NITROGENASE, NODULE ENZYMES AND CARBOHYDRATE COMPONENTS OF COPADA (*CRATYLIA FLORIBUNDA*, BENTH) RELATED TO SOIL FERTILITY EFFECTS ON REGROWTH VIGOR AND NODULATION WITH AN OXISOL OF BRAZIL

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CHAPTER I

INTRODUCTION

It is estimated that tropical America has 850 million hectares of acid, infertile oxisols and ultisols. In Brazil these soils comprise 68% of the country, i.e., 572.71 million ha (51); 150 million ha being under cerrado vegetation (136) and the remaining under forest.

Owing to the low fertility status of these soil resources (154, 241), land development under these conditions starts usually with pasture based-beef production (223). However, a good understanding of the factors influencing forage production in the tropics is required in order to improve the presently low carrying capacity of the native vegetation. Hutton (120) has recognized that lack of knowledge of soil nutrient deficiences, lack of well adapted legumes with pest resistance and tolerance to highly acid soils, legume seed inoculation, selection of grasses, dry-season forage, reduction of pasture establishment costs and adequate seed supplies of grass and legume cultivars are some of the problems that agricultural scientists must face in Latin America.

Apparently in this region more work has been done with the forage grasses than with forage leguminous plants. The improved Panicum maximum cv. colonião has been found to be well adapted to soils with lower Al^{3+} saturation and better fertility status, with Brachiaria

decumbens tolerating more stressed soils. Cenchrus ciliares appears to be tolerant to some water stress and Melinis menutiflora and Hyparrhenia rufa are planted or naturalized over large areas of Brazil. Special conditions such as heavier soils or water logging can be overcome by Cenchrus dactylon ecotypes and Echinochloa polystacya, Brachiaria mutica, Paspalun plicatum or Hemathria altinima respectively (120).

The search for a leguminous forage that will form stable persistent combination with these grasses has been difficult, since no legume has been found to be well adapted to the low fertility status of the oxisol-ultisol lands or to consorciate with the grass for long periods of time. Several factors have presently contributed to the failure of grass-legume pastures and the following considerations have been made by Roberts (215): the grass-legume species are non-compatible, species are not adapted to local ecological factors and the use of more grass seed than legumes in the seed mixture. He further states that the usually slow starter legume cannot compete with the improved high yielding grass, although this problem can be avoided by a good management practice (120).

Very encouraging results with grass-legume pastures are reported during the first year or two, but the legumes tend to disappear afterwards, due to a variety of reasons such as poor adaptation to acid soils, inadequate mineral nutrition, intolerance to insect and disease attacks, and overgrazing (223).

Anthony and Harris (12) have found that grasses have a wider range in nutritive value than legumes, but nonetheless have assigned three important roles for inclusion of a legume in pasture swards:

symbiotically fixed N contributes to increased grass production, the grazing season can be extended in some areas, and the legume may complement the nutritive value of the grass. Supporting these concepts Santhirasegaram (224) has pointed out that the amount of nitrogen fixed by the legume is linearly related to its dry matter yield and that the productivity of animals is also linearly related to the content of legume herbage in the pasture. Thus, the use of legume in pastures is to provide protein to the grazing animal and N to the associated grass.

Although Latin America is the richest germoplasm source of tropical forage legumes (223) most of the leguminous species tested under improved pasture conditions in this part of the world were derived from indigenous material selected and bred in Australia. Sanchez and Isbell (222) have compared edaphic and climatic data obtained in these two continents and presented evidence indicating that results attained under Australian conditions are not likely to be transferable to Latin America.

Thus, these two factors have prompted an intensification in Latin America of research aiming towards the finding of leguminous species more adapted to local conditions. It is believed that establishment of permanent grass-legume pastures will be truly a breakthrough in the development of the cattle industry of this part of the world.

The forage legume, Cratylia floribunda Benth, used in these experiments is one of the many species indigenous to South America, with an overlooked forage potential.

Hooker and Jackson (113) list six species of Cratylia, all of them native in Brazil, Bolivia and northern Argentina. Apparently C. floribunda is well adapted to the Brazilian conditions since it has

been observed to be native in Bahia (166), Minas Gerais (240), Ceara, Piaui, Maranhão, Mato Grosso, Territorio do Acre, Para (70, 110), Amazonas (226), Rio de Janeiro, São Paulo, Espirito Santo, and Parana (159). Within Minas Gerais, Maxwell (159) lists Cratylia as native in Santa Barbara, Bento Rodrigues, Ponte Nova, Viçosa, Col. Pacheco, Tesfilo Otoni, Serro and Barra do Rio Piranha.

Present observations indicate that this genus can adapt itself to the poor soils of the humid tropics of Amazonia (226), the severe aridic conditions of Piaui (70) and perhaps Bahia, and to northern Minas Gerais, characterized by having a hydric deficit during 4-6 months of the year.

These characteristics along with its vigorous regrowth after de-topping (205) prompt the author to a more detailed study of this leguminous plant, as a contribution to the evaluation of native Brazilian forage species.

The soil used in these experiments unless otherwise specified was a dark red latosol (Typic Eutrustox) already described by Epamig et al. (77) and Purcino (204).

Dark red latosols are important soil resources within the Brazilian cerrado ecosystem. Comprising 17.9 million ha of land dark red latosols exhibit a wide range of physical and chemical characteristics, and have been correlated with the Acrustox, Haplustox (136), and Eutrustox (77) great group of the U. S. Soil Taxonomy.

The soil was collected in northern Minas Gerais where Cratylia floribunda is indigenous within the native vegetative cover.

The area is characterized as forest and transition to cerrado with semi-aridic conditions with a hydric deficit ranging from 200 to 600 mm between April and October (77).

The principal objective of these studies was to evaluate the effects of soil fertility treatments on regrowth, nodulation, nodule enzymes and carbohydrates of Cratylia floribunda Benth, grown under defoliation stress in a greenhouse environment. A secondary objective was to study the nitrogen fixation mechanism of this plant as influenced by de-topping and nodule age and size.

CHAPTER II

LITERATURE REVIEW

The Genus Cratylia

Six species of Cratylia occur in Brazil (113): C. desvauxii, Tul., C. floribunda, Benth, C. hypargyraea, Mart. ex Benth, C. mollis, Mart. ex Benth, C. nuda, Tul. and C. spectabilis Tul. Cratylia floribunda has also been referred to as C. niteus, Benth and according to Menezes (166) has sometimes been included in the genus Dioclea. The vernacular name Copada has been used in Minas Gerais, but in Bahia the plant is known as camaratuba, cava or cavani. Of these six species, C. floribunda and C. nuda are being evaluated by Epamig (240) as potential forage legumes. When introduced in field observation plots, C. floribunda only flowered in the second year after sowing but apparently was highly drought tolerant. Common to many other forage legumes, C. floribunda is a slow starter but shows vigorous regrowth after clipping. Under field conditions in its native habitat in Minas Gerais, the plant was sensitive to diseases and pest injury, particularly nematodes (240). However when introduced in the northeast section of the state where the plant has not been found within the native vegetation, it produced high forage yields, with large numbers of rootings observed at stem internodes. Again it flowered only after 20-24 months and was drought tolerant (239), although sensitive to nematodes.

In Stillwater (OSU) mature plants cultivated in 20 kg cylinders with a Psammentic Paleustalf (Eufaula) under greenhouse conditions, vigorously recovered from insect injury (usually spider mites, family Tetranychidae) after clipping. Fungus disease and nematodes were not observed.

Ongoing experiments in Brazil (239) with C. floribunda include studies concerned with yield potential, photosynthesis, seed germination, drought resistance and hay production.

Cratylia nuda grown in field conditions has an advantage of flowering and producing seeds within 12 months.

The genus Cratylia (sub family Papilionaceae) has been described as both creeping and scandent vines, with an extensive root system set at stem internodes in contact with the soil. Rapid dissecation of leaves has been observed by the author with clipped runners, but the large root system and stems seem to confer drought hardiness to the plant. In the Brazilian Amazomia Duck (70) observed large native plants with a number of pink-purple flowers. The leaves are trifoliate, alternate, with elliptical leaflets. Leaf margins are repand with an acute leaf apex and obtuse base. Leaf surfaces are glabrous and membranous with silvery tones in the lower epidermis, and arcuate venation.

