STUDIES OF GLUTAMINE SYNTHETASE IN *LOTUS* SPECIES AND THE OCCURRENCE OF ROOT GS2 IN OTHER LEGUMES

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JANET WOODALL

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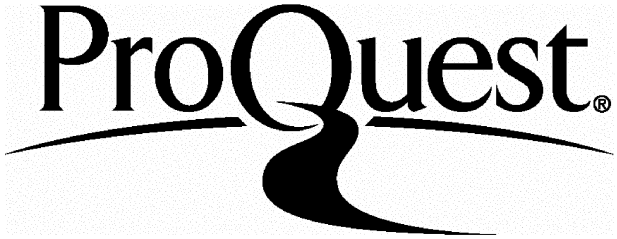

This thesis is dedicated to the memory

of my father

Douglas E. Woodall

ABSTRACT

The main body of this thesis is an analysis of the octameric enzyme of ammonia assimilation, glutamine synthetase (GS), in the model legume *Lotus japonicus* and its close relative *L. corniculatus*. Both species were shown to contain GS1 (cytosolic) and plastidic GS2 isoforms in differing amounts in all tissues. Two-dimensional electrophoresis revealed four non-glycosylated, isoelectric variants of GS1 and four variants of GS2, and evidence for the existence of four distinct GS genes in *L. corniculatus* was obtained using the polymerase chain reaction.

The root GS2 isoform was present in plants grown with NO_3^- as the sole N source and absent in plants grown on NH_4^+. To test the hypothesis that there may be a correlation between the presence of GS2 in roots and root nitrate assimilation, 55 temperate and tropical legume species (representing 38 genera from 21 different tribes) were surveyed for their site of nitrate assimilation and for the presence or absence of root GS2.

Root GS2 was found to be present in each of the 31 species with temperate origins, but absent from all of the tropical papilionoid legumes (17 species) and from four of the seven tropical non-papilionoid species. Based on *in vivo* and *in vitro* NR activities, some of the temperate legumes were probably shoot nitrate assimilators, and some of the tropical legumes were capable of assimilating nitrate in their roots. However, there was a positive correlation between the total GS activities and NR activities in the roots of the temperate species indicating a relationship between the two functions. The identification of some tropical species

that can assimilate NO_3^- in their roots, but which lack GS2, indicates that in these species at least, GS1 can assimilate the NH_4^+ derived from NO_3^- reduction, providing evidence that the functions of the two isoforms can overlap.

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ABBREVIATIONS

ADH	alcohol dehydrogenase
AEC	3-amino-9-ethyl carbozole
APS	ammonium persulphate
BIS	*N,N'*-methylene-bis-acrylamide
BPB	bromophenol blue
BSA	bovine serum albumin
DCD	dicyandiamide
DMF	dimethylformamide
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EtBr	ethidium bromide
FAD	flavin adenine dinucleotide
GABA	gamma-aminobutyrate
GOGAT	glutamate synthase
GS	glutamine synthetase
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HPI	hexose phosphate isomerase
NAD^+ (NADH)	nicotinamide-adenine dinucletide (reduced)
$NADP^+$ (NADPH)	nicotinamide-adenine dinucletide phosphate (reduced)
NEDD	*N*-(napthyl)-ethylene diamine dihydrochloride
NiR	nitrite reductase
Nonidet P40	ethylphenolpoly (ethyleneglycolether)
NR	nitrate reductase
PAS	periodic acid Schiff
PBS	phosphate-buffered saline
PCA	principle components analysis
PCR	polymerase chain reaction
PVPP	polyvinypolypyrollidine
PVDF	polyvinylidene difluoride
RuBisCO	ribulose biphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
TEMED	*N,N,N',N'*-tetramethylethylene diamine
TBS	Tris-buffered saline
Tween 20	polyoxyethylesorbitan monolaurate
VSP	vegetative storage protein

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CHAPTER 1: INTRODUCTION

It is necessary to characterize any organism used as a laboratory model and transgenic host in terms of its physiology, biochemistry and molecular biology. The characterization of nitrogen metabolism is especially important for a model legume, and this thesis is primarily concerned with the biochemistry of glutamine synthetase in the model legume *L. japonicus*, and its close relative *L. corniculatus*.

1.1 THE MODEL LEGUME

1.1.1 Transformation and regeneration of legume species

The importance of many legume species lies in their capacity, in symbiosis with *Rhizobium* species, to fix atmospheric nitrogen. The ability to grow in very poor soils, and the high protein content of the grain and fodder reflects this N-fixing capability, and renders many legumes of enormous ecological importance as pioneer species, and of great socioeconomic value.

The biochemistry and molecular biology of legume nitrogen metabolism has been a very active field of research in recent years. The molecular biology of some legume genes has been investigated using easily manipulated non-legume species as transgenic hosts. For example, the expression of *Pisum sativum* and *Glycine max* glutamine synthetase (GS) isoforms has been analyzed using fusions of their promoter sequences and the ß-glucuronidase (GUS) reporter gene in transgenic *Nicotiana tabacum* (Edwards *et al.*, 1990; Miao *et al.*, 1991). However, given the biochemistry of legume nitrogen metabolism and the consequent morphological and physiological adaptations, the use of non-legume transgenic systems may be

limited. There is an obvious need for a legume species that as well as being suitable for classical genetic and biochemical techniques, can be used as a transgenic host.

Although several legume species have been successfully transformed, many are recalcitrant to regeneration (for reviews see Tepfer, 1990, and van Wordragen and Dons, 1992). This recalcitrance has been overcome in some species, including *G. max* (Chee *et al.*, 1989), *P. sativum* (Pounti-Kaerlas *et al.*, 1989), *Medicago* species (Deak *et al.*, 1986; Kuchuk *et al.*, 1990) and *Lotus* species (Petit *et al.*, 1987; Handberg and Stougaard, 1992), but their usefulness for routine laboratory work may be limited by other factors. Plant size, length of life cycle, breeding characteristics, ploidy number and other genome characteristics, must be taken into consideration, as well as the complexity and speed of the transformation and regeneration procedure. The most successful systems have been developed for small forage legumes from the genera *Medicago* and *Lotus*. Major differences in nodule morphology and physiology between the two genera, the former having indeterminate nodules, the latter, determinate, have indicated a role for both, but although the *Medicago varia* system has been successfully employed to analyze *P. sativum* GS-GUS fusions (Brears *et al.*, 1991), *Lotus corniculatus* has so far been the legume most widely used for transgenic research.

1.1.2 *Lotus corniculatus*

Lotus corniculatus, common Bird's-foot trefoil, is a perennial herbaceous legume, widespread throughout Europe extending south to North and East Africa, and east to Russia and the Himalayas (Jones and Turkington, 1986). It is the most

variable species in the genus, and is important as a fodder crop, so that many cultivars and varieties have been developed for this application.

The cultivar Leo has a short generation time (12 weeks) and is capable of producing thousands of seeds. An easy and rapid transformation/regeneration system was developed for *L. corniculatus* cv. Leo (Petit *et al.*, 1987; Stougaard *et al.*, 1987) whereby the target DNA is integrated into the T-DNA of the Ri plasmid which is then reintroduced into *Agrobacterium rhizogenes*. Wounded stem tissue is infected with the transformed bacterium, and transgenic plants are regenerated from the hairy roots which develop. Alternatively, a 'short-cut' system can be used in which the hairy roots are left on the stem and the original root system is excised to produce a chimaeric 'hairy root' plant. The hairy roots can then be nodulated with *Rhizobium*. The first application of this system was to investigate *G. max* leghaemoglobin regulatory sequences using the chloramphenicol acetyltransferase (CAT) reporter gene (Stougaard *et al.*, 1986), and a modified binary transformation system has since been used extensively to analyze GS promoter-GUS fusions, notably of *Phaseolus vulgaris* (Forde *et al.*, 1989; Shen *et al.*, 1992) and *G. max* (Miao *et al.*, 1991, Marsolier *et al.*, 1993).

Unfortunately, *L. corniculatus* is tetraploid (4n = 48) and not self-fertile, characteristics that preclude its usefulness in classic genetic studies, e.g. mutant isolation and segregation. It is also possible that the hairy-root oncogenic phenotype conferred to the transformed plants may interfere with aspects of development and enzyme function. Therefore an alternative model species was needed, which was diploid and self-fertile, and for which an *Agrobacterium tumefaciens* transformation system using disarmed (non-oncogenic) vectors could

be developed.

1.1.3 *Lotus japonicus*

The small perennial, *Lotus japonicus*, native to the E. Himalayas, S. China, N. Korea and Japan (Jones and Turkington, 1986) is a self-compatible diploid and has been found to contain a very small genome. The 0.5 pg haploid genome in *L. japonicus* is comparable to 0.2 pg in *Arabidopsis thaliana*, and in contrast to 4.9 pg haploid genome found in *Pisum sativum* (Handberg and Stougaard, 1992). Although *L. japonicus* is self-compatible, a crossing technique has been described (Grant *et al.*, 1962) that allows for classical genetic studies. The natural variant of *L. japonicus*, biovar Gifu, originally from Japan, was found to have the shortest generation time of several variants that were studied (Handberg and Stougaard, 1992) and set seed profusely. Handberg and Stougaard (1992) developed a regeneration and transformation system for this biovar using *A. tumefaciens* gene transfer, and it is hoped that this transgenic system will overcome some of the difficulties associated with the *L. corniculatus* / *A. rhizogenes* system.

1.2 THE ROLE OF GLUTAMINE SYNTHETASE

Glutamine synthetase catalyses the addition of inorganic NH_4^+ to organic glutamate to make glutamine (see Figure 1.1). In higher plants it is the major enzyme of NH_4^+ assimilation (Miflin and Lea, 1980) and can be regarded as the interface between carbon and nitrogen metabolism. The glutamine may either be for transport or used to transaminate α-ketoglutarate to form two glutamate

molecules, a process catalyzed by glutamate synthase (GOGAT). As glutamate is the substrate of GS, the pathway is a cycle. NH_4^+ is evolved through a variety of processes, e.g. photorespiration, the phenyl propanoid pathway and transamination of amino acids, as well as being taken up directly from the soil or generated by NO_3^- reduction or N_2-fixation (for a review see Joy, 1988).

1.2.1 There are different isoforms of GS

There is evidence that NH_4^+ is phytotoxic (Mehrer and Mohr, 1989; Hecht and Mohr, 1989; Vollbrecht *et al.*, 1989; Magalhaes and Huber, 1991) so that NH_4^+ assimilation can also be regarded as detoxification (Givan, 1979). Although NO_3^- may be transported around a plant to the site of assimilation, and may accumulate to millimolar levels, NH_4^+ must be assimilated where it arises, and this is achieved through different GS isoforms the composition of which depends upon subunit composition. Most green plants contain two isoforms, the cytosolic GS1 and plastidic GS2. Cytosolic GS1 is found throughout the plant although usually most abundant in the roots and in the root nodules of legumes. All available evidence indicates that there is no difference between the leaf and root cytosolic isoform (Lara *et al.*, 1984a; Tingey *et al.*, 1987; Hirel *et al.*, 1987).

1.2.2 Cytosolic GS is involved in providing glutamine for transport

Several lines of evidence indicate a role of at least one cytosolic isoform in supplying glutamine for transport. Cytosolic GS is associated with vascular tissue in *P. sativum*, and its mRNA has been found to accumulate in organs where large amounts of nitrogen are mobilized, e.g. cotyledons (Walker and Coruzzi, 1989).

Vascular-specificity was also seen in the expression of *Pisum sativum* GS1 promoter-GUS fusions in transgenic *N. tabacum* (Edwards *et al.*, 1990) and *Medicago varia* (Brears *et al.*, 1991).

Further evidence of the role of GS1 in providing transportable glutamine is the increase in GS1 mRNA and protein during protein remobilization in senescing leaves of both *Raphanus sativus* and *Oryza sativa* (Kawakama and Watanabe, 1988; Kamachi *et al.*, 1991). The autumnal increase in GS1 protein and activity in deciduous trees confirms the importance of this isoform during remobilization of nitrogen resources (Pearson and Ji, 1994). It is also understood that the high expression of GS1 isoforms in tissues with high metabolic activity that lack large numbers of chloroplasts, such as anther theca, pollen and pulvini (Marsolier *et al.*, 1993) as well as in roots and nodules, reflects the need for cytosolic GS during all stages of development.

Recent studies using immunocytological methods have confirmed the vascular localization of GS1 (Kamachi *et al.*, 1992; Periera *et al.*, 1992; Carvalho *et al.*, 1992). However these results are at variance with earlier results obtained with protoplasts, which indicated GS activity in the cytosolic fraction of mesophyll cells in *Pisum sativum* (Wallsgrove *et al.*, 1979) and *Hordeum vulgare* (Wallsgrove *et al.*, 1980). The presence of GS1 in mesophyll cells, as well as in vascular tissues, has recently been confirmed by immunocytological localization (Kozaki *et al.*, 1991; Alison Tobin, personal communication).

1.2.3 Chloroplast GS assimilates ammonium derived from photorespiration

The relative activities of the two isoforms in shoots of many species when separated by ion exchange chromatography suggested four natural groupings to McNally *et al.* (1983). Group 1 consisted of plants with only GS1 activity, the achlorophyllous non-photosynthetic parasites; Group 2 contained plants with only GS2 activity, including many members of the Solanaceae; Group 3, plants with mainly GS2 activity, including many C3 plants; and Group 4 those with equal GS1 and GS2 activity, mainly C4 plants. The apparent correlation between the presence of GS2 and the photorespiratory strategy of plants in each of these groups led McNally *et al.* (1983) to suggest that chloroplastic GS2 assimilated the ammonia produced by photorespiration even though that NH_4^+ pool arises in the mitochondria. This has since been confirmed primarily by the use of barley photorespiratory mutants (Wallsgrove *et al.*, 1987; Blackwell *et al.*, 1987; Blackwell *et al.*, 1988; Freeman *et al.*, 1990). Further support is that the GS2 promoter in *Pisum sativum* (Edwards *et al.*, 1990), *Phaseolus vulgaris* (Cock *et al.*, 1992), *Oryza sativa* (Kamachi *et al.*, 1992), *Solanum tuberosum* (Pereira *et al.*, 1992) and *Nicotiana tabacum* (Carvalho *et al.*, 1992), directs expression to photosynthetic cells.

1.2.4 The roles of GS1 and GS2 can overlap in some species

Edwards *et al.* (1990) have proposed that the spatial separation of GS1 and GS2 indicates that they have non-overlapping roles. However, the existence of species that do not contain leaf GS1 activity e.g. *Sinapsis alba* (Höpfner *et al.*, 1991) suggests that GS2 can perform the same functions as the GS1 isoform in the

shoots of at least some species. Conversely, it has recently been reported that a fully photosynthetic species, *Pyrola media*, is deficient in GS2 activity and protein but has particularly high GS1 activity (30-40 μmol.h^{-1}.g^{-1}fwt) (Pearson, Woodall and Havill, in preparation). This suggests that GS1 must compensate for the lack of GS2 in this species and assimilate the NH_4^+ derived from photorespiration.

1.3 THE BIOCHEMISTRY OF GLUTAMINE SYNTHETASE

Glutamine synthetase is a 380-400 kD protein made up of eight identical or nearly identical subunits. The eight GS2 subunits are slightly larger (43-45 kD) than the GS1 subunits (37-43 kD) in a wide range of angiosperms (Forde and Cullimore, 1989; Pearson, Woodall and Havill, in preparation), and gymnosperms (Vézina and Margolis, 1990; Pearson, Woodall and Havill, in preparation). Separation by ion exchange HPLC, and by isoelectric focusing has revealed several slightly different subunit types, and molecular analysis of GS genes has demonstrated the existence of small families of genes encoding the different GS polypeptides found at the biochemical level.

1.3.1 Chloroplast GS

Many plants possess a single nuclear GS2 gene encoding a precursor polypeptide that is transported into the chloroplast, where simultaneous removal of the N-terminal transit peptide results in the mature subunit (Lightfoot *et al.*, 1988; Walker and Coruzzi, 1989). In addition to the N-terminal transit peptide of 56-66 amino acid residues, the GS2 polypeptide also possesses a highly conserved C-

terminal extension of 16 amino acids. This is not, however, thought to be essential for the transport of GS2 into the plastid (Lightfoot *et al.*, 1988; Forde and Cullimore, 1989).

1.3.2 Root plastid GS

The association of some GS activity with isolated plastids of *Pisum sativum*, *Vicia faba*, *Hordeum vulgare* and *Zea mays* roots has been noted by several authors (Miflin, 1974; Barratt, 1980; Emes and Fowler, 1983; Sakakibara *et al.*, 1992b). Vézina *et al.* (1987) recovered over 50% of total GS activity in isolated *P. sativum* and *Medicago media* plastids prepared from root tips. The *P. sativum* GS2 promoter directed expression of the GUS reporter gene to both leaf and root in transgenic tobacco (Edwards *et al.*, 1990) suggesting that the root plastid enzyme is the same as the leaf enzyme.

Transcripts for GS2 have been detected at low levels in the roots of some species, e.g. *Glycine max* (Hirel *et al.*, 1987) and *Phaseolus vulgaris* (Bennett and Cullimore, 1989), but the corresponding polypeptide was undetectable. However, both GS2 mRNA and protein have been identified in nodules of *P. vulgaris* (Lightfoot *et al.*, 1988; Bennett and Cullimore, 1989) and *G. max* (Hirel *et al.*, 1987). The induction of root plastid GS by NO_3^- will be discussed in Chapter 1.4.

1.3.3 Cytosolic GS

Cytosolic GS1 polypeptides are encoded by a small family of nuclear genes in many species (Sakamoto *et al.*, 1989; Peterman and Goodman, 1991; Sakakibara *et al.*, 1992a; Stanford *et al.*, 1993), although at least one species,

Sinapsis alba, appears not to contain any GS1 genes (Höpfner *et al.*, 1991). *Phaseolus vulgaris* has three expressed GS1 genes designated *gln-α*, *gln-ß* and *gln-γ* (Forde and Cullimore, 1989) plus an additional gene, *gln-ϵ*, which seems to be silent (Forde *et al.*, 1989; Shen, 1991). The α and ß polypeptides are found in all tissue types, although expression of *gln-ß* is very much higher in roots (Lara *et al.*, 1983; Gebhardt *et al.*, 1986). Studies with *gln-ß* promoter-GUS fusions in transgenic *L. corniculatus* have shown that this promoter confers levels of expression in roots 20 to 140-fold higher than in shoots (Forde *et al.*, 1989). The ß polypeptide is 5 to 10-fold more abundant than the α form in mature root tissue, whilst the α polypeptide predominates in embryonic tissue (Ortega *et al.*, 1986; Swarup *et al.*, 1990).

In *P. sativum* there are also three expressed GS1 genes. Two are nearly identical 'twin GSn genes' with 99% nucleotide identity, and encode the GS3A and GS3B subunits. The third gene, encodes a related but distinct subunit, GS299, having 86% nucleotide identity with the other two genes (Walker and Coruzzi, 1989). All three gene products are found throughout the plant in varying proportions. The GS299 subunit predominates in the root, whereas the twin GSn subunits are expressed more strongly in immature tissue, and nodules (Tingey *et al.*, 1987; Walker and Coruzzi, 1989; Edwards *et al.*, 1990). The GS3A subunit is present at a consistently higher level than GS3B especially in the cotyledons, and is expressed exclusively in phloem tissue (Edwards *et al.*, 1990; Brears *et al.*, 1991).

1.3.4 Nodule GS

High GS activities are required to deal with the high fluxes of NH_4^+ produced during N_2 fixation, a requirement met by induction of a nodule-specific GS subunit, and/or an increase in non-specific GS subunit(s). Indeed nodules contain the highest level of GS of all plant organs, up to 2% total soluble protein (Forde and Cullimore, 1989). In *P. vulgaris*, the *gln-γ* gene is expressed at high levels in the infected cells of nodules, and although originally thought to be nodule-specific (Cullimore *et al.*, 1984; Gebhardt *et al.*, 1986) both the mRNA and protein have now been detected in stems, petioles and green cotyledons (Bennett *et al.*, 1989). A nodule-specific cDNA has been identified in *Medicago sativa* (Dunn *et al.*, 1988), and *Lupinus luteus* (Konieczny *et al.*, 1988) although whether they are expressed exclusively in that organ has been questioned (Forde and Cullimore, 1989; Coruzzi, 1991). Nodules of *L. luteus* were also found to have high activities of a root GS1 isoform (Konieczny *et al.*, 1988). This is a strategy also adopted by *P. sativum*, where no nodule-specific isoforms have been detected but both GS3A and GS3B are at their highest levels of expression in the nodules (Tingey *et al.*, 1987; Walker and Coruzzi, 1989), GS3A being confined to the nodule meristem. A nodule-specific polypeptide has been found in *G. max* (Sengupta-Gopalan and Pitas, 1986; Roche *et al.*, 1993), but an NH_4^+-enhanced expression of two cytosolic isoforms found throughout the plant has also been shown to occur in the central infected zone of *G. max* nodules, including the uninfected cells (Hirel *et al.*, 1987; Miao *et al.*, 1991; Marsolier *et al.*, 1993; Roche *et al.*, 1993).

1.3.5 Composition of the GS holoenzyme

Spatial or temporal separation of the subunits may restrict composition of the octameric holoenzyme. The compartmentalization of the mature GS2 polypeptides in the plastid results in an isoform of homogeneous composition. Spatial separations are also important in nodules. In *P. vulgaris* the nodule GS isoforms are mainly composed of the γ and ß subunits in varying proportions i.e. $ß_8$, $ß_7γ_1$, $ß_6γ_2$, *etc*. The $ß_8$ enzyme is, however, more abundant than would be expected by a purely random association of ß and γ subunits. The reason for this appears to be that both are expressed in the central zone of the nodule, but only ß in the outer cortex and vascular regions (Forde *et al.*, 1989; Chen and Cullimore, 1989; Teverson, 1990)(see Forde and Woodall, 1994, for more details)

The changes in expression of GS during development also create heterogenous octamers, for example, the α polypeptide is more abundant than the ß polypeptide in plumules of soaked *P. vulgaris* seeds, so that the octameric form contains a high percentage of α. As the plumule develops, the ß subunit type becomes more abundant and is the major constituent of GS1 in mature plants (Bennett and Cullimore, 1989).

1.3.6 Isoelectric focusing variants of GS

When GS subunits are separated by isoelectric focusing (IEF) several charge variants can be seen. When known, the number of isoelectric variants of GS1 corresponds to the number of gene products expected. This correlation does not however exist between the number of GS2 variants and expected number of gene products. For example in *P. vulgaris* there is only one identified GS2 gene

(Lightfoot *et al.*, 1988) but four isoelectric variants (Lara *et al.*, 1984). Two possible explanations for the extra GS2 variants are that they are extraction artefacts, or that the single gene product undergoes post-translational modification.

1.3.7 Artefacts of protein extraction and isoelectric focusing

Steps required for isoelectric focusing (IEF) include solvation, denaturation and reduction. Urea is often used as the denaturant. If the urea solution is not freshly made from deionized urea, or if the sample is heated with the urea, then charge artefacts can result through carbamylation of amine and sulfhydryl groups in the protein.

Cysteine residues may be variably oxidized during IEF resulting in charge variants differing only by the oxidation state of the cysteines. Alkylation is sometimes employed to stabilize the thiol groups after reduction. Unfortunately, treatment with iodoacetamide, the alkylating agent often used, can itself result in charge variants (O'Connell and Brady, 1981).

1.3.8 Post-translational modification of GS

There are many possibilities for post-translational modification of proteins as there are plenty of reactive hydroxyl, amino, sulfhydryl, carboxyl, imidazole, guanidinium and tyrosol groups in amino acid sidechains. The most common modifications include phosphorylation, adenylation, limited proteolysis and glycosylation. Adenylation of GS is thought to be a major regulatory process in enteric bacteria (Magasanik, 1988) but is not thought to be relevant in plants (Forde and Cullimore, 1989). The GS2 isoform is synthesized in the cytosol but

must be transported into the chloroplast. As such the nascent protein possesses an N-terminal transit peptide that is cleaved upon crossing the chloroplast membrane. Two size variants of GS2 have been reported in *P. vulgaris*, thought to be the result of the use of two different cleavage sites by the processing peptidase, a situation known to exist in other nuclear-encoded chloroplast precursors (Robinson and Ellis, 1984; Mishkind *et al.*, 1985)

Glycosylation, where one or more carbohydrate residue is covalently attached to a protein, is the most widely distributed and varied post-translational modification. Several plant enzymes that have different isoforms have been shown to be differentially glycosylated. Examples include glycosidases from *P. sativum* leaves and α-amylases from cereal seeds (Gaudreault and Beevers, 1983; Miyata and Akazawa, 1982). It has also been suggested that plant proteins that are transported from the cytosol to another compartment are glycosylated to facilitate transport (Sengupta *et al.*, 1981; Faye *et al.*, 1984), a situation frequently reported for mammalian cells (Blobel and Dobberstein, 1975).

In 1984, Nato *et al.* found that the four IEF variants of purified GS2 from *N. tabacum* all appeared to be glycosylated. Several carbohydrate residues were identified (mannose, galactose, glucose, glucosamine and galactosamine) comprising 5% of the enzyme. However other experiments failed to find sugars linked to GS2 in either *P. sativum* (Tingey and Coruzzi, 1987), *Spinacia oleracea* (Ericson, 1985) or *N. plumbaginifolia* (Tingey *et al.*, 1987).

1.4 REGULATION OF GS

It can be seen from Chapter 1.3 that the appearance of different GS subunits depends largely on developmental or tissue-specific cues. Superimposed on this is a variety of environmental factors that can play a significant role in expression.

1.4.1 Light and GS1

Light has little effect on the accumulation of GS1 transcripts and proteins, although Sakamoto *et al.* (1990) reported that the expression of cytosolic GS in imbibing *Lactuca sativa* seeds increased upon illumination, an adaptation to the light-requiring manner of germination. Swarup *et al.* (1990) also noted that light had the effect of increasing the abundance of *gln-β* and *gln-γ* transcripts in *P. vulgaris* cotyledons, and suggested that this reflected the differing physiological status of seedlings germinated in the light or dark.

1.4.2 Light, photorespiration and GS2

As might be expected in enzymes involved in the photorespiratory pathway, light is the major factor in regulating GS2 gene expression. This is believed to be mainly a direct response to phytochrome (Schmidt and Mohr, 1989; Becker et al, 1992), but also through light-induced changes in chloroplast development and metabolism (Chandler *et al.*, 1985; Edwards and Coruzzi, 1989; Edwards *et al.*, 1990; Cock *et al.*, 1991; Peterman and Goodman, 1991). In the C4 plant, *Z. mays*, GS2 increases in response to light only in the bundle sheath cells and not in the mesophyll; an adaptation to the location of the photorespiratory apparatus

(Sakakibara *et al.*, 1992a). An interesting variation on this theme is that gymnosperms can develop green plastids in the absence of light (Possingham, 1980), and although Emlinger and Mohr (1992) reported a light-induced increase in the 43 kD GS subunit of *Pinus sylvestris*, Canovas *et al.* (1991) have shown that the major 43 kD GS band in cotyledons and needles of *Pinus pinaster* is unaffected by light. This 43 kD subunit was ascribed as cytosolic in the paper, but as a smaller subunit was also identified it seems likely that this smaller protein was GS1, and the larger protein, which was found in separated chloroplasts, was GS2. This is supported by the results of Vézina and Margolis, 1990, who found a predominant 44 kD GS subunit and a less abundant 40 kD subunit in *Pinus banksiana*, and by Canton *et al.*, 1993, who identified a GS1 cDNA in *P. sylvestris* which was predicted to encode a 39.5 kD protein.

1.4.3 Diurnal rhythm

The GS activity in *L. esculentum* leaves was reported to show a diurnal rhythm, increasing during the day and decreasing at night (Canovas *et al.*, 1986). This was supported by Becker *et al.* (1992) who found a two fold higher level of GS2 mRNA in *L. esculentum* leaves at the beginning of the light period than at the end, although the levels of protein and activity remained the same. Furthermore, Rhodes *et al.* (1976) found that dark-grown, NH_4^+-fed *Lemna minor* had decreased GS activity that *in vivo* was fully recoverable in the light, but which *in vitro* could be reactivated by Mg^{2+}. Both Mn^{2+} and Mg^{2+} influence GS activity in bacteria, and it was suggested that Mg^{2+} (levels of which were influenced by light/dark transitions) may mediate the diurnal rhythm found in *L. esculentum*, although

thioredoxin was also implicated.

1.4.4 Nitrogen source

Almost any nitrogen compound can be considered as a potential end product of NH_4^+ assimilation, and many act as inhibitor of GS activity *in vitro* (Stewart *et al.*, 1980), but their significance *in vivo* has not been established. There are many examples, however, of nitrogen source influencing GS expression.

Ammonia - In *Lemna minor* (Rhodes *et al.*, 1976) and in the embryonic axes of *Lupinus luteus* (Ratajczak *et al.*, 1981) total GS activity decreased in plants supplied with NH_4^+ as their sole source of nitrogen. This decrease correlated with an increase in free glutamine levels, and a mechanism was proposed whereby the native octameric enzyme is reversibly dissociated into two inactive tetramers (Stewart *et al.*, 1980). A tetrameric form of GS has been identified in the leaves of *Beta vulgaris* (Mack, 1988), although it was found to be active. Hoelzle *et al.* (1992) have more recently suggested that an inactive form of GS protein might be present in N-limited plants.

Monitoring GS mRNA levels in plants grown in the absence or presence of NH_4^+, and a comparison of GS mRNA levels in nodules of *Fix*$^-$ mutants and wild-type *Rhizobium* has indicated that induction of the 'nodule-specific' GS1 subunits in *P. vulgaris* (Padilla *et al.*, 1987; Cock *et al.*, 1990) *P. sativum* (Walker and Coruzzi, 1989; Brears *et al.*, 1991) *M. sativa* (Dunn *et al.*, 1988) and *G. max* (Roche *et al.*, 1993) is not due to NH_4^+ production during N_2 fixation, but is more likely to be developmental. It is possible, however, that NH_4^+ is required for full

induction to wild-type levels (Cock *et al.*, 1990; Shen, 1991).

Exogenous NH_4^+ has been shown to directly induce expression of a GS1 gene found in the roots and nodules of *G. max* (Hirel *et al.*, 1987; Miao *et al.*, 1991). Using this GS1 promoter to direct GUS expression in transgenic *L. corniculatus*, Miao *et al.* (1991) were able to demonstrate a three-fold increase in GUS activity in plants supplied with 10 mM NH_4^+, while applications of NO_3^-, asparagine or glutamine had little or no effect. Marsolier *et al.* (1993) located the NH_4^+-responsive elements of the gene to the region between 3.5 and 1.3 kb from the transcription start site, although this promoter was unresponsive to NH_4^+ treatment in transgenic *N. tabacum*.

Nitrate - The reduction of NO_3^- through NO_2^- to NH_4^+ is mediated by cytoplasmically located nitrate reductase (NR) and plastidic nitrite reductase (NiR), and it is now well established that both enzymes are induced as a primary response to NO_3^- (Redinbaugh and Campbell, 1991; Pelsey and Caboche, 1992). Nitrate is known to directly affect several other related systems including NO_3^- uptake and transport (Redinbaugh and Campbell, 1991). Nitrate has also recently been shown to increase both ferredoxin and NADPH-dependent ferredoxin-$NADP^+$ oxidoreductase located in the root plastids of *P. sativum* (Bowsher *et al.*, 1993), both of which are required for the transfer of reductant to NiR. As GS is the next enzyme along the pathway of NO_3^- reduction, its response to NO_3^- is of great interest, especially since it has not been established which isoform is responsible for assimilating NH_4^+ from NO_3^- reduction, although the plastid GS2 seems a likely candidate because of the plastidic location of NiR.

Externally applied NO_3^- has a positive, though minor, effect on GS2 of *Sinapsis alba* and *Helianthus annuus* seedlings, and acts synergistically with light (Schmidt and Mohr, 1989; de la Haba *et al.*, 1992), and it was suggested by Weber *et al.* (1990) that the appearance of *S. alba* NiR and GS2 in the presence of light and NO_3^- are synchronized. It has been observed that in *H. annuus* seedlings pre-treated with tungstate, which inhibits NR and thereby prevents the reduction of NO_3^- to NH_4^+ (Deng *et al.*, 1989), NO_3^- still stimulates GS synthesis (de la Haba *et al.*, 1992).

Barratt (1980) reported the appearance of an extra isoform of root GS in NO_3^--grown *V. faba*, which migrated alongside the leaf GS2 isoform, and which was absent from NH_4^+-grown plants. Furthermore Vézina and Langlois (1989) demonstrated a specific increase in pea root plastidic GS2 protein and activity in plants grown on NO_3^- as compared to NH_4^+ or nitrogen-free medium. In neither instance was it known if the induction was a primary or secondary response to NO_3^-. In *Z. mays* roots, mRNA and protein of a plastidic GS was shown to increase upon nitrate application (Sakakibara *et al.*, 1992a). The GS2 in leaf mesophyll cells also increased up to 50-fold with nitrate, whereas the bundle sheath GS2 and the cytosolic GS were unaffected. The authors noted that although the photorespiratory enzymes are located in the bundle sheath cells, those of nitrate assimilation are found predominantly in mesophyll cells. The accumulation of GS2 transcripts in the roots of *Z. mays* has also recently been shown to be a primary response to NO_3^- (Redinbaugh and Campbell, 1993).

1.5 NITRATE ASSIMILATION IN TROPICAL AND TEMPERATE LEGUMES

The possibility that GS2 may be the isoform involved in assimilating NH_4^+ derived from NO_3^- reduction (see Chapter 5), raises the question whether the presence of root plastidic GS2 in legumes is correlated with root nitrate assimilation.

1.5.1 Root versus shoot nitrate assimilation

Nitrate must be reduced to NH_4^+ before it can be assimilated into amino acids, but although most higher plants can assimilate NO_3^- in either the roots or the shoot, the division of the process between the two sites varies greatly among species.

Although many legumes and non-legumes carry out a substantial proportion of their NO_3^- assimilation in the shoot regardless of external NO_3^- concentration, temperate legumes and some non-legumes (including many cereals, grasses and temperate woody species) growing in NO_3^- concentrations expected in non-agricultural soils (less than 1 mM) carry out 30-50% of their assimilation in the root. However, as the NO_3^-concentration increases to that of agricultural soils (1-20 mM) shoot assimilation becomes more important (Andrews, 1986a; Wallace, 1986; Andrews *et al.*, 1992), implying that when an increase in the rate of NO_3^- uptake does not induce a further increase in root activity, more NO_3^- is transported to the shoot for reduction there.

Several factors other than species differences have been found to influence the partitioning of NO_3^- assimilation, including cultivar (Sutherland *et al.*, 1985; Andrews, 1986a), age (Sprent and Thomas, 1984), development of competing

sinks (Selamat and Gardner, 1985; Sung and Sun, 1990), temperature (Deane-Drummond *et al.*, 1980; Sutherland *et al.*, 1985), light intensity (Emes and Bowsher, 1991) and seasonal variation (Sung and Sun, 1990; Oaks, 1992).

1.5.2 Why does the site of nitrate assimilation vary?

There is much speculation about the purpose of these differences in NO_3^- assimilation partitioning. The observation that many plants export NO_3^- to the leaves, reduce it and transport the reduced nitrogen back to the roots, led to the suggestion that NO_3^- reduction and processing, with its reductant, energy and carbon requirements, may be more economical when localized in proximity to photosynthesis (Andrews, 1986a; Stewart *et al.*, 1987). It has also been observed that the leaves of pioneer or colonizing species, i.e. species that tend to utilize NO_3^- in preference to NH_4^+, generally exhibit a large capacity to assimilate NO_3^-, whereas leaves of understorey plants show low levels of NR activity and little capacity to assimilate NO_3^- (Stewart *et al.*, 1988, 1990). The stimulation of leaf growth by NO_3^- is a well-documented phenomenon, and Sprent and Thomas (1984) have suggested that nitrate transferred to the shoot rather than being reduced in the root, may be the osmotic driving force for cell expansion as well as being used for growth following reduction. Smirnoff and Stewart (1985) also suggest that the accumulation of NO_3^- in plants of desert and semi-arid regions may play a protective role, in that when stomata are closed during water stress, NO_3^- reduction may help dissipate excess photochemical energy and minimise photoinhibition.

Shoot assimilation can be an advantage if the available photon flux density (PFD) exceeds that required to saturate photosynthesis. However, if pH regulation

is taken into account, the energetic differences between shoot and root assimilation may be small (Sprent and Thomas, 1984). There is also evidence that shoot assimilation may be disadvantageous at low temperatures. When a cold-sensitive and a cold-tolerant cultivar of *Vicia faba* were compared, more NO_3^- assimilation occurred in the sensitive cultivar. If the cold-tolerant cultivar was supplied with a great excess of NO_3^- so that a large amount had to be transported to the shoot, it showed temperature stress effects (Sutherland *et al.*, 1985). A decrease in temperature has also been shown to cause a decrease in the amount of NO_3^- transported to the shoot of *Hordeum vulgare* and *Vicia faba* (Andrews, 1986a).

1.6 DIFFERENCES BETWEEN TEMPERATE AND TROPICAL LEGUMES

In order to discuss the possible reasons for differences in the partitioning of nitrate reduction between temperate and tropical legumes it is necessary to describe what is meant by the terms tropical and temperate. It is also important to become familiar with the legume tribes and to review their evolutionary relationships, their radiation and adaptation to different climates, and to discuss why nitrate nutrition is important to plants that can fix atmospheric nitrogen.

1.6.1 Tropical, subtropical and temperate biomes

The geographic tropics lie between 23.5° north and south, the area between which sunlight is received at 90° at noon at different times during the year. Several tropical biomes exist depending on their rainfall and temperature (see Figure 1.2). Tropical areas with a mean annual rainfall below 500 mm, where legumes will not grow without irrigation, form the extra-tropical biomes of desert and semi-desert

(Cox and Moore, 1985). Although there are no widely accepted limits to the subtropics they are usually defined as having a mean annual air temperature of 21°C or higher, broadly corresponding to latitudes 30° north and south. Tropical biomes are further modified by altitude. Air temperature falls on average by 0.6°C per 100 m rise in height, so that mountains in the tropics may be classed as temperate (Cox and Moore, 1985). The daylength of course remains tropical, and these areas experience considerable diurnal variation in temperature not found at higher latitudes. The term 'temperate' in this thesis and elsewhere implies regions that by default lie outside the tropics, encompassing an enormous range of climates from desert to arctic tundra (Isbell, 1978; Cox and Moore, 1985). Figure 1.2 shows the world distribution of major biomes, and draws the distinction between tropical (from now on including subtropical) and temperate regions as defined above.

1.6.2 The Leguminosae

The Leguminosae is one of the largest and most important families of angiosperms, second only to the Orchidaceae and Compositae in size, and to the Gramineae in economic importance. It is a very diverse family, comprised of approximately 650 genera containing 18,000 species, ranging from tiny ephemerals to forest giants and found from the Equator to dry, cold deserts. There are three subfamilies of which two (the Caesalpinioideae and Mimosoideae) are almost entirely tropical, whilst members of the third subfamily (the Papilionoideae) are found both in both tropical and temperate regions.

The Leguminosae were among the first angiosperms to evolve, and

evidence supports an early separation of legumes from the other flowering plants (the current view of the evolution of the major legume groups is shown in Figure 1.4). The most archaic of the three subfamilies are the Caesalpinioideae, thought to have evolved in the late Cretaceous period. The other two families are believed to have diverged from the Caesalpinioideae before the end of the Cretaceous, and were well defined by the middle Eocene (Crepet and Taylor, 1985).

Warm and wet zones in Africa appear to have been the primary site of evolution of the earliest lines of the Caesalpinioideae, and migration to other landmasses was fairly unconstrained as there were few climatic or geographic barriers. This is reflected in the scattered world distribution of the surviving relic genera which can be found in both warmer temperate regions and in tropical zones. These relic genera have retained generalized characteristics for possibly 60 million years, moving into more equable areas as climate and habitats altered rather than adapting (Raven and Polhill, 1981). The tropical woody legumes now represented by the main part of the Caesalpinioideae and Mimosoideae, and the primitive woody members of the Papilionoideae also have a fairly scattered distribution with a bias toward Africa and S. America. This is thought to reflect the occurrence of a substantial water barrier between Africa and Eurasia, and the close proximity of Africa and S. America in the Paleogene (Cox and Moore, 1985).

The advanced genera of all three subfamilies arose by aggressive bursts of expansion and migration into habitats of relatively modern origin, coincidental with climatic change that began in the mid-Tertiary period. These new habitats, savanna and grassland, desert, mountain top and tundra, required specializations resulting in

numerous discrete groups of legumes with narrow ranges of variation allowing many sharply defined genera, and abundant survival to the present (Raven and Polhill, 1981). The evolution and biogeography of the Papilionoideae is discussed in Chapter 6.

1.6.3 Adaptation to temperate life

Radiation into many of the recently formed temperate habitats necessitated adaptation to low temperature, changing daylengths and seasons, younger soils, and drought (Polhill *et al.*, 1981; Raven and Polhill, 1981). The main effect of cool temperatures on tropical legumes is the interruption and/or delay to reproductive development (t'Mannetje *et al.*, 1978). Their distribution to cooler latitudes and altitudes is prevented by the inability to set seed before the parent plants are killed. Temperate species can harden as temperatures gradually fall, whereas most tropical species cannot. Temperatures less than 10°C affect chill-sensitive tropical legumes in several ways. Although some subtropical legumes can tolerate cool temperatures by the formation of apical meristems near the ground, or by winter dormancy, many tropical legumes will die if subjected to a 20/6°C day/night regime for more than 10 days. It is thought that membrane physiology holds the key to adaptation to cold temperate climates (Lyons and Breidenbach, 1979; Quinn, 1988). The cell membranes of some chill-sensitive species have been found to undergo a transition from liquid-crystal to gel-liquid at between 8-10°C (McKersie and Thompson, 1978; Inoue, 1978; Clarkson *et al.*, 1988), a property that is dependent on the degree of saturation of the membrane phospholipids.

The ancient, highly weathered soils of the tropics tend to be very acidic,

have low concentrations of phosphorous, magnesium and calcium, and high concentrations of manganese and aluminium (Isbell, 1978; Munns, 1978). Acid soils can be tolerated by many tropical and temperate legumes, but the temperate species in particular cannot cope with toxic levels of aluminium and manganese. The soils in temperate regions tend to be much younger, reflecting recent geological activity, fertile, relatively base-rich, and often calcareous, indeed the almost entirely temperate tribes, the Loteae, Coronilleae, Vicieae and Trifolieae, are all calcicole (Allen and Allen, 1981). Domesticated crops of tropical origin, e.g. *Phaseolus* spp. and *Arachis hypogaea*, have also had to adapt to these base-rich, fertile soils (Adams and Pipoly, 1978).

The evolutionary advances of the host plants to the herbaceous habit, novel soils and temperate climates, provoked adaptation in the rhizobial partner. It is thought that a group of slow-growing ancestral-type rhizobia (the cowpea miscellany) symbiosed promiscuously with the primitive, woody leguminous genera thriving on the leached acid soils of the tropics during the upper Cretaceous and lower Tertiary. An adaptive bacterial mechanism to growth in these acid soils is to produce and secrete alkali, a trait to be seen in present day members of the cowpea miscellany. Faster-growing, acid-producing rhizobial strains evolved along with the host plants, and group specificities developed (Norris, 1972).

1.7 NOT ALL LEGUMES ARE NODULATED

Nitrate is as important to many legumes as it is to other plants because not all legumes bear nitrogen-fixing nodules. There are many reasons why nodulation may not occur.

1.7.1 Biotic factors preventing nodulation

Some legume species are incapable of nodulation, including 70% of the species in the Caesalpinoideae, 4% of the Mimosoideae and 2% of the Papilionoideae (mainly members of the Swartzieae). In legumes capable of N_2-fixation, the temperate papilionoid legume-*Rhizobium* relationship tends to be very specific. This means that the correct rhizobial symbiont must be present for effective nodulation to occur. Resistance and susceptibility of legumes to nodulation may vary between species in the same genera, or between strains of the same species. For example, some African species of *Trifolium* cannot symbiose with European strains of *Rhizobium trifoli*, and European *Trifolium* species cannot symbiose with African strains (Allen and Allen, 1981). One group of *P. sativum* cultivars cannot be nodulated by European strains of *R. leguminosarum* but can by strains from the Middle East, resistance being due to a single allelic variation. Another pea group is nodulated by rhizobia from both regions, an ability conferred by the presence in the bacteria of the plasmid pRL5JI (Kalloo, 1993).

Not only must the specific symbiont be present for effective nodulation, there must be a sufficient density of them in the soil. For example, most soils in temperate regions and some tropical soils, do not contain *R. japonicum* unless *G. max* has previously grown there. Good nodulation of land new to *G. max*

cultivation requires 10^5-10^6 viable rhizobia per seed (Brar and Carter, 1993).

1.7.2 Soil composition may prevent nodulation

Many soil factors can inhibit the formation of effective nodules. Nitrate inhibits nodulation and will be discussed later. Calcium is necessary for nodulation, whereas aluminium inhibits it by nature of its phytotoxicity, and by suppressing calcium uptake (Munns, 1978). Rhizobia also have limited pH tolerances and, even if the bacteria can grow, soils with low pH may inhibit the nodulation process. Several nodulation processes are affected by soil temperature, including root hair formation, root hair binding, infection thread formation, nodule initiation and growth, leghaemoglobin content and nitrogenase activity (Baudoin, 1993). Infection may be inhibited at mean diurnal temperatures greater than 24°C (Som and Hazra, 1993). The survival of free-living rhizobia in soils may also be affected by temperature, and lack of soil water may prevent their migration to the root zone of potential hosts (Som and Hazra, 1993).

1.7.3 Temporary loss of nodules

Legumes may experience the temporary absence of nodules. In seedlings there may be a delay before the first effective nodules are formed (Selamet and Gardner, 1985; Sprent and Thomas, 1984), and mature N_2-fixing legumes may abscind their nodules for a variety of reasons. Deciduous legumes shed their nodules along with their leaves, and any form of stress, including drought and flooding, may also cause nodule abscision, especially if accompanied by foliage loss (Allen and Allen, 1981).

Host vigour is also a factor influencing the formation of effective nodules. The structure and physiology of nodules may be altered by the effect of disease on the plant nutritional status, or through root exudates produced by virus-infected plants. Nematodes are known to produce nodule-suppressing factors in addition to their negative effect on host vigour and insects or their larvae may eat nodule tissue, eg the larvae of the *Sitona* weevil.

1.7.4 Nodulated legumes can still assimilate nitrate

Nodulation and N_2-fixation are very energy-demanding processes and only take place when supplies of combined nitrogen are low. It is energetically more favourable to assimilate nitrate and it is well documented that nitrate in the soil will inhibit nodulation. Nodules that have already been formed are capable of NO_3^- assimilation (Vézina *et al.*, 1988; Caba *et al.*, 1990; Hervas *et al.*, 1991; Arrese-Igor *et al.*, 1991), but if exposure to NO_3^- is prolonged then the nodules will senesce and abscind. For a discussion on possible mechanisms of inhibition by NO_3^-, see Vessey and Waterer (1992).

1.8 AIMS

The aim of this project was to characterize the subunit composition and activity of GS isoforms in *L. japonicus* and *L. corniculatus*, along with the distribution of subunit types in different tissues. The possibility of induction of GS subunits by NO_3^- or NH_4^+ was also to be explored, with special reference to root plastidic GS, along with the effects of these nitrogen sources on other aspects of nitrogen metabolism.

Reports of GS2 in the roots of three temperate legume species, and its NO_3^- -inducibility in at least two of these species, together with the lack of published evidence for GS2 in roots of any tropical legumes, suggested a possible correlation with the reported differences between tropical and temperate legumes in their partitioning of NO_3^- assimilation between root and shoot (Andrews, 1986a). This thesis aimed to investigate the occurrence of GS2 in the roots of a diverse range of temperate and tropical legumes, and the distribution of NO_3^- assimilation between root and shoot.

FIGURE 1.1 The GS-GOGAT pathway of ammonium assimilation in plants and the synthesis of asparagine

The major sources of NH_4^+ in a plant are shown, as are the requirements for ATP.

Enzyme abbreviations are -

AS	asparagine synthetase
AspAT	aspartate aminotransferase
GOGAT	glutamate synthase
GS	glutamine synthetase
NiR	nitrite reductase
NR	nitrate reductase

FIGURE 1.1

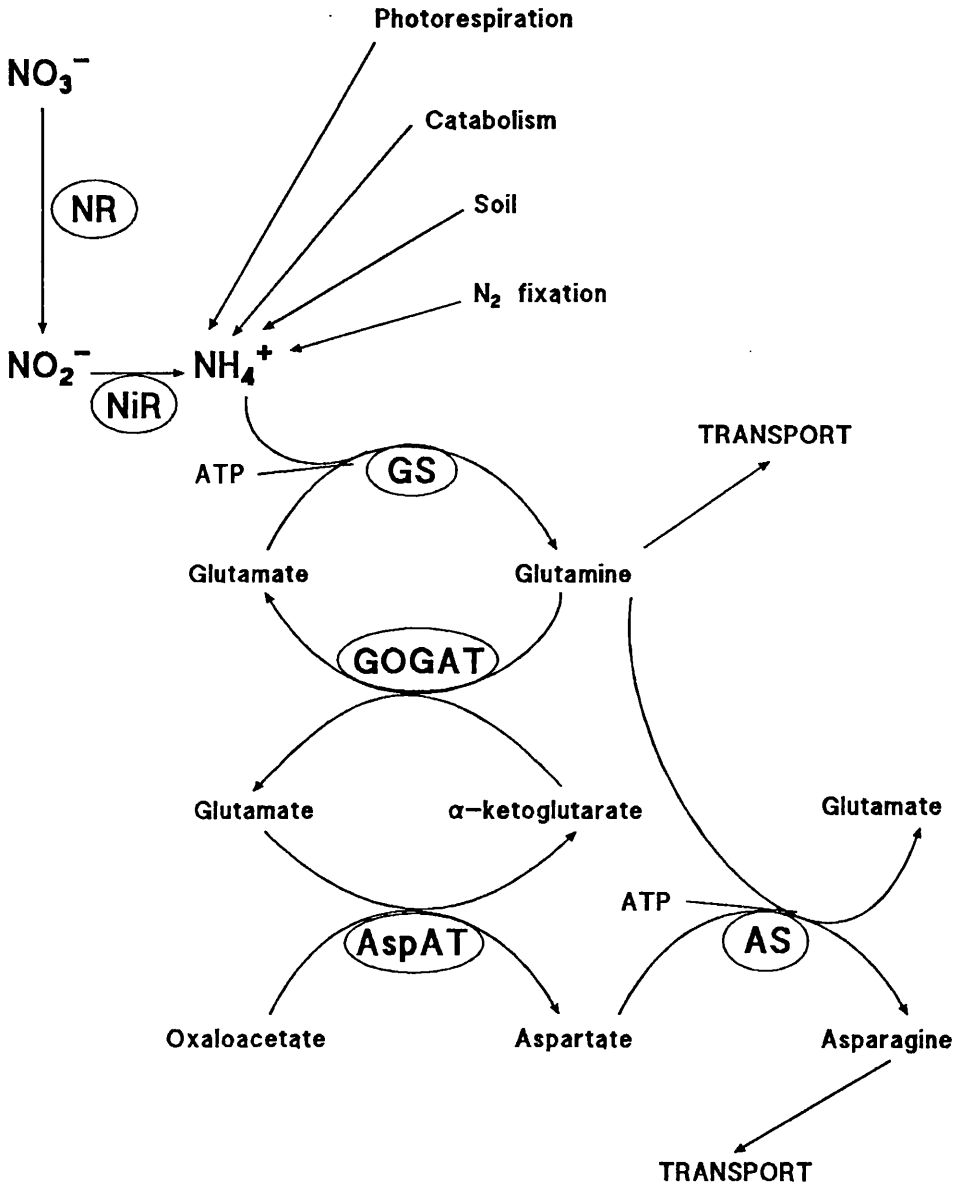

Photorespiration
NO_3^-
Catabolism
NR
Soil
N_2 fixation
NO_2^-
NH_4^+
NiR
TRANSPORT
ATP
GS
Glutamate
Glutamine
GOGAT
Glutamate
α–ketoglutarate
Glutamate
ATP
AS
AspAT
Oxaloacetate
Aspartate
Asparagine
TRANSPORT

FIGURE 1.2 **Distribution of the major terrestrial biomes of the world**

Redrawn from Cox and Moore (1985).

