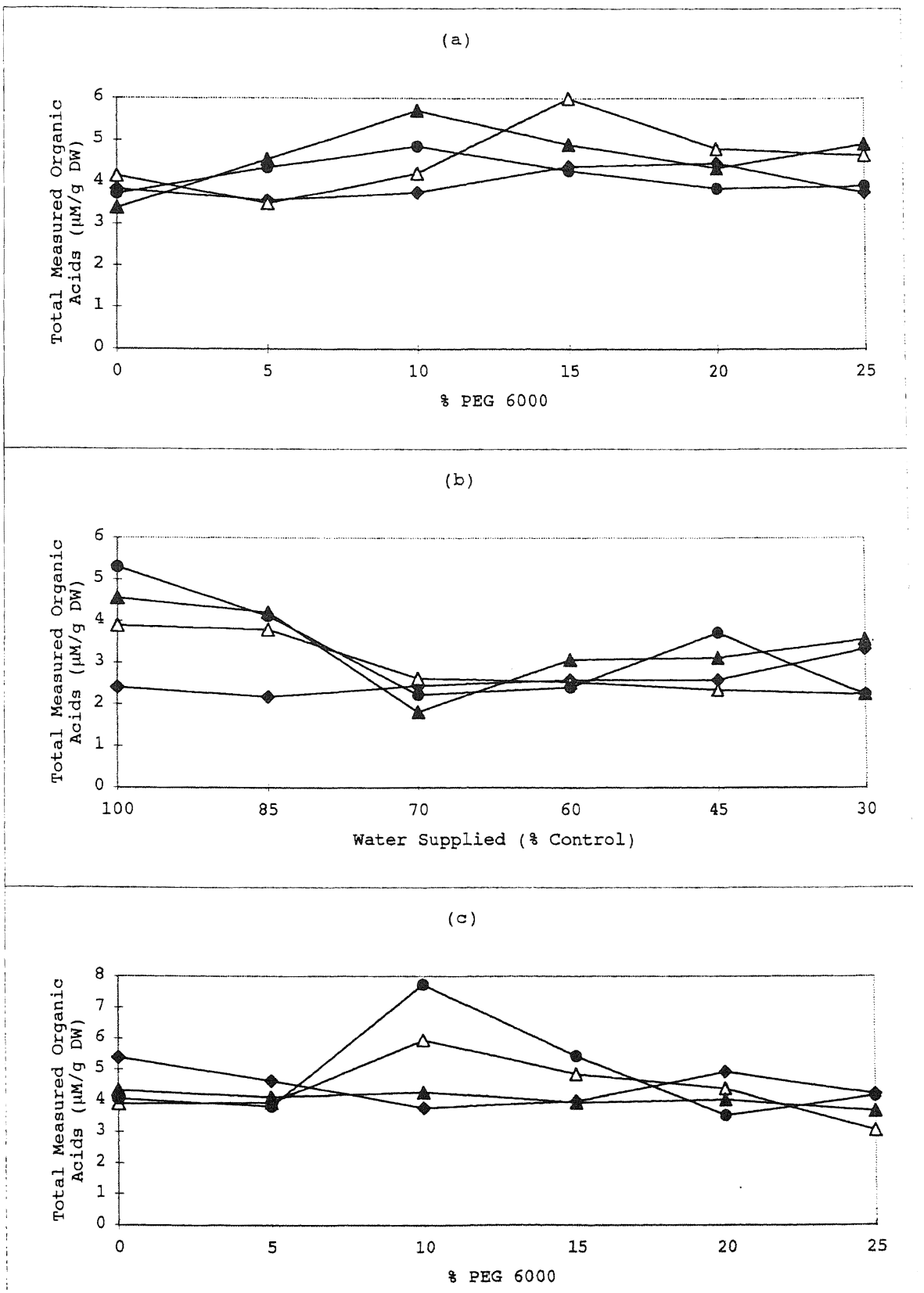


Fig. 5.13 Total measured organic acid concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

Table 5.1 Organic acid concentrations (μM gDW^{-1}) in the leaves of non-nodulated *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
CITRATE	0.57	0.30	0.27	0.54	0.30	0.81
PYRUVATE	1.46	1.63	1.84	1.56	1.23	1.34
MALATE	2.60	3.40	6.20	2.90	2.60	3.20
2-OXOGLUTERATE	15.90	17.20	11.60	19.20	14.90	16.70
TOTAL	20.53	22.53	19.91	24.20	19.03	22.05
(ii) '1/10 NITRATE'						
CITRATE	0.36	0.49	0.23	0.77	0.80	0.29
PYRUVATE	1.85	1.90	1.82	2.70	2.40	1.25
MALATE	2.90	3.30	4.90	4.10	2.40	4.40
2-OXOGLUTERATE	16.40	16.90	15.90	10.20	14.90	16.50
TOTAL	21.51	22.59	22.85	17.77	20.50	22.44
(iii) '1/2 NITRATE'						
CITRATE	0.31	0.39	0.65	0.84	0.60	0.64
PYRUVATE	1.20	2.50	1.32	1.27	1.91	1.26
MALATE	2.60	3.40	2.30	2.70	2.90	3.90
2-OXOGLUTERATE	12.90	13.80	13.40	12.30	15.60	11.80
TOTAL	17.01	20.09	17.67	37.84	21.01	17.60
(iv) 'COMBINED NITROGEN'						
CITRATE	0.78	0.84	0.79	0.84	0.56	0.68
PYRUVATE	2.01	1.47	1.57	1.24	1.29	2.60
MALATE	3.80	3.50	3.90	2.50	3.60	2.10
2-OXOGLUTERATE	14.10	15.90	11.90	16.40	15.60	12.00
TOTAL	20.69	21.71	18.16	20.98	21.05	17.38

Table 5.2 Organic acid concentrations ($\mu M\ gDW^{-1}$) in the roots of non-nodulated *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
CITRATE	0.37	0.56	0.41	0.56	0.68	0.54
PYRUVATE	1.40	1.23	1.49	1.41	1.47	1.26
MALATE	0.46	0.26	0.56	0.71	0.51	0.34
2-OXOGLUTERATE	1.60	1.50	1.30	1.70	1.80	1.60
TOTAL	3.83	3.55	3.76	4.38	4.46	3.74
(ii) '1/10 NITRATE'						
CITRATE	0.50	0.62	0.72	0.64	0.77	0.85
PYRUVATE	1.27	0.60	1.36	3.10	2.10	1.24
MALATE	0.58	0.47	0.34	0.46	0.23	0.44
2-OXOGLUTERATE	1.80	1.80	1.80	1.80	1.70	2.10
TOTAL	4.15	3.49	4.22	6.00	4.80	4.63
(iii) '1/2 NITRATE'						
CITRATE	0.33	0.45	0.10	0.61	0.96	0.26
PYRUVATE	1.40	2.50	3.40	2.40	1.27	1.27
MALATE	0.45	0.19	0.61	0.39	0.61	0.47
2-OXOGLUTERATE	1.20	1.40	1.60	1.50	1.50	2.90
TOTAL	3.38	4.54	5.71	4.90	4.34	4.90
(iv) 'COMBINED NITROGEN'						
CITRATE	0.49	0.81	1.69	0.43	0.50	0.54
PYRUVATE	1.42	1.82	1.72	1.12	1.83	1.47
MALATE	0.21	0.42	0.25	0.23	0.12	0.29
2-OXOGLUTERATE	1.60	1.30	1.20	2.50	1.40	1.60
TOTAL	3.72	4.35	4.86	4.28	3.85	3.90

Table 5.3 Organic acid concentrations ($\mu M\ gDW^{-1}$) in the leaves of nodulated *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
CITRATE	0.67	0.94	0.68	1.94	0.78	0.23
PYRUVATE	0.90	0.46	0.68	2.63	2.90	3.08
MALATE	2.30	2.10	2.90	4.10	2.70	2.70
2-OXOGLUTERATE	12.50	16.90	17.00	16.70	17.90	12.00
TOTAL	16.37	20.40	21.26	25.37	24.28	18.01
(ii) '1/10 NITRATE'						
CITRATE	0.35	0.55	1.09	0.55	0.62	0.45
PYRUVATE	1.31	2.69	2.57	2.75	2.57	2.57
MALATE	4.10	2.80	3.80	3.70	2.60	3.50
2-OXOGLUTERATE	11.00	12.50	13.60	13.10	13.50	16.40
TOTAL	16.76	18.54	21.06	20.10	19.29	22.92
(iii) '1/2 NITRATE'						
CITRATE	0.98	0.94	0.43	0.46	0.67	0.45
PYRUVATE	1.77	0.53	0.70	3.01	3.04	3.45
MALATE	3.60	3.50	2.70	2.60	3.10	3.20
2-OXOGLUTERATE	16.50	14.30	15.70	19.10	16.70	15.80
TOTAL	22.85	19.27	19.53	25.17	23.51	22.90
(iv) 'COMBINED NITROGEN'						
CITRATE	0.85	1.01	0.64	0.77	0.25	0.26
PYRUVATE	0.73	1.43	1.53	3.42	2.96	3.47
MALATE	3.10	3.60	2.90	3.40	2.90	2.60
2-OXOGLUTERATE	13.20	15.40	16.20	14.90	17.20	13.40
TOTAL	17.88	21.44	21.27	22.49	23.31	19.73

Table 5.4 Organic acid concentrations (μM gDW^{-1}) in the roots of nodulated *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
CITRATE	0.55	0.62	0.60	0.33	0.45	0.90
PYRUVATE	0.65	0.69	1.11	1.49	1.56	1.42
MALATE	0.98	0.27	0.48	0.34	0.33	0.64
2-OXOGLUTERATE	0.23	0.60	0.26	0.42	0.24	0.37
TOTAL	2.41	2.18	2.45	2.58	2.58	3.33
(ii) '1/10 NITRATE'						
CITRATE	0.62	0.64	0.68	0.82	0.66	0.65
PYRUVATE	2.75	2.57	0.82	0.89	0.90	0.91
MALATE	0.26	0.46	0.56	0.61	0.56	0.45
2-OXOGLUTERATE	0.25	0.12	0.56	0.21	0.23	0.23
TOTAL	3.88	3.79	2.62	2.53	2.35	2.24
(iii) '1/2 NITRATE'						
CITRATE	0.63	0.61	0.76	0.95	0.97	0.74
PYRUVATE	3.01	3.04	0.55	1.27	1.31	1.53
MALATE	0.54	0.33	0.27	0.51	0.38	0.67
2-OXOGLUTERATE	0.36	0.23	0.23	0.34	0.46	0.61
TOTAL	4.54	4.21	1.81	3.07	3.12	3.55
(iv) 'COMBINED NITROGEN'						
CITRATE	0.72	0.45	0.74	0.33	0.91	0.21
PYRUVATE	3.42	2.96	1.00	1.59	1.77	1.57
MALATE	0.68	0.26	0.35	0.27	0.65	0.22
2-OXOGLUTERATE	0.48	0.45	0.14	0.22	0.39	0.24
TOTAL	5.30	4.12	2.23	2.41	3.72	2.24

Table 5.5 Organic acid concentrations (μM gDW^{-1}) in the leaves of 'spiked' *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
CITRATE	0.44	0.62	0.42	0.27	0.54	0.65
PYRUVATE	1.20	2.75	2.57	1.80	2.40	2.12
MALATE	4.10	2.40	3.80	3.90	2.40	4.60
2-OXOGLUTERATE	17.50	15.70	19.40	16.40	14.30	15.40
TOTAL	23.24	21.47	26.19	22.37	19.64	22.77
(ii) '1/10 NITRATE'						
CITRATE	0.62	0.34	1.10	0.77	0.21	0.54
PYRUVATE	1.50	2.70	2.10	2.70	1.60	2.73
MALATE	4.60	5.40	3.70	3.40	2.50	3.90
2-OXOGLUTERATE	11.30	16.70	15.60	15.70	12.70	12.30
TOTAL	18.02	25.14	22.50	22.57	17.01	19.47
(iii) '1/2 NITRATE'						
CITRATE	0.54	0.36	0.55	0.21	0.29	0.48
PYRUVATE	1.40	1.60	1.50	3.00	2.10	0.90
MALATE	2.80	3.90	3.90	3.40	3.80	2.50
2-OXOGLUTERATE	12.50	16.90	16.40	13.90	17.80	15.10
TOTAL	17.24	22.76	22.35	20.51	23.99	18.98
(iv) 'COMBINED NITROGEN'						
CITRATE	0.78	0.32	0.56	0.89	0.54	0.61
PYRUVATE	1.80	1.60	1.80	1.90	1.40	1.50
MALATE	3.40	3.40	6.10	2.70	2.50	2.90
2-OXOGLUTERATE	16.10	19.40	13.80	14.50	16.10	18.40
TOTAL	22.08	24.72	22.26	19.10	20.54	23.41

Table 5.6 Organic acid concentrations ($\mu M\ gDW^{-1}$) in the roots of 'spiked' *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
CITRATE	0.47	0.89	0.39	0.63	0.50	0.64
PYRUVATE	2.70	2.10	1.41	1.42	1.38	1.45
MALATE	0.80	0.25	0.26	0.22	0.35	0.32
2-OXOGLUTERATE	1.40	1.40	1.70	1.70	2.70	1.80
TOTAL	5.37	4.64	3.76	3.97	4.93	4.21
(ii) '1/10 NITRATE'						
CITRATE	0.63	0.45	0.69	0.43	0.46	0.32
PYRUVATE	1.60	1.50	3.40	2.40	1.27	1.43
MALATE	0.46	0.39	0.45	0.51	0.25	0.30
2-OXOGLUTERATE	1.20	1.60	1.40	1.50	2.40	1.00
TOTAL	3.89	3.94	5.94	4.84	4.38	3 05
(iii) '1/2 NITRATE'						
CITRATE	0.43	0.46	0.56	0.35	0.45	0.24
PYRUVATE	1.50	1.27	1.72	1.12	1.83	1.45
MALATE	0.59	0.28	0.38	0.56	0.45	0.88
2-OXOGLUTERATE	1.80	2.10	1.60	1.90	1.30	1.10
TOTAL	4.32	4.11	4.26	3.93	4.03	3.67
(iv) 'COMBINED NITROGEN'						
CITRATE	0.48	0.49	3.66	0.98	0.61	0.69
PYRUVATE	1.50	1.37	1.80	1.60	1.50	1.47
MALATE	0.27	0.56	0.48	0.44	0.21	0.60
2-OXOGLUTERATE	1.80	1.40	1.80	2.40	1.20	1.40
TOTAL	4.05	3.82	7.74	5.42	3.52	4.16

deficits. That nitrogen assimilation was maintained during water deficits in *V. faba* is also inferred by the nitrogenous osmotica concentrations which were found to increase significantly during water deficits (section 4.4), and by the maintenance of nitrogen assimilation which was observed in *V. faba* during water deficits (see section 6.4).

In addition to their requirement in nitrogen assimilation, organic acids are also osmotic solutes (Raven & Smith, 1976), and organic acid accumulation is reportedly correlated with plant water deficit tolerance in *Gossypium hirsutum* L. (Tompa *et al*, 1986).

However figs. 5.12 & 5.13 and anova analyses reveal that organic acid concentrations did not alter significantly during water deficits in either non-nodulated or in 'spiked' *V. faba* when supplied with any of the pre-specified forms of medium nitrogen nutrition. While total organic acids increased significantly in the leaves of nodulated *V. faba* during water deficits, and decreased significantly in the roots (malate was the organic acid which was most significantly affected by water deficits in nodulated *V. faba*; tables 6.3 & 6.4) the changes exhibited were low in terms of actual concentrations (particularly when compared with the concentrations of total soluble carbohydrates and total amino acids which accumulated during water deficits; figs. 4.3 - 4.6), inferring that although such changes were statistically significant it is unlikely that they were physiologically significant in terms of osmotic adjustment. Alterations in organic acid concentrations in nodulated *V. faba* during water deficits may reflect the reported dependence of nitrogenase activities on maintained carbohydrate availabilities (Pate *et al*, 1979; Dekhuijzen *et al*, 1981; Schilling, 1983; Gonzalez *et al*, 1995; Caba *et al*, 1998), rather than water deficit tolerance adaptations *per se*.

That organic acid concentrations were maintained in nodulated and in non-nodulated *V. faba* during water deficits may reflect the fact that net photosynthesis was maintained until moderate water deficits were imposed and may have provided substrates for osmotic adjustment, and that starch concentrations also decreased and may also have provided substrates for osmotic adjustment (figs. 5.8 & 5.9).

In summary *V. faba* does not rely on organic acid accumulation to facilitate nitrogen assimilation or osmotic adjustment during water deficits.

5.4 CONCLUSION

Figs. 5.2 to 5.7 and anova analyses reveal that glucose; sucrose; and reducing sugars accumulated to significantly increasing concentrations in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and that sucrose; glucose; and reducing sugars accumulated significantly during water deficits (as previously reported in other plant species, Wilson *et al*, 1980; Hanson & Hitz, 1982).

Carbohydrate accumulation commenced prior to net photosynthesis decreases, inferring that net photosynthesis may have contributed some substrates towards osmotic adjustment during moderate water deficits (Miflin, 1974; Munns & Weir, 1981; figs. 3.21 & 3.22).

Figs. 5.8 & 5.9 and anova analyses reveal that starch concentrations decreased significantly during water deficits, and figs. 5.10 & 5.11 and anova analyses reveal that amylase activities increased significantly during water deficits in both the leaves and roots of *V. faba* (when supplied with all forms of medium nitrogen nutrition), inferring that some carbon skeletons for osmotic adjustment may have been produced via starch degradation (as reported in other species: Jones *et al*, 1980; Bussis &

Heinke, 1998; Clifford *et al*, 1998) particularly as net photosynthesis decreased during water deficits (fig. 3.22). That some carbon skeletons for osmotic adjustment may have been provided via a reduction in the incorporation of photosynthates into starch is also inferred by the data (as previously reported in *L. minor* Stewart, 1972a).

That carbohydrate and starch concentrations were maintained at significantly greater concentrations in nodulated than in non-nodulated *V. faba* throughout water deficits infers that nitrogen fixation may not be as susceptible to water deficits as is classically supposed (see introduction, pg. 19; Plies-Balzer *et al*, 1995; Serraj & Sinclair, 1997). Significantly greater net photosynthesis (fig. 3.22), and significantly greater concentrations of total soluble carbohydrates; starch; sucrose; fructose; and glucose were maintained in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits, inferring that carbohydrate metabolism may have been maintained in nodulated *V. faba* during water deficits. This may have contributed to the inferred maintenance of nitrogenase activity throughout water deficits, as decreasing photosynthate and reductant availabilities have previously been implicated as potential causes of nitrogen fixation decreases during water deficits (Schilling, 1983; Walsh, 1995; Epron, 1997; Clifford *et al*, 1998), see introduction, pg. 17.

Figs. 5.12 & 5.13 and anova analyses reveal that leaf and root organic acid concentrations were not significantly affected by either the form or the concentration of the supplied nitrogen source, and that organic acid concentrations did not alter significantly in nodulated or in non-nodulated *V. faba* during water deficits. Section 4.4 concluded that *V. faba* exhibited sufficient osmotic adjustment during water deficits to allow the

maintenance of RWCs until severe water deficits were imposed (figs. 3.16 & 3.17), indicating that *V. faba* did not rely upon the accumulation of organic acids during water deficits to act as osmotic solutes. Indeed it has previously been reported that organic acids accumulate during water deficits in some but not in all plant species (Ford & Wilson, 1981).

Furthermore intrinsic organic acid concentrations may have been above a threshold concentration required for the maintenance of nitrogen uptake and assimilation in *V. faba*. Increases in pyruvate during water deficits (as a substrate for alanine production) in suspended *Lycopersicon esculentum* cells have previously been inferred (Rhodes *et al*, 1986), however the demand for carbon skeletons for the maintenance of nitrogen assimilation and osmotica production may have been too great to result in the exhibition of organic acid accumulation in *V. faba*. The inference is that any potential increases in organic acid concentrations which may have been expected in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (due to increased net photosynthesis, fig. 3.22) may have been utilised in increased nitrogen assimilation and osmotic solute production (fig. 6.1; as discussed in chapter six) and in increased growth (as exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with medium ammonia additions; section 3.4). The continued utilization as opposed to storage of organic acids may partly explain the exhibition of maintained organic acid concentrations in *V. faba* during water deficits (figs. 5.12 & 5.13).

In summary the data presented in this chapter illustrates that sucrose, glucose and reducing sugar concentrations increased significantly in the leaves and roots of *V. faba* during water deficits. Significantly greater

individual sugar concentrations accumulated in the leaves and roots of *V. faba* when supplied with increasingly concentrated medium nitrogen (particularly when ammonia additions were included in the medium). Starch concentrations decreased in the leaves and roots of *V. faba* during water deficits. Amylase activities increased in the leaves and roots of *V. faba* during water deficits, inferring that starch degradation may have contributed some substrates towards carbohydrate accumulation during water deficits. However carbohydrate accumulation preceded starch degradation, inferring a role for the direct incorporation of photosynthates into osmotica during water deficits. Furthermore amylase activities plateaued during water deficits, and carbohydrate accumulation continued during severe water deficits, during which low starch concentrations were exhibited by *V. faba*, further inferring the direct accumulation of photosynthates as osmotica.

Leaf and root organic acid concentrations were not significantly affected by increasing water deficits, or by the form or concentration of the supplied nitrogen source.

CHAPTER SIX

ASPECTS OF NITROGEN METABOLISM IN NON-NODULATED; NODULATED; AND AMMONIA 'SPIKED' *V. faba* WHEN SUPPLIED WITH VARIOUS FORMS AND CONCENTRATIONS OF MEDIUM NITROGEN NUTRITION, DURING INCREASING WATER DEFICITS.

Loss of nitrate reductase activity from leaves is considered to be one of the more sensitive of physiological responses to water deficits, more so than for example stomatal response (Hsiao *et al*, 1976).

6.1 INTRODUCTION

This chapter is concerned with aspects of nitrogen metabolism in *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during water deficits.

It has been reported that the largest variation in the flow of photosynthetically fixed carbon is that incorporated into amino acids, inferring that the controlling factor in amino acid synthesis may be the availability of reduced nitrogen (Steer, 1973; Bassham *et al*, 1981), and therefore that maintained nitrogen assimilation during water deficits may potentially result in the production of increased concentrations of nitrogenous osmotica. Further potential benefits are associated with maintained nitrate reduction during water deficits, as NR activities are reportedly correlated with growth, grain yield and protein production in other plant species (Hageman, 1979; Srivastava, 1980; Muller & Janiesch, 1993). However while the (shoot) activities of the nitrogen assimilatory enzymes GS and GDH may not decrease during water deficits (Taylor *et al*, 1982), it is classically considered that NR activities decrease significantly during

even moderate) water deficits (Mattas & Pauli, 1965; Hsiao, 1973; Hsiao *et al*, 1976; Rajagopal *et al*, 1977; Wellburn *et al*, 1996; Foyer *et al*, 1998). Indeed NR activities reportedly halve in *Nicotania plumbaginifolia* within four days of water deficit imposition (Ferrario-Mery *et al*, 1998).

However a small number of workers have described NR as a relatively stable enzyme, the activity of which is reportedly more affected by disruption to nitrate transporters (e.g. Aslam & Huffaker, 1984) than to enzyme denaturation *per se*. A small metabolic pool of nitrate is accessible to NR, while a large vacuolar storage pool (containing up to fifty eight per cent of the total cellular nitrate, Grandstadt & Huffaker, 1982) is separate from metabolic sites (Shaner & Boyer, 1976), and influences anion/cation balances, and osmotic adjustment (Martinoia *et al*, 1981). NR activities are reportedly positively correlated with 'nitrate flux', which may have a greater regulatory influence on NR activities than substrate or NADH availabilities (Shaner & Boyer, 1976). Decreased nitrate fluxes (caused by e.g. cooled roots) reportedly result in reduced NR activities despite maintained organ nitrate concentrations (Shaner & Boyer, 1976; Smirnoff *et al*, 1984), while increased nitrate fluxes reportedly result in increased NR activities even during water deficits (Smirnoff *et al*, 1985; Ladley, 1990). A constitutive NR, expressed in the absence of nitrate has also been identified (Clement *et al*, 1978; Remmler & Campbell, 1986; Rufty *et al*, 1989; Andrews *et al*, 1990).

Glutamate dehydrogenase (GDH) has been detected in *V. faba* seeds and seedlings (Stewart & Rhodes, 1977), however the high kM of GDH for ammonia, plus evidence from labelling experiments indicate a deaminating role for GDH, and the glutamine synthetase / glutamate synthase (GS GOGAT) system is considered the primary route of plant ammonia assimilation (Lea & Joy, 1995; Stewart *et*

al, 1995). GDH is loosely membrane associated and is localised in the leaves (evidence indicates low levels of chloroplastic GDH, Miflin, 1974), and particularly in root mitochondria, which may represent a compartment where GDH can successfully compete for ammonia (Emes & Fowler, 1979; Sadunishvili *et al*, 1996), inferring a potential nitrogen assimilatory role for (particularly root) GDH when ammonia concentrations are high (Durzan & Steward, 1967; Rhodes *et al*, 1976).

GS isoenzymes may be (i) cytosolic - GS1, or (ii) chloroplastic/plastidic - GS2 (Lee & Stewart, 1978; Forde & Woodall, 1995). Root-specific and nodular GS isoforms have also been described (Forde & Woodall, 1995). Genetic evidence indicates that GS isoenzymes are spatially separated in many species, inferring non-overlapping 'roles' (Kamachi *et al*, 1991; see table 6.1, pg. 172). The activities of GS isoenzymes may alter throughout the life-cycles of plants, as determined by nitrogen assimilatory requirements (Kamachi *et al*, 1991; Pearson & Ji, 1994; see table 6.1, pg. 172). However only GS2 was qualified in *V. faba* (see appendix III, pg. 298, for an example GS profile). Thus in *V. faba* GS2 was solely responsible for ammonia assimilation throughout water deficits. GS2 has previously been reported to be the dominant GS isoenzyme in other C3 leguminous species (McNally *et al*, 1983; McNally & Hirel, 1983), and may assimilate photorespiratory ammonia and the ammonia which is produced via nitrate reduction (Pearson & Ji, 1994).

Previous work has described reductions in amino acid incorporation into protein (Mattas & Pauli, 1965; Dhindsa & Bewley, 1976b), and increases in protein degradation (Hsaio, 1976; Paleg & Aspinall, 1981; Riccardi *et al*, 1998), in other plant species during water deficits, which may contribute

Table 6.1 Previously reported properties and 'roles' of plant GS isoforms

GS1 - CYTOSOLIC GS (McNally *et al*, 1983)	**GS2 -CHLOROPLASTIC/PLASTIDIC (& GOGAT)** (Forde & Woodall, 1995)
Reportedly thermally stable; (Guiz *et al*, 1979; McNally *et al*, 1983b; Chandler *et al*, 1985).	Reportedly thermally unstable above 45°C, and inactive above 60°C (Guiz *et al*, 1979; McNally *et al*, 1983b; Chandler *et al*, 1985).
However Chandler *et al*, (1985), reported that the chloroplastic GS2 isoform from *Panicum maximum* and *Panicum miliaceum* was more heat stable than the cytosolic isoform; and the chloroplastic GS2 isoform from *Nicotiana tabacum* is also relatively heat stable (perhaps as it is glycosylated), Nato *et al*, (1984)	
Subcellular location in the cytosol indicates a potential role for GS1 in photorespiratory nitrogen assimilation (Keys *et al*, 1978; McNally & Hirel, 1983)	When photorespiration is suppressed in *P.sativum* & *P. vulgares* grown at elevated CO_2 concentrations, GS2 (but not GS1 mRNA decreases (Cock *et al*, 1991) inferring a role for GS2 in photorespiratory ammonia assimilation (however this is not a short-term response)
Wallsgrove *et al*, (1983), suggest that GS1 would access a high ATP:ADP ratio and may have an important role in photorespiratory ammonia assimilation	McNally *et al*, (1983) & Pearson & Ji, (1994) suggested that GS2 may be primarily responsible for photorespiratory ammonia assimilation, and probably in the assimilation of ammonia produced during nitrate reduction (indeed many C3 species exhibit very low GS1 activities
Woo & Osmond, (1982), suggest that both GS1 and GS2 may assimilate photorespiratory ammonia	
GS1 activity remains constant during senescence whereas GS2 decreases, inferring an important role for GS1 in the synthesis of glutamine for export (in rice; Kamachi *et al*, 1991) GS2 predominates early in the season while GS1 becomes more active towards senescence, inferring a storage/ tanslocation role for GS1 (in temperate deciduous trees; Pearson & Ji, 1994)	Chloroplastic GS activity is three to four fold higher in C3 than in C4 species (and often predominates in C3 legumes McNally *et al*, 1983)
Two GS isoenzymes have previously been isolated in *V. faba* (Barratt, 1980)	

substrates towards the production of nitrogenous osmotica. Indeed earlier quantification of polyribosome levels indicated that proline synthesis into proteins may be inhibited during water deficits (Stewart, 1981; Rhodes *et al*, 1986), particularly in rapidly growing tissues (Bewley, 1981), possibly as growing tissues contain more membrane-associated polyribosomes which may exhibit increased water deficit sensitivity than non-bound polyribosomes (Stewart, 1981).

The concentrations of the nitrogen assimilation products (individual amino acids and allantoin) were also quantified and are discussed in this chapter. Transaminases shuttle amino groups between appropriate acceptors allowing the production of the approximately twenty amino acids required for protein synthesis (Stryer, 1988). The activities of key transaminases (which influence which amino acids accumulate) are discussed, as related to the metabolic derivation of each amino acid and to the potential 'roles' of individual amino acids in plant metabolism.

6.2 MATERIALS & METHODS

6.2.1 NITRATE ASSAY (after Sloan & Sublett, 1966)

Cadmium was prepared by placing zinc rods in 20% $CdSO_4$. 1 ml of plant sample (see section 2.6.1) was added to 2 ml buffer (22.25 ml 35% NH_3 & 21.68 g NH_4Cl per litre) with 1 ml 1M $MgCl_2$ (which removes phosphate). 1 g of cadmium was added. Solutions stood at room temperature for 3 hours (with agitation every thirty minutes). 1 ml was then removed and added to 1 ml sulphanilic acid (1% sulphanilic acid in 3M HCl) with 1 ml N-(Naphyl) ethylene diamide dichloride (0.02%). After 20 minutes OD was read at 540nm, and concentrations were determined using a KNO_3 standard curve.

6.2.2 NITRATE REDUCTASE ASSAY (after Havill *et al*, 1974; Stewart & Orebamjo, 1979)

NR activities were quantified in the leaves, roots, and nodules of *V. faba* at the end of the water deficit regime. Approximately 1 g of tissue was cut into fine strips and was placed in a thunberg tube which contained 5 ml of assay mixture (assay mixture; 0.5M KNO_3 dissolved in 1 l 0.1M KH_2PO_4 (pH 7.5) with 1 ml propanol added). Air was dislodged by vacuum infiltration to prevent an under-estimation of NR activities (Canvin & Woo, 1979; Yoneyama, 1981), and root samples were maintained in a vacuum. The samples were incubated in the assay mix in the dark at 25 °C for 1 hour. 1 ml of assay solution was then added to 1 ml sulphanilic acid (1% sulphanilic acid in 3M HCl) and 1ml NED (0.02% alpha-naphthyl ethylene diamine dihydrochloride in water). After 35 minutes OD was determined at 540nm.

6.2.3 GS & GDH ENZYME EXTRACTIONS

Extractions were performed on ice using cooled laboratory hardware. 0.5 - 1 g fresh plant material was finely crushed using liquid nitrogen and a pestle and mortar. 5 ml extraction buffer was added, which contained 10 mM Mg SO_4; 5 mM glutamic acid; 1 mM EDTA disodium salt; 1 mM dithiothreitol; 1 mM glutathione; 2 mM mercaptoethanol; and 2% soluble polyvinylpyrrolidine; in 25 mM Tris-HCl at pH 8.0. 1 µg Tween 90 was added. The mixture was then agitated and passed into a centrifuge tube (through double muslin) and spun down at 30000 g for 10 minutes (5 °C). Final liquid volumes were recorded.

6.2.4 GLUTAMINE SYNTHETASE TRANSFERASE ASSAY (after Rhodes *et al*, 1975).

A GS assay mix was prepared which contained: 6.05 g Trizma base; 5.7 g Glutamine; 0.695 g hydroxyl amine; 0.2775 g $MnCl_2$; and 0.07 g ADP mixed in

50ml distilled water (pH 6.4). A sodium arsenate solution was prepared which contained: 8 g sodium arsenate; 1.2 g Tris-Cl buffer; 100 ml distilled water (pH 6.4).

Enzyme extracts were used in part to perform crude assays. A second portion of each extract was loaded onto a DEAE-sephacel column for GS isoform separation.

GS CRUDE ASSAY PROCEDURE

Four test tubes, each containing 0.8 ml assay mix and 0.1 ml sodium arsenate solution were pre-incubated at 30°C. 0.1 ml of enzyme extract was added to each tube. Reactants were incubated for 5; 10; 15; or 30 minutes, after which time reactions were stopped using 1 ml $FeCl_3$ additions (80 g Trichloroacetic acid; 52 g $FeCl_3$; and 160 µl 3M HCl in 2 l with distilled water; filtered through Whatman's No. 1 general purpose filter paper). OD was determined at 500nm, and GS activities determined using a GS standard.

SEPARATION OF GS ISOFORMS

1.5 X 15 cm DEAE-Sephacel columns were prepared and equilibrated with 25 mM Tris-Cl buffer prior to use. Extraction buffer was pumped through the columns for 30 minutes prior to loading. The enzyme extract was loaded onto the column at a flow rate of 18 ml $hour^{-1}$. Once loaded, the flow rate was reduced to 9 ml $hour^{-1}$ and the column was flushed for 5 minutes with extraction buffer. A 0.75 M KCl solution (dissolved in 25 mM Tris-Cl buffer) was dripped into the buffer which flowed through the column in order to separate the isoforms, which were eluted in discreet fractions into sixty tubes using a fraction collector. The fractions were individually assayed for GS activities, the reactions being stopped after five minutes. Comparisons with a GS standard allowed profiles to be produced (see appendix III), which highlighted the activities of the GS

isoforms within the nitrogen and water deficit regimes.

Differences in the thermostability of GS1 & GS2 isoenzymes are useful in their identification (Guiz *et al*, 1979; McNally *et al*, 1983b; Chandler *et al*, 1985; see table 6.1, pg. 172). Accordingly GS isoform extracts were assayed at increasing temperatures, and were thus identified.

6.2.5 GLUTAMATE DEHYDROGENASE ASSAY (after Taylor & Havill, 1981)

950 µl 100mM tris-acetate buffer; 100 µl 1.5M ammonium chloride; 100 µl 10mM calcium chloride; 100 µl 4mM NADH; and 50 µl enzyme extract (extracted as for GS analysis; section 6.2.4) were placed in a cuvette. 100 µl 150mM α-ketogluterate was added to start the reaction. Disappearance of NADPH was followed at 340nm.

6.2.6 TOTAL AMMONIA ASSAY (after McCullough, 1967)

Solution 1 contained 10 g phenol; 50mg sodium nitroprusside l^{-1} distilled water; solution 2 contained 5 g NaOH; 10 ml Sodium Hypochlorite; 53.7 g Na_2HPO_4 l^{-1} distilled water. 2.5 ml of each solution was added to 0.5 ml plant extract (see section 2.6.1). Following incubation at 37°C for 35 minutes, OD was measured at 625nM, and concentrations determined using a standard curve.

6.2.7 PROTEIN ASSAY (after Lowry *et al*, 1951; Taylor & Havill, 1981)

1 ml alkaline copper solution (50 ml 2% Na_2CO_3 in 0.1M NaOH, mixed with 50 ml 0.5% $CuSO_4.5H_2O$ in 1% Na/K tartrate), was added to 0.2 ml fresh plant extract (in potassium phosphate buffer pH 7.4). The samples were whirrli-mixed and then left to stand for 15 minutes. 0.1 ml Folin-Ciocalteu reagent (diluted 1:10) was added, with rapid mixing. OD was determined at 750nm after 45 minutes, and concentrations determined using a standard curve.

6.2.8 GC AMINO ACID ANALYSIS

Plant extracts were prepared according to section 2.6.2. Amino acids were separated from other solutes on Dowex 50+ acidic ion exchange resin (50 X 8 400 mesh on 0.5 X 5 cm) columns, and then eluted with 6 M NH_4 OH. Samples were rotary evaporated and then re-suspended in 1ml double distilled water. Neutral/basic amino acids were then separated from acidic amino acids on Dowex 1 acetate strongly basic ion exchange resin columns (1 X 8 400 mesh on 0.5 X 5 cm), neutral and basic amino acids being eluted with 7.5 ml distilled water; acidic amino acids were eluted with 7.5 ml 2M acetic acid. The eluents were rotary evaporated and then re-suspended in 1 ml distilled water, all fractions being stored at -20°C until required.

The relative instability of the GC derivatives dictated that derivatisation and analysis occurred on the same day. Amino acids were transformed into N (O-S), heptafluorobutyl isobutyl esters (n-HFBI esters). 0.25 ml amino acid extract was placed in a 1 ml reacti-vial and dried at room temperature under a steady stream of nitrogen (g). 0.2 ml dichloromethane was then added, and the mixture re-dried under nitrogen. This process was repeated. The residues were then re-suspended in an acetylchloride : 2 methyl 1-propanol mixture (3:10 v/v). The vials were sealed and then heated to 200°C for 30 minutes. After cooling, and a further evaporation to dryness under nitrogen (g), 0.05 ml heptafluorobutyric anhydride was added. The vials were re-sealed, and heated at 250°C for 10 minutes. After cooling and evaporating to dryness under nitrogen (g), the final residue was re-suspended in 0.5 ml ethyl acetate/acetic anhydride, (1:1 v/v).

An SGE 25QC2/BPX 5, 25 m long column was utilised for amino acid analysis, with a 0.25 micron. film thickness; an I.D. of 0.22 mm; and an O.D. of 0.33 mm; with a non-polar bonded phase with 5 % phenyl equivalent modified

siloxane.

1 µl of derivatised amino acid sample was injected into a Perkin Elmer 8420 capillary gas chromatograph. Injector temperature was 350°C. The initial column temperature (80°C) was maintained for 4 minutes and then raised to 250°C at a rate of 8°C min^{-1} for neutral/basic amino acids, and 16°C min^{-1} for acidic amino acids. The carrier gas was air / nitrogen / hydrogen throughout. Pressure was maintained at 10 Pa/min. Detection involved flame ionisation at 350°C.

6.2.9 ALLANTOIN ASSAY (after Fujihara *et al*, 1987; Resines *et al*, 1993)
0.5 ml NaOH (5M) was mixed with 1 ml plant sample (prepared as described in section 2.6.1), and boiled vigorously for 7 minutes. Samples were then incubated at 20°C for 30 minutes, and then 5 ml 5M HCl was added, followed by two extra drops. 5 ml phenylhydrazine solution (0.33%) was added and the samples were then boiled for 2 minutes, before being placed in an ice slurry bath for a further 3 minutes. 1.5 ml 3M HCl was added to the samples, followed by 0.5 ml potassium ferricyanide (1.67%). Sample volumes were made up to 15 ml with distilled water. OD was recorded at 500nm, after 20 minutes, and concentrations were determined using a standard curve.

6.2.10 ALANINE AMINOTRANSFERASE (after Hedley & Stoddart, 1971)
0.2M DL-alanine was prepared in 0.1 M tris buffer (pH 7.4). A 0.002M α-ketogluterate; 50 ml lactate dehydrogenase; 100 µl NADH solution was prepared in 0.1M tris buffer pH 7.4. 1 ml of each of the above was pipetted into 1 ml plant sample (fresh pulverised plant material in potassium phosphate buffer pH

7.4 (with 1 mM EDTA)). OD was determined at 546nm, (after blanks had been run omitting the alanine substrate). Changing ODs were measured (representing the disappearance of NADH).

6.2.11 ASPARTATE AMINOTRANSFERASE (after Hedley & Stoddart, 1971)

0.1M l-aspartate was prepared in 0.1M tris buffer (pH 7.4). A 0.002M α-oxogluterate; 50 µl malate dehydrogenase; 100 µl NADH solution was prepared in 0.1M tris-buffer (pH 7.4). 1 ml of each of the above was pipetted into 1 ml fresh plant extract (fresh pulverised plant material in potassium phosphate buffer pH 7.4 (with 1 mM EDTA)). OD was determined at 546nm (after blanks had been run omitting the aspartate substrate). Changing ODs were measured (representing the disappearance of NADH).

6.2.12 ASPARAGINE SYNTHETASE (after Rogenes, 1975; Scott & Farnden, 1976; Joy & Ireland, 1990)

0.5 ml distilled water and 0.1 ml plant extract (fresh pulverised plant material in potassium phosphate buffer pH 7.4 (with 1 mM EDTA)) were added to 0.5ml assay mix, which contained: 50 mM Tris-HCl buffer (pH 7.8); 10mM glutamine; 5mM ATP; 100 mM aspartate; 10mM magnesium sulphate; 2mM DDT; and 0.1mM EDTA. OD was determined at 340nm, (after blanks had been run omitting the glutamine and aspartate substrates), and changing ODs were measured.

6.2.13 HOMOSERINE DEHYDROGENASE

0.1 ml 4mM NADP; 0.1 ml 100mM homoserine; 0.2 ml 50mM Tris buffer (pH 8.4 with 5mM EDTA); and 0.5 ml distilled water were pipetted into a cuvette. 0.1 ml plant extract (fresh pulverised plant material in potassium phosphate buffer pH 7.4 (with 1 mM EDTA)), was added, and the rate of disappearance of NADP followed at 340nm (after blanks had been run omitting the homoserine

substrate).

6.3 RESULTS & DISCUSSION

6.3.1 PRIMARY NITROGEN ASSIMILATION

6.3.1.1 NITRATE REDUCTASE ACTIVITIES

Low nodular NR activities were recorded (as previously reported, Raven & Sprent, 1993), and therefore nodular NR activities are not presented. Figs. 6.2 & 6.3 and anova analyses reveal that significantly lower leaf and root NR activities were recorded in 'no nitrate' supplied as compared with medium nitrogen supplied non-nodulated *V. faba*, which may have been attributable to constitutive NR activities (Clement *et al*, 1978; Remmler & Campbell, 1986; Rufty *et al*, 1989). However it is possible that trace medium nitrogen concentrations may have resulted in NR induction in 'no nitrate' supplied *V. faba* (pg. 30), particularly as some earlier workers have been unable to detect constitutive NR activities in *V. faba* (Andrews *et al*, 1990).

NR activities increased significantly in the leaves and roots of *V. faba* as the concentration of the supplied nitrogen source increased. This is in agreement with earlier reports that nitrate net uptake rates (Ourry *et al*, 1995), and the concentration and activity of NR reportedly increase in *V. faba* (Andrews *et al*, 1984; Sutherland *et al*, 1985), and in other plant species (Bennet *et al*, 1964; Alfredi & Hewitt, 1964; Beevers & Hageman, 1969; Nicholas *et al*, 1976; Stewart & Orebamjo, 1979; Smirnoff *et al*, 1984; Somers *et al*, 1983; Sprent & Thomas 1984; Remmler & Campbell, 1986; Andrews, 1986; Rhoden *et al*, 1987; Martinez & Cerda, 1989; Campbell, 1988; Galangau *et al*, 1988; Solomonsen & Barber, 1990; Redinbaugh & Campbell, 1991; Pelsey & Caboche, 1992; Min *et al*, 1998), within a few hours of nitrate application (Oaks *et al*, 1972).

Fig. 6.1 Amino Acid Biosynthesis (adapted from Bryan, 1976).

Nitrate Reduction

NO^{3-} + NADH + H^+ → NO^{2-} + NAD^+ + H_2O

NO^{2-} + $3H_2O$ + $2H^+$ (& light) → NH_4^+ + $1.5O_2$ + $2\ H_2O$

PHOTOSYNTHESIS** → GLUCOSE

PHOTORESPIRATION

Glucose 6 Phosphate

Fructose 6 Phosphate

Fructose 1, 6 Bisphosphate

Dihydroxyacetone Phosphate

Glyceraldehyde 3 Phosphate

1 ,3 Bisphosphoglycerate

3 phosphoglycerate → SERINE → CYSTEINE / GLYCINE

2 phosphoglycerate

Phosphoenolpyruvate

PYRUVATE — 1 → ALANINE, GLUTAMATE

PYRUVATE → VALINE, LEUCINE

CITRIC ACID

CIS ACONITATE

ISOCITRATE

ALPHA-KETOGLUTERATE

SUCCINYL coA

SUCCINATE

FUMARATE

MALATE

OXALOACETATE*

pH STAT

GDH**
NH4+ + NADPH + H + ALPHA-KETOGLUTARATE - L-GLUTAMATE + NADP + H2O

GS GOGAT***
NH4+ + ATP + ALPHA-KETOGLUTARATE - **L-GLUTAMATE** + NADP+ + ADP + Pi

** *(affected by nitrogen source)*

Assimilation of Ammonia by GS GOGAT***

NH_4^+ + Glutamate —(ATP ADP)→ Glutamine

Glutamine + α-ketoglutarate —(NADH NAD)→ 2 X Glutamate

reduced ferredoxin (photosystem I)

NAD(P)H

2 - oxogluterate

Assimilation of ammonia by GDH**

NH_4^+ + α-ketogluterate + NADPH (or NADH) + H^+ → L-glutamate + $NADP^+$ (or NAD^+) + H_2O

ERYTHROSE 4 PHOSPHATE *(from pentose phosphate pathway (which produces NADPH))*

TYROSINE

TRYPTOPHAN

PHENYLALANINE

RIBOSE-5-PHOSPHATE *(from the pentose phosphate pathway)* ← GLUTAMINE

HISTIDINE

CITRULLINE

ORNITHINE

ARGININE

GLUTAMATE → PROLINE

5 → GLUTAMINE

+ OXALOACETATE* — 2 → alpha-ketogluterate

ASPARTATE

GLUTAMINE

+ NH4+ — 3 → GLUTAMATE

ASPARAGINE

LYCINE

HOMOSERINE — 4 ← + pyruvate

METHIONINE

THREONINE

VALINE

ISOLEUCINE

LEUCINE

1 = ALANINE AMINOTRANSFERASE, **2** = ASPARTATE AMINOTRANSFERASE, **3** = ASPARAGINE SYNTHETASE, **4** = HOMOSERINE DEHYDROGENASE, **5** = GS GOGAT

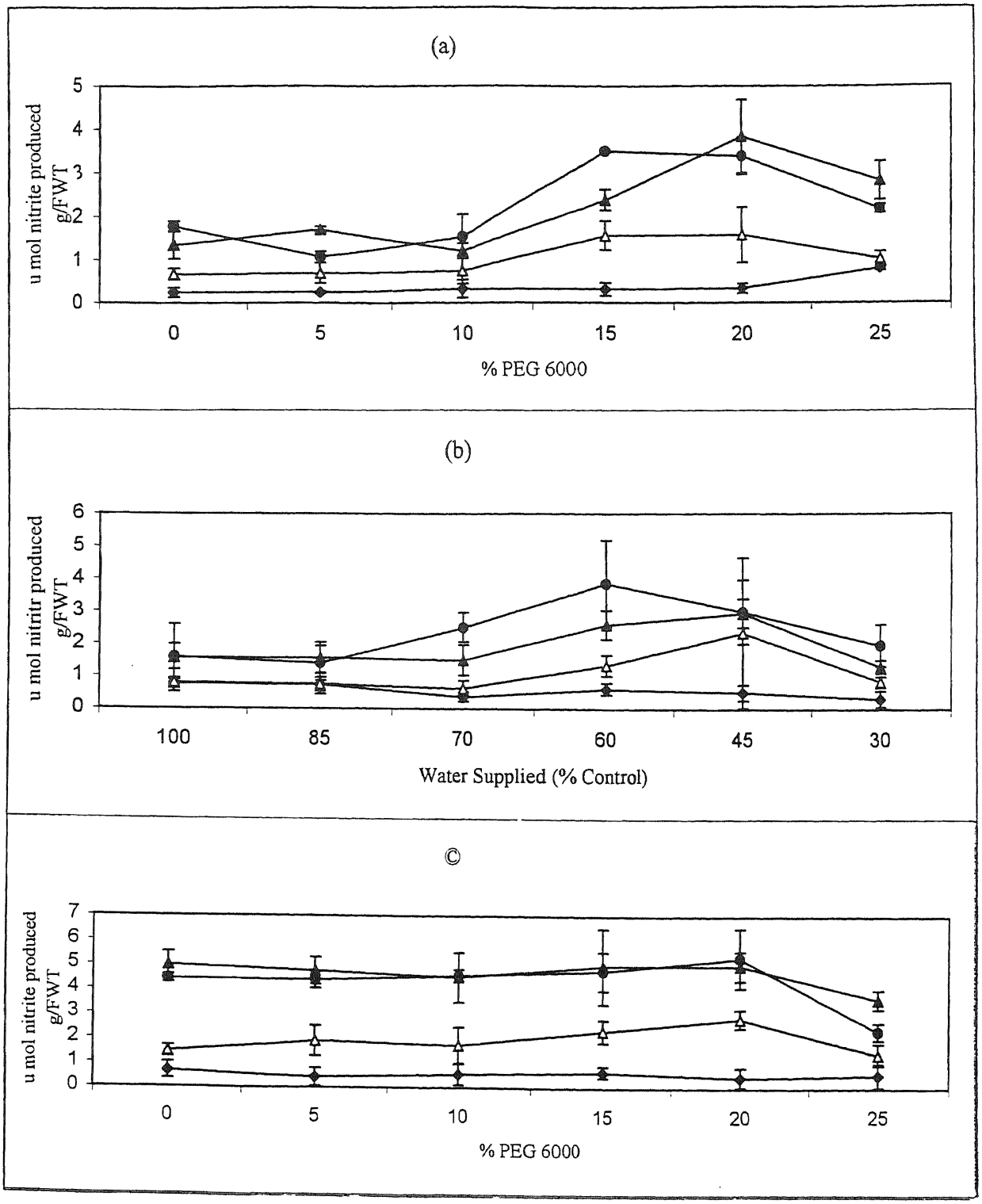


Fig. 6.2 NR activities in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

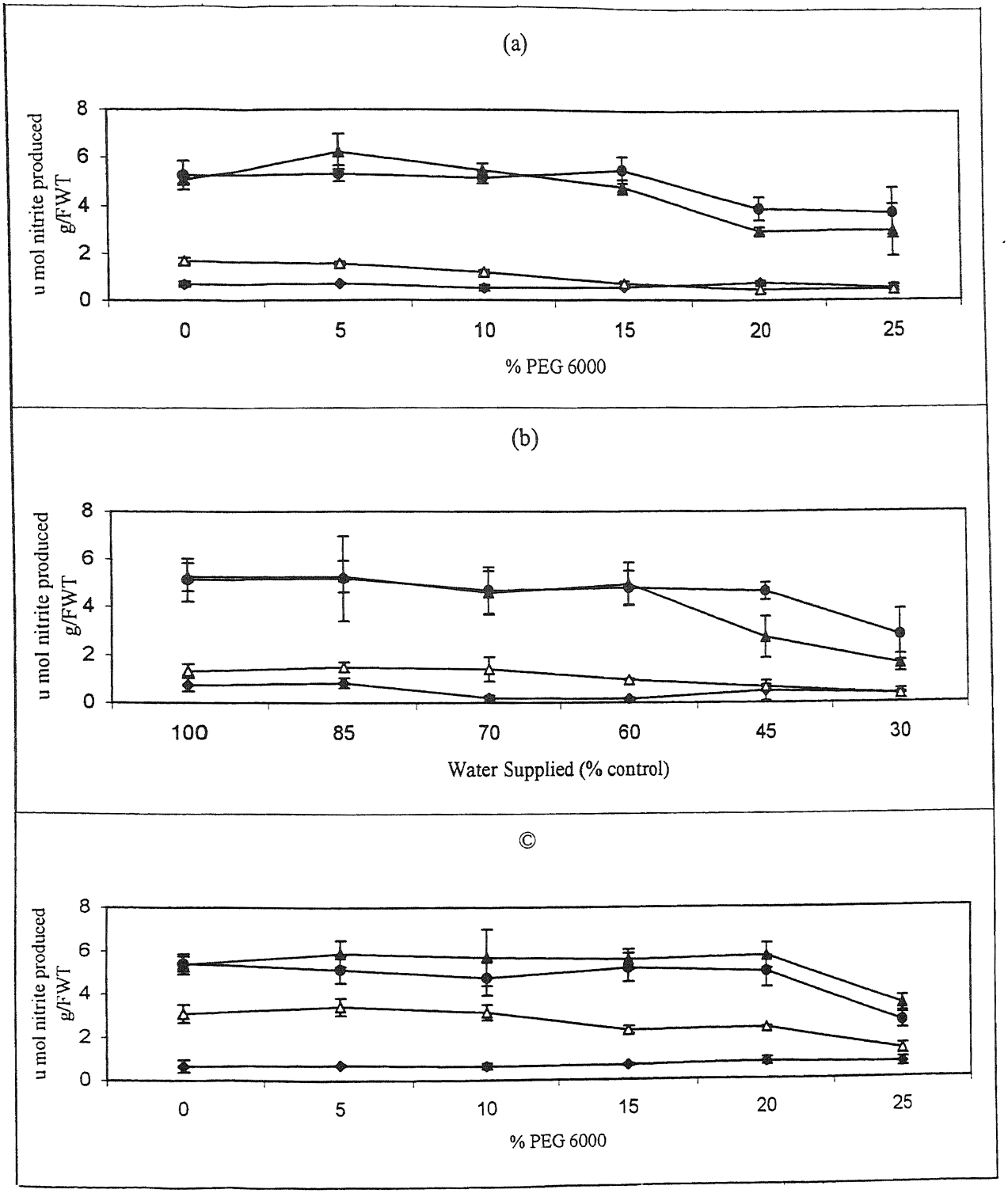


Fig. 6.3 NR activities in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

Figs. 6.2 & 6.3 illustrate that non-nodulated 'combined nitrogen' supplied *V. faba* maintained NR activities at values which were comparable with those exhibited by '1/2 nitrate' supplied *V. faba*; and anova analyses reveal that significantly greater NR activities were exhibited in the leaves and roots of 'spiked' than of 'non-spiked' *V. faba*. Indeed while some earlier workers have reported decreasing nitrate uptake in other plant species in the presence of (even low concentrations of) medium ammonia (Minotti *et al*, 1969b; Smith & Thompson 1971; Stewart 1972; Breteler & Smit, 1974: Miflin *et al*, 1980), and during pH and cation concentration increases (Townsend, 1969; Schrader, 1978), contrasting earlier literature describes maintained nitrate uptake in other plant species when supplied with a medium ammonia source (Orebamjo & Stewart, 1975a; Smirnoff *et al*, 1984).