The genus Cratylia (167) posses mucilaginous cells in the epidermis which also contains arranged rod-shaped crystals. The hypoderm is present in the upper side of the leaf.

The central layers of the mesophyll are occupied by cells containing little chlorophyll and often filled with taniniferous contents which are brown colored in dried material. Secretory elements

containing protein, mucilage and tanin are also found in the pith and phloem.

Usually the cork arises from the sub epidermis or between this and the sixth cell layer.

Very little is presently known about the forage potential of the genus Cratylia. In 1967 Hymowitz (121) listed 10 accessions of Cratylia as introduced in a nursery at IRI Research Institute in Brazil but did not further comment about this plant. Menezes (166) has suggested that the specie floribunda thrives in poor soils and produce good forage in Bahia and Hetch (110) has observed that in Paragominas (Para, Brazil) this species is an expontaneous legume in the brush community invading decaying formed pasture after onset of soil fertility limitations to the developed forage species. She further states that the tolerance of viney-type plants to variable light conditions almost preadapts them for pasture conditions. The observation pointing to its vigorous regrowth after clipping (205) tolerance to water stress (239, 240, Castelo Branco, L. J. 1978, personal communication) and probably to low soil fertility (110) are most encouraging.

Although nothing is presently known about its adaptation to consortiation with a grass, it can be speculated that the viney-type growth would be favored with a tufted-type grass such as Panicum maximum. In this case the spaces among the grass tufts would permit rooting of the legume internodes in contact with the soil, thus favoring the water stress hardiness of the plant.

Galli (86) in 1958 was probably the first scientist to conduct investigations concerning nodulation of Cratylia floribunda. In this

experiments he found that C. floribunda was not cross innoculated by any of the other rhizobium strains tested.

Later, in 1977, Purcino and Lynd (203) using a pouch culture technique were able to nodulate this plant using a Rhizobium leguminosarum culture isolated from nodules of Strophostyles sp. Conducting further experimentation with C. floribunda (205) they found that total vegetative growth of stems and leaves as dry matter from 154 day age nonclipped plants was 59% oq that from defoliated plants clipped at 14 day intervals. Compared with corresponding day-age nonclipped plants, the defoliated plants had 13.2% greater production with 26.9% protein; stems per plant increased from 5.7 to 15.3 with decreased stem length to 1.68 m from 5.6 m. Protein content of the stems, nitrogenase activity, glutamate-oxaloacetate transaminase (GOT) activity levels, and 2-oxoglutarate (αKG) levels of nodules decreased with plant aging. Regrowth after defoliation produced higher mature nodule number per plant, and increased both immature nodule number and weight. Clipping increased the glutamate dehydrogenase (GDH) activity levels of immature nodules from 0.2 g to 0.39 enzyme units per gram of nodule. Glutamate oxaloacetate transaminase levels of mature nodules closely parallel nitrogenase and 2-oxoglutarate levels with correlation r = 0.96. Activity levels of glutamate-pyruvate transaminase (GPT) and glutamate dehydrogenase of nodules did not significantly change with plant day-age in this study.

The Biology of N Fixation in Leguminous Plants

As early as 1932, Fred, Baldwin and McCoy (85) in a work considered classic today, recognized that the volume of publications in the field

of biological N fixation was enormous. However, modern techniques such as ^{15}N enrichment determination by either mass spectrometry or emission spectrometry and the acetylene-ethylene assay for nitrogenase (Nase) activity measurements have tremendously encouraged research on this subject after their work. A recent bibliography review (108) on ^{15}N research conducted between 1942 and 1968, focusing on the problems of biological N fixation, lists over 300 citations. The discovery of the acetylene (C_2H_2) reduction by nitrogen fixing preparations from Clostridium pasterianum by Dilworth (66) in 1966 and the determination of ethylene (C_2H_4) formation by gas chromatography as an assay for nitrogenase activity by Hardy and Knights (99), with the later evaluation of the technique under laboratory and field conditions (100), has also greatly contributed for increasing the number of publications on this subject. It is estimated (103) that over 400 papers were written on N fixation utilizing the C_2H_2-C_2H_4 technique within the first 9 years that followed publication of these papers. A recent monograph by Burns and Hardy (39) on the molecular biology, biochemistry and biophysics of N fixation lists 752 references, up to 1975.

Thus, this literature review will be rather selective, and emphasis will be given to papers more directly related with the work presented in this report.

Environment Factors and N Fixation

The process of N fixation is influenced by every factor that can affect the host plant, the survival of effective rhizobium strains in the soil, the process of infection with nodule initiation and growth, and the biochemistry of N fixation and ammonia assimilation into amino

acids. Some of these environmental interactions are briefly summarized as follows:

Soil Reaction. The effects of soil reaction on survival of the rhizobium, nodule formation and nodule functioning have recently been reviewed by Vincent (269, 270) and Munns (180). The toxic aluminum effects on legume nutrition, generally found in soils with pH<5 were described by Rorison (217).

According to Vincent (269) soil acidity is likely to be a major factor restricting the occurrence of rhizobia in the soil, though species differ considerably in their sensitivity. Norris (187) has proposed that cowpea-type rhizobia in general metabolize with production of an alkaline reaction and, thus, can neutralize some acidity in the soil. On the other hand, Vicieae and Trifolieae rhizobia further decreases the pH of their growing medium, thus requiring higher pH for competitive survival. Munns (180) discussing data from Loneragan and Dowling (151) and his own (178) points that an interaction of calcium and pH on nodulation of Trifolium subterraneum and Medicago sativa exists. It is clear in these data that increased nodule number/plant were obtained at low pH only upon addition of calcium, and that addition of this element had negligible effect on nodulation where pH>5.5. For Loneragan and Dowling (151) did not detect death of rhizobium growing in medium depleted of calcium, it appears that the Ca effect was on H-ion concentration. In this regard they also found that Mg does not substitute for calcium.

Under conditions of soil acidity Al^{+++} and Mn^{++} are the most soluble elements in the soil solution, decreasing the availability of the other nutrients, especially phosphorus and molybdenum. The toxic

effect of aluminum is characterized by rapid uptake of Al^{+++} ions which saturates the cell free spaces of the cortex, inhibiting further root growth (217), therefore impairing nodulation. The aluminum precipitation of phosphate in the external medium or in the root cortex or the inhibition of active phosphate uptake decreases the availability of energy for cell division and other plant metabolic activities with negative effects on nodule development.

Nutrition. Although the failure of legumes in mixed sward pastures in Latin America has been blamed on its higher sensitivity to nutrient deficiencies than grasses (120), legumes as a family are not considered to be more nutritionally demanding than other plants (180). Comparisons among legumes, grasses and other dycotyledous have indeed demonstrated that these plants do not have largely different nutrient requirements when the former are grown asymbiotically (7, 15, 16, 152, 153, 194). Some leguminous species are also known to tolerate some acidity, and nodulate quite well where soil pH = 4.7 (179).

It is usually accepted that high levels of NO_3^- in the soil will inhibit nodulation. Convincing data supporting this was obtained by Wilson (273) in 1917 using a split root technique to demonstrate that soybean root exposed to 4 mM NO_3^- did not nodulate, whereas the roots not exposed to nitrate nodulated normally. It is apparent that some rhizobium strains growing in the presence of NO_3^- will produce NO_2^-, which in turn inhibits the synthesis of indole-3-acetic acid (IAA), an auxin produced by the rhizobium, and required during the process of nodule formation (189, 253).

In this regard the metabolism of nitrogen, sulfur and zinc is likely to be interrelated. It has been observed that accumulation of sulfite metabolites in one month old wheat seedlings depressed the synthesis of serine a precursor of tryptophan, an essential intermediate in the IAA biosynthetic pathway (252). The influence of zinc on tryptophan synthesis has also been suggested (221). The classical effect of IAA is to promote cell enlargement although it can also induce root formation (264).

Another possible inhibitory mechanism of nitrate in nodule formation has been proposed by Ljunggren and Fahreus (82, 150). According to these authors, nitrate inhibits the induction of polygalacturonase by rhizobia at the root surface, and they considered that activity of this enzyme was essential for infection to occur.