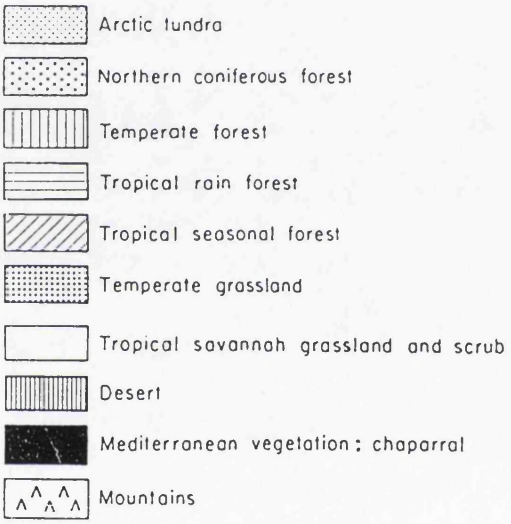


FIGURE 1.2

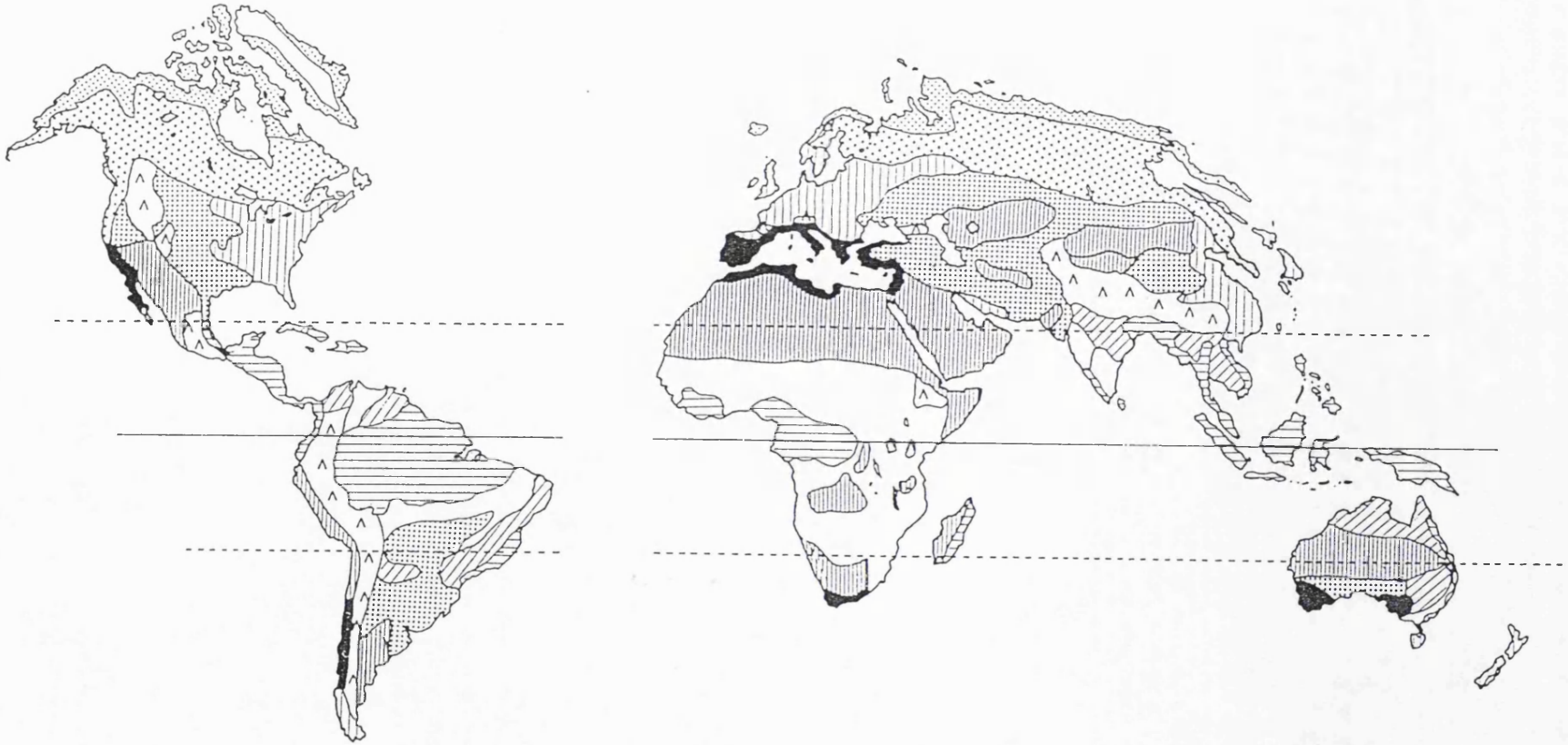


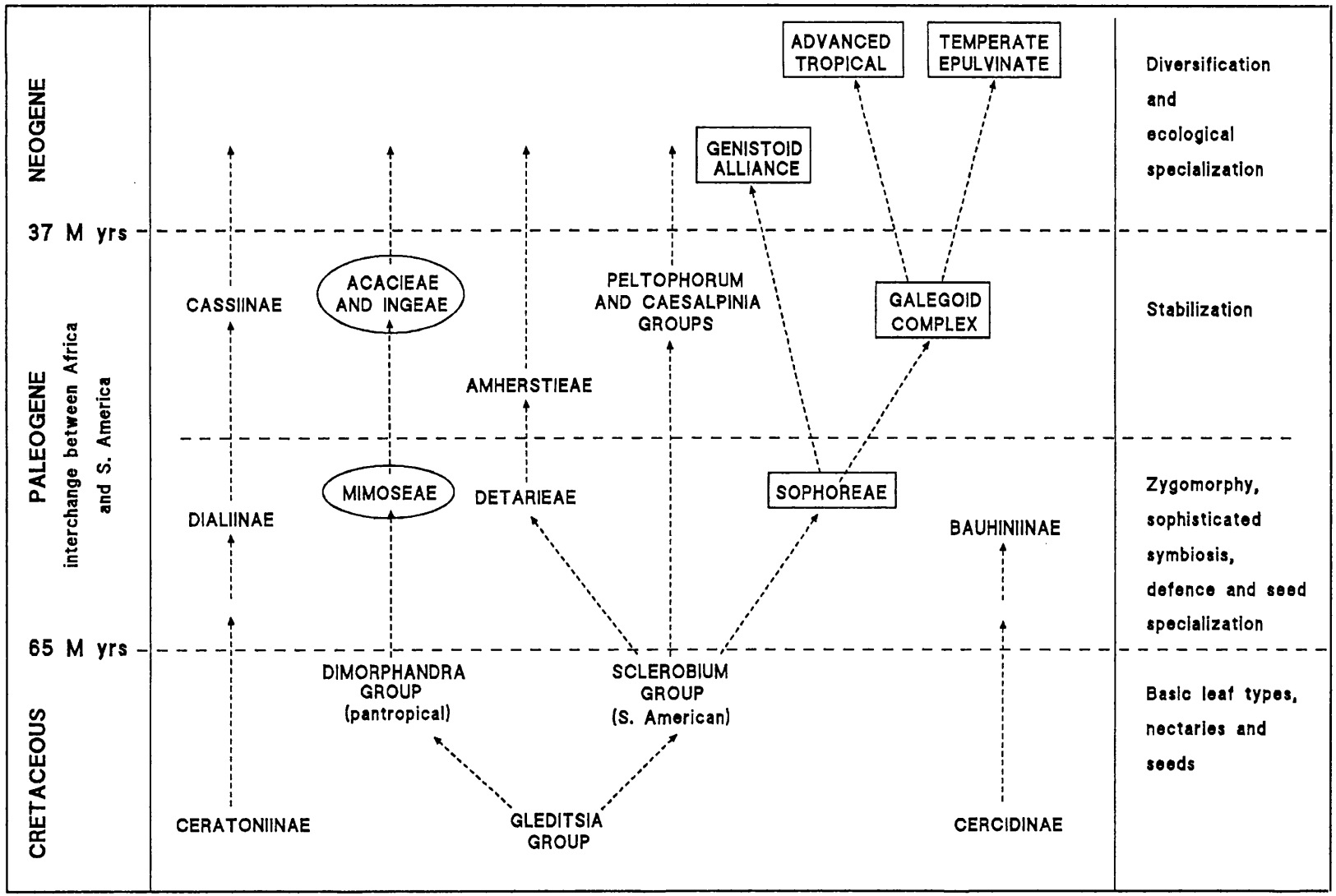
FIGURE 1.3 Putative divergence of the main groups of the Leguminosae showing periods of specialization

Derived from Polhill, Raven and Stirton (1981).

Components of the Papilionoideae are shown in square boxes and components of the Mimosoideae in eliptical boxes. The remaining groups belong to the Caesalpinoideae.

Angiosperms evolved during the early Cretaceous period with legumes first seen in the late Cretaceous. The Paleogene is comprised of the early Tertiary epochs, the Paleocene, Eocene and Oligocene. The Neogene includes the fourth and the last epochs of the Tertiary period (the Miocene and the Pliocene) and the first and the most recent epochs of the Quarternary period (Pleistocene and Holocene). Most temperate habitats were formed during the Neogene period.

FIGURE 1.3



Redrawn from Polhill, Raven and Stirton (1981)

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS AND SUPPLIERS

All organic and inorganic chemicals were purchased from Sigma and BDH respectively unless otherwise stated. Radioactive nucleotides where supplied by Amersham International. Suppliers' addresses can be found in Appendix I, and Appendix II contains a list of plant species and sources.

2.2 PLANT CULTURE CONDITIONS

Seeds were scarified and/or soaked as recommended by the supplier then surface-sterilized by soaking for 5 min in 75% (v/v) ethanol and for 2-3 min in a 7% (v/v) hypochlorite solution (0.7-1.0% available chlorine). After rinsing thoroughly with sterile distilled water (SDW) they were placed onto sterile filter paper in a Petri dish and germinated in the light. Seedlings were then transferred to trays or pots of sterile vermiculite. Unless otherwise stated, plants were grown at 22°C/18°C, under a 16/8 h light/dark photoperiod at 300 μE m^{-2} s^{-1} and 70% humidity, and watered every 2-3 d with nutrient solution containing 5 mM NH_4^+ and 8 mM NO_3^-.

Nutrient solutions were as described by Handberg and Stougaard (1992), made up as a nitrogen-free 40 x stock solution containing 12 mM $MgSO_4$, 3 mM $CaCl_2$, 6 mM KH_2PO_4, 0.2 mM Fe-EDTA, and 40 x micronutrients (containing l^{-1}: 48 mg $MnSO_4$; 48 mg H_3BO_3; 16 mg $CuSO_4$; 16 mg $ZnSO_4$; and 8 mg Na_2MoO_4).

This was stored at 4°C. The 1 x nutrient working solution was made up in distilled H_2O as required from the 40 x stock to which was added the relevant nitrogen source supplied either as KNO_3 or $(NH_4)_2SO_4$, and the pH was adjusted to 6.8-7.0 with KOH.

2.3 MEASUREMENT OF TRANSPIRATION

Clingfilm was used to mask the soil, pot and saucer to prevent evaporation. Loss of water through plant shoots was measured by weighing the plant and pot at 2-3 h intervals.

2.4 EXTRACTIONS AND ASSAYS

2.4.1 Plant harvest and tissue extraction

Plant leaves, stems and roots were harvested on ice, weighed into 0.4 g quantities, and either frozen in liquid nitrogen until required or stored overnight at -80°C. Frozen tissue was ground in a 1:10 (w/v) ratio of extraction buffer containing 50 mM Tris-HCl pH 8.5, 10 mM EDTA, 7.9 $\mu g.ml^{-1}$ flavin adenine dinucleotide (FAD), 4.75 $\mu g.ml^{-1}$ leupeptin, 0.21 $\mu g.ml^{-1}$ $NaMoO_4$, 1% (v/v) Nonidet, 3% (w/v) polyvinylpolypyrollidone (PVPP), 0.1% (w/v) dithiothreitol (DTT). These extracts were used for protein estimation (2.4.3), for the *in vitro* NR assay (2.4.4), for the GS assay (2.4.7), and for both one-dimensional and some two-dimensional electrophoreses (2.6.1 and 2.6.2).

2.4.2 Sap collection

The shoot was cut off below the first set of leaves and the cut end rinsed with dH_2O and patted dry. Sap was collected by pipette from stems that bled, and measured for NO_3^- content (2.4.9) and analyzed by HPLC (2.4.12).

2.4.3 Protein assay

Protein assays were performed according to the protocol of Bradford (1976), using a kit supplied by Bio-Rad. Five μl of extract was added to a cuvette containing 795 μl Bradford reagent diluted 1:14 with double dH_2O (ddH_2O). After thorough mixing the cuvettes were allowed to stand for 5 min before reading the absorbance at 595 nm. Standard curves were obtained using bovine serum albumin.

2.4.4 Nitrate reductase assay (*in vitro*)

The assay for NR activity (EC 1.6.6.1/1.6.6.2) was based on Wallace (1986). Tissue extract (50-100 μl) was added to assay buffer (50 mM K phosphate buffer pH 7.5, 10 mM KNO_3, 0.2 μM NADH) in a total volume of 500 μl, and incubated at 30°C for 1 h, after which the reaction was stopped with 500 μl 1% (w/v) sulphanilic acid (1 $g.l^{-1}$ sulphanilamide in 15% v/v HCl). Control tubes were set up containing 500 μl 1% sulphanilic acid to immediately stop the reaction, and 500 μl 0.02% N-(napthyl)-ethylene diamine dihydrochloride (NEDD). The colour was allowed to develop for approximately 20 min, then the tubes were centrifuged at 13000 x g for 10 min to pellet the protein precipitate. The absorbance of the supernatant was read at 540 nm (an absorbance of 1.0 = 6.66 nmol NO_2^- in a 1 cm light path). Samples were assayed with a half or double the amount of tissue

extract to test for proportionality of the reaction.

2.4.5 Nitrate reductase assay (*in vivo*)

In vivo assays for NR activity (EC 1.6.6.1/1.6.6.2) were performed according to Stewart and Orebamjo (1979). Leaf, stem or root tissue (0.2 g) was cut into approximately 2 mm pieces and placed into 5 ml incubation medium (1.5 mM KNO_3 and 1.5% v/v isopropanol in 50 mM K phosphate buffer, pH 7.6). The tissue was vacuum-infiltrated with the incubation medium by alternately imposing and releasing a vacuum two or three times. The sample was then incubated under vacuum and in the dark for 1 h at 25°C. When incubation was complete the tubes where inverted several times to ensure even distribution of evolved NO_2^- throughout the bathing medium. A 500 μl aliquot of the medium was added to 500 μl 1% (w/v) sulphanilic acid and 500 μl 0.02% (w/v) NEDD (see 2.4.4). The absorbance at 540 nm was read after 20 min.

2.4.6 Nitrite reductase assay

Nitrite reductase (EC 1.7.7.1) activity, which was used as a plastidic marker for plastid fractionation (see 2.5.1) was measured by the method of Wray and Filner (1970) using methyl viologen as the electron donor. The assay mixture contained 33 mM K phosphate buffer (pH 7.5), 2 mM KNO_3, 1 mM methyl viologen, 11.6 mM $Na_2S_2O_4$ and plastid fraction in a final volume of 1 ml. The reaction was started by gently adding 0.2 ml $Na_2S_2O_4$ (10 $mg.ml^{-1}$) in 0.29 M Na bicarbonate. Following incubation at 25°C for 20 min, the reaction was stopped by vigorously vortexing the tube contents until the blue dye was oxidized. Following

dilution of the reaction with dH_2O, a 500 μl aliquot of the medium was added to 500 μl 1% (w/v) sulphanilic acid and 500 μl 0.02% (w/v) NEDD (see 2.4.4), and the absorbance at 540 nm was read after 20 min.

2.4.7 Glutamine synthetase semi-biosynthetic assay

The semi-biosynthetic assay for GS (EC 6.3.1.2) was based on Stewart *et al.* (1988). Tissue extract (100 μl) was added to 50 μl 0.4 M ATP, 300 μl 50 mM Tris-HCl pH 7.6 and 50 μl of GS assay buffer (50 mM Tris-HCl pH 7.6, 150 mM $MgSO_4$, 300 mM glutamic acid, 40 mM hydroxylamine-HCl). Following incubation at 30°C for 1 h the reaction was stopped with 500 μl $FeCl_3$ solution (20 g trichloroacetic acid and 13 g $FeCl_3$ dissolved in 500 ml 8% v/v HCl). Control tubes were set up as above but with 50 μl Tris-HCl buffer in place of the ATP. Tubes were centrifuged for 10 min before reading the supernatant absorbance at 500 nm (an absorbance of 1 = 3 μmol glutamylhydroxylammonium in a 1 cm light path). Samples were assayed for proportionality as described in 2.4.4.

2.4.8 Tissue ammonium assay

The ammonium assay was based on McCulloch (1967). Tissue was extracted overnight at 4°C in 100% methanol in a 1:10 ratio. The extract was clarified by mixing 100 μl with 50 μl 10% (w/v) sodium tungstate solution and then adding 50 μl 0.5 M H_2SO_4. After centrifugation 700 x g for 10 min, 100 μl of supernatant was added to 500 μl of phenol solution (1% w/v phenol and 0.005% w/v Na nitroprusside) and 500 μl alkaline solution containing 125 mM NaOH, 344 mM Na_2HPO_4 and 1 ml Na hypochlorite solution (10-14% available

chlorine). After incubation at 37°C for 35 min, the absorbance was read at 625 nm. Standard curves were obtained for each determination, and some samples were spiked with a known quantity of NH_4^+ to assure the absence of interfering metabolites.

2.4.9 Nitrate determinations

Nitrate was assayed both by the Cadmium reductor method and by a novel method employing *Aspergillus* NR. The two methods were compared for accuracy, sensitivity and reproducibility.

Cadmium reductor method - The method of Sloan and Sublett (1966) was used. Cadmium was obtained by placing zinc rods in 20 % (w/v) $CdSO_4$ and collecting the mossy growth that appeared over several hours. The reduced cadmium was stored at 4°C under 0.4 M ammonium buffer (0.78% NH_4^+). Tissue was extracted in 100% methanol in a 1:10 ratio for 24 h at 4°C. A 100 μl aliquot of the methanol extract was added to 300 μl 0.4 M ammonium buffer and 100 μl $MgCl_2$, and approximately 0.1 g of reduced cadmium was added. Controls were set up without the cadmium. After incubation at room temperature for 30 min the tube contents were mixed and 30 μl added to 470 μl ddH_2O. Aliquots (500 μl) of sulphanilic acid and NEDD were added (see 2.4.4) and the absorbance read at 540 nm after 20 min.

Aspergillus NR method - A novel method for assaying NO_3^- was developed by modifying the Boehringer Mannheim protocol for analysis of NO_3^- in food (1989). Tissue was extracted in 100% methanol in a 1:10 (w/v) ratio for 24 h at 4°C. An aliquot (200 μl) was clarified according to the Boehringer Mannheim protocol by adding 50 μl Carrez I solution (0.36 g $K_4Fe(CN)_6$ in 10 ml dH_20) and 50 μl of Carrez II reagent (0.72 g $ZnSO_4$ in 10 ml). After thorough mixing each sample was centrifuged at 13,000 x g for 10 min and the supernatant transferred to a fresh tube.

A 25 μl aliquot of the clarified supernatant, suitably diluted (see later) was made up to 0.5 ml with assay buffer (50 mM K phosphate buffer pH 7.6, 10 μM NADPH, 2 μM FAD) and 5 μl of 4.5 $U.ml^{-1}$ NR from *Aspergillus* spp. (Boeringer Mannheim) made up according to suppliers instructions. The sample was then incubated at room temperature for at least 1.5 h to allow all of the nitrate to be reduced. Aliquots (500 μl) of 1% sulphanilic acid and 0.02% NEDD (see 2.4.4) were added and the absorbance at 540 nm measured after approximately 20 min. The clarified extract was diluted with dH_2O to give a final absorbance at 540 nm of between 0.1 and 1.0 (undiluted for ammonium-grown plants, 1 in 50 dilution for 20 mM nitrate-grown plants).

2.4.10 A comparison of methods for measuring NO_3^- concentration

The novel *Aspergillus* NR method was compared with the cadmium reductor method by assaying NO_3^- in tissue extracts both before and after being spiked with a known amount of NO_3^-. *L. japonicus* was grown on 20 mM NO_3^-, 0.2 mM NO_3^- or 5 mM NH_4^+, and it can be seen that the values obtained by the

Aspergillus NR method were greater, and more consistent than with the cadmium reductor method (Figure 2.1A). When the tissue samples were spiked with a known amount of NO_3^-, the recoveries using the cadmium reductor method, obtained by comparing the observed values with the expected values, were 88% ± 5 for the leaf, 89% ± 7 for the stem, and 88% ± 9 for the root. However, the recoveries obtained by the *Aspergillus* NR method were 100% ± 2, 102% ± 4 and 103% ± 3 respectively.

The Boehringer Mannheim protocol required that the reaction be followed directly by monitoring the depletion of NADPH at 340 nm. The newly developed method was shown to be consistent whether detected colorimetrically or by following the depletion of NADPH (Figure 2.1).

2.4.11 Measurement of chlorophyll

Leaf tissue was extracted in 80% acetone in a 1:5 (w/v) ratio, in the dark overnight. The concentrations of chlorophylls *a* and *b* in the extract were measured by the method of Arnon (1949).

2.4.12 High pressure liquid chromatography

Sap samples or tissue extracted in methanol (see 2.4.8) were dried under N_2 gas, resuspended in SDW to 25-50% of the original volume, and filtered through a 0.45 μm HVLP membrane (Millipore). These extracts were analyzed by high pressure liquid chromatography (HPLC) at University College, London, following the methods of Benson and Hare (1975) and Jarret *et al.* (1986) whereby the extracts were derivatized in for 2 min with 50 mM *O*-phthaldialdehyde in 50 mM

borate buffer, pH 9.5, containing 2% methanol and 20 mM ß-mercaptoethanol. Derivatized samples were injected into a C-18 column with 5 μm spherical packing and a length of 12 cm. Amino acids were eluted with a gradient of two buffers starting with 20% methanol and 80% buffer (50 mM Na acetate, 50 mM Na phosphate, 2% methanol and 2% v/v tetrahydrofuran), to 65% methanol in SDW. Absorbance was monitored at 340 nm and concentrations calculated with reference to a standard mix (Sigma) and using an internal standard of homoserine.

2.5 PREPARATION OF ROOT PLASTIDS

2.5.1 Root plastid separation (I)

Root plastids were initially obtained by the method of Vézina and Langlois (1989). A discontinuous sucrose gradient was prepared in a 30 ml corex centrifuge tube by layering 10 ml of 68% sucrose in buffer A (100 mM tricine, pH 8, 0.1% BSA, 1 mM $MgCl_2$ and 6 mM ß-mercaptoethanol) onto 10 ml 50% sucrose solution in buffer A. This was left at 4°C overnight for the gradient to form. Roots of *L. japonicus* plants grown on 5 mM KNO_3 were gently ground in one volume of extraction buffer (1.2 M sucrose, 100 mM tricine, pH 7.8, 1% polyvinyl-pyrrolidine, 0.1% BSA, 1 mM $MgCl_2$ and 6 mM ß-mercaptoethanol). This was filtered through 2 layers of muslin and the brei centrifuged at 1000 x g (4°C). The supernatant was layered on top of the discontinuous sucrose gradient and centrifuged for 20 min at 15,000 x g in a swing-out rotor. Samples were taken by syringe and needle from different depths in the gradient, and assayed for nitrite reductase (plastidic marker), nitrate reductase (cytosolic marker) and GS (see

Chapter 2.4.4, 2.4.6 and 2.4.7 for the enzyme assays).

2.5.2 Root plastid separation (II)

The separation of root plastids was achieved using a method based on Emes and England (1986). Plants were placed in the dark for 48 h prior to harvest and watered with nutrient solution containing 10 mM NO_3^-. Thoroughly cleaned roots were very finely chopped in 1 to 2 volumes of buffer A (50 mM Tricine-NaOH, pH 7.9, 350 mM sorbitol, 1 mM EDTA, 2 mM $MgCl_2$, 0.1% DTT) plus 0.1% BSA and 1.5% PVPP. The resulting mash was filtered through 2 layers of muslin and the brei was centrifuged for 1 min at 200 x g at 4°C. The supernatant was carefully underlaid with more than 1 volume 10% v/v Percoll (in 50 mM Tricine-NaOH, pH 7.9, 350 mM sorbitol). After centrifugation at 4000 x g for 5 min at 4°C, the supernatant was carefully removed so as not to disturb the plastid-enriched pellet which was subsequently resuspended in 2 ml buffer A. This plastid-enriched fraction was then centrifuged at 5000 x g for 3 min at 4°C. Finally the pellet was resuspended in a further 2 ml buffer A, and assayed for marker enzymes (Chapter 2.5.3) and the presence of GS.

2.5.3 Marker enzyme assays

All assays were in 350 mM sorbitol in a final volume of 1 ml. The extinction coefficient for NAD(P)H is 6.22 mM for a 1 cm light path. A measure of intactness of the organelles was obtained by following the change in absorbance before and after bursting them with 10 μl of 10% Triton X-100. This was calculated using the equation

$$\frac{I - B}{I} \times 100$$

where I = intact plastids and B = burst plastids.

Samples were also assayed which had been burst immediately to ensure that there was no loss of activity over the time course of the assay.

Plastidic marker (hexose phosphate isomerase) - Hexose phosphate isomerase activity was measured according to Bowsher *et al.* (1989). Extract was added to 250 mM glycylglycine, pH 7.5, containing 10 mM $MgCl_2$, 0.39 mM $NADP^+$ and 1 U glucose-6-phosphate dehydrogenase. The reaction was initiated with 0.1 ml 5 mM frutose-6-phosphate (F-6-P) and the change in absorbance at 340 nm followed both before and after the addition of detergent. Controls were minus F-6-P and minus extract.

Cytosolic marker (alcohol dehydrogenase) - Alcohol dehydrogenase activity was measured according to MacDonald and ap Rees (1983). Extract was added to 50 mM Tris-HCl pH 8.5, containing 1 mM NAD^+, and the reaction was initiated by the addition of 15 μl 100% ethanol. The reduction of NAD^+ was followed at 340 nm before and after the addition of Triton X-100.

2.6 PROTEIN ELECTROPHORESIS, BLOTTING AND STAINING

2.6.1 SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) using the Mini-Protean II electrophoresis system from Bio-Rad. The gels were 0.75 mm thick and composed of a 10% separating gel (0.375 M Tris-HCl, pH 8.8) and a 4% stacking gel (0.125 M Tris-HCl, pH 6.8). The gels were run for 45 min at 150 V (constant voltage) in 25 mM Tris-HCl buffer, pH 8.3, containing 192 mM glycine, 0.1% (w/v) SDS.

Tissue extracts (see 2.4.1) were diluted 1 in 4 with sample buffer containing 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 0.02% SDS, 1.25 μl of 1% bromophenol blue (BPB) ml^{-1} and 50 $\mu l.ml^{-1}$ ß-mercaptoethanol. The samples were then heated at 95°C for 5 min, and placed on ice until ready to load.

2.6.2 Two-dimensional SDS-Polyacryamide gel electrophoresis

Two dimensional SDS-PAGE was carried out according to O'Farrell (1975).

GS extraction buffer for 2-D electrophoresis - Tissue for 2-D electrophoresis was extracted as described in 2.6.1, but in the following modified buffer: 50 mM Tris-HCl buffer pH 8.5, 10 mM EDTA, 1% (v/v) Nonidet, 15 mM $MgSO_4$, 10% (v/v) ethanediol, 3% PVPP, 20 mM Na diethyl dithiocarbamate, 20 mM ß-mercaptoethanol and 0.1% DTT.

First dimension isoelectric focusing gels - Isoelectric focusing (IEF) was performed using the Mini-Protean II 2-D system (Bio-Rad). The IEF capillary gels were cast containing 9.2 M urea, 2% (v/v) Triton X-100, 1.6% (v/v) 5/7 Biolyte ampholyte (Bio-Rad) and 0.4% (v/v) 3/10 Biolyte ampholyte (Bio-Rad). The gels were pre-run in 20 mM NaOH catholyte and 10 mM H_3PO_4 anolyte at 200 V for 10 min, 300 V for 15 min and 400 V for 15 min.

Tissue extracts were prepared by incubating at room temperature for 30 min with an equal volume of sample buffer (9.5 M urea, 2% (v/v) Triton X-100, 5% (v/v) ß-mercaptoethanol, 1.6% (v/v) 5/7 ampholyte, 0.4% (v/v) 3/10 ampholyte). Upon completion of the gel pre-run both anolyte and catholyte were replaced, and the sample loaded (5-15 μg total protein in a 25-100 μl volume). The samples were overlaid with 30 μl overlay buffer (9 M urea, 0.8% (v/v) 5/7 ampholyte, 0.2% (v/v) 3/10 ampholyte, and 2.5 μl 1% BPB ml^{-1}) and the gels run at 200 V for 10 min, 300 V 15 min, 400 V 15 min, 500 V 10 min, and 750 V for 3 h, or at 300 V overnight.

Gels were extruded into equilibration buffer (62.5 mM Tris-HCl pH 6.8, 2.3% SDS, 10% (v/v) glycerol, 1.25 μl 1% BPB ml^{-1} and 5% ß-mercaptoethanol) and either immediately run in the second dimension or frozen and stored at -20°C.

Second dimension SDS-PAGE gels - The second dimension was run using a 10% SDS-PAGE separating gel as described in 2.6.1. A shallow 4% stacking gel was cast containing one marker well using the Bio-Rad 2-D comb.

2.6.3 Protein electrotransfer

Immobilon PVDF membrane (Millipore) was cut to approximate 9 x 7 cm rectangles, wetted in methanol for 5-10 s then washed in 500 ml dH_2O for 5 min and equilibrated in transfer buffer (Tris-HCl, pH 8.3, 192 mM glycine and 15% methanol in 25 mM) for at least 5 min.

SDS-PAGE gels (2.6.1), having been equilibrated with transfer buffer for 20 min, were electroblotted onto activated PVDF membrane with the Minitransfer cell (Bio-Rad). Electroblotting was complete after 1 h 15 min at 90 V (constant voltage).

2.6.4 Gel and membrane staining protocols

Membrane immunostaining - Immunodetection was performed with the anti-rabbit ExtrAvidin-Peroxidase kit (Sigma). The primary antisera were a rabbit anti-GS raised against purified GS from *Phaseolus vulgaris* nodules and a rabbit anti-ribulose bisphosphate carboxylase (RuBisCO) antiserum raised against the RuBisCO large subunit prepared from *Triticum aestivum* leaves.

Membranes were blocked overnight in TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl) plus 5% BSA fraction V, then after washing briefly in TBS, were incubated for 2 h with the primary antiserum diluted in buffer A (TBS containing 1% BSA) to 1 in 400 for the GS antiserum, or 1 in 1000 for the RuBisCO antiserum. The membranes were washed four times in wash buffer (TBS containing 1% BSA, 0.05% (v/v) polyoxyethylesorbitan monolaurate (Tween 20)) and incubated for 1 h in biotinylated anti-rabbit IgG diluted 1 in 800 with TBS and 1% BSA. The blots were then washed again and rinsed in TBS before incubation for

1 h in ExtrAvidin peroxidase diluted 1 in 1000 with buffer A, and finally washed and rinsed.

Blots were developed in 3-amino-9-ethyl carbazole (AEC) solution. The AEC solution was prepared by adding 2.5 ml of a 0.8% (w/v) AEC dissolved in dimethyl formamide to 47.5 ml 50 mM Na acetate pH 5, and just before use adding 25 μl H_2O_2. After washing the membranes in several changes of dH_2O, they were air dried, immediately photographed and stored in protective sleeves in the dark.

Silver staining - Silver staining was performed using the Bio-Rad Silver Stain Kit following the Bio-Rad bulletin No. 1089. The highest quality reagents and ddH_2O were used except for the methanol (traces of formaldehyde apparently help the staining process).

Essentially an SDS-PAGE minigel was fixed in 40% (v/v) methanol and 10% (v/v) acetic acid, then incubated twice in 10% (v/v) ethanol and 5% (v/v) acetic acid. The gel was then bathed in 10% (v/v) Bio-Rad oxidiser solution, washed twice in ddH_2O, incubated in 10% (v/v) Bio-Rad silver reagent, and rinsed briefly in ddH_2O. The gel was then immersed in developer solution until the protein spots had developed sufficiently, and the reaction stopped with 5% (v/v) acetic acid. The gels were washed and stored in dH_2O.

2.6.5 Glycoprotein deglycosylation and detection protocols

Glycoprotein staining - The GlycoTrack kit from Oxford GlycoSystems was used to stain glycoproteins immobilized on a membrane. The blotted membrane was washed in phosphate buffered saline (PBS containing 50 mM Na phosphate buffer pH 7.2, and 150 mM NaCl), incubated in 10 mM Na periodate solution in the dark for 20 min, washed three times in PBS, and incubated for 1 h in hydrazide solution. After three washes in TBS (see 2.6.4, membrane immunostaining) the membrane was incubated in blocking reagent for about 2 h. After a further TBS wash the membrane was incubated with the streptavidin-alkaline phosphatase conjugate solution for 1 h, and washed again with TBS. The glycoproteins were visualized in freshly made NBT/BCIP, and once the blue-brown colour had developed sufficiently, the membrane was rinsed several times in dH_2O and allowed to air dry.

Deglycosylation - Samples prepared using the extraction buffer for 2-D electrophoresis (2.6.2) were deglycosylated using endoglycosidase F/*N*-glycosidase F (Boehringer Mannheim). Briefly, 10-50 μg of protein in 25 μl extraction buffer was denatured by boiling with 0.4% SDS for 2 min, whereupon an equal volume of 2 x incubation mix (containing 100 mM NaH_2PO_4 pH 7.2, 50 mM EDTA and 10 $\mu l.ml^{-1}$ ß-mercaptoethanol) was added. This was boiled again for 2-5 min, cooled on ice, then incubated at 37°C overnight with 0.4 U endoglycosidase F/*N*-glycosidase F.

2.7 NUCLEIC ACID EXTRACTION AND PURIFICATION

2.7.1 DNA extraction and purification

Total DNA extraction - DNA was extracted by the method of Dellaporta *et al.* (1983) whereby plant tissue was ground in liquid N_2 to a fine powder then added to 100 mM Tris-HCl pH 8, 50 mM EDTA, 500 mM NaCl and 10 mM ß-mercaptoethanol. This was shaken vigorously with 1 ml of 20% SDS, and incubated at 65°C for 10 min, after which the mixture was shaken vigorously with 5 ml of 5 M K acetate and left on ice for at least 20 min.

Following centrifugation at 9000 x g for 30 min the supernatant was filtered through Miracloth into a clean tube containing 10 ml isopropanol, mixed and incubated at -20°C for 30 min. After centrifugation at 9000 x g for 30 min, the pellet was air dried and redissolved in 0.7 ml 50 mM Tris-HCl pH 8, 10 mM EDTA, phenol extracted and ethanol precipitated using standard methods (see Sambrook *et al.*, 1989), and finally resuspended in 100 μl 10 mM Tris-HCl pH 7.6, 1 mM EDTA (TE) containing 20 μl of 10 $mg.ml^{-1}$ RNase.

Plasmid DNA purification - Plasmid DNA was purified from cells by using the Magic Miniprep kit (Promega, following Technical Bulletin No. 117). Cells from an overnight culture were pelleted by centrifugation at 3200 x g and resuspended in 200 μl 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 $\mu g.ml^{-1}$ RNase A. The cells were then lysed with 200 μl 0.2 mM NaOH, 1% SDS. Following neutralization with 200 μl 2.55 M K acetate pH 4.8, the sample was centrifuged at 13,000 x g and the pellet discarded. Magic Miniprep DNA purification resin (1 ml) was added to the supernatant and syringed onto a Magic Miniprep column. The column was

washed with 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 2.5 mM EDTA, 50% (v/v) ethanol, and the DNA eluted with 50 μl TE heated to 65°C.

Prep-A-Gene DNA purification - DNA restriction fragments were purified using the Prep-A-Gene kit (Bio-Rad). Binding buffer was added to the sample DNA (1 μg 5 μl^{-1}) to approximately 40 $\mu l.\mu g^{-1}$ DNA, and 8 μl Prep-A-Gene matrix added. The sample was incubated at room temperature for 10 min with gentle mixing, centrifuged for 30 s at 13000 x g, and the supernatant discarded. The DNA pellet was rinsed twice by resuspending in 50 x pellet-volumes of binding buffer, than washed 3 times in 50 x pellet-volumes of wash buffer. The DNA-matrix was pelleted at 13000 x g for 30 s, and the bound DNA eluted by incubation for 5 min at room temperature in 1 x pellet volume of elution buffer. Following centrifugation at 13000 x g, the supernatant containing the DNA was removed and the pellet back-extracted with another pellet volume of elution buffer, and the two supernatants pooled.

2.7.2 Direct extraction of polyA$^+$ RNA

Magnetic oligo-dT Dynabeads (Dynal) were used to purify polyA$^+$ RNA from plant tissue following the method of Jakobsen *et al.* (1990). Leaf tissue (0.2 g) was ground in liquid N_2 to a fine powder and 1.5 ml lysis buffer (0.1 M Tris-HCl pH 8, 0.5 M LiCl, 10 mM EDTA, 1% SDS, 5 mM DTT, and 7.5 U of the RNase inhibitor, RNasin) was added. After centrifugation at 13000 x g the supernatant was added to 2 mg Dynabeads suspended in 100 μl lysis buffer, and gently mixed. The polyA$^+$ RNA was allowed to anneal to the beads for 5 min on

ice. The beads were then collected on the side of a tube using a magnet and the supernatant was aspirated off. The beads were resuspended and washed 4 times in 0.15 M LiCl, 10 mM Tris-HCl pH 8, 1 mM EDTA, 0.1% SDS, and the polyA$^+$ RNA was eluted by incubation for 2 min at 55°C in 100 μl SDW that had been made RNase-free by treatment with diethyl pyrocarbonate (DEPC).

2.7.3 Electrophoresis of DNA

The separation of DNA fragments larger than 500 bp was by electrophoresis in a 1.2% agarose gel for 2-3 h at 5 V cm^{-1}, or overnight at 1 V cm^{-1}, in 1 x TBE containing 5 μg.ml^{-1} ethidium bromide. DNA fragments smaller than 500 bp were separated on an 8% polyacrylamide gel run for 4-5 h at 7 V cm^{-1}. These gels were stained for 20-30 min in 1 μg.ml^{-1} ethidium bromide and destained for 45-60 min in dH_2O.

Gels were viewed on a long wavelength (300-360 nm) UV transilluminator, and photographed using a Polaroid Cu5 Land camera with a yellow filter. An exposure time of 0.125 s to 0.5 s with Polaroid film type 667 was used.

2.7.4 Recovery of DNA from gels

DNA was recovered from agarose and polyacrylamide gels by electroelution according to McDonnell *et al.* (1977). The required band of DNA was cut out of the gel with a new razor blade and placed into a section of dialysis tubing sealed at both ends with a mediclip and containing 1 ml of filtered, sterile electroelution buffer (5 mM Tris-HCl pH 8, 2.5 mM acetic acid). The same buffer was used for electrophoresis (it was allowed to contact the bag but not cover it) and the DNA

electroeluted at 15 V cm^{-1}. After 2 h the current was reversed for 1 min to remove any DNA stuck to the dialysis tubing. The DNA was then precipitated with ethanol and the pellet redissolved in 20 μl TE.

2.8 POLYMERASE CHAIN REACTION (PCR) PROTOCOLS

2.8.1 Synthesis of single-stranded cDNA

Leaf polyA$^+$ RNA (2 μg) and 0.4 μg oligo dT_{12-18} primers were mixed in a final volume of 17 μl, denatured at 65°C for 10 min and annealed at room temperature for 10 min. The cDNA synthesis reaction was set up containing the annealed RNA-primers, 0.2 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 50 mM Tris-HCl pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 5 mM DTT, 1 μCi [^{32}P]-dATP, 1.25 U RNasin (Amersham), and 30 U AMV reverse transcriptase (N.B.L. Life Sciences). This was incubated at 42°C for 60 min, following which 2 μl were removed for scintillation counting and the remainder ethanol precipitated and resuspended in 50 μl TE. The 2 μl reserved for scintillation counting was spotted onto a Whatman DE81 filter, washed 6 times with 0.5 M Na_2HPO_4, twice with SDW and twice with 95% (v/v) ethanol. The filter was then dried, placed in Cocktail 'O' and counted.

2.8.2 Oligonucleotide preparation

Oligonucleotides B (5'CAC ATT GCT GCA GAT GGA GAA G3') and C (5'ACA TAT GGA TCC ATG TTA GAA GCA G3') were designed from two highly conserved regions of plant GS genes, that flank intron 11. For cloning purposes, oligonucleotide B was designed to contain a *Pst*1 site while

oligonucleotide C was designed to contain a *Bam*H1 site. The oligonucleotides were purified by a modified procedure of Sawadogo and Van Dyke (1991) whereby 85 μl of NH_4OH (35% NH_3) was vortexed for 15 s with 25 μl oligonucleotide solution and 1.1 ml butan-1-ol. After centrifugation at 15000 x g for 1 min the pellet was resuspended in 100 μl SDW and a further 1 ml butan-1-ol added, vortexed, and spun. The pellet was finally redissolved in 100 μl SDW and the concentration adjusted to 5 $\mu g.\mu l^{-1}$.

2.8.3 'Hot start' PCR

The PCR reaction was set up using the GeneAmp PCR kit (Perkin Elmer Cetus) whereby 0.6 $pmol.\mu l^{-1}$ of each primer, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM TTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin were added to the target DNA in a final volume of 50 μl. This was overlaid with 50 μl mineral oil and heated to 94°C for 2 min prior to addition of 1 U AmpliTaq DNA polymerase (Perkin Elmer Cetus). The annealing and elongation temperatures and the number of cycles for each amplification are stated in the results Chapter. The most suitable annealing temperatures were calculated by using the equations published by Breslauer *et al.* (1986).

Upon completion 100 μl chloroform was added to remove the mineral oil and the aqueous phase recovered. The PCR product was ethanol precipitated and resuspended in 10 μl TE.

2.9 CLONING OF PCR PRODUCTS AND RECOMBINANT SELECTION BY COLONY HYBRIDIZATION

The procedures used were from Sambrook *et al.* (1989).

2.9.1 Ligation

The products of the PCR reaction were digested with *Pst*1 (Gibco BRL) and *Bam*H1 (Boehringer Mannheim), and the DNA fragments purified using the Prep-A-Gene kit (Chapter 2.7.1) and ligated in 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5% (w/v) polyethylene glycol 8000, 1 mM ATP, 1 mM DTT, 25 ng pUC9 (cut with *Pst*1 and *Bam*H1), 25 ng target DNA, and 1 U T4 DNA ligase (Pharmacia) in a total volume of 5 μl. The reaction was incubated overnight at 14°C, 45 μl of TE was added and it was stored at -20°C.

2.9.2 Transformation

Aliquots (1 μl and 5 μl) of the ligated DNA were diluted to 25 μl. The vector pUC9 (50 pg) was also diluted to 25 μl and used a negative control. 50 μl aliquots of competent *E. coli* cells (from J. Boxall) were transferred to the diluted ligation mix and this was placed on ice for 30 min. The samples were then heat-shocked for 5 min at 37°C and pipetted into 2% Bacto-tryptone, 1% yeast extract, 0.1% NaCl, 0.02% glucose, pH 7, and incubated at 37°C with shaking for 1 h. Following centrifugation at 3200 x g for 2.5 min, the pellets were resuspended in 50 μl 0.5% NaCl, 0.5% yeast extract, pH 7.4. The transformation products were spread onto 1.5% L-agar plates (1% NaCl, 0.5% yeast extract, 1% Bacto-tryptone, 100 $\mu g.ml^{-1}$ ampicillin, pH 7.4) with 50 μl of 2 % X-gal spread on top. This was incubated overnight at 37°C.

2.9.3 Colony transfer and liberation of bound DNA

Four nylon Hybond-N (Amersham) membranes were placed onto four L-agar plates (see 2.9.2 transformation) and 100 white recombinant colonies picked onto each filter and simultaneously onto a master plate. A blue colony containing non-recombinant plasmid was used as a negative control. Plates were incubated at 37°C overnight. The master plate was stored at 4°C and the filters treated to liberate the DNA from the colonies by treatment twice with 0.5 M NaOH for 2-3 min, twice with 1 M Tris-HCl (pH 7.4) for 5 min and once with 0.75 ml 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4) for 2-3 min (Grunstein and Hogness, 1975). Filters were then baked at 80°C for 2 h, and stored in 2 x SSC at 4°C.

2.9.4 Colony hybridization

The baked membranes were prehybridized for 3 h at 65°C in hybridization buffer (50 mM Na phosphate buffer, pH 6.5, 5 x SSC, 10 0.2% SDS, 250 $\mu g.ml^{-1}$ herring sperm DNA and 5 x Denhardt's solution containing 0.2% w/v, 0.2% Ficoll, 2% polyvinylpyrrolidone. Hybridization was at 65°C overnight in fresh buffer containing labelled probe (see Chapter 2.9.5). The hybridized membranes were washed for 1 h in 5 mM Na phosphate buffer, pH 7, 1 mM EDTA and 0.2% SDS at 50°C. The membrane was autoradiographed at -75°C between two intensifying screens at for 2-7 d.

2.9.5 Radioactive labelling of probe DNA

DNA fragments from a PCR reaction using *L. corniculatus* total DNA as template, were separated by gel electrophoresis and electro-eluted. The four fragments corresponding to the 200 bp, 300 bp, 500 bp and 600 bp products (see Chapter 4) were radiolabelled with ^{32}P using the nick translation kit supplied by Amersham International. A reaction was set up containing 0.1 μg target DNA, 1 nmol dCTP, 1 nmol dTTP, 1 nmol dGTP, and 1.5 U of DNA polymerase I in a total volume of 30 μl. This was added to 20 μCi [α-^{32}P]-dATP (Amersham) at 1 x 10^8 cpm.μg^{-1} DNA specific activity, and incubated at 15°C for 2-3 h, after which the labelled DNA was separated from unincorporated radioactivity by Sephadex G50 chromatography.

The specific activity of probes was estimated by the Cerenkov method. A 1 μl aliquot in a 1.5 ml Eppendorf tube was placed inside a glass scintillation vial and counted in a Kontron BETAmatic II liquid scintillation counter set for counting tritium. The specific activity, expressed as Ci.mmole^{-1} DNA, assumed an approximate efficiency of 30% counting.

2.9.6 'Colony' PCR

A cocktail stick was used to transfer a bacterial colony to 50 μl of colony lysis buffer (1% Triton X-100, 20 mM Tris-HCl pH 8.5, 2 mM EDTA) and the mixture was heated to 95°C for 10 min. Following centrifugation for 2 min at 3200 x g, 2.5 μl of the supernatant was added to PCR reagents (2.8.3)

2.10 DNA SEQUENCING

DNA sequencing was by the dideoxy method (Sanger, 1977) using the Sequenase Kit (USB), following the suppliers' protocol.

2.10.1 Sequencing reactions

Annealing double-stranded template and primer - Recombinant plasmid (4 μg) was denatured at room temperature for 5 min in 0.18 M NaOH, then ethanol precipitated. Universal primer (0.5 pmole) in 1μl SDW was added to rehydrate the DNA, followed by 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl, to give a final volume of 10 μl. This was heated for 2 min at 65°C, allowed to cool slowly to room temperature, then stored on ice.

Labelling reaction - The annealed template-primer was increased to 22 μl with 10 mM DTT, 1.5 μM dGTP, 1.5 μM dCTP, 1.5 μM TTP, 0.55 μCi [α-^{35}S]-dATP at 500Ci.mmol^{-1}, and 6.5 U Sequenase T7 DNA polymerase (USB), and incubated at room temperature for 2-5 min.

Termination reaction - Aliquots (3.5 μl) of the completed labelling reaction were transferred to the four termination solutions each containing 33.3 μM dGTP, 33.3 μM dATP, 33.3 μM dCTP, and 33.3 μM TTP, and either ddGTP, ddATP, ddCTP or ddTTP at 3.3 μM. The reactions were incubated at 37°C for 2-5 min, then quenched with 4 μl Stop solution (95% (v/v) formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The four reactions were stored at -20°C if necessary, and boiled before loading.

2.10.2 Sequencing gel

A 6% acrylamide gel was made up containing 1 x TBE and 8 M urea, and pre-run in 1.5 x TBE at up to 2000 V until the temperature of the gel had reached 50°C. The four sequencing reactions were loaded and the gel run to keep the temperature at 50°C (about 50 W) for 90 min. The gel was fixed in 10% (v/v) methanol and 10% (v/v) acetic acid, vacuum dried at 60°C, and autoradiographed.

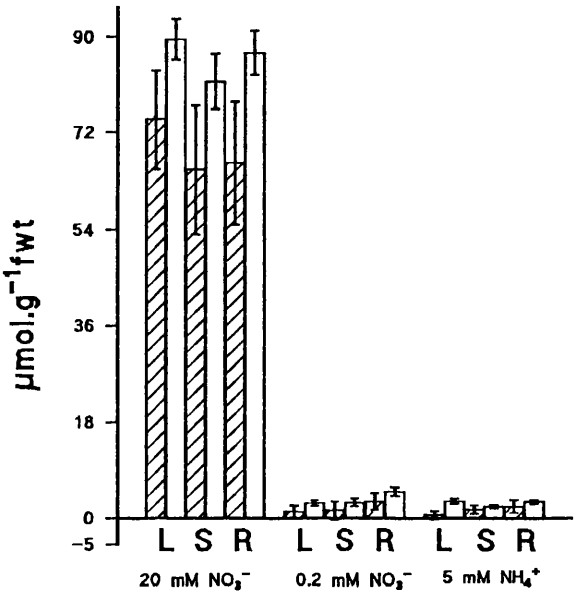
FIGURE 2.1 **Comparison of the cadmium reductor method and the *Aspergillus* NR method of nitrate determination**

A) Concentration of NO_3^- in the leaf (L), stem (S) or root (R) of *L. japonicus* grown on either 20 mM NO_3^- 0.2 mM NO_3^- or 5 mM NH_4^+, measured by the cadmium reductor method (///) or the *Aspergillus* NR method (□) and detected colorimetrically.

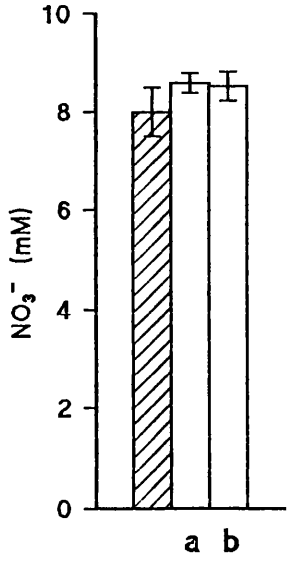
B) The NO_3^- concentration in an 8.5 mM NO_3^- solution measured by the cadmium reductor method (///) or the *Aspergillus* NR method (□) detected colorimetrically (a) or by following the depletion of NADPH (b).

FIGURE 2.1 **A Comparison of methods for measuring nitrate concentration**

A) Tissue NO_3^- measurements using two different assays



B) 8 mM NO_3^- assayed by three different methods



CHAPTER 3: GS ISOFORMS IN *L. JAPONICUS* AND *L. CORNICULATUS*

3.1. INTRODUCTION

3.1.1 GS activity and subunit composition

Total GS activities vary enormously between species, although in general, the shoot activity exceeds that of the root due to the role of GS2 in re-assimilating the large amounts of NH_4^+ evolved from photorespiration. For example, the shoot and root GS activities in the legume, *Trifolium repens*, collected from the field, were 96 μmol.h^{-1}.g^{-1}fwt, and 4 μmol.h^{-1}.g^{-1}fwt respectively, and in field-grown *L. corniculatus* activities were 54 and 14 μmol.h^{-1}.g^{-1}fwt respectively (Pearson, Woodall and Havill, in preparation).

Evidence from a wide variety of legume and non-legume species shows that GS2 subunits are approximately 43-45 kD in molecular weight, whereas the GS1 subunits are 37-43 kD (Forde and Cullimore, 1989; Pearson Woodall and Havill, in preparation). Shoots normally contain either just GS2 or both GS1 and GS2 subunits when separated by SDS-PAGE or column chromatography. When both isoforms are present the GS2 usually predominates, although GS1 activity and protein have been shown to become proportionally greater in deciduous species towards the end of the year (Pearson and Ji, 1994). Nitrogen-fixing nodules have been shown to express all of the GS gene products, including GS2, and in some cases a nodule-specific or nodule-enhanced cytosolic GS in addition to the root GS1 isoform (Forde and Cullimore 1989). Most plant roots express only GS1, although in the temperate legumes, *Pisum sativum*, *Medicago media* and *Vicia*

faba, and the cereals, *Zea mays* and *Hordeum vulgare* a GS2-like protein has also been found (Miflin, 1974; Emes and Fowler, 1979; Barratt, 1980; Vézina *et al.*,1987; Sakakibara *et al.*, 1992b). In *P. sativum*, *M. varia* and *Z. mays*, this root GS2-like protein has been confirmed to be plastidic (Vézina and Langlois, 1989; Sakakibara *et al.*, 1992b). In this chapter, the isoforms of GS in different tissues of *L. corniculatus* and *L. japonicus* were analyzed by one- and two-dimensional electrophoresis. Upon discovery of a root GS2-like isoform, compartmentation of the root isoforms was analyzed by obtaining plastid-enhanced extracts. The possibility of GS glycosylation was also investigated.

3.1.2 Glycosylation and GS

The carbohydrate moieties of glycoproteins are generally attached via the hydroxyl groups of serine or threonine residues (*O*-linked glycans), or the amide N of asparagine sidechains (*N*-linked glycans). *O*-glycans do not appear to require unique amino acid sequences, whereas *N*-glycans, which are widespread in plant glycoproteins, seem to occur at Asn-X-Ser or Asn-X-Thr sites, where X can be any amino acid except proline (Thomas and Creighton, 1983). These sequences are not the only determining factors as some proteins possess one or more of these potential sites but are not glycosylated (e.g. elastase), whereas other proteins may occur in both glycosylated and unglycosylated form (e.g. bovine pancreatic ribonuclease). Also, proteins that are fully glycosylated at one site may possess additional non-glycosylated sites (e.g. ovalbumin and deoxyribonuclease) (See Thomas and Creighton, 1983, for further information).

Nato *et al.* (1984) found evidence for glycosylation in *Nicotiana tabacum*, but Ericson (1985), Tingey and Coruzzi (1987) and Tingey *et al.* (1987) who all used different methods to Nato *et al.*, could not find any evidence for the glycosylation of GS in *P. sativum*, *N. plumbaginifolia* or *Spinacea oleracea* (see Chapter 1.3.9 for further details).

All known plant GS1 and GS2 amino acid sequences were compared and found to contain highly conserved Asn-X-Ser/Thr sites (see Table 3.1). The GS1 sequences had these conserved amino acid sequences at sites 11, 185 and 251 residues from the N-terminus (including a GS1 sequence of *L. japonicus*). The GS2 sequences had conserved sites at residues 185, 271 and 272 from the N-terminus of the mature peptide (i.e. following removal of the *ca* 17 amino acid transit peptide). It was thought that these potential *N*-glycosylation sites in different degrees of glycosylation may account for the charge variants seen in IEF separations. Consequently, *L. japonicus* GS was analyzed for carbohydrate content.

3.1.3 Methods for detection of glycosylation

There are several methods for detecting glycosylation. These methods can be divided into three categories, thymol-H_2SO_4 based, periodic acid-Schiff (PAS), and lectin-based methods (Gander, 1984). The PAS method employed by Nato *et al.* (1984) to detect glycan residues on GS2, uses periodic acid to oxidize and cleave the secondary alcohols of glycosyl residues to dialdehydes which are allowed to react with dansyl hydrazine to form a Schiff base (Gander, 1984). This allows detection of all glycan residues, but the PAS method is not very sensitive, its limits of detection being 40 ng of sugar residue. The thymol-H_2SO_4 is even less

sensitive, detecting only 50 ng glycoprotein. The method employed by Tingey and Coruzzi (1987) and Tingey *et al.* (1987), whereby blotted proteins were stained by reaction with lectins linked to horseradish peroxidase is much more sensitive, but the specificity of the binding can be a disadvantage and knowledge of the sugar residues is often required. Wheat germ agglutinin and Concanavalin A, the two lectins used in these papers, recognize several monosaccharides (e.g. α-D-glucose, α-D-mannose, ß-D-acetylglucosamine) but their ability to recognize these residues in complex oligosaccharides will vary. Also, detection of glycans by lectins presupposes that the recognised binding sites are available and unaltered, which may not be the case with blotted proteins.

3.1.4 The role of light in regulation of GS2

Light is known to dramatically increase synthesis of GS2 in the leaves of many species, including *Pisum sativum* (Edwards and Coruzzi, 1989) and *Phaseolus vulgaris* (Cock *et al.*, 1991). It was thought a possibility that light, penetrating the growth medium, might be responsible for inducing expression of the GS2-like isoform in the roots of these species. Light, passing through soil, is known to have a direct effect on root gene expression, for example, in inhibiting expression of dark-induced asparagine synthetase in roots (Tsai and Coruzzi, 1990), although the degree to which light penetrates soil varies greatly according to soil type (Mandoli *et al.*, 1990). The possibility of the root GS2-like protein being induced by light penetrating the growth medium was also investigated, using the light-inducible RuBisCO protein as a marker for light induction.