Furthermore while ammonia nutrition has previously been reported as inhibitory for NR activities in some plant species (Orebamjo & Stewart, 1975a; Clement *et al*, 1978; Srivastava, 1980; Schrader & Thomas, 1981; Yandow & Klein, 1986; Lee & Drew, 1989; Raper *et al*, 1991; Muller & Janiesch, 1993), and may reportedly result in the production of greater concentrations of a NR inhibiting protein (Stewart *et al*, 1974; Schrader & Thomas, 1981), contrasting earlier literature describes increases in NR protein, and in NR and NiR activities in some other plant species when supplied with 'combined nitrogen' as opposed to with nitrate or with ammonia nutrition (Oaks *et al*, 1977; Rigano *et al*, 1979; Somers *et al*, 1983; Lillo & Henriksen, 1984; Guerrier, 1991). Indeed figs. 6.2 & 6.3 and anova analyses reveal that leaf NR activities were significantly greater in 'spiked' than in 'non-spiked' *V. faba*, a further indication that medium ammonia additions do not result in inhibited NR activities in this species.

Three-fold greater shoot NR activities have previously been recorded in

Clematis vitalba when supplied with ammonia as opposed to with nitrate nutrition (Bungard *et al*, 1999).

Anova analyses reveal that NR activities were significantly greater in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*. Although nitrogen fixation (and medium ammonia additions) do not result in increased root nitrate concentrations (Sprent, 1980), correlations have previously been reported between NR and GS activities in nitrogen exporting plant parts (Hofstra *et al*, 1985). The inference is that NR activities may have increased concurrently with the quantified GS activity increases which were exhibited in nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba*, and in 'spiked' as opposed to 'non-spiked' *V. faba* (figs. 6.6 & 6.7), see also Bungard *et al*, (1999).

The significantly greater NR activities exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition may reflect the greater levels of net photosynthesis (fig. 3.22) and the significantly greater carbohydrate concentrations (figs. 4.3 & 4.4) which were also exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. Phloem sap carbohydrate supplies are reportedly regulatory for nitrate uptake (Imsande & Touraine, 1994), and may therefore influence nitrate 'flux', which as discussed is reportedly regulatory for NR activities (Shaner & Boyer, 1976). NR is reportedly continuously synthesized and degraded as affected by nitrate availabilities (Somers *et al*, 1983). Correlations have previously been reported between NR activities and both ambient photosynthesis (Ferrario-Mery *et al*, 1998; Foyer *et al*, 1998) and sucrose concentrations (Cheng *et al*, 1992). Additionally to being a requirement for nitrate uptake, carbon skeletons and reductants are required for the maintenance of NR activities and for amino acid production

(Stryer, 1988), and may be supplied directly via photosynthesis (Miflin, 1974).

NR activities reportedly decrease rapidly during water deficits (Mattas & Pauli, 1965; Hsiao, 1973; Hsaio *et al*, 1976; Rajagopal *et al*, 1977; Hanson & Hitz, 1982; Wellburn *et al*, 1996; Ferrario-Mery *et al*, 1998; Foyer *et al*, 1998). However figs. 6.2 & 6.3 illustrate that NR activities were maintained in *V. faba* until water deficits became severe, inferring that decreasing NR activities may not have been attributable to the effects of water deficits *per se*. Indeed maintained NR activities during water deficits have previously been reported in a small number of studies involving other plant species (Smirnoff *et al*, 1985; Ladley, 1990).

Nitrate is xylem translocated utilising the transpiration stream (Ziegler, 1975), and although xylem tensions may reportedly be independent of transpiration (Wei *et al*, 1999; using direct pressure probe measurements), nitrate uptake is reportedly dependant on water flow during low water fluxes (Shaner & Boyer, 1976; Boyer, 1985). Stomatal conductances, and by inference possibly transpiration and nitrate fluxes were maintained at increasingly great values in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (fig. 3.21); the same concentrated nitrogen nutrition which resulted in the exhibition of the greatest NR activities. However significant stomatal closure occurred during severe water deficits, even in *V. faba* which were supplied with concentrated nitrogen nutrition (fig. 3.21). The inference is that decreasing NR activities during severe water deficits may not have been attributable to water deficit effects *per se*, but to decreased stomatal conductances and by inference decreased nitrate fluxes (Shaner & Boyer, 1976; Smirnoff *et al*,

1985), and due to limitations in photosynthate availabilities (Foyer *et al*, 1998) which would only became apparent in *V. faba* during moderate to severe water deficits (fig. 3.22), and would therefore coincide with decreasing NR activities. Decreased nitrate fluxes during water deficits may have effectively limited NR activities in *V. faba*, as leguminous NR is reportedly characterised as high activity-low affinity (Stewart & Orebamjo, 1979).

Indeed some of the earlier literature which has reported that NR activities decrease during slight to moderate water deficits cited methodology which involved rapid water deficit imposition (e.g. Ferrario-Mery *et al*, 1998, imposed water deficits in five days). Rapid water deficit imposition reportedly results in decreased stomatal conductances and photosynthetic activities (Jones & Rawson, 1979; Richardson & McCree, 1985), and therefore potentially in decreased nitrate fluxes; carbohydrate levels; and reductant levels which as previously discussed are considered regulatory for NR activities (Shaner & Boyer, 1976; Somers *et al*, 1983; Foyer *et al*, 1998). Decreasing NR activities have previously been reported in *T. durum* when severe water deficit imposition was employed, whereas gradual water deficit imposition resulted in the exhibition of maintained NR activities (Smirnoff *et al*, 1985). Therefore earlier researchers may have attributed the effects of nitrate flux and photosynthate limitations on NR activities to water deficits.

While all of the tissues of *V. faba* are able to synthesise the enzyme complement for nitrate assimilation (Smirnoff & Stewart, 1985), *V. faba* reportedly predominantly root assimilates nitrate (Durzan & Stewart, 1983; Sprent & Thomas, 1984; Andrews *et al*, 1984; Sutherland *et al*, 1985), reducing only excess nitrate in the shoots (Andrews, 1986). Figs. 6.2 & 6.3 confirm that greater NR activities were recorded in the roots than in the

leaves of *V. faba* irrespective of the nitrogen regime, and that nitrate above a threshold value (around 6 μM nitrite /gFW/hr; figs. 6.4 & 6.5) was reduced in the leaves. Greater relative proportions of nitrate were shoot reduced in *V. faba* which were supplied with increasingly concentrated medium nitrate nutrition, with implications for internal energy balance, and hence for water deficit tolerance adaptations (Sutherland *et al*, 1985). Indeed earlier work has indicated that less energy may be required for leaf than for root nitrate assimilation (Sprent & Thomas, 1984), as leaf assimilation has lower associated sucrose transport costs (Gutschick, 1981). Furthermore excess photosynthates, reductants, and ATP may occur in the leaves (Sprent, 1980), and carbohydrate and reductant availabilities may reportedly limit nitrogen assimilation (Hanish ten Cate & Breteler, 1981; Ferrario-Mery *et al*, 1998).

However organic acid synthesis costs may complicate energy calculations as root NR allows easier disposal of excess OH^- ions (Raven & Smith, 1976; see section 5.1, pg. 135; section 5.3.2, pg. 154).

Shoot NR is reportedly more water economical than root NR, as shoot NR utilises photoreduction rather than root respiratory driven reduction (Sprent & Thomas, 1984; Smirnoff & Stewart, 1985). However nitrate uptake reportedly results in respiration enhancement (Redinbaugh & Campbell, 1991; Bowsher *et al*, 1991), and hence in increased electron donor concentrations (Dry *et al*, 1981), which are required both for nitrate and for ammonia assimilation, inferring that reductant availabilities may be increased in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. Furthermore, as protein concentrations (and therefore possibly synthesis) decreased during increasing water deficits (figs. 6.24 & 6.25), NADH availabilities may have increased, resulting in a partial alleviation

of root reductant shortages during water deficits.

Photosynthesis was maintained in *V. faba* until water deficits became moderate to severe (particularly in *V. faba* when supplied with '1/2 nitrate' and with 'combined nitrogen' nutrition; figs. 3.22). Reductants would therefore be required for CO_2 fixation in the leaves of *V. faba* (even during moderate water deficits) reducing the advantages associated with shoot nitrate reduction (Canvin & Atkins 1974; Smirnoff *et al*, 1984; Hogh-Jensen *et al*, 1997) in this species. Shoot reduction may incur advantages in plants adapted to receive high photo flux densities, however *V. faba* is adapted to temperate regions and is therefore less likely to exhibit excessive leaf photosynthate and reductant availabilities (Deane-Drummond *et al*, 1980).

It has been reported that the location of nitrate reduction does not significantly affect growth or nitrogen concentrations and appears to be a purely species-specific phenomenon (Lexa & Cheeseman, 1997). Capital costs may favour root nitrate assimilation in plants which frequently utilise ammonia or N_2 as principle nitrogen sources (such as *V. faba*), and may accordingly predominantly utilise root primary nitrogen assimilatory enzymes, and may 'expect' organic nitrogen delivery to the shoots. Such species may be metabolically and biochemically biased towards root nitrogen assimilation (see introduction; pg. 10). However figs. 6.2 & 6.3 illustrate that in non-nodulated *V. faba* root NR activities decreased during less severe water deficits than leaf NR activities. This may denote a more sensitive response of root NR to gradually decreasing photosynthate and reductant availabilities (fig. 3.22), inferring that the leaf assimilation of excess nitrate may result in the maintenance of nitrate reduction during more severe water deficits than would occur if nitrate was solely reduced

in the roots of *V. faba*, for reasons outlined on pg. 188.

6.3.1.2 NITRATE

Nitrate may be stored in vacuoles in the non-reduced form, thereby conserving energy (Cram, 1974; Pate, 1983; Monson *et al*, 1994). Figs. 6.4 & 6.5 and anova analyses reveal that nitrate concentrations were maintained in the following order in non-nodulated *V. faba* with respect to medium nitrogen nutrition: 'combined nitrogen' = '1/2 nitrate' > '1/10 nitrate' > 'no nitrate'. Earlier reports have related that 'combined nitrogen' nutrition may result in decreased nitrate uptake (Minotti *et al*, 1969b; Smith & Thompson 1971; Stewart 1972; Breteler & Smit, 1974). However figs. 6.18 & 6.19 illustrate that medium ammonia additions did not result in the exhibition of lower nitrate concentrations than those which were exhibited in *V. faba* which were supplied solely with (equimolar) nitrate nutrition (in agreement with earlier work on *Lemna minor* (Orebamjo & Stewart, 1975a), and on *G. max* (Bourgeais-Chaillou *et al*, 1992)).

Figs. 6.4 & 6.5 and anova analyses reveal that nitrate accumulated significantly in the leaves and roots of *V. faba* during water deficits. The tonoplast is not highly permeable to nitrate, making it an ideal osmotic solute (Shaner & Boyer, 1976). Plant tissue nitrate concentrations are generally in the range of 0-140 μM/gDW and account for 0-10% of total plant nitrogen concentrations (Lorenz, 1973). High nitrate concentrations were exhibited in *V. faba* (figs. 6.4 & 6.5). Nitrate concentrations increased significantly in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. A potential for maintained nitrate 'fluxes' and hence NR activities is thus inferred in *V. faba*, particularly when supplied with concentrated medium nitrogen nutrition, as nitrate may reportedly be

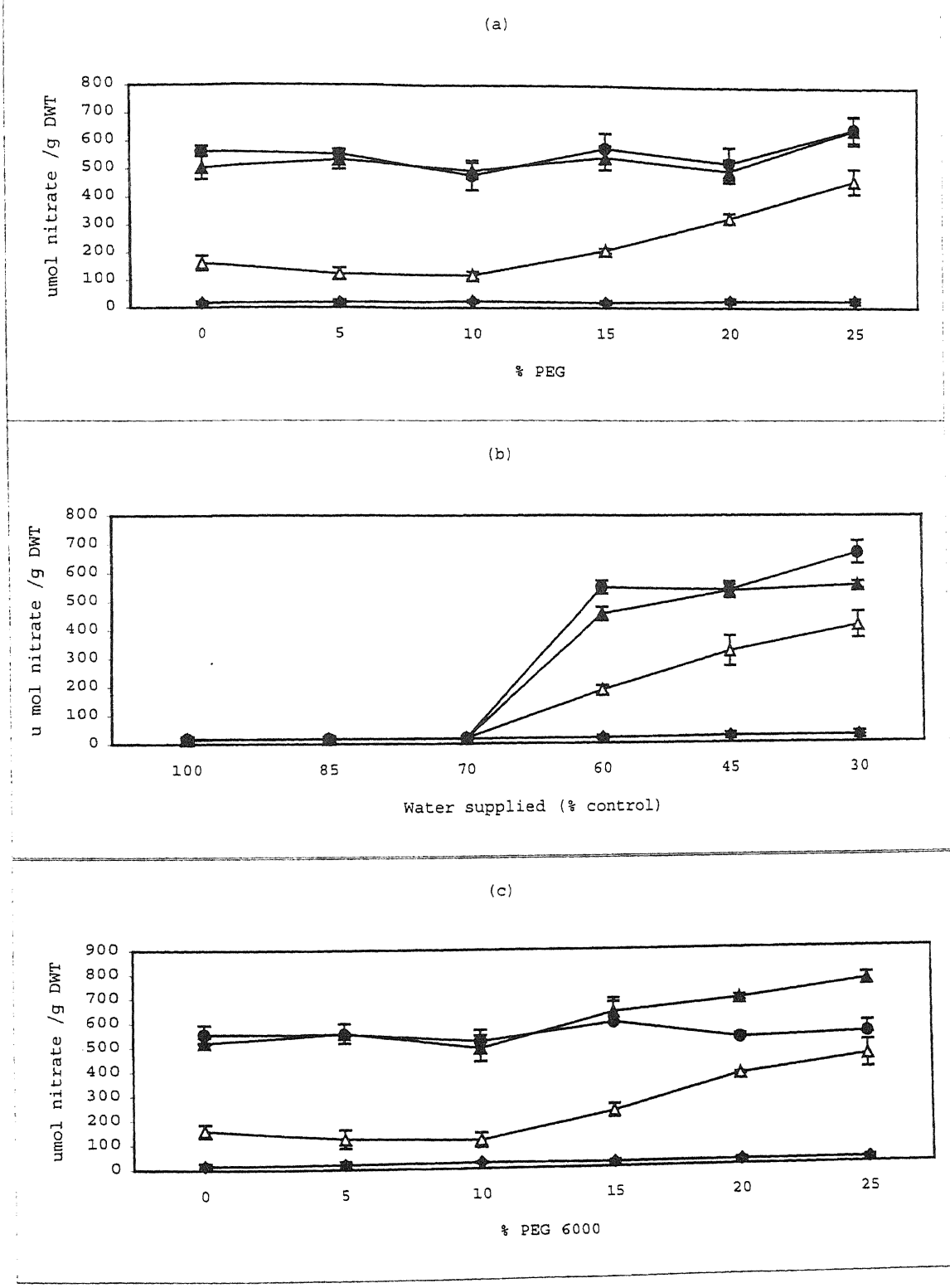


Fig. 6.4 Nitrate concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

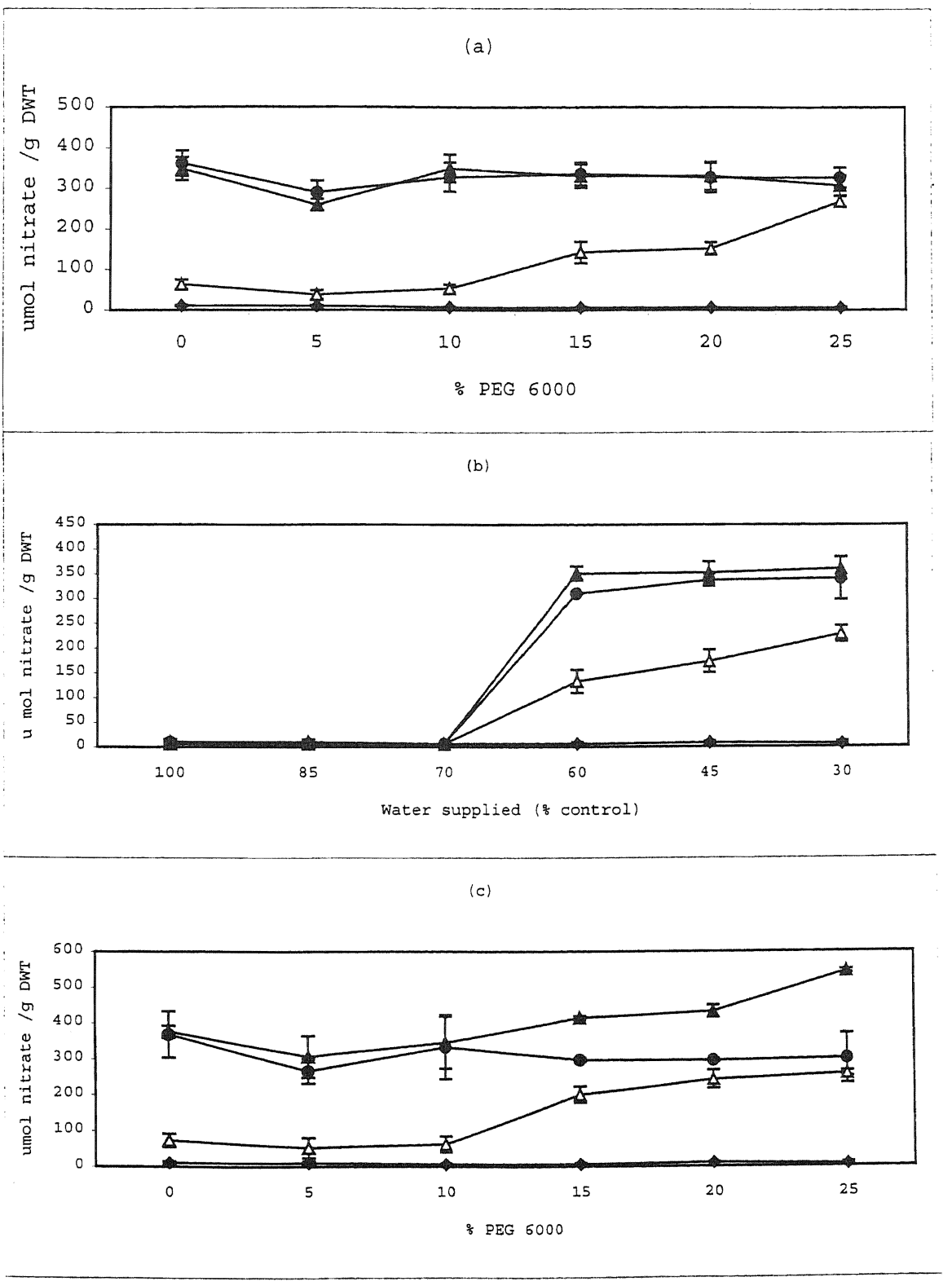


Fig. 6.5 Nitrate concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

mobilized from storage to metabolic pools during water deficits resulting in maintained NR activities (Shaner & Boyer, 1976 a&b; Chapin *et al*, 1988).

Figs. 6.4 & 6.5 and anova analyses reveal that the nitrate concentrations recorded in nodulated and in non-nodulated 'no nitrate' supplied *V. faba* were not significantly different. Similarly ammonia 'spiked' and 'non-spiked' *V. faba* accumulated similar nitrate concentrations. Nitrogen fixation and ammonia assimilation do not result in nitrate production (Sprent 1980; fig. 6.1), and figs. 6.6 - 6.9 illustrate that nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba*, and 'spiked' as opposed to 'non-spiked' *V. faba* exhibited greater GS and GDH activities; a reflection of the resultant increased ammonia assimilation.

An inverse relationship between foliar NR activities and leaf nitrate concentrations has previously been reported in *G. max* (Tolley-Henry & Raper, 1991). However fig. 6.4 illustrates that the highest leaf nitrate concentrations were exhibited in non-nodulated *V. faba* when supplied with 'combined nitrogen' and with '1/2 nitrate' nutrition, the same nitrogen nutrition which resulted in the maintenance of the greatest NR activities throughout water deficits (figs. 6.2 & 6.3), indicating that nitrate accumulation during water deficits was not attributable to decreasing NR activities in this species. Furthermore nitrate accumulation commenced prior to NR activity decreases (which occurred only during severe water deficits; figs. 6.2 & 6.3), and may represent storage of excess nitrate, and osmotic adjustment.

For high-nitrate plants such as *V. faba* a logic exists for relating reduced nitrogen to dry matter, and nitrate to the tissue water in which it is dissolved (Cardenas-Navarro *et al*, 1998); as reinforced by the concurrent

maintenance of increasing RWCs (figs. 3.16 & 3.17) and increasing nitrate concentrations (figs. 6.4 & 6.5) in *V. faba* when supplied with increasingly concentrated medium nitrate nutrition during water deficits.

6.3.1.3 GLUTAMINE SYNTHETASE (GS) ACTIVITIES

Figs. 6.6 & 6.7 and anova analyses reveal that significantly greater GS activities were exhibited in the leaves and roots of non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. GS activities were exhibited in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' = '1/2 nitrate' > '1/10 nitrate' > 'no nitrate' nutrition (in agreement with earlier work on *P. sativum* and *G. max*; Emes & Fowler, 1979; Ortega *et al*, 1999). Indeed root GS is reportedly induced co-ordinately with NR induction (within thirty minutes of nitrate treatment in *Z. mays*, Redinbaugh & Campbell, 1993), and may thus represent a primary response to environmental nitrogen (Hofstra *et al*, 1985; Redinbaugh & Campbell, 1991; Redinbaugh & Campbell 1993).
Figs. 6.6 & 6.7 and anova analyses reveal that significantly greater root GS activities were exhibited in 'spiked' than in 'non-spiked' *V. faba*. Ammonia nutrition has previously been reported to result in greater GS activities than nitrate nutrition in other plant species (Arnozis *et al*, 1988). GS2 activities may be directly induced by nitrogen, and they may also reflect the increased sucrose concentrations which were exhibited in *V. faba* when supplied with increasingly concentrated nitrogen nutrition (figs. 5.2 & 5.3), and which also reportedly result in increased GS2 induction (Oliveira & Coruzzi, 1999). Indeed GS activities reportedly increase as light levels increase (Edwards & Corruzzi, 1989: Elmlinger & Mohr, 1992), and this effect is apparently not caused by increases in photorespiration (Cock *et al*, 1991), but may be partly attributable to

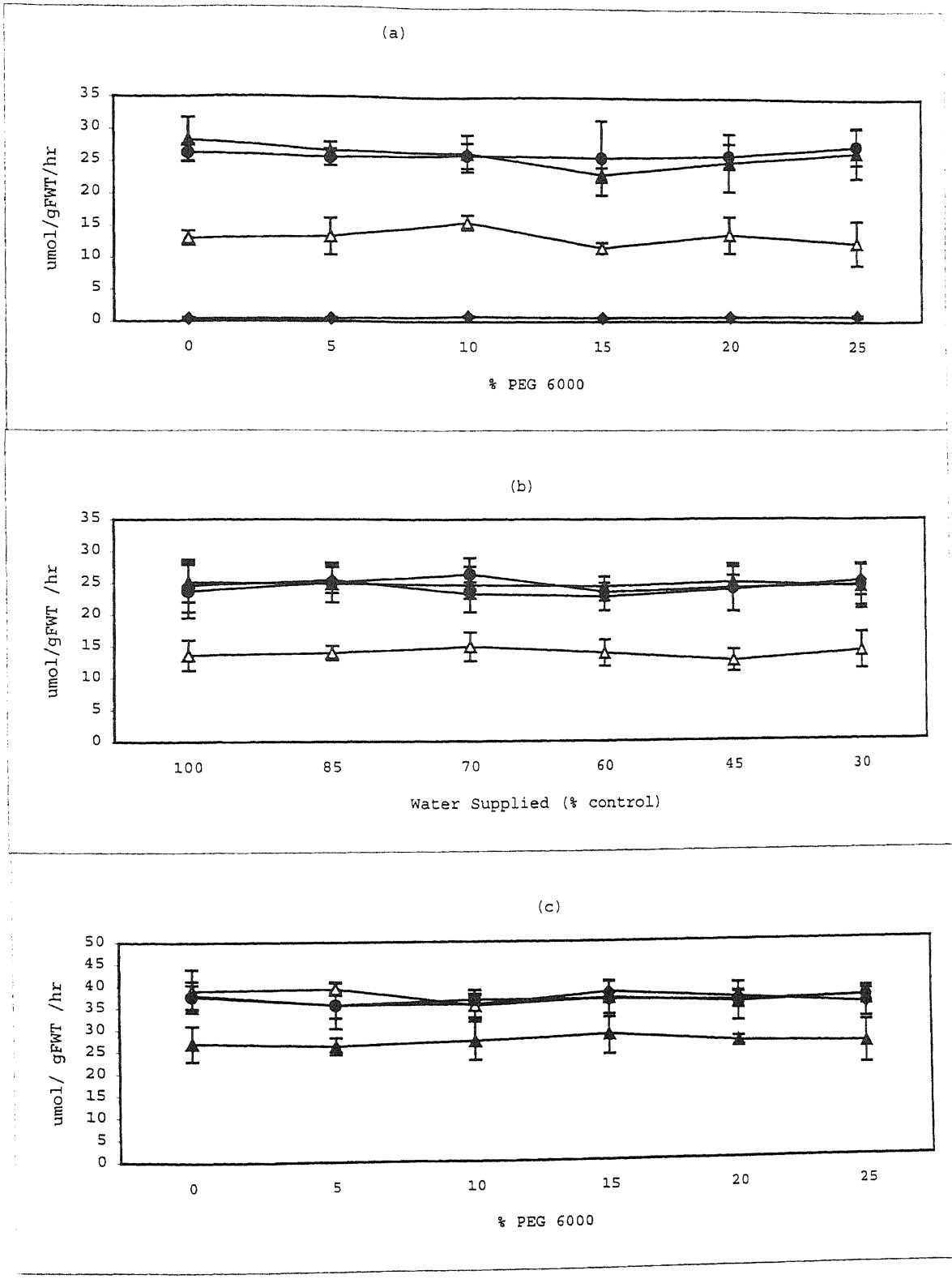
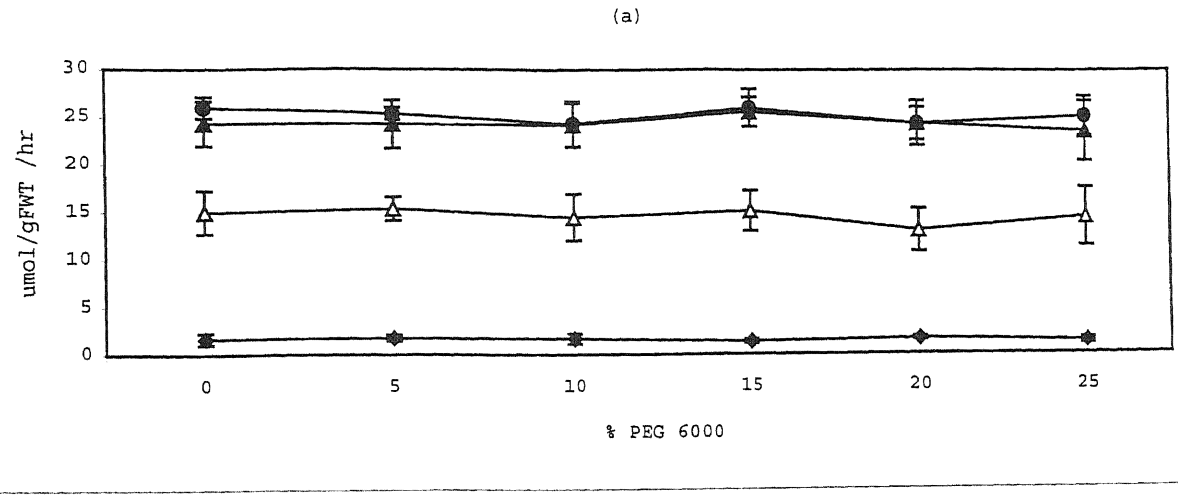
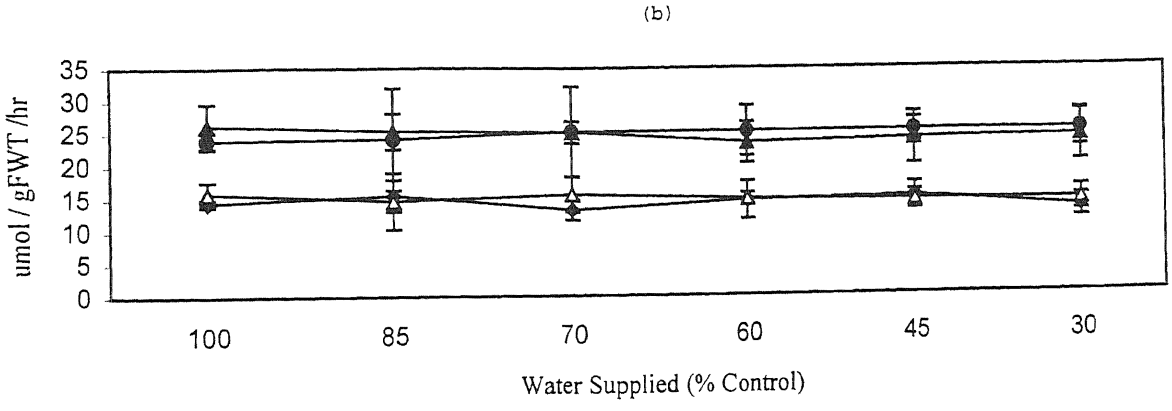


Fig. 6.6 GS activities in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'





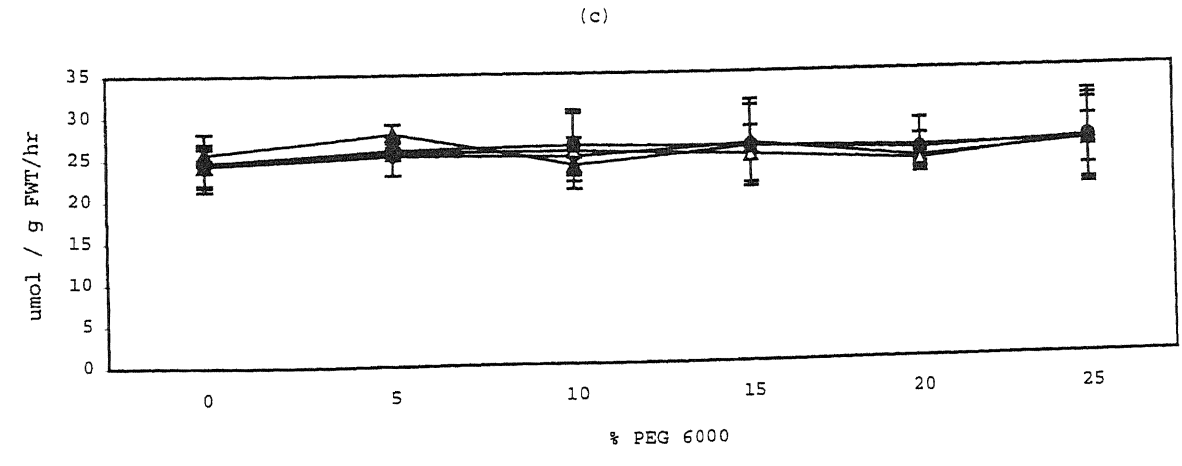


Fig. 6.7 GS activities in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

increased carbon skeleton availabilities and ATP concentrations (Ortega *et al*, 1999; see section 6.3.1.4).

However GS activities were not significantly greater in the leaves of 'spiked' as opposed to 'non spiked' *V. faba* when '1/2 nitrate' and 'combined nitrogen' formed the basic nutrition, perhaps as such *V. faba* exhibited such high intrinsic root GS activities that primary nitrogen assimilation did not require increased leaf GS activities. Although fig. 6.7 illustrates that root GS activities were not significantly greater in 'spiked' than in 'non spiked' '1/2 nitrate' supplied *V. faba*, fig. 6.9 illustrates that root GDH activities were significantly greater in 'spiked' than in 'non-spiked' '1/2 nitrate' supplied *V. faba* (see 6.3.1.4). Increased root GS activities in *V. faba* when supplied with increasingly concentrated medium nitrogen (and particularly with ammonia) nutrition may reflect increased ammonia assimilation (and de-toxification); roots being the predominant site of primary ammonia assimilation. Very little ammonia is shoot translocated (Min *et al*, 1998), inferring that the greater leaf GS activities exhibited in 'spiked' than in 'non-spiked' 'no nitrate' and '1/10 nitrate' supplied *V. faba* may also reflect increased photorespiration, which may have been greater in 'spiked' *V. faba* (as net photosynthesis increased in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; fig. 3.22). During water deficits actual rates of photorespiration reportedly decrease (Boyer, 1971), however relative rates of photorespiration to photosynthesis reportedly increase (Lawlor & Fock, 1975), an adaptation which may result in reduced photoinhibition.

Abundant GS2 encoding polypeptides have previously been recorded in developing nodules (Bennet *et al*, 1986; Cock *et al*, 1991), however root as

opposed to nodular GS is reportedly primarily responsible for the assimilation of ammonia in nodulated plants (Scott & Farnden, 1976; Cullimore *et al*, 1983). Figs. 6.6 & 6.7 and anova analyses reveal that significantly greater GS activities were exhibited in the leaves and roots of nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba*, which may reflect the assimilation of fixed nitrogen.

(Particularly shoot) GS activities are reportedly relatively insensitive to water deficits (Taylor *et al*, 1982). Accordingly figs. 6.6 & 6.7 and anova analyses reveal that GS activities were maintained in the leaves and roots of *V. faba* during water deficits (in agreement with work on other plant species, Taylor *et al*, 1982).

6.3.1.4 GLUTAMATE DEHYDROGENASE (GDH) ACTIVITIES

Figs. 6.8 & 6.9 and anova analyses reveal that GDH activities increased significantly in the leaves and roots of non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. GDH activities were exhibited in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate' nutrition (in agreement with earlier reports which indicate that GDH activities may increase in other plant species when supplied with increasing ammonia availabilities; Durzan & Steward, 1967; Barash *et al*, 1975; Rhodes *et al*, 1976; Taylor & Havill, 1981). Furthermore figs. 6.8 & 6.9 and anova analyses reveal that significantly greater leaf and root GDH activities were exhibited in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*, inferring a potential 'role' for GDH in the assimilation of the ammonia produced via nitrogen fixation. Figs. 6.8 & 6.9 and anova analyses reveal that significantly greater GDH activities were recorded in

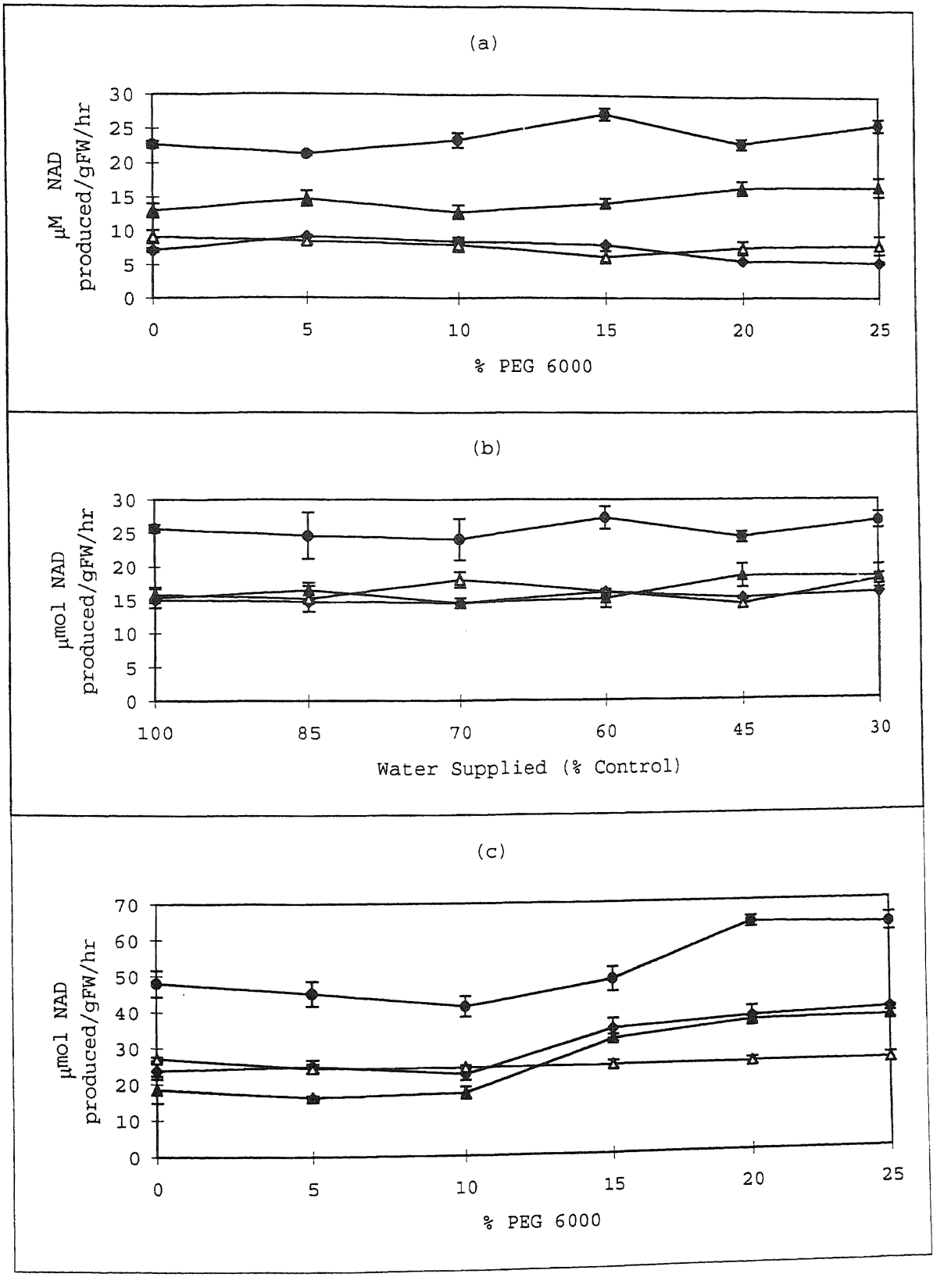


Fig. 6.8 GDH activities in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

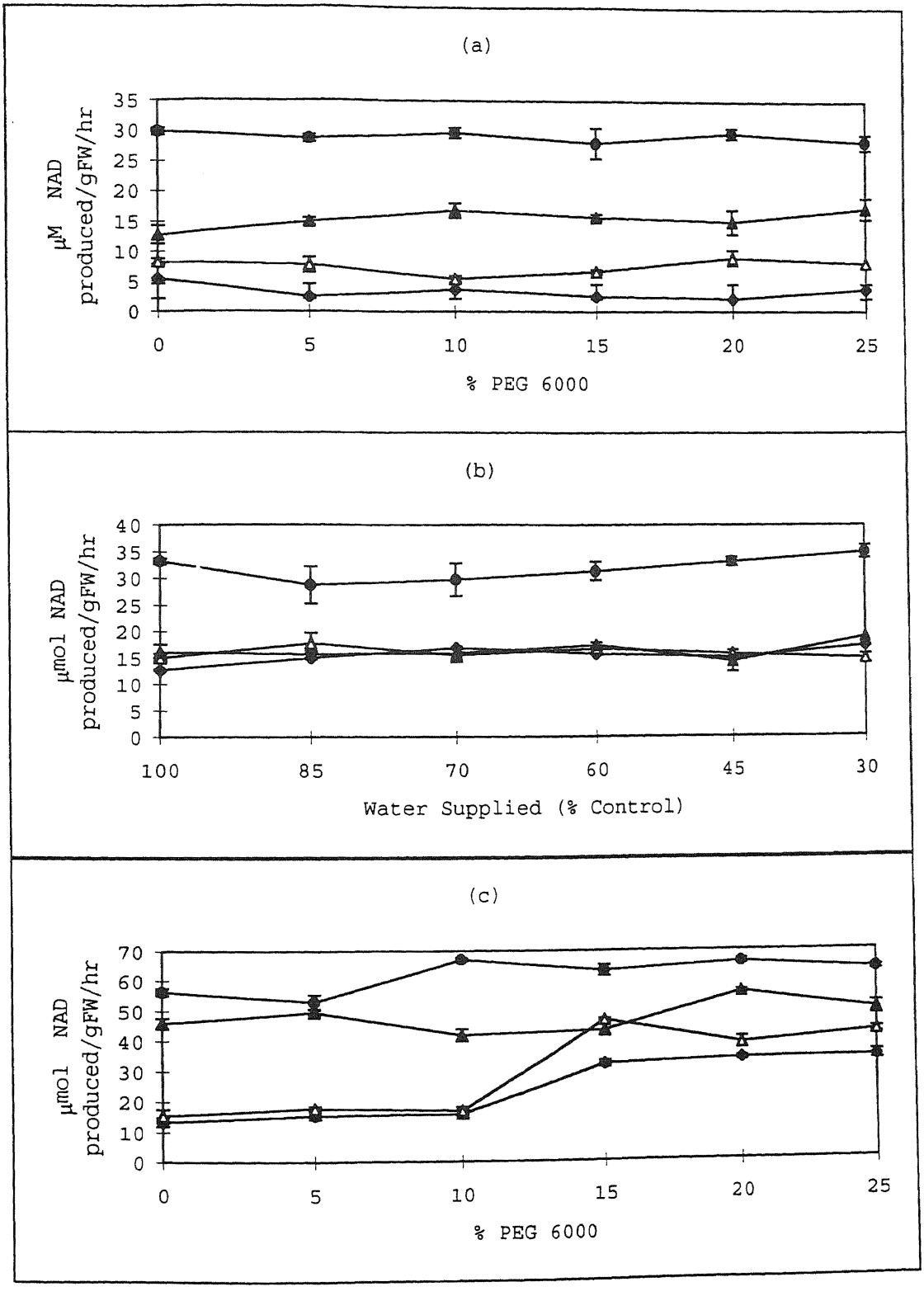


Fig. 6.9 GDH activities in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

the roots and leaves of 'spiked' than of 'non-spiked' *V. faba*. While root GS activities were merely maintained at similar values in 'spiked' as compared with 'non-spiked' '1/2 nitrate' supplied *V. faba* (fig. 6.7), GDH activities were significantly greater in the leaves and roots of 'spiked' than of 'non-spiked' '1/2 nitrate' supplied *V. faba* (figs. 6.8 & 6.9), indicating GDH activities increased in order to assimilate the additional ammonia 'spike'. Indeed increasing exogenous ammonium concentrations have previously been reported to result in the exhibition of increasing root and shoot GDH activities (Barash *et al*, 1975; Taylor & Havill, 1981; Arnozis *et al*, 1988), as interpreted as adaptive responses to avoid ammonia toxicity (Rhodes *et al*, 1976; Taylor & Havill, 1981). High medium ammonia concentrations may also reportedly result in the exhibition of reduced root GS & GOGAT activities (Rhodes *et al*, 1976; Taylor & Havill, 1981, in other plant species).

Figs. 6.8 & 6.9 and anova analyses reveal that GDH activities were maintained in the leaves and roots of non-nodulated and nodulated *V. faba* throughout water deficits. Water deficits did not result in significantly increased glutamine concentrations (tables 6.2 - 6.7). However greater glutamine concentrations were exhibited in 'spiked' than in 'non-spiked' *V. faba,* and greater glutamine concentrations may have contributed to the relatively greater increases in GDH as opposed to GS activities which were exhibited in 'spiked' *V. faba,* as high glutamine concentrations may reportedly inhibit GS and exert positive control on GDH activities (Rhodes *et al*, 1976).

Furthermore GDH is reportedly inhibited by high concentrations of ATP while GS has an ATP requirement, and is reportedly stabilised by ATP (Stewart &

Rhodes, 1977; Rhodes *et al*, 1979; Stryer, 1988; Lam *et al*, 1996; Ortega *et al*, 1999). The inference is that as ATP concentrations may decrease during water deficits (due to decreasing photosynthesis, fig. 3.22, and the continuing maintenance of plant water deficit tolerance adaptations e.g. osmotic adjustment - see section 4.4), the assimilation of ammonia via the GDH pathway may increase.

Indeed GDH activities increased during water deficits in the leaves and roots of 'spiked' *V. faba*. Increasing GDH activities were strongly correlated with increasing water deficits in 'no nitrate' and '1/10 nitrate' supplied 'spiked' *V. faba* (see appendix II a). 10-20% of total ammonia assimilation has previously been attributed to assimilation by GDH in other plant species during water deficits (Rhodes *et al*, 1986), and GDH activities have previously been reported to increase (three-fold) during severe water deficits (Kaur *et al*, 1985).

Increasing GDH activities in 'spiked' *V. faba* may enable increased nitrogen assimilation during severe water deficits, during which slight decreases in NR activities were apparent (figs. 6.2 & 6.3), inferring that potential benefits in terms of maintained nitrogen assimilation may be incurred in *V. faba* when supplied with medium ammonia additions during water deficits.

It has been reported that GDH may operate primarily in the direction of glutamate oxidation, and may thus provide skeletons for the TCA cycle during periods of carbohydrate limitation. Indeed proteolysis and GDH activities reportedly increase with the concurrent release of ammonia and metabolically active amino acids during periods of sucrose depletion in *Daucus carota* cells (Robinson *et al*, 1992), and GDH activities reportedly decrease when sucrose is supplied to such sucrose-deficient cells. However while figs. 6.10 & 6.11 illustrate that significantly greater ammonia

concentrations were recorded in 'spiked' than in 'non-spiked' *V. faba*, and in 'combined nitrogen' supplied than in '1/2 nitrate' supplied *V. faba*, sucrose deficiencies were not exhibited by *V. faba*, and the employed GDH assay (section 6.2.10) quantifies the GDH reaction in the direction of ammonia assimilation. Amino acid accumulation continued until water deficits were severe (20-25% PEG; 45-30% Control Water; figs. 4.5 & 4.6), by which stage protein concentrations had more than halved (figs. 6.12 & 6.13), inferring that maintained nitrogen assimilation may have contributed substrates towards osmotic adjustment. While GS activities were merely maintained during water deficits (figs. 6.6 & 6.7; albeit at greater activities in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and in 'spiked' than in 'non-spiked' *V. faba*), root and leaf GDH activities increased in 'spiked' *V. faba* during water deficits, inferring a 'role' for the GDH mediated assimilation of ammonia in *V. faba* during water deficits.

6.3.2 TOTAL AMMONIA

Figs. 6.10 & 6.11 and anova analyses reveal that total ammonia concentrations were significantly greater in non-nodulated *V. faba* when supplied with medium ammonia additions (i.e. with 'combined nitrogen', as opposed to with 'no nitrate', '1/10 nitrate', or '1/2 nitrate' nutrition); in 'spiked' as opposed to 'non-spiked' *V. faba* (and in nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba*).