A more direct effect of NH_4NO_3 on the mechanism of N fixation has also been reported. Addition of NH_4NO_3 to the nutrient solution in which Pisum sativum was infected with Rhizobium leguminosarum strain PRE, decreased the nitrogenase activity of intact nodules (32). It has been suggested that the decrease of the nitrogen-fixing capacity was caused by a decrease of the leghemoglobin content of the root nodules, and not by repression of the nitrogenase synthesis (32, 48). Supporting this theory is the observation made by Houwaard (115) in which the amount of nitrogenase in pea nodules is not diminished when nitrogenase activity of intact plants is reduced by addition of ammonium chloride. Houwaard also found similar results studying detached nodules (116).

Nevertheless, a long term repression of nitrogenase synthesis has been demonstrated upon addition of NH_4^+ to cultures of Azotobacter

chroococcum (73, 227), Klebsiella pneumoniae (259) and Clostridium (54), with only a partial inhibition being observed in free-living rhizobium (260).

According to Munns (180) the earlier concept that nitrate suppresses nodulation and nitrogen fixation by improving the plants nitrogen nutrition or increasing its nitrogen: carbohydrate ratio must be dismissed. Supporting his point of view is the fact that in Phaseolus, nitrate fed through the cut end of excised roots either increased nodulation or reduced it only slightly, whereas nitrate in the external solution reduced it significantly (44, 207).

However under field conditions the use of "starter-nitrogen" seems to be a common practice, mainly because it is a well known fact that symbiotically fixed N alone will not allow optimum soybean yields (184), supposing other factors are not limiting growth. A possible support for this is given by Gibson (91). In a seedling dependent on seed reserves for nitrogen supply there will be competition between shoots, roots and developing nodules for substrate such as free amino acids, with the strength of the competition being dependent on the environmental conditions. The level of free amino acids in the root will decline as the seedling develops (118), and competition for these substrates will place overall plant development, including nodule development, under a degree of stress. Supplementation with combined nitrogen should alleviate this stress at appropriate levels of supplementation. Increased photosynthesis should then increase the amount of available carbohydrates for nodule growth (193).

The number of publications reporting the effects of combined nitrogen on symbiotic N fixation both in the field and pots is large.

Disagreement of results obtained have not permitted that a clear cut conclusion be achieved.

Allors and Bartholomew (3) in 1955 determined that symbiotic N fixation was proportional to the total plant N and N uptake, and decreased with increasing N absorption from high rates of N fertilizer. However, 4 years later these same authors (4) reported that legumes respond in growth and N uptake to additions of inorganic N, and that increased growth, sometimes caused increases in N fixation. When nitrogen applied exceeded that necessary for growth increase, it tended to replace the symbiotic process.

Results demonstrating a beneficial effect of combined nitrogen on yields of soybean (184), yellow lupin (256) and cowpea (81) have been presented in the literature. A more common concept is that added inorganic nitrogen will decrease N fixation proportionally to the amount of fertilizer N added to the soil. Such observations have been made for soybean (4, 98, 107, 255, 256, 257, 271) ground nut (4), lucerne (4), lespedeza (4), ladino clover (4, 161), birdfoot trefoil (4), lupinus (135, 257), Vicia faba (140, 175, 257), alfalfa (161), garden peas (257), centrosema (83), and stylosanthes (83).

Apparently several noncontrolled effects have contributed to complicate the interpretation of these data. Host species (and cultivars within a specie) bacterial strains, type of root system, the form, level and placement of combined nitrogen and environmental conditions must be considered for interpretation of these results (91). The influence of soil texture (83), growth temperature and light intensity (83) has also been determined to influence the effect of combined inorganic nitrogen on symbiotic N fixation.

In 1947, Thornton (255) conducted some excellent research enlightning these problems. He determined that addition of inorganic ^{15}N to soybeans at time of planting significantly reduced the number of nodules per plant, and that this added N was found in the tops and roots. However, when ^{15}N was added at mid-season the greater amount was found in the seeds, and the use of inorganic nitrogen to well nodulated plants significantly increased yields. This finding is in agreement with modern research indicating that nitrogenase activity decreases during flowering due to a shortage of available carbohydrates (142, 145), and thus cannot meet the high nitrogen requirements of the plant during this stage (104, 192). Pulse crops, especially soybeans, require more N and carbohydrates for seed production than any other crop, according to the studies of Sinclair and de Wit (234).

Experiments carried out two decades later (251) with cowpea have indicated that N supply to the seeds depends largely upon post-flowering symbiotic fixation and/or uptake of inorganic nitrogen. It is thus possible to integrate N fixation with mineral N to produce optimum yields, and maximize symbiotic N fixation.

More recently, results with soybeans (62, 105, 143) have also indicated that applied fertilizer N when properly managed need not be inhibitory to the symbiotic N fixing process, and can supplement fixed N when nitrogenase activity is low.

Based on this review, it is apparent that addition of small amounts of inorganic N for vigorous seedling growth before onset of nodulation (107) or application of nitrogen during the pre-flowering stage (255), when the plant N requirement is high and nitrogenase activity is low,

can benefit crop yields in soils of low available nitrogen. Nonetheless, if the soil is well supplied with available residual N, it is likely that soil and fixed N will produce optimum yields (272) without any supplementation. The use of large amounts of fertilizer N during nodule formation inhibits nodulation and nitrogenase activity.

This conclusion is graphically represented in Figure 1 (modified from Pate and Dart, 1961, reference 199) for two strains of bacterium. The more efficient SU 41.237 formed more nodules on barrel medic (Medicago tribuloides Desr.) when supplemented with less than 0.5 mg N/plant, but nodulation was sharply inhibited if more than 10 mg N was added to the medium when planted.

On the other hand, the less efficient strain SU 277.7, was not strongly influenced by combined nitrogen levels up to 20 mg N/plant.

Several physiologically important roles are ascribed to the element P. Phosphates are found as moieties of the nucleotides and are therefore a part of the mechanism of genetic information transfer by the chromosomes (134). According to the fluid-mosaic model of Singer and Nicolson (235) biological membranes are formed by a fluid phospholipid bilayer with globular protein molecules penetrating into either side or extending entirely through the membrane. Phosphorous is present in the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) which are carriers of reducing power (H^+) for a myriad of metabolic reactions. Moreover, the hydrolysis of adenosine triphosphate (ATP) as represented below,

$$ATP + HOH \rightleftharpoons ADP + Pi$$

is exergonic and liberates 7.3 Kcal mol^{-1}, which is used for many anabolic processes (147). These facts suggest a widespread presence of