3.2 RESULTS

3.2.1 GS activity measurement in *L. japonicus* and *L. corniculatus*

There are three different types of GS assay, the biosynthetic (ATP + L-Glu + NH_3 ---> L-Gln + ADP + Pi), the semi-biosynthetic (ATP + L-Glu + NH_2OH ---> y-glutamylhydroxymate + ADP + Pi) and the transferase (L-Gln + NH_2OH ---> y-glutamylhydroxymate + NH_3). The biosynthetic and semi-biosynthetic assays are both synthetase reactions, proceeding in the same direction as believed to occur *in vivo* and thought to closely reflect endogenous activities. The transferase assay, which proceeds in the opposite direction, may produce activities several times higher than the other two methods, but lacks any obvious physiological significance. The semi-biosynthetic method, which is quick and easy to perform, was adopted for this thesis.

Total GS activities in both species varied widely depending on growth conditions. In *L. japonicus* the leaf activities were from 50 to 130 $\mu mol.h^{-1}.g^{-1}$ fwt, the stem activities were from 35 to 87 $\mu mol.h^{-1}.g^{-1}$ fwt for the stem and from 20 to 60 $\mu mol.h^{-1}.g^{-1}$ fwt for the root. The activities in tissues of *L. corniculatus* were lower than those in *L. japonicus* (30-60 $\mu mol.h^{-1}.g^{-1}$ fwt, 15-40 $\mu mol.h^{-1}.g^{-1}$ fwt and 15-30 $\mu mol.h^{-1}.g^{-1}$ fwt for the leaf, stem and root respectively). More detailed measurements are given in subsequent chapters.

3.2.2 GS isoforms in *L. japonicus* and *L. corniculatus*

The GS subunits in *L. japonicus* and *L. corniculatus* leaf, stem and root, were detected on western blots by immunostaining using polyclonal antiserum raised against *Phaseolus vulgaris* nodule GS (Cullimore *et al.*, 1983). This

antiserum is known to react with GS1 and GS2 subunits of both angiosperms and gymnosperms (Freeman *et al.*, 1990; Pearson, Woodall and Havill, in preparation; Vézina and Margolis, 1990).

Polypeptides of approximately 39 kD and a 45 kD which cross-reacted with anti-GS antibodies were detected in extracts of leaf, stem and root of NO_3^--grown *L. corniculatus* and *L. japonicus* (Figure 3.1A and C). The relative proportions of the two polypeptides differed however in the three organs, with the leaf having the 45 kD polypeptide in greater amounts and roots the 39 kD polypeptide. Based on their molecular weights and the prevalence of the larger polypeptide in leaves, the 39 kD and 45 kD polypeptides are likely to correspond to subunits of GS1 and GS2 respectively. Both were also found in nodules of *L. corniculatus* (not shown). A cross-reacting 50 kD polypeptide was also apparent in leaf and stem extracts, and was identified as the large subunit of ribulose bisphosphate carboxylase-oxygenase (RuBisCO) by immunostaining another blot with antibodies raised against wheat RuBisCO (Figure 3.1B). The detection of RuBisCO by anti-GS antiserum has been noted by other authors (Canovas *et al.*, 1991; Elmlinger and Mohr, 1992), but no pre-immune serum was available to determine whether the cross-reaction was due to the anti-GS antibodies or to pre-existing antibodies in the rabbit serum.

3.2.3 Two-dimensional electrophoretic analysis of GS

Two-dimensional separations of GS isoforms revealed that GS1 in extracts of root, shoot and nodule of *L. corniculatus* was resolved into three major and one minor charge variants, whilst there were three charge variants of GS2 in the

nodule, with an extra one or two in the root and leaf at the more acidic end of the IEF gel (Figure 3.2). Both species had the same number of GS charge variants in the leaf; four (possibly five) GS2 variants and four GS1 variants (Figure 3.2A and D) with the variants of both isoform-types at the more acidic end of the IEF gel being less abundant than those at the basic end. Root extracts of *L. corniculatus* had identical charge variants of both GS1 and GS2 to the leaf extracts, although the three GS1 variants at the basic end of the gel were much more abundant in the root. The pattern was identical in *L. corniculatus* nodule extracts except that there were lower levels of the GS2 variants, with the spot nearest the acidic end of the gel being barely detectable. In the *L. japonicus* root extracts only three GS1 spots and three GS2 variants were detectable (Figure 3.2B and D). This may have been due to the very high levels of the two GS1 variants nearest the basic end.

3.2.4 Are GS2 or GS1 glycosylated?

Although the number of GS genes in either species is not known it was thought that at least some of the GS2 isoelectric variants, and possibly the GS1 variants, might be due to glycosylation. The question of glycosylation was investigated in two ways; by staining for glycoproteins and by enzymatic removal of *N*-glycans from protein extracts (to see if this altered the number or mobility of the GS charge variants).

N-glycosidase was used to remove the *N*-linked carbohydrates enzymatically from *L. japonicus* protein extracts to see if this reduced the number of GS2 spots apparent on 2-D gels. *N*-glycosidase hydrolyzes the Asn-glycan link and has the advantage over other glycosidases of retaining activity in the presence of SDS and

other detergents, thereby allowing denaturation of proteins to expose the substrate and increasing the deglycosylation rate considerably. Commercial preparations of *N*-glycosidase F also contain endo-ß-*N*-acetylglucosaminidase F activity, a combination which deglycosylates a wide variety of high mannose and complex oligosaccharides glycoproteins (Plummer *et al.*, 1984). However, O-linked glycoproteins are not digested by this enzyme, so it was decided to stain western blots for non-specific glycosylation both before and after *N*-glycosidase F treatment.

Separations by SDS-PAGE of increasing amounts of *L. japonicus* leaf protein were stained for carbohydrate using the GlycoTrack glycosylation system (Figure 3.3). The GlycoTrack system was used because it combined the PAS method of oxidizing all categories of glycan through reaction with hydrazide. Sensitivity is increased through the hydrazide being linked to biotin, facilitating detection of the glycan residues with a streptavidin-alkaline phosphatase conjugate. Several dark bands of positive staining were apparent (Figure 3.3A and D), but two of these putative glycoproteins were shown to be artefacts by the control blot (Figure 3.3B). The area corresponding to the RuBisCO large subunit was entirely unstained, therefore not glycosylated, but the area corresponding to GS protein (between the RuBisCO and the 39.8 kD marker) contained both stained and unstained regions. Even when blots were immunostained for GS on top of the glycosylation stain (Figure 3.3C), it was still not obvious whether the GS polypeptides coincided with bands that stained for carbohydrate.

To help clarify the matter, 2-D gels were run with larger amounts of total protein (12 μg as opposed to 2 μg), then stained for glycosylation (Figure 3.4A),

and overstained for GS (Figure 3.4C). An aliquot of the protein extract was digested with *N*-glycosidase F prior to 2-D electrophoresis, then also stained for glycans (Figure 3.4B), and overstained for GS (Figure 3.4D). Four or five individual spots of glycosylated protein can be seen in the region on blot A, corresponding to the more acidic GS charge variants on blot C, but even though these spots disappeared when digested with *N*-glycosidase F (blot B) indicating removal of the carbohydrate moieties, the GS charge variants remained unaltered (blot D).

3.2.5 Plastid Separation

To determine whether the GS2-like 45 kD protein detected in root extracts was indeed plastidic, two methods were used to try and obtain plastid-enriched fractions of *L. japonicus* roots. Initially, the method used by Vézina and Langlois (1989) was adopted whereby a root homogenate was centrifuged through a discontinuous sucrose-gradient, and different layers assayed for the marker enzymes, cytosolic NR and plastidic NiR as well as for GS activity (Table 3.2). This procedure met with little success as the fractions that contained high NiR activity (plastidic marker) also had high NR activity (cytosolic). It was encouraging to note, however, that the fraction with the highest NiR activity (fraction 4) also contained the highest GS activity and, upon blotting, this fraction was seen to contain not only 39 kD subunit but also a 45 kD polypeptide (Figure 3.5A, lane 4).

More success was achieved using the method of Bowsher *et al.* (1989), where the sample was centrifuged through a cushion of 10% Percoll, and the

plastid-enriched pellet assayed for the cytosolic enzyme, alcohol dehydrogenase (ADH) and the plastidic enzyme, hexose phosphate isomerase (HPI). The plastids were tested for degree of intactness (latency) by the activity of HPI both before and after the addition of detergent. The plastid latency was 51.5%, with the HPI activity being 573 $\mu mol.h^{-1}.mg^{-1}$ after rupturing the plastids. Although the ADH activity in the crude extract (following the initial 200 x g centrifugation step) was 0.75 $\mu mol.h^{-1}.mg^{-1}$ protein, no ADH activity was detected in the plastid fraction. GS activity in the plastid fraction (100 $\mu mol.h^{-1}.mg^{-1}$ protein) was more than 30-fold higher than that in the crude extract (3 $\mu mol.h^{-1}.mg^{-1}$ protein), and when western blotted, the 45 kD polypeptide, which was undetectable in the crude extract, was present in the plastid-enriched fraction (Figure 3.5B). A 39 kD band was detected in both the crude extract and the plastid fraction.

3.2.6 The role of light in the induction of root GS2

It had been observed that the large subunit of RuBisCO, as well as GS1 and GS2, could be detected in the roots of *L. japonicus*, especially when grown in sterile-culture medium (see Figure 3.1B). This indicated that light, penetrating the rooting medium, may indeed affect gene expression.

In one experiment, *L. japonicus* seeds were germinated in the light and then transferred to transparent pots containing vermiculite. The roots of one set of plants were masked by foil and black beads whilst the other set were left exposed to the light. These plants were supplied with 10 mM NO_3^- and placed in a growth chamber with a 20°C/15°C, 16/8 h day/night regime. The 16 h day employed in the growth chamber during this experiment was calculated to be sufficient to

saturate the GS2 phytochrome response in leaves, as the fluence rate of 300 μE.m^{-2}.s^{-1} would require only 11.3 h for a 10% photoconversion of phytochrome (Kuhlemeier *et al.*, 1987; Mandoli *et al.*, 1990).

In plants that had their roots exposed to light, two polypeptides of approximately 50 and 55 kD could be detected on blots probed with RuBisCO antibody, with the smaller protein predominating (lane 1, Figure 3.6A). In roots masked from light the 55 kD polypeptide was present at the same staining intensity as the exposed roots, but the 50 kD polypeptide was barely visible (lane 3, Figure 3.6A). There was no difference in expression of either of the two GS isoforms (Lanes 3 and 4, Figure 3.6A).

In another experiment, capillary matting was placed over a tray of vermiculite to exclude all light. Seeds of *L. japonicus* were germinated in the dark at 20°C within small holes made in the capillary matting, and not brought into light until the seedlings had established themselves. The plants were then grown under the same day/night regime as above, and supplied with 5 mM NO_3^-. In this way the roots were masked completely from light during germination and into maturity. In these plants GS2 was still present (lane 7, Figure 3.6B), although the RuBisCO was no longer detectable (lane 4, Figure 3.6B).

There are two possible reasons why the antibody raised against wheat RuBisCO recognized two polypeptides: either the RuBisCO large subunit was subject to proteolytic degradation; or the antibody also recognised the RuBisCO large-subunit binding protein (M. Parry, personal communication). Both polypeptides are similar in size (50-60 kD), and both have been detected at low levels in etiolated leaves of *P. sativum*. However upon illumination the RuBisCO

protein is induced to a much greater degree than the binding protein (Musgrove and Ellis, 1986; Lennox and Ellis, 1986). This suggests that the 50 kD protein detected in *L. japonicus* root extracts was RubisCO, amounts of which were much higher in the light-grown roots, and that the 55 kD polypeptide which was largely unaffected by light, was the binding protein.

3.3 DISCUSSION

3.3.1 *Lotus* roots contain a GS2-like polypeptide

The GS polypeptides in both species were of a similar size to those found in other higher plants (Forde and Cullimore, 1989), and the predomination of GS2 in the leaf was to be expected to enable assimilation of the possible high levels of photorespiratory NH_4^+ in these two C3 species (McNally *et al.*, 1983). Although the GS1 polypeptide predominated in the root, there was also a large amount of a GS2-like protein in that organ in plants grown on NO_3^--containing media as had been noted to occur in other temperate legumes (Barratt, 1980; Emes and Fowler, 1983; Vézina *et al.*, 1987).

3.3.2 Two-dimensional electrophoresis reveals several isoelectric variants of GS1 and GS2

The isoelectric focusing of GS subunits in the leaf of the two species grown in culture (on a medium containing 8 mM NO_3^- and 5 mM NH_4^+) produced a similar pattern of variants: four GS1 and four GS2 variants in leaf extracts, and the same number in the root of *L. corniculatus*, but probably only three of each subunit-type in *L. japonicus* root extracts. This agrees well with the number of GS

isoelectric variants found in other legumes, e.g. four GS2 and three GS1 variants in *P. vulgaris* (Lara *et al.*, 1984). The fewer GS charge variants in *L. japonicus* roots than in *L. corniculatus* roots did not appear to be the result of proportionally less being loaded onto the gel, but seemed to reflect genuine differences between the two species. It may be relevant that *L. japonicus* was much more difficult to grow in culture than *L. corniculatus*, and when grown in pots in a growth chamber, four GS1 and at least three GS2 charge variants could be resolved in the *L. japonicus* root (see Chapter 5). The resolution of the major GS IEF variants at the basic end of the charge gradient in both *Lotus* species is compatible with the finding that upon the addition of pea leaf pre-GS2 protein to isolated chloroplasts, the cleaved peptide co-migrates with the more basic of the IEF variants (Tingey *et al.*, 1988).

The two-dimensional separation of *L. corniculatus* nodule extracts indicated that this species does not contain a nodule-specific polypeptide, but that it employs all of the GS subunit-types that are found in the root, including the GS2 polypeptide, to assimilate the NH_4^+ derived from N_2-fixation. This is similar to the situation in *P. sativum* (Tingey *et al.*, 1987; Walker and Coruzzi, 1989), but contrasts with *P. vulgaris*, *M. sativa* and *Lupinus luteus* which have been found to contain a 'nodule-specific' subunit-type (Lara *et al.*, 1983; Dunn *et al.*, 1988; Konieczny *et al.*, 1988).

3.3.3 Why are there so many isoelectric variants of GS?

The different charge variants were shown to not be due to differential glycosylation since an enzymatic treatment that removed the sugar moieties of *N*-

linked glycoproteins did not alter the number or the mobilities of the GS charge variants on 2-D gels (Figure 3.4). This does not agree with Nato *et al.* (1984) who found evidence for glycosylation in *Nicotiana tabacum*, but is in accordance with the findings of Ericson (1985), Tingey and Coruzzi (1987) and Tingey *et al.* (1987), who found no evidence for the glycosylation of GS in *P. sativum*, *N. plumbaginifolia* or *S. oleracea*.

The GS1 charge variants in *L. corniculatus* and *L. japonicus* may all prove to be products of different genes, as has been found in *P. vulgaris* GS1 (Lara *et al.*, 1984) and *P. sativum* (Tingey *et al.*, 1987). However, this is unlikely to be the explanation for the GS2 variants, where only one gene has ever been identified in several species. The GS2 variants may therefore be post-extraction artefacts or due to a different type of post-translational modification. Four GS2 isoelectric forms have been found in other plants, extracted by different methods and in different laboratories, e.g. *P. vulgaris* (Lara *et al.*, 1984) and *N. plumbaginifolia* (Hirel *et al.*, 1984; Nato *et al.*, 1984). Although this does not rule out extraction artefacts it makes it a less likely explanation. Phosphorylation of plant GS has not been investigated, and may prove worthwhile, especially as NR activity has been shown to be rapidly modulated by phosphorylation and dephosphorylation (Kaiser and Spill, 1991; Kaiser *et al.*, 1992; Glaab and Kaiser, 1993). Another likely explanation that remains to be examined is the presence of more than one cleavage site used by the processing peptidase when GS2 in transported into the chloroplast. Robinson and Ellis (1984) showed this to be the case for the RuBisCO small subunit by incorporating amino acid analogues into the precursor protein during translation in a cell-free system and testing the products for import by isolated

chloroplasts and processing by the purified stromal processing enzyme, an approach that could also be applied to GS2.

3.3.4 Is the root GS2-like polypeptide located in plastids?

The occurrence of a putative root GS2 has been noted in other temperate legumes (Barratt, 1980; Emes and Fowler, 1983; Vézina *et al.*, 1987), and although it was shown to be plastidic in *P. sativum* and *M. media* (Emes and Fowler, 1983; Vézina and Langlois, 1989), it was necessary to confirm the plastidic nature of the GS2-like protein in *L. japonicus*.

It proved difficult to obtain a plastid-enhanced fraction of *L. japonicus* roots. Bowsher *et al.* (1989) obtained preparations of pea root plastids that were greater than 75% intact based on the latency of hexose phosphate isomerase. However extraction of plastids is very species-dependent, with starch-rich tissues perhaps only achieving 30% latency (M. Emes, personal communication). The method of Bowsher *et al.* (1989) applied to *L. japonicus* roots, produced a plastid fraction that was 52% intact.

The plastid-enriched fraction had a greater than 30-fold increase in GS activity compared to the crude extract, but both a 39 kD and a 45 kD polypeptide were found in SDS-PAGE separations. It was puzzling that the 45 kD subunit was not detected in the crude extract in these experiments. This may be due to the inefficient nature of the extraction technique used for plastid purification, whereby the roots are finely chopped and the plastids allowed to gently migrate into the bathing medium. It is likely that the plastids are present in too low a number in this crude extract for detection of any GS2.

Polypeptides of approximately 39 kD and 41 kD were also found in plastid-enriched fractions of *P. sativum* roots by Vézina and Langlois (1989). As there is no evidence to support a plastidic location for GS1, the presence of a 39 kD protein in the plastid fraction may have been due either to cytosolic contamination of the plastid fraction, or to the binding of GS1 to the cytosolic side of the plastid membrane. The lack of alcohol dehydrogenase activity in the plastid fraction suggested very little cytosolic contamination, and although the presence of other organelles was not assayed for, there is no evidence for GS isoforms in other cell compartments. This would be clarified by using marker enzymes for the other organelles. It would also be interesting to digest intact and ruptured plastids with a protease, to see if the intact plastids offered any protection to the 39 kD polypeptide.

The occurrence of the plastid GS protein in the roots of temperate leguminous species, including *L. corniculatus* and *L. japonicus*, will be discussed in Chapter 6.

3.3.5 The GS2 polypeptide in *L. japonicus* roots is not light regulated

Expression of the GS2 subunit in roots of *L. japonicus* was entirely unaffected by light (Figure 3.6). In contrast, the large subunit of RuBisCO, another plastid protein, was light-inducible in roots under the same conditions. This was surprising given that light, acting directly through phytochrome, is a major factor enhancing GS2 in green tissues (Schmidt and Mohr, 1989; Becker *et al.*, 1992), although the light-inducibility of GS2 in *L. japonicus* leaves was not

specifically studied. There are several precedents for the light-independent expression of this isoform. The GS2 in *Z. mays* is light-inducible in the bundle-sheath cells which contain the photorespiratory apparatus, but is not affected by light in the mesophyll cells where the enzyme is induced by NO_3^- (Sakakibara *et al.*, 1992a). Gymnosperms can develop green plastids in the dark, and there is evidence to show that the appearance of GS2 in some gymnosperms is independent of light (Possingham, 1980; Canovas *et al.*, 1991). The GS2 protein in *P. vulgaris* can accumulate in dark-grown leaves, demonstrating activities only 25-35% less than those of light-grown leaves (Cock *et al.*, 1991). It appears likely, therefore, that light-independent factors, possibly through the development or maturation of root plastids, are the major influences on GS2 expression in *L. japonicus* roots. Nitrate has been implicated in the induction of root plastidic GS2, and this factor will be addressed in Chapter 5.

TABLE 3.1 ASN-X-SER AND ASN-X-THR MOTIFS IN GS cDNA SEQUENCES

GS1 TYPE

GENE	+11	+185	+251	Ref.
AtGSr1	+1---[]	[]	[]	1
AtGSr2	+1---[]	[]	[]	1
AtGSkb6	+1---[]	[]	[]	1
GmGS1	+1---[]	[]	[]	2
LsGS1	+1	[]	[]--------[]-	4
LjGSA9	+1---[]	[]	[]	5
LaGSn	+1---[]	[]	[]	6
MsGS1	+1	[]	[]	7
NpGS1	+1---[]	[]	[]------[]----	8
OsGS1root	+1---[]	[]	[]	10
OsGS1leaf	+1[]-[]	[]	[]	10
PvGS1PR1	+1---[]	[]	[]	11
PvGS1PR2	+1---[]	[]	[]	11
PsGS299	+1	[]	[]	13
PsGS341	+1---[]	[]	[]	13
ZmGS1	+1---[]	[]	[]	14
PsyGS1	+1----------[]	[]	[]	15

GS2 TYPE

	+185	+251	+272	
AtGSL1	**********+1-[]	[]	[]---[]-------*******	1
HvGS2	**********+1	[]	[]---[]-------*******	3
NtGS2	**********+1-[]	[]	[]---[]-------*******	9
OsGS2	**********+1	[]	[]---[]-------*******	10
PvGS2	**********+1	[]	[]---[]-------*******	12
PsGS185	**********+1	[]	[]---[]-------*******	13
SoGS2		--------[]	[]---[]-------*******	10
ZmGS2	**********+1	[]	[]---[]-------*******	14

[] = Asn-X-Ser and Asn-X-Thr sequences. The position downstream from +1 for the conserved sites are shown

**** = N and C terminal extensions

Abbreviations: At, Arabidopsis thaliana; Gm, Glycine max; Hv, Hordeum vulgare; Ls, Lactuca sativa; Lj, Lotus japonicus; La, Lupinus angustifolius; Ms, Medicago sativa; Np, Nicotiana plumbaginifolia; Nt, Nicotiana tabacum; Os, Oryza sativa; Ps, Pisum sativum; Psy, Pinus sylvestris; Pv, Phaseolus vulgaris; So, Spinacea oleracea; Zm, Zea mays.

References: 1, Peterman and Goodman, 1991; 2, Miao et al., 1991; 3, Freeman et al., 1990; 4, Sakamoto et al., 1990; 5, Stougaard, personal communication; 6, Grant et al., 1989; 7, Tischer et al., 1986; 8, Tingey and Coruzzi, 1987; 9, Becker et al., 1992; 10, Sakamoto et al., 1989; 11, Gebhardt et al., 1986; 12, Lightfoot et al., 1988; 13, Tingey et al., 1987; 14, Sakakibara et al., 1992; 15, Canton et al., 1993.

FIGURE 3.1 GS polypeptides and the RuBisCO large subunit in leaf, stem and root extracts of *L. corniculatus* and *L. japonicus*

Plants were grown in trays of vermiculite and watered with nutrient solution containing 5 mM NO_3. Plants were harvested at 35 d after germination. Two μg of soluble protein were loaded in each lane of a 10% SDS-PAGE gel. The GS polypeptides were detected with antibodies raised against *P. vulgaris* nodule GS, and the RuBisCO polypeptides detected with antibodies raised against the wheat RuBisCO large subunit.

A) Soluble protein extracts from the leaf (lane 1), stem (lane 2) and root extracts (lane 3) of *L.corniculatus* immunostained for GS. The corresponding GS activities were (in μmol.h^{-1}. g^{-1}fwt): leaf, 42; stem, 35; and root, 19.

B) Soluble protein extracts from the leaf (lane 1), stem (lane 2) and root (lane 3) of *L. corniculatus* immunostained for the RuBisCo large subunit.

C) Soluble protein extracts from the leaf (lane 1), stem (lane 2) and root (lane 3) of *L. japonicus* immunostained for GS polypeptides. The corresponding GS activities were (in μmol.h^{-1}. g^{-1}fwt): leaf, 68; stem, 49; and root, 27.

FIGURE 3.1

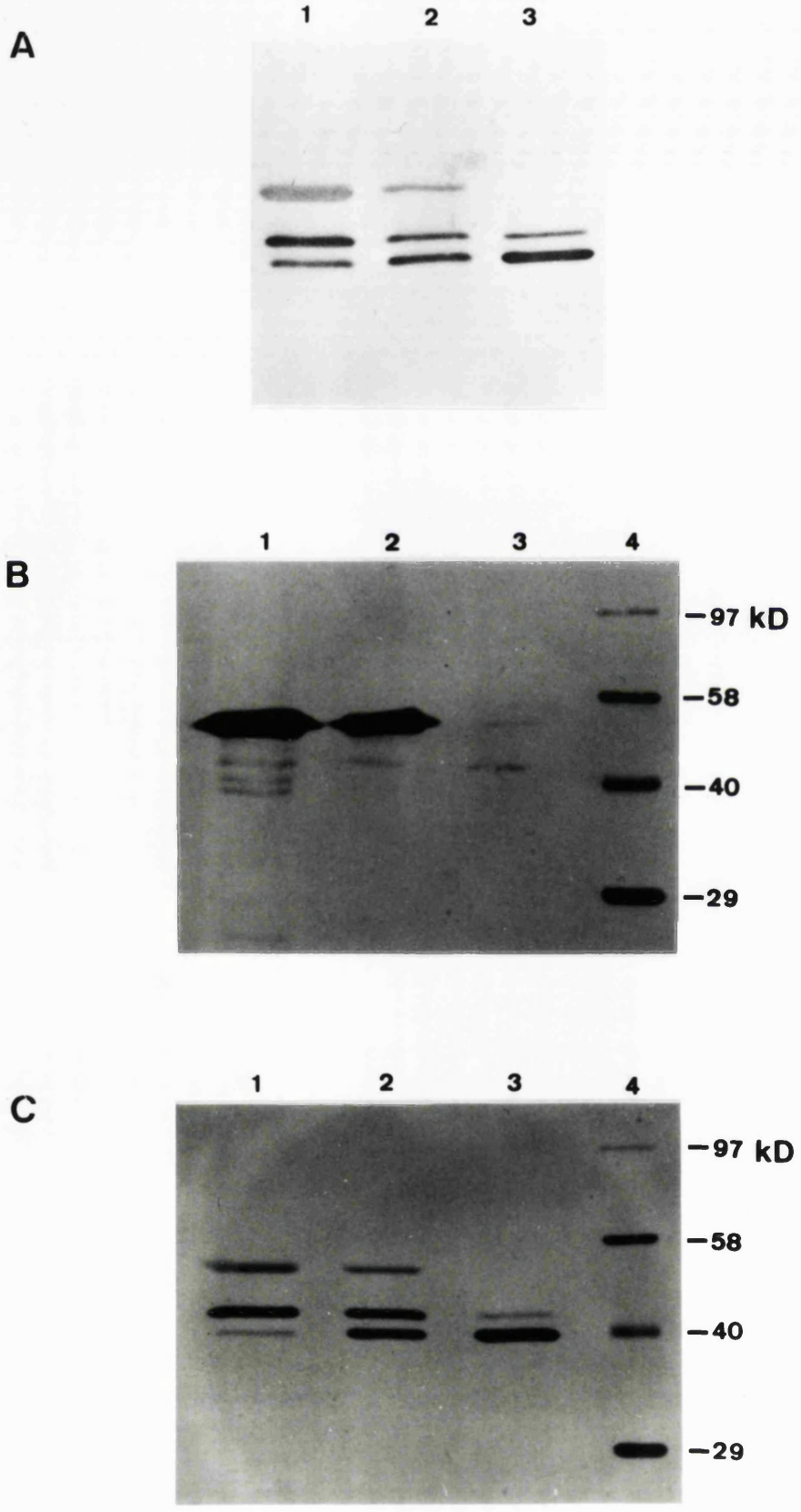


FIGURE 3.2 Two-dimensional separations of GS polypeptides in *L. corniculatus* and *L. japonicus* tissues.

Leaf and root extracts were from plants grown in sterile culture on 8 mM NO_3^- and 5 mM NH_4^+. Nodule extracts were from glasshouse-grown plants watered with an N-free medium. Six μg of soluble protein were loaded on to the isoelectric focusing gels (the first dimension). The (+) symbol indicates the anode, the (-) symbol, the cathode. A 10% SDS-PAGE gel was used in the second dimension. The GS polypeptides were detected with antibodies raised against *P. vulgaris* nodule GS.

A) Leaf extract from *L. corniculatus*.

B) Root extract from *L. corniculatus*.

C) Nodule extract from *L. corniculatus*.

D) Leaf extract from *L. japonicus*.

E) Root extract from *L. japonicus*.

FIGURE 3.2

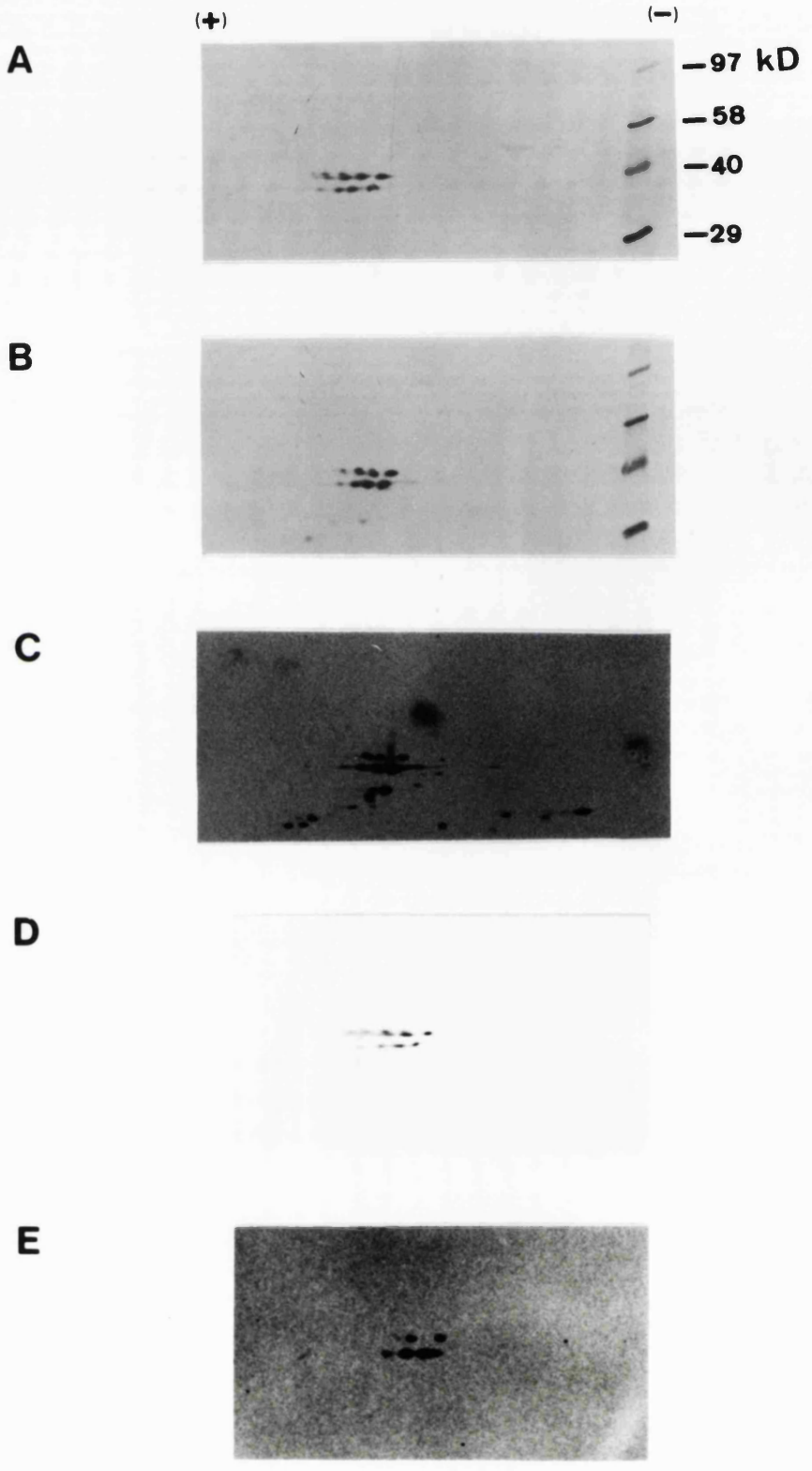


FIGURE 3.3 Total soluble proteins in the leaf of *L. japonicus* separated by SDS-PAGE and stained for glycan residues.

Soluble proteins were extracted from leaves of plants grown in sterile culture on 8 mM NO_3^- and 5 mM NH_4^+. Two μg of soluble protein were loaded in each lane of a 10% SDS-PAGE gel, and the blot was stained for the presence of carbohydrates (glycans) using the GlycoTrack system (see text for more details). Dark banding indicates the presence of glycan residues. Lane 1 in each gel contained molecular weight markers.

A) Lanes 2-8 were loaded respectively with 1 μg, 2 μg, 3 μg, 5 μg, 6 μg, 7 μg and 9 μg total soluble protein.

B) Control blot. As in caption A) but with the periodic acid oxidation step left out of the staining procedure.

C) Lanes 2-4 were loaded with 2 μg soluble protein, stained for the presence of glycans and then immunostained for GS polypeptides (see Figure 3.1 for details). Lanes 5-7 were loaded with 9 μg protein and stained only for the presence of glycans.

FIGURE 3.3

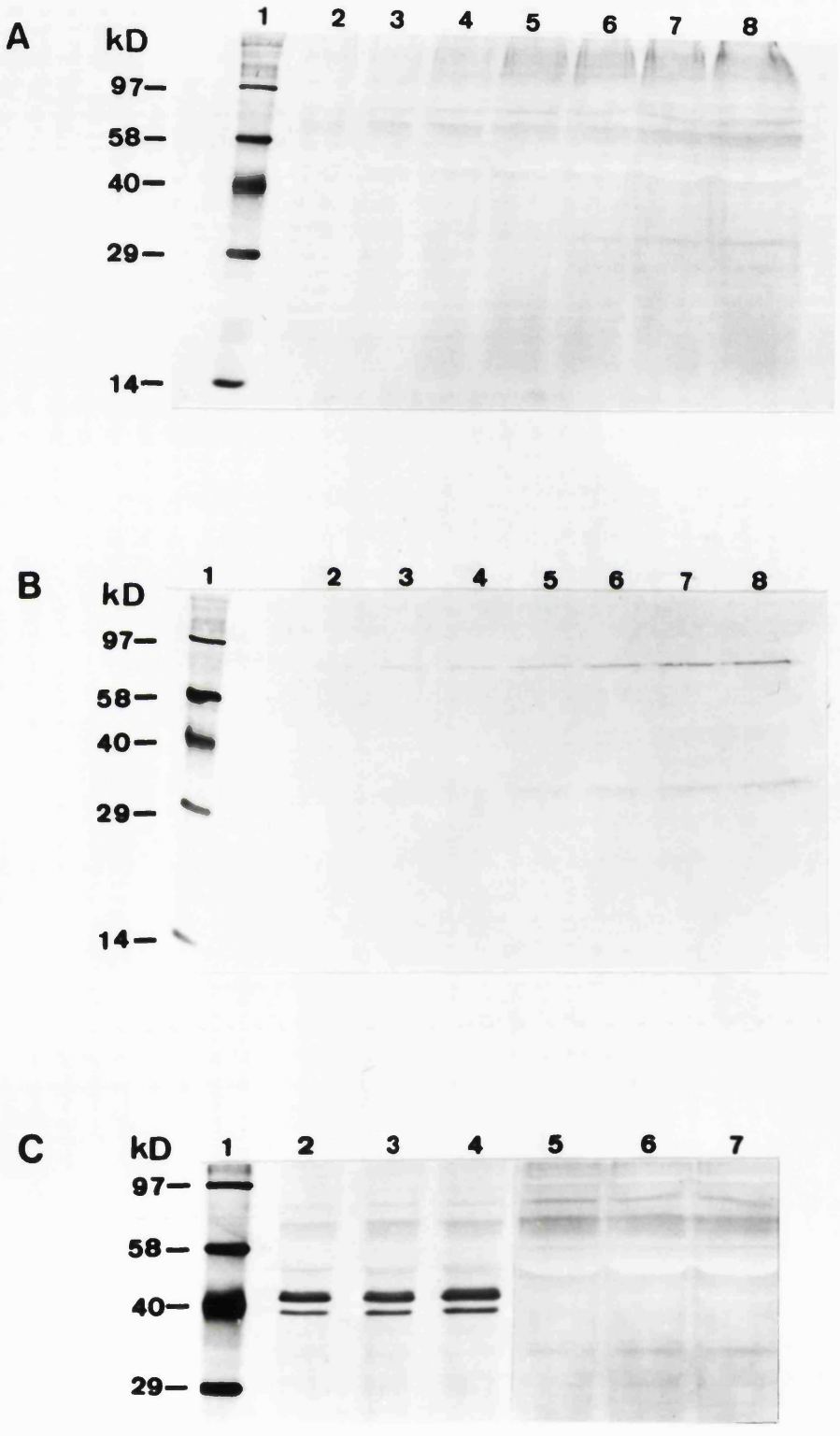

A
kD
97—
58—
40—
29—
14—
1 2 3 4 5 6 7 8
B
kD
97—
58—
40—
29—
14—
1 2 3 4 5 6 7 8
C
kD
97—
58—
40—
29—
1 2 3 4 5 6 7

FIGURE 3.4 Two-dimensional separations of soluble proteins in *L. japonicus* leaves stained for the presence of glycans and GS polypeptides.

Leaf and root extracts were from plants grown in sterile culture on 8 mM NO_3^- and 5 mM NH_4^+. Half of the soluble protein extract was deglycosylated with *N*-glycosidase. Twelve μg of either the deglycosylated or untreated extract were loaded on to the isoelectric focusing gels (the first dimension). The (+) symbol indicates the anode, the (-) symbol, the cathode. A 10% SDS-PAGE gel was used in the second dimension. Blots were stained for the presence of carbohydrates (glycans) using the GlycoTrack system and then immunostained for GS polypeptides using antibodies raised against *P. vulgaris* nodule GS.

A) Untreated extract stained for glycan residues.

B) Deglycosylated extract stained for glycan residues.

C) Blot A) immunostained for GS polypeptides following glycan detection.

D) Blot B) immunostained for GS polypeptides following glycan detection.

FIGURE 3.4

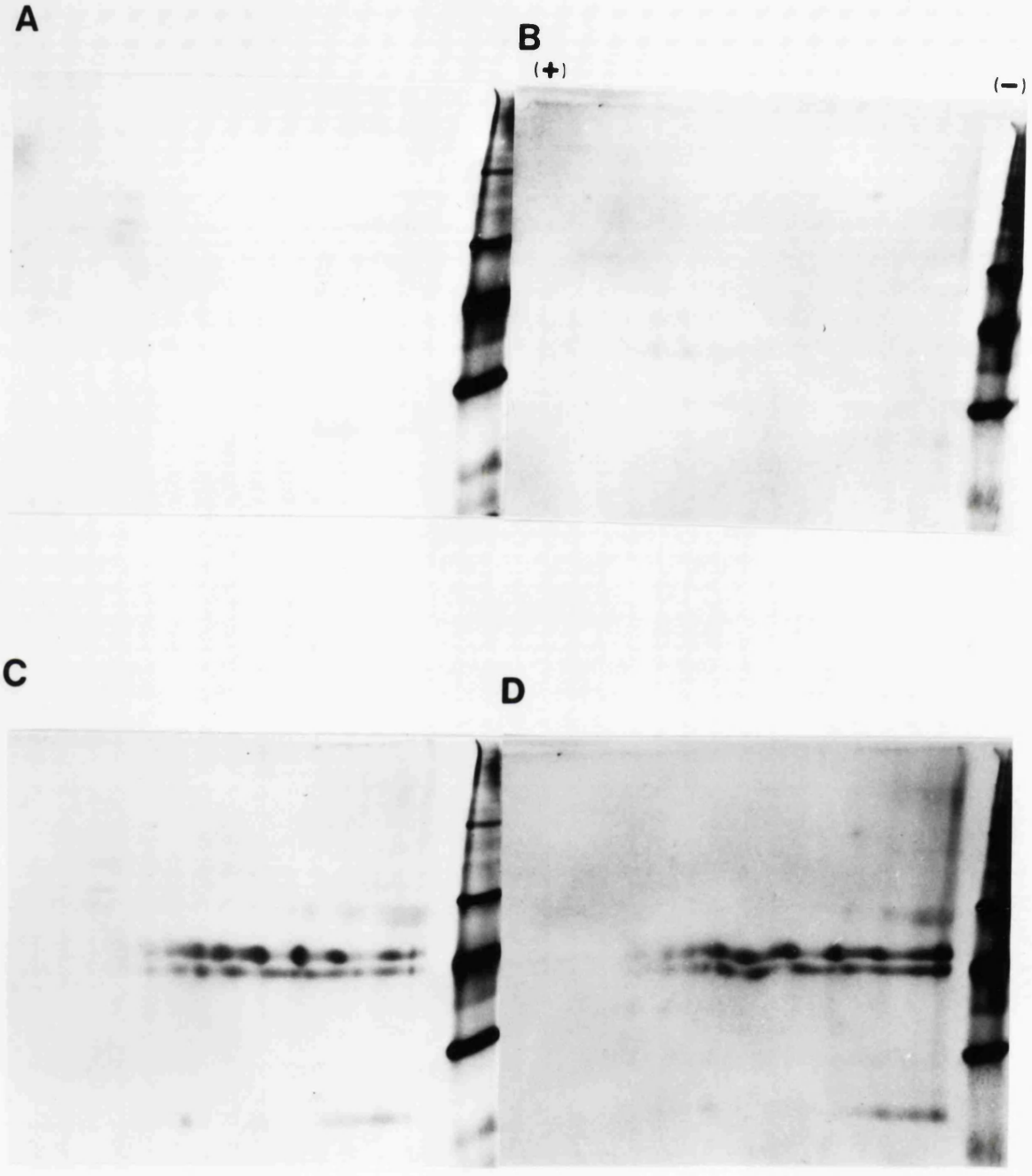


TABLE 3.2 **Marker enzyme activities of *L. japonicus* root fractions separated by sucrose density gradient.**

Plants were grown in vermiculite watered with 5 mM NO_3^-. For the 48 h days prior to harvest, plants were grown in darkness. Root extracts were centrifuged through a discontinuous sucrose gradient. The crude root extract and five fractions from the top to the bottom of the gradient were assayed for the marker enzyme activities and the activity of GS.

	ENZYME ACTIVITIES ($\mu mol.h^{-1}.mg^{-1}protein$)		
FRACTION	NiR (Plastidic)	NR (Cytosolic)	GS
Crude	46.80	0.43	31.61
1	2.70	0.47	24.23
2	4.21	0.72	13.61
3	4.14	0	13.25
4	35.17	4.57	42.84
5	8.21	3.42	23.83

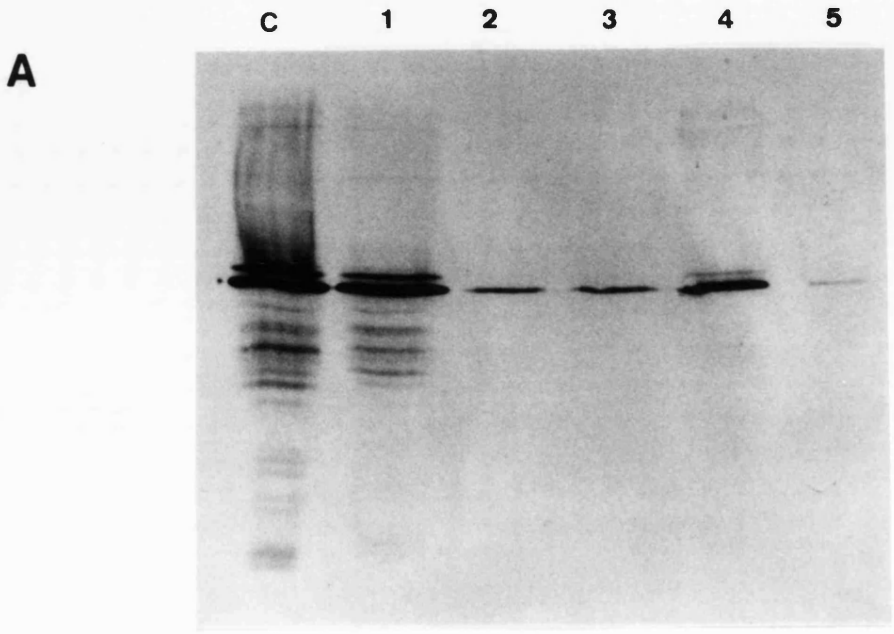
FIGURE 3.5 Isoforms of GS of *L. japonicus* in root extracts separated into plastid-depleted and plastid-enriched fractions

Plants were grown in vermiculite watered with 5 mM NO_3^- (blot A) or 10 mM NO_3^- (blot B). For the 48 h days prior to harvest plants were grown in darkness. Proteins were separated on a 10% SDS-PAGE gel and the GS polypeptides detected with antibodies raised against *P. vulgaris* nodule GS.

A) Roots were extracted and fractionated through a discontinuous sucrose gradient by the method of Vézina and Langlois (1989. Lane 1 contains 10 μl crude root extract; lanes 2-8 were loaded with 10 μl from one of five fractions taken from the top to the bottom of the gradient (the fraction at the top of the gradient is fraction 1). The GS activities of the crude root extract and the five fractions can be seen in Table 3.2, as can the activities of the plastidic and cytosolic marker enzymes.

B) Plastid-enriched fraction (lane 2) and crude extract (lane 3) of *L. japonicus* roots, obtained by the method of Bowsher *et al.* (1989). The corresponding GS activities were (in $\mu mol.h^{-1}.mg^{-1}$ protein): 100.5 for the plastids and 3.0 for the crude extract.

FIGURE 3.5



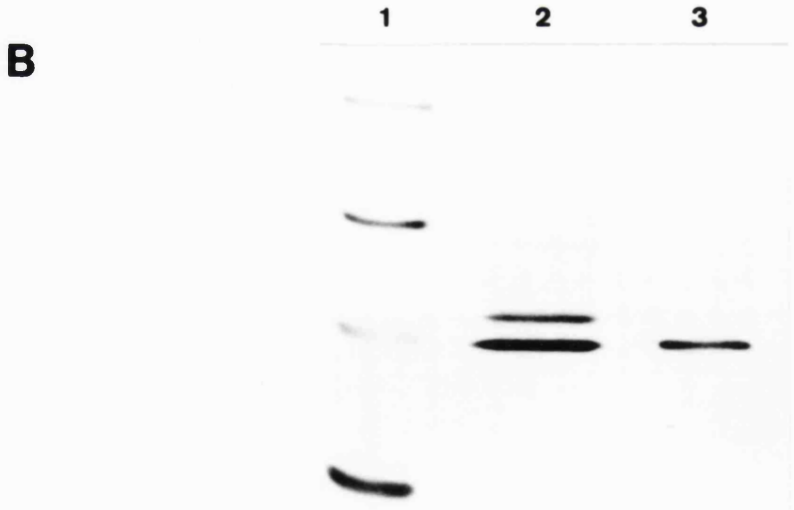
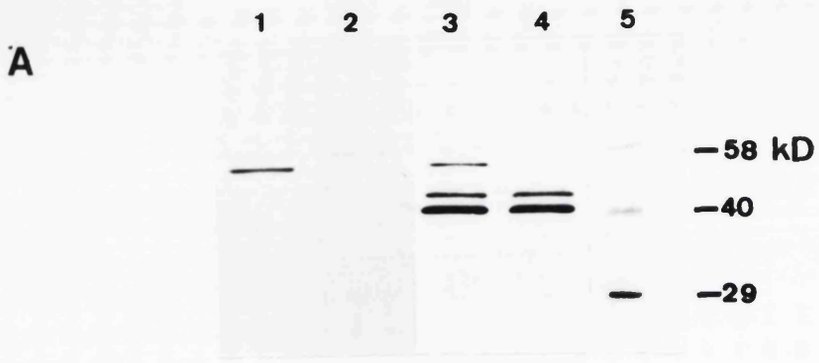


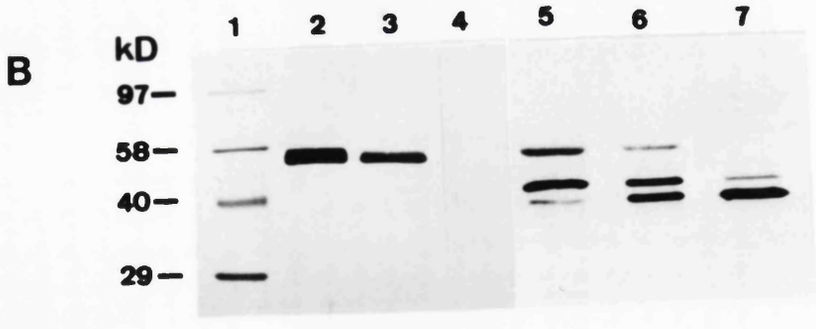
FIGURE 3.6 Effect of light on accumulation of the RuBisCO large subunit and GS polypeptides in *L. japonicus* roots

A) Total protein extracts (2 μg.lane^{-1}) of roots from 40 d old plants grown on 10 mM NO_3^-. Light-exposed roots (Lanes 1 and 3) and dark-grown roots (lanes 2 and 4) were probed with antibody raised against wheat RuBisCO (lanes 1 and 2) or with antibody raised against *P. vulgaris* nodule GS (lanes 3 and 4). The GS activity (μmol.h^{-1}.g^{-1}fwt) for the light-grown and dark-grown roots were 27.29 ± 0.16 and 28.54 ± 1.29 respectively. The protein concentrations were (in mg.g^{-1}fwt) 6.03 ± 0.15 and and 5.51 ± 0.36.

B) Total protein extracts of leaf (lanes 2 and 5), stem (lanes 3 and 6) and root (lanes 4 and 7) from 22 d old plants grown on 5 mM NO_3^-. Roots were masked from the light from germination onwards. Polypeptides (0.5 μg.lane^{-1}) were probed with antibody raised against wheat RuBisCO (lanes 2, 3 and 4) or with antibody raised against *P. vulgaris* nodule GS (1 μg protein per lane). The GS activities for the leaf, stem and root, were (in μmol.h^{-1}.g^{-1}fwt) 67.56 ± 1.98 , 48.69 ± 3.97 and 19.54 ± 0.43 respectively. Protein concentrations were (in mg.g^{-1}fwt) 34.85 ± 0.85, 23.66 ± 0.85 and 9.83 ± 0.59.

FIGURE 3.6





CHAPTER 4: IDENTIFICATION OF GS GENES IN *P. VULGARIS*, *L. CORNICULATUS* AND *L. JAPONICUS* USING PCR TECHNOLOGY AND SEQUENCING OF A PARTIAL GS CLONE FROM *L. CORNICULATUS*.

4.1 INTRODUCTION

4.1.1 The structure of plant genes

Most nuclear genes in plants are similar in structure to those of other advanced eukaryotes in containing coding regions (exons) and non-coding intervening regions (introns), as well as upstream and downstream non-transcribed regions, which may include important regulatory features. Although both the introns and exons are transcribed as part of the pre-mRNA, the introns are removed by splicing reactions and the exons are joined during the formation of mature mRNA. Transcripts often also contain untranslated 5' and 3' sequences of variable length. The generation of translatable mRNA usually involves post-transcriptional modifications such as the addition of a 7-methylguanosine cap at the 5' end, methylation of selected adenosine residues, and addition of a polyadenylic acid sequence at the 3' end, up to 200 bp in length.

4.1.2 Intron structure

With few exceptions, nuclear genes are interrupted by introns ranging in number from one to fifty, and are known to vary in length from 31 nucleotides to more than 50 kb. There are four categories of intron differing in structure, location and splicing mechanism. Although higher plants contain examples of pre-RNAs with each category of intron (e.g. nuclear pre-tRNAs, and the self-splicing introns

in some chloroplastic pre-tRNAs or mitochondrial pre-mRNAs) many introns from plant nuclear genes belong to the class III type.

Class III introns are AT-rich, contain a number of codons for termination of protein synthesis and may vary from a few dozen to hundreds of nucleotides. In higher plants, conserved sequence elements have been identified at the 5' and the 3' splice junctions (G/GTAAGT and PyPyPyPyPyPyPyPyPyPyPyRPyAG/G respectively, where / is the exon-intron boundary) (Krainer and Maniatis, 1988). Another important site for the splicing mechanism, the branchpoint, has been shown in *Saccharomyces cerevisiae* (UACUAACA) and animals (PyNPyURAPy) to lie within the intron near the 3' splice site. A consensus sequence identical to that of the branchpoint found in animals has been found in higher plants, but the function has not yet been demonstrated (Brown, 1986).

4.1.3 Gene families

Although some single-copy genes are known, many genes, including those for GS, belong to multigene families (Forde and Cullimore, 1989). These families are groups of related sequence which may differ in the coding region, the untranslated regions of the mRNA, and/or in the untranscribed regulatory regions of the chromosome. In contrast to the highly conserved exon regions, there is likely to be little or no selection pressure on intron sequences (except at the splice junctions and branch points) and they tend to be very variable in both sequence and length even between members of a gene family in one species.

4.1.4 The structure of plant GS genes

To date only one plant GS gene, a *Medicago sativa* GS1 gene, has been completely sequenced, the length of which is 3820 bp from the cap site to the polyadenylation site. It contains twelve exons ranging in length from 37-359 bp, and eleven AT-rich introns of 90-715 bp (Tischer *et al.*, 1986). The GS1 mRNAs from a wide range of dicotyledons and monocotyledons have an estimated size of 1.4-1.6 kb, with a coding region of approximately 1065 bp (Gebhardt *et al.*, 1986; Tischer *et al.*, 1986; Tingey and Coruzzi, 1987; Sakamoto *et al.*, 1989; Miao *et al.*, 1991; Peterman and Goodman, 1991; Sakakibara *et al.*, 1992a). However, GS2 mRNAs are approximately 200 bp larger than those of GS1, as a result of the N-terminal extension of approximately 56 amino acid residues, and the C-terminal extension of 16 residues (Lightfoot *et al.*, 1988; Sakamoto *et al.*, 1989; Freeman *et al.*, 1990; Peterman and Goodman, 1991; Becker *et al.*, 1992).

In the work described in this chapter, PCR (polymerase chain reaction) technology was used to try and amplify the eleventh intron and its flanking exon sequences in members of the GS gene family in *P. vulgaris*, *L. corniculatus* and *L. japonicus*, using primers designed from the flanking exon regions. Assuming that the intron length would vary in each member of a gene family, it was hoped to provide a rapid and convenient method to determine the number of GS genes in the two *Lotus* species. As the number of GS genes in *P. vulgaris* was known, this species was used to test the approach. A second verification of the method was through use of cDNA as the amplification template which was predicted to result in a product of a single, predictable size (i.e. that of the distance between the primers minus the variable intron length).

4.2 RESULTS

4.2.1 Using the polymerase chain reaction to identify the number of GS genes in *P. vulgaris*.

The PCR primers were designed from two strongly conserved nucleotide sequences which flank intron 11 (see Chapter 2.8.2). Primer B, which was 22 bases long and contained a *Pst*I site, was located approximately 830 nucleotides from the transcription start, and primer C, which was 25 bases long and contained a *Bam*HI site, was located at position 1010. The lowest optimum annealing temperature of primers B and C were ascertained by using the algorithm published by Breslauer *et al.* (1986).