Figs 6.10 & 6.11 and anova analyses reveal that total ammonia concentrations were maintained the leaves and roots of non-nodulated *V. faba* during water deficits, and that total ammonia concentrations increased significantly in the leaves of 'spiked' *V. faba* during water

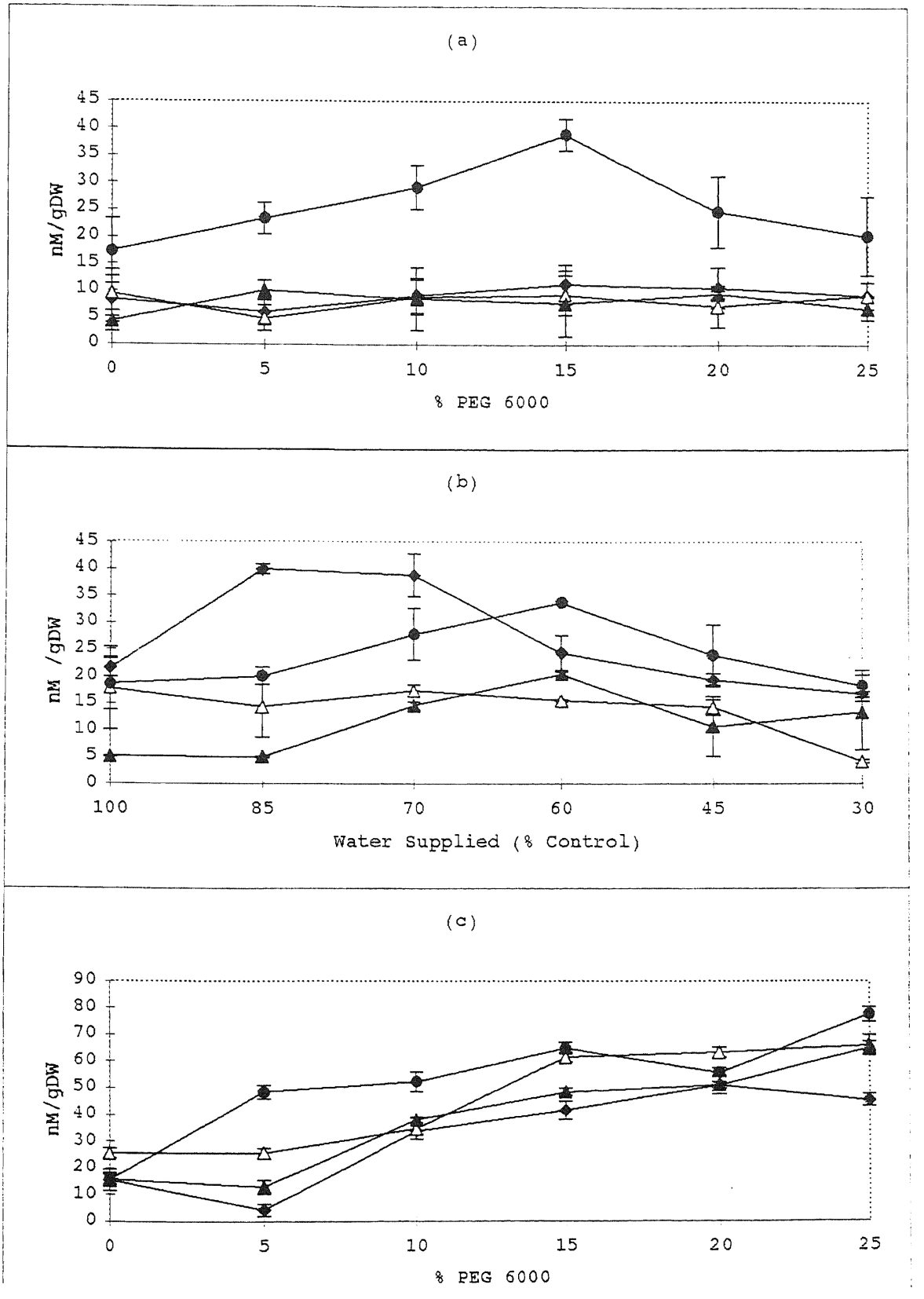


Fig. 6.10 Total ammonia concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

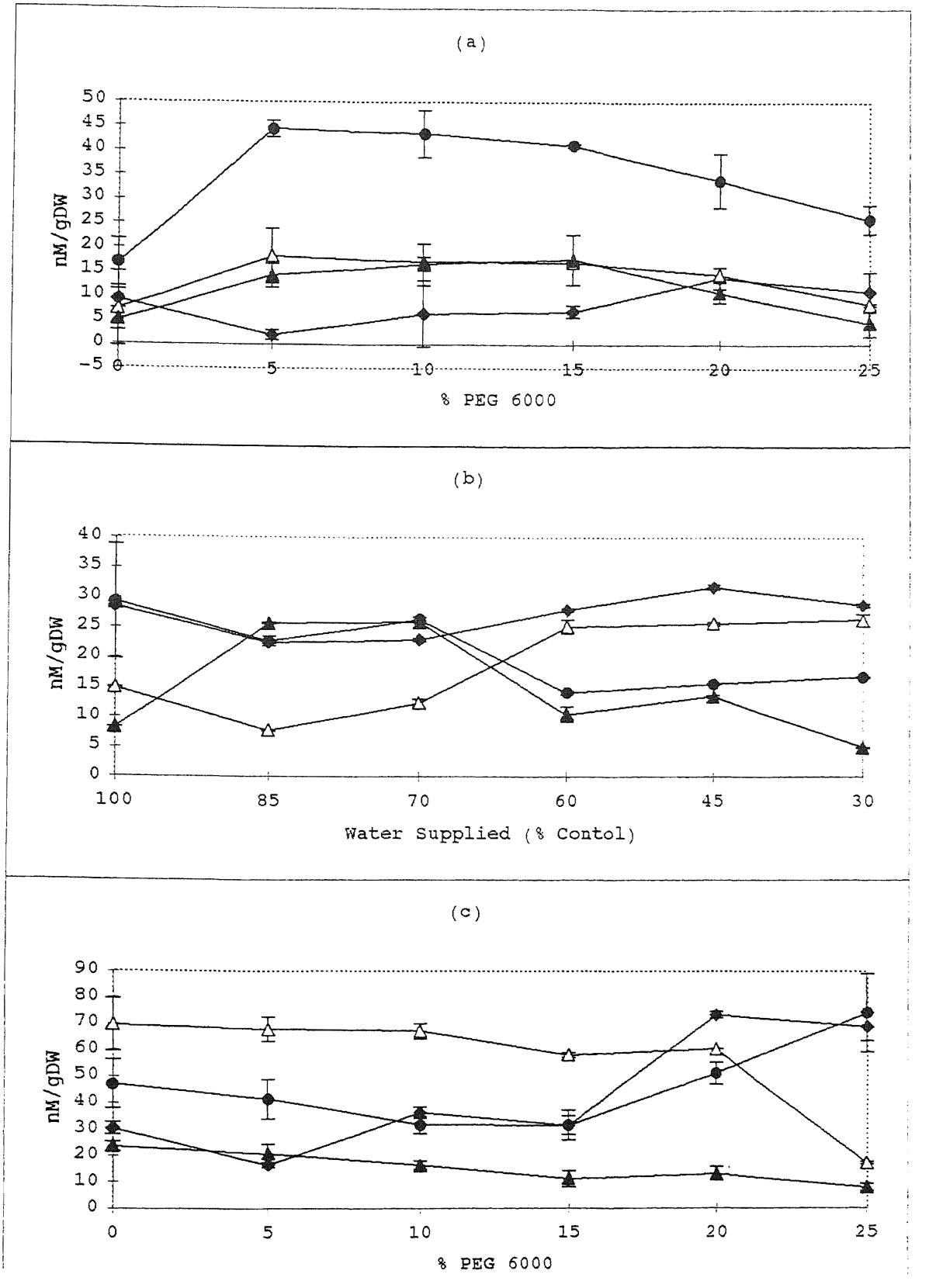


Fig. 6.11 Total ammonia concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

deficits.

It is thus apparent that increasing ammonia concentrations were exhibited in *V. faba* when supplied with increasingly concentrated medium ammonia additions. However ammonia toxicity symptoms (e.g. growth rate restrictions; wilting; leaf expansion and photosynthetic rate decreases; inhibited water uptake; and decreased leaf water potentials; Pill & Lambeth, 1977; Tolley-Henry & Raper, 1986) were not exhibited in *V. faba*. Greater net photosynthesis was maintained during water deficits in *V. faba* when supplied with 'combined nitrogen' as opposed to with nitrate nutrition (and potentially in 'spiked' than in 'non-spiked' *V. faba*, due to significantly increased CLAs; fig. 3.20), and therefore potentially more photosynthates and reductants were available to *V. faba* when supplied with medium ammonia additions, which may have resulted in the maintenance of sufficiently low ammonia concentrations that ammonia toxicity symptoms did not develop.

6.3.3 PROTEIN

Figs. 6.12 & 6.13 and anova analyses reveal that protein concentrations were significantly greater in the leaves and roots of non-nodulated *V. faba* when supplied with 'combined nitrogen' as opposed to with (equimolar) '1/2 nitrate' nutrition. Protein concentrations were exhibited in the following order with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate'. Indeed increased protein concentrations have previously been reported in *Z. mays* when supplied with 'combined nitrogen' as opposed to with nitrate or with ammonia nutrition (Domska, 1974). Furthermore ammonia nutrition has previously been reported to result in the exhibition of 4.3-fold higher soluble plant protein

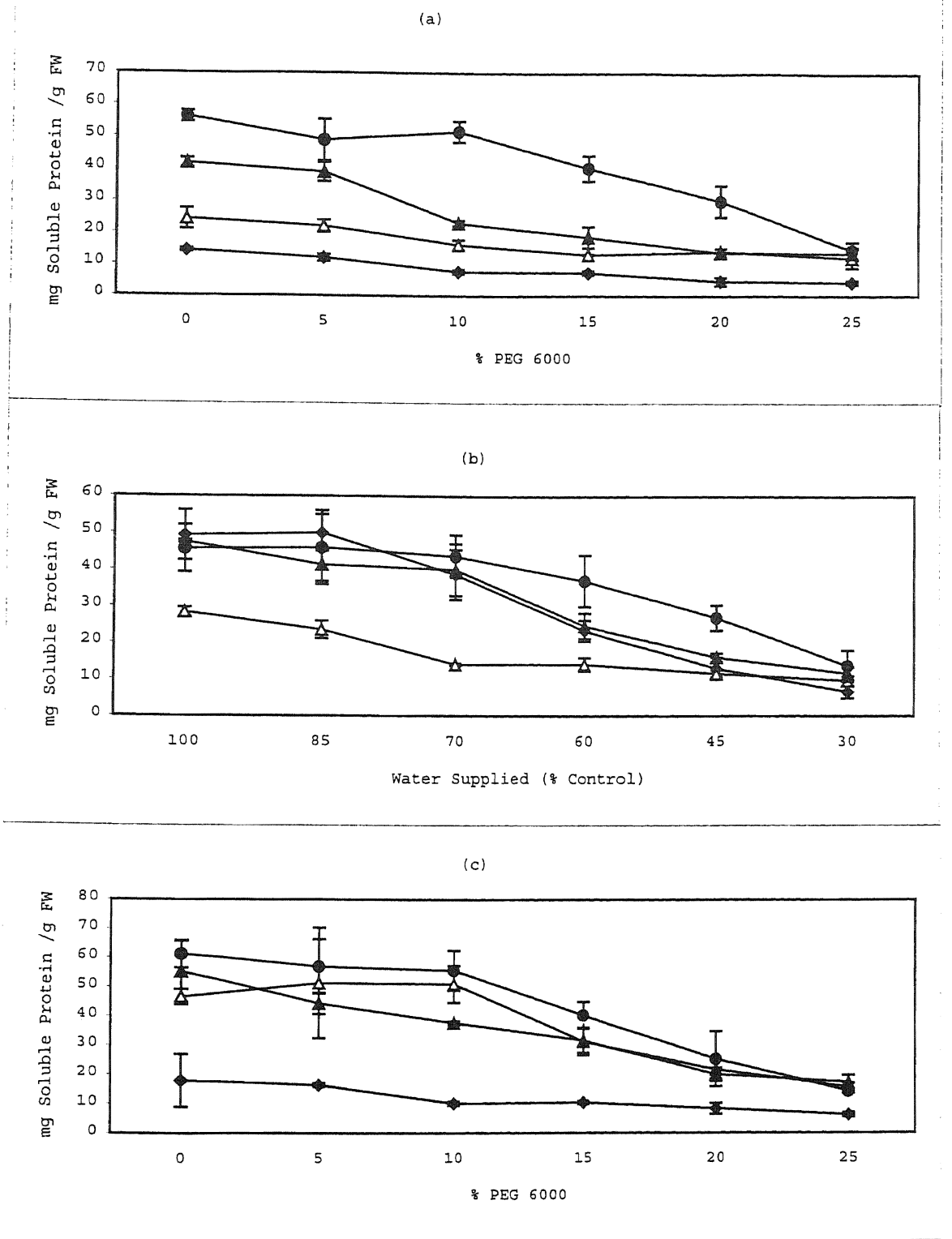


Fig. 6.12 Total soluble protein concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

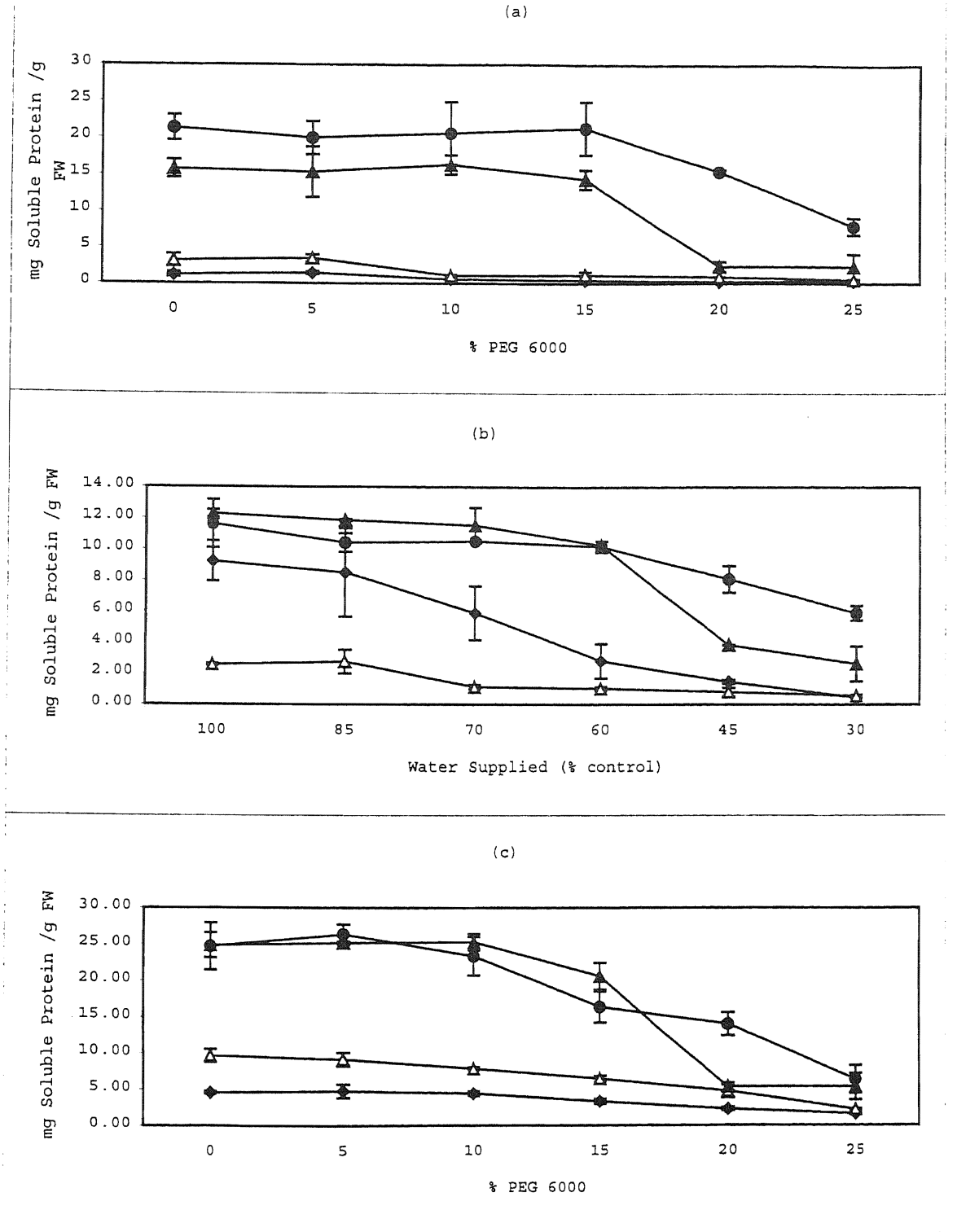


Fig. 6.13 Total soluble protein concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

concentrations than nitrate nutrition in *B. vulgaris* (Raab & Terry, 1994), and in greater nitrogen concentrations per percentage plant dry weight in *Casuarina equisetifolia* (Martinez-Carrasco *et al*, 1998). Greater protein concentrations in *V. faba* when supplied with medium ammonia additions may reflect the greater nitrogen assimilatory enzyme activities which were recorded in *V. faba* when supplied with medium ammonia additions (figs. 6.2; 6.3; 6.6 - 6.9), and the fact that ammonia is toxic (Raven, 1985) and requires rapid assimilation. Accordingly significantly greater protein concentrations were exhibited in the roots of 'spiked' than of 'non-spiked' *V. faba*, roots being the primary site of nitrogen assimilation in *V. faba*. Furthermore nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba* maintained significantly greater leaf and root protein concentrations, perhaps a reflection of the assimilation of fixed nitrogen (table 2.3).

Figs. 6.12 & 6.13 and anova analyses reveal that protein concentrations decreased significantly in the leaves and roots of non-nodulated *V. faba* during water deficits (in agreement with work on other plant species; Taylor *et al*, 1982; Wellburn *et al*, 1996). Decreasing protein concentrations may have contributed towards the production of nitrogenous osmotica in *V. faba* during water deficits (figs. 4.5 - 4.10; tables 6.2 - 6.7; Stewart, 1981). Decreasing protein concentrations may result in part from reduced protein requirements attributable to growth decreases during water deficits (see section 3.4). However earlier research involving nitrogen labelling has demonstrated that only fifty-four per cent of free amino acids and only five to fifteen per cent of proline are produced via proteolysis (Fukutuku & Yamada, 1984). Furthermore amino acid increases preceded protein decreases in *V. faba*, and continued when protein concentrations had decreased significantly (figs. 4.5; 4.6; 6.12;

6.13), indicating that reduced protein synthesis and/or enhanced protein degradation may not have solely accounted for nitrogenous osmotica production, inferring a 'role' for maintained primary nitrogen assimilation in nitrogenous osmotica production during water deficits in this species (figs. 6.2; 6.3; 6.6; 6.7; 6.8; 6.9).

6.3.4 TRANSAMINATION

Fig. 6.1 highlights four aminotransferases; (1) alanine aminotransferase; (2) aspartate aminotransferase; (3), asparagine synthetase; and (4) homoserine dehydrogenase; quantification of the activities of which provide information regarding which biochemical pathways are predominantly utilised in *V. faba* when subjected to the pre-specified nitrogen and water deficit regimes (tables 2.3 & 2.6; fig. 2.2).

6.3.4.1 ALANINE AMINOTRANSFERASE

glutamate + pyruvate ⟶ α-ketogluterate + alanine

glutamate + pyruvate ⟶ alanine + 2-oxogluterate

(Rhodes *et al*, 1986)

Figs. 6.14 & 6.15 and anova analyses reveal that significantly greater alanine aminotransferase activities were exhibited in the leaves and roots of non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. Furthermore nodulated 'no nitrate' supplied *V. faba* exhibited significantly greater leaf and root alanine aminotransferase activities than non-nodulated 'no nitrate' supplied *V. faba*; and 'spiked' *V. faba* exhibited significantly greater leaf and root activities than 'non-spiked' *V. faba*. This is consistent with the significantly greater alanine concentrations which were exhibited in the

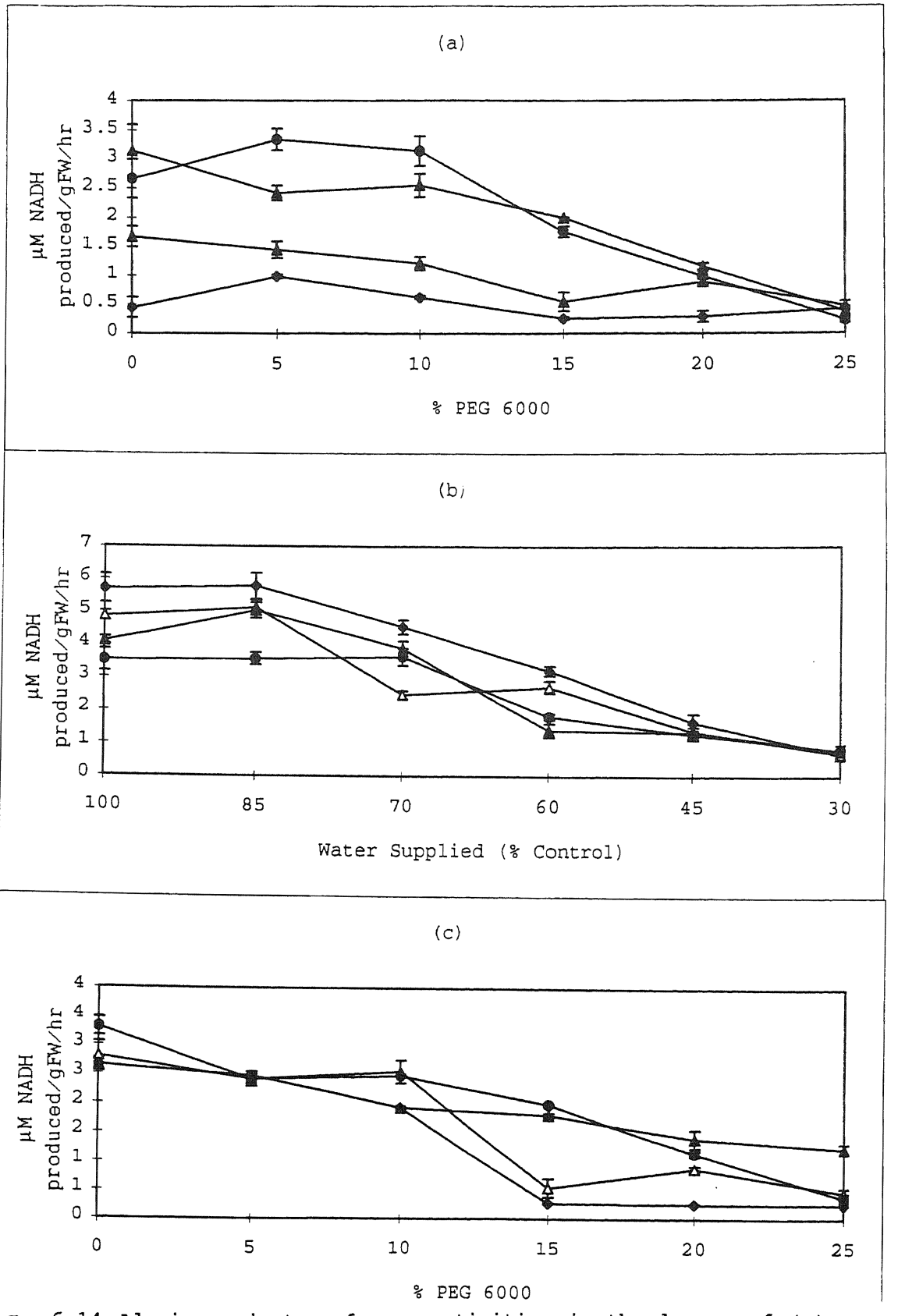


Fig. 6.14 Alanine aminotransferase activities in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

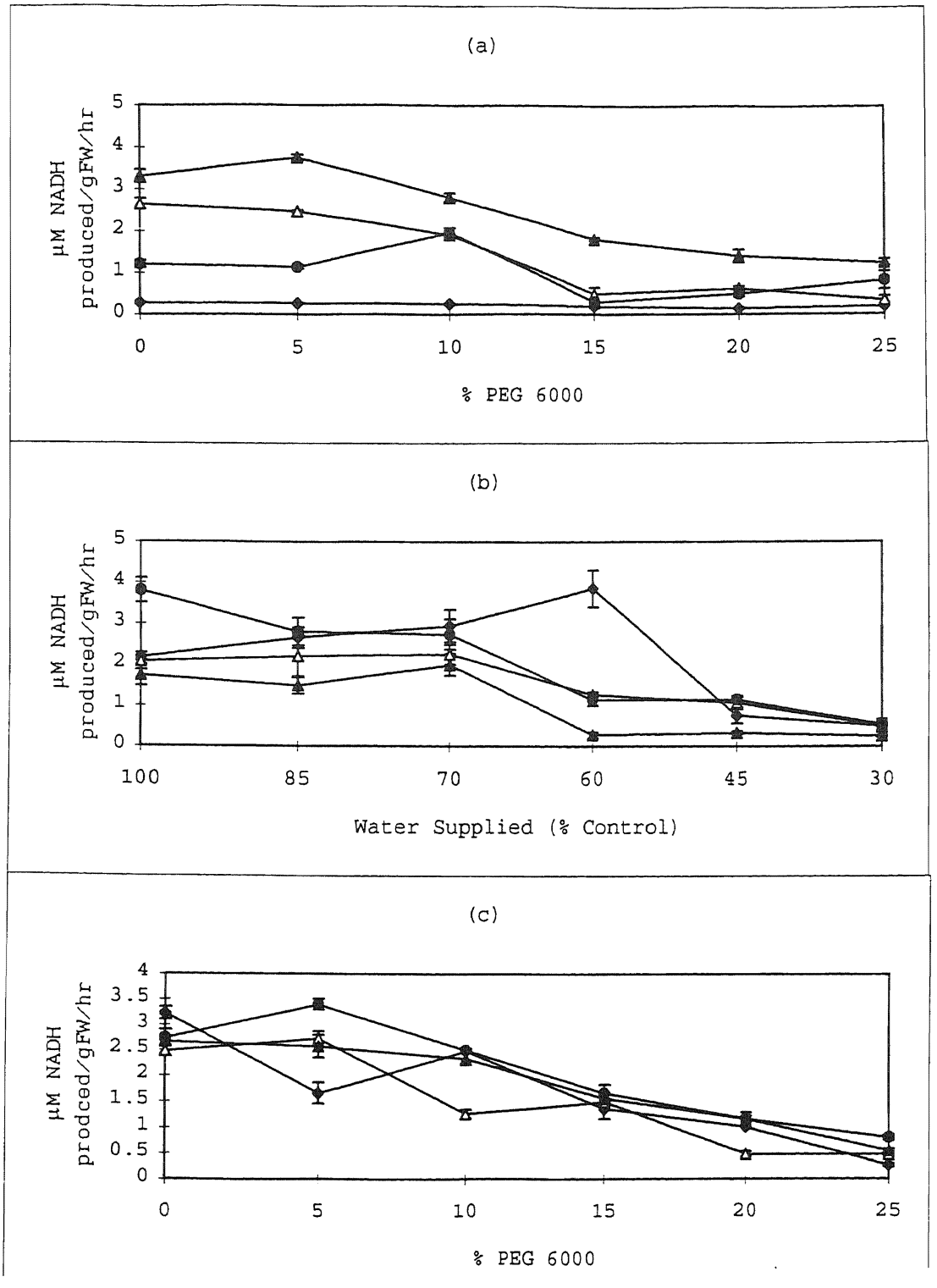


Fig. 6.15 Alanine aminotransferase activities in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

leaves and roots of *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (tables 6.2; 6.3; 6.6 & 6.7).

Alanine aminotransferase reportedly lacks *in vitro* amino acid substrate specificity (Bryan, 1976), however organic acids are compartmentalized (Oaks & Bidwell, 1970), inferring that pyruvate availability may regulate alanine aminotransferase activities. Fig. 3.22 illustrated increased net photosynthesis in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, inferring an increased capacity for pyruvate production in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition. Furthermore it has been highlighted that pyruvate and pyruvate kinase concentrations may increase in other plant species when supplied with increasingly concentrated medium nitrogen nutrition (Platt *et al*, 1977; Champigny & Foyer 1992), further inferring an increased potential for pyruvate utilization and therefore for alanine aminotransferase activities in plants when supplied with increasingly concentrated medium nitrogen nutrition. While significantly greater pyruvate concentrations were not exhibited in *V. faba* when supplied with increasingly concentrated nitrogen nutrition (tables 5.1 - 5.6), this may reflect an increased incorporation of the carbon skeletons of pyruvate into amino acids in *V. faba* when supplied with concentrated medium nitrogen nutrition (as supported by the data illustrated in figs. 4.5 & 4.6), as opposed to increasing pyruvate accumulation.

Figs. 6.14 & 6.15 and anova analyses reveal that alanine aminotransferase activities decreased significantly in the leaves and roots of *V. faba* during water deficits. This contrasts with the increasing alanine concentrations exhibited in the leaves and roots of non-nodulated and nodulated *V. faba* during increasing water deficits (tables 6.2 - 6.7).

However the actual concentrations of alanine which were exhibited accounted for very small fractions of the overall amino/imino acid pool during water deficits. Down-regulation of alanine aminotransferase may have been a regulatory response, as alanine accumulation may reportedly inhibit GS activities and hence ammonia assimilation (Miflin *et al,* 1980), and maintained nitrogen assimilation is required for the production of nitrogenous osmotica during water deficits (and was exhibited in *V. faba* during water deficits; figs. 6.2; 6.3; 6.7 - 6.9). Furthermore alanine accumulation may reportedly result in the exhibition of increased stomatal closure in *V. faba* and thereby result in decreased carbon acquisition (Sharma & Rai, 1989), and photoassimilates and reductants are also required for the maintenance of water deficit tolerance adaptations and nitrogen assimilation. It has previously been reported that alanine decreases may correspond with aspartate increases in some plant species (Bryan, 1976). Indeed alanine concentrations decreased as asparagine concentrations increased in *V. faba* (tables 6.2 - 6.7; see also fig. 6.1), and the data infers that glutamate may have been primarily converted to asparagine via aspartate during water deficits (fig. 6.1), particularly in *V. faba* which were supplied with concentrated medium ammonia nutrition.

6.3.4.2 ASPARTATE AMINOTRANSFERASE

Glutamate + Oxaloacetate ⟶ ketogluterate + aspartate

Figs. 6.16 & 6.17 and anova analyses reveal that aspartate aminotransferase activities were significantly greater in the leaves of non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; and that significantly greater aspartate aminotransferase activities were

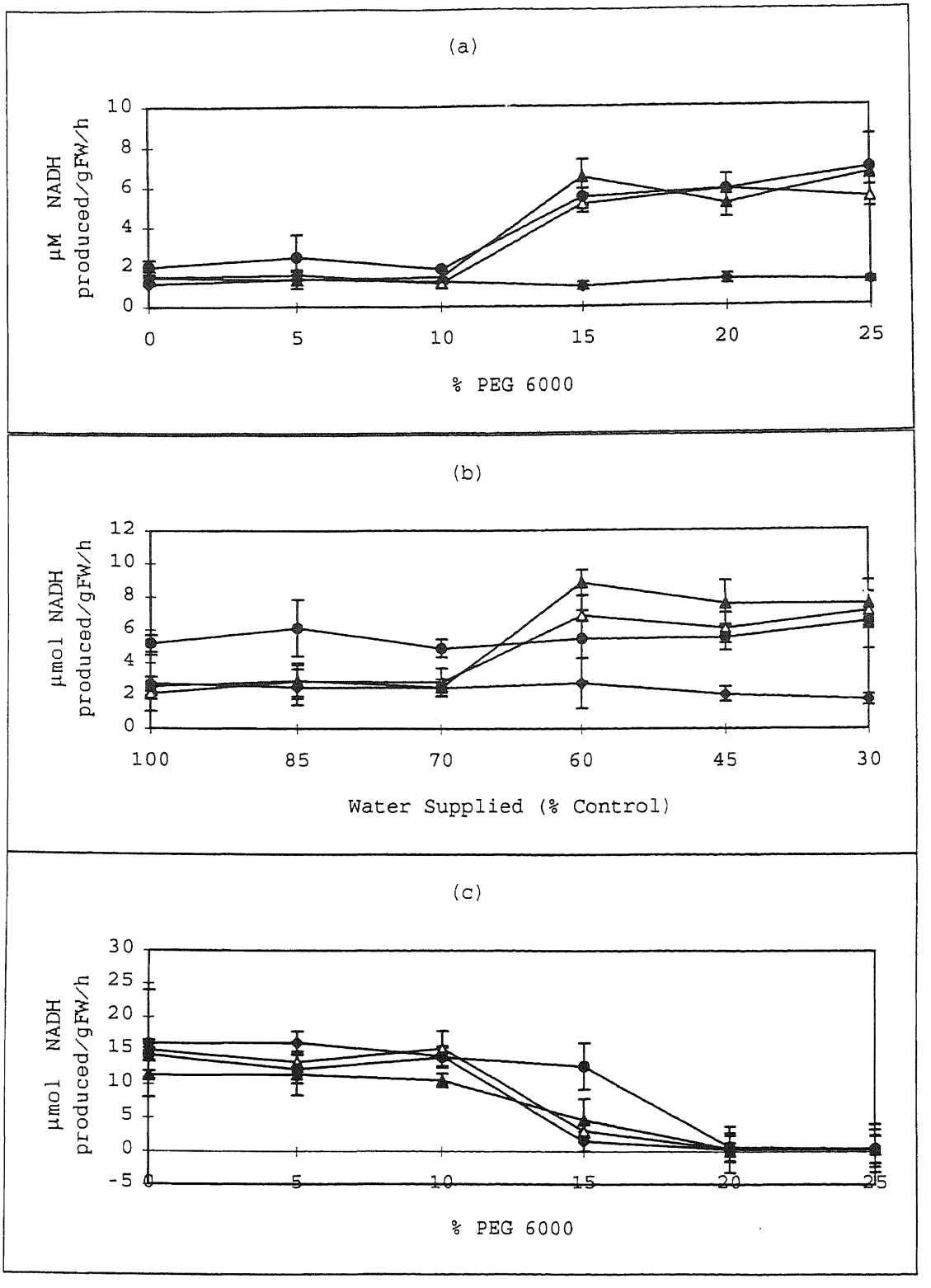


Fig. 6.16 Aspartate aminotransferase activities in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

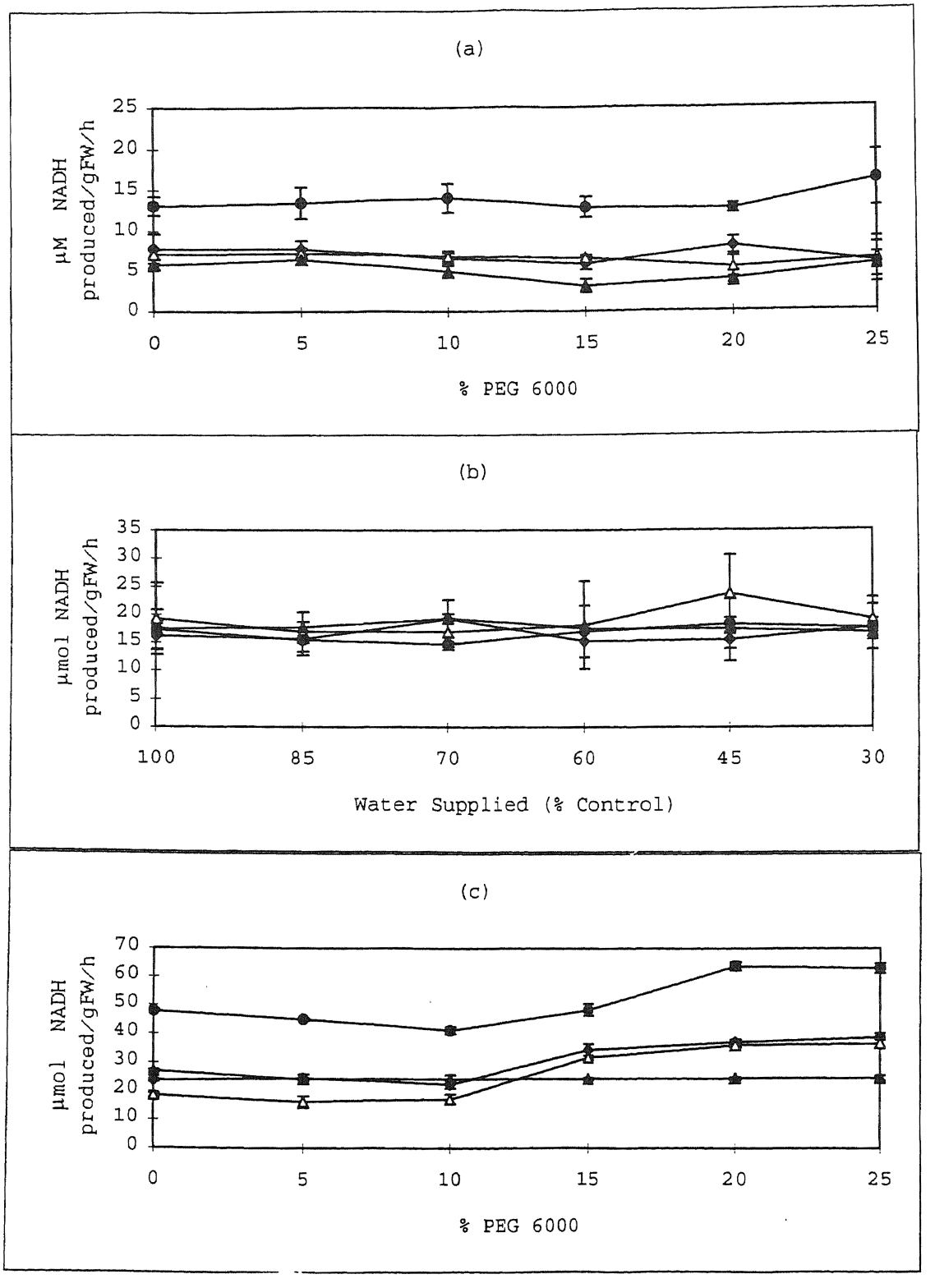


Fig. 6.17 Aspartate aminotransferase activities in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

exhibited in the roots (the site of primary ammonia assimilation) of non-nodulated *V. faba* when supplied with 'combined nitrogen', as opposed to with nitrate or with 'no nitrate' nutrition. Furthermore aspartate aminotransferase activities were significantly greater in the leaves and roots of nodulated as opposed to non-nodulated 'no nitrate' supplied *V. faba*; and in 'spiked' as opposed to 'non-spiked' *V. faba*. Aspartate has a high N:C ratio, and is a substrate in the production of asparagine, which has an even greater N:C ratio (carbon economy may be important during the water deficit mediated decreases in net photosynthesis; fig. 3.22). Aspartate accumulated to the greatest concentrations in 'spiked' (as opposed to 'non-spiked') *V. faba*, consistent with an ammonia de-toxification 'role' (Bryan, 1976).

Aspartate aminotransferase activities may reportedly decrease during water deficits (Kaur *et al*, 1985). However figs. 6.16 & 6.17 and anova analyses reveal that aspartate dehydrogenase activities increased significantly during water deficits in all but the roots of nodulated *V. faba*, where intrinsic activities were already high, and increases may not have been necessary to ensure the assimilation of glutamate into aspartate for the production of nitrogenous osmotica. Increased aspartate aminotransferase activities corresponded with aspartate (and asparagine) increases during water deficits (tables 6.2 - 6.7). However while aspartate concentrations increased in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition and during water deficits, it is apparent that these increases were not consistent within every nitrogen regime (tables 6.2 - 6.7; appendix II b). Aspartate increases were most apparent when ammonia was included in the medium. Furthermore the concentrations of aspartate accumulated were less than those of asparagine, inferring that the increased production of aspartate in *V. faba* both when supplied with increasingly concentrated nitrogen nutrition

and during water deficits may have represented a 'step' towards asparagine accumulation. This hypothesis is reinforced by the observation that asparagine accumulation occurred consistently in *V. faba*, and to greater concentrations than aspartate accumulation, and particularly in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and with medium ammonia additions) and during water deficits (tables 6.2 - 6.7).

6.3.4.3 ASPARAGINE SYNTHETASE

The glutamine dependant asparagine synthetase enzyme is generally accepted as the major plant route for asparagine synthesis (Lam *et al*, 1996; see fig. 6.1).

Glutamine + aspartate (+ ammonia) ⟶ glutamate + asparagine

Figs. 6.18 & 6.19 and anova analyses reveal that asparagine synthetase activities were significantly greater in the leaves and roots of non-nodulated *V. faba* when supplied with 'combined nitrogen' as opposed to with (equimolar) nitrate nutrition. Furthermore asparagine synthetase activities were significantly greater in the leaves and roots of nodulated as opposed to non-nodulated *V. faba*; and in the leaves and roots of 'spiked' as opposed to of 'non spiked' *V. faba*, throughout water deficits. Indeed fig. 6.1 illustrates that asparagine synthesis requires aspartate and glutamine (the synthesis of which involves direct ammonia assimilation), or potentially ammonia as substrates, and that asparagine synthesis therefore represents a useful ammonia de-toxification process within plants, particularly when supplied with concentrated nitrogen (and particularly with ammonia) nutrition.

Asparagine synthetase activities have previously been reported to decrease in *Vigna radiata* nodules during water deficits (Kaur *et al*, 1985); however this

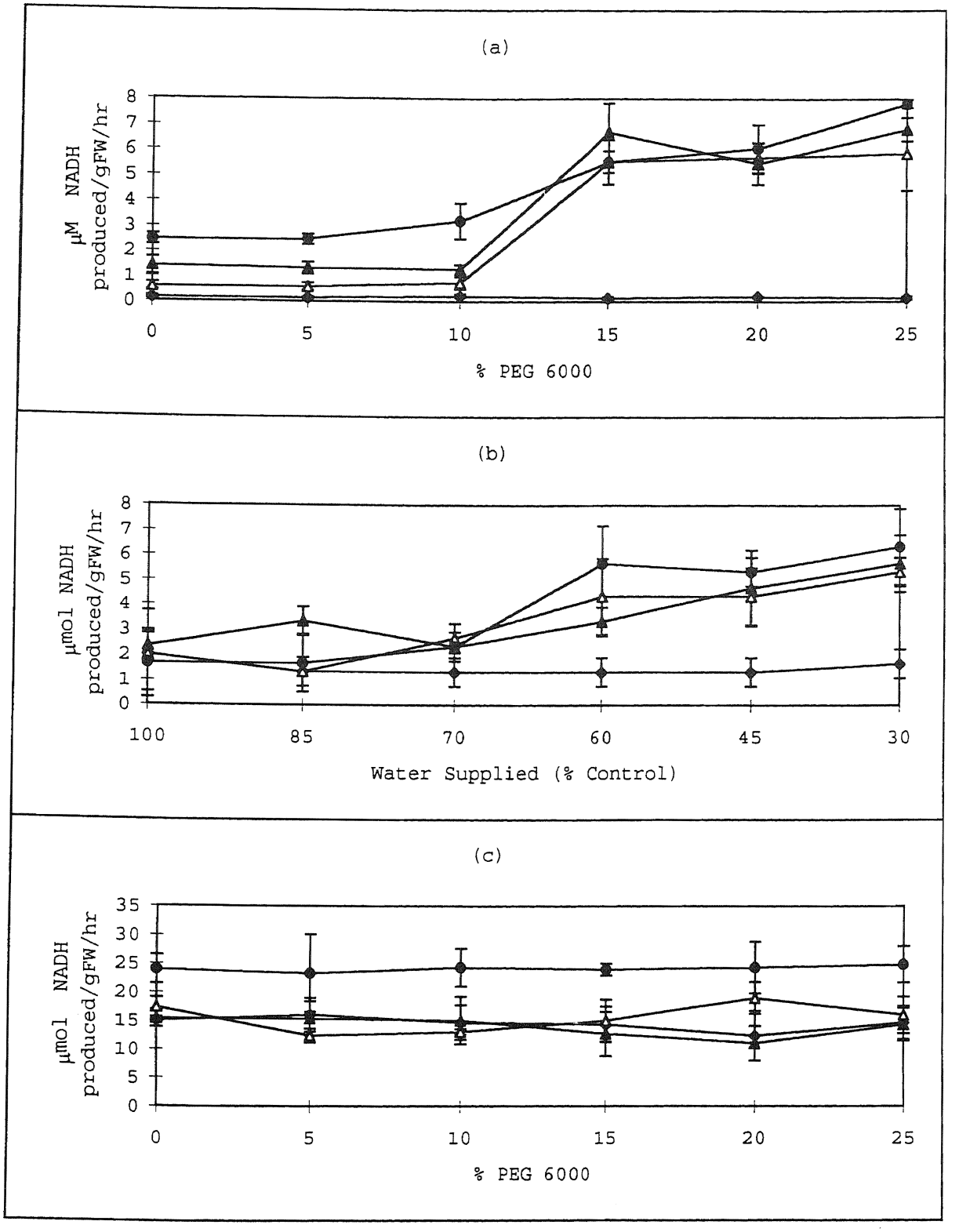


Fig. 6.18 Asparagine synthetase activities in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

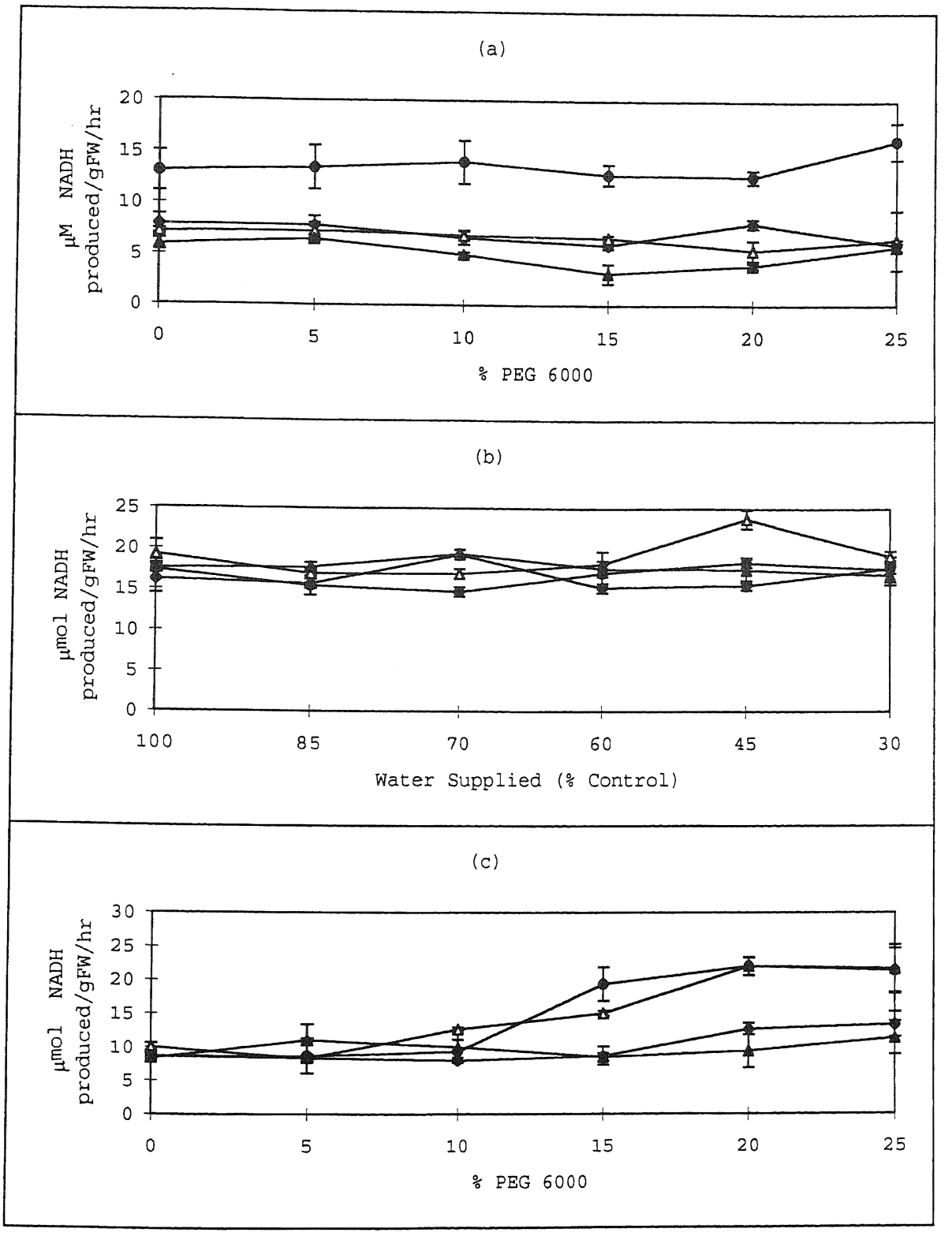


Fig. 6.19 Asparagine synthetase activities in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

is unexpected in the leaves and roots of *V. faba* which exhibited increased asparagine concentrations during water deficits (tables 6.2 - 6.7), particularly when supplied with medium ammonia additions. Accordingly figs. 6.18 & 6.19 illustrate increasing asparagine synthetase activities during water deficits, which were significant in all but the roots of nodulated *V. faba* and the leaves of 'spiked' *V. faba*, where high intrinsic asparagine synthetase activities may have adequately catalysed the assimilation of ammonia into asparagine throughout water deficits.

6.3.4.4 HOMOSERINE DEHYDROGENASE (HDH)

HDH is a regulatory enzyme (partly located in the chloroplasts) which is associated with the synthesis of several essential amino acids from aspartic acid (Bryan, 1976; see also fig. 6.1). HDH is sensitive to feedback inhibition by L-threonine in young plant tissue, whereas HDH within the older tissues of most plant species exhibits reduced inhibition (Bryan *et al*, 1979). That the feedback inhibition of HDH is not desensitized during growth in *V. faba* (Bryan *et al*, 1979) may account for the observation that when threonine concentrations did increase (i.e. in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; and in the roots of 'spiked' as opposed to 'non-spiked' *V. faba* (indicative of increasing nitrogen availabilities); and during water deficits (tables 6.2 - 6.7)), the actual concentrations of threonine which accumulated were relatively small, indicative of feedback inhibition of HDH.