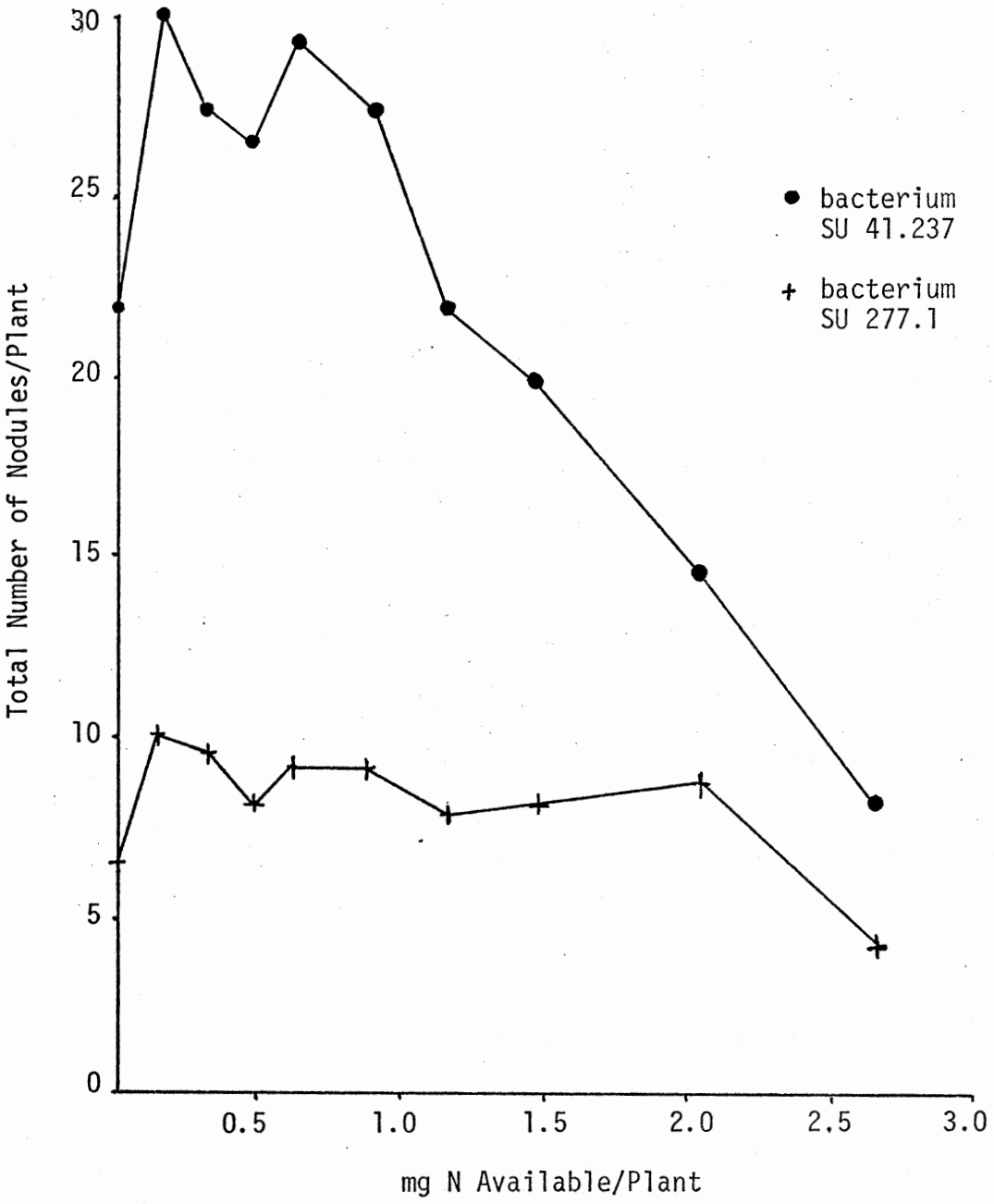


Figure 1. The Influence of Bacterial Strains on the Nodulation of 9 Weeks Old Seedlings of Medicago tribuloides Desr. to a Range of Ammonium Nitrate Applied at Sowing (modified from Figure 2 of Pate and Dart, 1961, ref. 199, p. 333)

P as an essential element in the functioning of the cell.

Nodulated legume plants have been shown to require more P than nonnodulating plants growing on combined nitrogen (162). This increased requirement for phosphate is apparently linked to the high energy requirement of the N fixation process (177), for nitrogenase requires ATP for activity. Figures quoted by Postgate (201) ranges from 20-30 ATP molecules/N_2 converted to $2NH_3$ in living anaerobes, 12-15 ATP/n_2 with enzymes, to about 5 ATP/N_2 for living Azotobacter. The role and stoichiometry of ATP in nitrogenase activity will be discussed in more detail later in this chapter.

Phosphorous is also a component of pyridoxyl phosphates essential coenzymes for transamination reactions (88). During transamination the amino group of an amino acid is transferred to the α carbon atom of an α keto acid bringing about the synthesis of new amino acids. Therefore the element P is essential for the assimilation of newly fixed N, which occurs by the action of the several transaminases (such as glutamate-oxaloacetate and glutamate-pyruvate transaminases) present in the nodule cytosol.

Nitrogenase, the enzyme that reduces N_2 to $2NH_3$ in the bacteroid cells of the nodules, contains a MoFe protein, and phosphorous has a beneficial effect on Mo uptake (247). The Australians have capitalized on this finding, and the success of permanent grass-legume mixed pastures there has been credited to the use of molybdenized superphosphate (120, 87).

Most soil areas of the world are usually lacking in available P for adequate plant growth. Many field and greenhouse studies have determined a beneficial effect of fertilization with this element on leguminous plant growth and on the mechanism of N fixation. In fact,

these experiments have determined that pulse and forage leguminous plants require high levels of P, more so than the cereals (262). Deficiency of P in the growing medium has been demonstrated to reduce nodulation in the field (185, 269) and in pot experiments (46, 269) and is suggested to inhibit nodule growth (262). Nevertheless, in some cases, the beneficial P effect has been noticed only when the soil reaction has been corrected to a more favorable value. In this regard, a strong interaction of lime and phosphorous on the nodulation and growth of white clover, was observed by Lowther and Adams (155) when the soil pH was 4.9.

Phosphorus fertilization has also been noticed to partially alleviate the negative effect of excessive heat on nodule weight of Glyine javanica L. in a tropical region (237, 238), but apparently it will not improve the efficiency of either efficient (206) or inefficient rhizobium strains (40).

Using a dark red latosol from Brazil, Purcino (206) conducted a detailed study of the effects of fertility treatments on growth, nodulation, nitrogenase activity and some enzymes of ammonia assimilation in two varieties of Psophocarpus tetragonolobus. A significant beneficial effect for P fertilization of this oxisol was observed with most of the parameters studied. Table I shows a summary of the results obtained. In these experiments the variety WB 21-8 Tinge was grown for 75 days with short day photoperiod, and produced flowers; the variety WB 12-11 Siempre was grown for 52 days with long day photoperiod, and did not flower. Shoot growth was increased in both varieties when P was added to the soil. Phosphorus also increased nodulation (nodule number and nodule weight) nitrogenase (Nase) activity,

TABLE I

PHOSPHORUS EFFECT ON SHOOT GROWTH, NODULATION, NITROGENASE ACTIVITY AND SOME COMPONENTS OF THE MECHANISM OF AMMONIA ASSIMILATION INTO AMINO ACIDS IN TWO VARIETIES OF PSOPHOCARPUS TETRAGONOLOBUS (ADAPTED FROM PURCINO, REF. 206)

	Nod. No.	Nod. Wt.	Nase	αKG	GDH	GS	GOGAT	GOT	GPT	PLP's	Shoot
					WB 21-8 Tinge Variety						
Without P	14.90	0.29	0.28	0.50	17.99	1.51	1.06	29.65	1.69	14.50	0.72
With P	21.94	0.75	0.94	0.40	23.65	3.14	2.09	59.21	2.20	16.10	0.96
P Effect	7.04**	0.46***	0.66**	-0.06#	5.66***	1.63***	1.03***	29.56***	0.51***	1.60**	0.24***
					WB 12-11 Siempre Variety						
Without P	7.06	0.78	1.01	0.24	2.19	2.73	1.01	6.51	1.09	13.03	1.12
With P	9.43	1.48	0.88	0.22	1.41	3.10	0.93	10.68	0.65	13.94	1.51
P Effect	2.37^{ns}	0.70***	-0.13^{ns}	-0.02^{ns}	-0.78***	0.37^{ns}	-0.08^{ns}	4.17***	-0.44***	0.91#	0.39***

See text for abreviations and details.

#, **, *** indicates that P effect is different from zero at $P \leq 0.1$, 0.01 and 0.001 respectively. ns = not significant. Shoot was expressed as g/pot, enzymes as U/mg protein, αKG as μ moles/g nodules and PLP's as μg/g nodules.

the activity of enzymes associated with NH_3 utilization, i.e., glutamate dehydrogenase (GDH), glutamine synthetase (GS), and glutamate synthase (GOGAT) as well as the aminotransferases, asparate transaminase (GOT) and alanine transaminase (GPT), of variety WB 21-8 Tinge. Less spectacular responses were observed on variety WB 12-11 Siempre, but beneficial effects were observed on nodule weight (nod. wt.), GDH, GOT and GPT. In both experiments P also increased the level of pyridoxyl phosphates (PLP's), essential coenzymes for GOT and GPT activity, and decreased alpha ketoglutarate (αKG) levels of WB 21-8 Tinge plants. The decrease on αKG levels was also determined to be associated with increased NH_3 utilization in the nodule cytosol of these plants; thus the P effect in this case seems to be that of increasing the rate of amination of free αKG instead of actually diminishing its presence within the nodules.

These results appear somewhat contrasting with the response of corn (Zea mays) to fertility treatments in this soil, under similar experimental conditions (204). For corn, it was observed that greatest yield increments were achieved when nitrogen and potassium were used. Response to phosphate fertilization was observed only when in combination with N and K. Thus, these experiments suggest that available P in the latosol from Jaiba, Minas Gerais, Brazil can support good corn yields but is inadequate for production of high legume grain yields with adequate levels of nitrogen fixation.

However, work conducted at Rothamsted Experimental Station in England has indicated that the higher P requirement of nodulated legumes can be economically met by using cheaper rock phosphate as a P source

and inoculating the plants with vesicular-arbuscular (VA) micorrhiza. A summary of the data obtained by Mosse and coworkers (174) using two cerrado soils from Brazil is presented in Table II.