It was reasoned that the products amplified by PCR from genomic DNA would be the length of intron 11 plus approximately 150 bp of the flanking coding region and the length of the two primers (47 bp). Variations in the length of intron 11 in each member of a GS gene family would therefore produce differently sized PCR products, and the total number of products should equal the number of genes in that family. It was known that the genome of *P. vulgaris* contains five GS genes (Forde and Cullimore, 1989), so total DNA from this species was initially used in a PCR amplification to test the protocol (Figure 4.1A, lane 4). As expected, five PCR products were obtained and these were approximately 280 bp, 380 bp, 440 bp, 510 bp and 610 bp in size. The 380 bp product was thought to represent the *gln*-ϵ gene, in which the intron is known to be 180 bp in length (B.G. Forde, personal communication).

4.2.2 Amplification by the polymerase chain reaction of the flanking region of intron 11 using cDNA from the leaf of *L. corniculatus*.

Figure 4.1B (lane 3) shows the PCR products generated with the two primers when the template was cDNA that had been synthesized from polyA$^+$ RNA from the leaf of *L. corniculatus* (Chapter 2.7.2 and 2.8.1). A single DNA fragment of the expected size (approximately 200 bp) was obtained. No product was obtained in a control reaction where the template DNA was omitted (lane 2). This result indicated that the consensus primers were indeed capable of amplifying GS sequence(s) from *L. corniculatus* with high specificity, although the exact identity of the amplified fragment was not investigated further at this stage.

4.2.3 Using the polymerase chain reaction to identify the number of GS genes in *L. corniculatus* and *L. japonicus*.

When total DNA from *L. corniculatus* was used as the template, amplification resulted in four to five DNA fragments, depending on the annealing temperature (Figure 4.1C). The three largest products (approximately 430 bp, 500 bp and 610 bp) were similar in size to three of the *P. vulgaris* products, whilst the major fragment was slightly larger than the 280 bp product found in *P. vulgaris* (see Figure 4.1A, lanes 3 and 4 for a comparison). At the lower annealing temperature a second major product of approximately 200 bp appeared (Figure 4.1C, lane 5), which was also apparent in the control reactions. A product of similar size to the *P. vulgaris* 380 bp fragment was not seen.

When total DNA from *L. japonicus* was used as template, the two major PCR products corresponded to the 300 bp fragment from *L. corniculatus* (Figure 4.1D, lanes 1 to 4), and the 430 bp product seen with both *P. vulgaris* and

L. corniculatus. Several larger products of 460 and 670 bp were also obtained, but despite attempts to optimize the PCR conditions by altering the Mg^{2+} concentration (Figure 4.1D), these larger products remained too indistinct to be identified.

4.2.4 Cloning and sequence analysis of *L. corniculatus* PCR products.

The DNA fragments of one amplification reaction (using a 50°C annealing temperature and *L. corniculatus* total DNA as template) were separated from the other reaction components using the Prep-A-Gene kit and ligated into pUC9. Transformed *E. coli* colonies were screened using radioactive probes synthesized from the purified 200 bp, 300 bp, 500 bp and 610 bp products of a separate PCR amplification of intron 11. Three-quarters of the colonies hybridized strongly to each of the 300 bp, 500 bp and 600 bp probes, but none of the colonies hybridized to the 200 bp probe, showing that this product had not been cloned and that it was unrelated to the other fragments. As each of the positive clones hybridized to three probes, twelve of the clones were screened by PCR, using the primers flanking intron 11 to enable identification of the cloned insert by size. It was found that all twelve clones contained the 300 bp insert (Figure 4.1E).

The nucleic acid sequence of the 300 bp PCR product was determined and was aligned with the corresponding region of the cDNA sequences available from other legumes, including a *L. japonicus* nodule GS cDNA (J. Stougaard, personal communication) and the genomic sequence from *M. sativa* (Figure 4.2). The legume sequences were also compared to other dicotyledonous and monocotyledonous GS sequences. As expected, the 300 bp *L. corniculatus* fragment contains 142 bp of GS coding sequence (excluding the 47 bp of primer

sequences), interrupted by a 106 bp intron. The number of mismatches between each pair of sequences was calculated as a percentage of the total number of overlapping bases (for percentage identities of the legume sequences see Table 4.1; for comparisons of the legume sequences with non-legume sequences, see Appendix III). The amino acid sequences of both the legumes and non-legumes were also compared (Figure 4.3, Table 4.2 and Appendix III).

Most of the mismatches in the coding regions were at the third base of the codons and therefore did not affect the amino acid sequence. The *L. corniculatus* GS sequence was much more closely related to the GS1 cDNAs than to the two leguminous GS2 cDNAs. Indeed there were no differences at the nucleotide level between the *L. corniculatus* genomic fragment and the *L. japonicus* GS1 cDNA over the region of overlap. The greatest similarities at both the nucleotide and amino acid level were between *L. corniculatus* cDNA and GS1 from *G. max*, *P. vulgaris* (*gln*-ß), *P. sativum* (pGS134) and the *M. sativa* GS1 gene (86-91% nucleotide identity and 96-98% amino acid identity).

The 5' splice junction (G/GTAAGC), 3' splice junction (TTGTGTTATTTGA.....AG/GGA), the putative branchpoint (ACTTAAT) and the surrounding areas in the *L. corniculatus* sequence are also shown aligned to homologous regions in intron 11 of *M. sativa* (Figure 4.2). The two splice junctions are identical in both species, although the 3' sequence in *L. corniculatus* is interrupted by seven additional bases (CTTGTGT). The significance, if any, of this is not known. Although there are three possible branchpoint sequences in the *L. corniculatus* intron, two of which align with *M. sativa* sequences, the site nearest the 3' end has been identified as the most likely branchpoint in *M. sativa*,

and is therefore assumed to also be the branchpoint in *L. corniculatus*.

4.3 DISCUSSION

4.3.1 Assessing the number of GS genes in *P. vulgaris*, *L. corniculatus* and *L. japonicus* by PCR technology

It is not known how many genes encode the four GS2 polypeptides and the four GS1 polypeptides seen in 2-D gel separations of *L. corniculatus* and *L. japonicus* protein extracts (see Chapter 3). Evidence from other legume species indicates the likelihood of only one GS2 gene, producing polypeptides that are differentially modified after translation, and at least three GS1 genes (Gebhardt *et al.*, 1986; Lightfoot *et al.*, 1988; Bennett *et al.*, 1989; Walker and Coruzzi, 1989; Stanford *et al.*, 1993).

A PCR approach was used to amplify the region of a GS gene between two primers which were designed from a consensus sequence found in exons 11 and 12, and containing intron 11 (assuming conservation of intron locations between *M. sativa* and other legume species). When total *P. vulgaris* DNA was used as the PCR template, five differently sized DNA fragments were amplified, including one of around 380 bp. It is known that there are five members of the GS gene family in *P. vulgaris* (Forde and Cullimore, 1989), and that intron 11 of one of these genes (*gln-ϵ*) plus the exon region between and including the flanking primers, is approximately 360 bp (B.G. Forde, personal communication). When *L. corniculatus* cDNA was used as the template, a single product of 200 bp resulted. This was the predicted length of the coding region lying between (and including) the two primers. These results suggested that this approach may indeed be a useful

way of estimating the number of GS genes in a particular species.

When total DNA from *L. corniculatus* was used as template, five major products of approximately 200 bp, 300 bp, 430 bp, 500 bp and 610 bp were obtained. The four largest fragments were of similar size to four of the products from *P. vulgaris*. The *P. vulgaris gln-ϵ* gene is thought to be a pseudogene as no evidence of a polypeptide or mRNA has been found (Forde and Cullimore, 1989), and it is interesting to note that the PCR product of 380 bp amplified from *P. vulgaris*, probably from the *gln-ϵ* gene, was absent from the *L. corniculatus* and *L. japonicus* amplifications. The 200 bp DNA amplified from the *L. corniculatus* genome was too small to encompass an intron, and only appeared at lower annealing temperatures, so is thought to be a spurious PCR product. Unfortunately, the technique was not as successful with *L. japonicus*, probably because the template DNA was not of sufficient quality. However, two DNAs similar in size to the 300 and 430 bp products of *L. corniculatus* could be resolved.

It is quite possible that the four amplification products do represent the number of GS genes in *L. corniculatus*. Several species have been shown to have only one GS2 gene and at least three genes encoding GS1 (Gebhardt *et al.*, 1986; Lightfoot *et al.*, 1988; Bennett *et al.*, 1989; Walker and Coruzzi, 1989; Stanford *et al.*, 1993). However, if there is only one GS2 gene and three GS1 genes in *L. corniculatus*, then post-translational modification must be responsible for at least one of the four GS1 polypeptides seen in 2-D gel separations as well as for the four GS2 polypeptides (Chapters 3.2.3 and 5.4.3).

4.3.2 The relationship of the GS sequence surrounding intron 11 in

***L. corniculatus*, to that of other species.**

Sequencing of the *ca* 300 bp product from *L. corniculatus* showed that it had indeed been amplified from a GS1 gene, containing a coding region of 159 bp flanking an intron of 106 bp (Figure 4.2). The amino acid sequence contained a region, NRGASIR, that is very similar to the substrate binding site of bovine glutamate dehydrogenase, DRGASIV, and has been proposed as part of the GS active site (Tischer *et al.*, 1986). The intron contained 3' and 5' splice sites, and a putative branchpoint sequence, ACTTAAT (Brown, 1986, Krainer and Maniatis, 1988).

Although the coding region of the *L. corniculatus* clone was probably only 15% of the full transcript length, it was possible to determine its relationship to cDNAs from other legume and non-legume species. The percentage identities obtained for the 159 base sequence were very similar to the identities found when complete cDNAs had been compared (see the figures in brackets in Table 4.1). However, the percentage identities of the deduced amino acid sequence were generally slightly higher than the complete translation products (the numbers in brackets in Table 4.2) but the trends were very similar.

The region shared less than 75% homology with the GS2 sequences from both legumes and non-legumes, but had much greater similarity to the GS1 sequences. The *L. corniculatus* cDNA was most closely related to GS1 expressed in both roots and nodules of *P. vulgaris* (*gln*-ß), *G. max* (pGS20), *P. sativum* (pGS134) and *M. sativa* (Gebhardt *et al.*, 1986; Miao *et al.*, 1991; Tingey *et al.*, 1987; Dunn *et al.*, 1988) and was identical to the *L. japonicus* cDNA obtained from both a nodule library (J. Stougaard, personal communication) and a root

library (D.T. Clarkson, personal communication). Therefore, the *L. corniculatus* genomic sequence is likely to correspond to the same GS1 gene as found in *L. japonicus*. In conclusion, although evidence from two-dimensional gels (Chapters 3.2.3 and 5.4.3) and the PCR reactions suggest otherwise, we have as yet no direct proof of the existence of multiple cytosolic GS1 genes in *L. corniculatus* or *L. japonicus*.

FIGURE 4.1 Products of the polymerase chain reaction initiated with primers designed from flanking regions of intron 11 in the GS gene.

The PCR reaction was set up as described in Chapter 2. Standard reaction conditions, unless otherwise stated, were 5 cycles of 94°C for 1 min (denaturation), 48°C for 2 min (annealing) and 72°C for 2 min (elongation), followed by 30 cycles with a 50°C annealing temperature. The concentration of Mg^{2+} was 1.5 mM unless otherwise stated. The primers recognized highly conserved sites in the exons which flank intron 11 in a number of GS gene sequences. The PCR products were separated on an 8% polyacrylamide gel in 1 x TBE buffer containing ethidium bromide (0.5 $\mu g.\mu l^{-1}$). Markers were either a 123 bp ladder, or *Hae*III fragments of pBR322.

A) *L. corniculatus* and *P. vulgaris* total DNA as template. Lane 1, 123 bp ladder markers; lane 2, PCR control reaction (minus template DNA); lane 3, *L. corniculatus* total DNA; lane 4, *P. vulgaris* total DNA.

B) *L. corniculatus* cDNA as template, made from leaf $polyA^+$ RNA. Lane 1, 123 bp ladder markers; lane 2, PCR control reaction (minus template DNA); lane 3, *L. corniculatus* cDNA.

C) Total leaf DNA from *L. corniculatus* as template, with the annealing reaction run at different temperatures; lane 1, 53°C; lane 2, 53°C control minus (template DNA); lane 3, 50°C; lane 4, 50°C control; lane 5, 47°C; lane 6, 47°C control; lane 7 123 bp ladder.

D) Total leaf DNA from *L. japonicus* as template. The reactions in each lane were run with different concentrations of Mg^{2+}; lane 1, 2 mM; lane 2, 1.5 mM; lane 3, 1.0 mM; lane 4, 0.9 mM; lane 5, 0.8 mM; lane 6, 0.7 mM; lane 7, 0.6 mM; lane 8, pBR322/*Hae*III markers.

E) Direct PCR of *E. coli* colonies (lanes 1-12) transformed with the PCR products amplified from *L. corniculatus* total DNA (seen in Figure 4.1C). Lane 13, 123 bp markers.

FIGURE 4.1

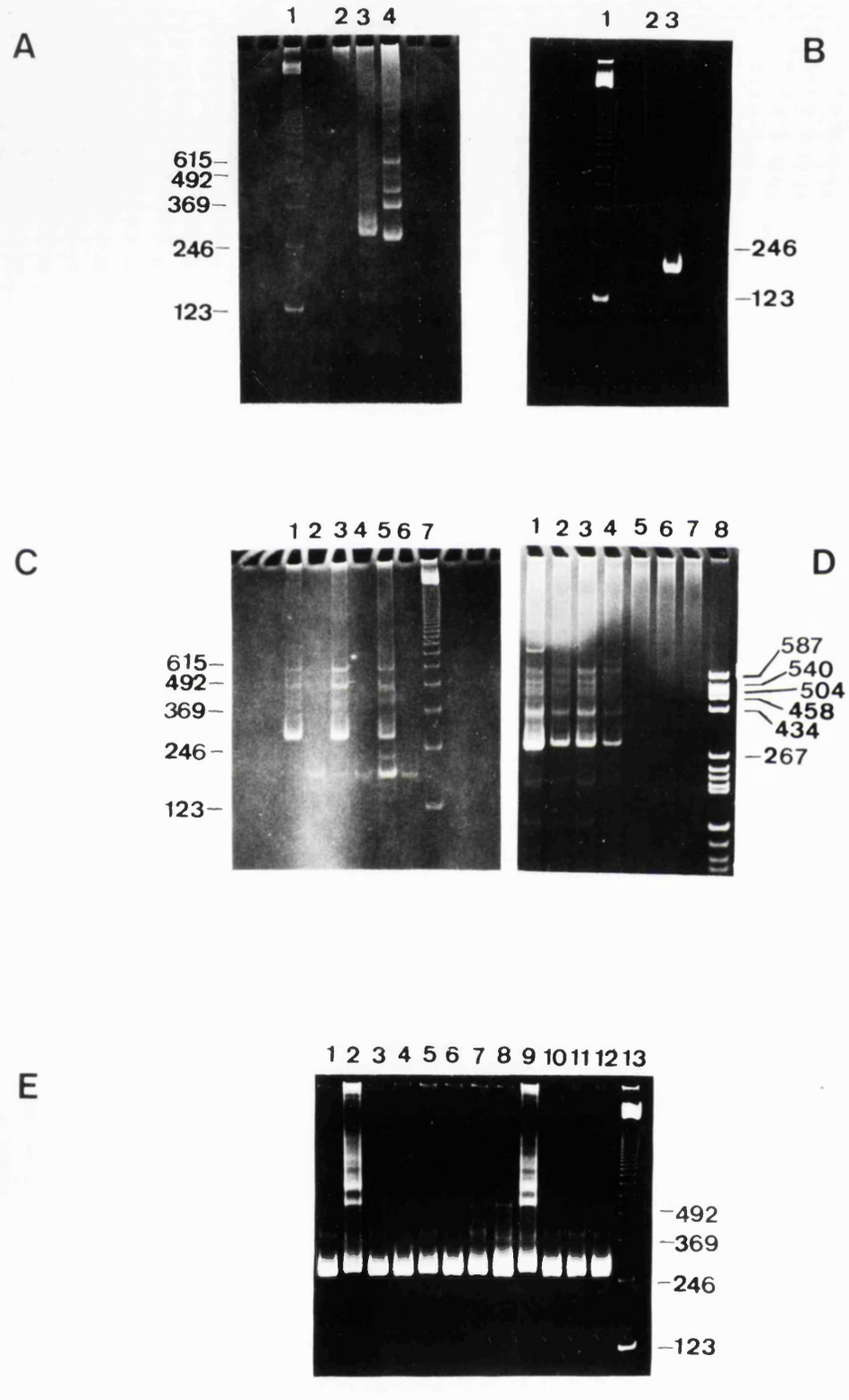

A
1 2 3 4
615
492
369
246
123
B
1 2 3
246
123
C
1 2 3 4 5 6 7
615
492
369
246
123
D
1 2 3 4 5 6 7 8
587
540
504
458
434
267
E
1 2 3 4 5 6 7 8 9 10 11 12 13
492
369
246
123

FIGURE 4.2 The nucleic acid sequence of intron eleven and the flanking region of a partial *L. corniculatus* GS clone, compared to that of other legumes.

The sequence of the 295 bp PCR product from *L. corniculatus* (Lc) is shown in full and the sequences from the other species are only show where they differ from it. The two primer sequences and the intron in *L. corniculatus* is shown in lower case. The intron is alligned with homologous regions in intron eleven of *M. sativa* (Ms1B). The conserved sequences at the 5′ and 3′ splice sites are in boxes, as are putative branch point sequences. The A residue of the branch point is marked with a dot.

Pv2 = *P. vulgaris* s/u (pcGS-δ1), Lightfoot *et al.*, 1988
Ps2 = *P. sativum* GS2 (pGS197), Tingey *et al.*, 1987
La1 = *L. angustifolius* GS1 (pGS5), Grant *et al.*, 1989
Gm1 = *G. max* GS1 (pGS20), Miao *et al*, 1991
Pv1A = *P. vulgaris* gln-α (pcGS-α1), Gebhardt *et al.*, 1986
Pv1B = *P. vulgaris* gln-ß (pcGS-ß1), *ibid*
Pv1C = *P. vulgaris* γ s/u (pcGS-γ1), Bennett *et al.*, 1989
Ps1A = *P. sativum* GS1 (pGS341), Tingey *et al.*, 1987
Ps1B = *P. sativum* GS1 (pGS134), *ibid*
Ms1A = *M. sativa* GS1 (pAnGS1), Dunn *et al.*, 1988
Ms1B = *M. sativa* GS1 gene, Tischer *et al.*, 1986
Mt1A = *M. trunculata* GS1 (MtGSa), Stanford *et al.*, 1993
Mt1B = *M. trunculata* GS1 (MtGSb), *ibid*
Mt1C = *M. trunculata* GS1 (MtGSc), *ibid*
Lj1 = *L. japonicus* GS1 (pGSA9) J.Stougaard, personal communication

FIGURE 4.2

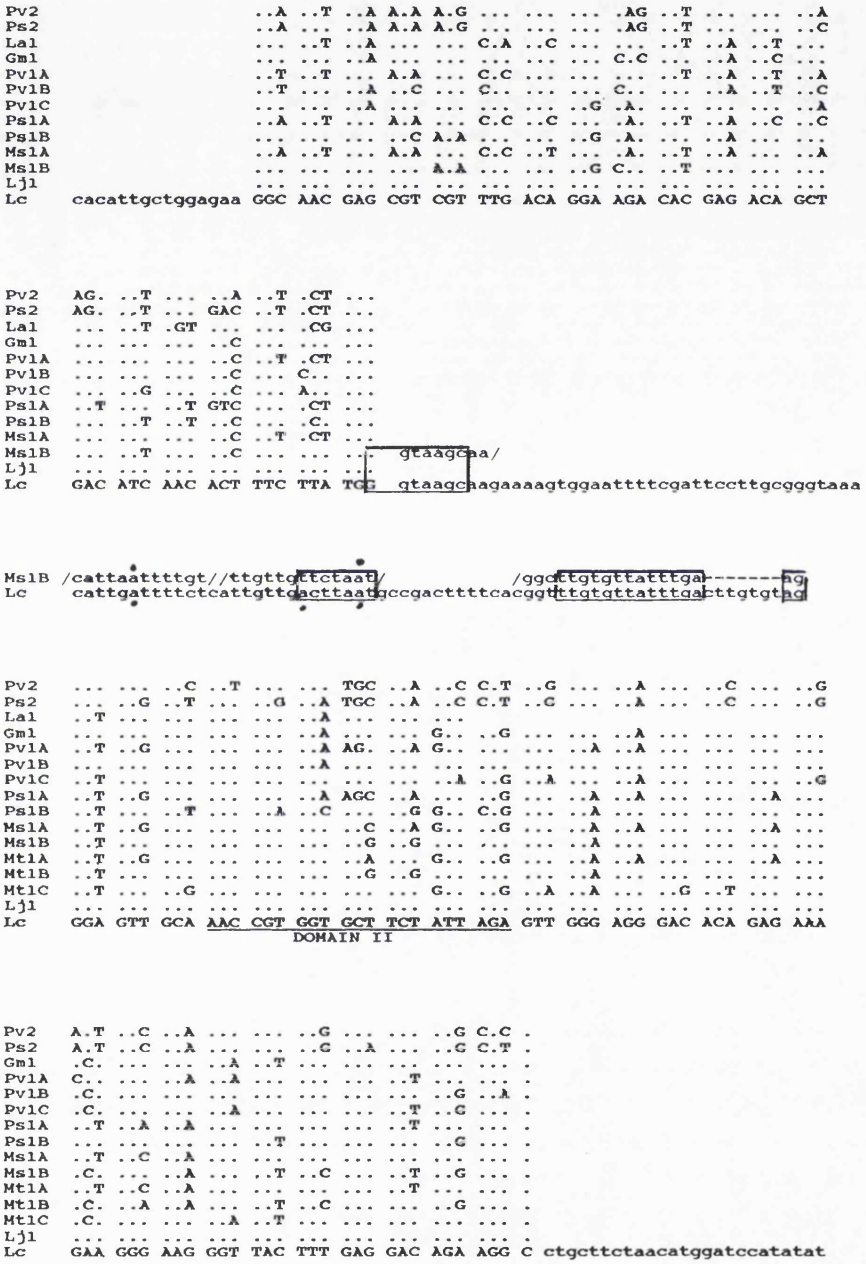

Pv2 ..A ..T ..A A.A A.G ... ... .AG ..T ... .A
Ps2 ..A ... ..A A.A A.G ... ... .AG ..T C
La1 ... ..T ..A ... ... C.A ..C ... .T ..A ..T ...
Gm1 ... .A ... ... C.C ... ..A ..C ...
Pv1A ..T ..T ... A.A ... C.C ... ...T ..A ..T ..A
Pv1B ..T ... ..A ..C ... C.. ... ... C.. ... ..A ..T ..C
Pv1C ... .A ... ...G .A. ... ...A
Ps1A ..A ..T ... A.A ... C.C ..C ... .A. ..T ..A ..C ..C
Ps1B ... ...C A.A ... .G .A. ... ..A ... ...
Ms1A ..A ..T ... A.A ... C.C ..T ... .A. ..T ..A ... ..A
Ms1B ... ... A.A ... .G C.. ..T ... ...
Lj1 ... ...
Lc cacattgctggagaa GGC AAC GAG CGT CGT TTG ACA GGA AGA CAC GAG ACA GCT
Pv2 AG. ..T ... ..A ..T .CT ...
Ps2 AG. ..T ... GAC ..T .CT ...
La1 ... ..T .GT ... ... .CG ...
Gm1 ... ...C ... ...
Pv1A ... ...C ..T .CT ...
Pv1B ... ...C ... C.. ...
Pv1C ... ..G ... ..C ... A.. ...
Ps1A ..T ... ..T GTC ... .CT ...
Ps1B ... ..T ..T ..C ... .C. ...
Ms1A ... ...C ..T .CT ...
Ms1B ... ..T ... ..C ... ... gtaagcaa/
Lj1 ... ...
Lc GAC ATC AAC ACT TTC TTA TGG gtaagcaagaaaagtggaattttcgattccttgcgggtaaa
Ms1B /cattaattttgt//ttgttgttctaat/ /ggcttgtgttatttga-------ag
Lc cattgattttctcattgttgacttaatgccgactttcacggttttgtgttatttgaattgtgtag
Pv2 ... .C ..T ... ... TGC ..A ..C C.T ..G ... ..A ... ..C ... ..G
Ps2 ... ..G ..T ... ..G ..A TGC ..A ..C C.T ..G ... ..A ... ..C ... ..G
La1 ..T ... ...A ... ...
Gm1 ... ...A ... ... G.. ..G ... .A ... ...
Pv1A ..T ..G ... ...A AG. ..A G.. ... .A ..A ... ...
Pv1B ... ...A ... ...
Pv1C ..T ... ...A ..G ..A ... ..A ... ...G
Ps1A ..T ..G ... ...A AGC ..A ... ..G ... ..A ..A ... .A ...
Ps1B ..T ... ..T ... ..A ..C ... ..G G.. C.G ... ..A ... ...
Ms1A ..T ..G ... ...C ..A G.. ..G ... ..A ..A ... .A ...
Ms1B ..T ... ...G ..G ... ...A ... ...
Mt1A ..T ..G ... ...A ... G.. ..G ... ..A ..A ... .A ...
Mt1B ..T ... ...G ..G ... ...A ... ...
Mt1C ..T ... ..G ... ... G.. ..G ..A ..A ... ..G ..T ... ...
Lj1 ... ...
Lc GGA GTT GCA AAC CGT GGT GCT TCT ATT AGA GTT GGG AGG GAC ACA GAG AAA
DOMAIN II
Pv2 A.T ..C ..A ... .G ... .G C.C .
Ps2 A.T ..C ..A ... .G ..A ... ..G C.T .
Gm1 .C. ... .A ..T ... ...
Pv1A C.. ... ..A ..A ... ...T ... .
Pv1B .C. ... ...G ..A .
Pv1C .C. ... .A ... ...T ..G ... .
Ps1A ..T ..A ..A ... ...T ... .
Ps1B ... ...T ... ...G ... .
Ms1A ..T ..C ..A ... ...
Ms1B .C. ... ..A ... ..T ..C ... ..T ..G ... .
Mt1A ..T ..C ..A ... ...T ... .
Mt1B .C. ..A ..A ... ..T ..C ... .G ... .
Mt1C .C. ... .A ..T ... ...
Lj1 ... ...
Lc GAA GGG AAG GGT TAC TTT GAG GAC AGA AGG C ctgcttctaacatggatccatatat

FIGURE 4.3 The amino acid sequence predicted from the *L. corniculatus* PCR clone compared to GS sequence from other species

The amino acid sequence of the 295 bp product amplified from total *L. corniculatus* DNA (Lc) is shown in full. The amino acid sequences from the other species are only shown where they differ from the *L. corniculatus* sequence. The underlined sequence is a putative substrate binding site (Tischer *et al.*, 1986).

Pv2 = *P. vulgaris* s/u (pcGS-δ1), Lightfoot *et al.*, 1988
Ps2 = *P. sativum* GS2 (pGS197), Tingey *et al.*, 1987
La1 = *L. angustifolius* GS1 (pGS5), Grant *et al.*, 1989
Gm1 = *G. max* GS1 (pGS20), Miao *et al*, 1991
Pv1A = *P. vulgaris* gln-α (pcGS-α1), Gebhardt *et al.*, 1986
Pv1B = *P. vulgaris* gln-ß (pcGS-ß1), *ibid*
Pv1C = *P. vulgaris* γ s/u (pcGS-γ1), Bennett *et al.*, 1989
Ps1A = *P. sativum* GS1 (pGS341), Tingey *et al.*, 1987
Ps1B = *P. sativum* GS1 (pGS134), *ibid*
Ms1A = *M. sativa* GS1 (pAnGS1), Dunn *et al.*, 1988
Ms1B = *M. sativa* GS1 gene, Tischer *et al.*, 1986
Mt1A = *M. trunculata* GS1 (MtGSa), Stanford *et al.*, 1993
Mt1B = *M. trunculata* GS1 (MtGSb), *ibid*
Mt1C = *M. trunculata* GS1 (MtGSc), *ibid*
Lj1 = *L. japonicus* GS1 (pGSA9) J.Stougaard, personal communication

FIGURE 4.3

```
Pv2   . . . . . K . . . . S . . . . S .     . . .
Ps2   . . . . . K . . . . S . D . . S .       . . .
Lal   . . . . . S . . S .       . . .
Gml   . . . . . .       . . .
PvlA  . . . . . S .       . . .
PvlB  . . . . . .       . . .
PvlC  . . . . . M . . . I .       . . .
PslA  . . . . . V . S .       . . .
PslB  . . . . . .       . . .
MslA  . . . . . S .       . . .
MslB  . . . . . .       . . .
Lj    . . . . . .       . . .

Lc    G N E R R L T G R H E T A D I N T F L W / G V A
```

```
Pv2   . . . C . . . . . N . . . . L . . . .
Ps2   . . . C . . . . . N . . . . L . . . .
Lal   . . . . . .
Gml   . . . . . V . . . . . A . . . . . .
PvlA  . . . S . V . . . . . Q . . . . . .
PvlB  . . . . . A . . . . . .
PvlC  . . . . . A . . . . . .
PslA  . . . S . . . . . D . . . . . .
PslB  . . . . . V . . . . . .
MslA  . . . . . V . . . . . D . . . . . .
MslB  . . . . . A . . . . . .
MtlA  . . . . . V . . . . . D . . . . . .
MtlB  . . . . . A . . . . . .
MtlC  . . . . . V . . . . . A . . . . . .
Lj    . . . . . .

Lc    N R G A S I R V G R D T E K E G K G Y F E D R R
```

TABLE 4.1 Percentage identity of the 159 bp nucleotide sequence surrounding intron 11 of various GS genes in eight legume species.

	Pv2	Ps2	Lal[a]	Gm1	PvlA	PvlB	PvlC	PslA	PslB	MslA	MslB	MtlA[a]	MtlB[a]	MtlC[a]	Ljl	Lcl
Pv2	100															
Ps2	92	100														
Lal[a]	68	65	100													
Gm1	71	71	82	100												
PvlA	72	72	80	82	100											
PvlB	71	71	83	91	82[80]	100										
PvlC	72[70]	71	78	88	79[79]	86[86]	100									
PslA	74	75	76	75	88	77	82	100								
PslB	72	72[69]	74	84	77	82	84	77[79]	100							
MslA	78	75	76	81	89	79	78	89	79	100						
MslB	72	70	78	84	78	84	86	75	88	79[81]	100					
MtlA[A]	72	70	*	83	86	81	82	89	80	97	83	100				
MtlB[A]	71	68	*	82	80	86	83	81	87	85	98	82	100			
MtlC[A]	67	62	*	88	81	84	87	77	86	80	83	80	83	100		
Ljl	75	72	83	91	82[73]	91[89]	89	78	86[86]	82	87	85	86	86	100	
Lc	75	72	83	91	82	91	89	78	86	82	87	85	86	86	100	100

[a] These sequences only overlapped with the others by 93 bp.

* These values are ignored as the two sequences only overlap by 27 bp

The superscript numbers are the percentage identities for the full length cDNAs

Underlined figures indicate strong similarity

Abbreviations are as in Figure 4.3

TABLE 4.2 Percentage identities of the 53 amino acids surrounding intron 11 of various GS genes in eight legume species.

	Pv2	Ps2	Lal[a]	Gm1	PvlA	PvlB	PvlC	PslA	PslB	MslA	MslB	MtlA*	MtlB*	MtlC*	Ljl	Lcl
Pv2	100															
Ps2	98	100														
Lal[a]	87	84	100													
Gm1	87	85	90	100												
PvlA	87	85	87	92	100											
PvlB	89	87	94	98	91^{88}	100										
PvlC	87^{78}	85	90	94	89^{86}	96^{90}	100									
PslA	89	89	90	91	92^{85}	92^{92}	91	100								
PslB	87	85^{78}	94	98	92	96	92	91^{89}	100							
MslA	87	85	94	94	92	92	91	92	94	100						
MslB	87	85	96	96	89	98^{95}	94	91^{97}	94	94	100					
MtlA[a]	84	84	*	94	90	90	90	87	94	100	94	100				
MtlB[a]	87	87	*	94	87	97	97	90	90	90	100	94	100			
MtlC[a]	84	84	*	84	90	94	94	87	94	94	94	94	94	100		
Ljl	89	87	94	96^{91}	91^{88}	98^{89}	94	92	98^{88}	92	96^{93}	90	94	90	100	
Lc	89	87	94	96	91	98	94	92	98	92	96	90	94	90	100	100

[a] These sequences overlapped with the others by only 31 residues.

* These values are ignored as the two sequences only overlap by 9 residues

The superscript numbers are the percentage identities for the amino acid sequences predicted from full length cDNAs

Underlined figures indicate strong similarity

Abbreviations are as in Figure 4.3

CHAPTER 5: THE EFFECT OF NITROGEN SOURCE ON GS ISOFORMS AND NITROGEN METABOLISM IN *L. JAPONICUS* AND *L. CORNICULATUS*.

5.1 INTRODUCTION

5.1.1 Nitrogen nutrition can alter expression of GS genes

Nitrogen source has been shown to affect expression of GS genes in various ways (see Chapter 1.4.4 and Forde and Woodall, 1994, for reviews). Of particular interest is that the GS2 polypeptide in root extracts of *P. sativum*, *M. media* and *V. sativa* is much more abundant when plants are grown on NO_3^-, but absent when NH_4^+ is the sole source of N (Barratt, 1980; Vézina and Langlois, 1989).

5.1.2 The amino acid composition in plants

The primary reaction in the biosynthesis of amino acids in plants is the amination of glutamate by GS to produce glutamine (see Figure 1.1), a reaction that can be regarded as the interface between C and N metabolism. Glutamate can also serve as an amino donor in transaminations reactions, e.g. to form aspartate, which can be converted further to asparagine, lysine, threonine, methionine and isoleucine. Glutamate is also a C and N source for the synthesis of many other metabolites, including arginine, proline, γ-aminobutyrate (GABA) and chlorophyll, and may also be a major export form of N under photorespiratory conditions (Madore and Grodzinski, 1984).

Glutamine is a major N transport compound in several species and is also used in many transamination reactions (with a concomitant regeneration of

glutamate), including the biosynthesis of asparagine from aspartate. Glutamine has also been implicated in feedback repression of NR genes (Deng *et al.*, 1991; Sechley *et al.*, 1992)

Asparagine is also a major N transport compound and is known to accumulate especially in plants faced with excess NH_4^+ rather than NO_3^-, e.g. during N_2-fixation, germination and senescence. Under these circumstances asparagine has been found to represent 86% of transported N (Joy, 1988). The degradative transamination of asparagine in leaves introduces N into the photorespiratory pathway, and is an important source of N and C during leaf development and in mature tissue. Asparagine is also degraded into NH_4^+ and aspartate through hydrolysis by asparaginase, a reaction which is only found in growing tissue, e.g. cotyledons and root tips (Sechley *et al.*, 1992).

The C:N ratio of asparagine (4:2) is higher than that of glutamine (5:2), so although the synthesis of asparagine requires more energy than the synthesis of glutamine (see Figure 1.1), it is a more economical compound for transport of N than glutamine. Also glutamine can be used by a wide variety of enzyme systems as mentioned above, and it is thought (Joy, 1988) that this high reactivity may account in part for the use of other compounds such as asparagine, as more common export products. N-labelling studies have shown that asparagine is rapidly synthesised by *L. japonicus* roots and is the major transport compound (D.T. Clarkson, personal communication).

Alanine is thought to be formed by transamination of pyruvate, with either glutamate, glutamine, serine, asparagine or GABA (which is a decarboxylation product of glutamate) as the N donor. Both alanine and GABA have been shown to

accumulate in response to several stresses (Streeter and Thompson, 1972; Good and Crosby, 1989), and alanine, along with asparagine, can be a major N donor during photorespiration. During photorespiration the peroxisome is a major source of glycine, which becomes a precursor of serine in a reaction that is localized in the mitochondria (Sechley *et al.*, 1992), although the chloroplast and the cytosol can also be important sites for synthesis of these two amino acids, indeed chloroplasts are the major site for biosynthesis of most amino acids within the cell (exceptions being asparagine and methionine). Of the remaining protein amino acids, cysteine is derived from serine whereas phenylalanine, tyrosine and tryptophan are all derived from the shikimate pathway. For a recent review see Sechley *et al.* (1992).

5.1.3 Vegetative storage proteins

Although all proteins can be said to store amino acids, their accumulation is usually governed by enzymatic or other metabolic activity. Therefore they have limited capacity for N storage. However it has recently been discovered that vegetative tissues may contain some polypeptides which have characteristics of storage proteins: they are abundantly expressed in vacuoles and are degraded to supply the nutritional requirements of other tissues (Staswick, 1990). These vegetative storage proteins (VSPs) were first found in *Glycine max* by Wittenbach (1983) who noted a protein composed of two glycosylated subunits of about 17 and 29 kD. These accumulated in the leaf to 10% of the total soluble protein during flowering, and declined during seed development. Removal of the seed pods led to accumulation to 50% of the total protein. This is one example of the preferential

loss of VSP as nutrients are translocated to new sinks, in this instance during during seed development. VSPs are found in all organs at different concentrations, and in leaves and stems at least have been found to be associated with vascular tissues (Bantroch et al., 1989). Putative VSPs are easily identified by comparing two-dimensional gel separations of proteins from plants grown on high or low N.

5.2 EXPERIMENTAL RATIONALE

This chapter examines the effect of NO_3^- and NH_4^+ on the GS isoforms and on N metabolism in *L. corniculatus* and *L. japonicus*, and investigates whether these two closely related species differ in their response. As a preliminary to investigating the effects of N source on GS, and to enable detection of NO_3^- contamination or nitrification in the NH_4^+ treatment, it was first necessary to characterize the NR activity in the leaf, stem and root of both species. These results can be seen in Chapter 5.3. In Chapter 5.4 plants were grown in various concentrations of NO_3^- or in NH_4^+, and the leaf, stem and root tissues were assayed for NR activity, and, along with the nutrient solutions and runoffs, assayed for NO_3^- concentration. Changes in the composition of subunits in the leaf, stem and root GS isoforms were followed by measuring total activity and by 1-D and 2-D SDS-PAGE. Tissue extracts were also analyzed for amino acid composition and comparisons were made between the two species, as well as between the different treatments. Two-dimensional separations of leaf proteins were also evaluated for the presence of a NO_3^--inducible vegetative storage protein (see Chapter 5.4.4).

5.3 PRELIMINARY ANALYSIS OF NR ACTIVITY IN TISSUES OF TWO *LOTUS* SPECIES

Most higher plants contain an NADH-specific nitrate reductase (NR), and some species (including *G. max*, *Z. mays*, *O. sativa*, and *H. vulgare*) have been found to contain an additional NAD(P)H-bispecific NR activity (Streit *et al.*, 1987; Redinbaugh and Campbell, 1981; Dailey *et al.*, 1982) which is predominantly present in the root. Both isoforms are induced by NO_3^-, although constitutive activities of NADH-NR have been described in several leguminous species (Andrews *et al.*, 1990).

The activity of NR can be assayed by *in vivo* or *in vitro* methods. The plant material in the *in vivo* assay remains fairly intact, and the endogenous electron donor is utilized. However, in the *in vitro* assay the enzyme is extracted and the NR activity measured in the presence of a large excess of exogenous reductant. Regardless of assay, the most sensitive method for detecting the NR activity is by colorimetric measurement of the product, NO_2^-. The NO_2^- is converted to a diazo-compound by reaction with sulfanilic acid which simultaneously stops the enzyme activity by lowering the pH. The diazo-compound then reacts with N-(1-naphthyl) ethylenediamine dehydrochloride to make an azo dye which can be measured spectrophotometrically at 540 nm.

5.3.1 A comparison of *in vitro* and *in vivo* NR activities.

Figure 5.1 shows the *in vivo* and *in vitro* NR activities in the leaf, stem and root of *L. japonicus* and *L. corniculatus* grown on 8 mM NO_3^- and 5 mM NH_4^+, either grown in sterile culture or in pots in a greenhouse. The results are the activity means of plants aged 25-55 days, between which the activities were

consistent. Both assays produced linear time courses of NO_2^- production, and modifications to the assay such as stopping the reaction by the addition of zinc acetate and PMS (Scholl *et al.*, 1974; Padidam *et al.*, 1991), did not enhance the colour development and the reaction was no longer linear with time (results not shown). The results presented in Figure 5.1A and B show that NR activities (assayed *in vivo* or *in vitro*) were very low in the leaves of both species grown in culture. The highest NR activities in each case were in the roots, with stems having intermediate *in vitro* NR activities. Based on the *in vivo* assay only, *L. corniculatus* stems had much higher activities than *L. japonicus* stems, similar to the root activities. The *in vitro* activities were in general higher than the *in vivo* activities in both species, but this was most pronounced for the stem of *L. japonicus* and the root of *L. corniculatus*. The most important difference between plants grown in sterile-culture and those grown in a greenhouse, was that the NR activity in the leaf was much higher when plants were grown in a greenhouse. The *in vitro* NR activity in leaves of *L. japonicus* grown in culture was only 20% of that in the greenhouse. The petiole activity was also lower (*ca* 27%) in culture-grown plants (not shown). There were differences in the light intensity and day length between these two growth conditions, but the difference in temperature (25°C in the growth chamber, *ca* 20°C in the greenhouse) may be the most significant factor (see Chapter 5.5.3 and Chapter 6).

5.3.2 Is NADPH-dependent NR activity detectable in either *Lotus* species?

The presence of NADPH-dependent NR activity was evaluated in the leaf, stem and root of *L. corniculatus* and *L. japonicus* using the *in vitro* method with

NADPH as the electron donor. Crude extracts of the leaves, stems and roots from plants grown in culture in NH_4NO_3 were incubated in assay buffer for 2 h, with aliquots removed every 15 min for determination of the NO_2^- formed. Two controls were set up either lacking exogenous reductant so that the presence of endogenous NAD(P)H could be assessed, or with enzyme extracts that had been previously boiled so as to gauge any non-enzymatic NO_2^- formation. There was no evidence for activity of this isoform in *L. japonicus*, but there was an indication of very low activity of NADPH-NR in the roots of *L. corniculatus* (Figure 5.2D). The two controls displayed the same kinetics in all three tissues, with a very low rate of NO_2^- accumulation over time. In the leaf and stem, the assay which contained NADPH had the same kinetics as the two controls, so that activity of an NADPH-NR was ruled out. However, the appearance of NO_2^- in the root extract was more than twice the rate of the controls, indicating a small but significant NADPH-NR activity. This activity was only *ca* 3% of the root NADH-NR activities (Figure 5.2A).

5.4 THE EFFECT OF NITROGEN SOURCE ON GS AND NITROGEN METABOLISM.

5.4.1 The concentration of nitrate in nutrient solutions and in run-off solutions following nutrient application.

The complete exclusion of NO_3^- from the system is a major problem when growing plants on a medium containing NH_4^+ as the sole source of N. Several precautions can be taken to prevent nitrification or contamination by NO_3^-. For example, the addition of nitrification inhibitors and sterilization of nutrient

solutions before application. It is also important to examine the system regularly for the presence of NO_3^-, either directly by assaying for NO_3^-, or indirectly by measuring the levels of the NO_3^--inducible enzyme, NADH-NR, in plant tissues.

Seedlings were established on vermiculite watered with Hornum medium containing 1 mM NO_3^-, and after 2-3 weeks, the nutrient solution was changed to 0.2 mM, 1 mM, 5 mM or 20 mM NO_3^--, or to 5 mM NH_4^+. The nutrient solutions were sterilized by autoclaving or filtering through a 0.2 μm membrane. The nitrification inhibitor dicyandiamide (DCD) was added (3% w/v), and the vermiculite thoroughly flushed through every morning with the treated nutrient solution. Plants were placed in a growth chamber with a 20°C/15°C, 16/8 h day/night regime.

A novel method of measuring NO_3^- (the *Aspergillus* NR method as described in Chapter 2.4.9 and 2.4.10) was used to routinely check the NO_3^- concentration in the nutrient solutions prior to application, and in the solution which drained from the pots following nutrient application (the run-off solution). In preliminary experiments it was found that this assay method was more accurate and consistent than the cadmium reductor method (Sloan and Sublett, 1966). The concentration of NH_4^+ in each solution was measured using the method of McCulloch (1967). In Table 5.1 it can be seen that NO_3^- could not be detected in the NH_4^+ solutions or in the runoff, indicating that the problem of contamination and nitrification was eliminated. Similarly, NH_4^+ could not be detected in the NO_3^- nutrient solutions or the run-offs.

The average NO_3^- concentration in the run-off from the 5 mM NO_3^- treatment showed that about 92% of the N was absorbed by the system. Slightly

less NH_4^+ was absorbed by the system treated with the 5 mM NH_4^+ nutrient solution (*ca* 82%). However, the run-off from the 20 mM NO_3^- treatment contained a slightly higher concentration of NO_3^- than was applied, indicating that these pots were NO_3^--saturated.

5.4.2 The effect of different nitrogen sources on *in vitro* NR activities and tissue nitrate concentrations

The *in vitro* NR activities and NO_3^- concentrations in the leaf, stem and root of *L. japonicus* and *L. corniculatus* were routinely measured to ensure that the NH_4^+ treatment was NO_3^--free. The NR activities and NO_3^- concentrations in both species grown on 5 mM NH_4^+ were at the limits of detection (Figure 5.3), but it is possible that these NR activities reflected the constitutive level of the enzyme in these tissues in accordance with Andrews *et al.* (1990). It was apparent that the NO_3^- concentrations and NR activities of *L. japonicus* grown on 5 mM NO_3^- were at their maximum, as they did not increase at the higher NO_3^- concentration (20 mM). This was in contrast to *L. corniculatus*, where plants grown on 20 mM NO_3^- had higher tissue NO_3^- concentrations and NR activities than plants grown on 5 mM NO_3^- (Figure 5.3B and D). It is significant that leaves of both species grown in 5 mM NO_3^- had high NO_3^- concentrations, so that the low NR activities in the leaves of these plants were not due to the failure of the NO_3^- to reach these tissues.

5.4.3 The effect of nitrogen source on GS activities and polypeptides

To assess the influence of NO_3^- and NH_4^+ on the GS1 and GS2 isoforms in *L. japonicus*, the GS subunits comprising the two isoforms were separated by SDS-

PAGE and western blotted (Figure 5.4A and C). The densities of the GS protein bands on both blots were estimated using a gel scanner, the peaks integrated, and the values plotted as a proportion of the value for GS2 in the leaf of plants grown in 5 mM NO_3^- (Figure 5.4B and D). The total GS activities in each tissue were also measured (Figure 5.5).

The most significant effect of the N source on GS was that GS2 was present in roots of plants grown on NO_3^- but not detectable in NH_4^+-grown roots. Both GS2 and GS1 polypeptides were present in the stems and leaves of *L. japonicus* under all three N-regimes, and the N source had little effect on the relative proportions of the two proteins in these tissues.

In *L. japonicus*, the absence of GS2 protein in the roots of plants grown on 5 mM NH_4^+ was reflected in the total GS activity, which was significantly less (at the 95% confidence level) than those grown on 5 mM NO_3^- (*ca* 50%; Figure 5.5A). The GS activity in roots grown on 0.2 mM NO_3^- was also significantly lower than those on 5 mM NO_3^-, and surprisingly, so too was the activity in roots grown on 20 mM NO_3^-. However there was no significant effect of the N source on leaf or stem GS activity. There was little or no change in GS activity in roots, stems or leaves of *L. corniculatus* grown under the three N conditions (Figure 5.5B).

When separated by 2-D electrophoresis (Figure 5.6), at least two of the charge variants that were detectable in roots of *L. japonicus* grown on 5 mM NO_3^- were also identifiable in roots grown on 0.2 mM NO_3^-, and in the radicle germinated on sterile distilled water. However most of the GS2 charge variants were absent in roots grown on NH_4^+, with the exception of the charge variant

closest to the more basic end of the IEF gradient, which was present in both *L. japonicus* and *L. corniculatus*. Therefore, either the GS2 polypeptide is present constitutively at low levels in the roots of plants grown on NH_4^+, or NO_3^- was not entirely excluded from the NH_4^+ treatment.

5.4.4 The presence of a putative vegetative storage protein in the leaves of *L. japonicus*.

The total proteins in the leaf tissue of *L. japonicus* plants grown on either 20 mM NO_3^- or 0.2 mM NO_3^- were separated by 2-D electrophoresis, and detected by silver staining (Figure 5.7). It was apparent that one protein, although present at low levels in leaves grown under the low NO_3^- regime, was expressed abundantly in the leaves grown on high NO_3^-. This protein was estimated to be approximately **22** kD in size, and may be a vegetative storage protein as described by Staswick (1990).

5.4.5 The nitrate and amino acid composition of both species grown on different nitrogen sources

The differences in enzyme activities between the different N treatments and between the two species, prompted further investigation of their respective N metabolism. High performance liquid chromatography was used to determine the amino acid composition of the leaf, stem and root of plants grown on either 20 mM NO_3^-, 5 mM NO_3^- or 5 mM NH_4^+. These were tabulated along with the concentrations of NO_3^- found in each tissue, and, in *L. japonicus*, the NH_4^+ levels (Table 5.2 and 5.3).

A) The major N compounds found in L. japonicus.

The major amino acid in all tissues of *L. japonicus* grown under all three N regimes was asparagine (Table 5.2). In plants grown on NH_4^+, the leaf, stem and root asparagine levels were (as a proportion of the total amino N) 63%, 54% and 85% respectively, and although the actual concentration of asparagine was much higher in tissues of *L. japonicus* grown on NH_4^+ than on NO_3^- (e.g. ten-fold higher in the roots of NH_4^+-grown plants), the percentage of total amino N represented by asparagine in plants grown on 5 mM NO_3^- was very similar to that in those grown on 5 mM NH_4^+ (e.g. 59%, 57% and 69% in leaf stem and root respectively). This implies that asparagine is the major N transport compound in *L. japonicus* regardless of N source.

The other major amino acids in the leaves and stems under all the N regimes were glutamate, aspartate, serine, GABA and alanine. The relative proportions of these amino acids (as a percentage of total amino N) were very similar in plants grown under each N regime. Glutamate levels were particularly high, comprising 13-24% of the total reduced N in the leaves and *ca* 10% in the stem in all three treatments, and the concentration of GABA was at its highest in the stem, comprising 4-8% of the total amino N.

The total concentration of amino N in the roots was 10-fold greater in the NH_4^+ treatment than under the two NO_3^- treatments, primarily due to the extremely high level of asparagine (85% of the total). The roots grown on NH_4^+ also had very high levels of glutamine (*ca* 8% of the total N), an amino acid not detected in roots of plants grown on NO_3^-, and present at levels six- to nine-fold higher than in NH_4^+-grown leaf and stem tissue. The other major amino acids in the roots were

glutamate, aspartate and serine, although they were present at lower levels than in the other tissues.

B) The major N compounds found in L. corniculatus.

As was the case in *L. japonicus*, asparagine was the predominant amino acid in *L. corniculatus* tissues (Table 5.3). However the proportion of amino N contributed by asparagine was actually higher in the leaf and stem of plants grown on 5 mM NO_3^- (40% and 64% respectively) than in those grown on 5 mM NH_4^+ (15% and 38%) and this was reflected in the concentrations of asparagine in these two tissues, i.e. greater than 3 μmol.g^{-1}fwt in NO_3^--grown plants and approximately 1 μmol.g^{-1}fwt in NH_4^+-grown plants. The asparagine content in the roots of all three treatments was very similar (75-83% of total amino N). The asparagine concentrations in the roots of the NO_3^--grown *L. corniculatus* plants were two- to four-fold higher than those in *L. japonicus*, but the concentration in NH_4^+-grown roots represented less than a third of what was found in roots of NH_4^+-grown *L. japonicus*.

The other major amino acids in the leaves and stems were, as in *L. japonicus*, glutamate, aspartate, serine, alanine and GABA. Alanine comprised up to 16% of the amino N in the leaves of *L. corniculatus*, in contrast to only 4-8% in leaves and stems of *L. japonicus*. Unlike in *L. japonicus*, where the concentration of GABA was highest in the stem (up to 8%), GABA was more predominant in the leaves of *L. corniculatus* representing 14% of the total amino N in leaves of NH_4^+-grown plants.

Although in *L. japonicus* the total concentration of reduced N was ten times

greater in NH_4^+-grown roots than in NO_3^--grown roots, the total reduced N in roots of *L. corniculatus* grown on all treatments were fairly similar as the asparagine concentrations did not differ significantly as was the case for *L. japonicus*. Glutamine levels in roots of *L. corniculatus* grown on NH_4^+ (13% of total N) were higher than was found in *L. japonicus* (8% of total N), and glutamine was again virtually undetectable in the NO_3^--grown plants.

C) The concentration of NO_3^- in both species

As might be expected, the concentration of NO_3^- in both species when grown on 20 mM and 5 mM NO_3^- was very high (see Table 5.2 and 5.3), comprising 90-96% of the total N (soluble in methanol) in *L. japonicus* and 83-96% in *L. corniculatus*. The NO_3^- concentrations in the leaf, stem and root of *L. corniculatus* grown on 5 mM NO_3^- were only 35-50% that found in *L. japonicus*. In plants grown on 20 mM NO_3^- the concentration of NO_3^- in the leaf of *L. corniculatus* was also 50% lower than that found in *L. japonicus*, but the stem and root NO_3^- concentrations in the two species were very similar. This suggests that in the 5 mM NO_3^- treatment the NO_3^- was either not being transported as efficiently to *L. corniculatus* shoots, or was being diluted by growth (*L. corniculatus* being much larger than *L. japonicus*), or was being assimilated rather than stored. The higher shoot NR activity in *L. corniculatus* (see Chapter 5.3.1) suggested that assimilation was the reason. In the NH_4^+ treatment, low levels of NO_3^- were found in tissues of both species, although these were just at the limits of detection.

5.4.6 Differences between the two species in protein concentration, fresh weight, dry weight and chlorophyll content

It was conjectured that some of the differences in tissue NO_3^- concentration and amino N content between *L. japonicus* and *L. corniculatus* might be a reflection of different water or protein contents. *L. japonicus* is a much smaller plant than *L. corniculatus* (Figure 5.8E and F), but there is little difference between the two species in protein content (Figure 5.8C and D), and although *L. japonicus* has a higher percentage of dry matter (Figure 5.8G and H), the greater water content in *L. corniculatus* is not enough to account for the differences in their N concentrations. It is also noteworthy that although *L. japonicus* leaves appear much darker green than those of *L. corniculatus*, the chlorophyll contents were very similar (Figure 5.8A and B).

5.5 DISCUSSION

5.5.1 The presence of root GS2 in nitrate-grown plants

Nitrate has been shown to induce GS2 in the roots of three legume species (Barratt, 1980; Vézina and Langlois, 1989). Evidence is presented in this chapter confirming the major role of NO_3^- in increasing the level of root GS2 in two more legume species, *L. japonicus* and *L. corniculatus*. The GS2 polypeptide was not entirely absent in roots grown on NH_4^+, as one of the isoelectric variants was consistently present (Figure 5.6). It is possible that low levels of GS2 are constitutively synthesised, or its presence may reflect the very low concentrations of NO_3^- that were found in the roots of both species grown on NH_4^+ (see Tables 5.2 and 5.3).