Figs. 6.20 & 6.21 and anova analyses reveal that HDH decreased significantly during water deficits in the leaves and roots of nodulated; non-nodulated; and 'spiked' *V. faba*. It is apparent that homoserine;

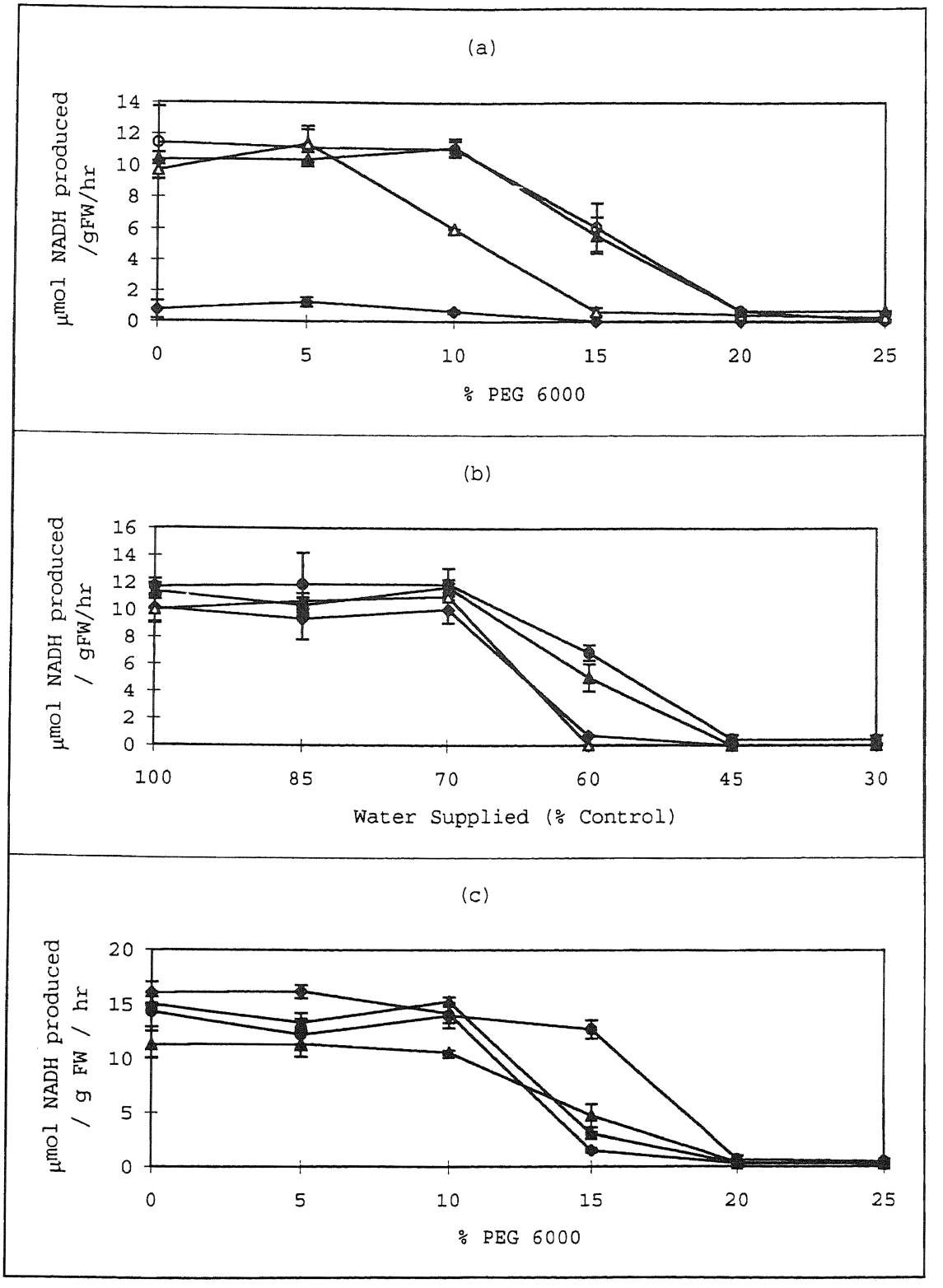


Fig. 6.20 Homoserine dehydrogenase activities in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

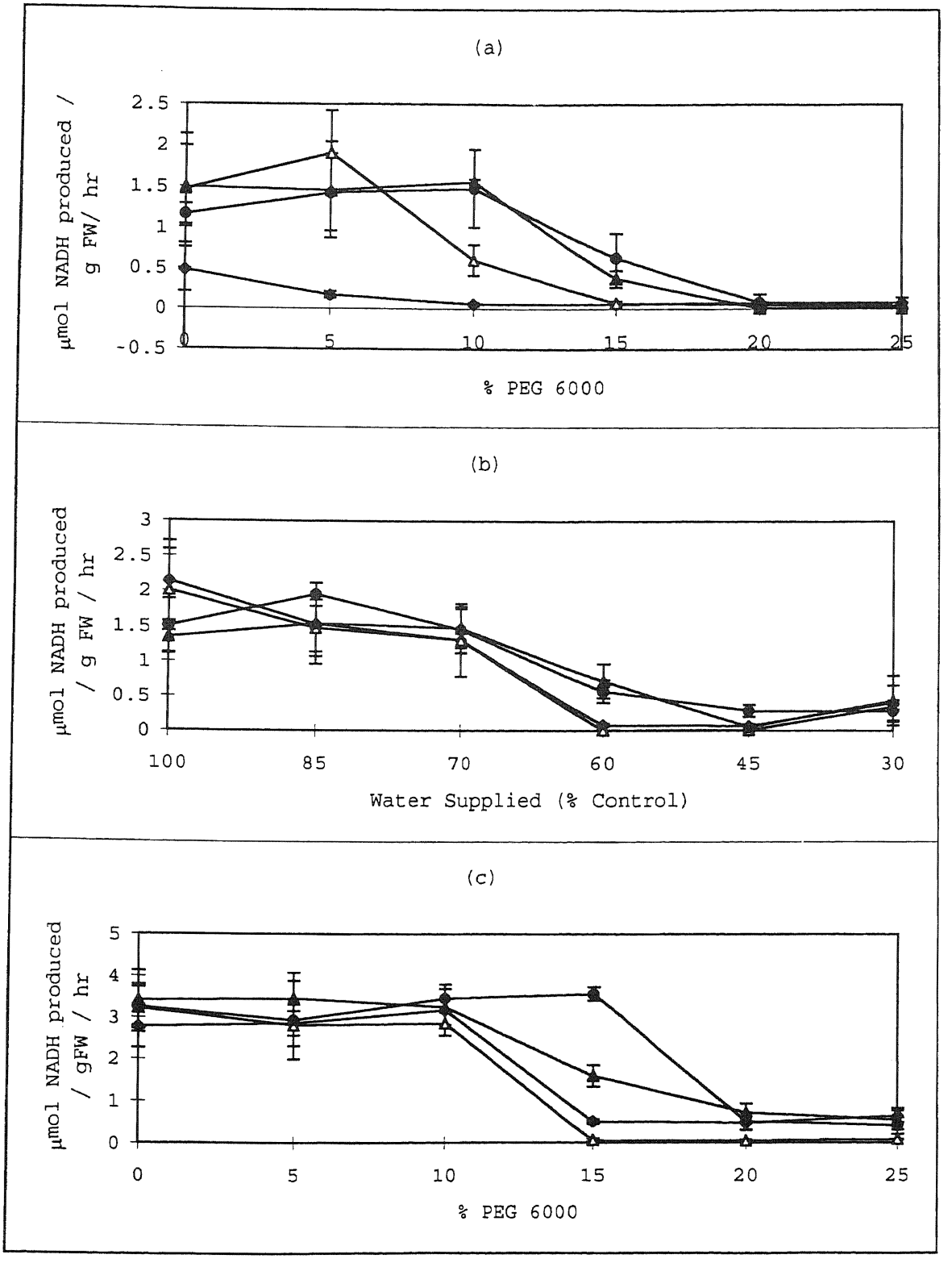


Fig. 6.21 Homoserine dehydrogenase activities in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

threonine; isoleucine; leucine and valine syntheses decreased, and asparagine synthesis increased during water deficits. The associated benefits of asparagine accumulation during water deficits are discussed in section 6.3.5, pgs. 233 - 235.

6.3.5 INDIVIDUAL AMINO ACIDS

Tables 6.2 & 6.3 and anova analyses reveal that significantly greater concentrations of proline; glutamate; glutamine; and asparagine were exhibited in the leaves and roots of non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition.

Glutamate; glutamine; aspartate; asparagine; and alanine accumulated to significantly greater concentrations in the leaves and roots of 'spiked' than of 'non-spiked' *V. faba*; and threonine accumulated to significantly greater concentrations in the roots only of 'spiked' than of 'non-spiked' *V. faba* (tables 6.6 & 6.7). Greater total amino acid concentrations have previously been recorded in *G. max* when supplied with ammonia than with nitrate nutrition (Chaillou et al, 1991). A potential ammonia de-toxification 'role' is inferred for the increased production of these amino acids, as the production of the amides of glutamate (and therefore of aspartate) involves the direct assimilation of ammonia into organic compounds, and the widespread availability of aminotransferases in plant cells reportedly enables ammonia to move rapidly into many products (Bryan, 1976; see also fig. 6.1). Furthermore fig. 6.1 illustrates that ammonia may be directly utilised in the production of asparagine. Although asparagine synthetase has a low km for glutamine and a high km for ammonia (Scott & Farnden, 1976; Miflin & Lea, 1977), asparagine production may be effective in the de-toxification of increasing ammonia concentrations. Greater amino

Table 6.2 Amino Acid Concentrations (mM gDW^{-1}) in the Leaves of non-nodulated *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
PROLINE	0.57	0.30	0.27	0.54	1.28	2.67
ALANINE	0.46	0.41	0.36	0.54	0.38	0.34
THREONINE	0.93	1.04	1.34	1.29	1.32	1.10
ASPARTATE	2.13	1.69	1.83	1.90	2.07	1.93
ASPARAGINE	5.03	4.90	4.55	4.70	4.73	4.73
GLUTAMINE	1.80	1.60	1.90	1.50	1.50	1.80
GLUTAMATE	0.60	0.90	0.70	0.30	0.90	0.50
TOTAL	11.52	10.84	10.95	10.77	12.18	13.07
(ii) '1/10 NITRATE'						
PROLINE	0.36	0.49	2.89	7.88	14.33	14.56
ALANINE	0.85	1.69	2.54	2.41	2.52	2.84
THREONINE	0.70	2.36	6.32	6.46	5.76	6.15
ASPARTATE	3.33	2.87	2.87	2.77	3.40	2.77
ASPARAGINE	11.06	12.44	12.21	24.85	26.13	23.45
GLUTAMINE	2.50	3.40	2.60	2.50	3.60	3.00
GLUTAMATE	1.50	1.90	1.40	1.30	1.70	2.00
TOTAL	20.30	25.15	30.83	48.17	57.44	54.77
(iii) '1/2 NITRATE'						
PROLINE	0.31	0.39	4.59	15.78	27.33	39.78
ALANINE	0.48	0.53	0.53	2.74	2.84	2.94
THREONINE	0.48	3.35	4.31	6.48	8.04	8.16
ASPARTATE	11.97	12.10	10.87	11.33	9.67	10.00
ASPARAGINE	12.37	11.84	12.39	27.05	26.33	26.09
GLUTAMINE	8.70	7.50	6.20	8.10	8.60	11.00
GLUTAMATE	6.80	6.90	6.60	5.40	5.30	6.90
TOTAL	40.81	42.61	45.49	76.88	88.11	104.87
(iv) 'COMBINED NITROGEN'						
PROLINE	0.78	0.84	3.42	28.00	39.67	47.22
ALANINE	0.54	1.47	1.57	3.52	2.99	3.24
THREONINE	0.04	1.75	2.51	6.06	8.09	8.25
ASPARTATE	10.37	11.10	11.47	11.87	12.43	12.20
ASPARAGINE	20.33	20.00	19.67	24.67	24.93	25.60
GLUTAMINE	7.90	7.20	8.90	9.10	6.80	10.10
GLUTAMATE	7.30	6.50	5.50	7.60	5.90	5.89
TOTAL	47.59	48.86	53.04	90.82	100.81	112.50

The concentrations of the amino acids which are listed in **bold** were significantly affected by increasing water deficits. The concentrations of the amino acids which are listed underlined were significantly affected by the supplied nitrogen source (Anova $\alpha = 0.05$).

Table 6.3 Amino Acid Concentrations (mM gDW^{-1}) in the Roots of non-nodulated *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
PROLINE	0.37	0.56	0.41	0.56	1.41	0.62
ALANINE	0.67	0.23	0.49	0.41	1.47	1.26
THREONINE	0.60	0.50	0.54	0.60	0.89	0.49
ASPARTATE	0.90	1.10	1.20	1.20	1.07	1.17
ASPARAGINE	1.16	1.55	1.43	2.13	1.73	1.40
GLUTAMINE	0.50	0.30	0.60	0.40	0.80	0.20
GLUTAMATE	0.20	0.30	0.15	0.26	0.25	0.19
TOTAL	4.40	4.54	4.82	5.56	7.62	5.34
(ii) '1/10 NITRATE'						
PROLINE	0.50	0.62	0.72	2.50	7.21	3.50
ALANINE	0.35	0.29	0.36	1.47	1.56	1.24
THREONINE	0.59	0.69	0.54	4.45	4.27	4.35
ASPARTATE	2.50	3.90	2.20	3.60	3.90	3.10
ASPARAGINE	6.64	5.34	6.29	12.07	12.18	11.74
GLUTAMINE	1.30	1.70	1.50	1.20	1.30	1.50
GLUTAMATE	0.80	0.60	0.90	0.40	0.50	0.60
TOTAL	12.68	13.14	12.51	25.69	30.92	26.03
(iii) '1/2 NITRATE'						
PROLINE	0.33	0.45	2.58	4.43	8.40	7.77
ALANINE	0.48	0.52	0.51	1.07	1.27	1.27
THREONINE	0.43	0.47	0.66	4.25	4.08	4.72
ASPARTATE	4.25	4.40	5.50	4.90	4.30	5.90
ASPARAGINE	8.20	9.62	9.99	12.27	13.90	13.83
GLUTAMINE	5.60	4.20	4.30	4.90	6.40	5.80
GLUTAMATE	2.70	3.10	3.00	2.60	2.90	2.50
TOTAL	21.99	22.76	26.54	34.42	41.28	41.79
(iv) 'COMBINED NITROGEN'						
PROLINE	0.49	0.81	1.69	4.99	13.13	17.21
ALANINE	0.73	0.82	0.72	2.12	1.83	1.47
THREONINE	0.48	0.60	0.50	3.43	4.77	4.60
ASPARTATE	4.67	5.67	5.33	4.33	5.33	4.67
ASPARAGINE	12.00	14.00	13.50	16.00	18.00	19.00
GLUTAMINE	5.90	5.80	5.70	6.90	7.70	5.60
GLUTAMATE	3.10	3.60	3.50	3.20	3.40	3.00
TOTAL	27.37	31.30	30.94	40.97	54.16	55.55

The concentrations of the amino acids which are listed in **bold** were significantly affected by increasing water deficits. The concentrations of the amino acids which are listed underlined were significantly affected by the supplied nitrogen source (Anova $\alpha = 0.05$).

Table 6.4 Amino Acid Concentrations (mM gDW^{-1}) in the Leaves of nodulated *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
PROLINE	0.67	0.94	0.68	1.94	11.23	18.41
ALANINE	0.63	0.46	0.68	2.63	2.90	3.08
THREONINE	0.54	0.74	0.47	4.11	3.91	4.39
ASPARTATE	4.67	3.67	4.50	4.20	5.50	5.90
ASPARAGINE	14.40	12.10	12.60	24.70	28.90	26.40
GLUTAMINE	15.90	12.50	16.50	16.40	17.60	19.20
GLUTAMATE	3.90	4.20	4.40	5.70	6.10	5.80
TOTAL	10.71	34.58	39.83	59.68	76.14	83.18
(ii) '1/10 NITRATE'						
PROLINE	0.35	0.55	1.01	7.13	8.41	6.00
ALANINE	0.64	0.69	2.57	2.63	2.90	3.08
THREONINE	0.49	0.51	0.45	4.22	4.40	4.88
ASPARTATE	4.20	5.10	4.90	4.60	5.10	5.00
ASPARAGINE	11.06	12.44	12.21	24.85	26.13	23.45
GLUTAMINE	15.10	12.30	14.20	12.90	14.70	14.20
GLUTAMATE	2.40	3.80	3.70	4.10	3.80	2.90
TOTAL	34.24	35.39	39.01	60.55	65.11	58.96
(iii) '1/2 NITRATE'						
PROLINE	0.98	0.94	0.43	8.67	12.77	28.14
ALANINE	0.62	0.53	0.70	3.01	3.04	3.45
THREONINE	0.61	0.46	0.62	3.95	3.28	5.12
ASPARTATE	4.90	4.30	4.20	5.70	4.90	5.80
ASPARAGINE	12.37	11.84	12.39	27.05	26.33	26.09
GLUTAMINE	18.00	19.30	16.90	18.00	18.40	16.80
GLUTAMATE	5.80	6.10	5.40	5.00	5.80	4.90
TOTAL	43.28	43.47	40.64	71.38	74.52	90.30
(iv) 'COMBINED NITROGEN'						
PROLINE	0.85	1.01	0.64	14.90	22.14	25.71
ALANINE	0.73	1.43	1.53	3.42	2.96	3.47
THREONINE	0.35	0.60	0.65	3.33	4.97	4.87
ASPARTATE	4.80	4.70	5.30	5.50	4.90	4.30
ASPARAGINE	10.33	20.00	19.67	24.67	24.93	25.60
GLUTAMINE	12.50	17.20	15.40	16.30	17.80	15.10
GLUTAMATE	6.50	6.30	5.80	5.90	4.70	6.10
TOTAL	36.06	51.24	48.99	74.98	82.40	85.15

The concentrations of the amino acids which are listed in **bold** were significantly affected by increasing water deficits. The concentrations of the amino acids which are listed underlined were significantly affected by the supplied nitrogen source (Anova $\alpha = 0.05$).

Table 6.5 Amino Acid Concentrations (mM gDW^{-1}) in the Roots of nodulated *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
PROLINE	0.55	0.62	0.60	1.11	8.47	7.9143
ALANINE	0.65	0.69	0.67	1.49	1.56	1.42
THREONINE	0.55	0.57	1.40	4.10	4.37	5.07
ASPARTATE	2.60	2.50	2.40	3.10	3.60	2.4
ASPARAGINE	6.40	7.40	6.10	18.50	18.90	17.2
GLUTAMINE	8.70	9.10	6.70	9.60	7.20	7.6
GLUTAMATE	2.60	2.40	2.80	2.10	2.10	2.8
TOTAL	22.05	23.28	20.67	40.00	46.20	44.4
(ii) '1/10 NITRATE'						
PROLINE	0.62	0.64	0.68	0.82	3.97	3.04
ALANINE	0.37	0.50	0.52	0.89	0.90	0.91
THREONINE	0.60	0.93	1.27	4.10	4.80	4.7
ASPARTATE	3.20	3.50	2.90	2.80	2.60	2.9
ASPARAGINE	6.40	6.50	5.60	10.40	13.20	12.6
GLUTAMINE	3.60	3.90	3.80	2.70	2.90	3
GLUTAMATE	1.70	1.60	1.90	2.00	1.50	2.1
TOTAL	16.49	17.57	16.67	23.71	29.87	29.25
(iii) '1/2 NITRATE'						
PROLINE	0.63	0.61	0.76	3.09	6.49	5.79
ALANINE	0.55	0.42	0.55	1.27	1.31	1.53
THREONINE	0.61	0.52	1.40	4.80	3.50	5.03
ASPARTATE	3.50	2.40	2.90	3.80	3.70	3.5
ASPARAGINE	9.40	10.20	8.60	12.10	14.80	13.6
GLUTAMINE	6.70	8.40	5.80	4.60	5.30	6.1
GLUTAMATE	2.20	2.10	3.80	2.90	2.70	2.5
TOTAL	23.59	24.65	23.81	32.56	37.80	38.05
(iv) 'COMBINED NITROGEN'						
PROLINE	0.72	0.45	0.74	5.33	9.14	14.286
ALANINE	0.87	0.82	1.00	1.59	1.77	1.57
THREONINE	0.60	0.80	1.42	2.93	5.20	5.17
ASPARTATE	3.70	3.40	3.50	2.20	2.50	3.1
ASPARAGINE	14.20	11.90	12.00	17.50	18.40	17.3
GLUTAMINE	8.10	5.70	7.90	7.10	6.70	6.8
GLUTAMATE	3.60	2.90	3.50	3.40	3.00	2.9
TOTAL	31.79	25.97	30.06	40.05	46.71	51.13

The concentrations of the amino acids which are listed in **bold** were significantly affected by increasing water deficits. The concentrations of the amino acids which are listed underlined were significantly affected by the supplied nitrogen source (Anova $\alpha = 0.05$).

Table 6.6 Amino Acid Concentrations (mM gDW^{-1}) in the Leaves of 'Spiked' *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
PROLINE	0.44	0.62	0.42	3.72	12.60	19.80
ALANINE	2.51	2.75	2.57	3.02	2.40	2.12
THREONINE	1.38	2.18	3.07	6.43	6.17	6.39
ASPARTATE	12.05	13.62	12.79	16.00	13.70	12.27
ASPARAGINE	13.72	12.62	12.12	15.00	12.37	13.93
GLUTAMINE	11.40	10.60	11.80	9.70	12.10	11.40
GLUTAMATE	5.80	5.70	6.90	4.80	4.70	4.90
TOTAL	47.30	48.09	49.67	58.67	64.04	70.81
(ii) '1/10 NITRATE'						
PROLINE	0.62	0.34	2.40	10.40	23.80	23.74
ALANINE	2.83	2.67	2.50	2.70	2.27	2.73
THREONINE	1.55	1.60	1.52	7.10	6.39	6.27
ASPARTATE	11.33	11.67	13.67	13.33	12.33	12.67
ASPARAGINE	24.67	26.33	24.67	25.33	23.33	22.67
GLUTAMINE	19.30	17.60	15.40	18.40	16.70	17.10
GLUTAMATE	8.30	8.90	7.50	7.10	8.90	8.70
TOTAL	68.60	69.11	67.66	84.36	93.72	93.88
(iii) '1/2 NITRATE'						
PROLINE	0.54	0.36	4.78	20.60	41.20	43.80
ALANINE	2.67	2.67	2.50	2.53	2.43	2.43
THREONINE	1.43	2.57	2.43	6.30	6.33	6.03
ASPARTATE	12.00	12.00	13.00	13.67	12.00	11.67
ASPARAGINE	22.33	23.67	25.00	24.67	24.00	23.00
GLUTAMINE	17.60	18.40	15.60	17.10	16.30	15.40
GLUTAMATE	9.10	7.80	6.90	8.10	8.20	10.00
TOTAL	65.67	67.47	70.21	92.97	110.46	112.33
(iv) 'COMBINED NITROGEN'						
PROLINE	0.78	0.32	3.28	30.20	41.50	41.90
ALANINE	2.83	2.67	2.57	2.90	2.73	2.70
THREONINE	1.95	2.23	2.40	5.27	6.20	6.40
ASPARTATE	12.67	12.67	11.33	13.33	14.00	11.00
ASPARAGINE	34.33	35.00	34.67	32.33	33.33	33.33
GLUTAMINE	18.90	22.40	17.90	16.90	18.50	17.60
GLUTAMATE	10.70	11.10	9.60	8.70	10.10	8.10
TOTAL	82.16	86.39	81.75	109.63	126.36	121.03

The concentrations of the amino acids which are listed in **bold** were significantly affected by increasing water deficits. The concentrations of the amino acids which are listed underlined were significantly affected by the supplied nitrogen source (Anova $\alpha = 0.05$).

Table 6.7 Amino Acid Concentrations (mM gDW^{-1}) in the Roots of 'Spiked' *V. faba* when supplied with various forms and concentrations of medium nitrogen nutrition during increasing water deficits.

	0% PEG	5% PEG	10% PEG	15% PEG	20% PEG	25% PEG
(i) 'NO NITRATE'						
PROLINE	0.47	0.89	0.39	3.44	6.84	10.66
ALANINE	1.47	1.51	1.41	1.42	1.38	1.45
THREONINE	1.38	1.25	1.03	4.90	4.86	5.45
ASPARTATE	11.00	11.00	10.33	14.33	12.33	12.33
ASPARAGINE	21.00	19.83	20.33	20.00	22.00	23.00
GLUTAMINE	2.50	2.40	3.90	5.80	4.20	4.90
GLUTAMATE	5.00	5.20	3.40	4.80	4.50	3.90
TOTAL	43.82	42.08	40.79	54.69	56.11	61.69
(ii) '1/10 NITRATE'						
PROLINE	0.63	0.45	0.69	6.28	7.62	7.16
ALANINE	1.60	1.57	1.53	1.70	1.67	1.43
THREONINE	1.37	1.47	1.23	4.70	5.63	5.67
ASPARTATE	14.00	13.00	11.67	12.00	12.67	11.33
ASPARAGINE	19.33	18.67	19.00	28.33	26.33	28.00
GLUTAMINE	12.60	9.80	11.20	12.40	14.10	9.80
GLUTAMATE	3.90	4.70	4.60	4.50	6.10	4.80
TOTAL	53.43	49.66	49.92	69.91	74.12	68.19
(iii) '1/2 NITRATE'						
PROLINE	0.43	0.46	0.56	4.82	9.46	16.18
ALANINE	1.50	1.27	1.63	1.70	1.67	1.45
THREONINE	1.40	1.67	1.40	3.40	6.00	5.37
ASPARTATE	7.60	8.90	7.40	8.70	6.40	5.80
ASPARAGINE	15.67	13.20	14.90	19.90	20.67	21.33
GLUTAMINE	11.00	11.80	8.90	12.50	11.20	12.40
GLUTAMATE	4.10	4.70	4.80	4.60	4.00	4.70
TOTAL	41.70	42.00	39.59	55.64	59.40	67.23
(iv) 'COMBINED NITROGEN'						
PROLINE	0.48	0.49	3.66	5.16	7.26	12.10
ALANINE	1.50	1.37	1.80	1.63	1.67	1.47
THREONINE	2.00	2.07	1.93	3.30	5.50	6.23
ASPARTATE	7.60	9.10	8.30	6.90	7.20	6.30
ASPARAGINE	18.33	17.33	18.67	26.67	28.67	25.67
GLUTAMINE	12.60	15.10	10.90	11.60	13.40	10.70
GLUTAMATE	5.20	5.80	4.50	4.90	5.00	4.70
TOTAL	47.71	51.26	49.76	60.16	68.70	67.17

The concentrations of the amino acids which are listed in **bold** were significantly affected by increasing water deficits. The concentrations of the amino acids which are listed underlined were significantly affected by the supplied nitrogen source (Anova $\alpha = 0.05$).

acid concentrations (particularly asparagine, Yoneyama & Kumazawa, 1975) have previously been reported in other plant species when supplied with medium ammonia as opposed to with medium nitrate nutrition (Durzan & Stewart 1967; Harada *et al*, 1968; Lorenz, 1973; Ikeda *et al*, 1974; Chaillou *et al*, 1991). Similarly glutamine; glutamate; aspartate; and asparagine accumulated to significantly greater concentrations in the leaves and roots of nodulated than of non-nodulated 'no nitrate' supplied *V. faba* (tables 6.2 - 6.5), which may reflect fixed nitrogen assimilation (table 2.3) in the former plant group.

Glutamate; glutamine; proline; aspartate; and asparagine are derived utilising α-ketoglutarate from the Kreb's cycle (Bryan, 1976; fig. 6.1), inferring that the production of these amino acids may prevent feed-back inhibition of glycolysis and the Kreb's cycle, particularly as amino acid utilization in protein synthesis may be reduced during water deficits (figs. 6.12 & 6.13). Indeed carbon flow may reportedly be distributed away from sucrose biosynthesis towards amino acid synthesis in plants when supplied with increasingly concentrated nitrate nutrition (McDonald & Davies, 1996). Amino acids are often synthesised in the chloroplasts (Wallsgrove *et al*, 1983), and when plants are supplied with increasingly concentrated medium nitrogen nutrition, relatively smaller fractions of the products of the light reactions and electron transport are consumed in CO_2 assimilation than in amino acid production, and the relative production of amino acids to carbohydrates is increased (McDonald & Davies, 1996). Sucrose phosphate synthase is reportedly de-activated, and phosphoenolpyruvate carboxylase activated in other plant species when supplied with increasingly concentrated medium nitrogen nutrition (Champigny & Foyer, 1992). Increased pyruvate and decreased

phosphoenolpyruvate have been reported in other plant species when supplied with medium ammonia (following labelling experiments) with an activation of pyruvate kinase by ammonia resulting in an increased transfer of photosynthetically incorporated carbon to amino acid synthesis at the expense of sucrose synthesis (Platt *et al*, 1977). The inference is that *V. faba* which are supplied with increasingly concentrated medium nitrogen (and particularly with ammonia 'spike') nutrition may possess a mechanism by which feedback inhibition of the Kreb's cycle is alleviated in favour of increased amino acid production. Such relatively increased amino acid production may have contributed towards the increasing compatible solute concentrations (proline (Samaras *et al*, 1995), and alanine and threonine (which reportedly have additive effects with proline, Samaras *et al*, 1995; Paleg *et al*, 1995), and glutamate (which together with ATP may protect GS from oxidative damage, Ortega *et al*, 1999); tables 6.2 - 6.7) which were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen, and particularly with ammonia 'spike' nutrition. As GS, NR and GOGAT may be located in the chloroplasts, it has been suggested that GS regulation may integrate carbon and nitrogen assimilation, and that GS activities may also regulate GDH activities (Rhodes *et al*, 1979).

That significantly greater amino acid concentrations were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition may also reflect the increased nitrogen assimilatory enzyme activities which were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (figs. 6.2 - 6.9).

Figs. 4.5 & 4.6 illustrate that total amino acid concentrations increased significantly during water deficits, and tables 6.2 & 6.3 and anova

analyses reveal that asparagine; proline; alanine; and threonine concentrations increased significantly in the leaves and roots of non-nodulated *V. faba* during water deficits. Asparagine and proline contributed the greatest concentrations to the quantified amino/imino acid pool in the leaves and roots of non-nodulated and nodulated *V. faba* during water deficits, with smaller contributions from aspartate and threonine. Increases in alanine; threonine; proline; and asparagine concentrations during water deficits have previously been reported in other plant species (Rajagopal & Sprent, 1977; Ranieri *et al*, 1989; Sharma & Rai, 1989; Bray, 1997).

Allantoin (see section 6.3.6; Miflin *et al*, 1980), asparagine and alanine are carbon economic (i.e. they have a high N:C ratio, Sprent, 1971; Lam *et al*, 1996). As such they may contribute to the carbon economy of *V. faba* during water deficits, as decreasing net photosynthesis and alanine and asparagine accumulation coincide during water deficits. Fig. 3.22 illustrates that net photosynthesis decreased during moderate water deficits (around 10% PEG), and tables 6.2 & 3 illustrate that (in non-nodulated *V. faba* which are supplied with '1/2 nitrate' or with 'combined nitrogen' nutrition) alanine accumulation commenced in the leaves during slight water deficits (5% PEG) and in the roots during moderate water deficits (10% PEG); while asparagine accumulation (which accounts for a large concentration of the total quantified amino acid pool) also commenced during slight to moderate water deficits in the leaves (5-10% PEG) and during moderate water deficits in the roots (15% PEG). Furthermore asparagine is closely related to glutamine and to aspartate, and the carbon skeleton of asparagine may be derived from or give rise to oxo-acids associated with the tricarboxylic acid cycle inferring that

metabolic flexibility may be maintained during asparagine accumulation (fig. 6.1; Bryan, 1976). Indeed earlier work involving protein fraction analyses revealed that asparagine may donate nitrogen to a wide range of amino acids, particularly glutamine and homoserine (Bryan, 1976; Miflin & Lea, 1975).

Asparagine reportedly functions in nitrogen storage and transport (Lees *et al*, 1968; Sprent, 1980), and is often exhibited in the xylem and storage organs of other leguminous species (Scott & Farnden, 1976; Miflin & Lea, 1977), accumulating for example when protein synthesis is limited (Pate & Gunning, 1972; figs. 6.12 & 6.13).

The 'roles' of asparagine contrast with those of glutamine, which is primarily involved in active metabolism. As such glutamine requires adequate photosynthate and water availabilities, and the ratio of glutamine : asparagine may reflect whether the metabolism of a plant is in 'storage' or 'active metabolic' mode (Lam *et al*, 1996). If excess carbohydrates are available within a plant, and therefore 2-oxogluterate is plentiful, the activities of GS and GOGAT reportedly increase, and asparagine synthesis decreases (Miflin & Lea, 1977), as asparagine synthetase is reportedly inhibited by ATP and 2-oxogluterate. However if ATP is limiting (e.g. potentially during the net photosynthesis decreases which occurred in *V. faba* during water deficits; fig. 3.22) and metabolism is slowed asparagine synthesis is stimulated, consistent with a storage 'role' for asparagine within plant physiology. Tables 6.2 - 6.7 and anova analyses reveal that while asparagine accumulated significantly in *V. faba* during water deficits, glutamine did not. This is important as glutamine is reportedly involved with the end product inhibition of GS (Rhodes *et al*, 1976), and thus a maintained potential for ammonia assimilation via the GS

GOGAT system is inferred in *V. faba* during water deficits (as illustrated in figs. 6.6 & 6.7). Indeed decreasing glutamine concentrations have previously been observed in other plant species which accumulate proline (another nitrogen storage compound; see section 4.4, pg. 129) during water deficits (Rhodes *et al*, 1986; see fig. 6.1).

As asparagine accumulation may reportedly enable the maintenance of greater stomatal conductances (Sharma & Rai, 1989), and hence increased carbon acquisition (provided that RWCs are maintained; figs. 3.16 & 3.17 reveal that RWCs were maintained during increasingly severe water deficits in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition), asparagine accumulation may enable increased photosynthate and reductant availabilities for maintained nitrogen assimilation and for increased osmotic adjustment during water deficits, and may result in an alleviation of metabolic perturbations during water deficits.

Glutamate concentrations increased significantly in the roots only of non-nodulated *V. faba* during water deficits (table 6.3), roots being the primary site of nitrogen assimilation in *V. faba* (Sutherland *et al*, 1985; see also figs. 6.3, 6.7 & 6.9), inferring that glutamate may have been transaminated in the leaves of non-nodulated *V. faba*. Glutamate accumulation has previously been reported as preventative against decreasing GS activities (Ortega *et al*, 1999), further inferring a capacity for maintained root GS activities in *V. faba* during water deficits (as were exhibited; fig. 6.7).

When 'total quantified' amino acid concentrations quantified utilising GC methodology (tables 6.2 - 6.7) are compared against total amino acid concentrations determined using spectrophotometric analyses (figs. 4.5 & 4.6) a shortfall is highlighted in the GC values, the contribution of which

to the total amino acid pool increases proportionally (up to four-fold) as water deficits increase. This shortfall is illustrated as 'non specified amino compounds' in figs. 6.22 & 6.23. The spectrophotometric assay detects amino compounds which may not be detected using the 'cleaned-up' samples analysed specifically via GC. 'Non specified amino compounds' may comprise non-quantified amino acids, short peptides, amines, and polyamines (Tabor & Tabor, 1976). Furthermore *Vicia* species reportedly contain non-protein amino acids which exhibit insecticidal properties (Bennet & Wallsgrove, 1994). Further work would allow qualification of these amino compounds and of their potential 'roles' during water deficits.

6.3.6 ALLANTOIN

Allantoin may reportedly be synthesized by nodules and by roots in other plant species (Pate, 1973), and is reportedly a major nitrogen transport compound (Pate, 1973; Twary & Heikel 1991). Figs. 6.24 & 6.25 reveal that allantoin accumulated to significantly greater concentrations in non-nodulated *V. faba* when supplied with 'combined nitrogen' as opposed to with nitrate or with 'no nitrate' nutrition; and with 'spiked' as opposed to with 'non-spiked' nutrition, consistent with the previously reported 'role' of allantoin in ammonia de-toxification, as allantoin is also carbon economic (has a high N:C ratio, Acer-Mothes, 1961; Thomas & Schrader, 1981).

The reported sensitivity of nitrogen fixation to water deficits has been associated with high shoot ureide accumulation which may reportedly limit nitrogen fixation via a concentration dependant feedback mechanism (Serraj & Sinclair, 1996; Purcall *et al*, 1998b). However figs. 6.24 & 6.25 reveal that allantoin was maintained at lower concentrations in nodulated 'no

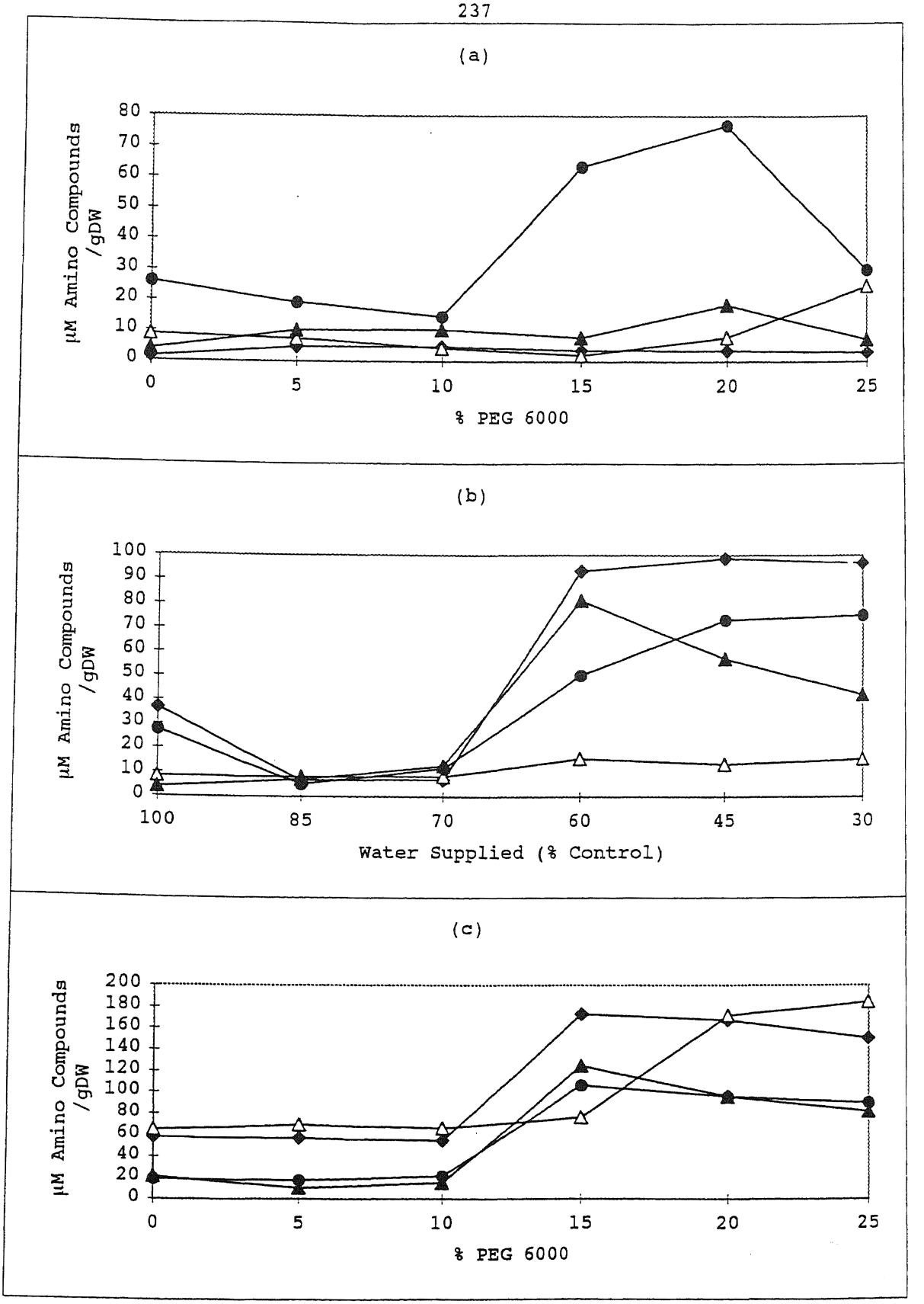


Fig. 6.22 Non-specified amino concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

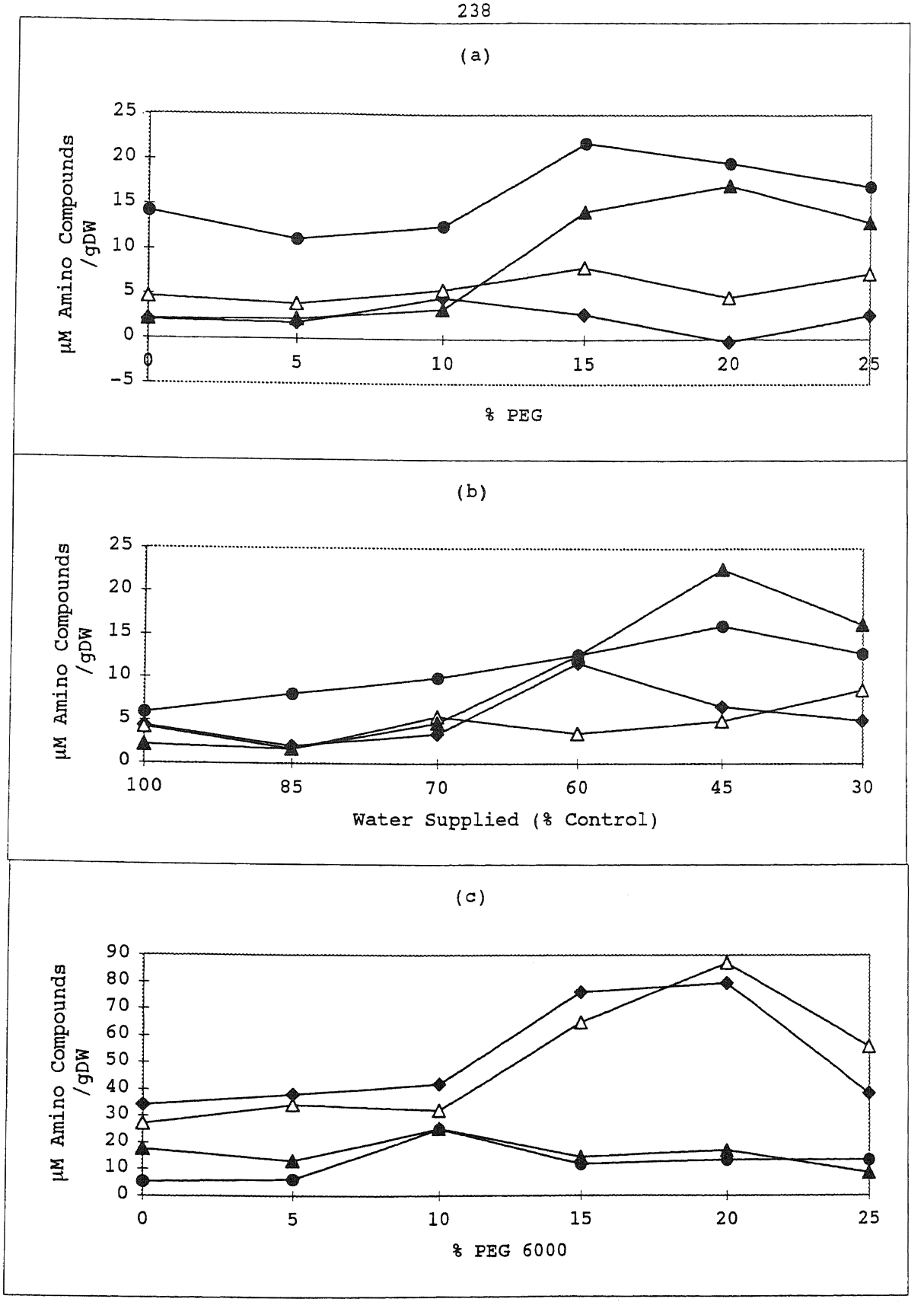


Fig. 6.23 Non-specified amino concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

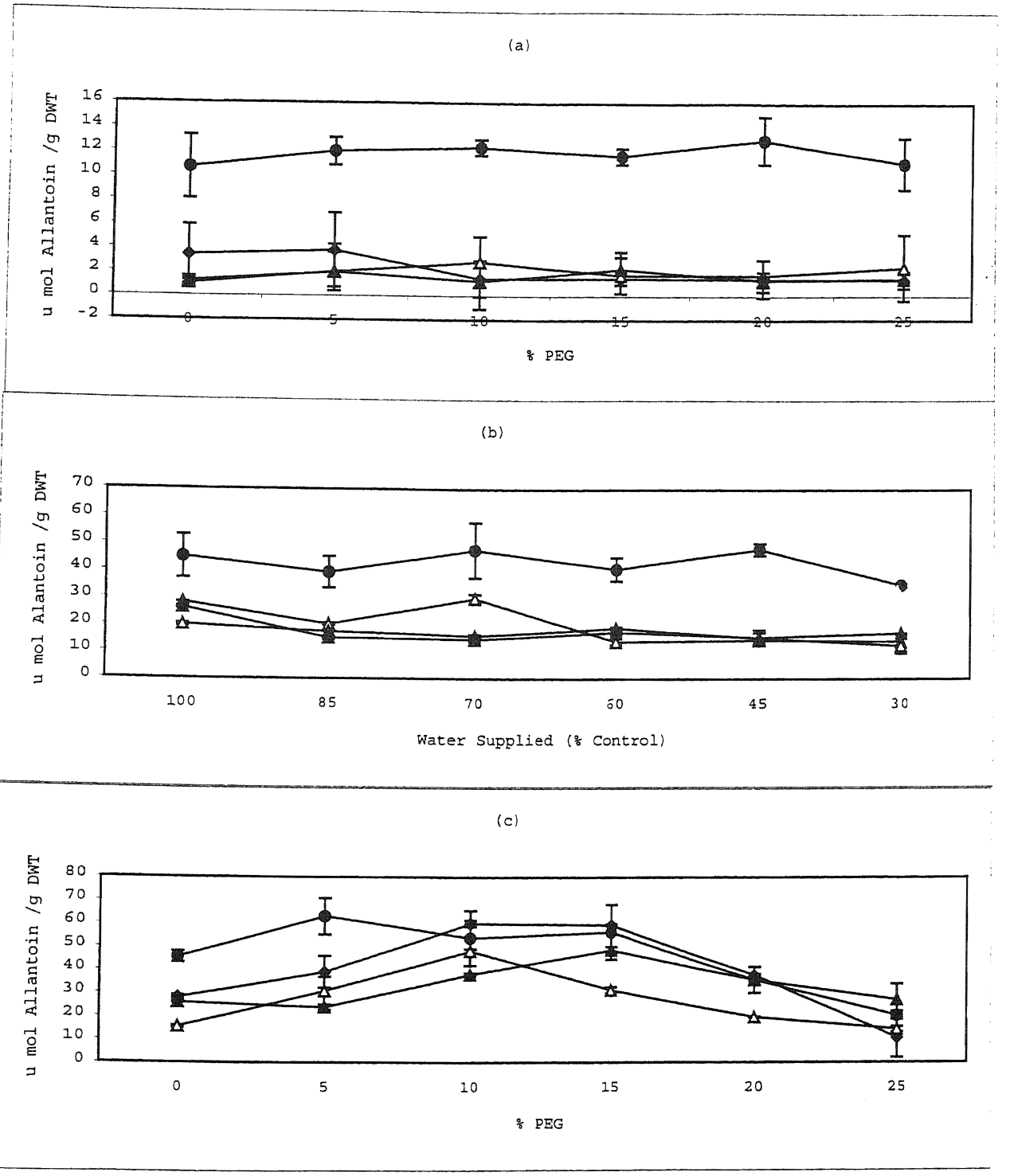


Fig. 6.24 Allantoin concentrations in the leaves of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; Δ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

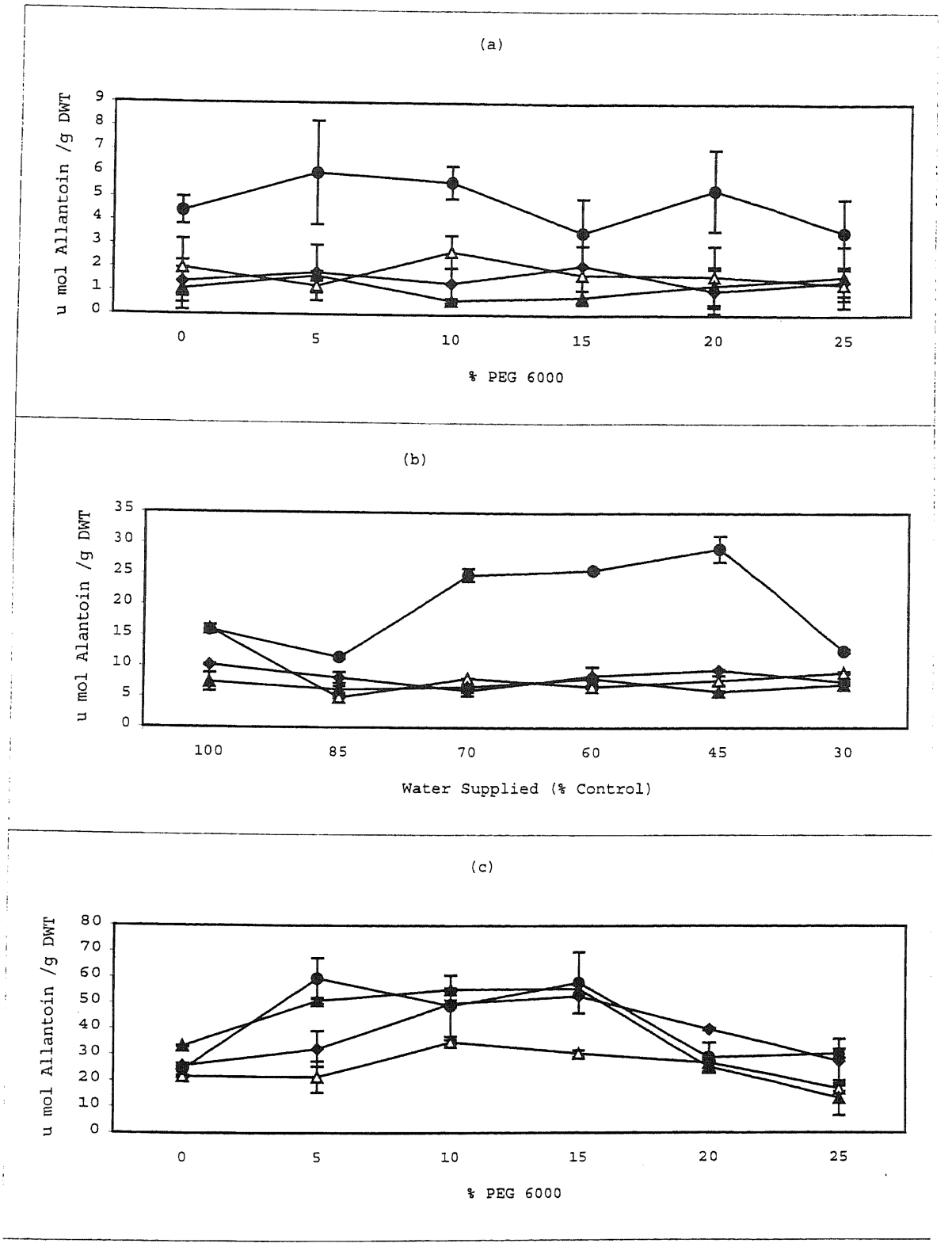


Fig. 6.25 Allantoin concentrations in the roots of (a) non-nodulated, (b) nodulated, and (c) 'spiked' *V. faba* during water deficits when supplied with various nitrogen sources where; ◆ = 'no nitrate'; △ = '1/10 nitrate'; ▲ = '1/2 nitrate'; ● = 'combined nitrogen'

nitrate' supplied *V. faba* than in 'spiked' *V. faba* throughout water deficits, and may not have resulted in the inhibition of Nase in this species (as inferred by the significantly greater growth and osmotic responses which were maintained in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits; see sections 3.4 & 4.4, and general discussion pg. 255). Indeed there is evidence to suggest that a metabolite of allantoin, as opposed to allantoin *per se* may be responsible for feedback inhibition of nitrogen fixation (Serraj *et al*, 1999).

Allantoin accumulation during water deficits has previously been reported in other plant species (Pate *et al*, 1969; Serraj *et al*, 1998). Following transport glutamine and asparagine may be readily utilised, however allantoin requires breakdown to CO_2 and ammonia (via urea) and glyoxylate, and therefore represents a circuitous way of transporting nitrogen. Furthermore almost three-times the amount of water is required to transport an equivalent amount of nitrogen as allantoin rather than as asparagine, which may limit allantoin transport during water deficits (Pate, 1973). However the relative insolubility of allantoin indicates a potential 'role' for allantoin as a nitrogen storage compound (Twary & Heikel, 1991). Figs. 6.24 & 6.25 and anova analysis reveal that allantoin concentrations were not significantly affected by water deficits in the leaves and roots of non-nodulated *V. faba*. Nodulated *V. faba* primarily exports asparagine as opposed to allantoin, and the metabolism of *V. faba* may be predisposed towards the synthesis of asparagine rather than allantoin. Furthermore the high proline and asparagine concentrations exhibited during water deficits may have negated a reliance on allantoin accumulation in non-nodulated 'non-spiked' *V. faba*.

Allantoin concentrations increased in the leaves and decreased in the roots of 'spiked' *V. faba*. Allantoin concentrations were significantly greater in 'spiked' than in 'non-spiked' *V. faba* and as such may have represented ammonia de-toxification, nitrogen storage and nitrogen transport products (Pate, 1973; Twary & Heikel, 1991) in *V. faba*, particularly when supplied with concentrated nitrogen nutrition, and particularly as allantoin is carbon economic (Sprent, 1980).

6.4 CONCLUSION

Proline; glutamine; glutamate; and asparagine concentrations were significantly greater in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; and glutamine; glutamate; asparagine; and alanine concentrations were significantly greater in 'spiked' than in 'non-spiked' *V. faba*, inferring ammonia de-toxification 'roles' for these carbon economic ('glutamate family') amino acids.

Asparagine; proline; alanine; and threonine accumulated significantly during water deficits. The reported 'roles' of asparagine (and allantoin, which was also exhibited at significantly greater concentrations in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition) as potential ammonia de-toxification, transport and storage compounds have been discussed.