The data clearly indicates there is a strong interaction between rock phosphate (RP) and the micorrhiza (ME_3). Plants in treatment RP + M(E_3) had increased nodulation, P content and higher nitrogenase activity (C_2H_2) red), than plants that only received RP, M(E_3) or the checks.

It has been observed that % P in the range of 0.28-0.30 is required for optimum red clover growth (172, 261), 0.17 for Stylosanthes humilis (9) and 0.20 for centrosema (174). Thus, these results suggest that neither RP nor M(E_3) alone can have optimum effect on these legumes, possibly with the exception of S. guyanensis. For both red clover, and centrosema the optimum % P in the plants was only attained by the RP + M(E_3) treatment.

Similarly, Crush (53) has found that in deficient soils VA micorrhiza strongly stimulated nodulation and growth of Centrosema pubescens, Stylosanthes guyanensis, Trifolium repens and Lotus pedunculatus. An interesting finding was that mycorrhiza's preferential growth was for Trifolium repens and not for the grass Lolium perenne.

Crush concluded in his paper that in his experiments P, and not N, was the factor limiting growth, and that VA micorrhiza helped the plants utilize more of the available soil P supply. Apparently micrrhizas increase the plant utilization of already available P in the soil, but are not capable of mobilizing the P fraction that is not soluble yet (173).

It is evident from these studies that utilization of legume crops

TABLE II

EFFECT OF ROCK PHOSPHATE AND VESICULAR-ARBUSCULAR MICORRHIZA ON FORAGE LEGUMINOUS PLANTS GROWN IN BRAZILIAN CERRADO SOILS (DATA FROM MOSSE, POWELL AND HAYMAN, REF. 174)

	Check	RP	$M(E_3)$	RP + $M(E_3)$
Clover				
Dry wt/shoot (mg)	10	29	64	145
% shoot P	0.03	0.08	0.15	0.30
Total shoot P (µg)	3	24	95	431
No. nodules/plant	0	1	3	55
µ moles C_2H_4/plant/hr	0	0.006	0.03	1.4
Centrosema				
Total dry wt/plant (mg)	87	221	265	666
% P (mean of shoot + root)	0.08	0.07	0.08	0.18
Total P/plant (µg)	61	149	205	930
Nod. fresh wt/plant (mg)	0	2	4	208
µ moles C_2H_4/plant/hr	0	0.005	0.012	0.93
Stylosanthes guiyanensis				
Dry wt/plant	44	473	448	659
Total P/plant (µg)	24	418	355	2404
No. of nodules	0	8	6	79
µ moles C_2H_4/plant/hr	0	0.17	0.04	2.13

All plants were inoculated with the appropriate Rhizobium strain.

RP = rock phosphate, $M(E_3)$ = micorrhiza type E_3

properly inoculated with effective strains of rhizobia along with an efficient VA micorrhiza can play an important role in the improvement of the cerrado soils of Brazil, which are poor in available P and nitrogen (154), but are located in a region where farmers can obtain rock phosphate.

Extremely scarce data is available in the literature relating symbiotic nitrogen fixation and potassium. The prevailing idea is that K does not play a major role in the process of symbiotic N fixation (180). This generalized conclusion was drawn by Munns (180) based on the work conducted by Andrews and Robins (10, 11) with several tropical and temperate legumes. However, a closer examination of the data presented by these authors does not support Munn's conclusion. In this series of experiments Andrews and Robins were concerned with the effect of potassium on the growth and chemical composition of the pasture legumes examined. They did not measure the K effect on either nodulation components (nodule number and nodule weight) or nitrogenase activity. On the other hand these authors were able to detect a K effect on the growth of all species tested. A beneficial effect for K application on alfalfa and red clover yields, two herbage legumes not tested by Andrews and Robins, was also observed by Smith and Smith working with a low K Typic Argiudoll (236).

Recent work by Mengel and colaborators (165) using Vicia faba grown in liquid culture has suggested that nitrogenase activity was increased with increasing K levels in the medium. These authors concluded that the effect of K on N fixation was by making more carbohydrates available for the nodules, thus providing a more abundant supply of reducing electrons and ATP. The role of K as a cofactor of carbohydrate

movement out of the leaves of entire plants of sugar cane has been evaluated by Hartt (106).

Recently it has also been determined that K has a beneficial effect on nodule number and nodule weight of winged bean plants (206). An increase in nitrogenase activity was also observed when the variety WB 21-8 Tinge was fertilized with this nutrient.

A striking feature of the K requirement of plants, is the fact that the K content of healthy plants is higher than its known functions in the plant can account for. Since the main function of this element seems to be that of a catalizer of several enzymes, this paradoxy apparently can be explained by the low affinity of K for organic ligands including the enzymes for which it is a cofactor (76). The maximum activity of these enzymes sometimes require as much as 50-100 mM K.

In recent years K has also been suggested to be part of the mechanism of stomata aperture control, and Humble and Hsiao have claimed that to date, this is the only recognized physiological process in plants specifically requiring K^+ (119).

Although, today our understanding of the K effects on symbiotic N fixation is very limited, this element in the future may be proved to play important roles in this mechanism since K^+ is necessary for glycolysis, oxidative phosphorilation, photophosphorilation and for adenine synthesis (78).

It is presently recognized that sulfur deficiency can limit both nodulation and N fixation (180). Apparently, this effect is caused by disturbances in the host plant metabolism and not directly on nodulation. Sulfur is required for protein synthesis from available nitrogen

(180), and in S-deficient plants nonprotein-N compounds tend to accumulate. This protein deficiency will cause visual N dificiency symptoms which are not ameliorated by N fertilization (111). However, low S-deficiency can lower plant protein synthesis without reducing plant growth (126). Because protein is a more limiting factor than fiber for the grazing animal, it is probably more beneficial to fertilize the legume with sulfur levels for maximum protein yield rather than maximum dry matter production (127).

A good review about glutathione (a sulfur containing tripeptide) effects on protein synthesis is presented by Kosower and Kosower (138). According to these authors, two major stages in protein synthesis are generally recognized: (a) initiation of polypeptide chains and (b) polypetide chain elongation (followed by termination and release). They have also determined that polypetide chain elongation only occurs in the presence of G-SH (reduced glutathione) and that G-S-S-G (oxidized glutathione) is inhibitory. Thus, it appears that protein synthesis is governed by the balance of the reduced and oxidized forms of glutathione in the cell.

Recent experimentation in this laboratory has indicated that in the dark red latosol from Brazil, S was required for nodule growth in two varieties of winged bean but did not affect the number of nodules formed (206). It can be suggested then, that the shortage of S inhibited protein synthesis, thus preventing cell division and consequent nodule growth.

Jager and Pahlich (123) have determined that shortly after pea seedlings were treated with SO_2, the concentration of glutamic acid and glutamine increased in both roots and shoots. This change was

correlated with the activity of the corresponding enzymes, glutamic acid dehydrogenase and glutamine synthetase. In a later work Pahlich (196) also determined that SO_2 fumigation can differentially affect levels of glutamate-oxaloacetate transaminase (GOT) within the same cell. He reported that SO_2 inhibited GOT activity in the mitochondria, while the cytoplasmic form of the enzyme was not affected.

Sulfate applied as $(NH_4)_2SO_4$ has been found to be an activator of glutamate dehydrogenase activity and the plotting of velocity versus rising concentrations of alpha ketoglutarate, follows a Michaelis-Menten type Kinetic. When $(NH_4)_2SO_4$ was replaced by NH_4Cl the kinetic response was sigmoidal (195).

Results in disagreement with the observation made above are presented by Kostir and colaborators (137). They found that 14-15 days old pea seedlings had decreased levels of glutamic acid and increased levels of alanine when treated with 0.1% of SO_2 fumigation. They also noted that pea seedlings subjected to high levels of SO_2 had low levels of sucrose and accumulated glucose and fructose.

Apparently these observations indicate that S may affect the activity of several enzymes of NH_4 assimilation, and therefore it may have a more direct effect on symbiotic N fixation.

The relation between S and indol acetic acid and the influence of this compound on nodule formation has been mentioned before.

Sulfur is also part of the molecule of several coenzymes such as thiamine, coenzyme A and biotin. The latter compound has been determined to favor Rhizobium growth in a chemically defined media (26).