5.5.2 NR activity, nitrate assimilation and the root GS2 isoform

Both species of *Lotus* had a considerable proportion of their NR activity in the root, and as this activity increased with increasing concentrations of exogenous NO_3^-, it seems likely that both species assimilate a major part of their NO_3^- in the root, although the stem tissue could also play a major part in NO_3^- assimilation in *L. corniculatus*. These results were in agreement with the results of Monza *et al.* (1989) who found 52% of total *in vivo* NR activity to be in the stem of *L. corniculatus* when grown on relatively high concentration of NO_3^- (5 mM NO_3^-), with a third of the total NR activity being found in the roots.

That a major part of the NO_3^- assimilatory capacity was found in the roots of both species was in accordance with the findings of Andrews (1986a), who suggested that root NO_3^- assimilation was a feature of legumes whose origins lie in temperate habitats, as opposed to shoot NO_3^- assimilation, which predominates in tropical legumes. The apparent correlation between root NO_3^- assimilation and a NO_3^--inducible GS2 in the root of *P. sativum*, *M. media*, *V. faba*, *L. corniculatus* and *L. japonicus* (all temperate species), was thought to be a strong indication that GS2 might assimilate the NH_4^+ derived from NO_3^- reduction (Forde and Woodall, 1994). Partitioning of NO_3^- assimilation, and its association with the presence of root GS2, is dealt with more fully in Chapter 6.

5.5.3 Temperature may affect the partitioning of NR activity

The distribution of *in vitro* NR activities in *L. japonicus* was slightly different in plants grown in sterile culture to those grown in pots in a greenhouse. Even though the plants were supplied with the same N source, significantly higher

NR activity could be detected in the leaves and petioles of *L. japonicus* (also apparent to a lesser extent in *L. corniculatus*) when grown in a greenhouse, whilst the root NR activity in L. corniculatus was higher in sterile culture. This may be associated with the fact that the temperature in the greenhouse, although variable, would have been lower than in the growth chamber (*ca* 20°C as opposed to 25°C). Evidence to support this idea is presented in Chapter 6.

5.5.4 Is *L. corniculatus* more stress tolerant than *L. japonicus?*

There was an indication that *L. japonicus* plants preferred growing on NO_3^- at concentrations less than 20 mM. The total fresh weight of plants grown on 20 mM NO_3^- were less than when grown on 5 mM NO_3^- or NH_4^+, even though the ratios of dry weight to fresh weight were very similar in all treatments. The concentrations of amino acids in the leaves of plants fed on 20 mM NO_3^- were also lower than in those grown on 5 mM NO_3^-. This was not the case with *L. corniculatus*, where plants grown on 20 mM NO_3^- had slightly higher fresh weights than those grown on 5 mM NO_3^- (although not as high as when grown on 5 mM NH_4^+), and similar amino acid concentrations in all tissues.

The higher fresh weight of *L. corniculatus* plants when grown on 5 mM NH_4^+, as opposed to 5 mM NO_3^-, may reflect different rates of uptake or more efficient utilization. The uptake of NO_3^- occurs against an electrochemical gradient and is energy dependent (Glass *et al.*, 1992; King *et al.*, 1992) unlike the uptake of NH_4^+ which has both an energy-dependent and an energy-independent uptake system. Consequently, uptake of NH_4^+ is not impaired if the rooting environment is lacking in O_2, but NO_3^- uptake is (Lewis, 1986). There was some evidence to

suggest that the root environment was experiencing mild hypoxia (shortage of O_2 due to saturation of the vermiculite water-holding capacity. This was inevitable as the vermiculite was flushed through with nutrient solution every day to prevent nitrification). When roots suffer a shortage of O_2 the most consistently observed change in amino acid composition is that GABA rapidly accumulates (Zemlianukhin and Ivanov, 1978; Sechley et al., 1992) due to a switch from protein synthesis to reductive amination. GABA was shown to be present at appreciable levels in the tissues of both species (Table 5.2 and 5.3).

5.5.5 *L. japonicus* probably has a vegetative storage protein in its leaves

Two-dimensional electrophoresis of protein extracts from leaves of *L. japonicus* plants showed the existence of a **22** kD polypeptide which represented the major protein species in plants grown on high NO_3^-, but which was absent or only present at low levels in plants grown on low NO_3^-. This pattern of synthesis is consistent with that of a VSP (Staswick, 1990) and the size of the polypeptide (*ca* **22** kD) is in accordance with the 17 kD subunit of the VSP found in *G. max* (Wittenbach, 1983). However, whilst the *G. max* VSP subunits are both known to be glycosylated, there was no evidence of the *L. japonicus* polypeptide being glycosylated in blots which have been stained for glycan residues (Figure 3.3 Chapter 3).

5.5.6 The major nitrogen transport compounds in the two species

Analysis of the amino acid composition in the leaf, stem and root grown under different nitrogen regimes revealed that asparagine was the major amino compound in both *Lotus* species, undoubtedly reflecting its role as an N transport compound (Sechley et al., 1992). Although glutamine is a major N transport compound in several root NO_3^- assimilators, it was only present in significant amounts in the roots *L. corniculatus* or *L. japonicus* plants grown on NH_4^+. This is in accordance with Maxwell *et al.* (1984) who found in ^{14}C-labelling studies that glutamine was the major labelled amino acid in the roots of nodulated *L. corniculatus* plants, and the third most abundant amino acid in the xylem sap.

5.5.7 Two closely related *Lotus* species differ in their distribution of asparagine

Although asparagine was the predominant amino acid in both *L. corniculatus* and *L. japonicus*, the two species differed greatly in asparagine concentration and distribution between tissues under the three N regimes. In *L. japonicus* the leaf, stem and root asparagine levels were (as a proportion of the total amino N) very similar in all treatments, although the actual concentration of asparagine was much higher in tissues of *L. japonicus* grown on NH_4^+ than on NO_3^- especially in the roots. This implies that asparagine is the major N transport compound in *L. japonicus* regardless of N source.

Unlike *L. japonicus*, the proportion of amino N contributed by asparagine in *L. corniculatus* was actually higher in the leaf and stem of NO_3^--grown plants than in those grown on NH_4^+, and the asparagine concentration in the roots was

similar in each treatment. Whereas the roots of *L. corniculatus* grown on NO_3^- contained up to four times the amount of asparagine than that found in *L. japonicus*, the asparagine concentration in the NH_4^+-grown roots represented less than a third of what was found in roots of NH_4^+-grown *L. japonicus*.

5.5.8 Two closely related legumes show marked differences in their nitrogen metabolism

The differences between *L. japonicus* and *L. corniculatus* in their responses to increasing NO_3^- and in their distribution of asparagine, were very surprising considering that the two species are closely related. The fact that *L. corniculatus* is a tetraploid and *L. japonicus* a diploid, may account for some of these differences. *L. corniculatus* also seemed to adapt more easily than its oriental relative to being grown in sterile culture and/or at higher concentrations of exogenous N, probably reflecting its adaptability and wide-ranging ecology. Grime *et al.* (1988) regards *L. corniculatus* as being one of the most wide-ranging of legumes, extending from maritime to montane habitats, withstanding fairly low to high pH and both fertile and infertile soils. This species is genotypically and phenotypically variable (Chrtkova-Zertova, 1973), with this variability being crucial in determining its ability to exploit different habitats.

FIGURE 5.1 **NR activities in the leaves, stems and roots of culture-grown and greenhouse-grown *L. japonicus* and *L. corniculatus***

The activities are the means of plants aged between 25-55 d grown on 8 mM NO_3^- and 5 mM NH_4^+.

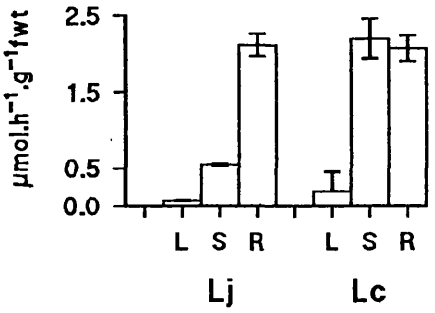
A) *In vivo* NR activities of plants grown in sterile culture

B) *In vitro* activities of plants grown in sterile culture

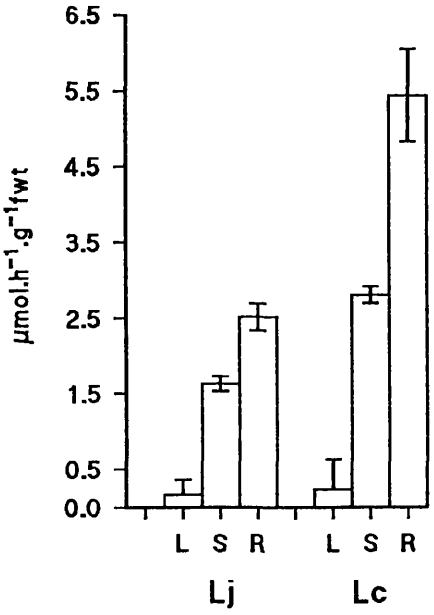
C) *In vitro* activities of plants grown in a greenhouse.

FIGURE 5.1

A) *In vivo* NR activity (culture-grown)



B) *In vitro* NR activity (culture-grown)



C) *In vitro* NR activity (greenhouse-grown)

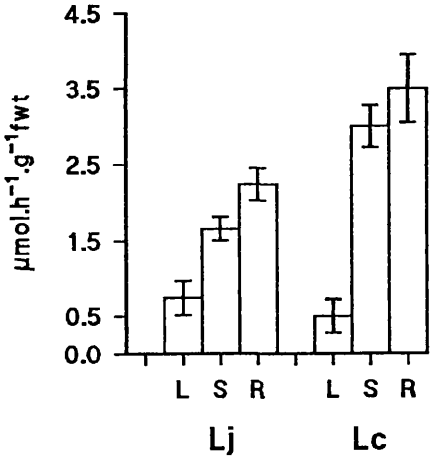


FIGURE 5.2 ***In vitro*** **NADH- and NADPH-NR activities in *L. corniculatus*, leaves, stems and roots**

Plants were grown in culture on 8 mM NO_3^- and 5 mM NH_4^+. Crude enzyme extracts were incubated for 2 h in assay buffer containing either NADH or NADPH. Aliquots were removed at 15 min intervals for NO_2^- determination.

A) NADH-NR activity in leaf (▼), stem (▲) and root (*).

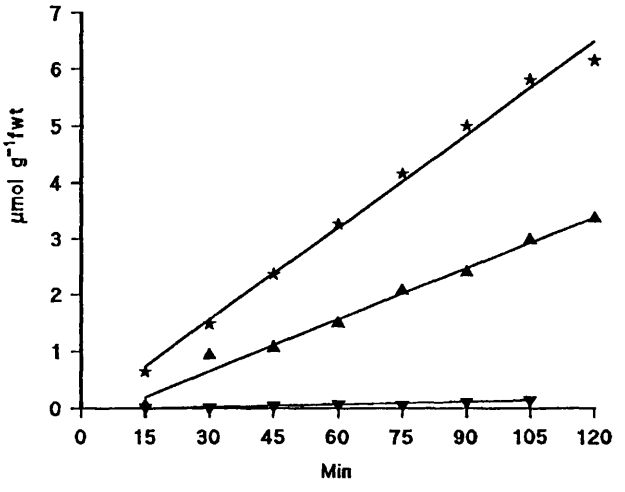
B) NADPH-NR activity in leaf extract (▼), compared with two controls. Control 1 (□) exogenous reductant was not added to the assay; in control 2 (o) the enzyme extract had been boiled for 10 min prior to the assay.

C) Stem NADPH-NR activity (▲) and compared with two controls. The controls were as in caption B).

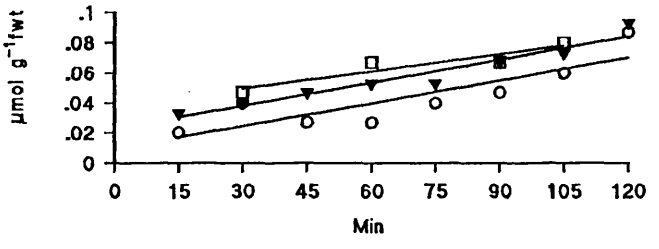
D) Root NADPH-NR activity (*) compared to two controls. The controls were as in caption B).

FIGURE 5.2

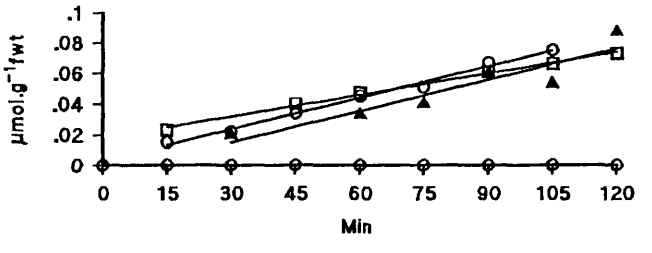
A) NADH–NR activity



B) Leaf NADPH–NR activity



C) Stem NADPH–NR activity



D) Root NADPH–NR activity

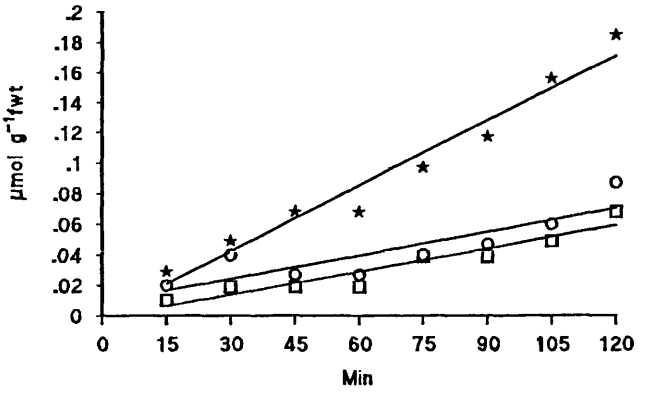


TABLE 5.1 **Concentration of nitrate and ammonium in nutrient solutions and runoffs**

The concentration of NO_3^- and NH_4^+ in nutrient solutions before application, and in the runoff solution collected from the vermiculite following nutrient application.

Nutrient Solution	NO_3^- Concentration	NH_4+ Concentration
20 mM NO_3^-	20.50 ± 1.37	ND
runoff	22.81 ± .39	ND
5 mM NO_3^-	4.99 ± .17	ND
runoff	.42 ± .08	ND
0.2 mM NO_3^-	.20 ± .01	ND
runoff	.11 ± .01	ND
5 mM NH_4^+	ND	5.08 ± .19
runoff	ND	.88 ± .34

ND = not detected.

FIGURE 5.3 The effect of different nitrogen sources on the *in vitro* NR activities and tissue nitrate concentration in *L. japonicus* and *L. corniculatus*

Plants were grown in a growth chamber with a 20°C/15°C, 16/8 h day/night regime, and watered daily with either 20 mM NO_3^-, 5 mM NO_3^-, 0.2 mM NO_3^-, or 5 mM NH_4^+.

A) NR activities in the leaves, stems and roots of *L. japonicus* plants

B) NR activities in the leaves, stems and roots of *L. corniculatus* plants

C) Tissue NO_3^- concentrations in the leaves, stems and roots of *L. japonicus* plants

D) Tissue NO_3^- concentrations in the leaves, stems and roots of *L. corniculatus* plants

FIGURE 5.3

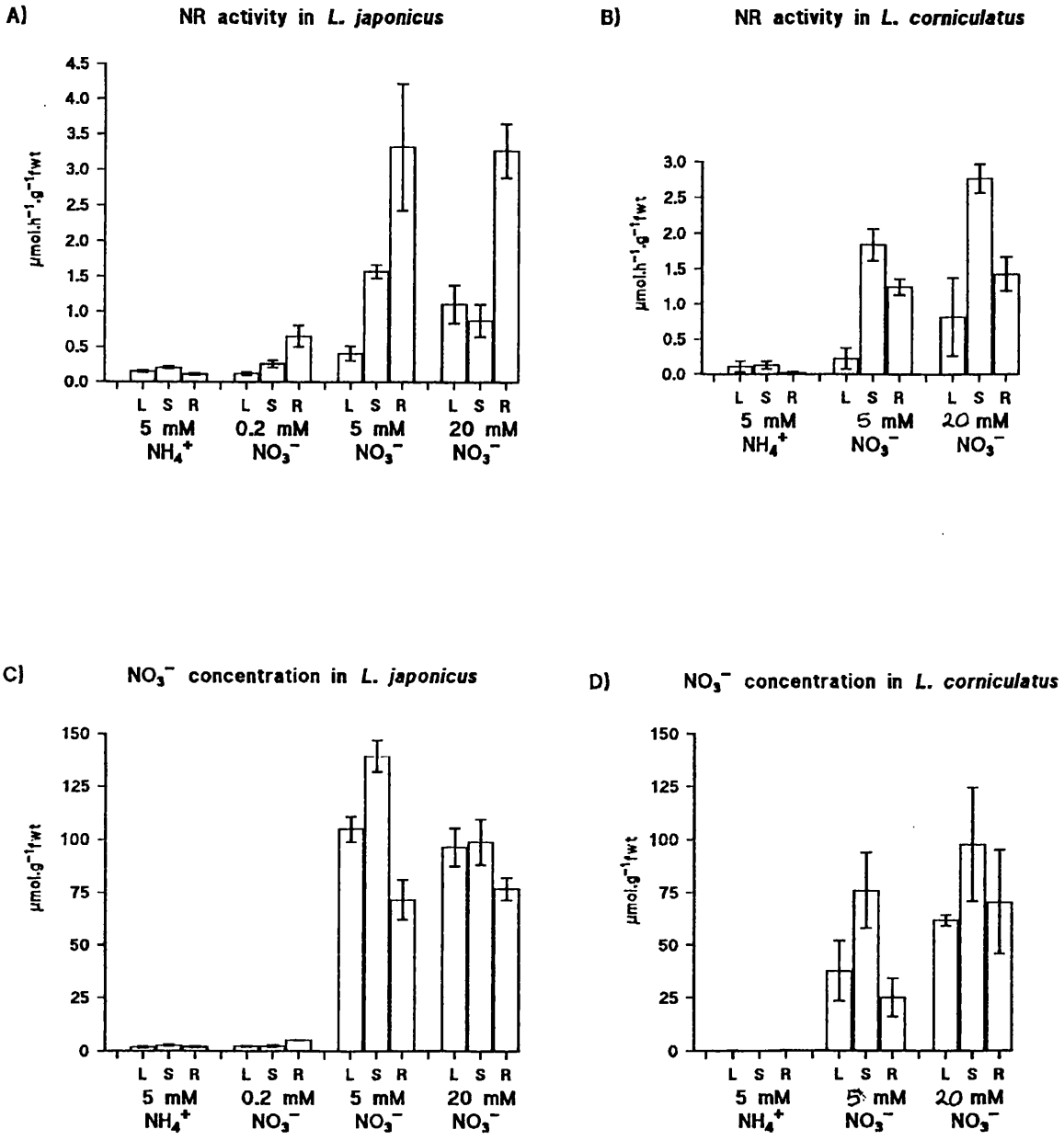

A) NR activity in L. japonicus
B) NR activity in L. corniculatus
C) NO3- concentration in L. japonicus
D) NO3- concentration in L. corniculatus
µmol.h-1.g-1fwt
µmol.g-1fwt
L S R
5 mM NH4+
0.2 mM NO3-
5 mM NO3-
20 mM NO3-

FIGURE 5.4 The effect of different nitrogen sources on the subunit composition of GS in the leaves, stems and roots of *L. japonicus*

Crude protein extracts were separated on a 10% SDS-polyacrylamide gel, and the western blots probed with antibody raised against *P. vulgaris* nodule GS.

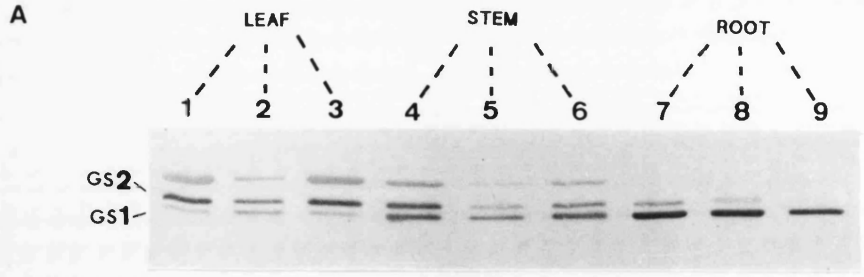
A) Western blot of leaves (lanes 1, 2 and 3), stems (lanes 4, 5 and 6) and roots (lanes 7, 8 and 9) of *L. japonicus* supplied with either 5 mM NO_3^- (lanes 1, 4 and 7), 0.2 mM NO_3^- (lanes 2, 5 and 8) or 5 mM NH_4^+ (lanes 3, 6 and 9). The gel was loaded with 1 μg of total protein. The GS activities (in nmol. h^{-1}.$lane^{-1}$) were respectively, 5.9, 4.6, 4.5, 4.2, 2.9, 3.5, 8.0, 5.2 and 3.9.

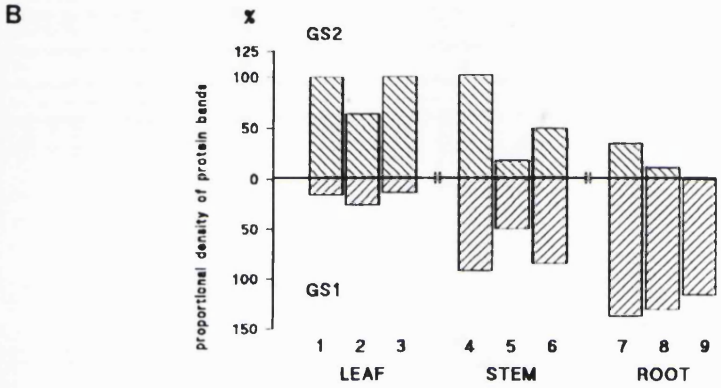
B) Proportional densities of the GS2 bands (above the abscissa) and the GS1 bands (below the abscissa) in the blot shown in A). A gel scanner was used to obtain density profiles of the protein bands in each lane. The RuBisCO peaks were ignored, but the areas under the GS peaks were integrated and the values used to calculate the relative proportions of the two isoforms relative to the GS2 band at 100%

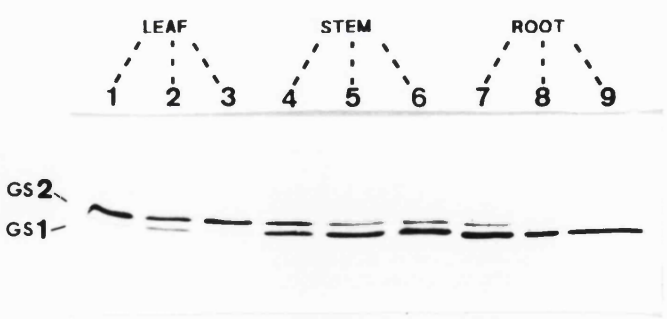
C) Western blot using the same extracts as in B), but loaded to give 5 nmol.h^{-1}.$lane^{-1}$ GS activity.

D) Proportional densities of the GS2 and GS1 bands in the blot shown in C). See caption B for more details.

FIGURE 5.4


A
LEAF
STEM
ROOT
1 2 3 4 5 6 7 8 9
GS2
GS1


B
x
GS2
125
100
50
0
50
100
150
GS1
proportional density of protein bands
1 2 3 4 5 6 7 8 9
LEAF
STEM
ROOT


C
LEAF
STEM
ROOT
1 2 3 4 5 6 7 8 9
GS2
GS1

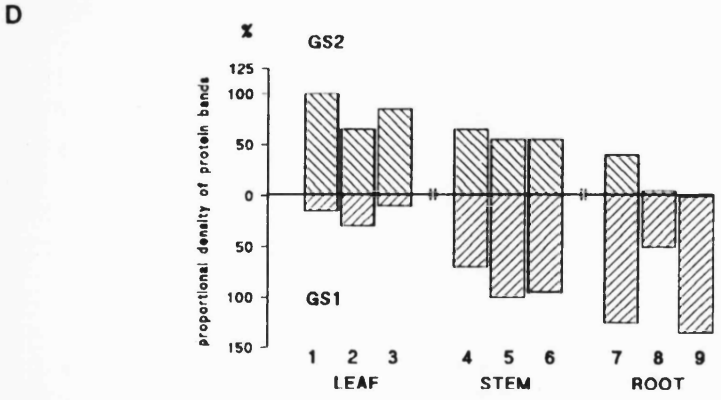

D
x
GS2
125
100
50
0
50
100
150
GS1
proportional density of protein bands
1 2 3 4 5 6 7 8 9
LEAF
STEM
ROOT

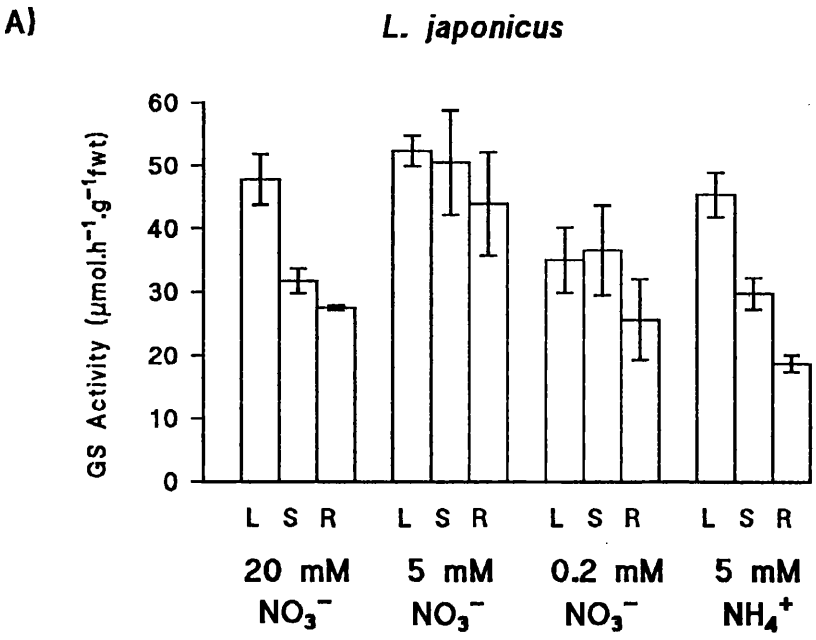
FIGURE 5.5 The effect of different nitrogen sources on the activity of GS in *L. japonicus* and *L. corniculatus*

Plants were grown in a growth chamber with a 20°C/15°C, 16/8 h day/night regime, and watered daily with either 20 mM NO_3^-, 5 mM NO_3^-, 0.2 mM NO_3^-, or 5 mM NH_4^+.

A) GS activities in leaves, stems and roots of *L. japonicus* plants

B) GS activities in the leaves, stems and roots of *L. corniculatus* plants

FIGURE 5.5


A)
L. japonicus
GS Activity (µmol.h⁻¹.g⁻¹fwt)
60
50
40
30
20
10
0
L S R L S R L S R L S R
20 mM NO3⁻
5 mM NO3⁻
0.2 mM NO3⁻
5 mM NH4⁺

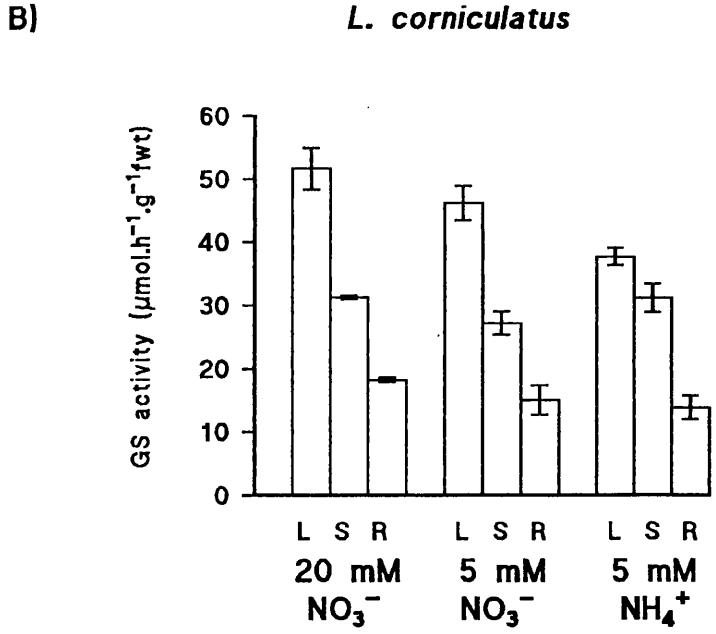

B)
L. corniculatus
GS activity (µmol.h⁻¹.g⁻¹fwt)
60
50
40
30
20
10
0
L S R L S R L S R
20 mM NO3⁻
5 mM NO3⁻
5 mM NH4⁺

FIGURE 5.6 Two dimensional separations of root GS subunits in *L. corniculatus* and *L. japonicus* grown on different nitrogen sources.

Plants were grown in a growth chamber with a 20°C/15°C, 16/8 h day/night regime, and watered daily with either 5 mM NO_3^-, 0.2 mM NO_3^-, or 5 mM NH_4^+ solutions.

Crude protein extracts (6 μg) were separated by isoelectric focusing, then on a 10% SDS polyacrylamide gel, and the western blots probed with antibody raised against *P. vulgaris* nodule GS.

A) Root of *L. corniculatus* grown on 5 mM NO_3^-. The GS activity was 32 $nmol.h^{-1}$.

B) Root of *L. corniculatus* grown on 5 mM NH_4^+. The GS activity was 27 $nmol.h^{-1}$.

C) Root of *L. japonicus* grown on 5 mM NO_3^-. The GS activity was 48 $nmol.h^{-1}$.

D) Root of *L. japonicus* grown on 0.2 mM NO_3^-. The GS activity was 31 $nmol.h^{-1}$.

E) Root of *L. japonicus* grown on 5 mM NH_4^+. The GS activity was 32 $nmol.h^{-1}$.

F) Radical of *L. japonicus* germinated on sterile ddH_2O. The GS activity was 25 $nmol.h^{-1}$.

FIGURE 5.6

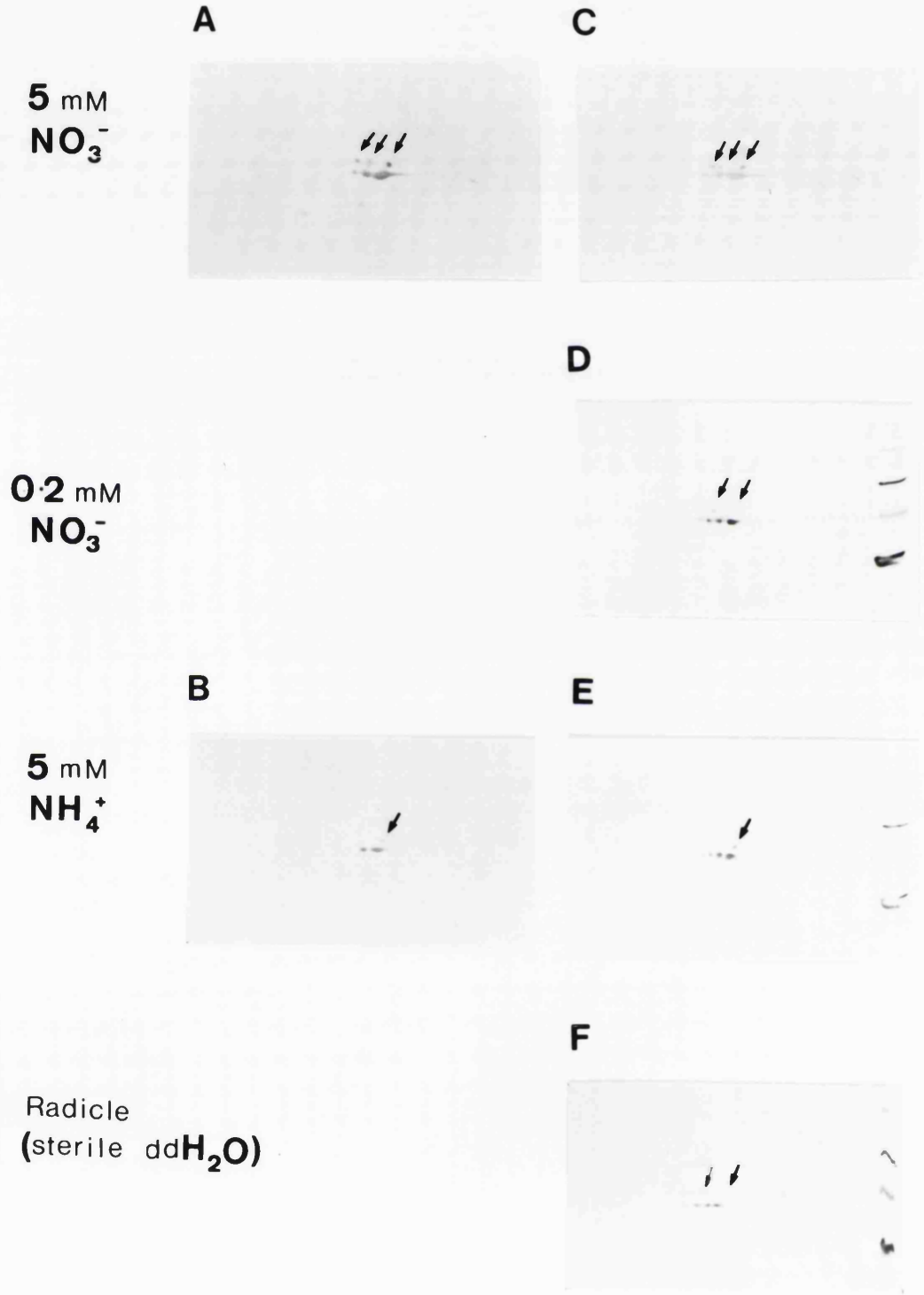


FIGURE 5.7 Two-dimensional separation of the leaf total proteins of *L. japonicus* grown on high or low nitrate

Plants were grown in a growth chamber with a 20°C/15°C, 16/8 h day/night regime, and watered daily with either 20 mM NO_3^- or 0.2 mM NO_3^- solutions.

The crude protein extracts (12 μg) were separated by isoelectric focusing, then on a 10% SDS polyacrylamide gel. Proteins were identified by silver staining. The arrows mark a putative vegetative storage protein.

A) Leaf proteins from plants grown on 20 mM NO_3^-

B) leaf proteins from plants grown on 0.2 mM NO_3^-.

FIGURE 5.7

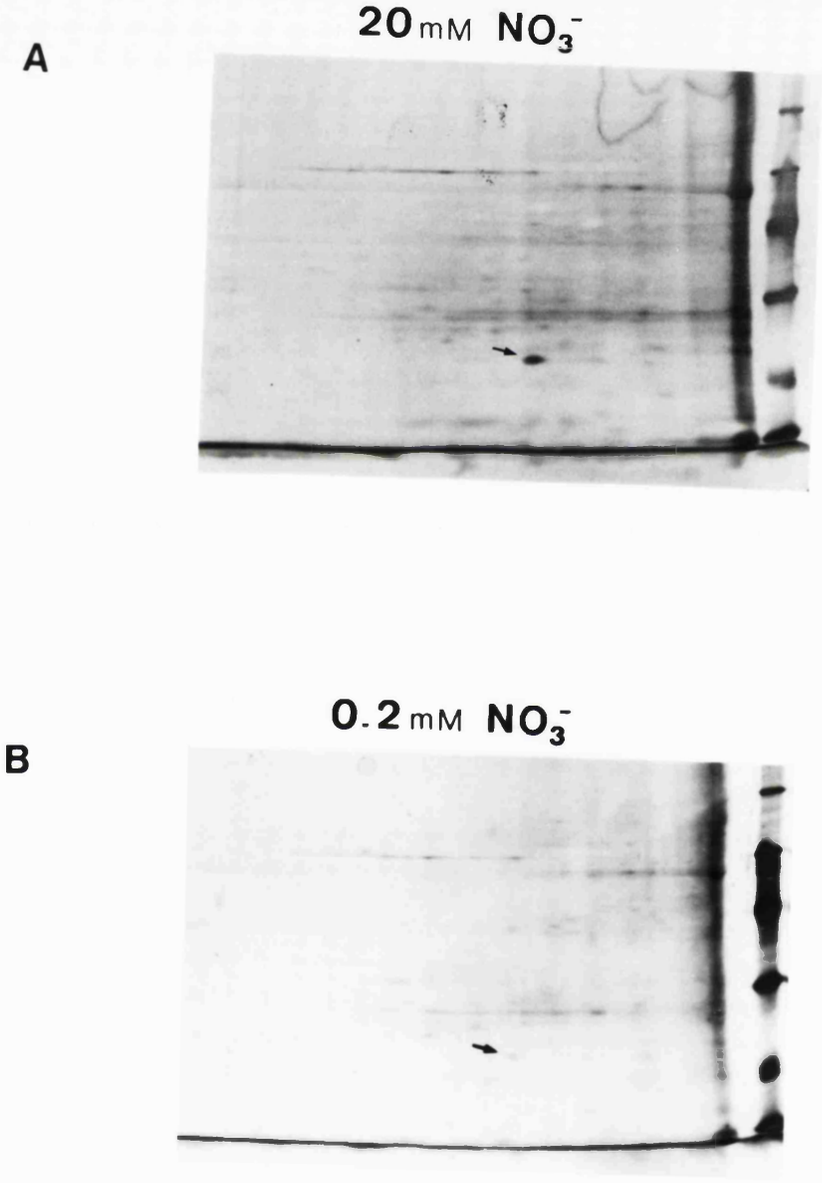


TABLE 5.2 Composition of amino acids, and the concentrations of ammonium and nitrate in leaves, stems and roots of *L. japonicus* grown under three nitrogen regimes

Plants were grown in a growth chamber with a 20°C/15°C, 16/8 h day/night regime, and watered daily with either 20 mM NO_3^-, 5 mM NO_3^-, or 5 mM NH_4^+ solutions. Tissues were extracted in methanol and the amino acids separated by HPLC. The *Aspergillus* NR method was used to determine the concentration of NO_3^-

ND = not detected.

TABLE 5.2

Tissue	Amino Acid	Concentrations (μmol.g^{-1}fwt) 20 mM NO_3^-	5 mM NO_3^-	5 mM NH_4^+
LEAF	Asp	.57 ± .19	.62 ± .10	.93 ± .07
	Glu	1.21 ± .26	1.70 ± .16	2.28 ± .30
	Asn	1.83 ± .40	6.96 ± 1.16	11.05 ± 3.34
	Ser	.56 ± .19	.67 ± .01	.71 ± .16
	Gln	.04 ± .06	.08 ± .01	.46 ± .24
	Thr	.14 ± .03	.26 ± .03	.38 ± .04
	Ala	.37 ± .14	.45 ± .05	.76 ± .18
	Ile	.02 ± .01	.10 ± .01	.11 ± .02
	Gly	ND	.05 ± .06	.13 ± .10
	Gaba	.01 ± .02	.16 ± .13	.28 ± .21
	others	.02	.08	.13
	NH_4^+	.24 ± .13	.76 ± .03	.23 ± .20
TOTAL REDUCED N		5.01	11.89	17.45
	NO_3^-	122.70 ± 8.93	105.09 ± 5.88	1.34 ± .43
STEM	Asp	.60 ± .24	.56 ± .12	.51 ± .04
	Glu	.75 ± .27	.93 ± .19	.72 ± .04
	Asn	4.54 ± 2.28	5.04 ± 1.21	4.45 ± .45
	Ser	.48 ± .23	.45 ± .06	.40 ± .01
	Gln	.10 ± .05	.20 ± .02	.29 ± .24
	Thr	.22 ± .09	.21 ± .04	.15 ± .02
	Ala	.70 ± .29	.56 ± .08	.66 ± .04
	Ile	.07 ± .05	.05 ± .01	.05 ± .01
	Gaba	.41 ± .13	.39 ± .15	.66 ± .09
	others	.06	.03	.01
	NH_4^+	.40 ± .02	.48 ± .02	.35 ± .04
TOTAL REDUCED N		8.33	8.90	8.25
	NO_3^-	129.89 ± 10.67	139.49 ± 7.48	2.85 ± .37
ROOT	Asp	.25 ± .04	.17 ± .02	.28 ± .19
	Glu	.53 ± .06	.32 ± .11	.27 ± .13
	Asn	2.09 ± .37	2.45 ± .78	26.54 ± 4.85
	Ser	.13 ± .02	.11 ± .02	.33 ± .04
	Gln	ND	ND	2.62 ± 1.65
	Thr	.04 ± .03	.02 ± .03	.10 ± .01
	Ala	.10 ± .01	.06 ± .05	.15 ± .04
	Ile	.02 ± .01	.03 ± .03	.01 ± .01
	Gaba	ND	ND	.09 ± .06
	others	.06	.03	.01
	NH_4^+	.09 ± .01	.36 ± .03	1.01 ± .670
TOTAL REDUCED N		3.31	3.55	31.41
	NO_3^-	57.88 ± 5.30	71.80 ± 9.65	.41 ± .43

TABLE 5.3 Composition of amino acids, and the concentrations of nitrate in leaves, stems and roots of *L. corniculatus* grown under three nitrogen regimes

Plants were grown in a growth chamber with a 20°C/15°C, 16/8 h day/night regime, and watered daily with either 20 mM NO_3^-, 5 mM NO_3^-, or 5 mM NH_4^+ solutions. Tissues were extracted in methanol and the amino acids separated by HPLC. The *Aspergillus* NR method was used to determine the concentration of NO_3^-.

ND = not detected.

TABLE 5.3

Tissue	Amino Acid	Concentration (μmol.g^{-1}fwt) 20 mM NO_3^-	5 mM NO_3^-	5 mM NH_4^+
LEAF	Asp	.91 ± .05	.62 ± .05	.39 ± .09
	Glu	1.79 ± .13	1.73 ± .10	1.09 ± .30
	Asn	3.73 ± .52	3.18 ± .21	.51 ± .10
	Ser	.98 ± .04	.69 ± .31	.27 ± .06
	Gln	.18 ± .13	.05 ± .06	.05 ± .05
	Thr	.22 ± .03	.30 ± .01	.10 ± .07
	Ala	1.62 ± .23	.47 ± .05	.41 ± .20
	Ile	.03 ± .02	.05 ± .04	.02 ± .01
	Gly	.10 ± .07	.07 ± .05	ND
	Gaba	.56 ± .33	.67 ± .23	.46 ± .23
	others	.07	.07	.05
TOTAL REDUCED N		10.19	7.90	3.35
	NO_3^-	61.90 ± 2.59	37.68 ± 14.29	.37 ± .09
STEM	Asp	.43 ± .02	.41 ± .05	.21 ± .01
	Glu	.64 ± .04	.65 ± .08	.35 ± .03
	Asn	2.33 ± 1.07	3.42 ± 1.94	.88 ± .13
	Ser	.22 ± .10	.22 ± .06	.14 ± .01
	Gln	.11 ± .05	.04 ± .05	.06 ± .04
	Thr	.06 ± .02	.09 ± .02	.06 ± .01
	Ala	.42 ± .24	.19 ± .11	.30 ± .06
	Ile	.04 ± .02	.03 ± .00	.04 ± .02
	GABA	.28 ± .05	.27 ± .10	.26 ± .03
	others	.02	.03	.04
TOTAL REDUCED N		4.55	5.35	2.34
	NO_3^-	97.69 ± 26.75	75.88 ± 17.90	ND
ROOT	Asp	.41 ± .09	.25 ± .06	.14 ± .05
	Glu	.40 ± .18	.29 ± .08	.29 ± .10
	Asn	7.01 ± 1.81	4.41 ± 2.38	7.16 ± 3.48
	Ser	.20 ± .03	.12 ± .06	.18 ± .09
	Gln	.03 ± .03	ND	1.20 ± 1.32
	Thr	.07 ± .02	.03 ± .01	.07 ± .04
	Ala	.16 ± .01	.07 ± .04	.11 ± .06
	Ile	.07 ± .08	ND	ND
	Arg	.34 ± .03	.08 ± .06	ND
	Gaba	.13 ± .08	.06 ± .06	.30 ± .26
	others	.07 ± .05	ND	.03 ± .01
TOTAL REDUCED N		8.89	5.31	9.48
	NO_3^-	70.41 ± 24.58	25.15 ± 9.18	.24 ± .10

FIGURE 5.8 The effect of different nitrogen sources on chlorophyll content, protein content, fresh weight and dry weight of *L. japonicus* and *L. corniculatus*

Plants were grown in a growth chamber with a 20°C/15°C, 16/8 h day/night regime, and watered daily with either 20 mM NO_3^-, 5 mM NO_3^-, 0.2 mM NO_3^-, or 5 mM NH_4^+ solutions.

A) Concentration ($\mu g.g^{-1}fwt$) of chlorophyll a and chlorophyll b in the leaves of *L. japonicus*

B) Concentration ($\mu g.g^{-1}fwt$) of chlorophyll a and chlorophyll b in the leaves of *L. corniculatus*

C) Protein content ($mg.g^{-1}fwt$) in the leaf, stem and root of *L. japonicus* plants

D) Protein content ($mg.g^{-1}fwt$) in the leaf, stem and root of *L. corniculatus* plants

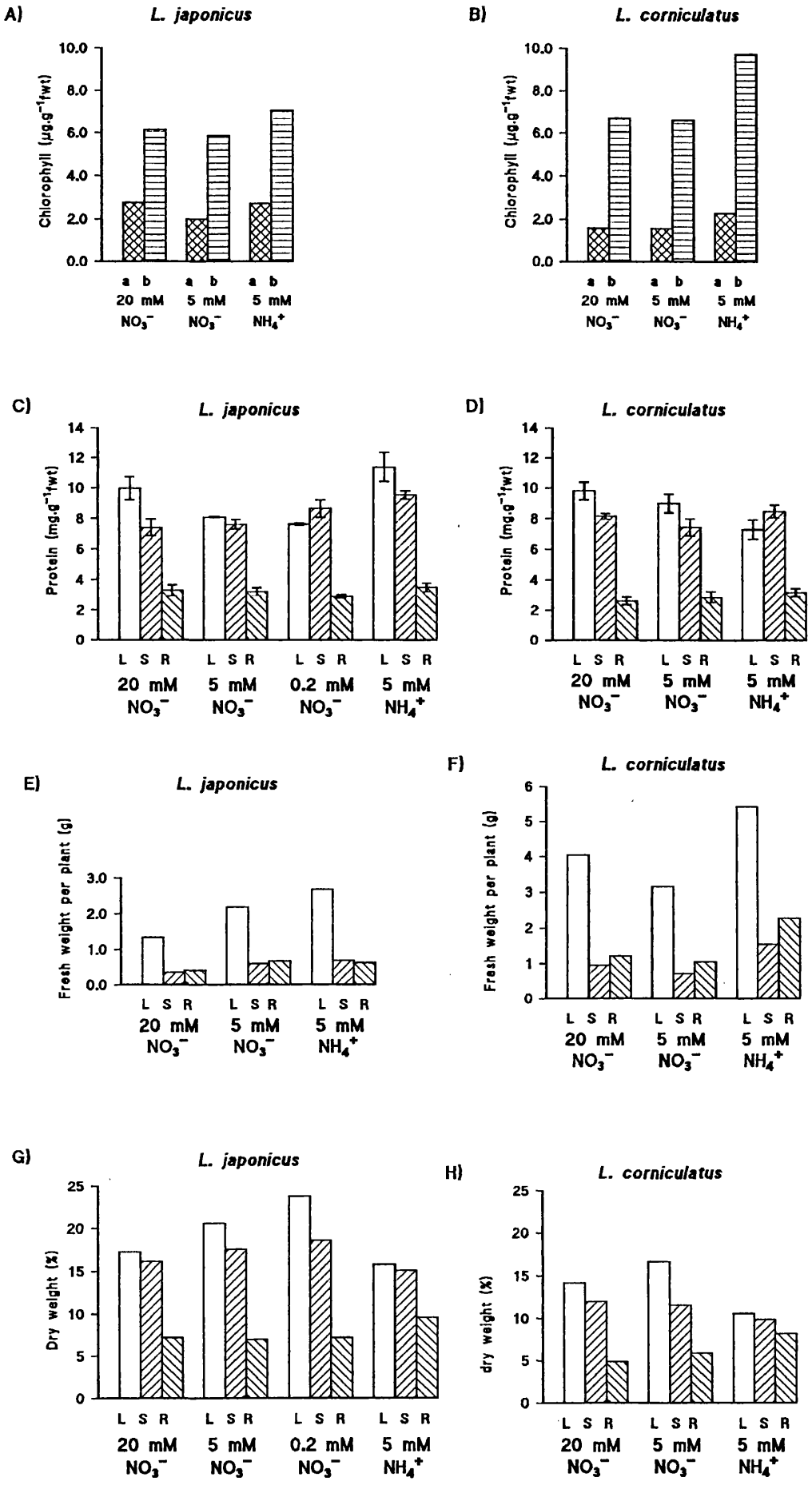
E) Fresh weight of the leaf, stem and root per *L. japonicus* plant

F) Fresh weight of the leaf, stem and root per *L. corniculatus* plant

G) Dry weight of the leaf, stem and root of *L. japonicus* as a percentage of the fresh weight.

H) Dry weight of the leaf, stem and root of *L. corniculatus* as a percentage of the fresh weight.

FIGURE 5.8


A)
L. japonicus
Chlorophyll (µg.g⁻¹fwt)
10.0
8.0
6.0
4.0
2.0
0.0
a b
20 mM NO3⁻
a b
5 mM NO3⁻
a b
5 mM NH4⁺
B)
L. corniculatus
Chlorophyll (µg.g⁻¹fwt)
10.0
8.0
6.0
4.0
2.0
0.0
a b
20 mM NO3⁻
a b
5 mM NO3⁻
a b
5 mM NH4⁺
C)
L. japonicus
Protein (mg.g⁻¹fwt)
14
12
10
8
6
4
2
0
L S R
20 mM NO3⁻
L S R
5 mM NO3⁻
L S R
0.2 mM NO3⁻
L S R
5 mM NH4⁺
D)
L. corniculatus
Protein (mg.g⁻¹fwt)
14
12
10
8
6
4
2
0
L S R
20 mM NO3⁻
L S R
5 mM NO3⁻
L S R
5 mM NH4⁺
E)
L. japonicus
Fresh weight per plant (g)
3.0
2.0
1.0
0.0
L S R
20 mM NO3⁻
L S R
5 mM NO3⁻
L S R
5 mM NH4⁺
F)
L. corniculatus
Fresh weight per plant (g)
6
5
4
3
2
1
0
L S R
20 mM NO3⁻
L S R
5 mM NO3⁻
L S R
5 mM NH4⁺
G)
L. japonicus
Dry weight (%)
25
20
15
10
5
0
L S R
20 mM NO3⁻
L S R
5 mM NO3⁻
L S R
0.2 mM NO3⁻
L S R
5 mM NH4⁺
H)
L. corniculatus
dry weight (%)
25
20
15
10
5
0
L S R
20 mM NO3⁻
L S R
5 mM NO3⁻
L S R
5 mM NH4⁺

CHAPTER 6: DIFFERENCES IN GS ISOFORMS AND SITE OF NITRATE ASSIMILATION BETWEEN TEMPERATE AND TROPICAL LEGUMES

6.1 INTRODUCTION

The discovery of a GS2 isoform that was induced by NO_3^- in the roots of the *L. japonicus* and *L. corniculatus*, together with previous reports of NO_3^--inducible GS2 in three other temperate legumes (*P. sativum*, *M. media* and *V. faba*; Barratt, 1980; Vézina *et al.*, 1987), but not in the tropical species *P. vulgaris* and *G. max* (Bennett and Cullimore, 1989; Hirel *et al.*, 1987) suggested that root GS2 may be restricted to temperate legumes and that it might be correlated with the ability to assimilate NO_3^- in the root. As the majority of temperate legumes, and many tropical ones, are in the Papilionoideae sub-family, it was decided to survey a range of legumes representative of the different tribes of the Papilionoideae for the presence of root GS2 and for the partitioning of NO_3^- assimilation between root and shoot.

6.1.1 Evolution of the tropical papilionoid tribes

Figure 6.1 shows the affiliations of the tribes in the Papilionoideae (see Polhill *et al.*, 1981, and Raven and Polhill, 1981, for more details). The tropical tribe Swartzieae lies on the borderline between the Caesalpinoideae and the Papilionoideae sub-families. The Sophoreae, found in both warm temperate and tropical regions of the Old and the New Worlds, are a diverse group sharing characteristics with many of the other tribes.

Many tropical legumes possess pulvini, organs found at the base of the petiole conferring movement to leaves. The Millettieae, Robinieae and Dalbergieae

are considered to be the core tribes of these pulvinate legumes. Broadly speaking the more advanced tropical tribes with a bias toward the Old World, such as Indigofereae, can be grouped with the Millettieae, as can the pantropical Phaseoleae, whereas the New World tribes, e.g. Amorpheae and Aeschynomeneae, can be linked to the Robinieae and Dalbergieae.

6.1.2 Evolution of the temperate papilionoid tribes

The Galegeae, thought to be a temperate offshoot of the Millettieae, are separated along with several of the other familiar temperate herbaceous tribes, including the Vicieae, Trifolieae and Loteae, as the epulvinate series. The loss of the pulvini in these legumes is accompanied by several morphological changes including closure of the vascular system, and the presence of phloem transfer cells, which make the epulvinate legumes obligately herbaceous. The basic Galegeae design is the ground plan of the other epulvinate legumes although there are no obvious connections between the different tribes.

The temperate tribes, the Podalyreae, Liparieae, Mirbelieae, Bossiaeeae, Genisteae and Thermopsideae, are separated from the other tribes and grouped together as the genistoid alliance. The Podalyreae and Liparieae from the South African Cape, and the Mirbelieae and Bossiaeeae from Australia are thought to have evolved independently from the ancient, northern hemisphere tribes, the Genisteae and Thermopsideae. All are centred in areas of Mediterranean climate and possibly survive as relics of a larger group.

In the temperate Northern hemisphere the legume flora is relatively poor, partly due to isolation from the centres of early divergence (Africa and S.

America), and because of extinctions following the deteriorating climate apparent since the Oligocene. However the tribes that could adapt to a more rigorous north temperate climate proliferated extensively from stock centred in Mexico and the Sino-Himalayas. Much of the temperate N. American flora is essentially Eurasian, including *Lotus* (Loteae), *Trifolium* (Trifolieae), *Astragalus* (Galegeae), *Vicia* and *Lathyrus* (Vicieae) and *Thermopsis* (Thermopsideae). Some northern temperate species, especially *Astragalus* and *Lupinus* (Genisteae), reached South America along the mountain chains formed in recent times and spread extensively.

There were probably no legumes in Australia prior to the collision of the Australian tectonic plate and the Asian plate 15 million years ago. Australia was dominated until 10 million years ago by temperate rain forest, and the ancestors of the Australian flora that prefer relatively dry areas, e.g. *Acacia* and *Senna* (genera in the Mimosoideae and Caesalpinoideae subfamilies respectively), and the papilionoid genera, *Hardenbergia* (Phaseoleae), *Clianthus* (Galegeae), and *Bossiaea* (Bossiaeeae), must have existed in pockets of sclerophyllous vegetation on infertile soils before the drying of the climate allowed their proliferation.