'Roles' for asparagine in metabolic flexibility, in the maintenance of stomatal opening, and in the prevention of feedback inhibition of the TCA cycle, and for asparagine and allantoin in the carbon-economic transport of nitrogen were also hypothesized. Proline and asparagine contributed the greatest concentrations to the quantified amino acid pool during water

deficits, with smaller contributions (in terms of concentration) from threonine, alanine and aspartate. Accordingly while greater aminotransferase activities were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (supporting the greater nitrogen assimilation which was exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; figs. 6.2; 6.3; 6.6; 6.7; 6.8; 6.9), aspartate and asparagine synthetase activities increased during water deficits, while homoserine dehydrogenase and alanine aminotransferase activities decreased (see fig. 6.1). Amino acid and aminotransferase data contrasts with the earlier work of Rhodes *et al*, (1986) who noted that proline, alanine, valine, leucine and γ-aminobutyrate contributed the greatest concentrations to the amino acid pool when suspended cells of *Lycopersicon esculentum* were treated with 25% PEG. Indeed Rhodes *et al* (1986) concluded that there was little influx of glutamate into the aspartate pathways during water deficits, and although threonine, asparagine and lycine concentrations were slightly increased during water deficits, the rates of synthesis of these amino acids were lower in *L. esculentum* suspended cells when treated with 25% PEG than when treated with 0% PEG. Proline did accumulate significantly in *V. faba* during water deficits, however the lack of aspartate family amino acid accumulation in *L. esculentum* cells may be explained in part as legumes reportedly exhibit greater asparagine and allantoin concentrations than non-legumes (Scott & Farnden, 1976; Sprent, 1980). Furthermore the suspended *L. esculentum* cells had unrestricted nitrogen and carbon supplies (Rhodes *et al*, 1986), whereas *V. faba* exhibited decreasing net photosynthesis during water deficits (fig. 3.22), which may have necessitated an accumulation of the carbon economic osmotic solutes such as

asparagine, alanine, and allantoin.

Further work would allow qualification of 'non-specified' amino compounds and of their 'roles' during water deficits.

Significantly greater protein concentrations were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with 'combined nitrogen' and with ammonia 'spike' nutrition, an important nutritional and economic consideration for a leguminous crop (Snoad, 1981). However protein concentrations decreased significantly during water deficits (being maintained at significantly greater concentrations in non-nodulated *V. faba* when supplied with 'combined nitrogen' as opposed to with equimolar nitrate nutrition; and in the roots of 'spiked' as opposed to 'non-spiked' *V. faba*), and may have provided substrates towards the production of nitrogenous osmotica. However amino acid accumulation preceded protein decreases, and continued when protein concentrations had decreased significantly, inferring a 'role' for primary nitrogen assimilation in the production of nitrogenous substrates.

Increased nitrogen uptake is inferred in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, as such *V. faba* exhibited significantly greater stomatal conductances, and therefore potentially increased transpiration and nitrate fluxes, which as discussed are reportedly regulatory for nitrate reductase activities (Shaner & Boyer, 1976), and increased nitrogen assimilation may result in increased nitrate uptake (Imsande & Touraine, 1994). The significantly greater NR activities exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition may also have been attributable to NR induction (Somers *et al*, 1983; Andrews *et al*, 1984; Sutherland *et al*, 1985), and may have

reflected the increased levels of net photosynthesis (fig. 3.22) and carbohydrate accumulation (4.3 & 4.4) which were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and which are reportedly required for nitrate uptake (Imsande & Touraine, 1994), and are reportedly correlated with NR activities (Cheng *et al*, 1992; Foyer *et al*, 1998). Additionally *V. faba* supplied with increasingly concentrated medium nitrogen nutrition may have experienced alleviated feedback inhibition of nitrogen assimilation due to an abundance of carbon skeletons, which are required for amino acid production (Miflin, 1974), and for nitrate uptake (Imsande & Touraine, 1994).

NR activities were maintained until water deficits became severe, perhaps decreasing then in response to increasing stomatal closure (fig. 3.21, which may have resulted in decreased nitrate fluxes, Shaner & Boyer, 1976), and to decreases in net photosynthesis (fig. 3.22). That decreasing stomatal conductances and net photosynthesis as opposed to water deficits *per se* may have been responsible for the observed decreases in NR activities is supported by the following factors: (i) maintained NR activities during (gradually imposed) water deficits have previously been reported in a small number of studies involving other plant species (Smirnoff *et al*, 1985; Ladley, 1990); (ii) stomatal conductances; net photosynthesis; and NR activities were all maintained at the greatest values in *V. faba* when supplied with the most concentrated medium nitrogen nutrition (figs. 3.21; 3.22; 6.2 & 6.3), and decreases in NR activities were only exhibited during severe water deficits, and as such coincided with decreases in stomatal conductance and net photosynthesis, which as discussed are reportedly regulatory for nitrate uptake (Shaner & Boyer, 1976; Imsande & Touraine, 1994), and for maintained NR activities (Foyer *et al*, 1998); and (iii) NR activities were maintained in *V. faba* during

moderate water deficits indicating that NR was not sensitive to water deficits *per se*.

V. faba exhibited high leaf and root nitrate concentrations throughout water deficits (figs. 6.4 & 6.5), which may have contributed to the maintenance of high NR activities, as nitrate may reportedly be mobilized from storage to metabolic pools during water deficits resulting in maintained nitrate fluxes, and hence NR activities (Shaner & Boyer, 1976 a&b; Chapin *et al*, 1988).

The accumulation of compatible solutes (proline; alanine; and threonine; Nash *et al*, 1981; Paleg *et al*, 1985; Ortega *et al*, 1999; see pg 232), which accumulated to increasing concentrations in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition may have afforded some protection towards NR (and other enzymes, and may have enabled maintained NR turnover) during water deficits, particularly in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; and with nodulated as opposed to with non-nodulated 'no nitrate' nutrition; and with 'spiked' as opposed to with 'non-spiked' nutrition. The protective effects of compatible solute accumulation have previously been discussed (pgs. 16; 103; 104; 232).

GS activities increased significantly in *V. faba* as the concentration of the supplied nitrogen source increased; while GDH activities were exhibited in the following order in the leaves and roots of non-nodulated *V. faba* with respect to medium nitrogen nutrition: 'combined nitrogen' > '1/2 nitrate' > '1/10 nitrate' > 'no nitrate', inferring an ammonia de-toxification 'role' for GDH. Greater NR activities have previously been recorded in *C. vitalba* when supplied with ammonia as opposed to with nitrate (Bungard et al, 1999) and NR and GS activities have previously

been shown to be correlated in *Urdica dioca* (Hofstra *et al*, 1985). Indeed greater GS; GDH; and NR activities were exhibited in the leaves and roots of nodulated than of non-nodulated 'no nitrate' supplied *V. faba*; and in the leaves of 'spiked' as opposed to 'non-spiked' *V. faba*, perhaps attributable to increased ammonia assimilation.

Furthermore GS activities were maintained, while GDH activities were either maintained or increased during water deficits (figs. 6.6- 6.9). It is apparent that *V. faba* exhibited significantly greater nitrogen assimilatory enzyme activities when supplied with increasingly concentrated medium nitrogen nutrition. That greater concentrations of the products of primary nitrogen assimilation (glutamate; asparagine; glutamine; and allantoin) were exhibited in non-nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition; in nodulated than in non-nodulated 'no nitrate' supplied *V. faba*; and in 'spiked' than in 'non-spiked' *V. faba* supports the observation that increasingly concentrated medium nitrogen nutrition (particularly with medium ammonia additions) results in the exhibition of greater nitrogen assimilatory enzyme activities. Continued nitrogen assimilation during water deficits may result in an alleviation of photoinhibition (Smirnoff & Stewart, 1985).

GOGAT is reportedly unaffected by feedback mechanisms (Miflin & Lea, 1977). However the accumulation of nitrate and amino acids (particularly glutamine; arginine; alanine; asparagine; and aspartic acid) in root phloem cells may reportedly result in reduced nitrate NURs (Gojon *et al*, 1998; Gessler *et al*, 1998); reduced ammonia absorption (Lee *et al*, 1992); and reduced Nase, NR, and GS activities (Steer, 1973; Rhodes *et al*, 1975; Rhodes *et al*, 1976; Stewart & Rhodes, 1977b; Deng *et al*, 1991; Parson *et al*, 1993; Garcia-Fernandez *et al*, 1995; Min *et al*, 1998; Serraj *et al*,

1998; Oliveira & Coruzzi, 1999), via a 'satiety' model (Imsande & Touraine, 1994). Increasing carbohydrate metabolite concentrations reportedly result in the exhibition of increasing GS activities, while increasing amino acid concentrations reportedly antagonise GS induction and activities and therefore GS activities appear to be regulated by the C:N ratio (Oliveira & Coruzzi, 1999).

However total amino acid concentrations, and NR; GS; and GDH activities all increased significantly in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition.

Maintained NR activities concurrent with amino acid accumulation have previously been reported in other plant species (Beevers & Hageman, 1969), and some workers have concluded that the availability of ammonia rather than amino acids may regulate GS activities (Kanamori & Matsumoto, 1974; Wallsgrove *et al*, 1977; Miflin *et al*, 1980), as appears to be the case for *V. faba*.

Furthermore it is glutamine which is primarily attributed as being inhibitory for GS activities (Rhodes *et al*, 1975; Rhodes *et al*, 1976), and glutamine concentrations did not increase in *V. faba* during water deficits. Previously reported inhibitory effects of amino acid accumulation on nitrogen assimilation appear to have been overridden by continued nitrogen supplies in *V. faba*, possibly as excess amino acids may be compartmentalized in vacuoles during water deficits (Rhodes *et al*, 1986). Asparagine did accumulate significantly during water deficits. However asparagine is primarily considered inhibitory for Nase rather than for medium nitrogen assimilatory enzyme activities (Serraj *et al*, 1999), and significantly greater growth; stomatal conductances and net photosynthesis; osmotic adjustment; amino acid concentrations; aminotransferase activities; protein concentrations; NR activities; GS activities; GDH activities; and

total ammonia concentrations were maintained in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits, inferring that increasing asparagine concentrations may not have resulted in the inhibition of Nase activities during water deficits in this species. Asparagine may reportedly have a small inhibitory effect on GS2 in *Oryza sativa* (Hirel & Gadal, 1980b), however GS activities did not decrease as asparagine activities increased in *V. faba*. Although high asparagine concentrations were recorded, amino acids may reportedly concentrate in the cytoplasm and vacuoles during water deficits (Rhodes *et al*, 1986), indicating that phloem amino acid concentrations may have remained low. Indeed the fact that asparagine accumulation may inhibit further asparagine synthesis *in vitro*, but not *in vivo* infers that asparagine may be stored away from the site of synthesis (for example in vacuoles, Miflin & Lea, 1977), and further infers that asparagine may represent an effective ammonia de-toxification product as it does not appear to be end-product inhibited. If asparagine is stored in the vacuoles rather than phloem translocated during water deficits, then its effect on nitrate uptake would be minimised (Imsande & Touraine, 1994). Proline is also produced via glutamate (fig. 6.1) and therefore may also represent an ammonia de-toxification product (Barnett & Naylor, 1966; Shobert, 1977; Aspinall & Paleg, 1981; Pulich, 1986; see section 4.4).

GS activities are reportedly high when low concentrations of ammonia are available, and may reportedly decrease as ammonia concentrations increase, whereas GDH activities reportedly increase as ammonia availabilities increase (Rhodes *et al*, 1976). Indeed figs. 6.6 - 6.9 illustrate that 'spiked' as opposed to 'non-spiked' '1/2 nitrate' and 'combined nitrogen' supplied *V. faba* exhibited significantly greater increases in GDH activities than in GS activities. It has been proposed that as ammonia is

toxic and plants have little control over ammonia uptake it is unlikely that a mechanism which unilaterally shuts off ammonia assimilation at the GS level would evolve, but rather that carbon may limit GS activities in plants which are supplied with concentrated medium ammonia nutrition (Miflin *et al*, 1980). However greater net photosynthesis and carbohydrate concentrations were exhibited in *V. faba* when supplied with increasingly concentrated medium ammonia additions (section 3.4). Accordingly GS activities were maintained or increased in *V. faba* when supplied with the most concentrated medium ammonia additions (by around 11% in the roots and 0% in the leaves of 'spiked' as compared with 'non-spiked' 'combined nitrogen' supplied *V. faba*), but not to the extent that GDH activities increased (by 50% in the roots and by >40% in the leaves of 'spiked' as compared with 'non-spiked' 'combined nitrogen' supplied *V. faba*). Thus an ammonia assimilatory 'role' is inferred for GDH in *V. faba* when supplied with increasingly concentrated medium ammonia additions. GDH activities also increased in 'spiked' *V. faba* during water deficits, inferring that medium ammonia additions may be especially beneficial during severe water deficits, when slight decreases in NR activities were recorded (figs. 6.2 & 6.3). That increasing GDH activities during water deficits may have been mediated via ATP decreases (Stewart & Rhodes, 1977; Rhodes *et al*, 1979; Stryer, 1988; Lam *et al*, 1996) was hypothesized. Increasing GDH activities in *V. faba* when supplied with medium ammonia additions (and during water deficits; figs. 6.8 & 6.9), may have contributed towards the increased amino acid (particularly proline and asparagine) concentrations which were exhibited in *V. faba* when supplied with medium ammonia additions (and during water deficits; tables 6.2 - 6.7), as previously reported in other plant species (Venekamp, 1989).

Amino acid accumulation continued until severe water deficits were imposed

(20-25% PEG; 45-30% Control Water; figs. 4.5 & 4.6), by which stage protein concentrations had more than halved (figs. 6.12 & 6.13). While GS activities were merely maintained during water deficits (figs. 6.6 & 6.7; albeit at increasing activities in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and in 'spiked' than in 'non-spiked' *V. faba*), root and leaf GDH activities increased in 'spiked' *V. faba* during water deficits, further inferring a 'role' for the GDH mediated assimilation of ammonia in nitrogenous osmotica production in *V. faba* during water deficits.

In summary, significantly increasing proline, glutamine, asparagine, alanine and protein concentrations were exhibited by *V. faba* as the concentration of the supplied nitrogen source increased. Asparagine, proline, alanine and threonine concentrations increased significantly in *V. faba* as water deficits increased. Although plant protein concentrations decreased as water deficits increased, amino acid accumulation preceded protein decreases and continued during severe water deficits when protein concentrations were low, inferring a role for primary nitrogen assimilation in the production of nitrogenous osmotica during water deficits (as supported by enzymic data). Indeed GS and GDH activities were either maintained or else increased in the leaves and roots of *V. faba* throughout water deficits, and NR activities were maintained until water deficits became severe.

Furthermore the activities of the enzymes of primary nitrogen assimilation increased as the concentration of the supplied nitrogen source increased. Significantly greater leaf NR and leaf and root GS and GDH activities were exhibited by 'spiked' than by 'non-spiked' *V. faba*, and ammonia de-toxification 'roles' were inferred for GS, and particularly for GDH.

CHAPTER SEVEN

GENERAL DISCUSSION

> Much research can be done on stress characteristics and phenomena in plants and crops, but it must be related to principles of crop/soil management if it is to be useful to agriculture in drought-susceptible areas (Swindale & Bidinger, 1981).

V. faba infected with *Rhizobia* is an effective nodulator, and nitrogen fixation reportedly accounts for over eighty per cent of the total nitrogen content of *V. faba* when supplied with adequate irrigation (Richards & Soper, 1979; Ines-Minguez & Sau, 1989). However nitrogen fixation is reportedly more sensitive to water deficits than medium nitrogen assimilation; carbon assimilation; plant growth; and grain yields (Serraj *et al*, 1999). Decreasing nitrogen fixation has previously been reported in *V. faba* (Sprent 1972; Guerin *et al*, 1990), and in other plant species (Sprent, 1973; Streeter, 1998; Serraj *et al*, 1998; Soussi *et al*, 1998) during water deficits. Furthermore legumes reportedly sever symbiotic associations with rhizobia when medium nitrogen is available, as nitrogen fixation is reportedly energetically expensive (Schilling, 1983; see introduction, pg. 8), and water intensive (Sprent, 1981; see introduction pg. 17). Table 2.3 illustrates that *V. faba* exhibited fewer nodules when supplied with (even low concentrations of) medium nitrogen nutrition, than when supplied with 'no nitrate' nutrition. Approximately equal productivities have previously been recorded by *V. faba* whether reliant on nitrogen fixation or supplied with medium nitrogen nutrition during periods of adequate irrigation (Richards & Soper, 1979, Simon & Skrdleta, 1983). The inference is that the nitrogen fertilisation of adequately irrigated

V. faba might be wasteful in terms of human and economic resources, and could potentially result in environmental damage (due to the nitrate leaching, Raven, 1985).

A major research proposal in this study was that medium nitrate, 'combined nitrogen', and ammonia additions prior to and during water deficit imposition would provide alternative nitrogen sources to nitrogen fixation. As such methodology was designed to determine whether increasingly concentrated medium nitrogen applications would result in increasing productivities in *V. faba* (as previously described in other plant species (see fig. 7.1*)), particularly during water deficits.

However Plies-Balzer *et al*, (1995), reported that although nitrogenase activities decreased in *V. faba* when water deficits were imposed either continuously or during flowering, water deficits during pod-filling resulted in maintained nitrogenase activities and biomass production, inferring that nitrogenase activities *per se* may not be as susceptible to water deficits as is classically supposed. Furthermore a *G. max* cultivar ('Jackson'), has been identified as water deficit tolerant for nitrogen fixation (Serraj & Sinclair, 1997), further inferring that nitrogen fixation may not be as sensitive to water deficits in all plant species and cultivars as is classically supposed. It has been reported that growth and yield in *V. faba* (cv. 'Alfred') may not be limited by symbiotic nitrogen fixation during water deficits, as although nitrogenase activities reportedly decreased during continuous water deficits, total nitrogen contents remained unaffected (Plies-Balzer *et al*, 1995). Indeed medium 'combined nitrogen' applications (1.2g N / two plants as NH_4NO_3; 800mg N / two plants as starter N, and 400 mg N / two plants later) reportedly cannot

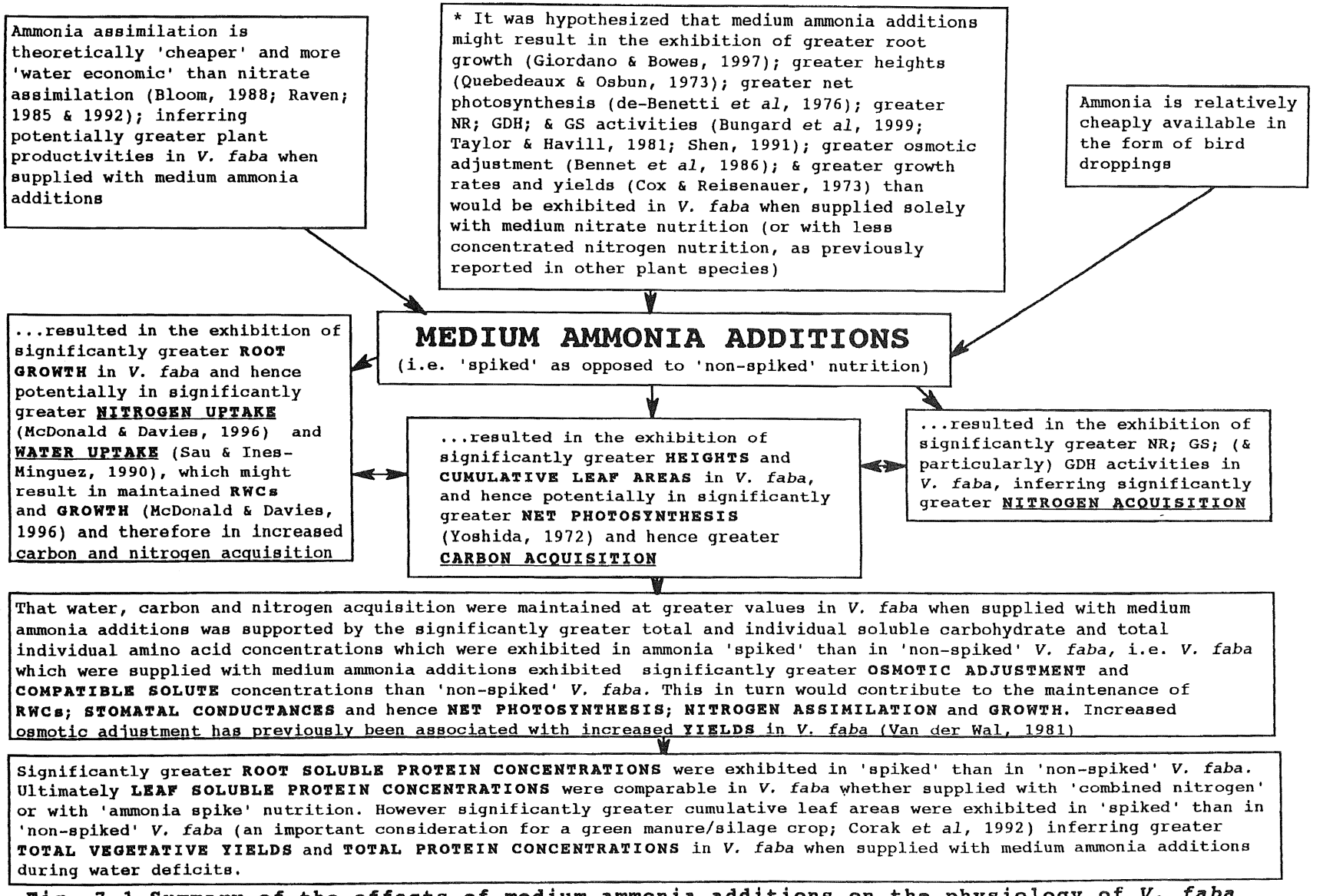


Fig. 7.1 Summary of the effects of medium ammonia additions on the physiology of *V. faba* during water deficits

compensate for the negative effects of water deficits on *V. faba* (cv. 'Alfred'), and reportedly do not result in improved plant aerial growth or total nitrogen concentrations (Plies-Balzer *et al*, 1995).

Thus earlier literature indicates that nitrogen applications may not result in improved productivities in *V. faba*, particularly during periods of adequate irrigation, but also during water deficits, particularly as medium nitrogen applications suppressed nodulation in *V. faba* (see table 2.3). Indeed significantly greater aerial fresh and dry organ biomasses; leaf and root RWCs; R:S increases; RGRs; NARs; CLAs; stomatal conductances; net photosynthesis; total and individual carbohydrate concentrations; starch concentrations; total and individual amino acid concentrations; allantoin concentrations; proline concentrations; glycine betaine concentrations; aminotransferase activities; ammonia concentrations; nitrogen assimilatory enzyme activities; and protein concentrations were maintained in nodulated than in non-nodulated 'no nitrate' supplied *V. faba* throughout water deficits. Thus the collective data infers that nitrogen fixation may not be as water deficit sensitive as is classically supposed (at least in 'Bunyards Exhibition'), and particularly during periods of vegetative growth.

Furthermore growth, stomatal conductances and net photosynthesis were maintained at greater values in nitrogen fixing *V. faba* (i.e. in nodulated 'no nitrate' supplied *V. faba*) than in '1/10 nitrate' (0.8 mM N) supplied *V. faba* (figs. 3.1 - 3.10; 3.21; 3.22), throughout water deficits. Accordingly figs. 4.3; 4.4; & 5.2 - 5.7 illustrate that significantly greater total soluble carbohydrate and individual carbohydrate concentrations were exhibited in nitrogen fixing *V. faba* than in '1/10 nitrate' supplied *V. faba*. Lower NR

activities and nitrate concentrations were exhibited in the leaves and roots of nitrogen fixing than of '1/10 nitrate' supplied *V. faba* (figs. 6.2 - 6.5). This was expected as nitrogen fixation does not result in increased nitrate availabilities. However greater GS activities were exhibited in the roots of nodulated 'no nitrate' than of '1/10 nitrate' supplied *V. faba* (fig. 6.7), inferring greater ammonia assimilation in nitrogen fixing *V. faba* than in *V. faba* which were supplied with low concentrations (0.8 mM N) of medium nitrogen nutrition. The greater net photosynthesis and carbohydrate concentrations which were exhibited in nitrogen fixing as opposed to in '1/10 nitrate' supplied *V. faba* may have contributed towards the exhibited greater root GS activities, as photosynthates and carbohydrates are required for nitrogen assimilation (Miflin *et al*, 1980). The greater GS activities exhibited in nitrogen fixing than in '1/10 nitrate' supplied *V. faba* may have contributed substrates towards the greater total and individual amino acid concentrations (figs. 4.5 & 4.6; tables 6.3 & 6.4); greater leaf and root 'non-specified amino' concentrations (figs. 6.22 & 6.23); greater leaf and root total osmolarities (figs. 4.11 & 4.12), greater plant growth (figs. 3.1 - 3.10); and greater leaf and root soluble protein concentrations (figs. 6.12 & 6.13) which were exhibited in nitrogen fixing (i.e. in nodulated 'no nitrate' supplied) *V. faba* than in '1/10 nitrate' supplied *V. faba*.

The indication is that < 0.8 mM medium nitrogen applications resulted in nitrogen fixation inhibition (see table 2.3), and ultimately in the exhibition of lower yields than would be exhibited by nitrogen fixing *V. faba*, even during water deficits. Previous workers have reported that *V. faba* exhibits greater biomasses when supplied with medium nitrogen than when reliant on nitrogen fixation during periods of adequate irrigation (Ines-Minguez & Sau, 1989 - 15.7 mol m^{-3} nitrate), however these

differences are reportedly less marked for *V. faba* than for any other legume grown under a controlled environment (Ryle *et al*, 1983); a further indication of the effectiveness of nitrogen fixation in *V. faba* (Richards & Soper, 1979). In summary greater growth or protein concentrations were not exhibited in *V. faba* when supplied with low medium nitrate concentrations (0.8 mM N) as opposed to when reliant on nitrogen fixing *V. faba* (figs. 3.1 - 3.10; 6.12; 6.13). Indeed environmental damage may result from superfluous applications of medium nitrate, as nitrate is prone to leaching (Raven, 1985).

However *G. max* reportedly exhibits increased nitrogen and biomass accumulation rates when supplied with more concentrated medium 'combined nitrogen', nitrate, or ammonia nutrition (336 kg/ha NH_4NO_3; 10 mM KNO_3; or 10 mM NH_4Cl) as opposed to when reliant on nitrogen fixation, both when supplied with adequate irrigation and during water deficits (Purcell & King, 1996).

Furthermore while increased seed yields ('seed number X average seed mass') were reportedly not exhibited in 'combined nitrogen' supplied as opposed to nitrogen fixing *G. max* during periods of adequate irrigation, an eighteen per cent seed yield increase was exhibited by *G. max* when supplied with medium 'combined nitrogen' nutrition, as opposed to when reliant on nitrogen fixation during water deficits (Purcell & King, 1996). Indeed *G. max* reportedly exhibits greater seed yields (2.5 per cent seed yield increases) when supplied with 'combined nitrogen' nutrition during water deficits, than when reliant upon nitrogen fixation and supplied with adequate irrigation (Purcell & King, 1996).

Similarly greater growth, stomatal conductances and net photosynthesis,

osmotic adjustment, and nitrogen assimilatory enzyme activities were exhibited in *V. faba* when supplied with (> 0.8 mM) medium nitrogen nutrition (i.e. with '1/2 nitrate'; 'combined nitrogen'; or with ammonia 'spike' nutrition; section 2.4), than when reliant on nitrogen fixation, both during water deficits and during periods of adequate irrigation.

Accordingly although organ fresh and dry weights; plant heights; NARs; leaf and root RWCs; and RGRs all decreased significantly during water deficits, all growth parameters were maintained at significantly increasing values in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N). It has been reported that *V. faba* does not exhibit greater aerial growth when supplied with 'combined nitrogen' nutrition, as opposed to when reliant on nitrogen fixation, both when supplied with adequate irrigation and during water deficits (Plies-Balzer *et al*, 1995). However fig. 3.8 illustrates significantly greater aerial biomasses in *V. faba* when supplied with > 0.8 mM medium nitrogen (i.e. with either 'combined nitrogen' or with '1/2 nitrate' nutrition) as opposed to when reliant on nitrogen fixation, both when supplied with adequate irrigation and during water deficits.

R:Ss increased significantly during water deficits inferring an increased capacity for water and nitrogen uptake (Sharp & Davies, 1979; McDonald & Davies, 1996), and were maintained at significantly increasing values in *V. faba* when supplied with decreasing medium nitrogen concentrations, reflecting the previously reported observation that plants growing in nitrogen deficient media may require increased root growth to enable adequate nitrogen uptake (McDonald & Davies, 1996; Hodge *et al*, 1999). However significantly greater root biomasses, heights and cumulative leaf areas were exhibited in

'spiked' than in 'non-spiked' *V. faba* (figs. 3.9; 3.20; 3.25), accordingly statistically similar R:Ss were exhibited in 'spiked' and in 'non-spiked' *V. faba* throughout water deficits.

RWCs were maintained at greater values in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (>0.8 mM N) during water deficits, perhaps attributable to the increased osmotic adjustment which was exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, as osmotic adjustment is reportedly correlated with RWC maintenance during water deficits (Singh & Gupta, 1983). However similar RWCs were exhibited in 'spiked' and in 'non-spiked' *V. faba* (figs. 3.16 & 3.17). This may be attributable to the fact that *V. faba* which were supplied with increasingly concentrated medium nitrate nutrition exhibited increasing nitrate concentrations, inferring that some additional nitrate was stored rather than assimilated. Nitrate may act as an effective vacuolar osmotic solute (Shaner & Boyer, 1976), and as such nitrate accumulation may have contributed towards the increasing RWCs which were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrate nutrition (figs. 3.16 & 3.17), as otherwise 'spiked' *V. faba* exhibited greater osmotic adjustment than 'non-spiked' *V. faba*. Conversely ammonia is toxic (Raven, 1985) and cannot be stored, and as such significantly greater plant heights, root biomasses, and CLAs were exhibited in 'spiked' than in 'non-spiked' *V. faba*, inferring that medium ammonia additions were metabolized. Accordingly similar RWCs were exhibited in 'spiked' and in 'non-spiked' *V. faba*. However the significantly greater plant heights, root biomasses, and cumulative leaf areas which were exhibited in *V. faba* when supplied with medium ammonia 'spikes' may have important physiological (and economic) implications, as summarized in fig. 7.1 (pg. 254) and discussed

below. Increased root growth (as was exhibited in 'spiked' as compared with 'non-spiked' *V. faba*) potentially results in increased substrate penetration and therefore potentially in increased water and nitrogen uptake (McDonald & Davies, 1996), and has been correlated with maintained water uptake in *V. faba* during water deficits (Sau & Ines-Minguez, 1990). Increased root biomasses may be particularly 'beneficial' to *V. faba*, as the roots of this temperate adapted species predominate in the top 30 cm of the medium (Hebblethwaite, 1982; see introduction pg 13; fig. 3.9). Accordingly *V. faba* may be inherently more susceptible to root dehydration than cereals, which reportedly exhibit deeper root growth (Sprent, 1973).

Although CLAS decreased significantly in 'non-spiked' *V. faba* during water deficits (fig. 3.20), anova analyses reveal that CLAs were significantly greater in 'spiked' than in 'non-spiked' *V. faba* throughout water deficits. The maintenance of high CLAs is reportedly associated with increasing overall photosynthetic capacities in *V. faba* (Van der Wal, 1981), and in other plant species (Yoshida, 1972), and has previously been strongly correlated with increasing dry matter yields and seed matter yields in *V. faba* (Hebblethwaite, 1984). Furthermore in climates which are characterised by limited intermittent rainfall, large CLAs may limit the evaporation of rain from the substrate surface throughout the growth period (Passioura, 1981), and may thus postpone water deficits. Maintained CLAs and heights infer increased capacities to intercept solar radiation, and therefore increased overall net photosynthetic capacities, and increased survival prospects upon water deficit alleviation (Yoshida, 1972), for *V. faba* when supplied with medium ammonia additions. While greater leaf areas were exhibited in 'spiked' than in 'non-spiked' *V. faba*, similar leaf biomasses were recorded by *V. faba* within both nitrogen schemes. Leaf thickness has previously been reported as variable in

Vigna subterranea L. (Collinson et al, 1997). It is also possible that 'spiked' *V. faba* may have contained less storage Rubisco than 'non-spiked' *V. faba* (Rubisco has been described as a nitrogen storage compound, Evans, 1975). Such a hypothesis is in agreement with the greater growth that was exhibited in 'spiked' than in 'non-spiked' *V. faba*.

Anova analyses reveal that similar relative growth rates, leaf area ratios, and net assimilation rates were exhibited in 'spiked' and in 'non-spiked' *V. faba*. It is thus apparent that medium ammonia additions did not result in the exhibition of increasing growth rates in *V. faba*. Furthermore figs. 3.7; 3.8 & 3.10 and anova analyses reveal that similar stem dry weights; aerial dry weights; and total dry weights were exhibited in 'spiked' and in 'non-spiked' *V. faba* throughout water deficits. However the growth which was significantly greater in 'spiked' than in 'non-spiked' *V. faba* was that of 'productive' rather than 'structural' tissue, i.e. significantly greater root biomasses, (heights) and CLAs were exhibited in 'spiked' than in 'non-spiked' *V. faba*. As such the potential for water, carbon, and nitrogen acquisition may be greater in *V. faba* when supplied with medium ammonia additions (see fig. 7.1), even during water deficits. This may have contributed towards the production of greater substrate concentrations for the production and maintenance of productive tissue biomasses, for greater osmotic adjustment and for greater nitrogen assimilation throughout water deficits than were exhibited in *V. faba* which were grown without medium ammonia additions.

Stomatal conductances were maintained at lower external water potentials in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N), and with 'combined nitrogen' as opposed to with equimolar '1/2 nitrate' nutrition. This may in

part reflect the increased RWCs which were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, as low leaf RWCs reportedly contribute to stomatal closure during water deficits in *V. faba* (Van der Wal, 1981), and in other plant species (Comstock & Mencuccini, 1998), and net photosynthesis often reflects stomatal conductance, particularly during slight to moderate water deficits (McDonald & Davies, 1996). Decreasing stomatal conductances infer decreasing transpiration and therefore decreasing water losses during water deficits, however figs. 3.22, 4.3 & 4.4 illustrate that net photosynthesis and therefore potentially carbon acquisition are simultaneously decreased. A compromise must be met within the physiology of the plant which results in the maintenance of RWCs (which may be achieved via partial stomatal closure), and simultaneously enables sufficient carbon acquisition for the maintenance of growth and of substrate provision for osmotic adjustment, and for maintained nitrogen uptake and assimilation during water deficits. Osmotic adjustment, RWCs and therefore stomatal conductances and net photosynthesis were maintained at increasing values in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N). Lower transpiratory water losses have previously been reported in nitrogen supplied as opposed to nitrogen fixing *V. faba* (Sau & Ines-Minguez, 1990). However greater stomatal conductances were maintained in nodulated *V. faba* when supplied with 'combined nitrogen' nutrition than when reliant on nitrogen-fixation (i.e. in nodulated 'no nitrate' supplied *V. faba*), during moderate water deficits (fig. 3.21). Such greater stomatal conductance maintenance may reflect the greater RWCs which were exhibited in 'combined nitrogen' supplied *V. faba* than in *V. faba* which were reliant on nitrogen fixation (figs. 3.16 & 3.17; Comstock & Mencuccini, 1998). The increased root growth exhibited in *V. faba* when supplied with

medium ammonia additions may have resulted in increased water (and nitrogen) uptake (McDonald & Davies, 1996), and therefore may also have contributed towards RWC maintenance (figs. 3.16 & 3.17). An increased potential for stomatal
conductance and therefore for carbon and nitrogen acquisition is therefore inferred in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N as indicated by the data, 3.21; 4.3; 4.4; 6.2; 6.3; 6.6 - 6.9). Increased net photosynthesis in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition may also reflect the fact that nitrogen is required for the production of (photosynthetic) enzymes.

Water deficit tolerance adaptations such as osmotic adjustment rely upon an optimum balance being achieved between water conservation and carbon (and nitrogen) uptake within the plant throughout water deficits. Fig. 7.1 (pg. 254) illustrates that *V. faba* which are supplied with medium ammonia additions may optimise carbon, nitrogen, and water uptake.

While 'spiked' and 'non-spiked' *V. faba* recorded similar rates of net photosynthesis per unit leaf area (fig. 3.22), the exhibition of significantly greater heights and CLAS by 'spiked' than by 'non-spiked' *V. faba* infer that greater total net photosynthesis (and hence carbon acquisition) may be exhibited by *V. faba* when supplied with medium ammonia additions (Yoshida, 1972; Clark *et al*, 1999). This was reflected in the significantly greater total soluble carbohydrate; sucrose; glucose; and reducing sugar concentrations which were exhibited in 'spiked' than in 'non-spiked' *V. faba* (figs. 4.3; 4.4; 5.2 - 4.7), throughout water deficits. Thus greater carbon acquisition was exhibited throughout water deficits in *V. faba* which were supplied with medium ammonia additions (as opposed to solely with nitrate);

and also in non-nodulated and nodulated *V. faba* which were supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N; see figs. 4.3; 4.4; 5.2 - 5.7).

Decreases in net photosynthesis coincided with growth decreases during water deficits, and hence photosynthate accumulation during water-deficit associated growth reductions would be minimal. The importance of decreasing starch concentrations (as occurred in *V. faba* during water deficits, figs. 5.8 - 5.11 (pgs. 148 & 149); and as previously reported in other plant species during water deficits, Jones *et al*, 1980; Bussis & Heinke, 1998; Clifford *et al*, 1998), (and of maintained nitrogen assimilation, see section 6.4) in the production of osmotica in *V. faba* during water deficits is thus highlighted. However carbohydrate accumulation was initiated prior to net photosynthesis decreases, and amylase activities plateaued, inferring that net photosynthesis may have contributed some substrates towards osmotic adjustment during slight to moderate water deficits (as previously reported in other plant species, Miflin, 1974; Munns & Weir, 1981).

It is interesting to note that although greater total potential net photosynthetic capacities (figs. 3.20; 3.22; 3.25) and greater total soluble carbohydrate concentrations (figs. 4.3 & 4.4) were exhibited in 'spiked' than in 'non-spiked' *V. faba*, statistically similar total organic acid and starch concentrations were exhibited in 'spiked' and in 'non-spiked' *V. faba* (figs. 5.8; 5.9; 5.12; 5.13). Such similar starch and organic acid concentrations may reflect a greater utilisation as opposed to storage of carbon skeletons in *V. faba* when supplied with medium ammonia additions. Ammonia cannot be stored, and as a toxin requires rapid assimilation (Raven, 1985), as reflected in the significantly greater growth maintenance (figs. 3.9; 3.20; 3.25) which was

exhibited in *V. faba* when supplied with 'combined nitrogen' as opposed to with equimolar '1/2 nitrate' nutrition; and with 'spiked' as opposed to with 'non-spiked' nutrition. Greater dry matter (and leaf area) production have previously been reported in *Casuarina equisetifolia* when supplied with ammonia as opposed to with nitrate (or with N_2) nutrition (Martinez-Carrasco *et al*, 1998), and in *T. aestivum* during saline 'stress' when supplied with ammonia as opposed to with nitrate nutrition (Hawkins & Lewis, 1993).

As such the metabolism as opposed to the storage of nitrogen and carbon compounds may have increased in *V. faba* when supplied with medium ammonia additions. Indeed whereas *V. faba* which were supplied with increasingly concentrated medium nitrate nutrition exhibited significantly increasing nitrate concentrations (which may have contributed towards the increasing RWCs which were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrate nutrition, as nitrate is an effective osmotic solute, Shaner & Boyer, 1976 (particularly as potassium, an osmotic solute, is the counterion for nitrate)), *V. faba* which were supplied with additional medium ammonia did not exhibit significantly greater RWCs, but rather exhibited significantly greater growth of 'productive' plant organs, consistent with the assimilation of, as opposed to the storage of the additional medium ammonia 'spike'. That productive growth, nitrogen assimilatory enzyme activities and osmotic adjustment were maintained at significantly increasing values in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and particularly with medium ammonia additions) may have resulted in an alleviation of 'sink size' feedback inhibition of photosynthesis (Krapp *et al*, 1993) and nitrogen assimilation (Imsande & Touraine, 1994), and may have contributed to the greater levels of net photosynthesis, nitrogen assimilation and productive growth which were

exhibited in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N), and particularly with medium ammonia additions, throughout water deficits. The organic acid concentrations of the leaves and roots of non-nodulated *V. faba* were unaffected by either the form or the concentration of the supplied medium nitrogen nutrition, or by water deficits. The inference is that organic acid concentrations may have been above a threshold level required to operate the 'pH stat', to stimulate nitrate uptake, and to provide carbon skeletons for nitrogen assimilation, and that *V. faba* accumulated sufficiently high concentrations of carbohydrates and amino/imino acids during water deficits to render organic acid accumulation superfluous as an osmotic adaptation. Maintained organic acid concentrations may denote the maintained utilization of carbon skeletons in nitrogen metabolism during water deficits.

Increasingly concentrated medium nitrogen nutrition may reportedly result in the exhibition of increased NR activities (Guerrier, 1991; Bungard et al, 1999); GS activities (Ortega *et al*, 1999); and GDH activities (Taylor & Havill, 1984) in other plant species. Indeed significantly greater NR; GS; and GDH activities were exhibited in non-nodulated and in nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N). Furthermore significantly greater GDH activities were exhibited in *V. faba* when supplied with 'combined nitrogen' than with equimolar '1/2 nitrate' nutrition (inferring an ammonia de-toxification 'role' for GDH), indicating greater nitrogen assimilation in *V. faba* when supplied with medium ammonia additions. Indeed significantly greater NR; GS; and GDH activities were exhibited in 'spiked' than in 'non-spiked' *V. faba*. NR activities may reportedly increase concurrently with ammonia assimilatory enzyme activities (Hofstra *et al*, 1985), and may be stimulated by medium

ammonia (Bungard *et al*, 1999).

In contrast to the bulk of previous literature, NR activities were maintained in the leaves and roots of *V. faba* until water deficits became moderate to severe (until RWCs dropped to below eighty per-cent, figs. 6.2; 6.3; 3.16; 3.17). Maintained NR activities during water deficits have previously been reported in a small number of studies involving other plant species (Smirnoff *et al*, 1985; Ladley, 1990). Maintained NR activities during (moderate to severe) water deficits may have resulted in the continued production of substrates for osmotic adjustment, and components for growth and yield (with which NR activities are reportedly positively correlated, Srivastava, 1980).

The increasing nitrate concentrations which were exhibited in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrate nutrition may have contributed towards maintained nitrate 'fluxes' and hence the increasing NR activities which were exhibited in *V. faba* when supplied with increasingly concentrated medium nitrate nutrition, even during water deficits, during which nitrate may reportedly be mobilized from storage to metabolic pools (Shaner & Boyer, 1976 a&b; Chapin *et al*, 1988). NR is an inducible enzyme which is reportedly continuously synthesized and degraded, and reportedly is synthesized at increasing rates as nitrate availabilities increase (Somers *et al*, 1983).

The decreasing NR activities exhibited in *V. faba* during severe water deficits may have been attributable to the exhibited decreases in net photosynthesis and stomatal conductance (figs. 3.21 & 3.22), as photosynthate availabilities (Foyer *et al*, 1998) and nitrate fluxes (which may be affected by transpiration and hence stomatal conductances during severe water deficits, Shaner & Boyer, 1976) are reportedly regulatory for

NR activities (Shaner & Boyer, 1976). Decreasing photosynthate availabilities may have become apparent in *V. faba* during severe water deficits (fig. 3.22), and as such would coincide with the exhibited NR activity decreases (figs. 4.3; 4.4). Indeed NR activities were maintained during moderate water deficits inferring that they were not sensitively affected by water deficits *per se*.

GS activities were maintained (figs. 6.6 & 6.7), while GDH activities were either maintained or else increased in 'spiked' *V. faba* during water deficits (figs. 6.8 & 6.9). That GDH activities increased in *V. faba* during water deficits infers that medium ammonia additions may incur benefits during severe water deficits, when slight NR activity decreases were exhibited in *V. faba* (figs. 6.2 & 6.3).

Increasing GDH activities during water deficits (as were exhibited in 'spiked' 'no nitrate' and '1/10 nitrate' supplied *V. faba*) may have been mediated via ATP decreases during water deficits (Stewart & Rhodes, 1977; Stryer, 1988; Lam *et al*, 1996; as discussed in section 6.3.1.4, pg. 201). Furthermore the significantly greater glutamine concentrations which were exhibited in the leaves and roots of 'spiked' than in 'non-spiked' *V. faba* may have contributed to the relatively greater GDH as opposed to GS activity increases which were exhibited in 'spiked' *V. faba*, as high glutamine concentrations may reportedly inhibit GS activities and exert positive control over GDH activities (Rhodes *et al*, 1976). Increased GDH activities may reportedly result in increased mitochondrial glutamate and/or α-ketogluterate production, and therefore potentially in increased amino acid (primarily proline and asparagine) synthesis (Venekamp, 1989), as supported by the data (figs. 6.8; 6.9; 4.5; 4.6; tables 6.2- 6.7). Thus

the increasing GDH activities which were exhibited in *V. faba* when supplied with medium ammonia additions (as exemplified by the greater GDH activities which were exhibited in *V. faba* when supplied with 'combined nitrogen' as opposed to with equimolar '1/2 nitrate' nutrition) may have contributed towards the significantly greater amino acid concentrations and hence osmotic adjustment which were exhibited in 'spiked' than in 'non-spiked' *V. faba* (section 4.4). Negative water potentials may reportedly decrease the kM (ammonia) of GDH and asparagine synthetase, resulting in increased GDH activities and asparagine production (Venekamp, 1989), as supported by the data. Photosynthetic decreases during water deficits may necessitate protein respiration and subsequent ammonia release, increasing the need for ammonia assimilation and proline / asparagine accumulation, the synthesis of which also results in H^+ removal during water deficits (Venekamp, 1989).

That *V. faba* supplied with increasingly concentrated medium nitrogen nutrition exhibited significantly greater primary nitrogen assimilatory enzyme activities was reflected in the significantly greater concentrations of the products of primary nitrogen assimilation (glutamate; asparagine; glutamine; allantoin) which were exhibited in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with medium ammonia additions. Indeed asparagine; proline; alanine; and threonine accumulated significantly during water deficits, and were exhibited at significantly increasing concentrations in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N), and particularly with medium ammonia additions (i.e. in 'spiked' *V. faba*). Some amino acids which accumulated significantly during water deficits were carbon economic (i.e. had high N:C ratios, for example asparagine and

allantoin, Sprent, 1980). Proline also accumulated significantly in *V. faba* during water deficits. The slow turnover of proline results in proline accumulation at a minimal cost of synthesis (Rhodes *et al*, 1986), and proline accumulation has previously been positively correlated with leaf tissue survival and with water deficit tolerance (Singh *et al*, 1973a), and also with post water deficit growth (Aspinall & Paleg, 1981) in other plant species. 'Roles' were hypothesized for individual accumulating amino acids in ammonia de-toxification; in nitrogen storage; in carbon economy; in metabolic flexibility; and in the prevention of feedback inhibition of the TCA cycle during water deficits (see section 6.3.5, pgs. 231 - 232). Significantly greater aminotransferase activities were also exhibited in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with ammonia 'spike' nutrition; a further reflection of the increased nitrogen metabolism which was exhibited in *V. faba* when supplied with medium ammonia additions.

It is thus apparent that nitrogen acquisition was greater in 'spiked' than in 'non-spiked' *V. faba*. Increased nitrogen assimilation is associated with yield increases (Hageman, 1979; Srivastava, 1980; Marschner, 1986; Muller & Janiesch, 1993), and with a reduced dependence on nitrogen fixation (Caba *et al*, 1998) in other plant species. Continued nitrogen assimilation may reportedly also result in an alleviation of photoinhibition during water deficits (Smirnoff & Stewart, 1985), which may contribute towards increased plant productivities and plant survival prospects upon re-hydration.

As water, carbon and nitrogen acquisition were greater in 'spiked' than in 'non-spiked' *V. faba*, more substrates were potentially available for

osmotic adjustment. Indeed significantly greater total soluble carbohydrates, amino acids and glycine betaine accumulated in 'spiked' than in 'non-spiked' *V. faba* during water deficits (see section 4.4). Significantly greater proline accumulated in non-nodulated and nodulated *V. faba* which were supplied with 'combined nitrogen' as opposed to with equimolar '1/2 nitrate' (or with less concentrated nitrogen) nutrition (figs. 4.7 & 4.8), and significantly greater total osmolarities were recorded in 'spiked' than in 'non-spiked' *V. faba* (figs. 4.11 & 4.12). Furthermore greater total soluble carbohydrate; total amino acid; proline; and glycine betaine concentrations were exhibited in the roots of non-nodulated and nodulated *V. faba* when supplied with 'combined nitrogen' as opposed to with equimolar '1/2 nitrate' (or with less concentrated nitrate) nutrition; and in 'spiked' than in 'non-spiked' *V. faba*. Increased root osmotic adjustment, as was exhibited in *V. faba* when medium ammonia additions were included during water deficits, further infers an increased capacity for water uptake (Boyer, 1985). Plants which accumulate greater concentrations of osmotic solutes reportedly extract more water during water deficits (Kumar & Singh, 1998; Collinson *et al*, 1997). Increased water uptake may have been particularly apparent in *V. faba* when supplied with medium ammonia additions as significantly greater root biomasses were exhibited in 'spiked' than in 'non-spiked' *V. faba*, and root growth is also reportedly correlated with water uptake in *V. faba* (Sau & Ines-Minguez, 1990).

The significantly greater concentrations of compatible solutes (sucrose; glycine betaine; proline; glutamate; alanine; and threonine; Nash *et al*, 1981; Paleg *et al*, 1985; Ingram & Bartels, 1996; Clifford *et al*, 1998; Ortega *et al*, 1999; see introduction, pg. 16 & section 6.3.5, pg. 232),

which were exhibited in non-nodulated (and nodulated) *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with medium ammonia additions, may have afforded some protection towards NR (and other enzymes, e.g. photosynthetic enzymes as compatible solutes may accumulate in the cytoplasm and in chloroplasts, Bussis & Heinke, 1998; Rhodes *et al* 1986; proline and glycine betaine may increase the heat stability of GS, Smirnoff & Stewart, 1985), and may therefore have contributed towards the maintenance of metabolism during increasingly severe water deficits in *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (and particularly with medium ammonia additions).

It is imperative for growth that plants maintain a threshold turgor during water deficits (Brownlee *et al*, 1999). That substantial osmotic solute concentrations; growth; and RWCs were all maintained at significantly increasing levels in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition during water deficits infers that solute accumulation as opposed to cellular volume decreases may have been responsible for the exhibited osmotic adjustment. Increasing osmotic adjustment (as was exhibited in non-nodulated and in nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition, and particularly with medium ammonia additions) during water deficits is reportedly correlated with increasing RWCs (Singh & Gupta, 1983); increasing stomatal conductances (and by inference carbon acquisition); increasing medium-water extraction (Kumar & Singh, 1998); and with the exhibition of increasing yields (Van der Wal, 1981; Rodriguez-Maribona *et al*, 1992) in other plant species.

Significantly greater cumulative leaf areas, plant heights, plant root growth, nitrogen assimilatory enzyme activities and total carbohydrate and amino acid concentrations were exhibited in *V. faba* when supplied with medium ammonia additions, even during periods of adequate irrigation, inferring that medium ammonia additions prior to water deficit imposition may pre-dispose *V. faba* to an increased tolerance to water deficits.