Ferredoxin, an important protein involved within electron transport systems contains equimolar concentrations of iron and sulfur (76).

The importance of S in the mechanism of symbiotic N fixation can hardly be overemphasized because S is a component of the nitrogenase molecule. The nitrogenase system from Azotobacter is made up of two different proteins: component I, contains 32 atoms of iron, 26 atoms of acid-labile sulfide and 2 atoms of molybdenum whereas component II has 4 atoms of iron and 4 atoms of acid-labile sulfide (38).

Calcium is essential for the infection of the root hairs by rhizobia. In subterranean clover (156) Ca levels that had no effect on plant fresh weight significantly increased the number of nodules per plant. In Medicago sp. (180) the Ca-sensitive stage occurs 1-3 days after inoculation and apparently coincides with the root hair curling stage. Interaction between Ca and PO_4^- levels in the soil are well known, as well as the increased availability of Mo at higher soil pH levels caused by liming materials such as $CaCO_3$.

The growth and survival of rhizobia has an essential requirement for Ca. Although Norris (186, 187) concluded that Rhizobium bacteria are not Ca sensitive microorganisms, his claims have been disproved (26). It has also been demonstrated that Ca-deficient Rhizobium trifolii (266) cells were swollen and vacuolated, indicating that the cell wall had lost rigidity, permitting an unusual amount of water penetration. Deficiency of Ca in the presence of sufficient Mg can also reduce the growth rate, the level of maximum growth and the proportion of viable cells for root hair infection (267).

Calcium is also required for the activity of both NADH and NAD^+ dependent reactions catalyzed by glutamate dehydrogenase from Lemma minor. Although the enzyme activity can be fully restored by Ca^{++} after inactivation by EDTA, only a partial effect was observed for

other divalent cations. In this study, Ehmke and Hartmann (75) suggested that Ca^{++} governs an equilibrium between a catalytically inactive (Ca^{++} free) and an active (Ca^{++} saturated) enzyme form. Inactivation by removal of Ca^{++} is related to an alteration in the binding characteristics or binding sequence of the substrate NH_4^+.

It has also been determined that Ca^{++}, Mg^{++} or Mn^{++}, but not Ba^{++}, Co^{++}, Sr^{++}, Ni^{++} or spermidine, have some physical and chemical effects on the properties of glutamine synthetase from Escherichia coli (230) The divalent cation saturated form of the enzyme is referred to as taut and the divalent cation-free form as relaxed. The relaxed form appears to have a asymmetric structure and is inactive, whereas the taut form is more compact, nearly spherical and active.

These results suggesting that Ca^{++} has an effect on the activity of both glutamate dehydrogenase and glutamine synthetase indicates the importance of proper calcium levels for the assimilation of symbiotically fixed nitrogen, since these are presently regarded as the main pathways for NH_3 incorporation into plant amino acids.

There is very little information in the literature about the possible roles of magnesium on nodulation and symbiotic fixation of nitrogen. The work conducted by Purcino (206) suggested that there were no beneficial effects on nodulation,N fixation and winged bean growth on the dark red latosol from Brazil when Mg was added as a fertilizer. Under the conditions that these experiments were conducted, Mg was not a limiting factor for the operation of the host-rhizobium system.

However, the rhizobium sensitivity for Mg deficiency has been demonstrated by the extensive work conducted by Norris (186, 187), and apparently this nutrient is required in greater concentration than calcium (269).

Magnesium is part of the chlorophyl molecule and thus can be said to be involved in the most important synthetic reaction on earth, namely, photosynthesis (88).

Magnesium is an activator of several enzyme systems, especially the ones that react with phosphor lated substrates (270). Nitrogenase activity is dependent on the presence of a Mg ATP complex and this energetic requirement is still puzzling today since no theoretical basis exists to explain the ATP requirement of the nitrogenase system (39).

The essentiality of molybdenum for symbiotic nitrogen fixation had been suspected long before isolation of the nitrogenase molecule (35). It had been observed in pot and field experiments that soils low in Mo were not capable of efficiently supporting the process of N fixation (8). Today Mo is known to be closely associated with N metabolism. The nitrogenase molecule contains several Mo^{5+} ions (38) which is also a structural component of the nitrate reductase enzyme (182, 188). If the nitrate reductase step is bypassed by fertilizing the plants with either nitrite or ammonia as a N source, the plant requirement for Mo is lowered (208). It is suspected that the Mo role is similar in both nitrogenase and nitrate reductase. Nitrogen (for nitrogenase) and NO_3^- (for nitrate reductase) during the process of reduction will enter into contact with the cation and the flow of electrons pass through the Mo ion itself (41). However, the ultimate electron donor for nitrogen reduction to ammonia seems to be ferredoxin (41) and for nitrate reduction both nicotiamide nucleotides (NADH and NADPH) are active in spinach leaf extracts, although ferredoxin can function with nitrate reductase of Anaebaena cylindrica (112).

Plants low in Mo have been observed to develop a large number of

small brown nonactive nodules (8).

The importance of Mo in the nutrition of leguminous plants has been thoroughly reviewed by Hewitt (111).

The first observations that cobalt might favor the process of nitrogen fixation was made by Holm-Hanson and co-workers (114) when they observed that blue-green algae capable of N fixation responded to cobalt additions. Later, in a series of sophisticated experiments, Evans and his associates (1) demonstrated the essentiality of Co for nodulated soybean plants. They observed that addition of 0.1 μg cobalt per liter of nutrient solution resulted in a 12-fold increase in the dry weight of shoots and 22-fold increase in their nitrogen content. The response to Co was specific and was not replaced by a series of other trace elements.

Apparently the biochemistry of Co deficiency is so closely related to N metabolism that Co deficiency symptoms of nodulated legumes cannot be distinguished from symptoms of N deficiency of nonnodulated legumes (159). It has been suggested that the Co-vitamin B12, is a coenzyme required for ribonucleotide reductase activity in rhizobia (52), with Co being also required for high hemoglobin metabolism (63, 275). These findings possibly explain why Ahmad and Evans (1) failed to demonstrate a cobalt requirement for nonnodulated soybean plants supplied with nitrate nitrogen. Similar results were also observed in Medicago sativa by Delwiche and co-workers (64).

Nodulation can be inhibited by boron deficiency (180) although the mechanism of B action is still not well understood. It is generally believed that B regulates carbohydrate metabolism (88) and that the plants lacking in this nutrient develop a shortage of carbohydrate for nodule

activity (269). A detailed account of B roles in plant metabolism is given by Gauch (88).

Some effects of copper on nodulation and N fixation have been reported. Yates and Hallsworth (279) observed that in nodulated clover, addition of Cu resulted in increased content of glutamic acid, alanine and ə-amino-n-butyric acid. They further noted that in isolated nodules the incorporation of ^{14}C-glucose into amino acids and protein was proportional to the Cu concentration in the medium. These authors concluded then that this nutrient is required for synthesis of ə-amino-n-butyric acid.

Copper deficiency has more recently been shown to influence nodulation and nitrogen fixation more severely than leaf growth in subterranean clover. In this work conducted by Cartwright and Hallsworth (45) it was postulated that a shortage of Cu reduced the activity of cytochrome oxidase, hence unduly increasing the oxygen concentration within the nodule and inhibiting nitrogenase activity.

Zinc deficiency primarily limits host plant growth and has a minor influence on nodulation or N fixation (180). Zinc has been suggested to be a constituent of glutamate dehydrogenase (88), an enzyme involved in the assimilation of ammonia, but as divalent cations are seldom strictly specific in their roles, the effect of Zn shortage on NH_3 assimilation is not known.

Iron is present in several plant enzyme systems and is closely related to the process of N fixation for it is a constituient of both nitrogenase (74) and leghhemoglobin, a protein, found exclusively in the nodules of leguminous plants (19). Iron is also present in the cytochromes and ferredoxin, proteins associated with oxidation and

reduction processes during photosynthesis and respiration (208).

Although manganese has been known to be required for O_2 evolution in photosystem II of photosynthesis (95) and to activate several enzymes of the tricarboxylic acid cycle (76) its effects on symbiotic N fixation are not known. Probably Mn will disturb the process of N fixation only if it becomes limiting for plant growth, thus having an indirect effect on the N metabolism of the plant.