The small New Zealand legume flora is thought to be mainly derived from the Millettieae through long-distance dispersal. Nearly all species belong to the endemic Carmichaelieae, which has spread since the late Pliocene into the newly available alpine habitats of New Zealand.

6.1.3 Determining the partitioning of nitrate assimilation

Although the presence of GS2 protein is quite easily assessed by western blotting and immunostaining, there is no easy way to determine the site of NO_3^- assimilation. Two common methods are the measurement of xylem sap NO_3^- concentration, and nitrate reductase (NR) activities. The former estimates the amount of NO_3^- being transported in the xylem sap as a percentage of the total nitrogen. Nitrate reductase assays determine the site of maximum activity of the NO_3^--inducible enzyme, either *in vivo* by vacuum-infiltrating chopped tissue with the assay medium, or by extracting the enzyme for an *in vitro* measurement. The *in vivo* method utilizes endogenous reductant, whilst the *in vitro* assay reflects the extractable activity where nothing is limiting. Of course, assaying for enzyme activities only establishes the potential of the system, and, as the true situation *in vivo* cannot be duplicated, such values are only approximations. Consequently, the distribution of NR activity does not unequivocally show the partitioning of NO_3^- reduction. It is generally accepted, however, that the major site of NR activity and induction is also the major site of NO_3^- reduction, especially if this agrees with xylem sap analyses (Smirnoff and Stewart, 1985; Andrews, 1986a; Wallace, 1986; Stewart *et al.*, 1993). Table 6.1 is collated from the literature and shows the NR activities and the percentage of xylem sap nitrogen represented by NO_3^-, in legumes grown either on low NO_3^- (less than 5 mM), or growing in the wild. Most of the legumes from tropical habitats seem to transport and reduce NO_3^- in the shoot whereas in the temperate species the root plays a more important role.

An added complication with using NR activities to gauge the site of NO_3^- assimilation is the possibility of constitutive NR activity being present. Andrews

(1986a) assayed 200 species of legume for constitutive NR activity, and although most species had no detectable constitutive activity, a substantial amount was detected in members of the Phaseoleae and Loteae (see Table 6.2).

In the work described in this chapter, partitioning of NO_3^- assimilation was assessed by assaying the tissue NO_3^- concentration and the *in vivo* and *in vitro* NR activities in leaf, stem and root of each legume. Analyses of the proportion of total N transported as NO_3^- in the xylem sap were also ascertained where possible. The data were analyzed with the CLUSTAN 3/PC package (Wishart, 1987), which has been used successfully for many biological analyses (Ingrouille *et al.*, 1990; Soares *et al.*, 1994).

It has been suggested that root NO_3^- assimilation has an advantage over shoot assimilation at low temperatures (Sutherland *et al.*, 1985). It was therefore decided to grow four of the temperate species, *L. corniculatus*, *L. japonicus*, *Vicia sativa* and *Melilotus alba*, at both 25/20℃ and 20/15℃ day/night to see if this affected the partitioning of NO_3^- assimilation.

6.2 RESULTS

Fifty-five species were surveyed, 47 of which were from the Papilionoideae (30 temperate, 17 tropical), four from the Caesalpinoideae (including one temperate species), and four from the Mimosoideae (see Table 6.2 for a list of species and the abbreviations used in the Figures). Plant species were chosen to include as diverse a range of mainly papilionoid legumes as possible, but the choice was limited by seed availability, so that many of the species analyzed were

pantropical weeds or cultivated plants from both tropical and temperate regions. However species indicative of many major biomes were sampled, including both lowland tropics, colder upland tropics, tropical savanna, chaparral, desert, and temperate grassland. Table 6.2 includes a summary of the biomes and geographical regions preferred by each species sampled in this chapter. The provenance of the seeds was ascertained wherever possible (see Appendix II which also includes taxonomic classification of the species).

The optimal growth temperature for most tropical plants is estimated to be between 25-30°C and for temperate plants between 20-25°C (McWilliam, 1978). Therefore the mixture of temperate and tropical species in this survey were grown at 25°C during the day, with a drop of 5°C during the night. However, it was observed that *L. japonicus* plants grown at 25°C in a growth room showed lower NR activity in the shoot than those grown in the lower temperatures in a greenhouse, although several of the other growth conditions were also different, *e.g.* light intensity and daylength (see Chapter 5.3.1). Therefore it was decided to grow four temperate legume species, *M. alba*, *L. corniculatus*, *L. japonicus* and *V. faba*, at 20°C (with a 15°C night) but when big enough, half were transferred for 10-14 d to 25/20°C.

All plants were watered with 1 mM NO_3^- solution every 2-3 d and on the day before sampling, and were harvested on ice about 5 to 7 h into the light period. Sub-samples were removed in triplicate for the *in vivo* NR assay and tissue NO_3^- analysis, with sub-samples also being immediately frozen in liquid N_2, to be subsequently extracted for *in vitro* NR and GS assays. The same extract was used for SDS-PAGE electrophoresis. Xylem sap was collected from plants which bled

when de-topped, and these were assayed for NO_3^- and for reduced N.

6.2.1 GS polypeptides in 55 species of legume

The 55 legume species (31 from temperate areas, 24 from the tropics) were assessed for the presence of GS2 in their non-nodulated roots by western blotting.

Each of the 30 temperate papilionoid species examined (26 genera from 15 tribes) possessed a root GS2. However none of the tropical papilionoid species (12 genera from 6 tribes) possessed detectable root GS2 when comparable amounts of extract were loaded on the gel (either on a total protein basis or on an activity basis). Examples can be seen in Figures 6.2 and 6.3, which include eight tropical species from five different tribes (including the non-papilionoid *Senna obtusifolia*), and eight temperate species from six different tribes. Most root extracts were run alongside shoot extracts (see Figure 6.3) to confirm that the slower moving band had the same mobility as the leaf GS2 from the same species.

Amongst the four members of the Caesalpinoideae that were analyzed, the GS2 isoform was undetectable in the roots of *S. obtusifolia* (Figure 6.2) or *Caesalpinia pulcherrima* (Figure 6.4) both tropical species. However, it was detectable in the one temperate Caesalpinoid legume, *Cercis siliquastrum*, as well as (faintly) in the tropical species, *Cassia fistula* (Figure 6.4).

All four of the Mimosoid legumes tested were tropical species, but GS2 could be detected in the roots of both *Mimosa pudica* (not shown) and *Acacia farnesiana* (Figure 6.4). It was not detected in *Prosopis juliflora* roots (not shown) or in *Albizia julibrissin*, although it should be noted that the proteins in extracts of the latter species were very degraded.

In some species the GS1 or GS2 bands appeared as a doublet (for an example of a GS1 doublet see *Genista aetnensis* root, Figure 6.2; and for a GS2 doublet, *Cassia fistula* root and leaf, Figure 6.4).

6.2.2 Distribution of GS activity between leaves, stems and roots

The activity of GS was assayed in the leaf, stem and root of 55 species of legume by the semi-biosynthetic method. The distribution of the GS activities was analyzed using CLUSTAN (Wishart, 1987) which separated the data into six clusters (see Figure 6.3). The data was initially standardized to give equal weighting to each measurement, then a principle components analysis (PCA) was carried out and scatter diagrams of the first few components constructed. A further multivariate analysis of the data was carried out independently of the PCA using Ward's Method, which attempts to find a set of clusters with the minimum total cluster variance, as the criterion for the fused data. The relationship of the species within each cluster was illustrated by a dendrogram (see Appendix Iv a), and as the clusters were calculated independently of the PCA, a comparison made by superimposing cluster circles onto the PCA scatter diagram was used to validate the two statistical methods (see Appendix Iv b and c). It was understood, however, that biological relevance of the clusters was the most important validation. Accordingly, histograms were constructed of the leaf, stem and root values of each species in each cluster in the order that they appear in the dendrograms (Figure 6.3).

The number of temperate and tropical species in each cluster was tabulated, as was the mean for the entire data set, and the means and T-values for each

variable (leaf, stem or root) in each cluster (Table 6.3). The cluster means for each variable, and their standard deviations were also added at the end of each histogram (Figure 6.3). The T-value is a comparison of the cluster mean with the mean for the entire data set, calculated by

(Mac - Mad) / Sad

where Mac = the mean for variable a in a cluster, Mad = the mean for variable a in the data set, and Sad = standard deviation for variable a in the data set. A large T-value indicates a variable whose mean is substantially greater than that of the other clusters (positive values) or much lower than the other clusters (negative values).

The widest division between the clusters was that between cluster 3 and the rest. This was due to the species in cluster 3 having higher than average activities in leaf, stem and root (note the large T-values in Table 6.3). There was another division between clusters 1, 4 and 6, and clusters 2 and 5. Clusters 1, 4 and 6 all had lower than average root activities (note the negative T-values in Table 6.3), with cluster 1 having average leaf but high stem activity, cluster 4 having low leaf and stem activities, and cluster 6 (a single species, the temperate legume *Bossiaea pulchella*) having very high leaf and low stem activities. Clusters 2 and 5 had higher than average root activities and low stem activities. There was no obvious division of the temperate and tropical species into separate clusters with respect to their GS activities.

6.2.3 Distribution of *in vivo* NR activity between the leaves, stems and roots of 46 species of legume.

The NR activities of 47 species were assayed by the *in vivo* method. Upon processing with CLUSTAN, there were six Ward's clusters that best fitted the data (Figure 6.4; Appendix IV d). These clusters were fairly well validated by PCA (Appendix IV e and f), although PCA group 1 appeared to be closer to group 2 than was suggested by Ward's clustering.

A large separation can be seen between cluster 6, *Coronilla varia*, and the other species. This is because *C. varia* had very high NR activities in all tissues, especially in the stem (note the Cluster means and the large T-values for cluster 6 in Table 6.4). The three species in cluster 4 are also widely separated from the other species as they displayed very high root NR activities (note the large Cluster mean and T-value for the root). However the temperate species *Lupinus nanus* and the tropical species *Arachis hypogaea* in cluster 4, possessed high shoot activity as well as high root activities (seen in Figure 6.4). The NR activity in the shoot of the remaining species in this group, the temperate legume *Carmichaelia aligera*, was very low due to this species having only a few very rudimentary leaves, although the flattened stems (phyllodes) are photosynthetic. This suggested the root to be the primary site of NO_3^- reduction in this species.

A further division was apparent between clusters 1 and 5, and clusters 2 and 3, with clusters 2 and 3 being quite closely related (see Appendix IV d). Members of clusters 1 and 5 were defined by relatively high leaf activity (note the Cluster mean and T-value). Cluster 1 also had high stem activity whereas cluster 5 had low stem and root activity. Clusters 1 and 5 together contained twelve tropical species and four temperate species, and it was assumed that the shoot played a

major role in NO_3^- assimilation in each of these species.

Clusters 2 and 3 had lower than average leaf and stem activities (note the negative T-values), but differed from one another by the root activity being slightly higher than average in cluster 2, but very much lower than average in cluster 3 (less than 0.145 μmol.h^{-1}.g^{-1}fwt). Cluster 3 contained several temperate, epulvinate legumes including *Trigonella foenum-graecum*, *Vicia sativa*, *V. faba*, *Astragalus glycyphyllos* and *Cicer arietinum*. However, several of the species in this cluster contained very little activity overall. This lack of induction, which was also apparent in some of the species in cluster 2, did not allow a reliable assessment of the partitioning of NR activity.

6.2.4 Distribution of *in vitro* NR activities between the leaves, stems and roots of 31 species of legume, and a comparison with the *in vivo* NR activities

The distribution of *in vitro* NR activity in the leaf, stem and root was obtained for 31 species of legume. The data separated into six Ward's clusters (Figure 6.5; Appendix Iv g, h and i), with a major division between clusters 1, 2 and 4, members of which had higher than average stem activities (see the large Cluster means and T-values in Table 6.5), and clusters 3, 5 and 6 which had lower stem activities (note the negative T-values). A further separation could be made between the species in clusters 1 and 2, all of which had low root activities (negative T-values), and cluster 4 which had high root activities. The species in cluster 3 differed from the others in having low leaf and high root activity, whereas those in cluster 5 had low leaf and root activities, and those in cluster 6, high leaf and low root activity.

Although 50% of the species had very similar *in vivo* and *in vitro* NR activities in all tissues, there were differences between the two measurements in the remaining sixteen species. The shoot *in vitro* activities in several species were higher than the corresponding *in vivo* activities. This was especially apparent in *S. obtusifolia*, *M. pudica*, *T. subterraneum*, *W. floribunda*, *S. grandiflora* and *I. pseudotincoria*, in each of which there was good agreement between the *in vivo* and *in vitro* root activities. Both shoot and root activities were higher *in vitro* in *T. caroliniana*, *R. pseudoacacia*, *P. calyptrata* and *L. corniculatus*, whilst *L. japonicus in vitro* root activity was higher but the shoot *in vitro* activity was not. The *in vitro* shoot activity was less in *C. varia*, *E. crista-galli*, *G. soja*, *G. max*, and *L. nanus*, although the root *in vivo* and *in vitro* activities were very similar.

However, none of these differences contended with the general trend of NR distribution in each species, for example 71% of the species that were grouped in predominantly temperate clusters on the basis of their *in vitro* NR activities (where more than two-thirds of the species were temperate, i.e. clusters 2, 3, 4 and 5), also clustered into predominantly temperate groups by their *in vivo* activities (Figure 6.4, clusters 2 and 6). In the predominantly tropical clusters 1 and 6, 83% of the species were also placed into tropical clusters on the basis of their *in vivo* NR activities (Figure 6.4, clusters 1 and 5).

6.2.5 The percentage of nitrate in the xylem sap of nine species

Xylem sap from four temperate species and five tropical species was assayed for NO_3^- and soluble amino-N. The proportion of NO_3^- being transported in the xylem sap was calculated as a percentage of the total soluble N, and these

values were found to correlate extremely well with the *in vivo* NR activities in the shoots of these species, especially if the data for *G. soya*, *L. nanus* and *T. caroliniana* were not included (Figure 6.8). The inclusion of the data for *G. soja* did not decrease the correlation very much ($r = 97$, $p = 9.8 \times 10^{-4}$ as opposed to $r = 99$ and $p = 2.4 \times 10^{-5}$), but if the data for *L. nanus* and *T. caroliniana* were included the correlation was reduced ($r = 0.072$ and $p = 0.042$). This was a result of the lower NR activities in the shoots of these two species compared to other temperate species which were transporting NO_3^- to the shoot, e.g. *R. pseudoacacia*. This might be because both species are members of the Genistoid alliance, a group which includes many species with very low NR activities, e.g. *C. scoparius* and *G. aetnensis*.

Of the temperate species, *L. nanus*, *T. caroliniana* and *R. pseudoacacia* were transporting over 80% of their xylem sap N as NO_3^-, as was the tropical legume *Mimosa pudica* and the wild soybean, *G. soja*. Xylem sap analysis revealed that *Hardenbergia violacea*, a species with very low overall NR activities, was transporting surprisingly high levels of NO_3^- (23 mM) in its xylem sap, comprising 93% of its total xylem N. Because of the apparent lack of NR induction in *H. violacea*, this species has been omitted from Figure 6.8.

Only one temperate species, *Podalyria calyptrata*, was found to be transporting a much smaller proportion of its xylem sap N as NO_3^--N (36%), which confirmed this species to be a root assimilator. However, the tropical species *Sesbania grandiflora*, and the domesticated soybean, *G. max* were also found to be transporting relatively low levels of NO_3^--N in their xylem sap (48% and 68% respectively), indicating a significant contribution of the root to NO_3^-

assimilation in these two tropical species.

6.2.6 Distribution of nitrate between the leaves, stems and roots of 55 species of legumes

The concentrations of NO_3^- in the leaf, stem and root were assayed by the *Aspergillus* NR method (Chapter 2.4.9). The species in the five Ward's clusters which best fitted the PCA can be seen in Appendix Iv j, k and l, and in Figure 6.6, with the cluster diagnostics in Table 6.6. Cluster 2, containing *Albizia julibrissin* and *Arachis hypogaea*, separated widely from the other species because of the very high NO_3^- concentrations in all tissues (note the high T-values). The remaining species divided into cluster 1 and a group containing clusters 3, 4 and 5. It can be seen that all of the 33 species in cluster 1 contained relatively low concentrations of NO_3^- in all tissues, especially those depicted in the cluster 1b. The concentration of NO_3^- in the stem of species in cluster 3 was higher than average, whereas, although the two species in cluster 4 differed greatly in their root NO_3^- concentration, they both possessed particularly high leaf activity. Cluster 5 had exceptionally high root NO_3^- concentration. There was no obvious reason for the differences in distribution of tissue NO_3^- between these species.

CLUSTAN analysis of the combined data for NR activities and tissue NO_3^- did not reveal any meaningful clusters. Indeed as most of the tissue NO_3^- is probably in the vacuole, a correlation between tissue NO_3^- concentrations and NR activity would not be expected. Likewise, there was no correlation between tissue NO_3^- concentration and GS activities.

6.2.7 Combined analysis of NR and GS activities

The CLUSTAN program was used to analyze the *in vivo* NR activities and the GS activities as a combined data set. There were six clusters that best fitted the combined data (see Appendix IV m, n and o), and the cluster diagnostics can be seen in Table 6.7. Although clusters 1, 2 and 3 were fairly well separated by Ward's clustering, the data overlapped in the PC analysis. However, the mean activities of the leaf, stem and root in each cluster (Table 6.7) indicated that separation into six clusters was valid.

The species in cluster 1 contained lower than average root NR and GS activities, and it is interesting to note that 10 of the 14 species were tropical, of which 7 belonged to the Phaseoleae (out of 9 sampled from that tribe), with the remaining 3 tropical species being the only non-papilionoid legumes in this data set.

Cluster 2, members of which had lower than average activities of both enzymes in leaf, stem and root, contained only 7 tropical legumes out of a total of 23, two of which, *S. grandiflora* and *T. vogelii*, were related to two of the temperate species, *R. pseudoacacia* and *W. floribunda* respectively.

The four temperate species in cluster 3 had higher than average activities of both enzymes in the root, but although the GS activity was also higher than the mean in the shoot, the stem and leaf NR activities were lower. The root NR and GS activities were particularly high in the two temperate and one tropical species in cluster 4, but there was no correlation between NR and GS activities in *E. crista-galli* (cluster 5). *Coronilla varia* (cluster 6) had higher than average activities of both enzymes in all tissues.

Table 6.8 shows the numbers of temperate and tropical species in each cluster which possessed a mean GS and NR activity higher or lower than the data mean. Thus, although 53% of the tropical species that had higher than average GS and NR activities in the leaf, only 19% of the temperate species did. Conversely, 26% of the temperate species and only 5% of the tropical species possessed high GS and NR activities in the root.

This indicated a relationship between the GS and NR activities, as well as a division between the temperate and tropical species. To analyze this possibility, the two enzyme activities were plotted against each other, but with separate graphs for the temperate and the tropical species (see Figure 6.7). The only significant correlations between the GS and NR activities were in the leaves of tropical species if the data for *E. crista-galli* were ignored (and to a lesser extent the leaves in the temperate species, ignoring *L. odoratus*) and in the roots of the temperate species (Figures 6.7a, b and f). If *E. crista-galli* was included in the calculation of the line of regression in the tropical leaves, the p value increased from 0.017 to 0.437 and the inclusion of *L. odoratus* in the regression calculation for temperate leaves resulted in a p value of 0.278 compared to 0.052. Although a possible outlier, the data for *C. varia* leaves was included because, if removed, either with or without the *L. odoratus* data, a negative slope was obtained but with an increased p value (0.148).

6.2.8 The effects of temperature on nitrate assimilation in four temperate species of legume

To examine the effect of temperature on NO_3^- assimilation, four species of temperate legume, *L. corniculatus*, *L. japonicus*, *M. alba* and *V. sativa*, were grown under two temperature regimes: either 20°C (day) and 15°C (night), or 25°C (day) and 20°C (night). Figure 6.9 shows the *in vivo* NR activities in the four species grown at both temperature regimes, and the *in vitro* NR activities of the two *Lotus* species.

The most profound difference in *in vivo* NR activity between the two temperature regimes was in the leaf and stem of *M. alba*, which had more than two-fold higher activities in plants grown at the lower temperature. The root activity was also significantly higher in plants grown at the lower temperature. There were no significant differences in *in vivo* NR activity between *V. sativa* plants grown under the two regimes, but the activity in the stems of *L. japonicus* plants grown at the lower temperature was significantly higher (*ca* 50%) than those of plants grown at 25/20°C. A similar result was found for *L. corniculatus* (significant only at the 90% confidence level).

Although *in vitro* NR activity was assayed for in all four species, the method did not work for *M. alba* and *V. sativa*, so that measurements were only obtained for the two *Lotus* species. The activities in the leaf and stem of *L. corniculatus* plants grown at the higher temperature were less than 50% of that in plants grown at 20/15°C, whereas the root activity at the higher temperature was 63% higher than in the plants grown at 20/15°C. A similar trend could be seen in *L. japonicus* in that the root activity in plants grown at 20/15°C was only 67% of

the root activity in plants grown at the higher temperature, whereas the stem activity at the lower temperature was double that at the higher temperature (significant only at the 90% confidence limit).

In the CLUSTAN analysis of *in vivo* NR activity both *L. japonicus* and *L. corniculatus* plants grown at either temperature regime, along with *M. alba* plants grown at the lower temperature, segregated with species possessing low shoot activity but high root activity (cluster 2, Figure 6.4). However, *V. sativa* grown at either temperature and *M. alba* plants grown at the higher temperature, separated into a cluster with low activities in all tissues (cluster 3).

The differences in the *in vitro* NR activities of *L. corniculatus* grown under the two temperature regimes affected its position in the Ward's clustering (Figure 6.5). The plants grown at the lower temperature segregated into a cluster of plants possessing low root and high stem activities (cluster 2) whereas plants grown at 25/20°C separated into a cluster with low stem and leaf activity but high root activity. The differences in activity between the *L. japonicus* plants grown at the two temperature regimes were not profound enough to place the plants into two different clusters.

There was a slight negative correlation between the concentration of NO_3^- in each tissue and the NR activities (not shown). For example, the NO_3^- concentration in the root of *L. corniculatus* plants grown at 20/15°C was greater than at the higher temperature, whereas the NR activities in the root were lower. In a similar fashion the concentration of NO_3^- in the shoot of *M. alba* grown at 20/15°C was reduced at the higher temperature, whereas the NR activity was increased. There was no apparent relationship between GS activities and NR activities in plants

grown under the two different temperature regimes, and no difference between the two treatments in GS subunit appearance in any of the four species (not shown).

6.2.9 The effects of temperature on transpiration in four temperate species of legume

Most of the NO_3^- in a plant is present as a slowly metabolized storage pool, and the induction of NR is thought to be dependent on a separate, 'active' pool of NO_3^- which responds directly to the external NO_3^- supply rather than on the total tissue NO_3^- concentration (King *et al.*, 1992; Sechley *et al.*, 1992). The flux of NO_3^- is related to the transpiration rate (Clarkson *et al.*, 1986) and it was conjectured that the higher temperatures in this experiment, although not very high, may have caused stomatal closure, thereby reducing NO_3^- flux and causing the lower NR activities outlined above. Therefore the transpiration rate was measured in the four species under both temperature regimes (Figure 6.9C). The transpiration rate was greater at the higher temperature regime in all four species. This showed that the lower NR activities apparent in the shoots of *M. alba*, *L. corniculatus* and *L. japonicus* plants grown at the higher temperature were not due to decreased transpiration through stomatal closure.

6.3 DISCUSSION

6.3.1 GS2 isoforms and activity

In this survey of 55 legume species, representing 38 genera from 21 different tribes, root GS2 was detected in each of the 30 temperate papilionoid species, but was absent from all of the 17 tropical papilionoid species. However,

the situation with the eight non-papilionoid species was not as straightforward. Although the one non-papilionoid temperate legume, *C. siliquastrum*, did possess the GS2 isoform, so too did tropical *C. fistula*, *M. pudica* and *A. farnesiana*. The isoform was not detected in the roots of the remaining four tropical non-papilionoid legumes. The small sample number of non-papilionoids precluded further interpretation of the occurrence of root GS2, except to say that in contrast to the tropical papilionoids, root GS2 was present in at least some tropical non-papilionoids.

So from the evidence presented here, caesalpinoid and mimosoid legumes may or may not have GS2 protein in their roots, and although tropical papilionoid species do not possess root GS2, those that may have originated in the tropics but can adapt to temperate habitats (e.g. the opportunistic weeds, *M. pudica* and *A. farnesiana*) and at least one temperate caesalpinoid (*C. siliquastrum*), do. Remarkably, expression of GS2 in the roots of temperate papilionoid species occurred even when the species concerned were closely related to tropical species that lacked the isoform, e.g. it was present in *W. floribunda* and *R. pseudoacacia* (temperate members of the Millettieae and Robinieae respectively) but absent from *T. vogelii* and *S. grandiflora* (tropical members of the Millettieae and Robinieae).

These findings are in agreement with earlier results that showed GS2 activity or protein to be present in root extracts of the temperate papilionoid legumes, *Vicia faba*, *P. sativum* and *Medicago media* (Barratt, 1980; Emes and Fowler, 1983; Vézina *et al.*, 1987; Vézina and Langlois, 1989), but absent from root extracts of the tropical papilionoid legumes, *Phaseolus vulgaris* and *Glycine max*, although GS2 transcripts were identified in the roots of these species (Bennett

and Cullimore, 1989; Hirel *et al.*, 1987). GS2 protein and activity have also been found in roots of some non-leguminous species which evolved in temperate areas, i.e. *Lycopersicum esculentum* (originating in the Andes) and the monocots *Hordeum vulgare* (from the Near East) and *Zea mays* (from warm temperate Central America)(Miflin, 1974; Becker *et al.*, 1992; Sakakibara *et al.*, 1992a; McGee, 1991). There is therefore reason to believe that the correlation between the presence of root GS2 and temperate origins may extend beyond the Leguminosae, although there is as yet no published evidence relating to the occurrence of root GS2 in tropical non-legume plants.

6.3.2 Both GS1 and GS2 polypeptides can appear as doublets.

It was noticed that both GS1 and GS2 can occur as doublets when separated by SDS-PAGE. A double GS2 band had also been found by Tingey *et al.* (1987), Lightfoot *et al.* (1988) and Becker *et al.* (1992) in *P. sativum*, *P. vulgaris* and *Lycopersicum esculentum* respectively. Although it is possible that GS2 may be encoded by more than one gene in at least some of these cases, only one GS2 gene has been found in those species most closely studied, including *P. vulgaris* and *P. sativum* (Lightfoot *et al.*, 1988; Tingey *et al.*, 1987), the non-legume dicots *L. esculentum*, *Nicotiana sylvestris* and *Sinapsis alba* (Becker *et al.*, 1992; Höpfner *et al.*, 1991), and the monocots *Zea mays* and *Hordeum vulgare* (Snustad *et al.*, 1988; Wallsgrove *et al.*, 1987). The appearance of GS2 protein as a doublet is consistent with there being differential cleavage of the precursor GS2 protein by the processing peptidase upon transport into the plastid as found with the RuBisCO small subunit (Robinson and Ellis, 1984; Mishkind *et al.*, 1985). In several of the

temperate species that had a GS2 doublet in the shoot, the root GS2 corresponded to only one of these bands.

The appearance of GS1 as a doublet had also been demonstrated by Hirel *et al.* (1987) and Marttila *et al.* (1993) in *G. max* and *H. vulgare*, and it is probable in these cases that the GS1 polypeptides corresponded to separate gene products, which were sufficiently different in size to be separated by SDS-PAGE.

6.3.3 Assessing the site of nitrate assimilation

Although the proportion of NO_3^--N transported in the xylem sap was evaluated whenever sap collection was possible (from only nine species), the main criterion for determining the major site of NO_3^- assimilation was the distribution of *in vivo* NR activity between root and shoot, as this was obtained for 47 of the sample species (Figure 6.4). The *in vivo* NR activity in the shoot was found to correlate fairly well with the percentage of xylem sap N transported as NO_3^- (Figure 6.8), and the *in vitro* activities, which were obtained from 32 species, were also generally found to reflect the *in vivo* activities (see Chapter 6.3.4). The presence of constitutive NR activity was a potential problem in gauging the predominant site of NO_3^- assimilation, although Andrews *et al.* (1990) in an extensive survey detected constitutive NR activity only in members of the Phaseoleae and Loteae, and nearly always only in the leaf.

Although the NR activities in *H. violacea* were very low in all tissues, data from xylem sap analysis along with field data of NR activities in the related species, *H. comptoniana* and *Kennedia prostrata* (Pate *et al.*, 1993; Stewart *et al.*, 1993) suggest shoot assimilation in this species. The temperate genistoid species,

G. aetnensis, *U. europaea*, *C. scoparius*, and *L. anagyroides* also had very low NR activities, which it was thought might reflect their known preference for acid, and therefore NO_3^--poor soils, i.e. they are probably mainly ammonium utilizers.

It was apparent that NR activities in the roots of both temperate and tropical species were generally very low compared to the shoot activities. However, whereas only 21% of the tropical species had root *in vivo* NR activities greater than the average (which was 0.214 μmol.h^{-1}.g^{-1}fwt), the NR activity in the roots of 63% of the temperate species exceeded the average. However, 74% of the tropical species showed higher than average shoot activities (the average being 0.973 μmol.h^{-1}.g^{-1}fwt), compared to only 33% of the temperate species. This was in accordance with the findings of Andrews (1986a).

6.3.4 Do any tropical legumes assimilate nitrate in their roots?

Almost all of the tropical species had high shoot NR activities, which was in accordance with published findings (see Table 6.2). However, four of the tropical species, *M. pudica*, *A. hypogaea*, *G. max* and *I. pseudotinctoria*, also had appreciable root NR activities. *G. max* was found to be transporting only 68% xylem sap N as NO_3^-, a figure comparable with published results (Andrews *et al.*, 1984a; Vessey and Layzell, 1987), confirming this species to be assimilating a large proportion of NO_3^- in its root. However *M. pudica* was shown to be transporting 88% of its xylem sap N as NO_3^- and therefore appears to be a shoot assimilator. The lack of information regarding xylem sap composition in *A. hypogaea* and *I. pseudotinctoria* prevented confirmation of their primary site of NO_3^- assimilation, but a fifth tropical species, *Sesbania grandiflora* was found to

be transporting only 48% of its xylem sap N as NO_3^-, which along with the low NR activity found in the shoot, suggested this species was also a root assimilator.

It is notable that three of these putative root assimilators are crop plants, *G. max* and *A. hypogaea* for grain, and the fast-growing tree *S. grandiflora*, for timber. Also, in contrast to the domesticated *G. max*, its wild progenitor species, *G. soja*, was a shoot assimilator. This suggested a link between domestication and root assimilation in tropical legumes. On the other hand, the non-domesticated pantropical species, *Dimorphandra mollis*, is probably a root assimilator (Stewart *et al.*, 1987) as is *Dalbergia brasiliense* (Stewart et al., 1993.) although *Dalbergia sissoo* in this survey was concluded to be a shoot assimilator.

6.3.5 The site of nitrate assimilation in the temperate species

The temperate species that clustered into groups containing high NR activities in the shoot were the herbaceous species *C. varia*, *G. officinalis* and *C. formosus*, the woody vine *W. floribunda*, and the tree *R. pseudoacacia*. The shoot activity was greater than 85% of the total activity in each species, with the leaf activities being 4- to 14-fold higher than those of the other temperate legumes. These species were therefore concluded to assimilate most of their NO_3^- in the shoot. Of the remaining temperate legumes, xylem sap NO_3^- analysis suggested that the related species, *Lupinus nanus* and *Thermopsis caroliniana* were also shoot assimilators. This was surprising as both *L. albus* and *L. angustifolius* have been shown previously to be root assimilators (Andrews *et al.*, 1984a; Andrews, 1986a). Several other temperate species, including *S. japonica*, *T. subterraneum*, *H. coronarium*, *M. alba*, *V. faba* and *A. glycyphyllos*, had relatively high activities

in the shoot (between 0.50 and 0.91 μmol.h^{-1}.g^{-1}fwt) indicating a degree of shoot assimilation, especially when coupled with the negligible root activities (less than 0.14 μmol.h^{-1}.g^{-1}fwt) found in *V. faba* and *A. glycyphyllos*.

Although the temperate legumes mentioned above probably assimilated a major part of their NO_3^- in the shoot, several of the temperate species that had higher than average root NR activities and low shoot activities, especially *L. corniculatus*, *L. japonicus*, *L. odoratus*, *P. calyptrata* and *C. aligera*, probably assimilated most of their NO_3^- in the root. Further evidence for this was that only 36% of the xylem sap N was being transported as NO_3^- in *P. calyptrata*. The NR activities in the roots of both *L. japonicus* and *L. corniculatus* were shown in the previous chapter to rise with increasing concentrations of exogenous NO_3^-, whereas the leaf activity remained low (although the stem became increasingly more important in *L. corniculatus*). This was in agreement with the results of Monza *et al.* (1989) who found that in *L. corniculatus* treated with 5 mM NO_3^-, 52% of the total *in vivo* NR activity was found in the stem, and 31% in the root.

6.3.6 The influence of temperature on NR activity

The finding that several members of the Vicieae and Trifolieae, notably *V. faba*, *M. alba* and *T. subterraneum*, appeared to be shoot assimilators was unexpected. Cultivars of *V. faba* have been reported that assimilate NO_3^- primarily either in the shoot or the root (Andrews *et al.*, 1984b), with shoot assimilation being associated with cold-sensitive cultivars, and root assimilation with cold-tolerant cultivars. Andrews and colleagues also found that if the cold-tolerant cultivar was grown at a higher temperature (25/15°C as opposed to 15/10°C) the

NR activity increased, especially in the leaf. So although the cultivar used in this experiment (Aquadulce Claudia) is cold-tolerant, its categorization as a shoot assimilator in the present study may be explained by the fact that it was grown at 25/20°C.

Four temperate species, *V. sativa*, *M. alba*, *L. corniculatus* and *L. japonicus*, were grown in either 25°C or 20°C daytime temperatures, and although the NR activities in *V. sativa* did not differ significantly between the two temperature regimes, the two treatments produced significantly different NR activities in *M. alba*, where the leaf, stem and root activities all significantly increased at the lower temperature, most notably the leaf activity which increased four-fold (see Figure 6.9). The NR activity in the shoots of *L. corniculatus*, and to a lesser extent *L. japonicus*, was also greater at the lower temperature. These results are in contrast to those of Andrews *et al.* (1984b), who found that the NR activity in *V. faba* was much lower in plants grown at 15/10°C than at 25/15°C especially in the leaves, although this was dependent on the cultivar. However, in agreement with Andrews and colleagues, the root activity in the two *Lotus* species decreased at the lower temperature. This contrasts with the results of Clarkson and Deane-Drummond (1983) who found a shift in emphasis of NO_3^- assimilation, including an increase in NR activity, to the roots of plants grown with cooler root systems, although this may explain the increased root activity in *M. alba* at the lower temperature (though not the increased shoot activity).

Some of the differences between the results described here and previous publications may be explained by the different growth systems used. Root temperature may be quite different to shoot temperature, and is known to influence

both NR activity and the pattern of translocation of NO_3^- (Rorison *et al.*, 1983; Clarkson *et al.*, 1986). Although the plants used by Andrews *et al.* (1984b) were pot-grown (as were the plants used in this thesis), the experiments of Clarkson and colleagues were performed on plants grown in flowing culture solutions, with the roots pretreated at 5°C before being adjusted to the experimental root temperature, whilst the shoots were kept at ambient temperature. The root temperatures in the pot-grown plants used for this thesis were probably similar to the shoot temperatures.

Why might NR activities, and presumably the partitioning of NO_3^- assimilation, be affected by temperature? The induction of NR is thought be dependent on an active pool of NO_3^-, which is dependent on the rate of transpiration, rather than on the storage pool of NO_3^- found largely in the vacuole (Clarkson *et al.*, 1986; King *et al.*, 1992). It was conjectured that the higher growth temperatures used in this experiment, although not very high, might have been causing stomata to close thereby reducing the rate of transpiration. However, the transpiration rates of the four species grown at the two temperature regimes were actually greater at the higher temperature (Figure 6.9), indicating that NO_3^- flux was not a limiting factor.

Temperature is known to affect enzymes in several ways. The post-translational processing of a polypeptide, as well as regulation at the transcriptional or translational level, may be altered by temperature (Ougham and Howarth, 1988). It is possible that the differences in NR activity in plants grown under the two regimes are a result of differential effects of temperature on phosphorylation, a post-translational modification known to regulate NR activity (Kaiser and Spill,

1991; Glaab and Kaiser, 1993). The Q_{10} of an enzyme can vary with the temperature and the extent of this variation can differ according to the enzyme, the substrate concentration and the species (Pollock and ap Rees, 1975; Hochachka and Somero, 1984). The differential effect of temperature on the component enzymes of a metabolic pathway can lead to a substantial redirection of metabolism. For example, temperature is known to effect partitioning of respiratory substrates, in that a decrease in temperature causes deactivation of three cold-labile enzymes (phosphofructokinase, glyceraldehydephosphate dehydrogenase and pyruvate kinase) directing photosynthetic C to sugars and away from respiration. This leads to phenomena such as cold induced sweetening of potatoes (Pollack and ap Rees, 1975; Dixon and ap Rees, 1980). It is possible therefore that NO_3^- was being redirected to assimilation and away from storage through temperature effects on metabolism other than upon NR activity.

6.3.7 What determines the partitioning of nitrate assimilation?

It is probable that several factors determine the site of NO_3^- assimilation. At high light intensities, when photosynthesis is saturated and there is no competition for ATP and reductant between NO_3^- and CO_2 assimilation, leaf NO_3^- assimilation may be an advantage since it can make use of surplus photochemical energy. Indeed most of the tropical species examined in this chapter were shoot assimilators, and several of the temperate shoot assimilators were plants of open habit, e.g. *Robinia pseudoacacia* and *Clianthus formosus*. However, energy and carbon gain are not always the factors limiting plant growth. The reduction of NO_3^- in the shoots of desert plants may help dissipate excess photochemical energy that

could otherwise be damaging (Smirnoff and Stewart, 1985). The results obtained also support the observation that pioneer or colonizing species, which utilize NO_3^- in preference to NH_4^+ (e.g. *M. pudica* and many *Phaseolus* species), tend to assimilate NO_3^- in their shoots. Conversely, the more persistent perennial species (e.g. *S. japonica*, *C. aligera*, and the *Lotus* species) show low levels of shoot NR activity and probably have less capacity to utilize NO_3^- (Al Gharbi and Hipkin, 1984; Stewart et al., 1988; 1990; 1993). However, none of the present theories completely explain the data, and it may prove that there is no strong selective pressure in favour of root or shoot assimilation.

6.3.8 Did root GS2 arise as an adaptation to young, base-rich soil?

The ancient, highly weathered soils of the tropics tend to be very acidic, with reduced inorganic N being a more usual N source than NO_3^-. The soils in temperate regions tend to be much younger, reflecting recent geological activity, and are relatively base-rich and often calcareous, factors which promote nitrification. However, many tropical soils are not acidic, and nitrification rates will be higher at the elevated temperatures found in the Tropics. Not surprisingly, some tropical legumes are adapted to these soils, e.g. several *Mimosa* species, and it may be important to note that GS2 was observed in root extracts of *Mimosa pudica*.

The more advanced temperate tribes, the Loteae, Coronilleae, Vicieae and Trifolieae, are all calcicole (Allen and Allen, 1981) and, as has been shown, several species in these tribes synthesize root GS2 in the presence of NO_3^-. However, temperate soils can be very acidic and NH_4^+-rich, and some temperate

legumes, e.g. *U. europaea* and *C. scoparius*, are calcifuge, whilst several species e.g. *T. subterraneum* will tolerate acid soils. So although adaptation to the predominantly base-rich, fertile soils has undoubtedly influenced species' responses to NO_3^- and NH_4^+, soil type *per se* does not reflect the present occurrence of root GS2 in temperate legumes.

Root GS2 is found in two monocots, *Z. mays* and *H. vulgare* (Miflin, 1974; Sakakibara *et al.*, 1992a) and in the non-legume dicot, *L. esculentum* (Becker *et al.*, 1992), all of which originate from temperate climes, as well as in the legume species described in this Chapter. Monocots and dicots diverged very early in angiosperm history (in the early Cretaceous), with the legumes separating very quickly from the other dicots (Friis *et al.*, 1989). This suggests that the presence of GS2 in roots was either the ancestral condition prior to the divergence of the monocots and dicots, a condition that was subsequently lost in at least the tropical papilionoid legumes, or it arose independently in many diverse plant families. All higher plants from temperate areas, including temperate legumes, are thought to have evolved from tropical ancestors, which suggests that the occurrence of root GS2 has arisen independently in members from different plant families as an adaptation to temperate climates. The two major centres of origin of temperate legumes (and many other plant groups), the Sino-Himalayan and Mexican regions, have experienced recent geological perturbation and generally have young soils. This supports the idea that root GS2 expression may have arisen independently in several different species, to assimilate NH_4^- derived from the more abundant NO_3^- encountered in the two centres of origin.

6.3.9 Does the presence of root GS2 correlate with root nitrate assimilation?

GS2 protein is found in the roots of all temperate legumes so far looked at and it is NO_3^--inducible in at least some of these species. Also there appears to be a positive correlation between the total GS activities and the NR activities in the roots of these temperate species, a relationship that is also found to a lesser degree in the leaves of the tropical legumes (see Figure 6.7a and f). This strongly supports the contention that root GS2 is important in the assimilation of NH_4^+ derived from NO_3^- reduction in many temperate legumes, and emphasises the importance of shoot NO_3^- assimilation in the tropical species. However, several of the temperate legumes that were sampled did not appear to assimilate appreciable NO_3^- in their roots, and at least three of the tropical species, which did not possess the GS2 polypeptide in root extracts, assimilated a large proportion of NO_3^- in their roots.

FIGURE 6.1 Tribes of the Papilionoideae

Derived from Corby, Polhill and Sprent (1983).

At least one member of each of the tribes in upper case lettering were sampled. Members of the tribes depicted in square boxes are predominantly temperate in origin, those in eliptical boxes have members of both temperate and tropical origin.

FIGURE 6.1

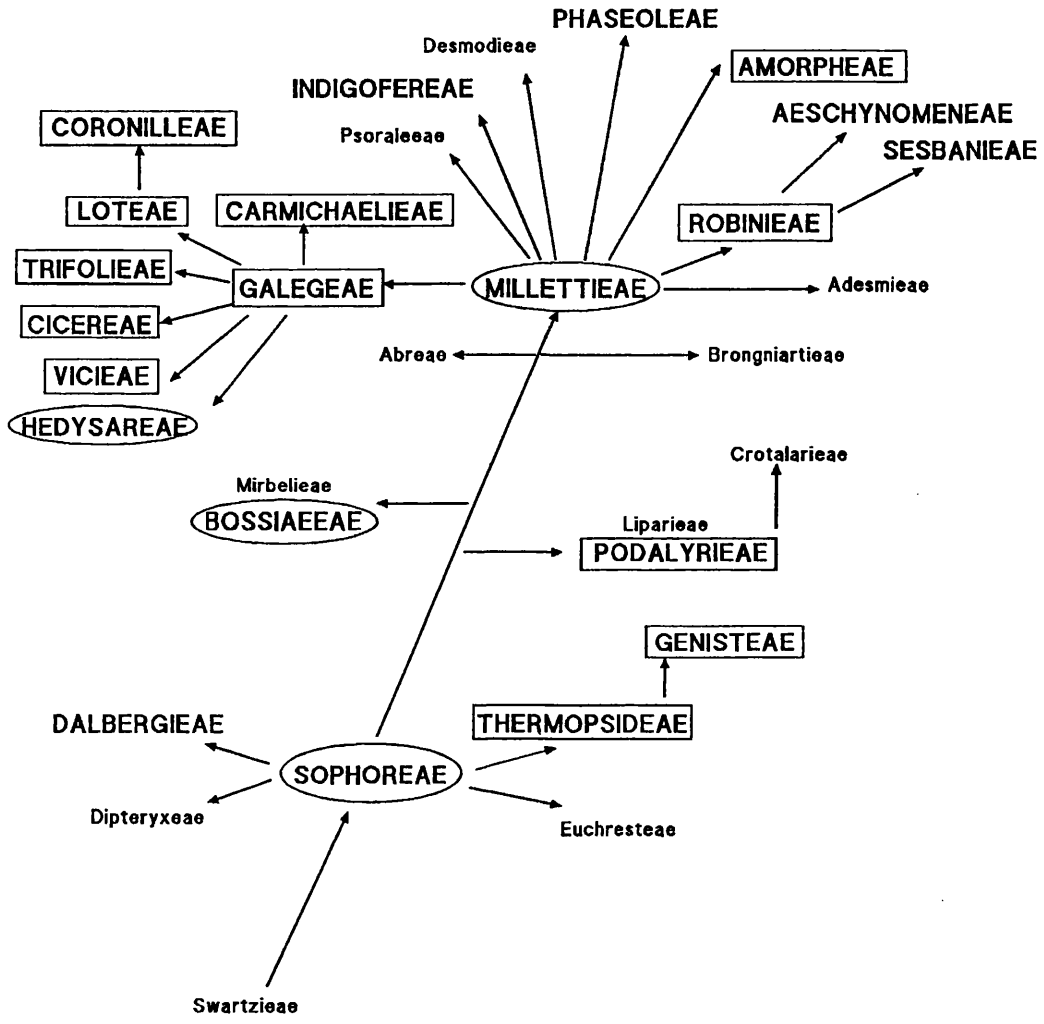

PHASEOLEAE
Desmodieae
AMORPHEAE
INDIGOFEREAE
AESCHYNOMENEAE
CORONILLEAE
Psoraleeae
SESBANIEAE
LOTEAE
CARMICHAELIEAE
ROBINIEAE
TRIFOLIEAE
GALEGEAE
MILLETTIEAE
Adesmieae
CICEREAE
Abreae
Brongniartieae
VICIEAE
HEDYSAREAE
Crotalarieae
Mirbelieae
BOSSIAEEAE
Liparieae
PODALYRIEAE
GENISTEAE
DALBERGIEAE
THERMOPSIDEAE
SOPHOREAE
Dipteryxeae
Euchresteae
Swartzieae

TABLE 6.1 **NR activities and the percentage of xylem sap nitrogen transported as nitrate in legumes grown on low nitrate**

The NR activities are *in vivo* except those marked # which are *in vitro* NR activities. All species are in the Papilionoideae except *Dimorphandra mollis* (Caesalpinoideae) and *A. pulchella* (Mimosoideae). Species marked * are of tropical origin

References:

1, Andrews *et al.* (1990);
2, Andrews *et al.* (1984a);
3, Andrews (1986a);
4, Andrews (1986b);
5, Al Gharbi and Hipkin (1984);
6, Hervas *et al.* (1991);
7, Oliver *et al.* (1983);
8, Stewart *et al.* (1993);
9, Pate *et al.* (1993);
10, Stewart and Orebamjo (1979);
11, Sutherland *et al.* (1985);
12, Vessey and Layzell (1987);
13, Stewart *et al.* (1987);
14, Monza *et al.* (1989);
15, Wallace (1986);
16, Andrews *et al.* (1984b);
17, Laurie and Stewart (1993);
18, Stewart *et al.* (1992).

TABLE 6.1 NR ACTIVITIES AND XYLEM SAP NITRATE CONTENT OF LEGUMES GROWN ON LOW NITRATE.

SPECIES	[NITRATE] IN GROWTH MEDIUM	NR ACTIVITIES ($\mu mol.h^{-1}.gfwt^{-1}$) LEAF	STEM	SHOOT	ROOT	% XYLEM SAP NITRATE	REF
Acacia pulchella*	field data			0.11	0.05		8,9
Cajanus cajan*	nitrate free	0.1-0.3	0		0		1
	1 mM	0.8	0.8		0.8	14-60%	2,3,4
	5 mM					20-66%	3,4
Cicer arietinum	1 mM	1.0-3.0	0.3-1.2		0.3-0.9		17
Dalbergia brasiliense*	field data	0.2			0.55		18
Daviesia divaricata	field data			0.04	0.05		8,9
Daviesia nudiflora	field data			0.07	0.08		8,9
Dimorphandra mollis*	5 mM			0.10	0.45		13
Erythrina crista-galli*	nitrate free	0.1-1.9	0		0		1
Erythrina senegalensis*	field data	50-278					10
Glycine max*	nitrate free	0.6-3.4	<0.4		0		1
	1 mM	1.8	1.5		0.9	58%	2
	2 mM					44%	12
Gompholobium tomentosum	field data			0.22	0.05		8,9
Hardenbergia comptoniana*	field data			0.79	0.04		8,9
Hovea trisperma*	field data			0.09	0.03		8,9
Inga flagelliformis*	field data	1.40			0.16		18
Jacksonia sternbergiana*	field data			0.16	0.18		8,9
Kennedia prostrata*	field data			0.90	0.15		8,9
Laburnum anagyroides	field data	0.1					5
Lotus corniculatus	5 mM	0.1-0.2	0.8		0.57		14
Lupinus albus	1 mM					14%	3
Lupinus angustifolius	1 mM	<0.20	0.2-1.2		3.2		2
Oxylobium capitatum	field data			0.13	0.04		8,9
Phaseolus vulgaris*	nitrate free	0.2-0.8	0		<0.2		1
	1 mM	<2.1	<0.9		<0.7	62%	2
	4 mM					61%	4
	5 mM	#7.74	#0.18		#0.10		15
Pisum sativum	1 mM	0.3-0.7	<1.5		1.3-1.8	33%	2,3
	2 mM	0.06	0.04		0.43		6
	4 mM	0.12	0.08		0.45		6
	5 mM			#3.7	#1.98		15
Robinia pseudoacacia	field data	0.4					5
Trifolium repens	1 mM					15%	3
Trifolium subterraneum	1 mM			0.21	0.15		7
	5 mM			2.46	1.74		7
Vicia faba	field grown	0.4	0.45		0.2-0.4		16
	1 mM	0.2	0.4		0.4		2
		0.12	0.43		0.45	7-11%	16
				0.1-0.2	0.2-0.4		11
	4 mM			0.2-0.7	0.4-0.7		11
Vigna radiata*	nitrate free	0.3-5.6	0		0		1

TABLE 6.2 Distribution of 55 species of temperate and tropical legume throughout the major biomes of the World.

SPECIES	BIOMES[1]										GEOGRAPHIC AREAS[1]
Af - *Acacia farnesiana*	1	2	3	4		6	7	8		10	SAm,(Global)
Aj - *Albizia julibrissin*		2	3	4				8	9	10	SAm,Af,(NAm)
Ac - *Amorpha canescens*			3			6	7	8	9		NAm
Ah - *Arachis hypogaea*		2	3	4	5		(7)		(9)	(10)	eSAm,(Global)
Am - *Arachis monticola*		2	3	4	5						eSAm
Ag - *Astragalus glycyphyllos*							7	8	9	10 11	Eu,As
Bp - *Bossiaea pulchella*				4			7	8	9	10	Au
Cae- *Caesalpinia pulcherrima*		2	3	4		6	7				pTr
Cc - *Cajanus cajan*	1	2	3	4		6					Af,As,(pTr)
Car- *Carmichaelia aligera*									9		NZ
Cas- *Cassia fistula*	1	2			5						As,(pTr)
Cer- *Cercis siliquastrum*							7	8	9	(10)	NAm,Eu,As
Ca - *Cicer arietinum*						6	7	8	9	10	As,Af,Eu
Cf - *Clianthus formosus*						6	7				Au,(As)
Cv - *Coronilla varia*							7	8	9	10	Eu
Cs - *Cytisus scoparius*								8	9	10	sEu,wAs,nAf
Ds - *Dalbergia sissoo*			3		5	6	7				As
Ecg- *Erythrina crista-galli*		2	3								CAm,SAm,(pTr)
Go - *Galega officinalis*					5			8	9	10	Eu
Ga - *Genista aetnensis*						6	7	8		(10)	Eu
Gm - *Glycine max*	1	2					(7)		(9)	(10)	As,(Global)
Gs - *Glycine soja*	1	2									As,(Global)
Hv - *Hardenbergia violacea*			3		5						Au
Hc - *Hedysarum coronarium*			3				7	8	9	10 11	Eu,nAf
Hip- *Hippocrepis comosa*								8	9	10	Eu
Ip - *Indigofera pseudotinctoria*	1	2	3	4							Af,NAm
La - *Laburnum anagyroides*							7	8		10	wEu,wAs,nAf
Lo - *Lathyrus odoratus*								8		(10)	Eu
Lc - *Lotus corniculatus*					5		7		9	10 11	Eu,As,Af,(Global)
Lj - *Lotus japonicus*					5		7	8	9	10	As,(NAm,SAm)
Lt - *Lotus tetragonolobus*							7	8	(9)	(10)	Eu,As
Ln - *Lupinus nanus*								8		10	Eu,NAm
Ma - *Melilotus alba*							7	8	9	10	Eu,Af,As,(Global)
Mp - *Mimosa pudica*			3	4	5						SAm,Af,As
Or - *Ononis repens*						6	7	8	9	10	Eu,Af,As
Pc - *Phaseolus coccineus*		2			5					(10)	CAm,(Global)
Pl - *Phaseolus lunatus*	1	2	3	4			7			(10)	CAm,SAm,(pTr)
Pv - *Phaseolus vulgaris*		2	3		5					(10)	C+SAm,(Global)
Pod- *Podalyria calyptrata*								8	(9)	(10)	sAf,(NAm,NZ)
Pj - *Prosopis juliflora*		2	3	4						(10)	CA,(pTr,NAm)

TABLE 6.2 Continued

SPECIES	BIOMES[1]	GEOGRAPHIC AREAS[1]
Rp - *Robinia pseudoacacia*	(8)9(10)	NAm,(Eu)
So - *Senna obtusifolia*	2 3 4	pTr
Sg - *Sesbania grandiflora*	1 2	pTr
Sj - *Sophora japonica*	10	As,NAm
Tv - *Tephrosia vogelii*	1 2 3 4	Af,(As)
Tc - *Thermopsis caroliniana*	5 7 8 9 10	Eu,As,NAm
Ts - *Trifolium subterraneum*	7 8 9 10	Eu,As,Af,(Global)
Tfg-*Trigonella foenum-graecum*	6 7 8 9	Eu,As,Af
Ue - *Ulex europaeus*	9 10	wEu,nAf
Vf - *Vicia faba*	(5)6 7 8 9 10	As,Af,Eu,(Global)
Vs - *Vicia sativa*	7 8 9 10 11	As,Eu
Vr - *Vigna radiata*	2 3 4 5	As,(pTr)
Vu - *Vigna unguiculata*	2 3 4 7	Af,(pTr)
Vus- *Vigna unguiculata* ssp. *sesquipedalis*	2 3 4 7	Af,(pTr)
Wf - *Wisteria floribunda*	10	As

1 = tropical rainforest
2 = sub tropical forest
3 = dry tropical scrub and thorn forest
4 = savanna
5 = tropical upland
6 = desert
7 = scrub, steppe and semi-desert
8 = Mediterranean sclerophyll and chaparral
9 = temperate grassland
10 = warm temperate forest
11 = cold temperate forest

Af = Africa
As = Asia
Au = Australasia
CAm = central America
Eu = Europe
NAm = N. America
NZ = New Zealand
pTr = pantropical
SAm = S. America

Prefixes: n = north
s = south
e = east
w = west

[1] Introduced species are in brackets

FIGURE 6.2 Western blots showing the occurrence of GS polypeptides in leaves, stems and roots of representative species of temperate and tropical legumes.