That greater GDH activities; osmotic adjustment; and inferred net photosynthesis were maintained in non-nodulated and nodulated *V. faba* when supplied with 'combined nitrogen' as opposed to with equimolar '1/2 nitrate' nutrition, and with 'spiked' as opposed to with 'non-spiked' nutrition during water deficits may also reflect the previously reported observation that ammonium assimilation may be more economical in terms of photons; water; and metal ions (Mg; Fe) per unit carbon assimilated per unit time than nitrate or N_2 assimilation, both in *V. faba* (Hebblethwaite *et al*, 1984; Sutherland *et al*, 1985), and in other plant species (Ines-Minguez & Sau, 1989; Raven & Sprent, 1993; Martinez-Carrasco *et al*, 1998). The inference is that *V. faba* supplied with medium ammonia additions may incur fewer metabolic perturbations during water deficits, due to a more efficient 'water economy', which would in turn result in increased nitrogen and carbon assimilation capabilities, and hence in an increased capacity for osmotic adjustment during water deficits (see fig. 7.1). The total energy expenditure of a plant which assimilates nitrate (which requires more assimilation steps than ammonia assimilation) is reportedly around fifteen per cent of the plant's total energy production, while ammonia assimilation reportedly expands between two and five per cent of the total energy production of a plant (Bloom, 1988), inferring that more 'energy' may be available to plants when supplied with some medium ammonia additions. Increasing medium ammonia additions may result in increased ammonia

and nitrate uptake (Ourry *et al*, 1997).

The introduction highlighted previous reports which concluded that medium ammonia nutrition may result in the exhibition of plant toxicity problems (Tolley-Henry & Raper, 1986; see pg. 6). However as a legume *V. faba* is adapted to receive nitrogen from the roots via nitrogen fixation, via nitrate reduction (which is also predominantly a root phenomenon in *V. faba*, Sutherland *et al*, 1985; figs. 6.6 & 6.7), or via ammonia assimilation (which is also a root phenomenon). Indeed similarities are noted when nitrogen fixation and ammonia assimilation are compared, for example xylem sap compositions are similar in ammonia supplied and in nitrogen fixing legumes (Baker *et al*, 1997), and ammonia assimilation and nitrogen fixation both result in the production of H^+ ions (Raven, 1985). The entire physiology of *V. faba* may be predisposed towards root assimilation, and ammonia toxicity symptoms were not exhibited in 'spiked' *V. faba* (which were supplied with ammonia additionally to nitrate). Indeed Troelstra *et al*, (1992) working with *Myrica gale*, a species which predominantly assimilates nitrate in the roots and is also capable of nitrogen fixation, reported that RGRs were exhibited in the following order with respect to medium nitrogen nutrition: 'ammonia'> 'combined nitrogen' > 'nitrate' = 'atmospheric nitrogen'.

However the collected data contrasts with that of Raab & Terry (1994) who reported that less (root and particularly) shoot growth, and lower leaf areas were exhibited in *Beta vulgaris* when supplied with ammonia as opposed to with nitrate nutrition, as attributed to the reported exhibition of lower osmolyte concentrations in *B. vulgaris* when supplied with ammonia than with nitrate nutrition.

Ammonia does not accumulate in many soils (with the possible exception of acidic soils), as ammonia may quickly be nitrified into nitrite and then

nitrate (by e.g. *Nitrosomonas* and *Nitrobacter*; Sprent & Thomas, 1984), indicating that even when medium ammonia additions are supplied, plants may predominantly utilise nitrate (Lewis, 1986). Nitrification of ammonia may have contributed towards the observed lack of ammonia-associated toxicity symptoms in *V. faba*, and towards the maintained NR activities exhibited by *V. faba* when supplied with medium ammonia additions (see also Bungard *et al*, 1999).

The collected data indicates improved productivities in non-nodulated and nodulated *V. faba* when supplied with medium nitrogen nutrition (> 0.8 mM N, and particularly with medium ammonia additions) during water deficits, as opposed to when reliant on nitrogen fixation, as attributable to increased capacities to maintain plant RWCs, carbon acquisition, nitrogen assimilation, and osmotic adjustment (fig. 7.1). Thus plant metabolism and growth were maintained at lower external water potentials in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N), and particularly with medium ammonia additions. Indeed turgor maintenance in *V. subterranea* during water deficits reportedly required growth reductions, the regulation of transpiration via stomatal control, and osmotic adjustment (Collinson *et al*, 1997; see fig. 7.1).

Ultimately figs. 6.12 & 6.13 illustrate that *V. faba* exhibited greater leaf and root protein concentrations when supplied with 'combined nitrogen' as opposed to with equimolar nitrate nutrition; and greater root protein concentrations (and CLAs) when supplied with ammonia 'spiked' as opposed to with 'non-spiked' nutrition. Increasing protein concentrations (and CLAs) represent important nutritional and economic considerations for this leguminous crop (Pirie, 1979; Snoad, 1981), particularly as the vegetative

yield of *V. faba* may be utilised as silage and green manure (Lawes, 1980; Corak *et al*, 1992). Protein concentrations decreased significantly during water deficits, however amino acid accumulation preceded protein decreases suggesting a 'role' for primary nitrogen assimilation in the production of nitrogenous osmotica, and protein concentrations were maintained at increasing values in non-nodulated and nodulated *V. faba* when supplied with increasingly concentrated medium nitrogen nutrition (> 0.8 mM N), and particularly with medium ammonia additions (figs. 6.12 & 6.13).

The collected data contrasts with the work of Plies-Balzer *et al*, (1995), who concluded that mineral nitrogen fertilization could not compensate for the negative effects of water deficits on the yields of nodulated *V. faba* (cv. 'Alfred'), and that it was necessary to establish optimal growth conditions for the exhibition of high dry matter and seed yields in *V. faba*. However increased dry matter yields were exhibited in *V. faba* (cv. 'Bunyards Exhibition') when supplied with medium nitrogen nutrition (> 0.8 mM N), as opposed to when reliant on nitrogen fixation, throughout water deficits.

V. faba is an effective nodulator, and <0.8 mM nitrogen resulted in nitrogen fixation inhibition, and in the exhibition of lower (vegetative) yields than were exhibited by nitrogen fixing V. faba
The collective data inferred that nitrogen fixation may not be water deficit sensitive in *V. faba* (cv. 'Bunyards Exhibition')
Significantly greater growth was exhibited by *V. faba* when supplied with increasingly concentrated medium nitrogen (> 0.8 mM N), as opposed to when reliant on nitrogen fixation
Ammonia 'spiked' as opposed to 'non-spiked' *V. faba* exhibited significantly greater root biomasses, heights, and cumulative leaf areas, and hence potential net photosynthesis and water uptake capacities were greater in nitrogen supplied (particularly ammonia 'spiked') *V. faba* than in nitrogen fixing *V. faba*
Ammonia 'spiked' as opposed to 'non-spiked' *V. faba* exhibited significantly greater (leaf and root) osmotic adjustment, and hence potential water deficit tolerance was greater in nitrogen supplied (particularly ammonia 'spiked') *V. faba* than in nitrogen fixing *V. faba*
Significantly greater nitrogen assimilatory enzyme activities (NR; GS; GDH) were exhibited by ammonia 'spiked' than by 'non-spiked' *V. faba*, and hence greater nitrogen assimilation was exhibited by medium nitrogen supplied (particularly ammonia 'spiked') *V. faba* as opposed to by nitrogen fixing *V. faba*
Nitrate reductase activities were not sensitive to gradually imposed water deficits
Significantly greater root protein concentrations, and cumulative leaf areas were exhibited by ammonia 'spiked' than by 'non-spiked' *V. faba*, both when supplied with adequate irrigation and during water deficits, inferring that economic advantages may be incurred by the provision of medium nitrogen (particlarly with medium ammonia additions) to *V. faba*

Table 7.1 Key Research Outcomes

Field trials would establish the applicability of the reported conclusions in agricultural scenarios, as controlled conditions do not fully mimic field conditions. In the field plants experience multiple biotic and abiotic influences, particularly in the radiant energy field, which influence the development of photoprotective systems (Wise *et al*, 1994),

and in potential medium nitrification (Sprent & Thomas, 1984). UVB radiation (as experienced in the field) results in increased stomatal closure (Nogues *et al*, 1998). Furthermore the inferred effects of medium ammonia additions on total carbon, nitrogen and water acquisition may decrease in the field due to planting density factors, competition from weeds etc.

However the yield benefits described for *V. faba* when supplied with medium ammonia additions are of interest as nitrate is susceptible to leaching in the field (Raven, 1985), rendering concentrated field nitrate applications environmentally undesirable. Furthermore if ammonia proves a beneficial medium supplicant in the field economic benefits may incur, as ammonia is cheaply available in the form of bird droppings.

Further work would also allow qualification of 'non-specified amino' compounds and of their 'roles' during water deficits. Water deficits induce an array of late embryogenesis abundant proteins (LEAs) in plants (e.g. dehydrin, Bray, 1997), which act like compatible solutes (Ingram & Bartels, 1996), sequestering ions which would otherwise be damaging when water contents are low (Close, 1996). 'Non specified amino' compounds may comprise LEAs; short peptides; non-quantified amino acids; amines; non-protein amino acids (Bennet & Wallsgrove, 1994); polyamines etc.

Water deficit tolerance adaptations may be significant considering predictions that global temperatures are rising. With expected increases in CO_2 concurrent with water deficits, stomatal resistance to CO_2 may result (Van Oosten & Besford, 1996), and plant growth and therefore sink strength may be reduced. However species which exhibit increased osmotic adjustment during water deficits may maintain stomatal opening (and therefore potentially carbon

acquisition, McDonald & Davies, 1996) during more severe water deficits if additional nitrogen (and specifically ammonia in *V. faba*) is supplied to maintain sink strength and to enable the production of osmotica (Sims *et al*, 1998), and to enable increased plant growth and increased potential plant yields.

APPENDIX I
LONG ASHTON MEDIA RECIPES

'NO NITRATE'

MACRONUTRIENTS:
$KHPO_4$, 4.0 mM;
$MgSO_4$, 1.5 mM;
$CaCl_2$, 4.0mM;
FeNa EDTA, 0.1 mM

MICRONUTRIENTS:
$MnSO_4$, 0.01 mM;
$ZnSO_4$, 0.001 mM;
$CuSO_4$, 0.001 mM;
HBO_3, 0.05 mM;
$NaMoO_4$, 0.0005 mM;
NaCl, 0.1 mM

'1/2 NITRATE'

MACRONUTRIENTS:
KNO_3, 2.0 mM;
$Ca(NO_3)_2$, 2.0 mM;
$KHPO_4$, 4.0 mM;
$MgSO_4$, 1.5 mM;
$CaCl_2$, 4.0mM;
FeNa EDTA, 0.1 mM

MICRONUTRIENTS:
$MnSO_4$, 0.01 mM;
$ZnSO_4$, 0.001 mM;
$CuSO_4$, 0.001 mM;
HBO_3, 0.05 mM;
$NaMoO_4$, 0.0005 mM;
NaCl, 0.1 mM

'1/10 NITRATE'

MACRONUTRIENTS:
KNO_3, 0.4 mM;
$Ca(NO_3)_2$, 0.4 mM;
$KHPO_4$, 4.0 mM;
$MgSO_4$, 1.5 mM;
$CaCl_2$, 4.0mM;
FeNa EDTA, 0.1 mM

MICRONUTRIENTS:
$MnSO_4$, 0.01 mM;
$ZnSO_4$, 0.001 mM;
$CuSO_4$, 0.001 mM;
HBO_3, 0.05 mM;
$NaMoO_4$, 0.0005 mM;
NaCl, 0.1 mM

'COMBINED NITROGEN'

MACRONUTRIENTS:
$KHPO_4$, 4.0 mM;
NH_4NO_3, 4.0 mM;
$MgSO_4$, 1.5 mM;
$CaCl_2$, 4.0mM;
FeNa EDTA, 0.1 mM

MICRONUTRIENTS:
$MnSO_4$, 0.01 mM;
$ZnSO_4$, 0.001 mM;
$CuSO_4$, 0.001 mM;
HBO_3, 0.05 mM;
$NaMoO_4$, 0.0005 mM;
NaCl, 0.1 mM

APPENDIX II

ANALYSIS OF VARIANCE (ANOVA)

Research involved comparisons of the effects of (a) four nitrogen sources, and (b) six levels of water deficit imposition on various parameters of plant metabolism (e.g. osmotic adjustment, enzyme activities etc.) within three overall nitrogen schemes (see fig. 2.1). Anova is a technique which partitions the variance in a set of data into several components in such a way that the contribution of each component to the overall data set may be assessed.

MAIN ASSUMPTIONS of ANOVA (after Kvanli *et al*, 1992)

1. Replicates must be independent and random from each population - the value of one observation must not influence any other value. Error variance was minimized by the employment of random sampling techniques, as discussed in section 2.7. For example one cultivar was used, one seed supplier was used, and similar sized seeds were selected. Furthermore all plants received identical treatment with the exception of the analysed factors i.e. water deficit regime and nitrogen regime (and medium physical state). Treatments were distributed in random blocks, and pots were well spaced to minimise mutual shading.

2. The observations / replicates from each population must follow (approximately) a normal distribution. Lack of normality is not critical and providing departure is not extreme, and replicates are taken (as in this research), and sample sizes are equal, the F test used in ANOVA is only slightly affected. Anderson-Darling calculations showed normality within samples, especially considering the large sample numbers.

3. The measured variable is continuous.

ANOVA determined whether variations in each data set were attributable to the nitrogen treatment, water deficit treatment, or were attributable to error variance (i.e. within-group variance, due to e.g. genetic differences between individuals).

$$\frac{\text{between groups (treatment/s) variance}}{\text{error variance}}$$

is the F distribution.

If a treatment had no effect, virtually all of the variance in the data would be attributable to error variance, and the variance ratio would be very low. H_o was rejected if 'F crit' < 'F calc'.

As two factors were of interest, (i) water deficits and (ii) nitrogen treatments, two-way ANOVA was employed, which tested:

(i) H_{oa} - the insignificance of water deficit for each measured parameter,

(ii) H_{ob} - the insignificance of nitrogen treatment for each measured parameter, and

(iii) $H_{o\ ab}$ - that there was no interaction between nitrogen source and water deficit for each measured parameter.

Calculations were performed using statworks and minitab software, which calculated 'F calc' values for factors (i), (ii), and (iii) above. These values were compared against tables of 'F crit' values for the various degrees of freedom (Fisher & Yates, 1963), with α set at 0.01, i.e. one in one-hundred sample means could be attributable to error for all data (except for individual amino acids data, where n was smaller, and α was accordingly set at 0.05 - i.e. one in twenty sample means could be attributable to error).

APPENDIX II (a) was constructed by comparing the data for each parameter within each nitrogen scheme (a-c fig 2.1; (a) non-nodulated *V. faba*, (b) nodulated *V. faba*, and (c) 'spiked' *V. faba*) separately. Within each nitrogen scheme the effects of all four nitrogen sources ('no nitrate', '1/10 nitrate', '1/2 nitrate', and 'combined nitrogen'), along with the effects of increasing water deficits were tested for the significance of effect on the measured parameter. The interactive effects of nitrogen source and water deficits were also evaluated. As such Appendix II (a) records the significance of the effects of different medium nitrogen sources, and of the effects of increasing water deficits on each parameter (within all three nitrogen schemes).

APPENDIX II (b) was constructed by comparing the effects of only two nitrogen sources. For each parameter data obtained from non-nodulated 'no nitrate' *V. faba* were compared against data obtained from nodulated 'no nitrate' *V. faba* during water deficits. It has been explained that any significantly different responses between *V. faba* from these two groups may be attributed to N-fixation. Thus, Appendix II (b) examines whether parameters differed significantly in N-fixing 'no nitrate' supplied *V. faba* as compared against non N-fixing 'no nitrate' supplied *V. faba* during water deficits.

APPENDIX II (c) was constructed by comparing all of the data for a specified parameter from non-nodulated 'non-spiked' *V. faba* against all of the data for that parameter from non-nodulated 'spiked' *V. faba*, during increasing water deficits. Thus appendix II (c) examines whether 'ammonia spike' nutrition resulted in significant differences in each parameter as compared against 'non spike' nutrition during increasing water deficits.

The following tables consist of 'F calc' and 'F crit' values following anova analyses. Appendix II (a) also gives correlation values for GDH activities with increasing water deficits.

Where the nitrogen source, water deficits, or interactions between the two are deemed to have significantly affected the measured parameter F values are given in **bold.**

APPENDIX II (a)

		N TREATMENT		WATER DEFICITS		INTERACTION		
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit	
LEAF	Non-nodulated	469.31	4.2	168.65	3.4	14.3	2.4	
FRESH WEIGHT	Nodulated	13.04	4.2	43.6	3.4	0.97	2.4	
	Spiked	208.28	4.2	85.1	3.4	7.43	2.4	
STEM	Non-nodulated	395.92	4.2	51.75	3.4	2.91	2.4	
FRESH WEIGHT	Nodulated	29.42	4.2	32.35	3.4	1.28	2.4	
	Spiked	246.94	4.2	37.95	3.4	2.84	2.4	
ABOVE-MEDIUM	Non-nodulated	776.92	4.2	169.87	3.4	11.66	2.4	
FRESH WEIGHT	Nodulated	24.72	4.2	46.57	3.4	1.12	2.4	
	Spiked	321.95	4.2	79.96	3.4	6.25	2.4	
ROOT	Non-nodulated	197.25	4.2	46.59	3.4	1.3	2.4	
FRESH WEIGHT	Nodulated	42.55	4.2	36.86	3.4	0.46	2.4	
	Spiked	241.85	4.2	55.98	3.4	2.07	2.4	
TOTAL	Non-nodulated	175.06	4.2	35.35	3.4	2.1	2.4	
FRESH WEIGHT	Nodulated	38.49	4.2	44.47	3.4	0.058	2.4	
	Spiked	336.16	4.2	81.26	3.4	4.34	2.4	
LEAF	Non-nodulated	363.43	4.2	68.57	3.4	8.01	2.4	
DRY WEIGHT	Nodulated	15.79	4.2	38.48	3.4	1.27	2.4	
	Spiked	199.82	4.2	45.76	3.4	5.41	2.4	
STEM	Non-nodulated	343.31	4.2	11.83	3.4	1.49	2.4	
DRY WEIGHT	Nodulated	22.6	4.2	12.76	3.4	0.99	2.4	
	Spiked	152.58	4.2	8.99	3.4	1.23	2.4	
ABOVE-MEDIUM	Non-nodulated	565.4	4.2	50.59	3.4	5.54	2.4	
DRY WEIGHT	Nodulated	22.9	4.2	27.78	3.4	0.92	2.4	
	Spiked	269.67	4.2	32.41	3.4	4.06	2.4	
ROOT	Non-nodulated	92.68	4.2	1.98	3.4	1.33	2.4	
DRY WEIGHT	Nodulated	41.78	4.2	13.62	3.4	0.44	2.4	
	Spiked	178.41	4.2	6.12	3.4	1.84	2.4	
TOTAL	Non-nodulated	317.1	4.2	19.69	3.4	3.09	2.4	
DRY WEIGHT	Nodulated	35.13	4.2	20.09	3.4	0.54	2.4	
	Spiked	208.51	4.2	19.98	3.4	2.34	2.4	
LEAF	Non-nodulated	27.81	4.2	110.62	3.4	3.97	2.4	
FW : DW	Nodulated	5.92	4.2	62.9	3.4	1.57	2.4	
	Spiked	16.08	4.2	117.12	3.4	3.69	2.4	

APPENDIX II (a)

		N TREATMENT		WATER DEFICITS		INTERACTION	
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit
STEM	Non-nodulated	18.29	4.2	43.32	3.4	1.08	2.4
FW : DW	Nodulated	13.01	4.2	55.34	3.4	1.6	2.4
	Spiked	0.96	4.2	1.22	3.4	1.01	2.4
ABOVE-MEDIUM	Non-nodulated	54.31	4.2	222.3	3.4	6.72	2.4
FW : DW	Nodulated	14.21	4.2	95.83	3.4	2.19	2.4
	Spiked	27.88	4.2	201.53	3.4	4.94	2.4
ROOT	Non-nodulated	82.94	4.2	347.4	3.4	11.71	2.4
FW : DW	Nodulated	13.59	4.2	100.37	3.4	1.26	2.4
	Spiked	28.71	4.2	183.77	3.4	4.76	2.4
TOTAL	Non-nodulated	7.68	4.2	32.04	3.4	9.71	2.4
FW : DW	Nodulated	7.59	4.2	57.86	3.4	1.26	2.4
	Spiked	5.9	4.2	41.49	3.4	3.76	2.4
R : S	Non-nodulated	1041.99	4.2	165.61	3.4	9.95	2.4
	Nodulated	6.11	4.2	24.19	3.4	1.97	2.4
	Spiked	175.29	4.2	40.33	3.4	1.47	2.4
LEAF RWC	Non-nodulated	81.48	4.2	341.94	3.4	10.05	2.4
	Nodulated	21.36	4.2	182.18	3.4	2.48	2.4
	Spiked	57.4	4.2	335.09	3.4	7.29	2.4
ROOT RWC	Non-nodulated	212.59	4.2	1015.1	3.4	27.7	2.4
	Nodulated	26.34	4.2	209.29	3.4	2.19	2.4
	Spiked	4.5	4.2	9.23	3.4	0.64	2.4
RELATIVE	Non-nodulated	1654.57	4.2	65.4	3.4	6.35	2.4
GROWTH RATE	Nodulated	1	4.2	1	3.4	1	2.4
	Spiked	294.83	4.2	66.2	3.4	4.43	2.4
HEIGHT	Non-Nodulated	42.08	4.2	14.81	3.4	8.45	2.4
	Nodulated	37.64	4.2	27.05	3.4	4.89	2.4
	Spiked	18.15	4.2	15.49	3.4	5.77	2.4
LEAF	Non-nodulated	8.56	4.2	1.92	3.4	0.99	2.4
AREA	Nodulated	2.62	4.2	2.4	3.4	0.23	2.4
RATIO	Spiked	18.33	4.2	3.96	3.4	0.74	2.4
CUMULATIVE	Non-nodulated	41.36	4.2	4.73	3.4	1.22	2.4
LEAF AREA	Nodulated	12.3	4.2	44.58	3.4	1.15	2.4
	Spiked	41.86	4.2	21.58	3.4	1.8	2.4

APPENDIX II (a)

		N TREATMENT		WATER DEFICITS		INTERACTION		
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit	
STOMATAL	Non-nodulated	1.07	4.2	1.06	3.4	1	2.4	
CONDUCTANCE	Nodulated	63.47	4.2	366.05	3.4	3.46	2.4	
	Spiked	1.01	4.2	1	3.4	1	2.4	
PHOTOSYNTHESIS	Non-nodulated	305.67	4.2	252.01	3.4	5.48	2.4	
	Nodulated	83.14	4.2	360.51	3.4	3.47	2.4	
	Spiked	119.1	4.2	196.54	3.4	2.64	2.4	
NET	Non-nodulated	344.45	4.2	15.15	3.4	2.05	2.4	
ASSIMILATION	Nodulated	41.05	4.2	11.5	3.4	0.6	2.4	
RATE	Spiked	327.55	4.2	8.72	3.4	1.57	2.4	
SOLUBLE	leaves non-nodulated	612.54	4.2	31.66	3.4	10.76	2.4	
CARBOHYDRATE	leaves nodulated	62.46	4.2	34.19	3.4	15.73	2.4	
	leaves spiked	409.64	4.2	132.28	3.4	21.01	2.4	
	roots non-nodulated	131.19	4.2	11.26	3.4	3.34	2.4	
	roots nodulated	19.17	4.2	23.74	3.4	2.68	2.4	
	roots spiked	86.96	4.2	26.01	3.4	7.15	2.4	
TOTAL	leaves non-nodulated	136.76	4.2	25.61	3.4	6.92	2.4	
AMINO	leaves nodulated	51.44	4.2	116.8	3.4	7.52	2.4	
ACIDS	leaves spiked	17.25	4.2	89.02	3.4	3.55	2.4	
	roots non-nodulated	153.92	4.2	31.51	3.4	4.59	2.4	
	roots nodulated	40.02	4.2	51.88	3.4	1.72	2.4	
	roots spiked	103.94	4.2	49.38	3.4	7.47	2.4	
PROLINE	leaves non-nodulated	148.98	4.2	157.22	3.4	31.08	2.4	
	leaves nodulated	13.95	4.2	63.58	3.4	5.37	2.4	
	leaves spiked	60.57	4.2	218.86	3.4	11.78	2.4	
	roots non-nodulated	54.43	4.2	67.75	3.4	14.13	2.4	
	roots nodulated	27.36	4.2	118.49	3.4	15.55	2.4	
	roots spiked	20.61	4.2	392.18	3.4	15.35	2.4	
GLYCINE	leaves non-nodulated	23.73	4.2	46.14	3.4	4.33	2.4	
BETAINE	leaves nodulated	20.13	4.2	509.84	3.4	10.47	2.4	
	leaves spiked	516.14	4.2	611.23	3.4	225.2	2.4	
	roots non-nodulated	38.22	4.2	45.59	3.4	8.16	2.4	
	roots nodulated	118.41	4.2	243.85	3.4	36.66	2.4	
	roots spiked	2720.62	4.2	3274.2	3.4	1545	2.4	

APPENDIX II (a)

		N TREATMENT		WATER DEFICITS		INTERACTION		
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit	
TOTAL	leaves non-nodulated	525.19	4.2	263.9	3.4	35.16	2.4	
OSMOLARITY	leaves nodulated	92.53	4.2	680.78	3.4	9.5	2.4	
	leaves spiked	480.34	4.2	623.17	3.4	46.04	2.4	
	roots non-nodulated	1152.95	4.2	692.28	3.4	89.91	2.4	
	roots nodulated	119.73	4.2	786.56	3.4	19.96	2.4	
	roots spiked	745.03	4.2	639.91	3.4	59.3	2.4	
SUCROSE	leaves non-nodulated	322.49	4.2	171.46	3.4	34.62	2.4	
	leaves nodulated	61.6	4.2	195.74	3.4	13.91	2.4	
	leaves spiked	324.58	4.2	341.3	3.4	56.55	2.4	
	roots non-nodulated	126.31	4.2	74.03	3.4	6.96	2.4	
	roots nodulated	156.62	4.2	150.11	3.4	15.64	2.4	
	roots spiked	97.28	4.2	41.97	3.4	5.65	2.4	
GLUCOSE	leaves non-nodulated	420.27	4.2	105.33	3.4	46.23	2.4	
	leaves nodulated	157.82	4.2	131.15	3.4	20.09	2.4	
	leaves spiked	899.97	4.2	215.95	3.4	53.59	2.4	
	roots non-nodulated	187.27	4.2	186.7	3.4	26.94	2.4	
	roots nodulated	83.61	4.2	229.41	3.4	14.97	2.4	
	roots spiked	376.02	4.2	155.5	3.4	26.55	2.4	
REDUCING SUGARS	leaves non-nodulated	244.54	4.2	78.15	3.4	37.44	2.4	
	leaves nodulated	5.27	4.2	142.14	3.4	2.18	2.4	
	leaves spiked	388.55	4.2	131.86	3.4	29.71	2.4	
	roots non-nodulated	100.91	4.2	104.06	3.4	10.44	2.4	
	roots nodulated	7.25	4.2	37.13	3.4	1.66	2.4	
	roots spiked	128.54	4.2	91.02	3.4	13.77	2.4	
STARCH	leaves non-nodulated	631.01	4.2	191.29	3.4	20.52	2.4	
	leaves nodulated	116.27	4.2	208.74	3.4	7.29	2.4	
	leaves spiked	41.91	4.2	16.06	3.4	1.04	2.4	
	roots non-nodulated	378.71	4.2	109.99	3.4	13.2	2.4	
	roots nodulated	26.57	4.2	165.98	3.4	4.62	2.4	
	roots spiked	316.26	4.2	150.44	3.4	16.36	2.4	
AMYLASE	leaves non-nodulated	3.91	4.2	89.43	3.4	1.14	2.4	
	leaves nodulated	0.25	4.2	81.09	3.4	0.39	2.4	
	leaves spiked	0.25	4.2	137.5	3.4	0.98	2.4	
	roots non-nodulated	1.31	4.2	110.31	3.4	1.86	2.4	
	roots nodulated	54.69	4.2	141.4	3.4	45.45	2.4	
	roots spiked	1.04	4.2	66.38	3.4	1.96	2.4	

APPENDIX II (a)

		N TREATMENT		WATER DEFICITS		INTERACTION		
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit	
TOTAL	leaves non-nodulated	0.21	3.03	0.97	2.64			
MEASURED	leaves nodulated	1.3	3.03	2.73	2.64			
ORGANIC	leaves spiked	0.84	3.03	1.62	2.64			
ACIDS	roots non-nodulated	1.94	3.03	2.13	2.64			
	roots nodulated	1.64	3.03	3.14	2.64			
	roots spiked	0.63	3.03	1.42	2.64			
2-OXOGLUTERATE	leaves non-nodulated	1.5	3.03	0.69	2.64			
	leaves nodulated	2.99	3.03	1.58	2.64			
	leaves spiked	1.76	3.03	0.93	2.64			
	roots non-nodulated	0.58	3.03	1.66	2.64			
	roots nodulated	0.47	3.03	0.11	2.64			
	roots spiked	0.36	3.03	0.91	2.64			
MALATE	leaves non-nodulated	0.65	3.03	1.32	2.64			
	leaves nodulated	0.7	3.03	1.41	2.64			
	leaves spiked	0.34	3.03	1.14	2.64			
	roots non-nodulated	2.71	3.03	0.36	2.64			
	roots nodulated	1.03	3.03	1	2.64			
	roots spiked	0.88	3.03	0.93	2.64			
PYRUVATE	leaves non-nodulated	0.99	3.03	0.14	2.64			
	leaves nodulated	1.12	3.03	8.95	2.64			
	leaves spiked	1.63	3.03	1.29	2.64			
	roots non-nodulated	1.14	3.03	0.87	2.64			
	roots nodulated	2.14	3.03	3.49	2.64			
	roots spiked	0.92	3.03	0.83	2.64			
CITRATE	leaves non-nodulated	2.56	3.03	1	2.64			
	leaves nodulated	0.79	3.03	1.41	2.64			
	leaves spiked	1.28	3.03	0.96	2.64			
	roots non-nodulated	1.8	3.03	1.39	2.64			
	roots nodulated	1.48	3.03	0.37	2.64			
	roots spiked	1.71	3.03	1.1	2.64			
THREONINE	leaves non-nodulated	7.18	2.33	6.69	2.64			
	leaves nodulated	0.22	2.33	111.32	2.64			
	leaves spiked	0.23	2.33	91.88	2.64			
	roots non-nodulated	4.91	2.33	9.42	2.64			
	roots nodulated	0.03	2.33	66.94	2.64			
	roots spiked	0.59	2.33	63.71	2.64			

APPENDIX II (a)

		N TREATMENT		WATER DEFICITS		INTERACTION		
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit	
GLUTAMINE	leaves non-nodulated	74.79	2.33	1.38	2.64			
	leaves nodulated	5.66	2.33	0.63	2.64			
	leaves spiked	40.13	2.33	1.7	2.64			
	roots non-nodulated	145.59	2.33	1.76	2.64			
	roots nodulated	14.72	2.33	1.54	2.64			
	roots spiked	39.59	2.33	0.93	2.64			
GLUTAMATE	leaves non-nodulated	144.48	2.33	0.67	2.64			
	leaves nodulated	11.36	2.33	0.27	2.64			
	leaves spiked	19.66	2.33	0.91	2.64			
	roots non-nodulated	565.1	2.33	2.97	2.64			
	roots nodulated	17.13	2.33	2.29	2.64			
	roots spiked	1.21	2.33	0.93	2.64			
ASPARTATE	leaves non-nodulated	285.29	2.33	0.08	2.64			
	leaves nodulated	0.17	2.33	1.05	2.64			
	leaves spiked	1.75	2.33	3.29	2.64			
	roots non-nodulated	57.35	2.33	0.7	2.64			
	roots nodulated	0.95	2.33	0.2	2.64			
	roots spiked	32.24	2.33	1.15	2.64			
ASPARAGINE	leaves non-nodulated	26.15	2.33	5.49	2.64			
	leaves nodulated	1.05	2.33	29.52	2.64			
	leaves spiked	321.79	2.33	0.82	2.64			
	roots non-nodulated	90.09	2.33	7.73	2.64			
	roots nodulated	9.8	2.33	14.03	2.64			
	roots spiked	8.39	2.33	10.37	2.64			
ALANINE	leaves non-nodulated	9.55	2.33	5.24	2.64			
	leaves nodulated	1.79	2.33	32.2	2.64			
	leaves spiked	1.55	2.33	2.45	2.64			
	roots non-nodulated	4.28	2.33	12.06	2.64			
	roots nodulated	21.57	2.33	36.39	2.64			
	roots spiked	2.07	2.33	2.05	2.64			
ALANINE	leaves non-nodulated	57.06	4.2	34.61	3.4	5.54	2.4	
AMINO-	leaves nodulated	23.92	4.2	193.71	3.4	6.03	2.4	
TRANSFERASE	leaves spiked	36.7	4.2	211.89	3.4	10.24	2.4	
	roots non-nodulated	436.88	4.2	147.34	3.4	27.51	2.4	
	roots nodulated	19.8	4.2	38.03	3.4	5.77	2.4	
	roots spiked	36.54	4.2	230.67	3.4	11.39	2.4	

APPENDIX II (a)

		N TREATMENT		WATER DEFICITS		INTERACTION		
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit	
ASPARTATE	leaves non-nodulated	96.72	4.2	114.76	3.4	14.83	2.4	
AMINO-	leaves nodulated	29.97	4.2	16.34	3.4	5.79	2.4	
TRANSFERASE	leaves spiked	87.85	4.2	0.79	3.4	2.13	2.4	
	roots non-nodulated	133.73	4.2	2.53	3.4	1.53	2.4	
	roots nodulated	1.27	4.2	0.43	3.4	0.51	2.4	
	roots spiked	65.76	4.2	67.88	3.4	13.62	2.4	
ASPARAGINE	leaves non-nodulated	217.14	4.2	140.04	3.4	18.14	2.4	
SYNTHETASE	leaves nodulated	19.38	4.2	16.34	3.4	3.06	2.4	
	leaves spiked	86.57	4.2	1.41	3.4	2.72	2.4	
	roots non-nodulated	162.86	4.2	3.69	3.4	2.99	2.4	
	roots nodulated	2.2	4.2	0.31	3.4	0.29	2.4	
	roots spiked	26.41	4.2	20.48	3.4	4.06	2.4	
HDH	leaves non-nodulated	268.53	4.2	307.53	3.4	35	2.4	
	leaves nodulated	4.83	4.2	54.76	3.4	1.43	2.4	
	leaves spiked	14.33	4.2	366.93	3.4	12.99	2.4	
	roots non-nodulated	13.75	4.2	28.98	3.4	3.92	2.4	
	roots nodulated	7	4.2	65.99	3.4	2.02	2.4	
	roots spiked	11.63	4.2	94.65	3.4	5.02	2.4	
PROTEIN	leaves non-nodulated	623.28	4.2	179.06	3.4	24.66	2.4	
	leaves nodulated	70.32	4.2	124.8	3.4	6.74	2.4	
	leaves spiked	122.3	4.2	77.21	3.4	5.9	2.4	
	roots non-nodulated	579.18	4.2	61.33	3.4	16.95	2.4	
	roots nodulated	360.17	4.2	121.37	3.4	14.23	2.4	
	roots spiked	474.18	4.2	150.44	3.4	23.9	2.4	
NRA	leaves non-nodulated	193.98	4.2	52.37	3.4	11.06	2.4	
	leaves nodulated	31.65	4.2	8.42	3.4	1.96	2.4	
	leaves spiked	223.69	4.2	9.28	3.4	2.27	2.4	
	roots non-nodulated	645.89	4.2	34.47	3.4	7.83	2.4	
	roots nodulated	218.66	4.2	17.31	3.4	3.79	2.4	
	roots spiked	278.7	4.2	17.77	3.4	2.96	2.4	
NITRATE	leaves non-nodulated	851.19	4.2	28.21	3.4	5.13	2.4	
	leaves nodulated	302.53	4.2	450.38	3.4	61.78	2.4	
	leaves spiked	1395.06	4.2	44.44	3.4	16.95	2.4	
	roots non-nodulated	1169.84	4.2	18.77	3.4	15.39	2.4	
	roots nodulated	377.7	4.2	546.92	3.4	81.19	2.4	
	roots spiked	552.89	4.2	21.57	3.4	10.15	2.4	

APPENDIX II (a)

		N TREATMENT		WATER DEFICITS		INTERACTION		
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit	
GSA	leaves non-nodulated	770.35	4.2	0.84	3.4	0.33	2.4	
	leaves nodulated	72.63	4.2	0.09	3.4	0.28	2.4	
	leaves spiked	0.28	4.2	0.59	3.4	0.2	2.4	
	roots non-nodulated	569.72	4.2	1.32	3.4	0.78	2.4	
	roots nodulated	85.74	4.2	0.46	3.4	0.34	2.4	
	roots spiked	35.27	4.2	0.61	3.4	0.29	2.4	
GDHA	leaves non-nodulated	396.85	4.2	0.71	3.4	3.9	2.4	
	leaves nodulated	44.92	4.2	1.25	3.4	0.76	2.4	
	leaves spiked	253.04	4.2	45.47	3.4	6.96	2.4	
	roots non-nodulated	227.25	4.2	0.18	3.4	0.57	2.4	
	roots nodulated	147.36	4.2	1.13	3.4	1.15	2.4	
	roots spiked	115.84	4.2	14.76	3.4	3.23	2.4	
		'NO	'1/10	'1/2	'COMBINED			
		NITRATE'	NITRATE'	NITRATE'	NITROGEN'			
GDHA	**leaves non-nodulated**	-0.70	-0.57	0.80	0.60			
CORRELATION	**leaves nodulated**	0.63	0.21	0.69	0.33			
COEFFICIENTS	**leaves spiked**	0.90	0.90	-0.53	0.78			
	roots non-nodulated	-0.49	0.16	0.70	-0.42			
	roots nodulated	0.70	-0.29	0.39	0.54			
	roots spiked	0.92	0.84	0.43	0.68			
ALLANTOIN	leaves non-nodulated	234.46	4.2	0.6	3.4	1.08	2.4	
	leaves nodulated	322.33	4.2	18.38	3.4	6.74	2.4	
	leaves spiked	63.86	4.2	96.35	3.4	12.84	2.4	
	roots non-nodulated	4.25	4.2	1.28	3.4	1.28	2.4	
	roots nodulated	1381.44	4.2	113.32	3.4	119.7	2.4	
	roots spiked	30.56	4.2	49.77	3.4	6.92	2.4	
TOTAL	leaves non-nodulated	106.63	4.2	1.34	3.4	0.51	2.4	
AMMONIA	leaves nodulated	3.88	4.2	1.12	3.4	15.37	2.4	
	leaves spiked	21.04	4.2	23.17	3.4	11.9	2.4	
	roots non-nodulated	16.27	4.2	3.41	3.4	2.4	2.4	
	roots nodulated	1.32	4.2	2.42	3.4	7.53	2.4	
	roots spiked	99.72	4.2	2.97	3.4	9.89	2.4	

APPENDIX II (b)

		N TREATMENT		WATER DEFICITS		INTERACTION	
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit
FRESH WEIGHT	Leaf	207.17	4.26	56.32	2.26	7.15	2.26
	Stem	170.72	4.26	33.06	2.26	4.32	2.26
	Above - medium	233.75	4.26	51.62	2.26	6.6	2.26
	Root	251.52	4.26	22.53	2.26	7.23	2.26
	Total	267.41	4.26	32.61	2.26	7.56	2.26
DRY WEIGHT	Leaf	251.49	4.26	32.96	2.26	8	2.26
	Stem	193.67	4.26	14.52	2.26	4.51	2.26
	Above - medium	261.21	4.26	22.99	2.26	6.98	2.26
	Root	328.71	4.26	5.72	2.26	9.64	2.26
	Total	310.31	4.26	11.48	2.26	8.78	2.26
FW : DW	Leaf	11.61	4.26	53.07	2.26	1.84	2.26
	Stem	10.3	4.26	13.87	2.26	0.8	2.26
	Above - medium	26.38	4.26	98.94	2.26	5.58	2.26
	Root	11.49	4.26	72.49	2.26	9.97	2.26
	Total	1.03	7.08	36.91	3.51	0.33	3.51
R:S		34.21	4.26	83.99	2.26	11.88	2.26
L RWC		63.75	4.26	199.59	2.26	10.61	2.26
R RWC		6.89	4.26	229	2.26	8.02	2.26
RGR		1548.3	4.26	31.42	2.26	19.84	2.26
HEIGHT		1.41	4.26	4.94	2.26	6.99	2.26
LAR		0.24	4.26	1.02	2.26	0.4	2.26
CLA		110.23	4.26	9.68	2.26	3.43	2.26
STOMATAL		59.69	4.26	101.96	2.26	3.7	2.26
CONDUCTANCE							
PHOTOSYNTHESIS		119.2	4.26	102.38	2.26	5.24	2.26
NAR		401.17	4.26	6.86	2.26	4.7	2.26
SOLUBLE	Leaves	995.68	4.26	8.77	2.26	8.87	2.26
CARBOHYDRATE	Roots	669.73	4.26	19.95	2.26	10.68	2.26
TOTAL	Leaves	839.27	4.26	66.06	2.26	64.58	2.26
AMINO ACIDS	Roots	505.62	4.26	16.93	2.26	19.62	2.26

APPENDIX II (b)

		N TREATMENT		WATER DEFICITS		INTERACTION	
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit
PROLINE	Leaves	27.36	6.61	16.02	6.61	9.63	2.26
	Roots	44.83	6.61	19.3	6.61	14.37	2.26
GLYCINE	Leaves	25.51	4.26	102.05	2.26	7.84	2.26
BETAINE	Roots	267.28	4.26	158.28	2.26	121.21	2.26
OSMOMETER	Leaves	792.83	4.26	166.15	2.26	72.75	2.26
	Roots	769.99	4.26	170.6	2.26	88.96	2.26
SUCROSE	Leaves	775.76	4.26	90.44	2.26	86.22	2.26
	Roots	868.04	4.26	92.81	2.26	15.78	2.26
GLUCOSE	Leaves	881.63	4.26	57.12	2.26	48.81	2.26
	Roots	323.35	4.26	56.26	2.26	22.92	2.26
REDUCING SUGARS	Leaves	213.49	4.26	42.63	2.26	25.95	2.26
	Roots	158.37	4.26	38.47	2.26	7.27	2.26
STARCH	Leaves	914.94	4.26	298.36	2.26	101.05	2.26
	Roots	887.24	4.26	30.61	2.26	21.76	2.26
AMYLASE	Leaves	2.25	4.26	48.68	2.26	0.92	2.26
	Roots	1.84	4.26	117.79	2.26	0.98	2.26
TOTAL	Leaves	0.08	4.48	1.32	3.2		
ORGANIC ACIDS	Roots	40.72	4.48	1.14	3.2		
2-OXOGLUTERATE	Leaves	0.07	4.48	0.7	3.2		
	Roots	164.45	4.48	0.82	3.2		
MALATE	Leaves	459.1	4.48	81.85	3.2		
	Roots	156.86	4.48	28.03	3.2		
PYRUVATE	Leaves	0.22	4.48	0.59	3.2		
	Roots	2.02	4.48	1.38	3.2		
CITRATE	Leaves	2.37	4.48	0.77	2.26		
	Roots	0.32	4.48	0.72	2.26		
NRA	Leaves	4.84	4.26	0.52	2.26	1.65	2.26
	Roots	9.91	4.26	8.59	2.26	2.84	2.26
NITRATE	Leaves	0.59	4.26	8.43	2.26	0.23	2.26
	Roots	0.7	4.26	0.89	2.26	0.2	2.26

APPENDIX II (b)

		N TREATMENT		WATER DEFICITS		INTERACTION	
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit
GSA	Leaves	736.64	4.26	1.29	2.26	0.98	2.26
	Roots	1698.8	4.26	0.56	2.26	0.65	2.26
GDHA	Leaves	55.83	4.26	1.09	2.26	1.37	2.26
	Roots	245.36	4.26	0.71	2.26	1.59	2.26
ALLANTOIN	Leaves	1585.3	4.26	52.79	2.26	40.83	2.26
	Roots	745.32	4.26	5.99	2.26	5.48	2.26
THREONINE	Leaves	2.33	6.61	1.14	6.61		
	Roots	6.36	6.61	1.1	6.61		
GLUTAMINE	Leaves	267.85	6.61	1.07	6.61		
	Roots	220.05	6.61	0.7	6.61		
GLUTAMATE	Leaves	106.86	6.61	0.8	6.61		
	Roots	76.18	6.61	0.6	6.61		
ASPARTATE	Leaves	85.78	6.61	1.56	6.61		
	Roots	64.57	6.61	0.94	6.61		
ASPARAGINE	Leaves	23.16	6.61	0.98	6.61		
	Roots	18.63	6.61	1.15	6.61		
ALANINE	Leaves	6.5	6.61	0.99	6.61		
	Roots	3.91	6.61	4.57	6.61		
ALANINE AMINOTRANSFERASE	Leaves	492.14	4.26	45.49	2.26	33.17	2.26
	Roots	201.87	4.26	15.83	2.26	14.64	2.26
ASPARTATE AMINOTRANSFERASE	Leaves	20.05	4.26	0.32	2.26	0.52	2.26
	Roots	133.81	4.26	0.56	2.26	1.09	2.26
ASPARAGINE SYNTHETASE	Leaves	41.35	4.26	0.26	2.26	0.35	2.26
	Roots	537.04	4.26	0.051	2.26	0.06	2.26
HDH	Leaves	474.55	4.26	122.57	2.26	87.86	2.26
	Roots	96.76	4.26	26.74	2.26	14.82	2.26
PROTEIN	Leaves	459.1	4.26	81.85	2.26	35.89	2.26
	Roots	156.86	4.26	28.03	2.26	17.2	2.26
TOTAL AMMONIA	Leaves	79.13	4.26	1.46	2.26	36.06	2.26
	Roots	21.18	4.26	16.87	2.26	7.34	2.26

APPENDIX II (c)

		N TREATMENT		WATER DEFICITS		INTERACTION	
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit
FRESH WEIGHT	Leaf	3.26	6.75	13.76	3.1	0.15	3.1
	Stem	0.24	6.75	9.24	3.1	0.57	3.1
	Above - medium	3.91	6.75	9.11	3.1	0.11	3.1
	Root	10.17	6.75	9.04	3.1	0.2	3.1
	Total	7.86	6.75	8.53	3.1	0.29	3.1
DRY WEIGHT	Leaf	3.43	6.75	7.68	3.1	0.16	3.1
	Stem	3.03	6.75	1.78	3.1	0.13	3.1
	Above - medium	3.65	6.75	4.11	3.1	0.13	3.1
	Root	8.73	6.75	0.77	3.1	0.28	3.1
	Total	5.78	6.75	3.01	3.1	0.22	3.1
FW : DW	Leaf	2.64	6.75	85.46	3.1	0.48	3.1
	Stem	1.47	6.75	1.54	3.1	1.05	3.1
	Above - medium	3.37	6.75	4	3.1	0.7	3.1
	Root	6.9	6.75	105.15	3.1	0.63	3.1
	Total	2.25	6.75	35.61	3.1	0.958	3.1
R:S		0.41	6.75	7.53	3.1	0.02	3.1
L RWC		5.93	6.75	114.16	3.1	0.55	3.1
R RWC		0.04	6.75	33.91	3.1	1.26	3.1
RGR		4.61	6.75	1.87	3.1	0.08	3.1
HEIGHT		104.56	6.75	6.27	3.1	3.44	3.1
LAR		4.03	6.75	2.69	3.1	1.24	3.1
CUMULATIVE LEAF AREA		26.21	6.75	7.01	3.1	1.09	3.1
STOMATAL CONDUCTANCE		0.78	6.63	0.96	3.1	1.04	3.1
PHOTOSYNTHESIS		0.2	6.63	42.01	3.1	0.37	3.1
NAR		3.42	6.75	1.25	3.1	0.13	3.1
NRA	Leaves	31.44	6.75	1.98	3.1	1.16	3.1
	Roots	2.85	6.75	2.03	3.1	0.31	3.1
NITRATE	Leaves	0.03	6.75	1.32	3.1	0.03	3.1
	Roots	0	6.75	0.98	3.1	0.1	3.1
GSA	Leaves	54.87	6.75	0.09	3.1	0.05	3.1
	Roots	146.66	6.75	0.05	3.1	0.12	3.1
GDHA	Leaves	137.22	6.75	2.6	3.1	2.3	3.1
	Roots	124.49	6.75	1.61	3.1	1.6	3.1

APPENDIX II (c)

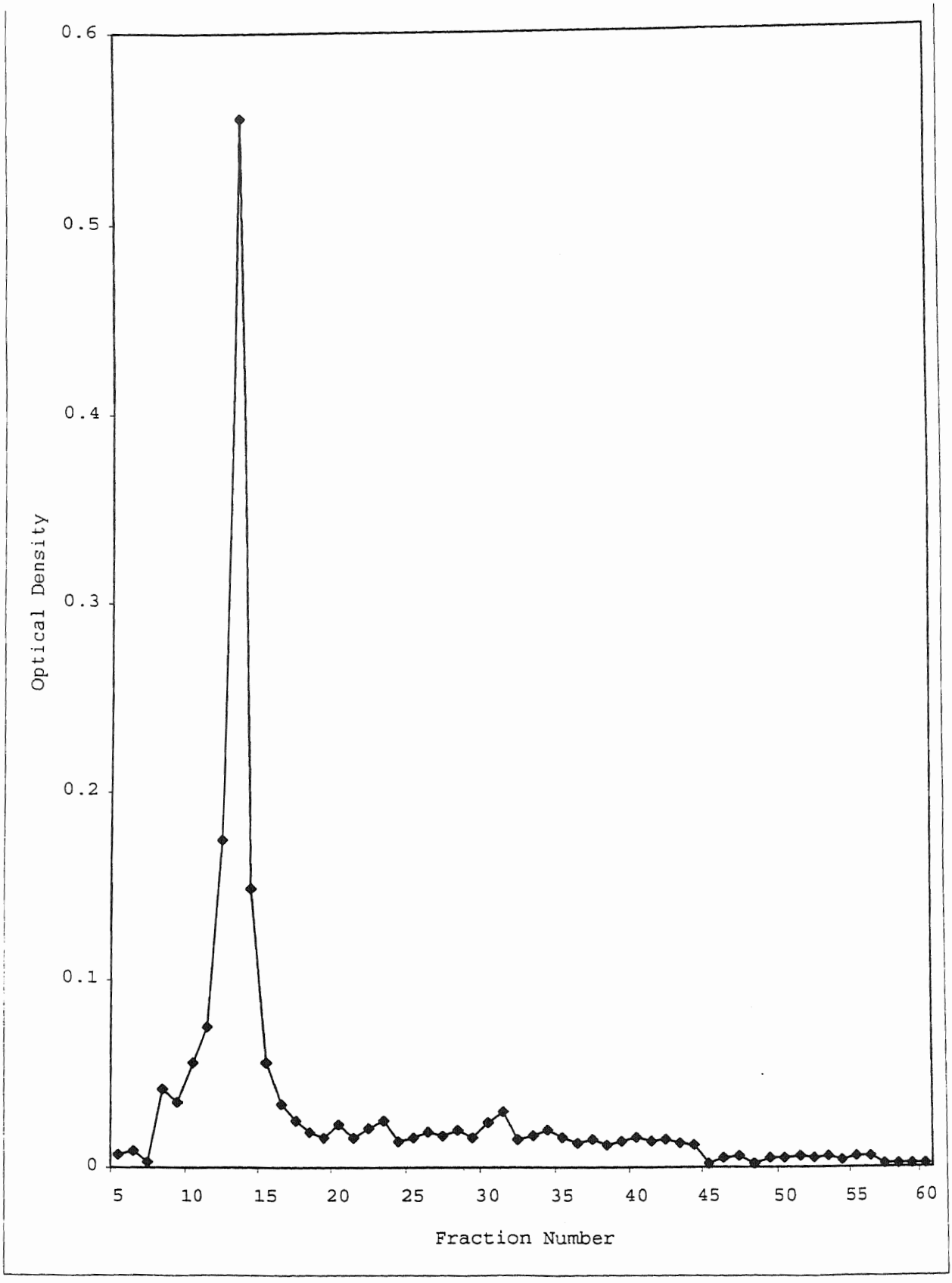
		N TREATMENT		WATER DEFICITS		INTERACTION	
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit
ALLANTOIN	Leaves	496.25	6.75	12.83	3.1	12.24	3.1
	Roots	457.39	6.75	10.37	3.1	8.52	3.1
TOTAL AMMONIA	Leaves	11.15	6.75	3.88	3.1	2.12	3.1
	Roots	33.84	6.75	0.58	3.1	1.73	3.1
SOLUBLE CARBOHYDRATE	Leaves	14.75	6.75	5.73	3.1	1.05	3.1
	Roots	19.66	6.75	4.87	3.1	0.61	3.1
TOTAL AMINO ACIDS	Leaves	192.48	6.75	25.01	3.1	5.16	3.1
	Roots	206.02	6.75	10.18	3.1	1.04	3.1
PROLINE	Leaves	1.93	7.41	36.8	3.61	0.4	3.61
	Roots	4.22	7.41	44.25	3.61	1.84	3.61
GLYCINE BETAINE	Leaves	13.85	6.75	9.06	3.1	7.2	3.1
	Roots	11.5	6.75	7.16	3.1	6.61	3.1
TOTAL OSMOLARITY	Leaves	4.07	4.04	23.94	2.45	0.32	2.45
	Roots	6.76	4.04	22.53	2.45	0.3	2.45
SUCROSE	Leaves	45.98	6.75	18.74	3.1	2.4	3.1
	Roots	40.03	6.75	13.24	3.1	1.01	3.1
FRUCTOSE	Leaves	59.04	6.75	7.24	3.1	3.56	3.1
	Roots	10.18	6.75	20.72	3.1	0.42	3.1
GLUCOSE	Leaves	27.53	6.75	6.47	3.1	1.36	3.1
	Roots	28.14	6.75	14.78	3.1	0.77	3.1
STARCH	Leaves	0.37	6.75	11.09	3.1	0.13	3.1
	Roots	0.77	6.75	12.28	3.1	0.21	3.1
AMYLASE	Leaves	5.36	6.75	196.15	3.1	1.11	3.1
	Roots	5.56	6.75	23.56	3.1	3.13	3.1
TOTAL ORGANIC ACIDS	Leaves	0.24	4.04	1.25	2.45	1.25	2.45
	Roots	0.17	4.04	2.28	2.45	0.94	2.45
2-OXOGLUTERATE	Leaves	1.95	4.04	0.85	2.45	0.61	2.45
	Roots	0.07	4.04	0.98	2.45	1.69	2.45
MALATE	Leaves	0.88	4.04	2.47	2.45	0.21	2.45
	Roots	0.3	4.04	0.83	2.45	0.44	2.45
PYRUVATE	Leaves	2.62	4.04	0.9	2.45	0.48	2.45
	Roots	0.02	4.04	1.24	2.45	0.46	2.45
CITRATE	Leaves	0.44	4.04	0.78	2.45	0.93	2.45
	Roots	0.55	4.04	0.8	2.45	1.2	2.45

APPENDIX II (c)

		N TREATMENT		WATER DEFICITS		INTERACTION	
		Fcalc	Fcrit	Fcalc	Fcrit	F calc	F crit
THREONINE	Leaves	0.35	7.41	13.39	3.61	0.52	3.61
	Roots	21.29	7.41	25.99	3.61	0.68	3.61
GLUTAMINE	Leaves	106.64	7.41	0.11	3.61	0.22	3.61
	Roots	39.18	7.41	0.17	3.61	0.04	3.61
GLUTAMATE	Leaves	30.77	7.41	0.13	3.61	0.04	3.61
	Roots	76.18	7.41	0.17	3.61	0.11	3.61
ASPARTATE	Leaves	29.63	7.41	0.1	3.61	0.08	3.61
	Roots	79.95	7.41	0.13	3.61	0.17	3.61
ASPARAGINE	Leaves	9.55	7.41	0.48	3.61	0.61	3.61
	Roots	72.88	7.41	2.61	3.61	0.2	3.61
ALANINE	Leaves	21.9	7.41	1.91	3.61	2.41	3.61
	Roots	63.59	7.41	7.9	3.61	6.36	3.61
ALANINE	Leaves	7.33	6.75	31.49	3.1	1.15	3.1
AMINOTRANSFERASE	Roots	12.11	6.75	25.94	3.1	1.3	3.1
ASPARTATE	Leaves	508.43	6.75	1.29	3.1	1.63	3.1
AMINOTRANSFERASE	Roots	43.64	6.75	4.81	3.1	6.14	3.1
ASPARAGINE	Leaves	405.57	6.75	1.71	3.1	0.26	3.1
SYNTHETASE	Roots	138.6	6.75	3.88	3.1	5.47	3.1
HDH	Leaves	42.59	6.75	72.01	3.1	5.46	3.1
	Roots	158.54	6.75	51.25	3.1	10.11	3.1
PROTEIN	Leaves	2.35	6.75	27.83	3.1	0.04	3.1
	Roots	8.27	6.75	6.88	3.1	0.49	3.1

APPENDIX III

EXAMPLE GS PROFILE



APPENDIX IV

Photograph of *V. faba* growing hydroponically.