Only very recently has chlorine been conclusively associated with the Hill reaction of photosynthesis. This evidence was provided by Izawa and his colaborators (122) who showed that in alkaline pH, chlorine deficient plants had a disturbed photosystem II but a functional rate of cyclic photophosphorilation. However, because of its abundance in agricultural soils it is highly unlikely that Cl will ever become limiting insofar as to affect plant growth or symbiotic N fixation.

Several of the essential plant nutrients cited above under certain conditions can have harmful effects and impair nodulation and plant growth. However these aspects were recently reviewed (278) and thus will not be discussed here.

Moisture. The moisture content of the soil is an important factor influencing nodulation and rates of N fixation, as well as the metabolism of the host plant. Furthermore, the survival of rhizobia is greatly influenced by the relative humidity (RH) of the air. Vincent and his co-workers (268) determined that Rhizobium trifolii cells lost their viability when suspended in glass-distilled water and spread on glass beads, under both drying (0-20% RH) and nondrying (100% RH) conditions. High concentration of maltose (up to 27%) had some protective effect

against drying effects during rhizobia storage. They also noticed two phases under drying conditions; a rapid death phase caused by a rapid loss of water lasting for about 24 hours, and then a subsequent phase with reduced death rate. It has also been suggested that desiccated cells have a larger heat stability, although the practical importance of this is not known, since under field conditions rhizobium cells are more likely to be under an alternate wilting and drying regime, thus nullifying the stabilizing effect of a single desiccation (269).

Working with soybeans and making in situ determinations of acetylene reduction (nitrogenase activity) rates, net photosynthesis, dark respiration and transpiration, Huang and collaborators (117) observed no acetylene reduction in flooded soils and soils with water potential of -19.5 bars. The decrease in acetylene reduction upon soil desiccation was correlated with a decrease in both photosynthesis and transpiration, but not with dark respiration. Although dark respiration was also affected, it could not account for the reduction in nitrogenase activity.

Kuo and Boersma (141) determined an interaction between temperature and soil water suction on the efficiency of the nitrogen fixing mechanism with soybeans. They proposed that these effects could be explained by: (1) effects of temperature and water stress on enzyme activity, (2) effects of temperature and water stress on carbohydrate supply, and (3) effects of temperature and water stress on substances produced by the roots and required by nitrogen fixing organisms. When they measured the amount of N fixed per unit of CO_2 absorbed it was apparent that increased water suction, at all temperatures observed,

decreased the efficiency of the N fixation process.

Working with the forage, birdsfoot trefoil (Lotus corniculatus L.), McKee (160) has observed that inadequate soil moisture depressed nodulation more severely than top or root growth. He concluded in his work that nodulation of this forage legume may be retarded or completely inhibited on soils subject to desiccation or to alternating periods of moisture and drouth. One interesting observation was that at pH values below 6.0 the effects of desiccation were more severe than at pH above 6.0. In general, when soil pH was greater than 6.0, after a 6 week period of desiccation, there was a recovery in nodulation upon watering. On the other hand, however, if the pH was below 6.0, results were erratic, and the plants had fewer functioning pink nodules.

Sprent (243, 244, 245) has also conducted a series of experiments to determine the short term effects of water supply on detached soybean nodules. The noted that when nodules were only 80% fully turgid irreversible changes on acetylene and nitrogen reducing activities occurred, respiration rates diminished and gross structural changes took place. She speculated that such nodules are probably shed by the plant (243). Figure 2 shows her results indicating that less than fully turgid nodules, have reduced rates of nitrogenase activity (C_2H_2 red), probably due to a shortage of ATP and reductants.

When she examined the fine structure of the detached soybean nodules she noted that water stress differentially affected the noninfected (vacuolated) cells and the infected ones (nonvacuolated) (244). The infected nonvaculated cells could withstand a 60-70% water loss, but the noninfected vacuolated cells were damaged when only 20% water loss occurred. This resulted in breakage of the plasmodesmata

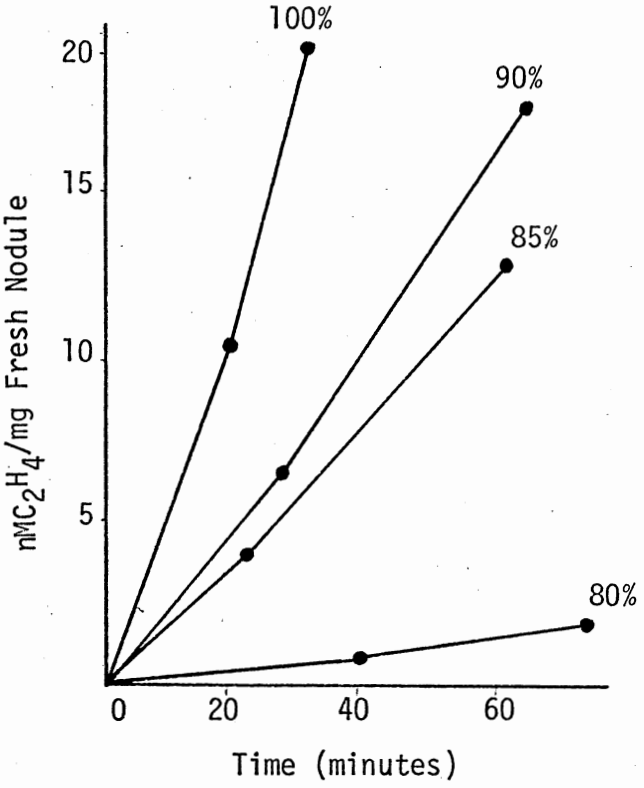


Figure 2. Effect of Different Levels of Nodule Turgidity on Nitrogenase Activity (C_2H_2 Red). (From Fig. 4 of Sprent, Ref. 243, p. 13)

connecting the noninfected with the infected cells, thus interrupting the transit of oxygen and carbon skeletons to the site of N fixation and NH_3 assimilation.

Non-electrolytes, such as mannitol, also depressed nitrogenase activity by osmoticaly withdrawing water from the nodules (245). However, water stress effects in saline soils are further complicated by the presence of salts. Bernstein and Ogata (31) observed that legumes dependent on symbiotically fixed N for growth are more sensitive to salinity than when supplied with combined nitrogen. In this respect a difference between Glycine max and Medicago sativa was also observed.

Avoidance of waterlogging is also important for setting of functional nodules (269) and Sprent (242) has proposed that free water will impair oxygen supply to detached nodules. Nodules of Vicia faba and Glycine max exhibited maximum nitrogenase activity when the soil was near field capacity (246). In disagreement with Sprent's data are the results obtained by Van Straten and Schmidt (263). These authors concluded from their experiments that a layer of water around the nodule will not impair gas diffusion as long as the nodule surface is undamaged. They further suggested that the lower levels of acetylene reduction observed by Sprent, were due to water penetration in the nodule wounds caused during detachment, thus filling the intercellular spaces.

Apparently the nodulation response to waterlogging is influenced by plant age, development stage of the host, and differs with different leguminous plants (65).

Temperature. Temperature is an important environmental factor influencing the symbiotic mechanism of N fixation. It has a large effect on rhizobia growth (55), nodule formation (269) and nitrogen fixation (210). It is also apparent that the influence of temperature on rates of N fixation will interact with host plant genera and bacterial strains (57, 89, 91). Vincent (269) has observed in his laboratory that the optimum temperature for plant growth may not be optimum for nodule setting. In Lotus and Stylosanthes, abnormalities of the infection threads were usually associated with high temperatures (209).

In a detailed study, Dart and Mercer (55) were able to determine that temperature significantly affected nodulation on both primary and secondary roots, fresh nodule weight per plant, and nodule size of Vignia sinensis. Their data also revealed that temperature markedly affected the distribution of starch within the nodules and leghemoglobin levels were lower at 21° and 36°C. At 21°C the symbiotic N fixation mechanism was not functional and growth was entirely dependent on supply of combined nitrogen. These authors concluded that temperature exerts a far greater influence than either ammonium nitrate or light on nodulation and on the overall growth pattern of the Vignia sinensis - Rhizobium association. Thus, rates of N fixation of different experiments cannot be compared if temperature was not a controlled factor.

Davidson and co-workers (60) have also presented data to indicate that temperature effect on single plants may not reflect the true situation under field conditions. Their data indicated that in contrast to results of studies with single plants (176), dry matter yield and nitrogen fixation on small swards of subterranean clover were

not lower at 12° than at 22°C.