Extracts were separated by SDS-PAGE (1μg total protein per lane) and electroblotted onto membranes which were then immunostained for GS polypeptides (see Figure 3.1 for more details).

A) Root extracts from six species of temperate legume. Lanes 1-6: *Robinia pseudoacacia*; *Genista aetnensis*; *Clianthus formosus*; *Podylaria calyptrata*; *Carmichaelia aligera*; and *Sophora japonica*. All of the species are members of the Papilionoideae.

B) Root extracts from five species of tropical legume. Lanes 1-5; *Senna obtusifolia*; *Erythrina cristagalli*; *Sesbania grandiflora*; *Indigofera pseudotinctoria*; and *Glycine soja*. All of the species are members of the Papilionoideae except for *S. obtusifolia* which belongs to the Caesalpinoideae.

C) Leaf extracts (lanes 1 and 5), stem extracts (lanes 2 and 6) and root extracts (lanes 3 and 7) from two temperate species; *Vicia faba* (lanes 1, 2 and 3) and *Lupinus nanus* (lanes 5, 6 and 7). The markers (lane 4) are 29, 39.8, 58.1 and 97.4 kDa. Both species are in the Papilionoideae.

D) Leaf extracts (lanes 1 and 5), stem extracts (lanes 2 and 6) and root extracts (lanes 3 and 7) from two tropical species; *Arachis hypogaea* (lanes 1, 2 and 3) and *Glycine max* (lanes 5, 6 and 7). The markers (lane 4) are 29, 39.8, 58.1 and 97.4 kDa. Both species are in the Papilionoideae.

E) Shoot (lanes 1, 3, 5 and 7) and root extracts (lanes 2, 4, 6, and 8) of four species of non-papilionoid legume. *Caesalpinia pulcherrima* (lanes 1 and 2); *Cercis siliquastrum* (lanes 3 and 4); *Cassia fistula* (lanes 5 and 6); and *Acacia farnesiana* (lanes 7 and 8).

FIGURE 6.2

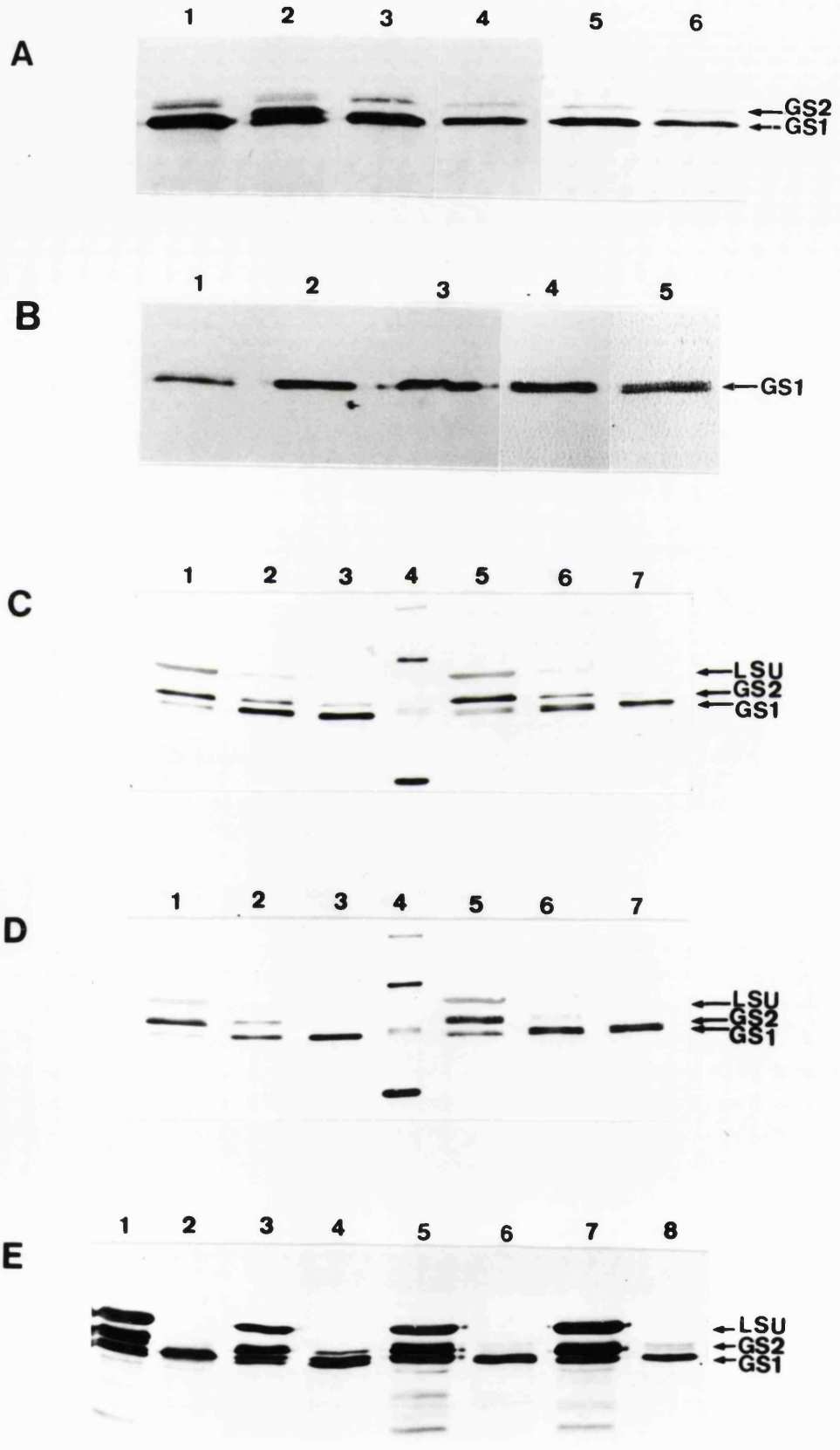

A
1 2 3 4 5 6
GS2
GS1
B
1 2 3 4 5
GS1
C
1 2 3 4 5 6 7
LSU
GS2
GS1
D
1 2 3 4 5 6 7
LSU
GS2
GS1
E
1 2 3 4 5 6 7 8
LSU
GS2
GS1

FIGURE 6.3 The activity of GS in the leaves, stems and roots of 55 species of legume separated into groups of similar activity by Ward's clustering.

The GS activity in leaf (□), stem (///) and root (\\\) extracts of the species in Ward's cluster 1, cluster 2, cluster 3, cluster 4, cluster 5 and cluster 6.

The arithmetic means and standard deviations of the activities in each cluster are also plotted. The cluster diagnostics are given in Table 6.3.

All plants were grown on 1 mM NO_3^-, in a 25°C 16 h day and a 20°C 8 h night, except for those marked with an asterisk which were grown in a 20°C day and a 15°C night.

The species' names have been abbreviated the key to which can be found in Table 6.2: the abbreviations in boxes indicate tropical species.

FIGURE 6.3

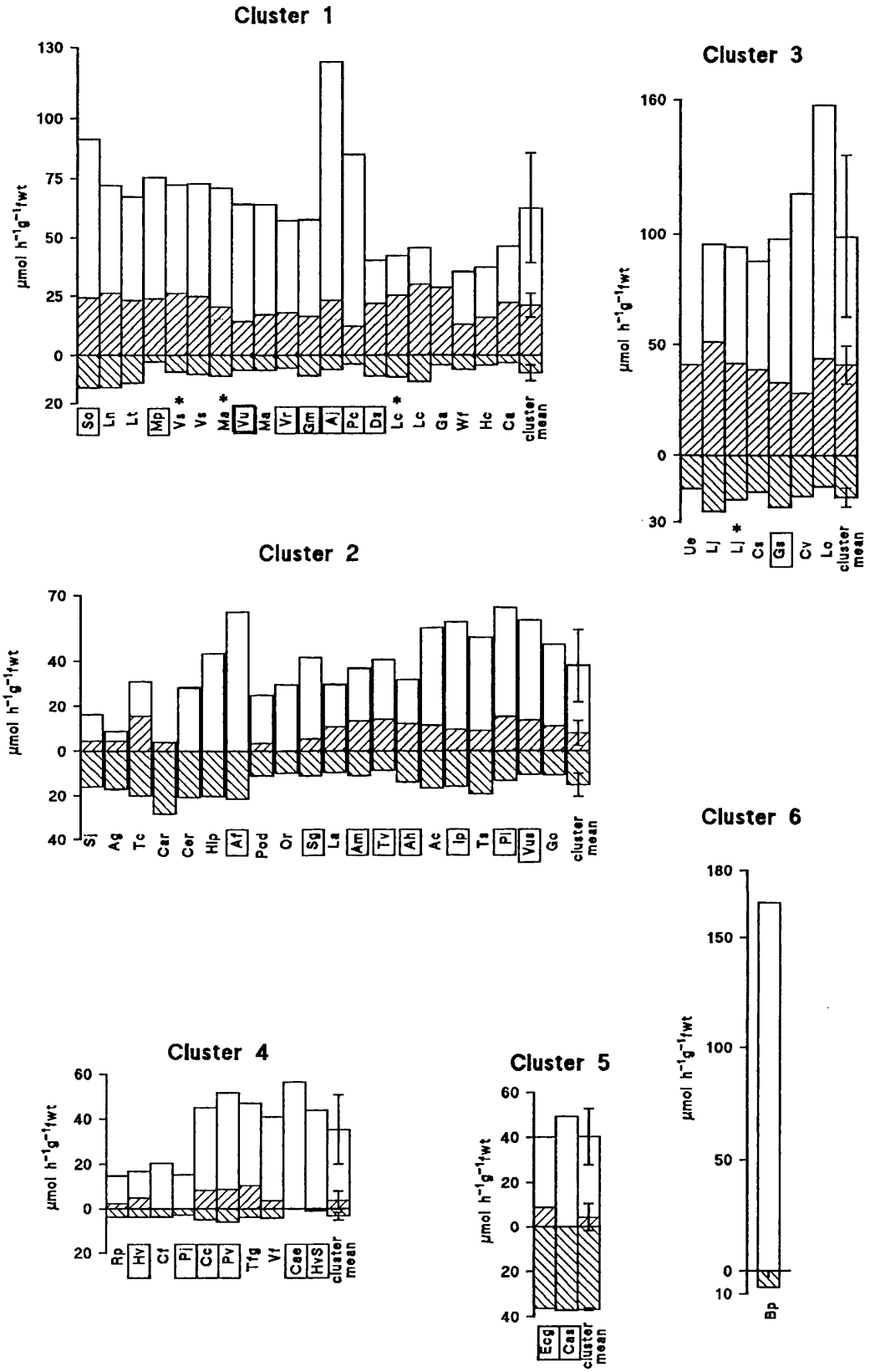

Cluster 1
Cluster 3
Cluster 2
Cluster 6
Cluster 4
Cluster 5
µmol h⁻¹g⁻¹fwt
cluster mean

TABLE 6.3 **Diagnostics for Ward's clustering of GS activities**

The number of tropical and temperate species, and the cluster diagnostics in each Ward's cluster plotted in Figure 6.3. Mean GS activities and T-values of the leaf, stem and root in each cluster, and for the entire data set.

CLUSTER	NUMBER OF SPECIES			CLUSTER MEAN (μmol.h^{-1}.g^{-1}fwt)	T-VALUE
	Temperate	Tropical			
1	9	8	Leaf	41.24	0.046
			Stem	21.17	0.459
			Root	7.19	-0.545
2	12	8	Leaf	30.38	-0.343
			Stem	7.80	-0.574
			Root	15.27	0.457
3	5	1	Leaf	57.85	0.640
			Stem	40.98	1.989
			Root	18.93	0.910
4	4	5	Leaf	31.41	-0.306
			Stem	3.89	-0.876
			Root	3.31	-1.026
5	0	2	Leaf	40.47	0.018
			Stem	4.40	-0.837
			Root	36.70	3.111
6	1	0	Leaf	165.60	4.492
			Stem	0.00	-1.176
			Root	7.07	-0.560

TOTAL NUMBER OF SPECIES			DATA SET MEAN (μmol.h^{-1}.g^{-1}fwt)
Temperate	Tropical		
31	24	Leaf	39.97
		Stem	15.23
		Root	11.59

FIGURE 6.4 ***In vivo*** **NR activity in the leaves, stems and roots of 46 species of legume separated into groups of similar activity by Ward's clustering.**

The NR activity in the leaf (□), stem (///) and root (\\\) of the species in six Ward's clusters. See the caption for Figure 6.3 for further details

FIGURE 6.4

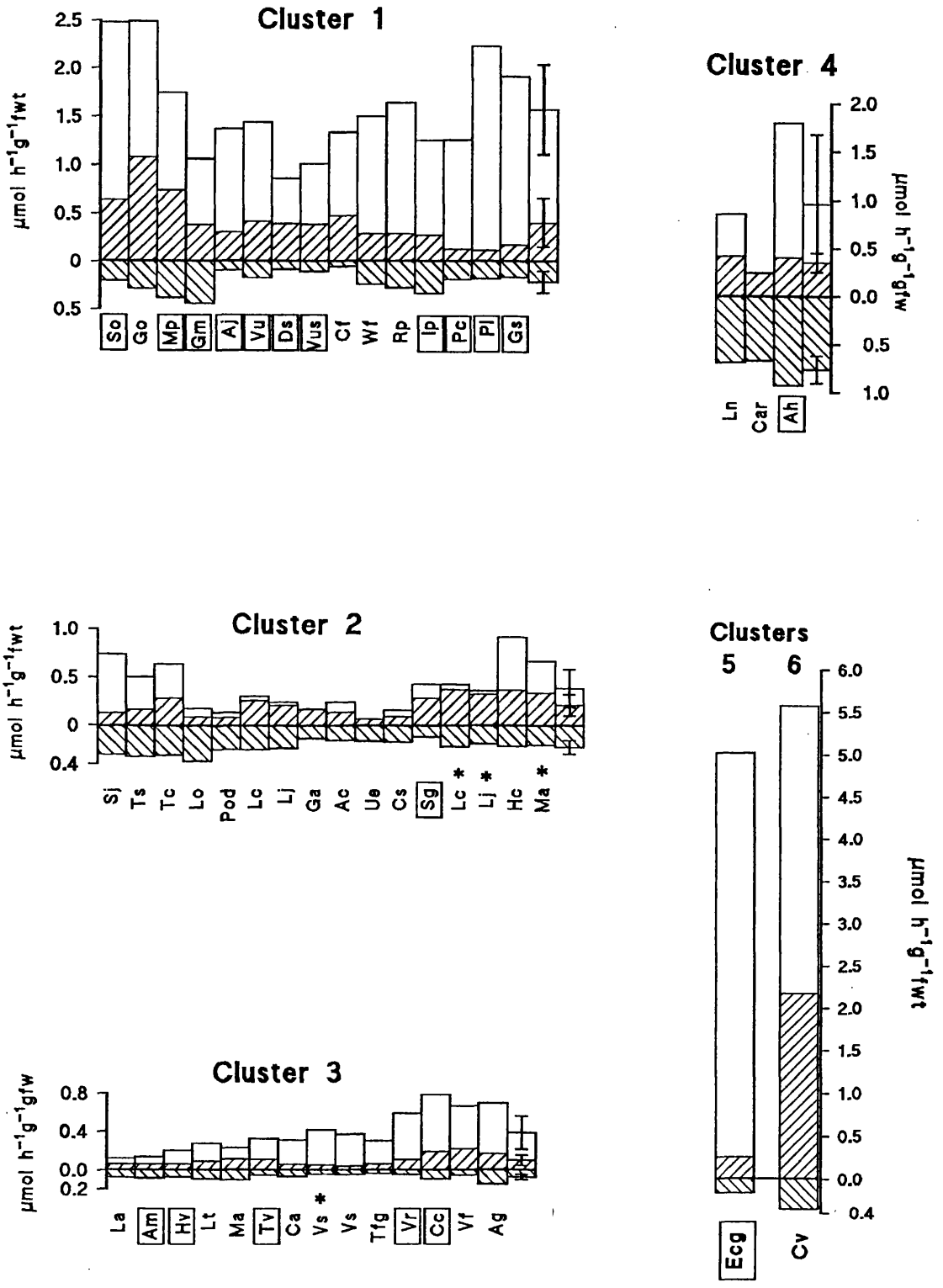

Cluster 1
μmol h-1g-1fwt
So Go Mp Gm Aj Vu Ds Vus Cf Wf Rp Ip Pc Pl Gs
Cluster 4
μmol h-1g-1gfw
Ln Car Ah
Cluster 2
μmol h-1g-1fwt
Sj Ts Tc Lo Pod Lc Lj Ga Ac Ue Cs Sg Lc* Lj* Hc Ma*
Clusters
5 6
μmol h-1g-1fwt
Ecg Cv
Cluster 3
μmol h-1g-1gfw
La Am Hv Lt Ma Tv Ca Vs* Vs Ttg Vr Cc Vf Ag

TABLE 6.4 **Diagnostics for Ward's clustering of *in vivo* NR activities**

The number of tropical and temperate species, and the cluster diagnostics in each Ward's cluster plotted in Figure 6.4. Mean *in vivo* NR activities and T-values of the leaves, stems and roots in each cluster, and for the entire data set.

CLUSTER	NUMBER OF SPECIES			CLUSTER MEAN (μmol.h^{-1}.g^{-1}fwt)	T-VALUE
	Temperate	Tropical			
1	4	11	Leaf	1.166	0.539
			Stem	0.407	0.358
			Root	0.214	0.001
2	12	1	Leaf	0.175	-0.574
			Stem	0.205	-0.243
			Root	0.230	0.093
3	8	5	Leaf	0.286	-0.449
			Stem	0.103	-0.547
			Root	0.071	-0.807
4	2	1	Leaf	0.611	-0.084
			Stem	0.354	0.212
			Root	0.762	3.112
5	0	1	Leaf	4.759	4.577
			Stem	0.264	-0.068
			Root	0.157	-0.319
6	1	0	Leaf	3.400	3.050
			Stem	2.178	5.636
			Root	0.350	0.776

TOTAL NUMBER OF SPECIES			DATA SET MEAN (μmol.h^{-1}.g^{-1}fwt)
Temperate	Tropical		
27	19	Leaf	0.686
		Stem	0.287
		Root	0.214

FIGURE 6.5 ***In vitro*** **NR activity in the leaves, stems and roots of 31 species of legume separated into groups of similar activity by Ward's clustering.**

The NR activity in the leaf (□), stem (///) and root (\\\) of the species in six Ward's clusters. See the caption for Figure 6.3 for further details

FIGURE 6.5

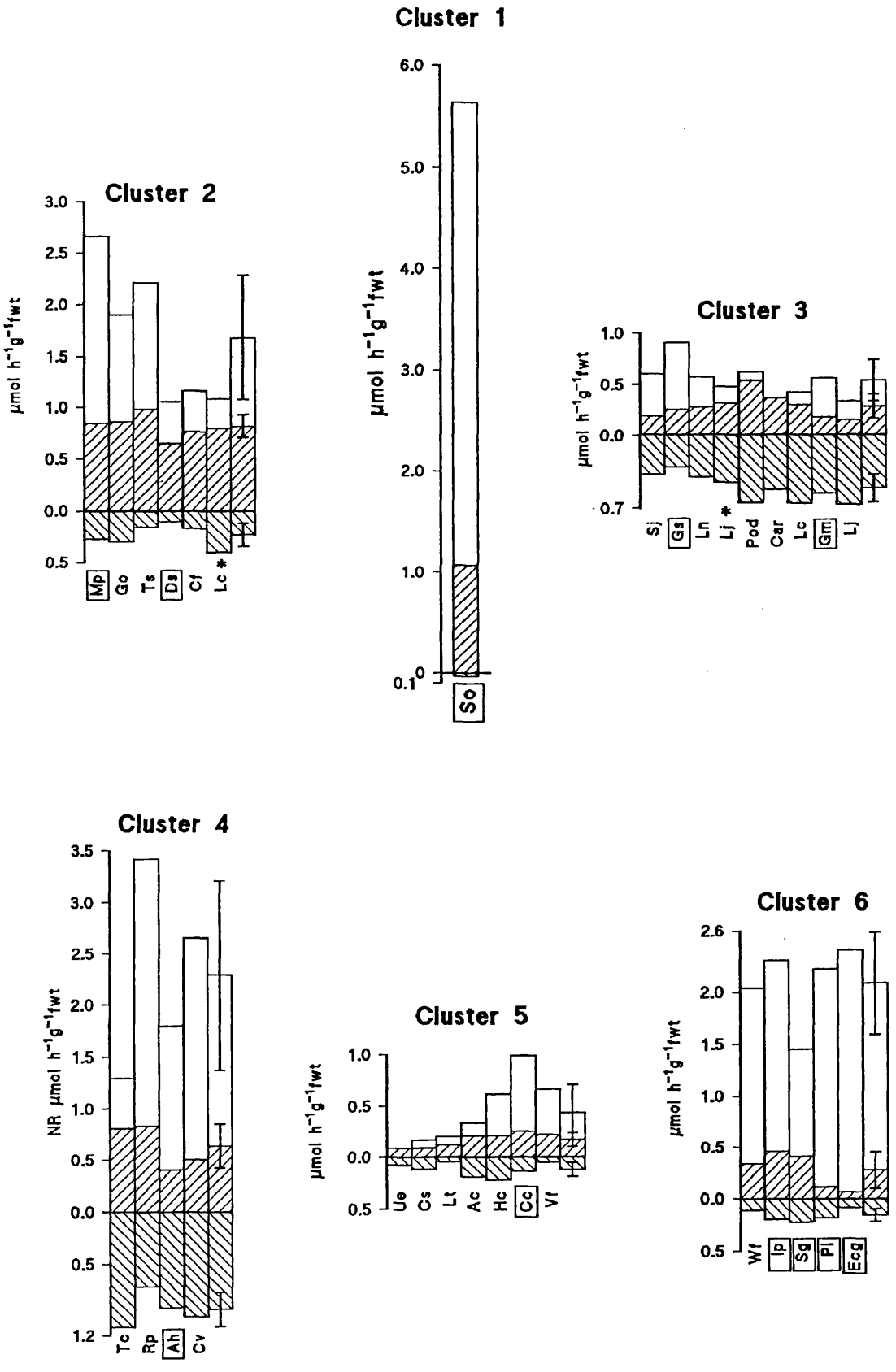

Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
µmol h⁻¹g⁻¹fwt
NR µmol h⁻¹g⁻¹fwt
Mp Go Ts Ds Cf Lc *
So
Sj Gs Ln Lj * Pod Car Lc Gm Lj
Tc Rp Ah Cv
Ue Cs Lt Ac Hc Cc Vt
Wf Ip Sg Pl Ecg

TABLE 6.5 **Diagnostics for Ward's clustering of *in vitro* NR activities**

The number of tropical and temperate species, and the cluster diagnostics in each Ward's cluster plotted in Figure 6.5. Mean *in vitro* NR activities and T-values of the leaves, stems and roots in each cluster, and for the entire data set.

CLUSTER	NUMBER OF SPECIES			CLUSTER MEAN (μmol.h^{-1}.g^{-1}fwt)	T-VALUE
	Temperate	Tropical			
1	0	1	Leaf	4.565	3.554
			Stem	1.067	2.161
			Root	0.039	-1.081
2	4	2	Leaf	0.863	-0.061
			Stem	0.816	1.315
			Root	0.233	-0.422
3	6	2	Leaf	0.256	-0.654
			Stem	0.287	-0.471
			Root	0.507	0.509
4	3	1	Leaf	1.655	0.713
			Stem	0.636	0.707
			Root	0.944	1.995
5	6	1	Leaf	0.268	-0.642
			Stem	0.168	-0.872
			Root	0.124	-0.792
6	1	4	Leaf	1.814	0.868
			Stem	0.276	-0.507
			Root	0.157	-0.681

TOTAL NUMBER OF SPECIES			DATA SET MEAN (μmol.h^{-1}.g^{-1}fwt)
Temperate	Tropical		
20	11	Leaf	0.897
		Stem	0.414
		Root	0.346

FIGURE 6.6 The nitrate concentrations in the leaves, stems and roots of 46 species of legume separated into groups of similar activity by Ward's clustering.

The concentration of NO_3^- in the leaves (□), stems (///) and roots (\\\) of species in six Ward's clusters. See the caption for Figure 6.3 for further details

FIGURE 6.6

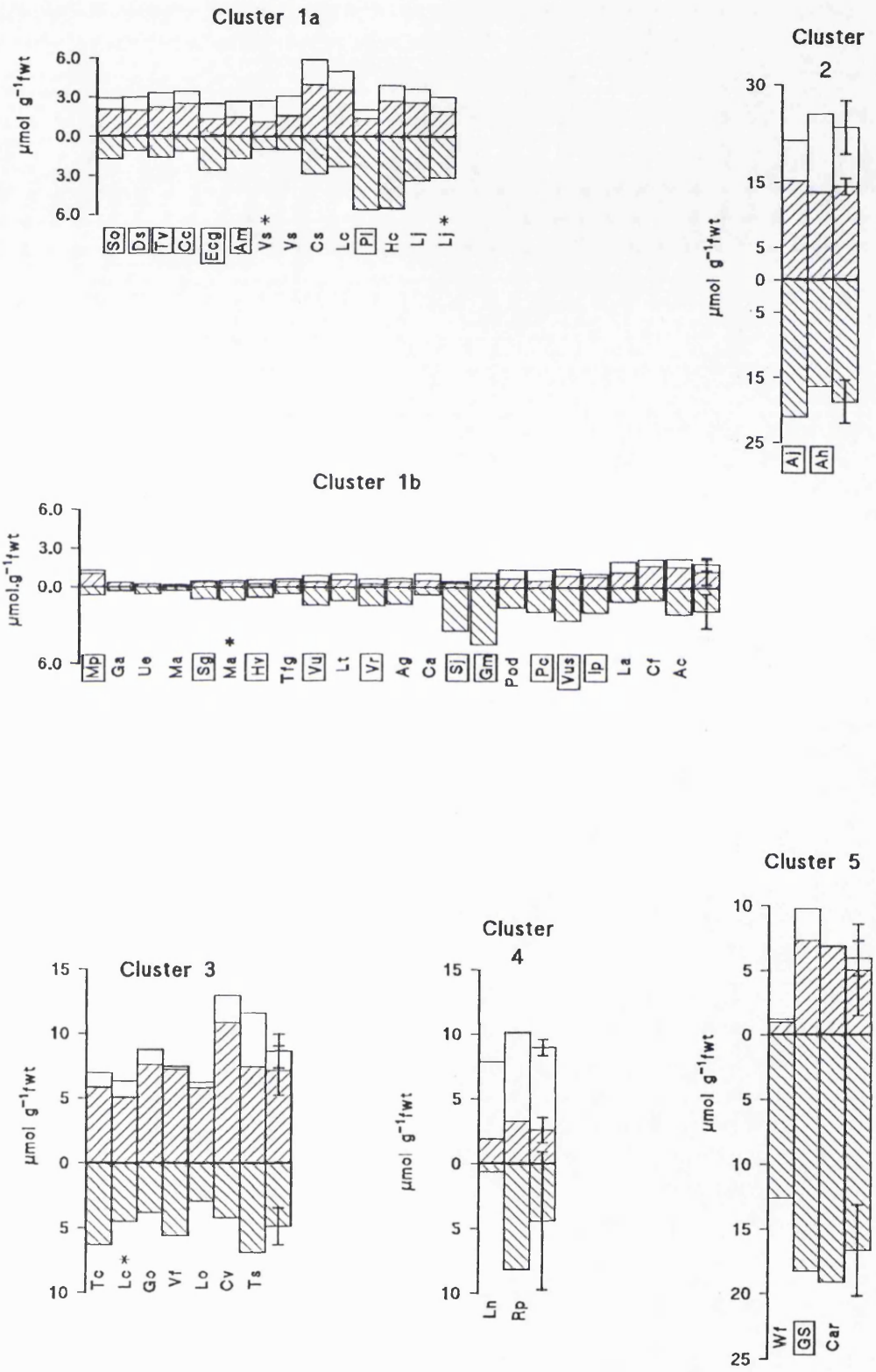

Cluster 1a
µmol g⁻¹fwt
6.0
3.0
0.0
3.0
6.0
So Ds Tv Cc Ecg Am Vs * Vs Cs Lc Pl Hc Lj Lj *
Cluster 2
30
15
5
0
5
15
25
Aj Ah
Cluster 1b
µmol.g⁻¹fwt
6.0
3.0
0.0
6.0
Mp Ga Ue Ma Sg Ma * Hv Tlg Vu Lt Vr Ag Ca Si Gm Pod Pc Vus Ip La Cf Ac
Cluster 5
10
5
0
5
10
15
20
25
Wf GS Car
Cluster 4
15
10
5
0
5
10
Ln Rp
Cluster 3
15
10
5
0
5
10
Tc Lc * Go Vf Lo Cv Ts

TABLE 6.6 Diagnostics for Ward's clustering of tissue nitrate concentrations

The number of tropical and temperate species, and the cluster diagnostics in each Ward's cluster plotted in Figure 6.7. Mean NO_3^- concentrations and T-values of the leaves, stems and roots in each cluster, and for the entire data set.

CLUSTER	NUMBER OF SPECIES			CLUSTER MEAN (μmol.g^{-1}fwt)	T-VALUE
	Temperate	Tropical			
1	17	16	Leaf	0.693	-0.320
			Stem	1.238	-0.472
			Root	1.797	-0.420
2	0	2	Leaf	9.051	3.575
			Stem	14.410	3.334
			Root	18.655	2.949
3	6	0	Leaf	1.504	0.058
			Stem	7.130	1.230
			Root	4.873	0.195
4	2	0	Leaf	6.382	2.331
			Stem	2.590	-0.085
			Root	4.410	0.103
5	2	1	Leaf	0.894	-0.227
			Stem	5.058	0.631
			Root	16.636	2.545

TOTAL NUMBER OF SPECIES			DATA SET MEAN (μmol.g^{-1}fwt)
Temperate	Tropical		
27	19	Leaf	1.380
		Stem	2.873
		Root	3.897

TABLE 6.7 **Diagnostics for Ward's clustering of the combined data for GS activities and *in vivo* NR activities**

The number of tropical and temperate species, and the cluster diagnostics in each Ward's cluster calculated from the *in vivo* NR activities and the GS activities. Mean GS activities and T-values of the leaves, stems and roots in each cluster, and for the entire data set.

CLUSTER	NUMBER OF SPECIES			CLUSTER MEAN (μmol.h^{-1}.g^{-1}fwt)		T-VALUE	
	Temp	Trop		NR	GS	NR	GS
1	4	10	Leaf	0.902	53.30	0.243	0.670
			Stem	0.320	19.82	0.100	0.028
			Root	0.172	8.97	-0.235	-0.310
2	16	7	Leaf	0.399	22.73	-0.322	-0.603
			Stem	0.206	13.52	-0.241	-0.420
			Root	0.162	9.55	-0.292	-0.232
3	4	0	Leaf	0.056	54.28	-0.707	0.711
			Stem	0.153	50.89	-0.398	2.231
			Root	0.248	15.82	0.196	0.627
4	2	1	Leaf	0.611	23.03	-0.084	-0.590
			Stem	0.354	12.79	0.202	-0.471
			Root	0.762	18.45	3.112	0.988
5	0	1	Leaf	4.759	31.67	4.577	-0.231
			Stem	0.264	8.80	-0.068	-0.754
			Root	0.157	36.30	-0.319	3.431
6	1	0	Leaf	3.400	89.67	3.050	2.187
			Stem	2.178	28.45	5.636	0.640
			Root	0.350	18.42	0.776	0.983

TOTAL NUMBER OF SPECIES			DATA SET MEAN (μmol.h^{-1}.g^{-1}fwt)	
Temp	Trop		NR	GS
27	19	Leaf	0.686	37.20
		Stem	0.287	19.43
		Root	0.214	11.24

TABLE 6.8 **The number of tropical and temperate species in the Ward's clusters diagnosed in Table 6.7 containing both NR and GS activities higher or lower than the data mean.**

	NR AND GS ACTIVITY	CLUSTER NUMBERS	NUMBER OF Temp	Trop	% Temp[a]	% Trop[b]
Leaf	Above mean	1 & 6	5	10	19%	53%
	Below mean	2 & 4	18	8	67%	42%
Stem	Above mean	1 & 6	5	10	19%	53%
	Below mean	2 & 5	16	8	59%	42%
Root	Above mean	3,4 & 6	7	1	26%	5%
	Below mean	1 & 2	20	17	74%	89%

[a] Total number of temperate species = 27
[b] Total number of tropical species = 19

FIGURE 6.7 The relationship between *in vivo* NR activities and GS activities in the leaves, stems and roots of temperate and tropical legumes.

A) GS activities plotted against *in vivo* NR activities for leaves of 19 species of tropical legume. The outlier marked Ecg (*Erythrina crista-galli*) is not included in the calculation of the line of best fit where $y = 20.11x + 27.05$; $r = 0.554$; $r^2 = 0.307$; $t = 2.667$; $p = 0.017$.

B) GS activities plotted against *in vivo* NR activities for leaves of 28 species of temperate legume. The outlier marked Lo (*Lathyrus odoratus*) is not included in the calculation of the line of best fit where $y = 9.85x + 28.79$; $r = 0.378$; $r^2 = 0.143$; $t = 2.042$; $p = 0.052$.

C) GS activities plotted against *in vivo* NR activities for stems of 19 species of tropical legume. The line of best fit is $y = 14.74x + 11.16$; $r = 0.381$; $r^2 = 0.145$; $t = 1.697$; $p = 0.108$.

D) GS activities plotted against *in vivo* NR activities for stems of 28 species of temperate legume. The line of best fit is $y = 0.40x + 19.65$; $r = 0.011$; $r^2 = 0.0001$; $t = 0.061$ $p = 0.952$.

E) GS activities plotted against *in vivo* NR activities for roots of 19 species of tropical legume. The line of best fit is $y = 3.83x + 10.06$; $r = 0.099$; $r^2 = 0.0098$; $t = 0.410$ $p = 0.687$.

F) GS activities plotted against *in vivo* NR activities for roots of 28 species of temperate legume. The line of best fit is $y = 24.553 + 6.34$; $r = 0.573$; $r^2 = 0.329$; $t = 3.767$; $p = 0.0008$.

FIGURE 6.7

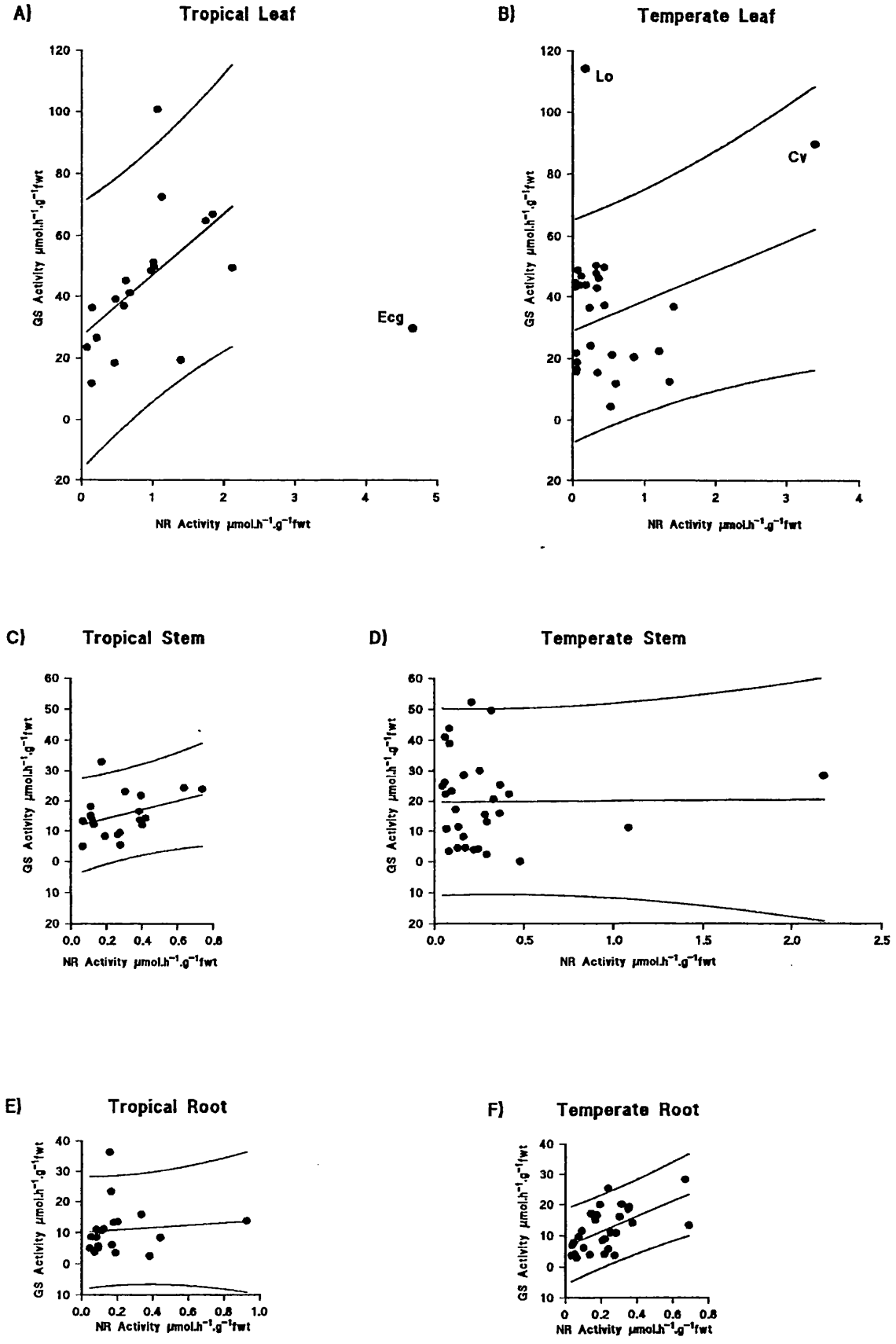

A) Tropical Leaf
B) Temperate Leaf
C) Tropical Stem
D) Temperate Stem
E) Tropical Root
F) Temperate Root
Ecg
Lo
Cv
GS Activity µmol.h⁻¹.g⁻¹fwt
NR Activity µmol.h⁻¹.g⁻¹fwt

FIGURE 6.8 The correlation between the percentage of xylem sap nitrogen transported as nitrate and the *in vivo* NR activities in the shoots of eight legume species.

The abbreviations are

Pod = *Podalyria calyptrata*
Sg* = *Sesbania grandiflora*
Gm* = *Glycine max*
Rp = *Robinia pseudoacacia*
Mp* = *Mimosa pudica*
GS* = *Glycine soja*
Ln = *Lupinus nanus*
Tc = *Thermopsis caroliniana*

Species marked with an asterisk are of tropical origin

Gs, Ln and Tc were not used in the calculation of the line of best fit where $y = 0.031x - 1.025$; $r = 999$; $r^2 = 999$; $t = 44.87$; $p = 2.4 \times 10^{-5}$

FIGURE 6.8

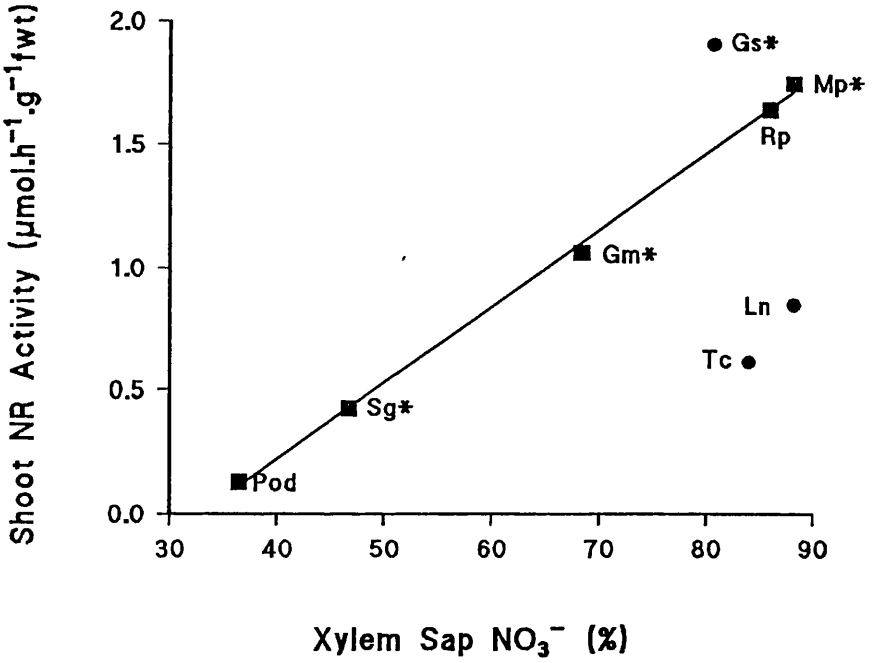

Shoot NR Activity (µmol.h⁻¹.g⁻¹fwt)
2.0
1.5
1.0
0.5
0.0
30
40
50
60
70
80
90
Xylem Sap NO₃⁻ (%)
Gs*
Mp*
Rp
Gm*
Ln
Tc
Sg*
Pod

FIGURE 6.9 The effect of temperature on NR activities and transpiration rates in the leaves, stems and roots of four temperate legumes

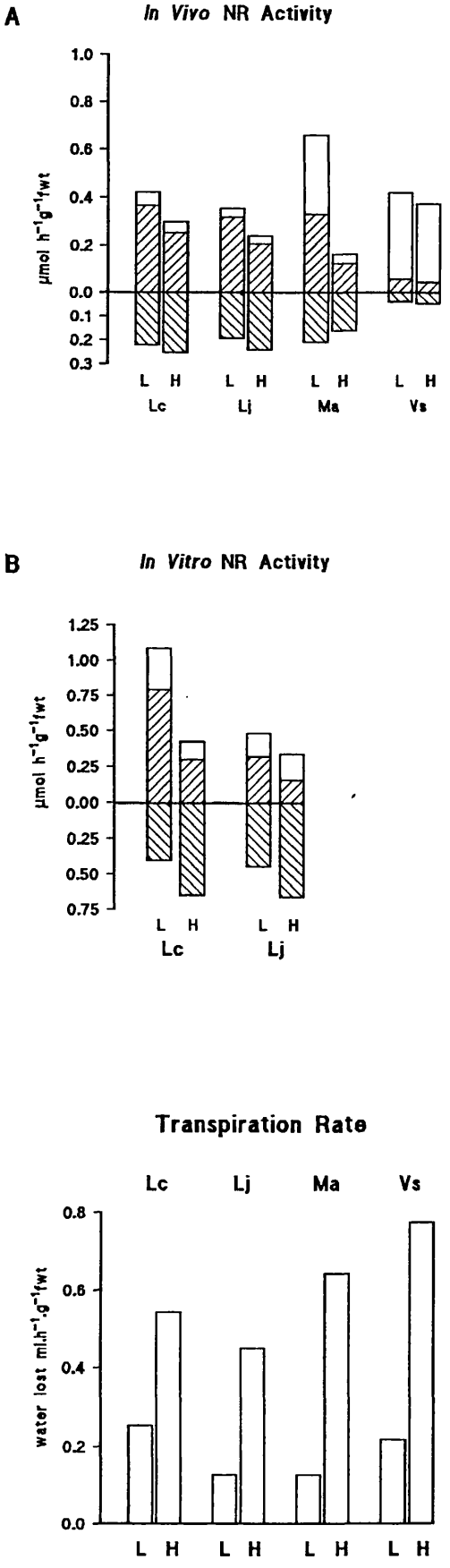
Plants were grown in a 20°C day and 15°C night temperature regime for 28 d, the plants were then either left under the same conditions for a further 10 d (L) or transferred to 25°C (day) and 20°C (night) for 10 d (H). Transpiration rate was estimated by weighing the plants in their pots at regular intervals. The pots were wrapped in clingfilm to prevent evaporation

A) *In vivo* NR activities in leaves (□), stems (///) and roots (\\\) of *L. corniculatus*, *L. japonicus*, *Melilotus alba* and *Vicia sativa*

B) *In vitro* NR activities in leaves (□), stems (///) and roots (\\\) of *L. corniculatus* and *L. japonicus*

C) Rates of transpiration in *L. corniculatus*, *L. japonicus*, *Melilotus alba* and *Vicia sativa*

FIGURE 6.9


A
In Vivo NR Activity
µmol h⁻¹g⁻¹fwt
1.0
0.8
0.6
0.4
0.2
0.0
0.1
0.2
0.3
L H
Lc
L H
Lj
L H
Ma
L H
Vs
B
In Vitro NR Activity
µmol h⁻¹g⁻¹fwt
1.25
1.00
0.75
0.50
0.25
0.00
0.25
0.50
0.75
L H
Lc
L H
Lj
C
Transpiration Rate
Lc
Lj
Ma
Vs
water lost ml.h⁻¹.g⁻¹fwt
0.8
0.6
0.4
0.2
0.0
L H
L H
L H
L H

CHAPTER 7: FINAL DISCUSSION

7.1.1 The biochemistry and molecular biology of GS in *L.corniculatus* and *L. japonicus*

A 39 kD polypeptide, presumed to correspond to cytosolic GS1 protein, was detected in roots and shoots of *L. corniculatus* and *L. japonicus*, with the shoots of both species also containing a 45 kD polypeptide, presumed to be the chloroplast GS2 polypeptide. This was in accordance with other investigations (Forde and Cullimore, 1989; Pearson, Woodall and Havill, in preparation). Both species were unusual, however, in synthesising the 45 kD GS2-like polypeptide in their roots, although this has been observed in a few other species (Barratt, 1980; Vezina *et al.*, 1987; Sakakibara, *et al.*, 1992b). Two-dimensional electrophoresis of shoot and root protein extracts consistently revealed four 39 kD and four 45 kD isoelectric variants in *L. corniculatus* (Figure 3.2). However, *L. japonicus* extracts revealed either three or four of each polypeptide type, depending on whether the plants were grown in culture or in a greenhouse. *L. corniculatus* (and probably *L. japonicus*) is similar to *P. sativum* in apparently not having a nodule-specific (or nodule-enhanced) GS polypeptide (Tingey *et al.*, 1987; Walker and Coruzzi, 1989), and dissimilar to *P. vulgaris*, *M. sativa* and *L. luteus*, which do (Lara *et al.*, 1983; Dunn *et al.*, 1988; Konieczny *et al.*, 1988).

It appears that in most plant species GS is encoded by a multigene family of a minimum of four functional genes encoding one plastidic and at least three cytosolic polypeptides (Forde and Woodall, 1994). So although the number of isoelectric variants of the 39 kD polypeptide in *L. corniculatus* and *L. japonicus*

might reflect the number of gene products, it seems likely that post-translational modification is responsible for at least some of the 45 kD isoelectric variants, and possibly some of the 39 kD variants. As glycan residues could not be detected in any of the GS polypeptides separated by one- and two-dimensional electrophoresis and stained for carbohydrates (see Chapter 3.2.4) glycosylation appears to be ruled out. This is in agreement with previous evidence that GS in *P. sativum*, *N. plumbaginifolia* and *S. oleracea* is not glycosylated (Ericson, 1985; Tingey and Coruzzi, 1987; Tingey *et al.*, 1987). Phosphorylation, which is a common means of regulating enzyme activity in plants (Budde and Chollet, 1988), now seems the most likely post-translational modification of GS, especially as NR has recently been shown to be rapidly activated by dephosphorylation (Kaiser and Spill, 1991; Glaab and Kaiser, 1993). As NO_3^- has been found to induce both NR and root GS2 expression (see Chapter 7.1.4), it may be relevant that NO_3^- has recently been found to activate a cytosolic protein kinase which modulates the activities of at least two key enzymes of assimilate partitioning, by activating phosphoenolpyruvate carboxylase and deactivating sucrose phosphate synthase (Champigny and Foyer, 1992).

A PCR approach, whereby intron 11 of GS genes, plus approximately 180 bp of the flanking exon region, were amplified, indicated a minimum of four GS genes in the *L. corniculatus* genome (Chapter 4). Although, as *L. corniculatus* is tetraploid, the number of GS genes per haploid genome may be less than this. Only one DNA fragment amplified from *L. corniculatus* was positively identified as being from a GS gene. The exon sequences in this fragment were identical to

part of the *L. japonicus* cDNA obtained from both a nodule library (J. Stougaard, personal communication) and a root library (D.T. Clarkson, personal communication). So although the number of isoelectric variants and PCR fragments suggest a family of GS genes, this is not yet proven.

7.1.2 Differences and similarities between the two species: will stress sensitivity limit the role of *L. japonicus* as the model legume?

It appears that both species assimilate most of their NO_3^- in the root since the NADH-NR activities in the root were at least one third of the total NR activities and increased markedly upon NO_3^- application (Chapter 5.4.2 and Figure 5.3). However, the NR activity in the stems of *L. corniculatus* plants was substantial in plants fed on high NO_3^-, as has been found by others (Monza *et al.*, 1989), and probably plays a major role in NO_3^- assimilation. The concentrations of NO_3^- in leaves, stems and roots of *L. corniculatus* were generally lower than in *L. japonicus*, probably reflecting the higher shoot NR activities in *L. corniculatus*. Also, although there was no evidence for a NADPH-dependent NR activity in *L. japonicus*, the roots of *L. corniculatus* had a small but significant activity (Chapter 5.3.2).

Asparagine was the major N transport compound in both species, in accordance with xylem sap analyses of *L. japonicus* (D.T. Clarkson, personal communication). The levels of asparagine (as a proportion of the total amino N) in tissues of *L. japonicus* were very similar whether plants were grown on NO_3^- or NH_4^+, although the actual concentration of asparagine was much higher in plants

grown on NH_4^+. However, in *L corniculatus* the asparagine content (as a proportion of total amino N) was greater in NO_3^--grown plants with the roots containing up to four times the amount found in roots of NO_3^--grown *L. japonicus*. The roots of *L. corniculatus* plants grown on NH_4^+ contained less than a third of the asparagine found in NH_4^+ *L. japonicus* roots.

Differences between the two species in NR activities, asparagine levels, and capacity for growth at very high NO_3^- concentrations (20 mM) are probably a reflection of *L. corniculatus* being tetraploid and more stress tolerant than its diploid relative (Chrtkova-Zertova, 1973; Grime *et al.* 1988). Stress sensitivity may be an important limiting factor in the preferential use of *L. japonicus* over *L. corniculatus* in transformation and regeneration techniques.

7.1.3 Small changes in temperature may have profound effects on assimilate partitioning

A difference of 5°C in the growing temperature resulted in significant differences in NR activities in three species of temperate legume (Chapter 6.2.8, Figure 6.9). In *M. alba*, the NR activities in the roots, stems and especially the leaves, were all much higher at the lower temperature (20°C day, 15°C night) even though the transpiration rate was faster at the higher temperature (25°C day, 20°C night). In *L. corniculatus*, and to a lesser extent *L. japonicus*, *in vitro* NR activity in the shoot was also greater at the lower temperature, but unlike *M. alba* the root activity was higher at the higher temperature. The NR activities in one species, *V. sativa*, were not affected significantly.

The influence of temperature on NR activity (if it is reflected *in vivo*) may confer greater flexibility in the more changeable temperate climates, allowing homeostasis in NO_3^- assimilation over a range of temperatures.

7.1.4 The root GS2 polypeptide in *L. corniculatus* and *L. japonicus*

The root GS2 polypeptide was confirmed as being plastidic in *L. japonicus* (Chapter 3.2.5), and unlike the leaf GS2 in most species was shown to not be regulated by light (Chapter 3.2.6). Synthesis of the root GS2 was found to be enhanced when grown on NO_3^- and almost entirely absent in plants grown on NH_4^+ (Chapter 5.4.3). This has also been found to be the case in three other legume species, *P. sativum*, *V. faba* and *M. media* (Barratt, 1980; Vezina *et al.*, 1987). As the leaf GS2 was not significantly regulated by NO_3^- it appears that if there is a single GS2 gene (as seems likely), it is regulated quite differently in roots and leaves.

Synthesis of several enzymes associated with NO_3^- metabolism, including NR, NiR, ferredoxin-$NADP^+$ oxidoreductase and the NO_3^- uptake and transport systems, are induced as a primary response to NO_3^- (Redinbaugh and Campbell, 1991; Pelsey and Caboche, 1992; Bowsher et al., 1993). The accumulation of GS2 transcripts in *Z. mays* roots has been shown to be a primary response to NO_3^-, and this seems likely to be the case for the root GS2 in the five temperate legume species.

7.1.5 The evolution of root GS2 in temperate legumes

A survey of 55 legume species, representing 38 genera from 21 different tribes (Chapter 6.2.1), revealed the presence of a GS2-like polypeptide in the roots of all of the species that originated in temperate climates (30 papilionoid, 1 caesalpinoid). In contrast, this polypeptide was absent from all of the 17 tropical papilionoid species that were sampled. Three of the 8 non-papilionoid species with tropical origins also had the root GS2 polypeptide, although these were opportunistic species known to have adapted to non-tropical climes. This was in agreement with other studies where GS2 activity or protein has been found associated with roots of temperate species, e.g the legumes, *V. faba*, *P. sativum* and *M. media*, and the non-legumes, *L. esculentum*, *H. vulgare* and *Z. mays* (Miflin, 1974; Barratt, 1980; Emes and Fowler, 1983; Vézina *et al.*, 1987; Vézina and Langlois, 1989, Becker *et al.*, 1992; Sakakibara *et al.*, 1992a), but where the polypeptide is absent from tropical species, e.g. *G. max* and *P. vulgaris* (although GS2 transcripts have been detected in roots of these species; Hirel *et al.*, 1987; Bennett and Cullimore, 1989).

Nearly all angiosperms that have adapted to temperate climates evolved from tropical species during the mid-Tertiary period, many millions of years after the divergence of monocots from dicots (early Cretaceous) and of legumes from non-legumes (late Cretaceous) (Friis *et al.*, 1989). Therefore, it is hypothesised that the occurrence of GS2 in the root is a trait that arose independently in the temperate legumes and the non-legume dicots and monocots as an adaptation to the young, base-rich soils of habitats which were forming during the Neogene (Polhill *et al.*, 1981).