(a) '1/2 nitrate' supplied *V. faba*; adequately irrigated (left) and supplied with 25% Peg (right)
(b) (b) *V. faba* growing hydroponically in randomized blocks

Acer-Mothes K, (1961), The metabolism of urea and ureides. *Can. J. Bot.* **39** 1785-1807

Alfidi MMRK & Hewitt EJ, (1964), The inducible formation and stability of nitrate reductase in higher plants. *J. Exp. Bot.* **15** 44 251-71

Allen S & Smith JAC, (1986), Ammonium assimilation in *Ricinus communis* grown with ammonium or nitrate as N source: the role of long distance transport. *J. Exp. Bot.* **37** 1599-1610

Andrews M, Sutherland J & Sprent JI, (1984a), Nitrate reduction in *V. faba* grown at different temperatures. In :World Crop series; *V. faba* agronomy, physiology, and breeding. (Eds. Hebblethwaite, Heath, Dawkins & Lockwood) pp 29-36 Martinus Nijhoff Pubs.

Andrews M, (1986), The partitioning of nitrate assimilation between root and shoot of higher plants. *Plant Cell & Environ.* **9** 511-19

Andrews M, (1986), Nitrate and reduced N concentrations in the xylem sap of *Stellaria media, Xanthium stromarium* and six legume species. *Plant Cell & Environ.* **9** 605-08

Andrews M, de Faria SM, McInroy SG, & Sprent JI, (1990), Constitutive nitrate reductase activity in the leguminosae. *Phytochemistry* **29** 49-54

Arnozis PA, Nelemans JA & Findenegg GR, (1988), Phosphoenolpyruvate Carboxylase Activity in Plants Grown with either NO_3^- or NH_4^+ as Inorganic Nitrogen Source. *J. Plant Physiol.* **132** 23-27

Arrese-Igor C, (1998), Nitrate entry and nitrite formation in the infected region of soybean nodules. *J. Exp. Bot.* **49** 318 41-48

Aslam M & Huffaker RC, (1984), Dependency of NR on soluble carbohydrates in primary leaves of barley under aerobic conditions. *Plant Physiol.* **75** 623-28

Aspinall D & Paleg LG, (1981), Proline accumulation: physiological aspects. In: Physiology and biochemistry of drought resistance in plants. Ed Paleg LG & Aspinall D pp 206-242 Academic Press

Atwell BJ, (1992) Nitrate and ammonium as nitrogen sources for lupins prior to nodulation. *Plant and Soil* **139** 247-251

Bacon MA, Wilkinson S & Davies WJ, (1998), pH-regulated leaf cell expansion in droughted plants is abscsisic acid dependant. *Plant Physiol.* **118** 1507-15

Barash I, Mor H, & Sadon T, (1975), Evidence for ammonium dependant *de novo* synthesis of glutamate dehydrogenase in detached oak leaves. *Plant Physiol.* **56** 856-58

Barker DB, (1966), Root environment acidity as a regulatory factor in ammonium assimilation by the bean plant. *Plant Physiol.* **41** 1193-99

Barratt DHP, (1980), Method for the detection of glutamine synthetase activity on starch gels). *Plant Science Letters* **18** 249-50

Baker A, Hill GF & Parsons R, (1997), Evidence for nitrogen feedback regulation of N2 fixation in *Alnus glutinosa* L. *J. Exp. Bot.* **48** 306 67-73

Barnett NM & Naylor AW, (1966), Amino acid and protein metabolism in Bermuda grass during water stress *Plant Physiol.* **41** 1222-230

Bassham JA, Larsen PO, Lawyer AL, & Cornwell KL, (1981), Relationships between nitrogen metabolism and photosynthesis. In: Bewley JD (Ed) Nitrogen and Carbon Metabolism pp 135-164 Martinus Nijhoff/ Dr W Junk Publishers

Bauer WD, (1981), Infection of legumes by rhizobia. *Ann. Rev. Plant Physiol.* **325** 407-49

Beevers L & Hageman RH, (1969), Nitrate reduction in higher plants. *Ann. Rev. Plant Physiol.* 20 495-522

de-Benedetti E, Forti G, Garlaschi FM, & Rosa L, (1976), On the mechanism of ammonium stimulation of photosynthesis in isolated chloroplasts. *Plant Science Letters.* 7 85-90

Bennet WF, Pesek J & Haniway JJ, (1964), Effect of nitrate and ammonium on growth of corn in sand culture. *Agron. J.* **56** 342-45

Bennet JM, Jones JW, Zur B & Hammond LC, (1986), Interactive effects of nitrogen and water relations of field grown corn leaves. *Agron. J.* **78** 273-80

Bennet RN & Wallsgrove RM, (1994), Tansley Review No. 72 Secondary metabolites in plant defence mechanisms. *New Phytol.* **127** 617-33

Bewley JD, (1980), Protein Synthesis. In: Physiology and Biochemistry of Drought Resistance in Plants. Paleg *et al,* (Eds.) Academic Press

Bjorkman T, (1981), Encyclopedia of plant physiology New Series Pirson & Zimmerman Eds. Vol J2a pp 57-107 Springer-Verlag Berlin ISBN 3-540-10763-0

Blackman VH, (1919), The compound interest law and plant growth. *Ann. Bot.* **23** 353

Blackman PG & Davies WJ, (1985), Root to shoot communication maize plants of the effects of soil drying. *J. Exp. Bot.* **36** 162 39-48

Bloom A, (1988), Ammonium and nitrate as nitrogen sources for plant growth. ISI Atlas of science: Animal & Plant Sciences. **0894-3761** 55-59

Bloom AJ, Randall LB, Meyerhoff PA & St Clair DA, (1998), The chilling sensitivity of root ammonium influx in a cultivated and wild tomato. *Plant Cell & Environ.* **21** 191-199

Blum A, (1983), Genetic and physiological relationships in plant breeding for drought resistance. In: Plant production and management under drought conditions. Stone JF & Willis WO (Eds.) pp 195-205 Amsterdam: Elsevier Science Publishers

Boggess SF, (1970), Associations between plant production and some physiological components of drought resistance in wheat. *Plant Cell & Environ.* **6** 219-25

Boggess SF, Paleg LG, & Aspinall D, (1975), Delta-pyrroline-5-carboxylic acid dehydrogenase in barley, a proline accumulating species. *Plant Physiol.* **56** 259-62

Borowitzka LJ, (1981),Ch 6 Solute accumulation and regulation of cell wall activity. pp 97-104 In: Physiology and biochemistry of drought resistance. Paleg & Aspinall Eds. ISBN 0 12 544 380 3 Academic Press

Bourgeais-Chaillou P, Perez-Alfocea F & Guerrier G, (1992),Comparative effects of N sources on growth and physiological responses of soybean exposed to NaCl stress. *J. Exp. Bot.* **43** 254 1225-1233

Bowsher CG, Long DM, Oaks A & Rothstein SJ, (1991), Effect of dark/light cycles on expression of nitrate assimilatory genes in maize shoots and roots. *Plant Physiol.* **95** 281-85

Boyer JS, (1968), Relationship of water potential to growth of leaves. *Plant Physiol.* **43** 1056-1062

Boyer JS, (1970), Differing sensitivity of photosynthesis to low leaf water potentials in corn and soybeans. *Plant Physiol.* **46** 236-39

Boyer JS, (1971a), Recovery of photosynthesis in sunflower after a period of low leaf water potential. *Plant Physiol.* **47** 816-820

Boyer JS, (1971b), Resistances to water transport in soybean, bean, and Sunflower. *Crop Sci.* **11** 403-407

Boyer JS, (1985), Water transport. *Ann. Rev. Plant Physiol.* **36** 473-516

Bray EA, (1997), Plant responses to water deficit. *Trends in Plant Science* **2** 2 49-54

Breteler H & Smit AL, (1974), Effect of ammonium nutrition on uptake of and metabolism of nitrate in wheat. *Neth. J. Agric. Sci.* **22** 73-81

Breteler H & Siegerist M, (1984), Effect of ammonium on nitrate utilisation by roots of dwarf bean. *Plant Physiol.* **75** 1099-1103

Briggs GE, Kidd F & West C, (1920b), A quantitative analysis of plant growth. *Ann. Appl. Biol.* **7** 202-223

Brown AD & Simpson JR, (1972), Water relations of sugar tolerant yeasts; the role of intracellular polyols. *J. Gen. Microbiol.* **72** 589-91

Brownlee C, Goddard H, Hetherington AM & Peake LA, (1999), Specificity & integration of responses: Ca_2^+ as a signal of polarity & osmotic regulation. *J. Exp. Bot.* **50** 1001-11

Bryan JK, (1976), Amino Acid Biosynthesis and its Regulation. In: Plant Biochemistry 3rd Ed. J Bonner & JE Varner Eds. pp 525-560 Academic Press

Bryan JK, Lissik EA, & Dicamelli CA, (1979), Changes in enzyme regulation during plant growth. In: Hewitt & Cutting Eds. Nitrogen assimilation of plants pp 423-30 Academic Press

Bungard RA, Wingler A, Morton JD, Andrews M, Press MC & Scholes JD, (1999), Ammonium can stimulate nitrate and nitrite reductase in the absence of nitrate in *Clemantus vitalba*. *Plant Cell & Environ.* **22** 859-66

Burlina A, (1985), 2-oxogluterate. In: Methods of enzymic analysis. Metabolites. 2 (Ed. Bergmeyer) Vol II 3 Edn. VCH Weinheim pp 2-12

Burris RH, (1974), Biological Nitrogen Fixation, 1924-1974. *Plant Physiol.* **54** 443-449

Bussis D, Kauder F & Heinke D, (1998), Acclimation of potato plants to polyethylene glycol-induced water deficit. I Photosynthesis and Metabolism. *J. Exp. Bot.* **49** 325 1349-60

Bussis D & Heineke D, (1998) Acclimation of potato plants to polyethylene glycol induced water deficit II contents and subcellular distribution of organic solutes. *J. Exp. Bot.* **49** 325 1361-70

Buttery BR, (1970), Effects of variation in Leaf Area Index on Growth of Maize and Soybeans. *Crop Sci.* **10** 9-13

Caba JM, Recalde L & Ligero F, (1998), Nitrate induced ethylene biosynthesis and the control of nodulation in alfalfa. *Plant Cell & Environ.* **21** 87-93

Campbell WH, (1988b), In: Molecular & genetic aspects of nitrate assimilation. Wray & Kingburn Eds. Oxford Uni. Press

Canny MJ, (1995), Apoplastic water and solute movement: new rules for an old space. *Ann. Rev. Plant. Physiol. & Plant Mol. Biol.* **46** 215-236

Canvin DT & Atkins CA, (1974), Nitrate, nitrite and ammonia assimilation by leaves; effect of light, carbon dioxide and oxygen. *Planta* **116** 207-224

Canvin DT & Woo KC, (1979), The regulation of nitrate reduction in spinach leaves. *Can. J. Bot.* **57** 1155-60

Cardenas-Navarro R, Adamowicz S & Robin P, (1998), Diurnal nitrate uptake in young tomato (*Lycopersicon esculentum* Mill.) plants: tests of a feedback based model. *J. Exp. Bot.* **49** 321 721-30

Cellier F, Conejero G, Breteler JC & Casse F, (1998), Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower. *Plant Physiol.* **116** 319-28

Chaillou S, Vessey JK, Morot-Gaudry JF, Raper CD, Henry LT & Boutin JP, (1991), Expression of characteristics of ammonium nutrition as affected by pH of the root medium. *J. Exp. Bot.* **42** 235 189-196

Champigny ML & Foyer C, (1992), Nitrate activation of cytosolic protein kinases diverts photosynthetic carbon from sucrose to amino acid biosynthesis. *Plant Physiol.* **100** 7-12

Chandler G, Ladley P, McNally SF, Patel M, Stewart GR, & Sumar N, (1985), The activity and isoform complement of glutamine synthetase in *Panicum* species differing in photosynthetic pathway. *J. Plant Physiol.* 121 13-21

Chaparro A, Maldonado JM, Diez J, Relimpio AM & Losada M, (1976), Nitrate reductase inactivation and reducing power and energy charge in *Chlorella* cells. *Plant Science Letters* **6** 335-342

Chapin III, FS, Walter CHS, & Clarkson DT, (1988), Growth response of barley and tomato to nitrogen stress and its control by abscisic acid, water relations and photosynthesis. *Planta* **173** 352-66

Cheng CL, Acedo GN, Cristinsin M & Conkiling MA, (1992), Sucrose mimics the light induction of Arabidopsis nitrate reductase gene transcription. *Proc. Natl. Acad. Sci.* USA **89** 1861-64

Chrispeels FS & Varner JE, (1967), Giberrelic acid-enhanced synthesis and release of amylase and ribonuclease by isolated barley alurone layers. *Plant Physiol.* **42** 398 - 406

Clark H, Newton PCD & Barker DJ, (1999), Physiological and morphological responses to elevated CO2 and a soil moisture deficit of temperate pasture species growing in an established plant community. *J. Exp. Bot.* **50** 331 233-42

Clement CR, Hopper MJ & Jones LHP, (1978), The uptake of nitrate by *Lolium perenne* from flowing nutrient solution. I. Effect of NO_3^- concentration. *J. Exp. Bot.* **29** 453-64

Clifford SC, Arndt SK, Corlett JE, Joshi S, Sankhla N, Popp M & Jones HG, (1998), The role of solute accumulation and changes in cell wall elasticity in drought tolerance in *Ziziphus mauritiana* (Lank.). *J. Exp. Bot.* **49** 323 967-77

Close TJ, (1996), Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiol. Plant.* **97** 795-803

Cock JM, Brock IW, Watson AT, Swarup R, Morby AP, & Cullimore JV, (1991), Regulation of glutamine synthetase genes in leaves of *Phaseolus vulgaris*. *Plant Molecular Biology* **17** 761-771

Cockbain AJ, (1981), Concerning Virus Diseases. In: *Vicia faba:* Agronomy, Physiology and Breeding. Hebblethwaite, Dawkins, Heath & Lockwood (Eds.) Martinus Nijhoff/Dr W. Junk ISBN 90-247-2964-5

Collins English Dictionary, (1995), Sinclair JM (Consultant). Harper Collins

Collinson ST, Clawson EJ, Azam-Ali SN & Black CR, (1997), Effects of soil moisture deficits on the water relations of bambara groundnut (*Vigna subterranea* L. Verdc.). *J. Exp. Bot.* **48** 309 877-84

Colmer TD & Bloom AJ, (1998), A comparison of NH_4 and NO_3 net fluxes along roots of rice and maize. Plant Cell & Environ. **21** 240-246

Comber M, (1999), Beans against cancer. *Health & Fitness* July 29
Comstock J & Mencuccini M, (1998), Control of stomatal conductance by leaf water potential in *Hymenoclea salsola* (T&G), a desert subshrub. *Plant Cell & Environ.* **21** 1029-38

Corak SJ, Smith MS & MacKown CT, (1992), Fate of 15N labelled legume and ammonium nitrogen sources in a soil-plant system. *Commun. Soil Sci. Plant Anal.* **23** (5&6) 631-642

Cox WJ & Reisenhauer HM, (1973), Growth and ion uptake by wheat supplied nitrogen as nitrate or ammonium or both. *Plant & Soil* **38** 363-80

Cram WJ, (1974), Effects of Cl^- on HCO_3^- and malate fluxes and CO_2 fixation in carrot and barley root cells. *J. Exp. Bot.* **25** 253-268

Cullimore JV, Lara M, Lea PJ & Miflin BJ, (1983), Purification and properties of two forms of glutamine synthetase from the plant fraction of *Phaseolus* root nodules. *Planta* **157** 245-53

Curioni P, Hartwig UA, Nosberger J & Schulier KA, (1999), Glycolytic flux in adjusted nitrogenase activity in nodules of detopped and argon treated alfalfa plants. *Plant Physiol.* **119** 2 445-54

Darling A, (1982), Preface in: Proceedings of faba bean conference held in Cairo, Egypt, March 7-11, 1981, World crops production, utilisation, and description. Hawtin & Webb (Eds.) Martinus Nijhoff Publishers.

Davies DD, (1973), Control of and by pH. Symposia for the society for experimental biology. **27** 513-29

Day JM & Legg, (1981), Water relations and irrigation response in the faba bean (*V.faba* L.) Ed. PD Hebblethwaite Butterworths. London

Dean JR & Clarke KW, (1980), Effect of low level nitrogen fertilization on nodulation, acetylene reduction, and dry matter accumulation in faba beans and three other legumes. *Can. J. Plant Sci.* **60** 121-30

Deane-Drummond CE, Clarkson DT, & Johnson CB, (1980), The effect of differential root and shoot temperature on the nitrate reductase activity assayed *in vivo* and *in vitro* in roots of *Hordeum vulgare* (Barley). *Planta* **148** 455-461

Dekhuijzen HM, Verkerke DR, & Houwers A, (1981), Physiological aspects of growth and development of *Vicia faba.* In: Faba bean improvement Ed Hawtin & Webb. pp 7-31 Kluwer Academic Publishers Group

Delwiche CC, (1951), The assimilation of ammonium and nitrate by tobacco plants. *J. Biol. Chem.* **189** 167-75

Demmig B & Winter K, (1985), Sodium, potassium, chloride, and proline concentrations of chloroplasts isolated from a halophyte *Mesembryanthemum crystallinum* L. *Planta* **168** 421-26

Deng M, Moureaux T, Leydecker MT & Caboche M, (1991), Effect of nitrogen on the regulation and circadian expression of tobacco NR. *Plant Physiol. Biochem.* **29** 1-9

Dhindsa RS & Bewley JD, (1976b), Water stress and protein synthesis. IV. Responses of a drought tolerant plant. *J. Exp. Bot.* **27** 513-23

Dighton J, (1991), Acquisition of nutrients from organic resources by mycorrhizal autotrophic plants. *Experientia* **47** 362-69

Dijkshoorn R, (1973), Chemistry and biochemistry of Herbage Ed Butler & Bailey (Eds.) 163-88 Academic Press

Doehlert DC, (1993), Sink Strength: dynamic with source strength. *Plant Cell & Environ.* 1027-28

Domska H, (1974), Zeszyty Naukowe Akademii Rolniczo Techniczne jul Olsztynie. **9** 85-96

Dry I, Wallace W & Nicholas DJD, (1981), Role of ATP in nitrate reduction in roots of wheat and pea. *Planta* **152** 234-38

Duc G & Picard J, (1982), Study of the fertility components in faba beans (*Vicia faba L.*) - variability among six different genotypes - effect of top and flower removal. In: Faba bean improvement Ed Hawtin & Webb. pp 283-298 Kluwer Academic Publishers Group

Dudel G & Kohl G, (1974), Uber die verteilung der Nitratreduktaseaktivat in Wurzel und Blatt bie *Hordeum vulgare* L. und ihre Abhangigkeit vo exogenen Nitratangebot Arch. *Acker-Pflanzenbau Bodenkd.* **18** 233-42

Durzan DJ & Stewart FC, (1967), The nitrogen metabolism of *Picea glauca* (Moench) Voss. and *Pinus banksiana* Lamb. as influenced by mineral nutrition. *Can. J. Bot.* **45** 695-710

Durzan DJ & Steward FC, (1983), Ch2 Nitrogen Metabolism. In: Ed. A Treatise. Plant Physiology Vol. III Nitrogen Metabolism. pp 55-265 Academic Press ISBN 0-12-668608-4

Edwards JW & Coruzi GM, (1989), Photorespiration and light act in concert to regulate the expression of the nuclear gene for chloroplast glutamine synthetase. *The Plant Cell* **1** 241-48

Elmlinger & Mohr, (1992), Glutamine synthetase in scots pine seedlings and its control by blue light and light absorbed by phytochrome. *Planta* **188** 396-402

Elowad HO & Hall EA, (1987), Influences of early and late nitrogen fertilisation on yield and nitrogen fixation of cowpea under well-watered and dry field conditions. *Field Crops Research* **15** 229-44

Elston J, Karamanos AJ, Kassam AH & Wadsworth RM, (1976), The water relations of the field bean crop. *Phil. Trans. R. Soc. London. ser. B.* **273:** 581-91

Emes & Fowler, (1979), The intracellular location of the enzymes of nitrate assimilation in the apices of seedling pea roots. *Planta* **144** 249-53

Epron D, (1997), Effects of drought on photosynthesis and on the thermotolerance of photosystem II in seedlings of cedar (*Cedrus atlantica* and *C. libani*). *J. Exp. Bot.* **48** 315 1835-41
Etherington JR, (1962), The growth of *Alopecurus pratensiol* and *Agrostis tenuis* in relation to soil moisture conditions. Ph.D. Thesis. University of London

Evans & Nason, (1953), *Plant Physiol.* **28** 233-54

Evans LT & Dunstone RL, (1970), Some physiological aspects of evolution in wheat. *Aust. J. Biol. Sci.* **23** 723-42

Evans LT, (1975), In: Crop Physiology Ed LT Evans 101-150 Cambridge Uni. Press

Evans JR, (1989), Photosynthesis and nitrogen relationships in leaves of C3 plants. *Oecologia* **78** 9-19

Fagena, (1974), Absorption of magnesium and it's effects on the uptake of phosphorus, potassium and calcium by intact groundnut plants. *Plant & Soil* **40** 313-20

Falisse A, Cors F, Biston R & Bartiaux-Thill N, (1984), In: Hebblethwaite PD, Dawkins TCK, Heath MC & Lockwood G (Eds.) *Vicia faba* Agronomy, Physiology and Breeding Martinus Nijhoff/ Dr W Junk Publishers, Netherlands. ISBN 90-247-2964-5

Faulkner JS & Steen RWJ, (1984), The potential of *V. faba* for protein. In: *Vicia faba*: Anatomy Physiology & Breeding World crops: production, utilisation, description. Vol. 10. (Eds. Hebblethwaite, Dawkins, Heath & Lockwood). Martinus Nijhoff/Dr. Junk 90-247-2964-5

Ferrario-Mery S, Valadier MH, & Foyer CH, (1998), Overexpression of nitrate reductase in tobacco delays drought induced decrease in nitrate reductase activity and mRNA. *Plant Physiol.* **117** 293-302

Fiscus EL & Markhart AH, (1979), Relationships between root system water transport properties and plant size in *Phaseolus*. *Plant Physiol.* **64** 770-73

Fisher RA & Yates F, (1963), Statistical tables for biological, agricultural, and medical research, Oliver & Boyd. Edinburgh.

Fitter AH, (1991), Costs and benefits of mycorrhiza; implications for functioning under natural conditions. *Experientia* **47** 350-55

Ford CW & Wilson JR, (1981), Changes in the levels of solutes during osmotic adjustment to water stress in leaves of four tropical pasture species. *Aust. J. Plant Physiol.* **8** 77-91

Forde BG & Woodall J, (1995), Glutamine synthetase in higher plants: molecular biology meets plant physiology In: Wallsgrove RM (Ed.) Amino acids and their derivatives in higher plants. pp 1-18 Cambridge University Press ISBN 0-521-45453-0

Foyer CH, Valadier MH, Migge A & Becker TW, (1998), Drought induced effects of nitrate reductase activity and mRNA on the coordination of nitrogen and carbon metabolism in maize leaves. *Plant Physiol.* **117** 283-92

Frauen M, Robbelen G & Ebmeyer E, (1981), Quantitative measurement of quality determining constituents in seeds of different inbred lines from a world collection of *V.faba*. *Vicia faba*, Physiology and Breeding. World Crops utilisation and description. Thompson R Ed. pp 279-283 Martinus Nijhoff.

Fujihara T, Orskov ER, Reeds PJ & Kyle DJ, (1987), The effect of protein infusion on urinary purine derivatives in ruminants nourished by intragastric nutrition. *J. Agric. Sci. Camb.* **109** 7-12

Evans LT, (1975), In: Crop Physiology Ed LT Evans 101-150 Cambridge Uni. Press

Evans JR, (1989), Photosynthesis and nitrogen relationships in leaves of C3 plants. *Oecologia* **78** 9-19

Fagena, (1974), Absorption of magnesium and it's effects on the uptake of phosphorus, potassium and calcium by intact groundnut plants. *Plant & Soil* **40** 313-20

Falisse A, Cors F, Biston R & Bartiaux-Thill N, (1984), In: Hebblethwaite PD, Dawkins TCK, Heath MC & Lockwood G (Eds.) *Vicia faba* Agronomy, Physiology and Breeding Martinus Nijhoff/ Dr W Junk Publishers, Netherlands. ISBN 90-247-2964-5

Faulkner JS & Steen RWJ, (1984), The potential of *V. faba* for protein. In: *Vicia faba*: Anatomy Physiology & Breeding World crops: production, utilisation, description. Vol. 10. (Eds. Hebblethwaite, Dawkins, Heath & Lockwood). Martinus Nijhoff/Dr. Junk 90-247-2964-5

Ferrario-Mery S, Valadier MH, & Foyer CH, (1998), Overexpression of nitrate reductase in tobacco delays drought induced decrease in nitrate reductase activity and mRNA. *Plant Physiol.* **117** 293-302

Fiscus EL & Markhart AH, (1979), Relationships between root system water transport properties and plant size in *Phaseolus*. *Plant Physiol.* **64** 770-73

Fisher RA & Yates F, (1963), Statistical tables for biological, agricultural, and medical research, Oliver & Boyd. Edinburgh.

Fitter AH, (1991), Costs and benefits of mycorrhiza; implications for functioning under natural conditions. *Experientia* **47** 350-55

Ford CW & Wilson JR, (1981), Changes in the levels of solutes during osmotic adjustment to water stress in leaves of four tropical pasture species. *Aust. J. Plant Physiol.* **8** 77-91

Forde BG & Woodall J, (1995), Glutamine synthetase in higher plants: molecular biology meets plant physiology In: Wallsgrove RM (Ed.) Amino acids and their derivatives in higher plants. pp 1-18 Cambridge University Press ISBN 0-521-45453-0

Foyer CH, Valadier MH, Migge A & Becker TW, (1998), Drought induced effects of nitrate reductase activity and mRNA on the coordination of nitrogen and carbon metabolism in maize leaves. *Plant Physiol.* **117** 283-92

Frauen M, Robbelen G & Ebmeyer E, (1981), Quantitative measurement of quality determining constituents in seeds of different inbred lines from a world collection of *V.faba*. *Vicia faba*, Physiology and Breeding. World Crops utilisation and description. Thompson R Ed. pp 279-283 Martinus Nijhoff.

Fujihara T, Orskov ER, Reeds PJ & Kyle DJ, (1987), The effect of protein infusion on urinary purine derivatives in ruminants nourished by intragastric nutrition. *J. Agric. Sci. Camb.* **109** 7-12

Fukutuku Y & Yamada Y, (1984), Sources of proline - nitrogen in water stressed soybean (*Glycine max.*) II Fate of ^{15}N labelled protein. *Physiol. Plant.* **61** 622-8

Galangau F, Daniel-Vedele F, Moureaux T, Dorbe MF, Leydecjer MT & Caboche M, (1988), Expression of leaf nitrate reductase genes from tomato and tobacco in relation to light-dark regimes and nitrate supply. *Plant Physiol.* **88** 383-88

Gallacher AE & Sprent JI, (1978), The effect of different water regimes on growth and nodule development of greenhouse-grown *Vicia faba. J. Exp. Bot.* **29** 109 413-423

Garcia-Fernadez JM, Lopez-Ruiz A, Alhama J, Roldan JM & Dapena JD, (1995), Effect of glutamine on glutamine-synthetase regulation in the green alga *Monoraphidium braunii. Planta* **195** 434-439

Gessler A, Schultze M, Schrempp S, & Rennenberg H, (1998), Interaction of phloem-translocated amino compounds with nitrate net uptake by the roots of beech (*Fagus sylvatica*) seedlings. *J. Exp. Bot.* **49** 326 1526-37

Ghashghaie J & Saugier B, (1989), Effect of nitrogen deficiency on leaf photosynthetic response of tall fescue to water deficit. *Plant Cell & Environ.* **12** 261-71

Gigon A & Rorison I, (1972), The response of some economically distinct plants to nitrate and ammonium nutrition. *J. Ecology* **60** 93-102

Gilbert GA, Gadush MV, Wilson C, & Madore MA, (1998), Amino acid accumulation in sink and source tissues of *Coleus blumei* Benth. during salinity stress. *J. Exp. Bot.* **49** 318 107-114

Giordano M & Bowes G, (1997), Gas exchange and C allocation in *Dunaliella salina* cells in response to the N source and CO_2 concentration used for growth. *Plant Physiol.* **115** 1049-56

Gojon A, Dapoigny L, Lejay L, Tillard P & Rufty TW, (1998), Effects of genetic modification of nitrate reductase expression on $15NO_3^-$ uptake and reduction in *Nicotiana* plants. *Plant Cell & Environ.* **21** 43-53

Gonzalez EM, Aparicio-Tejo PM, Gordon AJ, Minchin FR, Royeuela M & Arrese-Igore C, (1998), Water deficit effects on carbon and nitrogen metabolism of pea nodules. *J. Exp. Bot.* **49** 327 1705-14

Good NE, (1960), Activation of the Hill Reaction by amines. *Biochem. Biophys. Acta* **40** 502-17

Gordon AJ & James CL, (1997), Enzymes of carbohydrate and amino acid metabolism in developing and mature nodules of white clover. *J. Exp. Bot.* **48** 309 895-903

Goupil P, Loncle D, Druart N, Bellettre A, & Rambour S, (1998), Influence of ABA on nitrate reductase activity and carbohydrate metabolism in chicory roots (*Cichorium intybus* L.). *J. Exp. Bot.* **49** 328 1855-62

Gradjeda, (1990), Cinetica de la fijacion de nitrogeno en frijol comun, Tesis de mestria centro de investicacsion y eshidlos advanzados del IPN Unidad Irapuato Cito Mexico

Granier C & Tardieu F, (1999), Water deficit and spatial pattern of leaf development, variability in responses can be simulated using a simple model of leaf development. *Plant Physiol.* **119** 2 609-20

Grandstadt RC & Huffaker RC, (1982), Identification of the leaf vacuole as a major nitrate storage pool. *Plant Physiol.* **70** 410-13

Greaves MP & Derbyshire JF, (1972), The ultrastructure of the mucilaginous layer on plant roots. Soil Biological Biochemistry

Gregory FG, (1926), The effect of climatic conditions on the growth of barley. *Ann. Bot.* **40** 1 5-26

Griffiths DW & Bray EA, (1996), Shoot induction of ABA-requiring genes in response to soil drying. *J. Exp. Bot.* **47** 1525-31

Grossnickle SC & Russell JH, (1996), Changes in shoot water relations parameters of yellow cedar (*Chamaecyparis nootkatensis*) in response to environmental conditions. *Can. J. Bot.* **74** 31-9

Grzesiak S, Filek W & Koscielniak J, (1989), Influence of different soil moistures during the vegetative phase of development of field bean, (*Vicia faba* L. var. minor) on leaf water status, photosynthetic rate and plant growth. *J. Agron. & Crop Sci.* **162** 192-200

Guerin V, Trinchard JC & Rigaud J, (1990), Nitrogen fixation (C_2H_2 reduction) by broad bean (*Vicia faba* L.) nodules and bacteroids under water restricted conditions. *Plant Physiol.* **92** 595-601

Guerrier G, (1991), Nitrogen metabolism in Erica and soybean, two species differing by their sensitivity to inorganic N source. *Biologia plantarum* **33** 6 468-74

Guiz C, Hirel B, Shedlofsky G & Gadal P, (1979), Occurrence and influence of light on the relative proportions of two glutamine synthetases in rice leaves. *Plant Science Letters* **15** 271-277

Gutschick VP, (1981), Evolved Strategies in Nitrogen Acquisition by plants. *American Naturalist* **118** 607-637

Hafner H, Ndunguru, Bationo A & Marschner H, (1992), Effect of nitrogen, phosphorous and molybdenum applications on growth and symbiotic N2 fixation of groundnut in an acid sandy soil in Niger. *Fertiliser Research* **31** 69-77

Hageman RH, (1979), Integration of nitrogen assimilation in relation to yield. In: Nitrogen Assimilation of Plants Hewitt EJ & Cutting CV pp 591-611 Academic Press

Hamdi YA, (1982), Symbiotic Nitrogen fixation in faba beans. In: Faba bean Improvement Hawtin & Webb (Eds.) ISBN 9024725933 Holland pp 129-143

Hanish Ten Cate CH & Breteler H, (1981), The role of sugars in nitrate utilisation by dwarf bean. *Physiologia Plantarum* **52** 129-35

Hanson AD & Nelson CE, (1978), Water: adaptation of crops top drought-prone environments. In: Crop Improvement Carlson PS (Ed.) pp 77-152 Academic Press

Hanson AD & Hitz WD, (1983), Whole plant responses to water deficits: water deficits and nitrogen economy. In: Limitations to efficient water use in crop production. pp 331-343 ASA-CSSA-SSSA

Hanson AD & Hitz WD, (1982), Metabolic responses of mesophytes to plant water deficits. *Ann. Rev. Plant Physiol.* **33** 163-203

Harada A, Takaki H & Yamada Y, (1968), Effect of nitrogen sources on the chemical components in young plants. Soil Sci. & Plant Nut. **14** 47-55

Hardy RWF, Holsten RD, Jackson EK & Burns RC, (1968), The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiol.* **43** 1185-1205

Hardy RWF & Havela UD, (1976), Photosynthate as a major factor limiting nitrogen fixation by field grown legumes with emphasis on soybeans symbiotic N fixation in plants. In: Nitrogen fixation in plants Nutman (Ed.), **7** 421-39

Haynes R & Goh K, (1978), Ammonium and nitrate nutrition of plants. *Biol. Rev.* **53** 465-510

Haynes R & Goh K, (1978), Measurement of ammonium ions in flowing solution culture and diurnal variation. *Biol. Rev.* **53** 465-510

Havill DC, Lea JA & Stewart GR, (1974), Nitrate utilisation by species from acidic and calcareous soils. *New Phytologist* **73** 1221-1231

Hawkins HJ & Lewis OAM, (1993), Combination effect of NaCl salinity , nitrogen form, and calcium concentration on the growth, ionic content and gaseous exchange properties of *Triticum aestivum* L cv Gamtos. *New Phytol.* **124** 161-70

Heatherly LG & Elmore CD, (1986), Irrigation and planting date effects on soybean in clay soil. *Agron. J.* **78** 576-80

Hebblethwaite P (1982), The effects of water stress on the growth, development and yield of *Vicia faba* L. In: Proceedings of the international faba bean conference, Cairo, March 7-11, 1981. pp 165-75. Martinus Nijhoff / Dr. W. Junk. Netherlands.

Hebblethwaite PD, Dawkins TCK, Heath MC & Lockwood G, (1984), Faba bean research in Europe. In: *Vicia faba*: Anatomy Physiology & Breeding World crops: production, utilisation, description. Vol. 10. (Eds. Hebblethwaite, Dawkins, Heath & Lockwood). Martinus Nijhoff/Dr. Junk 90-247-2964-5

Heckenberger U, Roggatz U & Schurr U, (1998), Effect of drought stress on the cytological status in *Ricinus communis. J. Exp. Bot.* **49** 319 181-189

Hedley CL & Stoddart JL, (1971), Factors influencing alanine aminotransferase activity in leaves of *Lolium temulentum* L. *J. Exp. Bot.* **22** 71 239-48

Hirel B & Gadal P, (1980b), Glutamine synthetase in rice. A comparative study of the enzyme from roots and leaves. *Plant Physiol.* **66** 619-23

Hocking PJ & Meyer CP, (1991), Effects of CO_2 enrichment & N stress on growth and partitioning of nitrogen matter in wheat and maize. *Aust. J. Plant Physiol.* **18** 4 339-356

Hodge A, Robinson D, Griffiths BS & Fitter AH, (1999), Why plants bother: root proliferation results in increased nitrogen capture from an organic patch when two grasses compete. *Plant Cell & Environ.* **22** 811-20

Hofstra RJJ, Lanting L & de-Visser R, (1985), Metabolism of *Urdica dioca* as dependant on the supply of mineral nutrients. *Physiologia plantarum* **63** 13-18

Hogh-Jensen H, Wollenweber B & Schjoerring JK, (1997), Kinetics of nitrate and ammonium absorption and accompanying H^+ fluxes in roots of *Lolium perenne* L. and N_2 fixing *Trifolium repens* L. *Plant Cell & Environ.* **20** 1184-92

Hsaio TC, (1973), Plant Responses to water stress. *Ann. Rev. Plant Physiol.* **24** 519-70

Hsaio TC, Acevedo E, Fereres E & Henderson DW, (1976), Water stress, growth and osmotic adjustment. *Phil. Trans. R. Soc. London Ser B* **273** 479-500

Hsaio TC & Jing J, (1987), Leaf and root expansive growth in response to water deficits. In: physiology of cell expansion during plant growth. DJ Cosgrove & DP Knievel Eds. The American Society of Plant Physiologists. pp 180-192

Hunt R, (1978), Plant Growth Analysis. The Institute of Biology's Studies in Biology no. 96. Edward Arnold Pub.

Hurley M & Rowarth JS, (1999), Resistance to root growth and changes in the concentrations of ABA within the root and xylem sap during root-restriction stress. *J. Exp. Bot.* **50** 353 799-804

Ikeda M, Yamada Y & Harada T, (1974), Glucose metabolism in detached leaves of young tomato plants grown with ammonium and nitrate as nitrogen sources. *Soil Sci. & Plant Nut.* Japan **20** 185-94

Imsande J & Touraine B, (1994), Update on mineral nutrition: N demand and the regulation of nitrate uptake. *Plant Physiol.* **105** 3-7

Ines-Minguez MI & Sau F, (1989), Responses of nitrate-fed and nitrogen-fixing soybeans to progressive water stress. *J. Exp. Bot.* **40** 497-502

Ingram J & Bartels D, (1996), The molecular basis of dehydration tolerance in plants. *Ann Rev Plant Physiol. & Plant Mol. Biol.* **47** 377-403
Iturbe-Ormaetxe I, Escuredo PR, Arrese-Igor C & Becana M, (1998), Oxidative damage in pea plants exposed to water deficit or paraquat *Plant Physiol.* **116** 173-181

Jagtap V, Bhargava S, Streb P & Feierabend J, (1998), Comparative effects of water, heat, and light stresses on photosynthetic reactions in *Sorghum bicolor* (L.) Moench. *J. Exp. Bot.* **49** 327 1715-21

Jermyn MA, (1975), Increasing the sensitivity of the anthrone method for carbohydrate. *Analytical Biochemistry* **68** 332-335

Jeschke W, Wolf O & Hartung W, (1992), Effect of NaCl salinity on flows and partitioning of C, N, and mineral ions in whole plants of lupin, *Lupinus albus* L. *J. Exp. Bot.* **43** 251 777-88

Jones MM & Rawson HM, (1979), Influence of rate of development of leaf water deficits upon photosynthesis, leaf conductance, water use efficiency and osmotic potential in sorghum. *Physiologia plantarum* **45** 103-11

Jones MM, Osmond CB & Turner NC, (1980), Accumulation of solutes in leaves of sorghum and sunflower in response to water deficits. *Aust. J. Plant Physiol.* **7** 193-205

Jones MM Flowers TJ & Jones MB, (1989), Introduction: some terminology and common mechanisms. In: Plants Under Stress. University Press Cambridge

Jordan BR, He J, Chow WS & Anderson JM, (1983), Changes in mRNA levels and polypeptide subunits of ribulose 1,5-bisphosphate carboxylase in response to supplementary ultraviolet-B radiation. *Plant Cell & Environ.* **15** 91-98

Joy KW & Ireland R, (1990), Enzymes of Asparagine Metabolism. In: Methods of Plant Biochemistry. Vol 3 Ed. PJ Lea pp 287-295 Academic Press

Kamachi K, Yamaya T, Mae T, & Ojima K, (1991), A role for glutamine synthetase in the remobilization of leaf nitrogen during natural senescence in rice leaves. *Plant Physiol.* **96** 411-417

Kanamori T & Matsumoto H, (1974), Asparagine biosynthesis by *Oryza sativa* seedlings. *Phytochemistry* **13** 1407-12

Kassam AH, (1971), Some physical aspects of the water relations of *V. faba*, L. Ph.D. thesis, University of Reading

Kato T, (1980), Nitrogen assimilation by a citrus tree, Assimilation of labelled nitrate and ammonium by detached leaves in light and dark. *Physiology Planta* **50** 304-08

Katz A & Glantz MH, (1977), Rainfall statistics droughts and desertification in the Sahel. In: Desertification Ed MH Glantz pp 81-102 Westview Press, Boulder, Col.

Kaur A, Sheoran IS, & Singh R, (1985), Effect of water stress on the enzymes of nitrogen metabolism in mung beans (*Vigna radiata* Wilczeck) nodules. *Plant Cell & Environ.* **8** 195-200

Kemp CD, (1960), Methods of estimating the leaf area of grasses from linear measurements. *Ann. Bot.* **24** 491-500

Kennedy C & Eady RR, (1979), Regulation of nitrogen fixation: the effects of ammonium and oxygen on nitrogenase synthesis. In: Nitrogen assimilation of plants. Ed. Hewitt EJ & Cutting CV (Eds.) pp 73-89 Academic Press

Keys AJ, Bird IF & Cornelius MJ, (1978), Photorespiratory Nitrogen Cycle. *Nature* **275** 741-42

Kirkby EA & Hughes, (1970), Some aspects of ammonium and nitrogen nutrition in plant metabolism In Nitrogen nutrition of the plant. EA Kirkby (Ed.) pp 69-77 Waverley Press Leeds.

Kizirian H & Taha M, (1997), GCC-S Pulses Market: worth more than a hill of beans. http://www.fas.usda.gov/info/agexporter/1997/gcc5.html.

Klepper L, Flesher D & Hageman RH, (1971), Generation of reduced nicotinamide adenine dinucleotide for nitrate reduction in green leaves. *Plant Physiol.* **48** 580-90

Krajina VJ, Madoc-Jones S & Mellor G, (1973), Ammonium and nitrate in the nitrogen economies of some confers growing in the Douglas fir communities of the Pacific Northwest of America. *Soil Biol & Biochem.* **5** 143-147

Krapp A, Quick WP & Stitt M, (1991), Ribulose-1,5 bisphosphate carboxylase-oxygenase, other Calvin cycle enzymes and chlorophyll decrease when glucose is supplied to mature spinach leaves via the transpiration stream. *Planta* **186** 58-69

Krapp A, Hoffman B, Schafer C & Stitt M, (1993), Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates : a mechanism for the sink regulation of photosynthesis. The Plant Journal **3** 6 817-28

Kronzucker HJ, Siddiqi MY, Glass ADM & Kirk GJD, (1999), Nitrate-ammonium synergism in rice. A subcellular flux analysis. *Plant Physiol.* **119** 3 1041-46

Kueh JSH, Hill JM, Smith DJ & Bright SWJ, (1984), Proline biosynthesis in a proline accumulating barley mutant. *Phytochem.* **23** 2207-10

Kuiper D, (1993), Sink Strength: Established and regulated by plant growth regulators. *Plant Cell & Environ.* 1025-26

Kumar A & Singh DP, (1998), Use of physiological indices as a screening technique for drought tolerance in oilseed brassica species. *Annals of Bot.* **81** 3 413-20

Ladley PD, (1990), Water Deficit responses of *Geum urbanum* and *Geum rivale*. Ph.D.thesis. University College London

Lam HM, Coschigano KT, Oliviera IC, Melo-Oliveira R & Coruzzi GM, (1996), The molecular genetics of nitrogen assimilation into amino acids in higher plants. *Ann. Rev. Plant Physiol. & Plant Mol.* Biol. **47** 569-93

Lamprecht W & Heinz F, (1984), Pyruvate. In: Methods of Enzymic Analysis Vol VI Metabolites 1 (Ed. Bergmeyer) 3rd Edn. VCH Weinheim pp 570-7

Lawes DA, (1980), Opening Remarks in: Bond DA (Ed.) *Vicia faba* - feeding value, processing and viruses. Martinus Nijhoff Publishers

Lawlor DW & Fock H, (1975), Photosynthesis and photorespiratory CO_2 evolution of water stressed sunflower leaves. *Planta* (Berl.) **126** 247-258

Lawlor DW, (1995), The effect of water deficit on photosynthesis In: environment and plant metabolism; flexibility & acclimation (Ed. Smirnoff) pp 129-160 BIOS

Layzell DB Turpin DH & Elrifi IR, (1985), Effect of N source on the steady state growth and N assimilation of P-limited *Anabaena flos-aquae*. *Plant Physiol.* **78** 739-45

Lee JA & Stewart GR , (1978), Ecological aspects of N assimilation. In: Advances in Botanical Research. pp 2-41 Woolhouse HW (Ed.) pp 2-41

Lee RB, Purves JV, Ratcliffe RG & Saker LB, (1992), Nitrogen assimilation and the control of ammonium and nitrate absorption by maize roots. *J. Exp. Bot.* **43** 1385-96

Lee RB & Drew MC, (1989), Rapid, reversible inhibition of nitrate influx in barley by ammonium. *J. Exp. Bot.* **40** 741-52

Lees H *et al*, (1968), Studies on asparagine synthesis and utilisation in seedlings. *Biochem. Biophys.* **126** 539-46

Leigh RA, Ahmad N & Wyn Jones RG, (1981), Assessment of glycine betaine and proline compartmentation by analysis of isolated beet vacuoles. *Planta* **153** 34-41

Lewis OAM, (1986) Plants & Nitrogen. Edward Arnold Publishers ISBN: 0-7131-2899-2

Lewis OAM, Leidi EO & Lips SH, (1989), Effect of nitrogen source on growth response to salinity stress in maize and wheat. *New Phytol.* **111** 155-60

Lexa M & Cheeseman G, (1997), Growth and nitrogen reactions in reciprocal grafts of wild-type and nitrate reductase deficient mutants of pea (*Pisum sativum* L. var. Juneau). *J. Exp. Bot.* **48** 311 1241-1250

Li J, JLYR Lee & Assman SM, (1998), Cyclic AMP stimulates K^+ channel activity in mesophyll cells of *Vicia faba* L.. *Plant Physiol.* **106** 957-61

Ligero F, Caba JM, Lluch C & Olivares J, (1991), Nitrate inhibition of nodulation can be overcome by the ethylene inhibitor aminoethoxyvinylglycine. *Plant Physiol.* **97** 1221-1225

Lillo C & Henriksen A, (1984), Comparative studies of diurnal variations of nitrate reductase activity in wheat, oat, and barley. *Physiologia plantarum* **62** 89-94

Lips SH, (1979), Photosynthesis and photorespiration in nitrate metabolism. In: Nitrate Assimilation in Plants. Hewitt EJ & Cutting CV (Eds.) pp 445-450. Academic Press.