7.1.6 Is root GS2 accumulation a reflection of plastid development?

It is possible that the presence or absence of root GS2 may be correlated with the occurrence or abundance of root plastids, or possibly a particular type of root plastid. It is likely that a plastidic factor is required for synthesis of GS2, as has been found to be the case in NR accumulation which is prevented if plastids are blocked at an early stage of development (Börner *et al.*, 1986; Schuster *et al.*, 1989). Studies have shown that the activity of GS2 in the shoot is correlated with the differentiation of plastids in *L. esculentum*, *P. sativum* and in transgenic *N. tabacum* (Edwards and Coruzzi, 1989; Galvez, *et al.*, 1990; Kozaki *et al.*, 1991). It is also known that the level of plastid differentiation varies along the length of the root (M. Emes, personal communication), so the fact that GS2 in the roots of *P. sativum* is mainly found 4 cm behind the growing tip (Vézina and Langlois, 1989) suggests an association with plastids at a particular stage of development.

7.1.7 Root GS2 is not a prerequisite for root nitrate assimilation

Root NO_3^- assimilation was more common among the temperate legumes examined, although shoot assimilation was not unusual. The total GS activity in the roots of temperate species correlated well with the *in vivo* NR activities (Figure 6.7), which along with the NO_3^--inducibility of the GS2 polypeptide, suggests an association of the two functions.

The fact that some tropical legumes can reduce NO_3^- in their roots in the absence of root GS2 suggests that GS1 is able, at least in these species, to assimilate the evolved NH_4^+. It seems likely that the cytosolic isoform is present in

the same cells as the NH_4^+-evolving plastids, a situation supported by the finding that the abundance of GS1 may have been severely underestimated in non-vascular tissues (A. Tobin, personal communication), but movement of the NH_4^+ to neighbouring GS1-containing cells in the vasculature cannot be ruled out.

So it is clear that although the presence of root GS2 is very strongly associated with adaptation to temperate life amongst the legumes (and possibly other plant species), the partitioning of NO_3^- assimilation is not, and possession of a root GS2 is neither an indicator of, nor a requirement for a root assimilator.

APPENDIX I List of manufacturers' names and Addresses

Amersham International,
Lincoln Place,
Green End,
Aylesbury,
Buckinghamshire, UK.

BDH Ltd.,
P.O. Box 15,
Freshwater Road,
Dagenham,
Essex, UK.

BRL. (Bethesda Research Laboratories),
Gaithersburg,
Maryland, USA.

Bio-Rad,
Watford,
Hertfordshire, UK.

Boehringer Mannheim UK,
Bell Lane,
Lewes,
East Sussex, UK.

Hybaid Ltd.,
111-113 Waldegrave Road,
Teddington,
Middlesex, UK.

Millipore (UK) Ltd.,
The Boulevard,
Blackmoor Lane,
Watford,
Hertfordshire, UK.

N.B.L. (Life Sciences)
St. Petersburg,
Florida, USA.

Oxford Glycosystems,
Abingdon,
Oxfordshire, UK.

APPENDIX I continued

Perkin Elmer Cetus,
Norwalk,
Connecticut, USA.

Pharmacia Biotech Ltd.,
Davy Avenue,
Knowlhill,
Milton Keynes,
Bedfordshire, UK.

Sigma Ltd.,
Fancy Road,
Poole,
Dorset, UK.

Stratagene,
140 Cambridge Innovation Centre,
Cambridge Science Park,
Milton Road,
Cambridge, UK.

U.S.Biochemicals,
Cleveland,
Ohio, USA.

APPENDIX II Taxonomic classification of legume species in Chapter 6 and the provenance of the seeds

SUB-FAMILY TRIBE sub-tribe *species*	SEED PROVENANCE
CAESALPINIOIDEAE	
CASSIEAE	
**Cassia fistula* L.	[a]India
Senna obtusifolia (L.) Irw & Barneby	[i]Zimbabwe
CERCIDEAE	
**Cercis siliquastrum* L.	[a]Italy
CAESALPINIEAE	
Caesalpinia pulcherrima (L.) Sw.	[a]France
MIMOSOIDEAE	
MIMOSEAE	
**Mimosa pudica* L.	[a]not known
Prosopis juliflora L.	[a]U.S.A.
ACACIEAE	
Acacia farnesiana (L.) Willd.	[a]U.S.A.
INGEAE	
**Albizia julibrissin* Durazz.	[a]not known
PAPILIONOIDEAE	
SOPHOREAE	
Sophora japonica L. (syn. *Styphnolobium japonicum* (L.) Schott)	[a]Australia
DALBERGIEAE	
Dalbergia sissoo Roxb.	[a]India
PODALYRIEAE	
Podalyria calyptrata (Retz.) Willd.	[a]Australia
BOSSIAEEAE	
Bossiaea pulchella Meissn.	[a]Australia
THERMOPSIDEAE	
Thermopsis caroliniana L.	[a]U.S.A.

APPENDIX II continued

TRIBE sub-tribe *species*	SEED PROVENANCE
GENISTEAE	
Genistinae	
Genista aetnensis DC	[a]Australia
**Ulex europaeus* L.	[a]not known
Cytisus scoparius (L.) Link	[a]not known
**Laburnum anagyroides* Medicus	[d]England
Lupininae	
Lupinus nanus Dougl. ex Benth. cv. Pixie delight	[a]Holland
MILLETTIEAE	
Tephrosia vogelii Hook.	[g]Sri Lanka
Wisteria floribunda (Willd.) DC	[a]Japan
AMORPHEAE	
Amorpha canescens (Nutt.) Pursh.	[a]France
ROBINIEAE	
Robiniinae	
**Robinia pseudoacacia* L.	[a]Italy
Sesbaniinae	
Sesbania grandiflora (L.) Poir.	[a]India
AESCHYNOMENEAE	
**Arachis hypogaea* L.	[b]Nigeria
Arachis monticola Krap. & Rig.	[e]Argentina
PHASEOLEAE	
Cajaninae	
**Cajanus cajan* (L.) Millsp.	[g]Brazil
Erythrininae	
Erythrina crista-galli L.	[a]China
Glycininae	
Glycine max (L.) Merrill cv.Labrador	[b]India
Glycine soja Sieb. & Zucc. PI 378691	[f]Australia
Kennediinae	
Hardenbergia violacea (Schneev.) Stearn	[f]Australia
Phaseolinae	
**Phaseolus vulgaris* L. Y-21-W250	[b]not known
Phaseolus coccineus L. cv. Best of all	[a]Holland
Phaseolus lunatus L. cv Henderson	[b]U.S.A.
Vigna unguiculata (L.) Walp. cv. California	[h]U.S.A.
V. unguiculata ssp. *sesquipedalis* (L.) Verdc. cv. Liana	[a]Holland
Vigna radiata (L.) Wilczek	[a]E. Africa
INDIGOFEREAE	
Indigofera pseudotinctoria Matsum.	[a]China

APPENDIX II continued

TRIBE sub-tribe *species*	SEED PROVENANCE
GALEGEAE	
Astragalinae	
Astragalus glycyphyllos L.	[a]not known
Coluteinae	
Clianthus formosus (G. Don) Ford. & Vickery	[a]Australia
Galeginae	
**Galega officinalis* L.	[a]Holland
CARMICHAELIEAE	
Carmichaelia aligera Simpson	[a]New Zealand
HEDYSAREAE	
**Hedysarum coronarium* L.	[a]Holland
LOTEAE	
**Lotus corniculatus* L. cv. Leo	[b]not known
Lotus japonicus (Reg.) Larsen bv. Gifu	[c]Japan
Lotus tetragonolobus L.	[a]Malta
CORONILLEAE	
**Coronilla varia* L.	[a]not known
Hippocrepis comosa L.	[a]not known
VICIEAE	
**Vicia sativa* L.	[a]not known
Vicia faba L. cv. Aquadulce Claudia	[b]not known
Lathyrus odoratus L. cv. Snoopea	[a]not known
CICEREAE	
**Cicer arietinum* L. JG62	[b]not known
TRIFOLIEAE	
Ononis repens L.	[a]not known
Melilotus alba Medicus	[a]not known
Trifolium subterraneum L. cv. Clare	[b]not known
**Trigonella foenum-graecum* L.	[a]India

* = type species

[a], bought from a seed merchant; all other seeds were gifts from [b], Rothamsted; [c], Jens Stougaard, Univ. of Aarhus, Denmark; [d], Mrs. Jean Woodall; [e], ICRISAT; [f], GRIN; [g], the Krukoff Collection, Kew Gardens; [h], U.C.L; [i], collected by the author from Rekomeshe Tsetse Research Station, Zimbabwe.

APPENDIX IIIa Percentage identities of the 159 bp nucleotide sequence flanking intron 11 in non-legume dicotyledons and monocotyledons

	Hv2	Os2	At2	Hv1	Os1A	Os1B	Zm1A	Zm1B	Zm1C	Zm1D	Np1	Ls1	At1A	At1B	At1C
Hv2	100														
Os2	87	100													
At2	81	85	100												
Hv1	71	69	69	100											
Os1A	72	67	66	84	100										
Os1B	69	67	69	92	86	100									
Zm1A	70	67	69	92	87	94	100								
Zm1B	70	68	69	94	87	93	96	100							
Zm1C	72	67	67	84	95	87	86	87	100						
Zm1D	72	68	67	85	95	87	86	87	98	100					
Np1	69	67	67	77	76	76	75	74	79	77	100				
Ls1	69	73	70	69	74	70	70	69	74	72	81	100			
At1A	69	69	69	74	78	73	73	72	77	75	80	82	100		
At1B	68	67	69	73	78	73	72	70	78	77	82	82	93	100	
At1C	66	68	72	75	77	74	77	74	75	74	79	79	84	84	100

Underlined figures indicate strong similarity

Abbreviations
Hv2 = *Hordeum vulgare* GS2, Freeman *et al.*, 1990
Os2 = *Oryza sativa* GS2 (GS31) Sakamoto *et al.*, 1989
At2 = *Arabidopsis thaliana* GS2 (λAtgs11) Peterman and Goodman 1991
Hv1 = *H. vulgare* GS1 (pGS3), Marigo *et al.*, 1993
Os1A and Os1B = *Oryza sativa* GS1 (GS8 and GS28), Sakamoto *et al.*, 1989
Zm1A, Zm1B, Zm1C and Zm1D = *Zea mays* GS1 (pGS107, pGS112, pGS117 and pGS122) Sakakibara *et al.*, 1992
Np1 = *Nicotiana plumbaginifolia* GS1 (pGS1/pGS15), Tingey and Coruzzi, 1987
Ls1 = *Lactuca sativa* GS1 (pLGS11), Sakamoto *et al.*, 1990
At1A, At1B and At1C = *A. thaliana* GS1 (λAtgsr1, λAtgsr2 and λAtgskb6), Peterman and Goodman, 1991

APPENDIX IIIb Percentage identities of the 159 bp nucleotide sequence flanking intron 11 in dicotyledons and monocotyledons compared to that in eight legume species

	Hv2	Os2	At2	Hv1	Os1A	Os1B	Zm1A	Zm1B	Zm1C	Zm1D	Np1	Ls1	At1A	At1B	At1C
Pv2	77	78	77	69	69	70	69	68	67	65	73	74	71	72	72
Ps2	79	81	82	69	70	69	68	67	67	67	69	71	69	69	72
La1[a]	63	71	67	71	70	71	71	70	69	69	73	83	78	78	76
Gm1	71	69	65	72	76	73	72	70	75	74	79	84	81	80	80
Pv1A	66	68	68	69	69	69	68	69	71	70	75	82	79	79	82
Pv1B	67	68	66	73	77	72	72	70	74	74	79	84	81	81	78
Pv1C	70	71	69	75	75	74	71	69	74	73	81	79	79	80	75
Ps1A	67	70	73	69	70	68	66	67	69	68	73	79	75	75	79
Ps1B	71	70	72	82	74	73	72	71	73	73	77	82	78	78	75
Ms1A	67	68	67	72	71	69	69	68	71	69	81	82	77	78	82
Ms1B	70	69	70	71	74	70	69	68	72	72	74	85	79	80	76
Mt1A[a]	67	68	68	70	70	67	66	65	70	68	81	84	75	78	77
Mt1B[a]	65	67	70	71	73	68	68	67	71	70	76	87	81	81	81
Mt1C[a]	63	65	65	70	70	68	68	67	70	68	82	82	81	80	76
Lj1	70	72	71	74	76	74	73	72	75	68	81	84	81	81	81
Lc	70	72	70	74	76	74	73	72	75	74	81	85	82	82	82

[a] These sequences only overlapped with the others by 93 bp.
Underlined figures indicate strong similarity

Pv2 = *P. vulgaris* s/u (pcGS-61), Lightfoot *et al.*, 1988
Ps2 = *P. sativum* GS2 (pGS197), Tingey *et al.*, 1987
La1 = *L. angustifolius* GS1 (pGS5), Grant *et al.*, 1989
Gm1 = *G. max* GS1 (pGS20), Miao *et al*, 1991
Pv1A = *P. vulgaris* gln-α (pcGS-α1), Gebhardt *et al.*, 1986
Pv1B = *P. vulgaris* gln-β (pcGS-β1), *ibid*
Pv1C = *P. vulgaris* γ s/u (pcGS-γ1), Bennett *et al.*, 1989
Ps1A = *P. sativum* GS1 (pGS341), Tingey *et al.*, 1987
Ps1B = *P. sativum* GS1 (pGS134), *ibid*
Ms1A = *M. sativa* GS1 (pAnGS1), Dunn *et al.*, 1988
Ms1B = *M. sativa* GS1 gene, Tischer *et al.*, 1986
Mt1A = *M. trunculata* GS1 (MtGSa), Stanford *et al.*, 1993
Mt1B = *M. trunculata* GS1 (MtGSb), *ibid*
Mt1C = *M. trunculata* GS1 (MtGSc), *ibid*
Lj1 = *L. japonicus* GS1 (pGSA9) J.Stougaard, personal communication
Other abbreviations as in Appendix IIIa

APPENDIX 111c Percentage identities of the amino acid sequences predicted from the 159 bp nucleotide sequence flanking intron 11 in dicotyledons and monocotyledons

	Hv2	Os2	At2	Hv1	Os1A	Os1B	Zm1A	Zm1B	Zm1C	Zm1D	Np1	Ls1	At1A	At1B	At1C
Hv2	100														
Os2	96	100													
At2	94	96	100												
Hv1	81	81	83	100											
Os1A	85	85	85	89	100										
Os1B	83	83	83	96	92	100									
Zm1A	81	81	81	98	91	98	100								
Zm1B	81	81	81	98	91	98	100	100							
Zm1C	85	85	85	89	100	92	91	91	100						
Zm1D	85	85	85	89	98	92	91	91	98	100					
Np1	83	81	83	89	91	89	87	87	91	89	100				
Ls1	83	83	83	89	96	92	91	91	96	94	89	100			
At1A	83	83	83	89	94	91	89	89	94	92	89	82	100		
At1B	83	83	83	89	94	91	89	89	94	92	89	82	100	100	
At1C	83	83	85	94	92	94	92	92	92	91	92	79	94	94	100

Underlined figures indicate strong similarity

Abbreviations as in Appendix IIIa

APPENDIX IIId Percentage identities of the amino acid sequence predicted from the 159 bp nucleotide sequence flanking intron 11 in dicotyledons and monocotyledons compared to that in eight legume species

	Hv2	Os2	At2	Hv1	Os1A	Os1B	Zm1A	Zm1B	Zm1C	Zm1D	Np1	Ls1	At1A	At1B	At1C
Pv2	91	91	92	89	89	89	87	87	89	87	87	89	89	89	91
Ps2	92	91	92	87	87	87	85	85	87	85	85	87	87	87	89
La1[a]	87	84	84	90	94	94	94	94	94	94	87	94	90	90	90
Gm1	81	81	81	91	94	94	92	92	94	92	92	96	94	94	94
Pv1A	81	81	81	89	91	92	91	91	91	89	87	91	89	89	92
Pv1B	83	83	83	89	96	94	91	91	96	94	91	98	96	96	94
Pv1C	81	81	81	87	94	91	89	89	94	92	89	94	92	92	91
Ps1A	85	85	85	87	92	91	89	89	92	91	85	92	91	91	91
Ps1B	81	81	81	89	94	94	92	92	94	92	91	98	96	96	96
Ms1A	81	83	81	91	94	94	94	92	94	91	89	92	91	91	94
Ms1B	81	81	81	87	96	91	91	89	96	92	89	92	94	94	91
Mt1A[a]	81	81	81	92	90	90	92	87	90	92	94	90	90	90	94
Mt1B[a]	84	84	84	91	94	87	91	84	94	94	94	94	94	94	90
Mt1C[a]	81	81	81	92	90	90	92	87	90	92	97	90	90	90	94
Lj1	83	83	83	89	96	92	91	91	96	94	89	100	98	98	94
Lc	83	83	83	89	96	92	91	91	96	94	89	100	98	98	94

[a] These sequences only overlapped with the others by 31 amino acids

Underlined figures indicate strong similarity

Abbreviations are as in Appendix IIIa and IIIb

APPENDIX IV Ward's clusters and principle components analysis of GS and NR activities and tissue nitrate concentrations

The Ward's clusters for each data set are represented as a dendrogram and as circles superimposed onto scattergrams compiled from principle component analysis (PCA). The clusters which are referred to in Chapter 6 are numbered. The abbreviations of the species are defined in Table 6.2.

a) Dendrogram of the GS activities in the leaf, stem and root of 55 species of legume, analyzed by Ward's method.

b) Scattergram of GS activities in the leaf, stem and root of 55 species of legume analyzed by PCA.

c) Ward's clusters of the GS activities (as in caption a) superimposed onto the PCA scattergram (b).

d) Dendrogram of the *in vivo* NR activities in the leaf, stem and root of 46 species of legume, analyzed by Ward's method.

e) Scattergram of the *in vivo* NR activities in the leaf, stem and root of 46 species of legume analyzed by PCA.

f) Ward's clusters of the *in vivo* NR activities (as in caption d) superimposed onto the PCA scattergram (e).

g) Dendrogram of the *in vitro* NR activities in the leaf, stem and root of 31 species of legume, analyzed by Ward's method.

h) Scattergram of the *in vitro* NR activities in the leaf, stem and root of 31 species of legume analyzed by PCA.

i) Ward's clusters of the *in vitro* NR activities (as in caption g) superimposed onto the PCA scattergram (h).

j) Dendrogram of the NO_3^- concentrations in the leaf, stem and root of 46 species of legume, analyzed by Ward's method.

k) Scattergram of the NO_3^- concentrations in the leaf, stem and root of 46 species of legume analyzed by PCA.

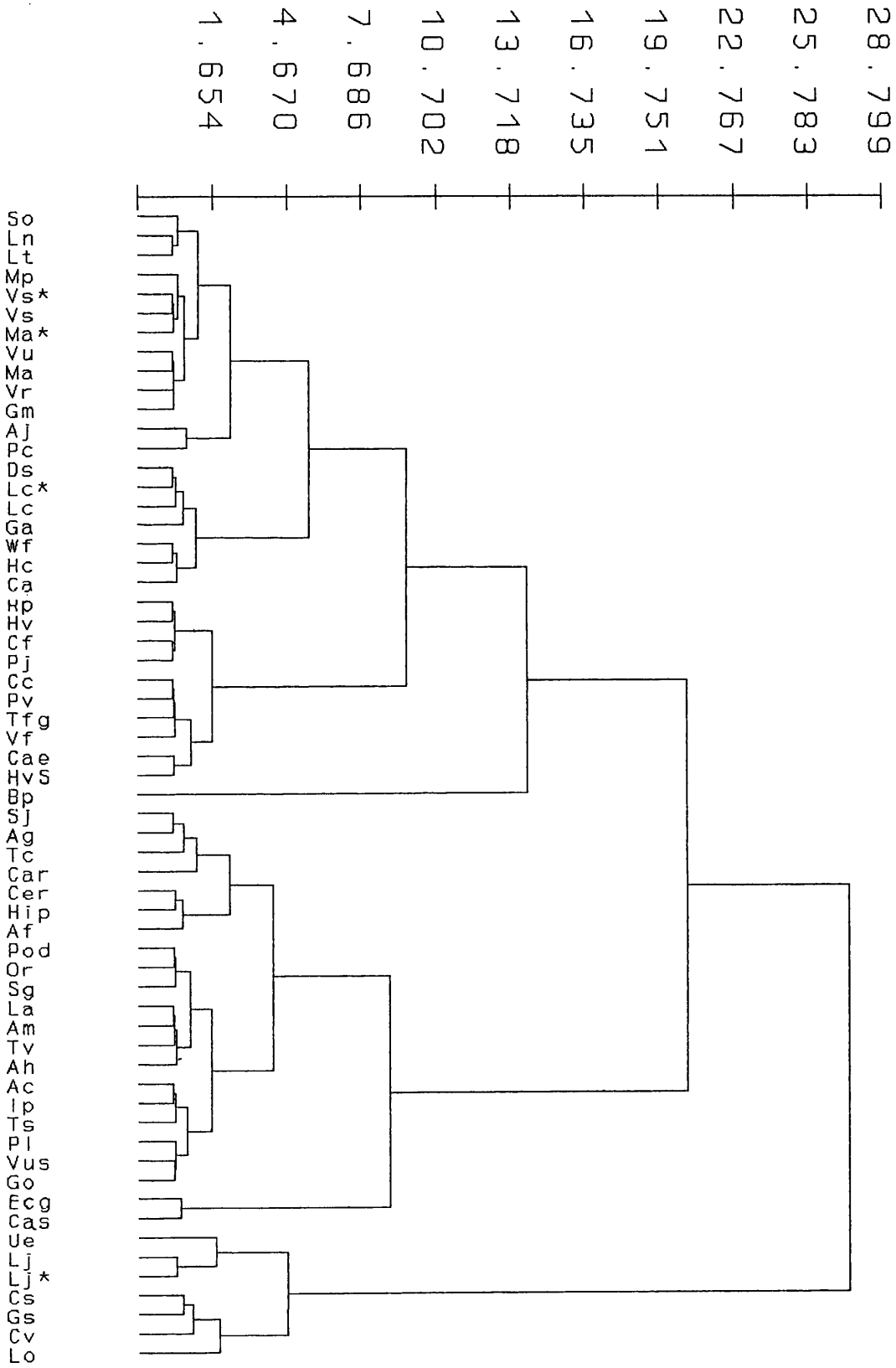
l) Ward's clusters of the NO_3^- concentrations (as in caption j) superimposed onto the PCA scattergram (k).

m) Dendrogram of the combined data for GS and *in vivo* NR activities in the leaf, stem and root of 46 species of legume, analyzed by Ward's method.

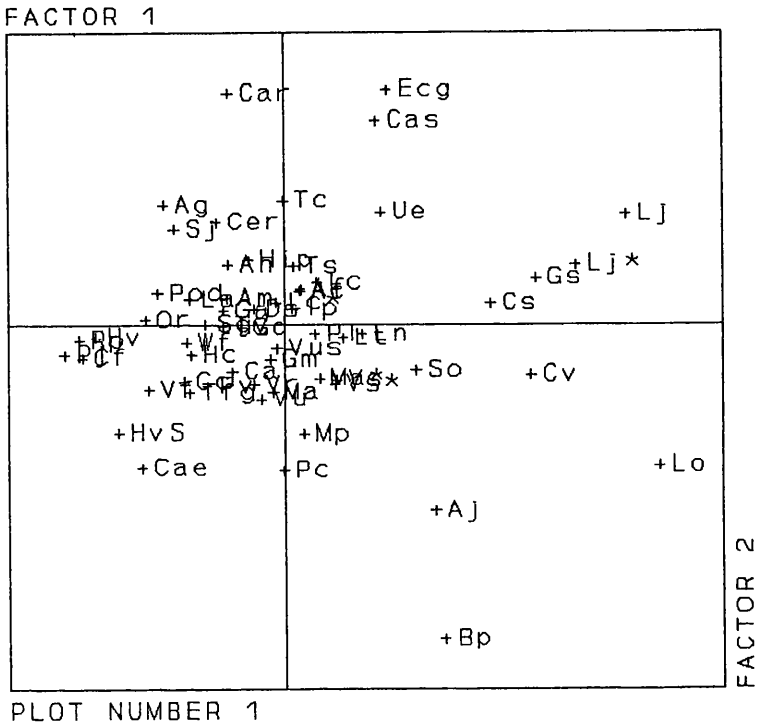
n) Scattergram of the combined data for GS and *in vivo* NR activities analyzed by PCA.

o) Ward's clusters of the combined data for GS and *in vivo* NR activities as in caption m) superimposed onto the PCA scattergram n).

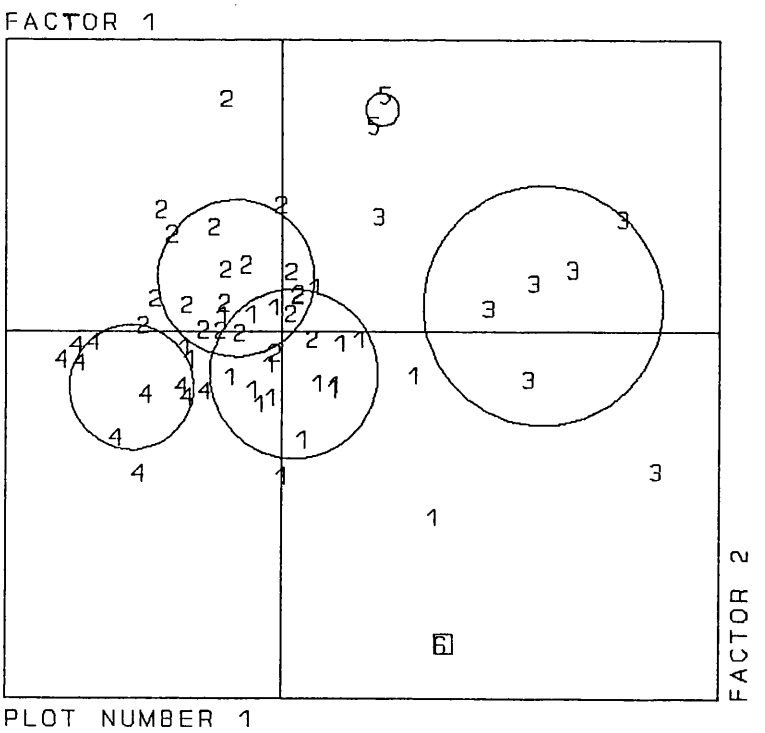
APPENDIX IVa,



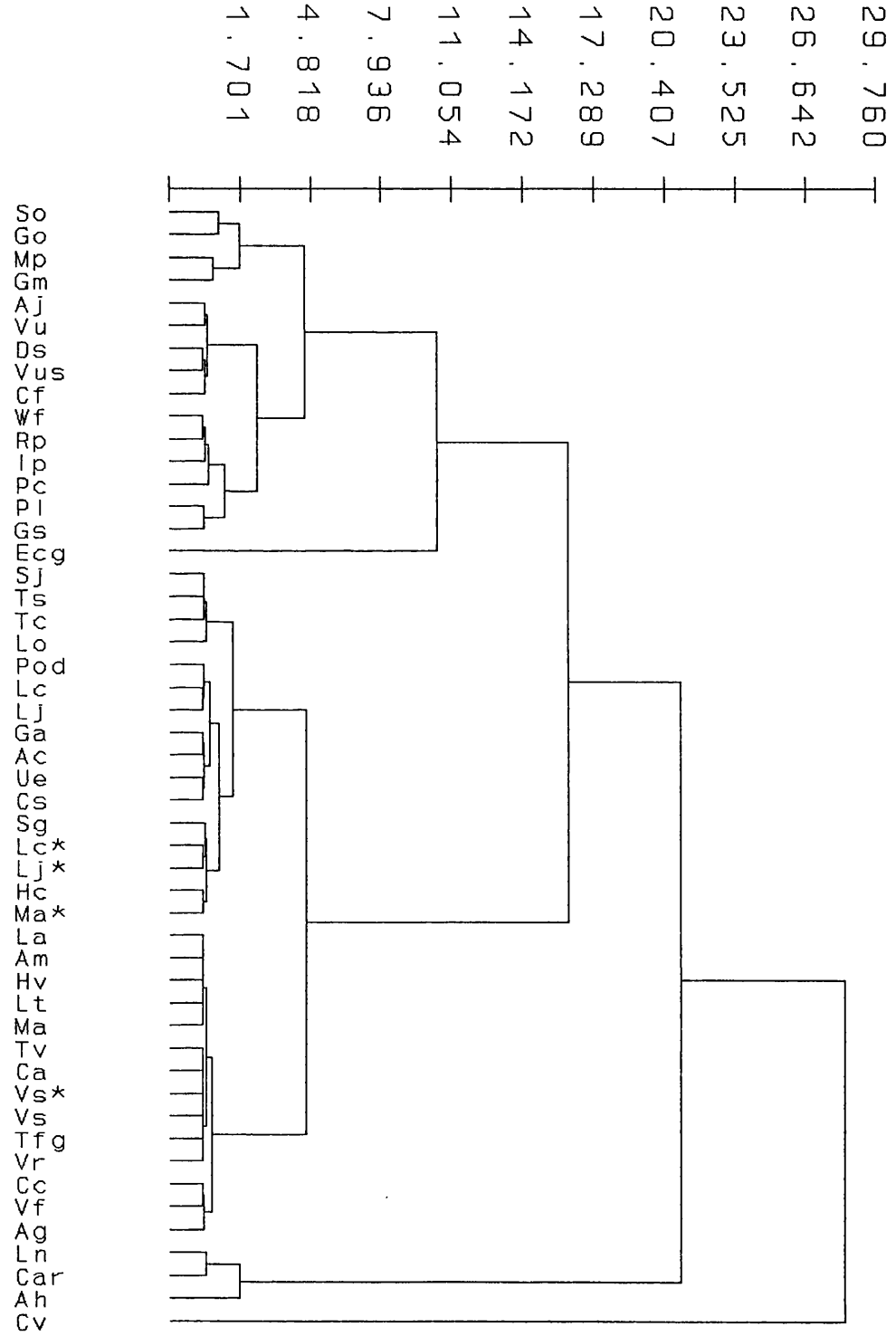
APPENDIX IVb



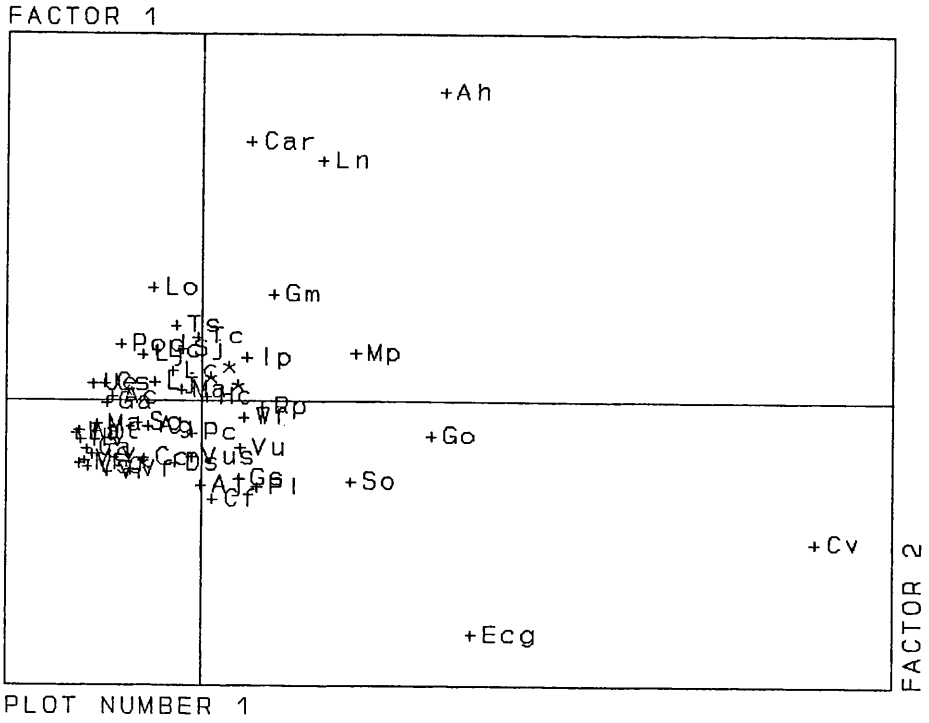
APPENDIX IVc



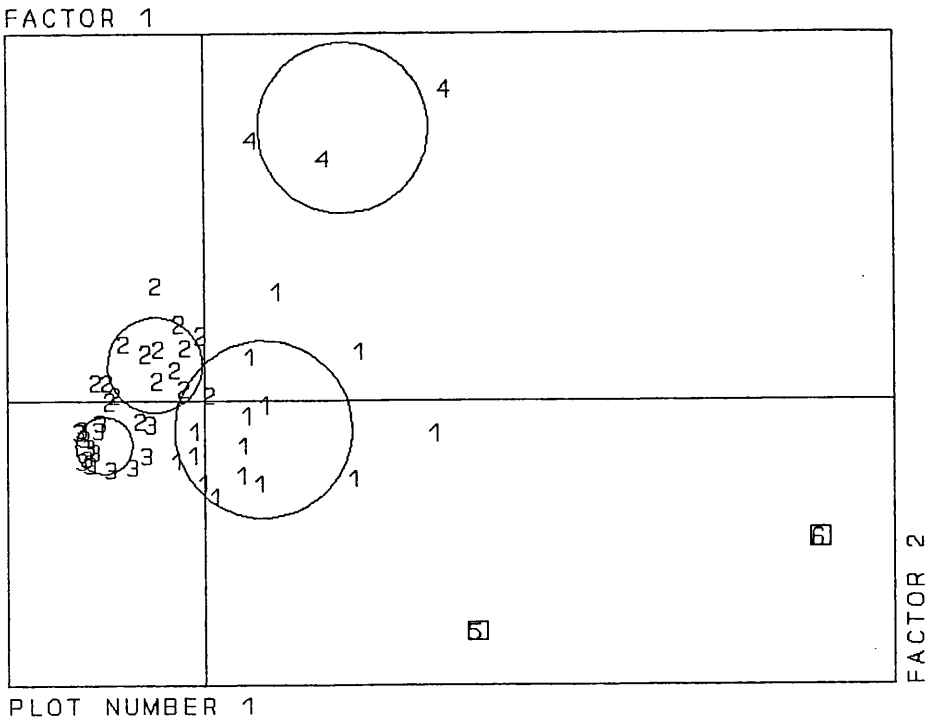
APPENDIX IVd



APPENDIX IVe



APPENDIX IVf



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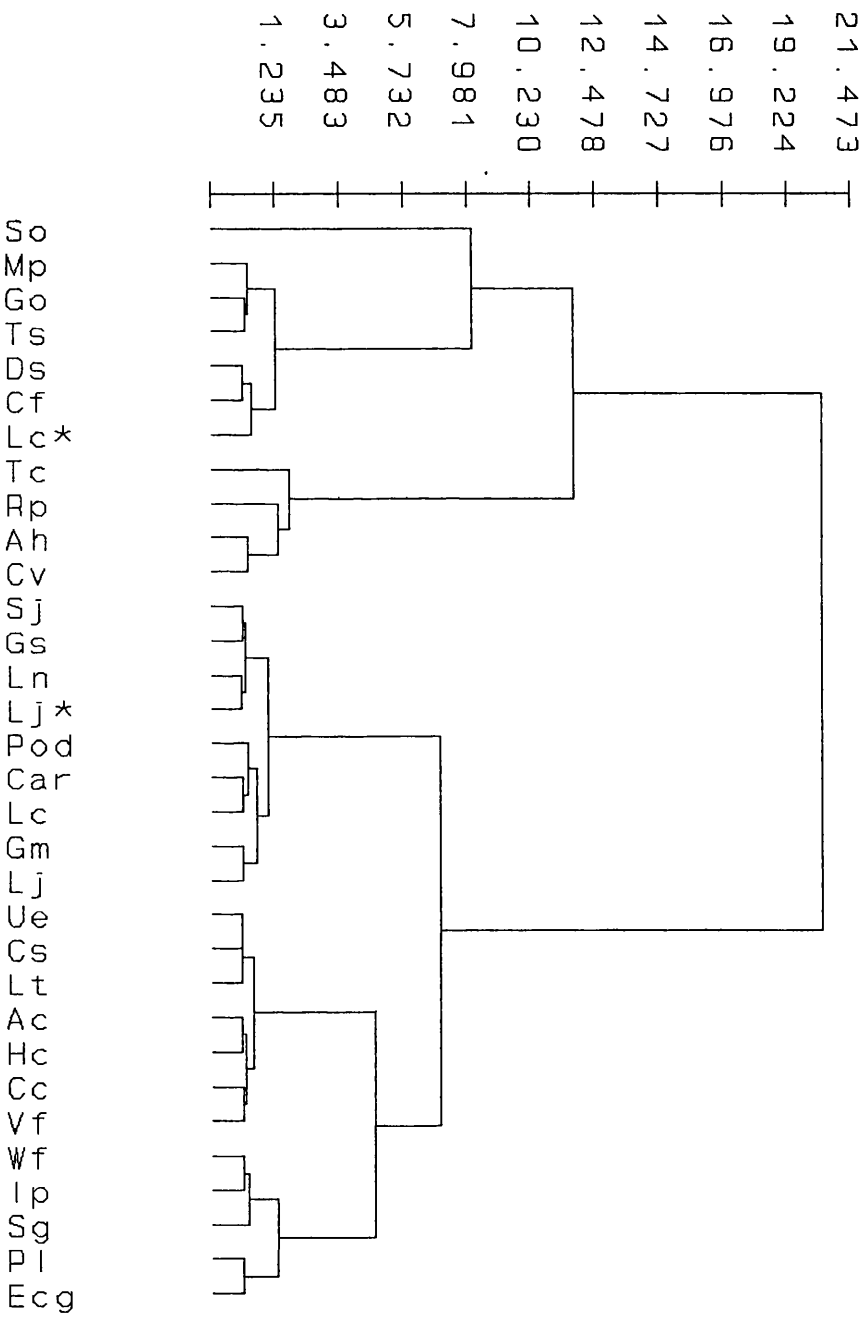

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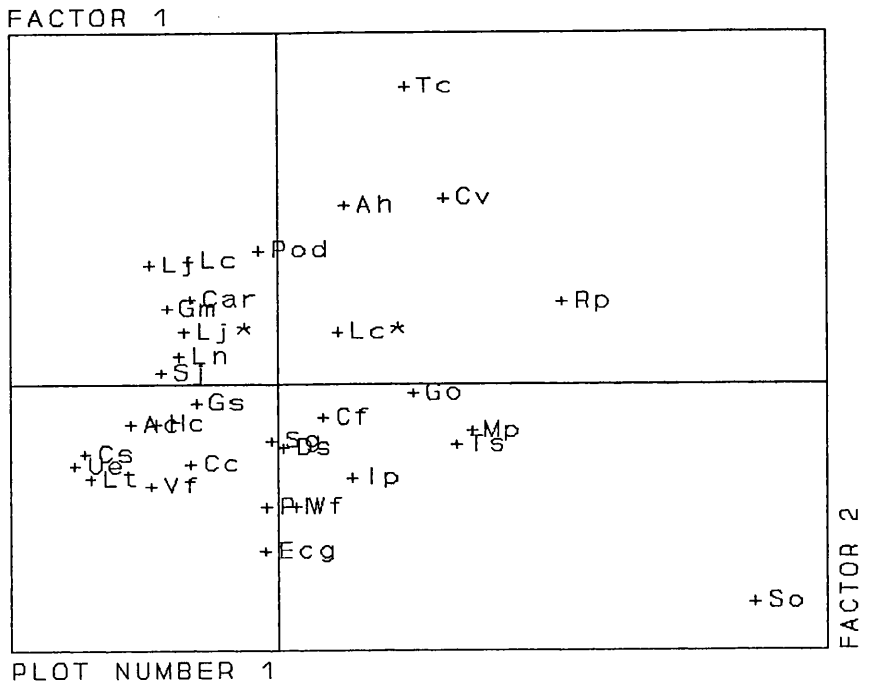

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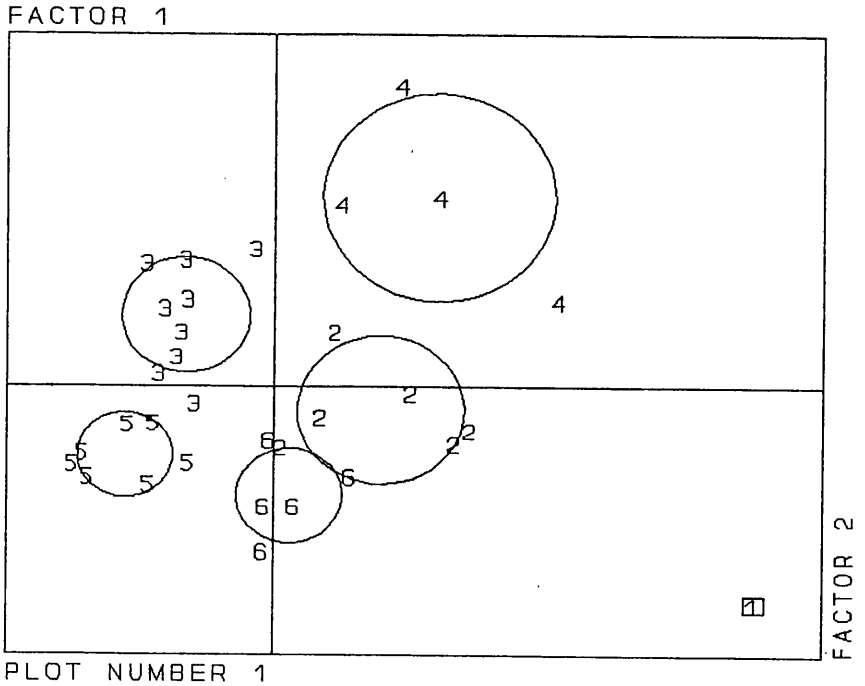

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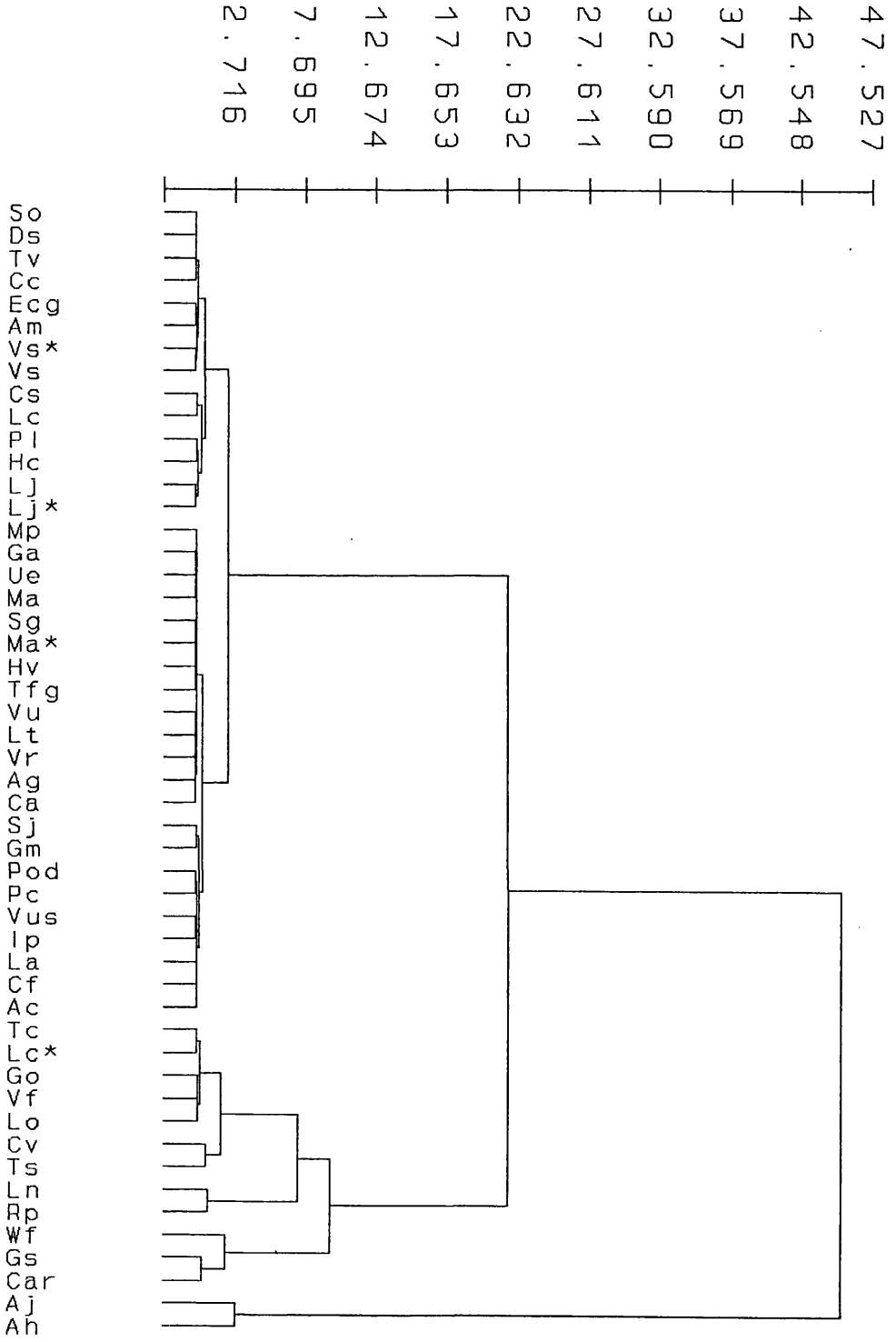

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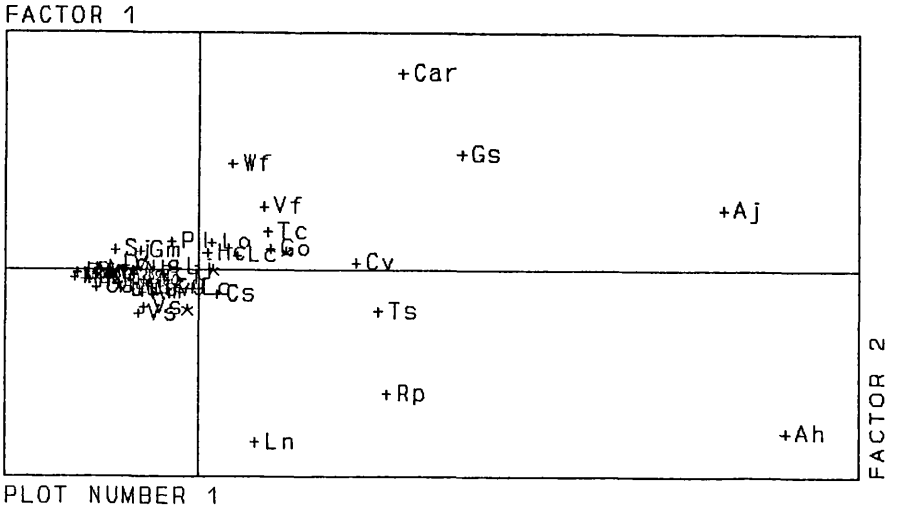

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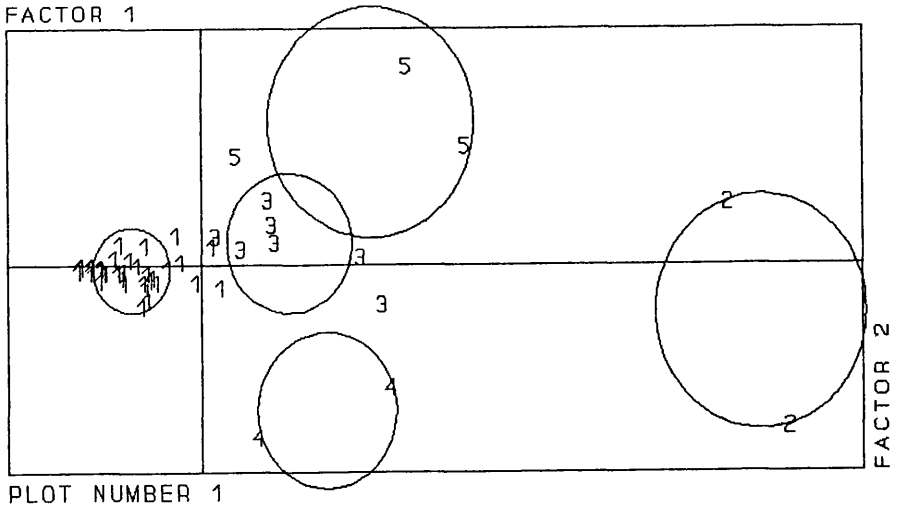

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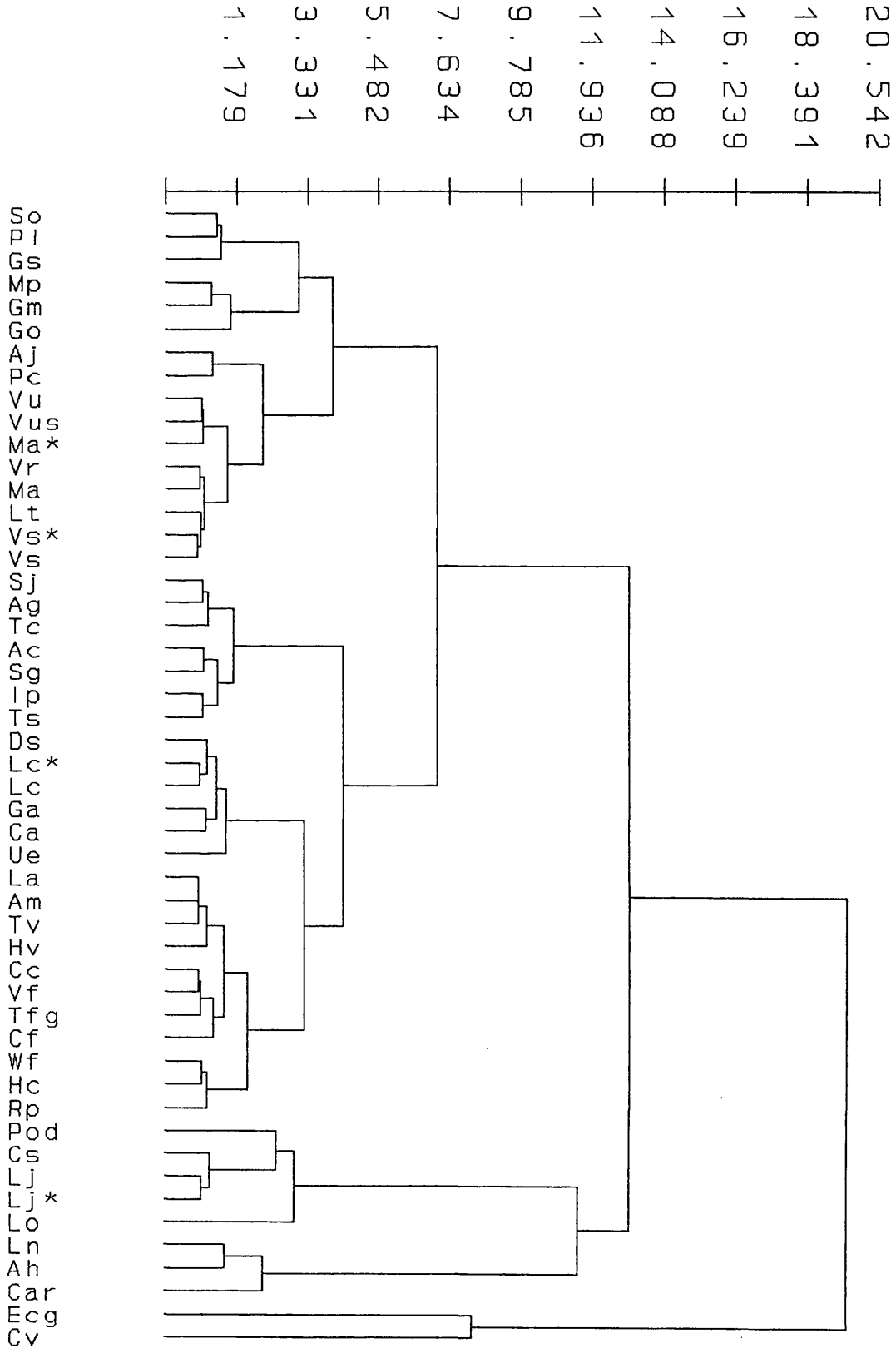

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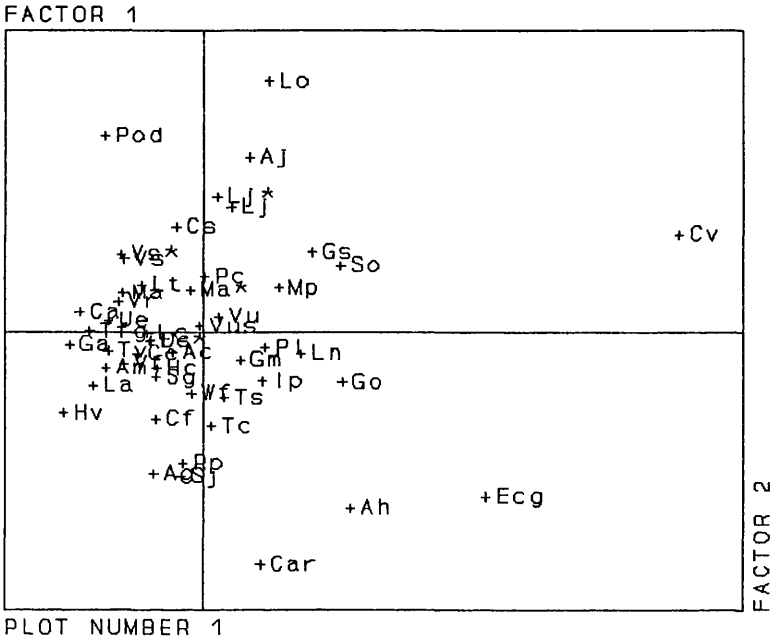

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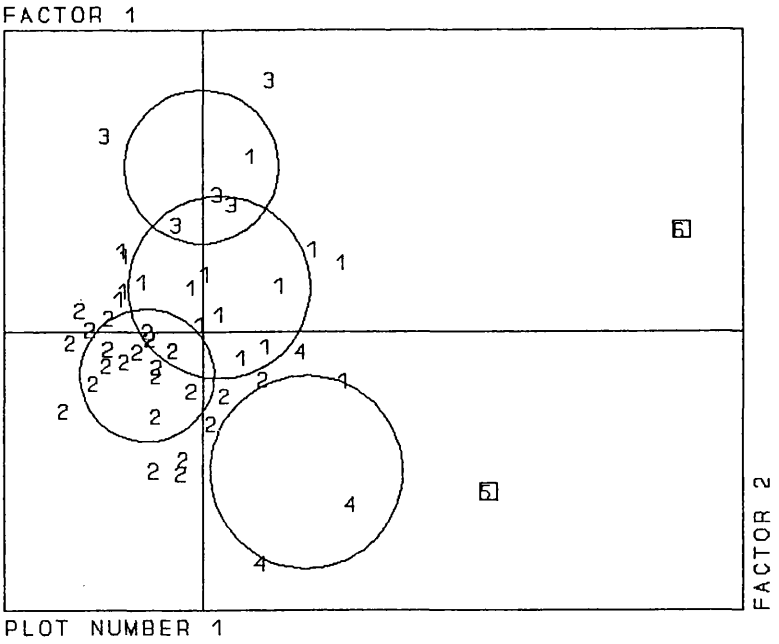

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