Lorenz H, (1973), Nitrate ammonium and amino acids in the bleeding sap of tomato plants in relation to form and concentration of nitrogen in the medium. *Plant & Soil* **45** 163-68 169-75

Losada M, Herrara J, Maldonado JM & Paneque A, (1973), Mechanism of nitrate reductase reversible inactivation by ammonia in *chlamydomonas*. *Plant Sci. Letters* **1** 31-37

Lowry OH, Rosebrough NJ, Lewis-Farr A, & Randall RJ, (1951), Protein measurement with the Folin Phenol Reagent. *Biol. Chem.* **193** 1 265-75

Ludlow MM & Ng TT, (1974), Water stress suspends leaf ageing. *Plant Science letters* **3** 235-240

McCready RM, Guggolz J, Silviera V & Owens HS, (1950), Determination of starch and amylase in vegetables. *Anal. Chem.* **22** 1156-58

McCree KJ, (1986), Whole plant carbon balance during osmotic adjustment to drought and salinity stress. *Aust. J. Plant Physiol.* **13** 33-43

McCulloch H, (1967), The determination of ammonia in whole blood by a direct colorimetric method. *Clinica. Chimica. Alca.* **17** 297-304

McCully ME, (1999), Root xylem embolisms and refilling. Relations to water potentials of soil, roots and leaves and osmotic potentials of root xylem sap. *Plant Physiol.* **119** 3 1001-8

McDonald AJS & Davies WJ, (1996), Keeping in touch: responses of the whole plant to deficits in water and nitrogen supply. In: Advances in Botanical Research Vol. 22 pp 229-300. Academic Press ISBN: 0-12-005922-3

McNally S & Hirel B, (1983), Glutamine synthetase isoforms in higher plants. *Physiol. Veg.* **21** 4 761-774

McNally SF, Hirel B, Gadal P, Mann AE & Stewart GR, (1983b), Glutamine synthetases of higher plants. *Plant Physiol.* **72** 22-25

Mairs H, (1996), The vegetarian society. Broad Beans. http://www.vegsoc.org/food/broad.html.

Mansfield TA & Davies WJ, (1981), Stomata & Stomatal Mechanisms. In: Physiology and biochemistry of drought resistance in plants. Paleg LG & Aspinall D (Eds.) Academic Press

Marchner H & Romheld V, (1983), *In vivo* measurement of root induced pH changes at the soil : root interface : effect of plant species and nitrogen source. *Z. Pflanzenphysiol.* **111** 241-51

Marques IA, Oberholzer MJ, & Erismann CH, (1983), Effects of different inorganic nitrogen sources on photosynthetic carbon metabolism in primary leaves of non-nodulated *Phaseolus vulgares* L.. *Plant Physiol.* **71** 555-61

Marschner H, (1986), Mineral Nutrition in higher plants. Academic Press London

Martinez V & Cerda A, (1989), Influence of N source on rate of Cl, N, Na, and K uptake by cucumber seedlings grown in saline conditions. *J. Plant Nut.* **12** 8 971-83

Martinez-Carrasco R, Perez P, Handley LL, Scrimgeour CM, Igual M, Molino IMD & de la Puente LS, (1998), Regulation of growth water use efficiency and 13C by the nitrogen source in *Casuarina equisetifolia* Forst. & Forst. *Plant Cell and Environ.* **21** 531-34

Martinoia E, Hecj U & Wiemken A, (1981), Vacuoles as storage compartments for nitrate in barley leaves. *Nature* **289** 292-294

Masle J & Passioura JB, (1988), The effect of soil strength on the growth of young wheat plants. *Aust. J. Plant Physiol.* **14** 643-56

McCready RM, Guggolz J, Silviera V & Owens HS, (1950), Determination of starch and amylase in vegetables. *Anal. Chem.* **22** 1156-58

McCree KJ, (1986), Whole plant carbon balance during osmotic adjustment to drought and salinity stress. *Aust. J. Plant Physiol.* **13** 33-43

McCulloch H, (1967), The determination of ammonia in whole blood by a direct colorimetric method. *Clinica. Chimica. Alca.* **17** 297-304

McCully ME, (1999), Root xylem embolisms and refilling. Relations to water potentials of soil, roots and leaves and osmotic potentials of root xylem sap. *Plant Physiol.* **119** 3 1001-8

McDonald AJS & Davies WJ, (1996), Keeping in touch: responses of the whole plant to deficits in water and nitrogen supply. In: Advances in Botanical Research Vol. 22 pp 229-300. Academic Press ISBN: 0-12-005922-3

McNally S & Hirel B, (1983), Glutamine synthetase isoforms in higher plants. *Physiol. Veg.* **21** 4 761-774

McNally SF, Hirel B, Gadal P, Mann AE & Stewart GR, (1983b), Glutamine synthetases of higher plants. *Plant Physiol.* **72** 22-25

Mairs H, (1996), The vegetarian society. Broad Beans. http://www.vegsoc.org/food/broad.html.

Mansfield TA & Davies WJ, (1981), Stomata & Stomatal Mechanisms. In: Physiology and biochemistry of drought resistance in plants. Paleg LG & Aspinall D (Eds.) Academic Press

Marchner H & Romheld V, (1983), *In vivo* measurement of root induced pH changes at the soil : root interface : effect of plant species and nitrogen source. *Z. Pflanzenphysiol.* **111** 241-51

Marques IA, Oberholzer MJ, & Erismann CH, (1983), Effects of different inorganic nitrogen sources on photosynthetic carbon metabolism in primary leaves of non-nodulated *Phaseolus vulgares* L.. *Plant Physiol.* **71** 555-61

Marschner H, (1986), Mineral Nutrition in higher plants. Academic Press London

Martinez V & Cerda A, (1989), Influence of N source on rate of Cl, N, Na, and K uptake by cucumber seedlings grown in saline conditions. *J. Plant Nut.* **12** 8 971-83

Martinez-Carrasco R, Perez P, Handley LL, Scrimgeour CM, Igual M, Molino IMD & de la Puente LS, (1998), Regulation of growth water use efficiency and 13C by the nitrogen source in *Casuarina equisetifolia* Forst. & Forst. *Plant Cell and Environ.* **21** 531-34

Martinoia E, Hecj U & Wiemken A, (1981), Vacuoles as storage compartments for nitrate in barley leaves. *Nature* **289** 292-294

Masle J & Passioura JB, (1988), The effect of soil strength on the growth of young wheat plants. *Aust. J. Plant Physiol.* **14** 643-56

Mattas RE & Pauli AW, (1965), Trends in nitrate reduction and nitrogen fractions in young corn (*Zea mays* L.) plants during heat and moisture stress. *Crop Sci.* **5** 181-184

Maynard DN & Barker AV, (1969), Studies on the tolerance of plants to ammonium nutrition. *J. Americ. Soc. Hort. Sci.* **94** 235-39

Meier CE, Newton RJ, Puryear JP, & Sen S, (1992), Physiological responses of loblolly pine (*Pinus taeda* L.) seedlings to drought stress: osmotic adjustment & tissue elasticity. *J. Plant Physiol.* **140** 754-60

Meinzer FC & Zhu J, (1998), Nitrogen stress reduces the efficiency of the C4 CO_2 concentrating system, and therefore quantum yield, in *Saccharum* (sugarcane) species. *J. Exp. Bot.* **49** 324 1227-34

Mengel K, Haghparast MR & Koch K, (1974), The effect of potassium on the fixation of molecular nitrogen by root nodules of *Vicia faba*. *Plant Physiol.* **54** 535-538

Michael G, Martin & Owassi I, (1970), The uptake of ammonium and nitrate from labelled ammonium nitrate in relation to the carbohydrate supply of the roots. In: Nutrition of Plants. Kirkby EA (Ed.) pp 22-29 Waverley Press Leeds

Miflin BJ, (1974), The location of nitrate reductase and other enzymes related to amino acid biosynthesis in the plastids of roots and leaves. *Plant Physiol.* **54** 550-555

Miflin BJ & Lea PJ, (1975), Glutamine and asparagine as nitrogen donors for reductant dependant glutamate synthesis in pea roots. *Biochem. J.* **149** 403-09

Miflin BJ & Lea PJ, (1977), Amino acid metabolism. *Ann. Rev. Plant Physiol.* **28** 299-329

Miflin BJ, Lea PJ, & Wallsgrove RM, (1980), 12. The role of glutamine in ammonia assimilation and reassimilation in plants In: Glutamine: metabolism, enzymology, and regulation. pp 213-34 Academic press. ISBN 0-12-506040-8

Min X, Siddiqi MY, Guy RD, Glass ADM & Kronzucker HJ, (1998), Induction of nitrate uptake and NRA in trembling aspen and lodgepole pine. *Plant Cell & Environ.* **21** 1039-46

Min X, Siddiqi MY, Guy RD, Glass ADM & Kronzucker HJ, (1999), A comparative study of fluxes and compartmentation of nitrate and ammonium in early successional tree species. *Plant Cell & Environ.* **22** 821-30

Minchin FR & Pate JS, (1973), The carbon balance of a legume and the functional economy of its root nodules. *J. Exp. Bot.* **24** 79 259-71

Minotti PL, Williams DC & Jackson WA, (1969b), Nitrate uptake by wheat as influenced by ammonium and other cations. *Crop Sci.* **8** 9-14

Mollering H, (1985a), L-(-)-Malate. In Methods of Enzymic Analysis. Vol VI Metabolites 1. (Ed. Bergmeyer) 3rd Edn. VCH Weinheim pp 39-47

Mollering H, (1985b), Citrate. In: Methods of Enzymic Analysis Vol II. Metabolites 2. (Ed. Bergmeyer) 3rd Edn. VCH Weinheim pp 2-12

Monson RK, Schulze ED, Freund M & Heilmeier H, (1994), The influence of nitrogen availability on carbon and nitrogen storage in the biennial *Cirsium vulgare* (Savi) Ten.II. The cost of nitrogen storage. *Plant Cell & Environ.* 17 1133-41

Morgan JM, (1984), Osmoregulation and water stress in higher plants. *Ann. Rev. Plant. Physiol.* **35** 299-319

Morgan JM & Condon, (1986), Water use, grain yield, and osmoregulation in wheat. *Aust. J. Plant Physiol.* **13** 523-32

Morris MD, (1974), What is famine? *Economic & Political Weekly* 9 44 1855-64

Muller EKH & Janiesch P, (1993), *In vivo* nitrate reductase activity in *Carex pseudocyperus* L.: the influence of nitrate-ammonium concentration ratios and correlation with growth. *J. Plant Nut.* **167** 1357-72

Munns R & Weir R, (1981), Contribution of sugars to osmotic adjustment in elongating and expended zones of wheat leaves during moderate water deficits at two light levels. *Aust. J. Plant Physiol.* 8 93-105

Myers J, (1980), On the algae: thoughts about physiology and measurements of efficiency. In: Plant productivity in the sea Falkowski PG (Ed.) pp 1-16 Plenum Press, New York

Nash D, Paleg LG & Wiskich JT, (1981), Effect of proline and betaine on the heat stability of some mitochondrial enzymes. *Aust. J. Plant Physiol.* 8

Nato F, Hirel B, Nato A & Gadal P, (1984), Chloroplastic glutamine synthetase from tobacco leaves. A glycosylated protein. *FEBS Letters* **175** 3 443-446

Nelson P & Selby L, (1974), The effect of nitrogen sources and corn levels on the growth and composition of sitka spruce and Scot's Pine. *Plant & Soil* **41** 573-588

Nicholas JC, Harper JE & Hageman RH, (1976), Nitrate reductase activity in soybeans (*Glycine max.* L.Merr) II Energy limitations. *Plant physiol.* **58** 736-39

Nobel PS, (1991), Phytochemical and environmental plant physiology San Diego: Academic Press

Noctor G, Arisi ACM, Jouanin L, Kunert KJ, Rennenberg H & Foyer CH, (1998), Review article: Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. *J. Exp. Bot.* **49** 321 623-47

Nogues S, Allen DJ, Morison JIL & Baker NR, (1998), Ultraviolet-B radiation effects on water relations, leaf development, and photosynthesis in droughted pea plants. *Plant Physiol.* **117** 173-181

Nonami H & Boyer JS, (1989), Turgor and growth at low water potentials. *Plant Physiol.* **89** 798-804

Nonami H & Boyer JS, (1990a), Primary events regulating stems growth at low water potentials. *Plant Physiol.* **93** 1601-09

Nonami H & Boyer JS, (1990b), Wall extensibility and cell wall hydraulic conductivity decrease in enlarging stem tissues at low water potentials. *Plant Physiol.* **93** 1610-19

Oaks A & Bidwell RGS, (1970), Compartmentation of intermediary metabolites. *Ann. Rev. Plant Physiol.* **21** 43-66

Oaks A, Wallace W & Stephens D, (1972), Synthesis and turnover of nitrate reductase in corn roots. *Plant Physiol.* **50** 649-54

Oaks A, Aslam M & Boesel L, (1977), Ammonium and amino acids as regulators of nitrate reductase in corn roots. *Plant Physiol.* **59** 391-94

Oghoghorie CGO & Pate JS, (1971), The nitrate stress syndrome of the nodulated field pea (*Pisum Arvense* L.). Techniques for measurement and evaluation in physiological terms. *Plant & Soil* Special Volume. 185-202

Oliveira IC & Coruzzi GM, (1999), Carbon and amino acids reciprocally modulate the expression of glutamine synthetase in *Arabidopsis*. *Plant Physiol.* **121** 301 - 309

Orebamjo TO & Stewart GR, (1975), Ammonium inactivation of nitrate reductase in *Lemna minor* L. *Planta* **122** 37-44

Ortega JL, Roche D, & Sengupta-Gopalan C, (1999), Oxidative turnover of soybean root glutamine synthetase *in vitro* and *in vivo* studies. *Plant Physiol.* **119** 1483-95

Osmond CB, Austin MP, Berry JA, Billings WD, Boyer JS, Dacey WH, Nobel PS, Smith SD & Winner WE, (1987), Stress psychology and the distribution of plants. *Bioscience* **37** 38-48

Othman WMW, Lie TA, Mennetje L, Wassink GY, Wan Othman WM, & Manetz LT, (1991), Low level phosphorus supply affecting nodulation, nitrogen fixation, and growth of cowpea, (*Vigna-unguiculata* L Walp). *Plant & Soil* **135** 1 67-74

Ourry A, Decau ML & Laine PH, (1995), Plant N uptake in relation to nitrate availability In: Lemaire & Burns (Eds.) Diagnostic procedure for crop N management INRA 2-7380-0757-0

Paleg LG & Aspinall (1981), The physiology and biochemistry of drought resistance in plants. Academic Press. Australia. ISBN 0-12-544380-3

Paleg LG, Stewart GR & Starr R, (1985), The effect of compatible solutes on proteins. *Plant & Soil* **89** 83-94

Palmer WC, (1974), Meteological drought. US weather bureau department Commer. Res. paper 45 US Governmant Printing Office Washington DC

Pankovic D, Sakac Z, Kevresan S & Plesnicar M, (1999), Acclimation to long term water deficit in the leaves of two sunflower hybrids: photosynthesis, electron transport and carbon metabolism. *J. Exp. Bot.* 50 330 127-38

Pankow W *et al*, (1991), The significance of mycorrhizas for protective ecosystems. *Experientia* **47** 391-94

Parson R, Stanforth A, Raven JA & Sprent JI (1993), Nodule growth and activity may be regulated via a feedback mechanism involving phloem nitrogen. *Plant Cell & Environ.* **16** 125-36

Passioura JB, (1981), Ch 3 Water collection by Roots. In: Physiology and Biochemistry of drought resistance. Paleg & Aspinall (Eds.) pp 39-53 Academic Press. ISBN 0 12 544 380 3

Passioura JB & Fry S, (1992), Turgor and cell expansion - beyond the Lockhart equation. *J. Plant Physiol.* **19** 565-76

Passioura JB, Condon AG, & Richards RA, (1993), Water deficits, the development of leaf area and crop productivity. In: Water deficits plant responses from cells to community. Smith JAC & Griffiths H (Eds) pp 253-263 Bios Scientific Publishers. ISBN 1 872748 06 6

Pate JS, Gunning BES & Briarty LG, (1969), Ultrastructure and functioning of the transport system of the leguminous root nodule. *Planta* **85** 11-34

Pate JS & Gunning BES, (1972), Transfer Cells. *Ann. Rev. Plant Physiol.* **23** 173-96

Pate JS, (1973), Uptake assimilation and transport of Nitrogen compounds by plants. *Soil Biol. Biochem.* **5** 109-119

Pate JS, Layzell DB & Atkins CA, (1979), Economy of carbon and nitrogen in a nodulated and non-nodulated (NO_3 grown) legume. *Plant Physiol.* **64** 1083-1088

Pate JS, (1983), Distribution of Metabolites In: Plant Physiology III Nitrogen Metabolism. Treatise A (Ed.) pp335-401 Academic Press ISBN 0-12-668608-4

Pearson J & Stewart GR, (1987), Development of a Metabolic Index of stress. Final Report Overseas development Admin. Research Pro. 1984-1987

Pearson J & Ji YM, (1994), Seasonal variations of leaf glutamine synthetase isoforms in temperate deciduous tress strongly suggests different functions for the enzymes. *Plant Cell & Environ.* **17** 1331-37

Pelsey F & Caboche M, (1992), Molecular genetics of nitrate reductase in higher plants. *Advances in Genetics* **30** 1-40

Peoples MB, Sudin MH & Herridge DF, (1987), Translocation of nitrogenous compounds in symbiotic and nitrate fed amide exporting legumes. *J. Exp. Bot.* **38** 567-79

Pill WG & Lambeth VN, (1977), Effects of NH_4 and NO_3 nutrition with and without pH adjustment on tomato growth, ion composition, and water relations. *J. Amer. Soc. Hort. Sci.* **102** 78-81

Pirie NW, (1979), The efficiency of protein production by different farming systems. In: nitrogen Assimilation of Plants Hewitt EJ & Cutting CV Eds.) pp 613-623 Academic Press

Platt SG, Plaut Z & Bassham JA, (1977), Ammonia regulation of carbon metabolism in photosynthesising leaf discs. *Plant Physiol.* **60** 739-742

Plies-Balzer E Kong T, Schubert S & Mengel K, (1995), Effect of water stress on plant growth, nitrogenase activity and nitrogen economy of four different cultivars of *V. faba*. *Eur. J. Agron.* 4 2167-73

Plummer DT, (1978), Quantitative determination of carbohydrates In: An introduction to practical biochemistry. 2nd Ed. McGaw Hill UK

Poljakoff-Mayber A, (1981), Ultrastructural consequences of drought. In: Physiology and Biochemistry of drought resistance in plants. Paleg LG & Aspinall D (Eds.) pp 389-403 Academic Press.

Postgate JR, (1974), New advances and future potential in biological N fixation. *J. App. Bact.* **37** 185-202

Postgate J, (1987), Nitrogen fixation 2nd Ed. Edward Arnold Publishers

Purcell LC & King CA, (1996), Drought and nitrogen source effects on nitrogen nutrition, seed growth, and yield in soybean. *J. Plant Nut.* **19** 6 969-93

Purcall LC, Vadez V, Sinclair CR, Serraj R & Nelson R, (1998b),Screen of soybean germplasm for N2 fixation drought tolerance,. 16th North American Symbiotic Nitrogen Fixation Conference. Cancun, Mexico Feb 1998.

Pulich WM, (1986), Variations in leaf soluble amino acids and ammonium content in sub tropical seagrasses related to salinity stress. *Plant Physiol.* **80** 283-286

Pustoviotova TN & Zholkevich VN, (1992), Main trends in the study of the effect of drought on physiological processes in plants. *Fixiologiya i Biolhimiya kul Turnykh Rastenii*

Quebedeaux B & Ozbun JL, (1973), Effects of ammonium nutrition on water stress, water uptake and root pressure in *Lycopersicon esculentum* Mill. *Plant Physiol.* **52** 677-9

Quartacci MF, Forli M, Vacchia D, Bochicchio A & Navari-Izzo F, (1997), Desiccation-tolerant *Sporobolus stapfianus* lipid composition and cellular ultrastructure during dehydration and rehydration. *J. Exp. Bot.* **48** 311 1269-79

Raab TK & Terry N, (1994), Nitrogen source regulation of growth and photosynthesis in *Beta vulgaris* L.. *Plant Physiol.* **105** 1159-66

Radin JW & Ackerson RC, (1981), Water relations of cotton plants under nitrogen deficiency. *Plant Physiol.* **67** 115-119

Radin J & Boyer, JS, (1982), Control of leaf expansion by nitrogen nutrition in sunflower plants. Role of hydraulic conductivity and turgor. *Plant Physiol.* **69** 771-75

Radin JW, Mauney JR, & Guinn G, (1985), Effect of N fertility on plant water relations and stomatal responses to water stress in irrigated corn. *Crop Sci.* **25** 110-15

Radoglou KM & Jarvis PG, (1993), Effects of atmospheric CO_2 enrichment on early growth of *Vicia faba*, a plant with large cotyledons. *Plant Cell & Environ.* **16** 93-98

Rajagopal V, Balasubramanian V & Sinha SK, (1977), Diurnal fluctuations in relative water content, nitrate reductase and proline content in water-stressed and non-stressed wheat. *Physiol. Plant.* **40** 69-71

Raniera P, Bernardi R, Lanese P & Soldatini GF, (1989), Changes in free amino acid content and protein pattern of maize seedlings under water stress. *Environ. & Exp. Bot.* **29** 3 351-57

Rao KP & Rains DW, (1976a), Nitrate absorption by barley. I. Kinetics. *Plant Physiol.* **57** 55-58

Raper CD, Vessey JK, & Henry LT, (1991), Increase in nitrate uptake by soybean plants during interruption of the dark period with low light intensity. *Physiol. Plant.* **81** 183-9

Raschke K, (1976), How stomata resolve the dilemma of opposing priorities. *Phil. Trans. R. Soc. Lond. B.* **273** 551-60

Raven JA & Smith FA, (1976), Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. *New Phytol.* **76** 415-431

Raven JA, (1985), Regulation of pH and osmolarity generation in vascular plants: a cost-benefit analysis in relation to efficiency of use of energy, nitrogen, and water. *New Phytol.* **101** 25-77

Raven JA, Wollenweber B, & Handley LL, (1992), A comparison of ammonia and nitrates as nitrogen sources for photolithotroph. *New Phytologist* **121** 1 19-32

Raven JA & Sprent JI, (1993), Nitrogen assimilation and it's role in plant water relations - In Water deficits plant responses from cell to community. Ed JAC Smith & H Griffiths Bios Publishers Environmental Plant Biology Series.

Read DJ, (1991), Myccorhizas in ecosystems. *Experientia* **47** 377-91

Redinbaugh MG & Campbell WH, (1991), Higher plant responses to environmental nitrate. Minireview. *Physiologia planta* **82** 640-650

Redinbaugh MG & Campbell WH, (1993), Glutamine synthetase and ferredoxin-dependant glutamate synthase expression in the maize (*Zea mays*) roots primary response to nitrate. *Plant Physiol.* **101** 1249-55

Remmler JL & Campbell WH, (1986), Regulation of corn leaf nitrate reductase II synthesis and turnover of the enzyme's activity and protein. *Plant Physiol.* **80** 442-47

Resines JA, Diez MT & Arin MJ, (1993), Statistical evolution of agreement between HPLC and colourimetic methods for analysis of allantoin in ruminants' urine. *J. Liquid Chromatography* **16** 13 2853-2859

Rhoden EG, Croy LI & Doolittle G, (1987), Seasonal and diurnal variation in nitrate reductase activity of cowpeas. *Plant & Soil* **102** 17-19

Rhodes D, Rendon GA & Stewart GR, (1975), The control of glutamine synthetase level in *Lemna minor*. *Planta* (Berl.) **125** 201-11

Rhodes D, Rendon, GA, & Stewart GR, (1976), The regulation of ammonia assimilating enzymes in *Lemna minor*. *Planta* (Berl.) **129** 203-10

Rhodes D, Sims AP, & Stewart GR, (1979), Glutamine synthetase and the control of nitrogen assimilation in *Lemna minor*. CV. In: Nitrogen assimilation of plants Ed Hewitt EJ & Cutting (Eds) pp 501-19 CV Academic Press

Rhodes D & Samaras Y, (1994), Genetic control of osmoregulation. In: Strange K Ed. Cellular & molecular physiology of cell volume regulation pp 347-61 CRC Press 0-8493-4448-4

Rhodes D, Handa S, & Bressan RA, (1986), Metabolic changes associated with adaptation of plant cells to water stress. *Plant & Soil* **82** 890-903
Riccardi F, Gazeau P, Vienne D, & Zivy M, (1998), Protein changes in response to progressive water deficit in maize. *Plant Physiol.* **117** 1253-63

Richards JE & Soper RJ, (1979), Effects of nitrogen fertiliser on yield, protein content, and symbiotic nitrogen fixation in faba beans *Vicia faba* var. Minor. *Agron. J.* **71** 5 807-11

Richardson SG & McCree KJ, (1985), Carbon balance and water relations of sorghum exposed to salt and water stress. *Plant Physiol.* **79** 1015-20

Rigano C, Fuggi A, Aliota G, di Martino Rigano V & Vona V, (1979), Reduction of nitrate to nitrite in vivo at non-physiological pH Effect of NH_4^+ 307-308 In: Nitrogen assimilation of plants Hewitt EJ & Cutting CV (Eds.) Academic Press

Rigaud J, (1981), Comparison of the efficiency of nitrate and N2 fixation in crop yield In: Nitrogen and carbon metabolism. JD Bewley (Ed.) Martinus Nijhoff/Dr W Junk Publishers

Robinson D, (1986), Limits to nutrient inflow rates in roots and root systems. *Physiologia plantarum* **68** 551-9

Robinson S, Stewart GR, & Starr, (1992), Regulation of glutamate dehydrogenase activity in relation to C limitation and protein catabolism in carrot cell suspension cultures. *Plant Physiol.* **98** 1190-195

Rodriguez-Maribona B, Tenorio JL, Conde JR & Ayerbe L, (1992), Correlation between yield and osmotic adjustment of peas *Pisum sativumi* under drought stress. *Field Crops Research* **29** 1 15-22

Rogenes SE, (1975) Glutamate dependant asparagine synthetase from *Lupinus luteus*. *Phytochemistry* **14** 1975-79

Rufty TW, Raper Jnr. CD & Jackson WA, (1983), Growth and nitrogen assimilation of soybeans in response to ammonium and nitrate nutrition. *Botanical Gazette* **144** 466-470

Rufty TW, Raper Jnr. CD & Huber SC, (1984), Alterations in internal partitioning of carbon in soybean plants in response to N stress. *Can. J. Bot.* **62** 501-508

Rufty TW, Israel DW & Volk RJ, (1984), Assimilation of $15NO_3^-$ taken up by plants in the light and in the dark. *Plant Physiol.* **76** 769-75

Rufty TW, MacKown CT & Volk RJ, (1989), Effects of altered carbohydrate availability on whole plant assimilation of $15NO_3^-$. *Plant Physiol.* **89** 457-63

Russell BL, Rathinasabapathi B & Hanson AD, (1998), Osmotic stress induces expression of choline monooxygenase in sugar beet and amaranth. *Plant Physiol.* **116** 859-65

Ryan PR & Walker NA, (1994), The regulation of ammonia uptake in *Chara austalis*. *J. Exp. Bot.* **45** 277 1057-67

Ryle GJA, Powell CE & Gordon AJ, (1979), The respiratory costs of nitrogen fixation in soybean cowpea and white clover. *J. Exp. Bot.* **30** 114 145-53

Sadunishvili T, Gvarliani N, Nutsubidze N & Kvestidze G, (1996), Effect of methionine sulphoximine on nitrogen metabolism and externally supplied ammonium assimilation in kidney bean. *Ecotox. & Environ. Safety* **34** 1 70-75

Salama A & Sinclair T, (1994), Soybean nitrogen fixation and growth as affected by drought stress and potassium fertilization. *J. Plant. Nut.* **17** 7 1193-1203

Salsac L, Chaillou S, Morot-Gaudry JF, Lesaint C & Jolvet E, (1987), Nitrate and ammonium nutrition in plants. *Plant Physiol. Biochem.* **25** 805-12

Samaras Y, Bressan RA, Csonka LN, Garcia-Rios MG, Paino D'Urzo M & Rhodes D, (1995), Proline accumulation during drought and salinity In: Environment & Plant Metabolism Flexibility & Acclimation (Ed Smirnoff) Bios Pub.

Sanchez-Rodriguez J, Perez P & Martinez-Carrasco R, (1999), Photosynthesis, carbohydrate levels and chlorophyll florescence-estimated intercellular CO_2 in water-stressed *Casuarina equisetifolia* Forst. & Forst. *Plant Cell & Environ.* **22** 867-73

Sangakkara UR, Hartwig UA, & Nosbuerger J, (1996), Soil moisture and potassium affect the performance of symbiotic nitrogen fixation in faba bean and common bean. *Plant & Soil* **184** 123-30

Sau F & Ines-Minguez M, (1990), Response to water stress and recovery of nitrate fed and N-fixing faba bean. *J. Exp. Bot.* **41** 230 1207-1211

Saxena MC, (1982), Physiological aspects of adaptation. In: Faba bean Improvement. Hawtin & Webb (Eds.) pp 145-159 Kluer Academic Publishers Group ISBN 9024725933

Schrader LE & Thomas RJ, (1981), Nitrate uptake, reduction, and transport in the whole plant, In: Nitrogen & Carbon Metabolism Ed JD Bewley M Nijhoff/Dr W Junk publishers

Schrader LE, (1978), Uptake, accumulation, assimilation, and transport of nitrogen in higher plants. In: Nitrogen in the environment. Vol 2 Nielsen DR & MacDonald JG (Eds.) pp 101-141 Academic Press

Schilling G, (1983), Genetic specificity of nitrogen nutrition in leguminous plants. *Plant & Soil* **72** 321-34

Scott DB & Farnden KJF, (1976), Ammonia assimilation in lupin nodules. *Nature*. **263** 703-05

Serraj R & Sinclair TR, (1996), Inhibition of nitrogenase activity and nodule oxygen permeability by water deficit. *J. Exp. Bot.* **47** 301 1067-73

Serraj R & Sinclair TR, (1997), Variation among soybean cultivars in dinitrogen fixation response to drought. *Agron. J.* **89** 963 - 69

Serraj R, Sinclair TR, & Allen, LH, (1998), Soybean nodulation and N_2 fixation response to drought under carbon dioxide enrichment. *Plant Cell & Environ.* **21** 491-500

Serraj R, Sinclair TR, & Purcell LC, (1999), Symbiotic N_2 fixation response to drought. *J. Exp. Bot.* **50** 331 143-55

Serraj R, Vadez V, Denison RF, & Sinclair TR, (1999), Involvement of ureides in nitrogen fixation inhibition in soybean. *Plant Physiol.* **119** 1 289-96

Shaner DL & Boyer JS, (1976a), Nitrate Reductase Activity in Maize (*Zea mays* L.) leaves. I. Regulation by nitrate flux. *Plant Physiol.* **58** 499-504

Shaner DL & Boyer JS, (1976b), Nitrate Reductase Activity in Maize (*Zea mays* L.) leaves. II Regulation by nitrate flux at low leaf water potential. Plant Physiol. **58** 505-09

Sharma UD & Rai VK, (1989), Modulation of osmotic closure of stomata, stomatal resistance and K+ fluxes by exogenous amino acids in *Vicia faba* L. leaves. *Biochem. Physiol. Pflanzen* **39** 491-93

Sharp RE & Davies WJ, (1979), Solute accumulation and growth by roots and shoots of water stressed maize plants. *Planta* **147** 43-9

Shorter Oxford English Dictionary (1983) 3rd Edn. Fowler HW & Fowler FG (original Eds). Clarendon Press London

Sheveleva E, Chmara W, Bohnert HJ, & Jensen RG, (1997), Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. *Plant Physiol.* **115** 1211-19

Shobert B, (1977), Is there an osmoregulatory mechanism in algae and higher plants. *J. Theor. Biol.* **68** 17-26

Sims DA, Seeman JR & Luo Y, (1998), Elevated CO_2 concentration has independent effects on expansion rates and thickness of soybean leaves across light and nitrogen gradients. *J. Exp. Bot.* **49** 320 583-91

Simon J & Skrdleta V, (1983), Biomass production in peas *Pisum sativum* and broad beans *Vicia faba* and symbiotic dinitrogen fixation as affected by ploughing or no-tillage and nitrogen fertiliser. *Soil Tillage Research* **49** 320 583-91

Simpson BB & Ogarzaly MC, (1995), Economic Botany : Plants in Our World. 2nd Ed. McGraw Hill Inc. pp 367-375

Sinclair TR & Ludlow MM, (1985), Who taught plants thermodynamics? The unfulfilled potential of plant water potential. *Aust. J. Plant Physiol.* 12 213-17

Singh TN, Paleg LG, & Aspinall D, (1973a), Stress metabolism III Variations in response to water deficits in the barley plant. *Aust. J. Biol. Sci.* 52 109-110

Singh TN, Paleg LG, & Aspinall D, (1973b), Stress metabolism I Nitrogen metabolism and growth in the barley plant during water stress. *Aust. J. Biol. Sci.* **26** 57-63

Singh BB & Gupta DP, (1983), Proline Accumulation and Relative Water Content in Soya Bean (*Glycine max.*) varieties under water stress. *Annals of Bot.* **26** 45-46

Sinha SK & Nicholas DJ, (1981), Nitrate Reductase In: Physiology and biochemistry of drought resistance. Paleg LA & Aspinall D (Eds.) pp 109-110 Academic Press

Sloan CH & Sublett BJ, (1966), Colorimetric method of analysis for nitrates in tobacco. *Tobacco Science* **10** 121-25

Smirnoff N, Todd P & Stewart GR, (1984), The Occurrence of nitrate reduction in the leaves of woody plants. *Annals of Botany* **116** 1539-1549

Smirnoff N, Winslow MD & Stewart GR, (1985), Nitrate reductase activity in leaves of barley (*Hordeum vulgare*) and durum wheat (*Triticum durum*) during field and rapidly applied water deficits. *J. Exp. Bot.* **54** 363-374

Smirnoff N & Stewart GR, (1985), Nitrate assimilation and retranslocation by higher plants. Comparative physiology and ecological consequences. *Physiology Plant.* **36** 169 1200-1208

Smirnoff N, (1995), Metabolic flexibility in relation to the environment In: Environment and plant metabolism flexibility & acclimation (Ed. Smirnoff) pp 1 - 15BIOS

Smith IK & Thompson JF, (1971), Purification and characterization of L-serine transacetylase and O-acetylserine sulfhydrylase from kidney bean seedlings *Phaseolus vulgares. Biochim. Biophys. Acta.* **227** 288-95

Snir N & Neumann PM, (1997), Mineral nutrient supply, cell wall adjustment and the control of leaf growth. *Plant Cell & Environ.* **20** 239-46

Solomonsen LP & Barber MJ, (1990), Assimilatory nitrate reductase: functional properties and regulation. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **41** 225-53

Somers DA, Kuo TM, Kleinhofs A, Warner RL & Oaks A, (1983), Synthesis and degradation of barley nitrate reductase. *Plant Physiol.* **72** 949-52

Soussi M, Ocana A & Lluch C, (1998), Effects of salt stress on growth, photosynthesis and nitrogen fixation in chick-pea (*Cicer arietinum* L.). *J. Exp. Bot.* **49** 3251329-1337

Sperry JS, Alder FR, Campbell GS & Comstock JP, (1998), Limitation of plants water use by rhizosphere and xylem conductance: results from a model. *Plant Cell & Environ.* **21** 347-359

Sprent JI, (1971a), Effects of water stress on N-fixation in root nodules. *Plant & Soil* **8** 372-375

Sprent JI, (1971b), The effects of water stress on N-fixing root nodules. I. The effects on the physiology of detached soybean nodules. *New Phytol.* **70** 9-17

Sprent JI, (1972), The effects of water stress on N-fixing root nodules. IV. Effects on whole plants of *Vicia faba* and *Glycine max*. *New Phytol.* **71** 443-450

Sprent JI, (1973), Growth and N-fixation in *Lupinus arboreus* as affected by shading and water supply. *New Phytol.* **71** 603-611

Sprent JI, (1976), Water deficits and N-fixing root nodules. Ch 7 in Water deficits and plant growth. Vol. IV. Ed. TT Kozlowski (Ed.) 74 461-463

Sprent JI, (1980), Root nodule anatomy, type of export product, and evolutionary origin in some *Leguminosae*. *Plant Cell & Environ.*

Sprent JI & Thomas RJ, (1984), Nitrogen nutrition of seedling grain legumes some taxonomic, morphological and physiological constraints. *Plant Cell & Environ.* **7** 637-45

Srivastava H, (1980), Regulation of nitrate reductase activity in higher plants. *Phytochemistry* **54** 923-29

Steer BT, (1973), Diurnal variations in photosynthetic products and nitrogen metabolism in expending leaves. *Plant Physiol.* **115** 1083-88

Stewart CR, (1971), *Plant Physiol.* **48** 792-94

Stewart CR, (1972a), Effects of proline and carbohydrate on the metabolism of exogenous proline by excised bean leaves in the dark, *Plant Physiol.* **50** 551-55

Stewart C, (1981), Proline Accumulation : Biochemical aspects In: Physiology and biochemistry of drought resistance in plants. Paleg LG & Aspinall D (Eds.) pp 311-319 Academic Press

Stewart GR, (1972), The regulation of nitrate reductase in *Lemna minor* L. *J. Exp. Bot.* **23** 171

Stewart GR, Lee JA, Orebamjo TO & Havill DC, (1974), Ecological aspects of nitrogen metabolism. In: Mechanisms of regulation of plant growth. Bieleski RL *et al*, (Eds.) pp 41-47 Bulletin of the Royal Society of New Zealand. Wellington.

Stewart GR & Rhodes D, (1977a), Control of enzyme levels in the regulation of nitrogen assimilation. In: Regulation of Enzyme Synthesis and Activity Smith H (Ed.) pp 1-22 Academic Press

Stewart GR & Lahrer F, (1980), Accumulation of amino acids and related compounds in response to environmental stress. In: Biochemistry of plants Vol 5 . Amino acids and their derivatives. p 609 Miflin BJ (Ed.) Academic Press

Stewart GR & Orebamjo TO, (1979), Some unusual characteristics of nitrate reduction in *Erythrina senegalensis* DC. *New Phytol.* **83** 311-19

Stewart GR, Shatilov VR, Turnbull MH, Robinson SA & Goodall R, (1995), Evidence that Glutamate Dehydrogenase plays a role in the Oxidative Deamination of Glutamate in seedlings of *Z. mays. Aust. J. Plant Physiol.* **22** 805-9

Straub PF, Shearer G, Reynolds PHS, Sawyer SA, & Kohl, DH, (1997), Effect of disabling bacteroid proline catabolism on the response of soybeans to repeated drought stress. *J. Exp. Bot.* **48** 311 1299-1307

Streeter JG, (1998), Effect of elevated calcium concentration in infected cells of soybean (*Glycine max* (L) Merr.) nodules on nitrogenase activity and N input to the plant. *J. Exp. Bot.* **1** 5 139-144

Stryer L, (1988,, Biochemistry, 3rd. Edn. WH Freeman Press. ISBN: 0-7167-1843-X

Stumpf D, (1984), Quantification and purification of quaternary ammonium compounds from halophyte tissue. *Plant Physiol.* **75** 273-74

Subramaniam V, (1975), Parched Earth - the Maharashtra Drought 1970-73 Orient Longman Bombay

Sutherland JM, Andrews M, McInroy S, & Sprent JI, (1985), The distribution of nitrate assimilation between root and shoot in *V. faba* L.. *Annals of Bot.* **56** 259-63

Swindale LD & Bidinger FR, (1981), Ch. 1 Introduction - the human consequences of drought and crop research priorities for their alleviation. In: Physiology and Biochemistry of Drought Resistance Paleg & Aspinall (Eds.) pp 2-13 Academic Press ISBN 0-12-544-380-3

Tabor C & Tabor H, (1976), 1,4-Diaminobutane (Putrescine), Spermidine, and Spermine. *Ann. Rev. Biochem.* **87** 53-62

Takacs E & Tecsi L, (1992), Effects of NO3-/NH4+ ratio on photosynthetic rate, nitrate reductase activity and chloroplast ultrastructure in three cultivars of red pepper (*Capsicum annuum* L.). *J. Plant Physiol.* **140** 298-305

Taylor AA & Havill DC, (1981), The effect of inorganic nitrogen on the major enzymes of ammonium assimilation in grassland plants. *New Phytol.* **90** 19-25

Taylor AR & Bloom AJ, (1998), Ammonium, nitrate, and proton fluxes along the maize root. *Plant Cell & Environ.* **21** 1255-63

Tabor CW & Tabor H, (1976), 1,4-Diaminobutane (Putrescine), Spermidine, and Spermine. *Ann. Rev. Biochem.* **87** 53-62

Taylor AA, de-Felice J & Havill DC, (1982), Nitrogen metabolism in *Poterium sanguisorba* during water stress. *New Phytol.* **90** 19-25

Thomas RJ & Schrader LE, (1981), Ureide metabolism in higher plants. *Phytochemistry* **20** 361-371

The Concise Oxford English Dictionary, (1990), Eighth Ed. 1st Ed. Ed. Fowler & Lowler, 8th Ed. Ed. RE Allen. Oxford University Press. ISBN 0-19-861200

Thompson JF, Stewart CR, & Morris CA, (1966), Changes in amino acid content of excised leaves during incubation. I. The effect of water content of leaves and atmospheric oxygen level. *Plant Physiol.* **41** 1578-84

Thompson R & Taylor H, (1981), Factors limiting growth and yield of *Vicia faba*. In: *Vicia faba*: physiology & Breeding. ISBN 90-247-2496-1 Martinus Nijhoff/Dr. W Junk 99 607-614

Thompson R *et al*, (1989), Light limited growth on ammonium versus nitrate; what is the advantage for marine phytoplankton? *Limnology & Oceanography* **34** 1014-24

Thorne GN, (1960), Variations with age in net assimilation rate and other growth attributes of sugar-beet, potato, and barley in a controlled environment. *Annals of Botany* **24** 95 356-71

Thorne GN, (1961), Effects of age and environment on Net Assimilation Rate of Barley. *Ann. Bot.* **25** 29-38

Tompa JD, Burke JJ, Quisenberry JE, & Wendt W, (1986) Effects of water stress on the organic acid and carbohydrate compositions of cotton plants. *Plant Physiol.* **82** 724-28

Tolley-Henry L & Raper CD, (1986), Utilization of ammonium as a N source effects of ambient acidity in growth and nitrogen. *Plant Physiol.* 569-593

Townsend, (1969), Influence of form of nitrogen and pH on growth and nutrient levels in leaves and roots of lowbush blueberry. *Can. J. of Plant Sci.* **50** 603-05

Trinchant JC & Rigaud J, (1981), Acetylene reduction and respiration of bacteroids isolated from french beans receiving nitrate. *Physiol. Plant.* **14** 1-12

Troelstra SR, Wagenaar R & Smant W, (1992), Growth of actinorhizal plants as influenced by the form of N with special reference to *Myrica gale* and *Alnus incana*. *J. Exp. Bot.* **81** 566-571

Tolley-Henry L & Raper CD, (1986), Nitrogen and dry matter partitioning in soybean plants during onset of and recovery from N stress. *Bot. Gaz.* **147** 392-99

Turner LB & Stewart GR, (1986), The effect of water stress upon polyamine levels in Barley, (*Hordeum vulgare* L.) leaves. *J. Exp. Bot.* **26** 159-86

Twary SN & Heikel GH, (1991), Carbon costs of dinitrogen fixation associated with dry matter accumulation in *Alfalfa*. *Crop Sci.* **37** 175 170-177

Vance CP, Heichel GH, & Barnes DK, (1984), In: Advances in Nitrogen Fixation Research. Veeger IC & Newton WE (Eds.) pp 565-71 Nijhoff/Junk Netherlands

Van der Wal AF, (1981), Drought tolerance definition and measurement. In: *Vicia faba*: physiology & Breeding. Thompson (Ed). pp 49-53 Martinus Nijhoff/Dr. W Junk ISBN90-247-2496-1

Van Oorschott JLP, (1955), Conversion of light energy in algael culture. Medededlling Landbouhougeschol Wageningen **55** 225-76

Van Oosten JJ & Besford RT, (1996), Acclimation of photosynthesis to elevated CO2 through feedback regulation of gene expression climate of opinion. *Photosynthesis Research* **48** 353-65

Venekamp JH, (1989), Regulation of cytosol acidity in plants under conditions of drought. *Physiol. Plant.* **43** 251 865-70

Verslues P & Sharp R, (1998), Proline accumulation in maize (*Zea mays* L.) . Primary roots at low water potentials II. Metabolic source of increased proline deposition in the elongation zone. *Plant Physiol.* **119** 1349-60

Vessey JK, Tolley Henry L, Raper CD Jr. & Henry LT, (1990), Nitrogen nutrition and temporal effects of enhanced carbon dioxide on soybean growth. *Crop Sci.* **30** 287-94

Vines HM & Wedding RT, (1960), Some effects of ammonia on plant metabolism and a possible mechanism for ammonium toxicity. *Plant Physiol.* **35** 820-25

Von Caemmerer S & Farquhar GD, (1981), Some relationships between the chemistry of photosynthesis and the gas exchange of leaves. *Planta* **153** 376-87

Vuylsteker C, Prinsen E, Boutin JP, Onckelen HAV & Rambour S, (1998), Evidence for nitrate reductase expression during initiation of lateral roots by NRA in chicory. *J. Exp. Bot.* **43** 251 789-95

Wakiuchi N *et al*, (1971), Changes of some enzyme activities of cucumber during ammonia toxicity. *Physiologia plantarum* **24** 248-53

Waldren RP & Treare ID, (1974), Free proline accumulation in drought stressed plants under laboratory conditions. *Plant & Soil* **40** 689-92

Wallace W & Pate JS, (1965), Nitrate reductase in the field pea (*Pisum arvense* L.). *Annals of Bot.* **46** 1253-59

Wallsgrove RM, Keys AJ, Lea PJ & Miflin BJ, (1983), Photosynthesis, photorespiration, and nitrogen metabolism. *Plant Cell & Environ.* **29** 655-671

Wallsgrove RM, Hiral E, Lea PJ & Miflin BJ, (1977), Studies on glutamate synthase from the leaves of higher plants. *J. Exp. Bot.* **28** 588-96

Walsh KB, (1995), Physiology of the legume nodule and its response to stress. *Soil Biol. & Biochem.* **27** 637-55

Walsh KB, Thorpe MR & Minchin PEH, (1998), Photoassimilate partitioning in nodulated soybean II. The effect of changes in photoassimilate availability shows that nodule permeability to gases is not linked to the supply of solutes or water. *J. Exp. Bot.* **49** 328 1817-25

Wang Z, Quebedeaux B & Stutte GW, (1995), Osmotic adjustment: effect of water stress on carbohydrates in leaves, stems and roots of apple. *Aust. J. Plant Physiol.*

Wann M & Raper CD, (1979), A dynamic model for plant growth; adaptation for vegetative growth of soybeans. *Crop Sci.* **19** 461-67

Wardlaw IF, (1993), Sink Strength: it's expression in the plant. *Plant Cell & Environ.* 1029-30

Wegner LH & Zimmerman U, (1998), Simultaneous recording of xylem pressure and trans-root potential in roots of intact glycophytes using a novel xylem pressure probe technique. *Plant Cell & Environ.* **133** 1 101-110

Wei C, Steudle E, & Tyree MT, (1999), Water ascent in plants: do ongoing controversies have a sound basis? *Trends in Plant Sci.* **4** 9 372-75

Weissman GS, (1964), Effect of ammonium and nitrate nutrition on protein levels and exudate composition. *Plant Physiol.* **39** 947-52

Wellburn FAM, Lau KK, Milling, P & Wellburn AR, (1996), Drought and air pollution affect nitrogen cycling and free radical scavenging in *Pinus halepensis* (Mill). *J. Exp. Bot.* **54** 136-41

Whittington J & Smith FA, (1992), Salinity induced malate accumulation in *Chara*. *J. Exp. Bot.* **8** 4 170-177

Wignarajah K, Jennings & Handley JF, (1975), The effect of salinity on growth of *Phaseolus vulgares* L. II Effect on Internal solute Concentration. *Ann. Bot.* **39** 1039-55

Willard HH, Merritt Jr. LL, Dean JA & Settle Jr. FA, (1988), Instrumental methods of analysis 7th Ed. Wadsworth Publishing Co. Belmont California. ISBN 0-534-08142-8

Winzer U, Albrecht SL & Bennet JM, (1992), Effect of water deficits on growth and nitrogen fixation of hairy indigo. 51th Annual meeting of the soil & crop science society of Florida 1991 Orlando USA 125-29 58 257

Wise RR, Frederick JR, Alm DM, Kramer DM, Hesketh JD, Crofts AR & Ort DR, (1994), Corrigendum. Investigation of the limitations to photosynthesis induced by leaf water deficit in field-grown sunflower (*Helianthus annus* L.) *Plant Cell Environ.* **13** 923-31

Woo KC & Osmond CB, (1982), Stimulation of ammonia and 2-oxoglutarate-dependant O_2 evolution in isolated chloroplasts by dicarboxylates and the role of the chloroplast in photorespiratory nitrogen recycling. *Plant Physiol.* **47**

Wood AJ, (1998), Betaine aldehyde dehydrogenase in sorghum. *Plant Physiol.* **69** 591-596

Worral V & Roughly R, (1976), The effect of moisture stress on infection of *Trifolium subterraneum* L. by *Rhizobium Trifolii* Dang. *J. Exp. Bot.* **5** 4

Wyn Jones RG & Storey R, (1981), Betaines. In: Physiology and Biochemistry of drought resistance of plants. Paleg LG & Aspinall D, (Eds.) pp 171-203 Academic Press

Wyn Jones RG, (1983), Chapter 3 . Phytochemical aspects of Osmotic Adjustment. In: Recent Advances in Phytochemistry Vol. 18 Proc. of 23th Annual Meeting of the Phytochemical Soc. of N. America, Tuscon, Arizona, July.

Yandow TS & Klein RM, (1986), Nitrate reductase of primary roots of red spruce seedlings. Effects of acidity and metal ions. *Plant Physiol.* **100** 1427-32

Yang C, Signer ER & Hirsch AM, (1992), Nodules initiated by *Rhizobium meliloti* exopolysaccharide mutants lack a discrete persistent nodule meristem. *Plant Physiol.* **81** 723-25

Yin ZH & Raven JA, (1997), A comparison of the impacts of various nitrogen sources on acid-base balance in C3 *Triticum aestivum* L. and C4 *Zea mays* L. plants. *J. Exp. Bot.*

Yoneyama T & Kumazawa K, (1975), A kinetic study of the assimilation of 15N labelled nitrate in rice seedlings. *Plant & Cell Physiol.* **16** 21-26

Yoneyama T, (1981), 15N studies on the *in vivo* assay of nitrate reductase in leaves; occurrence of underestimation of the activity due to dark assimilation of nitrate and nitrite. *Plant & Cell Physiol.* **22** 8 1507-20

Yoshida S, (1972), Physiological Aspects of Grain Yield. *Ann. Rev. Plant Physiol.* **49** 320 521-26

Ziegler H, (1975), Nature of transported substances. In: Encyclopedia of Plant Physiology - New Series. Zimmerman MH & Milburn JA (Eds.) Vol. 1 pp 59 - 100 Springer-Verlag Berlin

Zhang J, Nguyen HT & Blum A, (1999), Genetic analysis of osmotic adjustment in crop plants. *J. Exp. Bot.* **50** 332 291-302

Zohary D, (1977), Comments on the origin of cultivated broad bean, *Vicia faba* L. *Israel J. Bot.* **26** 39-40