Nitrogenase activity usually is not detected at temperatures around 0°C (39), but activity still remains at temperatures which can affect other aspects of the symbiosis (56). Apparently the effects of high temperatures on nitrogenase can be reversed by lower temperatures as demonstrated by Ranga Rao (210). When his Lotus and Stylosanthes plants were transferred from 15, 20, 25 and 30°C to 40°C nitrogenase activity ceased immediately, but upon return of the plants to their original temperatures, nitrogenase activity was reestablished within one hour.

Other Environmental Factors Affecting N Fixation. Several other environmental factors such as day length, light intensity and quality, phage, shading, agrochemicals, pests, diseases, biotic factors, defoliation and others, are recognized to influence the process of N fixation. For many of these factors are not of interest regarding the experiments described in this monograph, the readers will be referred to the reviews written by Gibson (91, 92), Vincent (269), and Lie (148) for more details on these topics.

The Rhizobium Host Plant Symbiosis

Apparently, the principal function of the bacteroid in the root nodule is to reduce nitrogen to ammonia. Why the bacteroid fixes more N than is required only for its own metabolic processes is a question largely unanswered. Apparently the sole purpose of the symbiont in the symbiotic N fixation is the production of NH_3 for the host (229). The metabolism of the nodules is harmonized with the host plant

metabolic processes (274), and those factors affecting plant growth will have an effect on the process of N fixation.

In this section the process of nodulation and the host-rhizobium relationship will be presented.

The free living rhizobia are gram negative, often motile, small rodlike bacteria (Rhizobiaceae) (128). Infection of the roots of most leguminous species seems to take place through its root hairs (190), although in Stylosanthes, infection has been observed to occur only through the openings created by emerging lateral roots (209). Similar type of infection has also been reported for Arachis hypogea by Allen and Allen (2). The infection process is specific and recognition of infection sites may involve the binding of specific legume lectins to unique carbohydrate structures found exclusively on the surface of the homologous rhizobial and legume symbiont (61).

Dazzo (61) has recently studied the biochemical basis for host specificity in the Rhizobium-clover root nodule symbiosis. He observed that clover root hairs, preferentially attract viable cells of Rhizobium trifolii or their capsular polysaccharides. A carbohydrate-containing protein called trifoliin plays a role in this specific recognition process. Apparently R. Trifolii and clover roots have similar overlapping carbohydrate receptors for trifoliin on their surfaces.

Competition experiments that Dazzo conducted showed that this carbohydrate receptor binds R. trifolii to clover root hairs, and that the recognition process was disturbed by the presence of fixed forms of nitrogen.

Red clover hairs, during infection, usually curl probably in response to presence of β-indoleacetic acid (190) secreted by the roots.

Apparently at this stage the enzyme polygalacturonase will weaken the root hair cell wall allowing penetration of the rhizobia. Only rarely more than one rhizobia strain will penetrate the root hair during infection (269). An infection thread, which is essentially a cellulose tube within the host cell, grows through the root hair cells and cortical cells of the developing nodule, and serves as a pathway for the invading bacteria (39). According to Nutman (190) the growth of the infection thread is controlled by the host cell nucleus. Formation of a new nodule takes place when the rhizobia pass through the infection threads and a group of mostly tetraploid cells in the cortex starts to divide. The mechanism of deposition of the rhizobia into the dividing tetraploid cells with its subsequent enclosure into a cytoplasmic membrane is not completely well understood yet. Within these cytoplasmic membrane vesicles, the bacteria will divide a few times, giving rise to vesicles containing up to eight organisms (39). As the nodule grows and develops the bacteria will undergo several physiological changes and will form the bacteroids. Bacteroids have the unique enzyme called nitrogenase capable of reducing atmospheric nitrogen into ammonia. The induction of leghemoglobin synthesis and presence of cytochrome oxidase are also changes accompanying the bacteroid formation.

An interesting series of electron micrographsis shown by Burns and Hardy (39) depicting changes in the soybean nodule structure caused by aging and the amount of μ moles C_2H_2 reduced to C_2H_4/g fresh nodule x day. In these soybean nodules, nitrogenase activity was observed 16 days after planting, peaked at about 34 days and apparently started to decrease when the plant entered the flowering and pod filling stage.

According to Jordan (128) a longitudinal section of mature nodules

reveal four zones of tissue differentiation: a basal meristematic zone, a central bacteroid zone, a peripheral cortical layer, and a vascular system. Pate and his co-workers (200) have studied the features of the nodule ultrastructure and recognized its importance in the functioning of the transport system of the leguminous root nodule.

Energy in the form of reductant and adenosine triphosphate essential requirements for nitrogenase activity (80). These factors are likely to be contributed by the plant photosynthetic process and Pate (197) has described the synchronization of host and symbiotic development in Pisum arvense. He observed in his experiments that leghemoglobin formation was rapid in all nodules just before cotyledon-nitrogen reserves exhausted, and nitrogenase activity commenced.

The rate of respiration of nodulated soybean, cowpea, and white clover has been observed to be 11-13% higher than for nonnodulated plants fertilized with abundant nitrogen. This superiority in terms of root respiration was generally associated with intense nitrogen fixation (220). On the other hand, based on theoretical considerations, Bergesen (29) concluded that the energy required for N fixation could reduce yield by 5-10%, but this would have no practical difference from nonnodulated legumes because they must expend some energy to reduce the fixed forms of fertilizer nitrogen into ammonia. Bergesen cited the data obtained by Gibson (90) to support his theoretical calculations. In his work Gibson (90) concluded that the carbohydrate requirements for symbiotic nitrogen fixation are similar to, or slightly greater than, those required for the assimilation of combined nitrogen. However, he also noted that in the early stage of growth, nodulated Trifolium subterraneum L. seedlings had a larger carbohydrate requirement than control plants.

Minchin and Pate (170) have determined the budget of the carbon economy in Pisum sativum L. Their results indicated that of the carbon gained photosynthetically by the shoot from the atmosphere 26% was incorporated directly into dry matter, 32% was translocated to the nodules, and 42% went to the root. Of the nodules' share, 5% was consumed in growth, 12% in respiration and 15% returned to the shoot via the xylem, as amino compounds generated in nitrogen fixation. On the other hand, Haystead and co-workers (109) have found that in white clover only 17% of the photosynthate is lost in nodulated root respiration.

Hardy and Havelka (101, 102) have demonstrated that soybean plants photosynthesizing in an atmosphere enriched with CO_2 showed an increased efficiency of the N fixation process. During the vegetative growth stage, these plants increased N fixation from 75 to 425 kg/ha whereas the absorption of soil nitrogen decreased from 220 to 85 kg/ha. In this case the process of symbiotic N fixation supplied 85% of the N requirements of the plant, as compared to the 25% of the control plants. According to these authors, nitrogenase concentration is not a factor limiting nitrogen fixation by soybeans provided temperature is in the range 20-35°C. However, we cannot take advantage of this fact because enrichment of the atmosphere with CO_2 is not economically feasible yet.

Several authors have studied the effect of photosynthesis on the process of N fixation and concluded that inhibition of shoot photosynthesis accounted for inhibition of nodule acetylene reduction (117, 144). Consequently plant defoliation, such as caused by a grazing animal, will have large effects on the symbiotic process.

Vance and co-workers (265) working with Medicago sativa L. determined that 70-80% shoot removal caused a 88% decline in acetylene reduction of detached root systems within 24 hrs. These plants then needed 18 days to recover to levels comparable to non-defoliated plants. An inverse relationship was observed in these plants between acetylene reduction and nitrate reductase activity.

Acetylene reduction was also observed to decrease in nodules of white clover after severe defoliation, and decreased availability of photosynthate was correlated with leghemoglobin degradation. In this study, conducted by Chu and Robertson (50) it took about 10 days for recovery of normal acetylene reduction levels.

The differential effect of shading and defoliation on nodule loss of three leguminous species is characterized on the work of Butler and collaborators (42). In their work, shading induced a marked loss of both roots and nodules of white clover, but on the other hand, little effect was observed for red clover. Apparently, Cratylia floribunda also does not have a strong tendency to shed nodules as a result of drastic reduction in available photosynthate (205).

Changes in rates of N fixation caused by the day/night regime also have been observed. Bach and collaborators (18) for instance, working with 8-10 week-old soybean plants exposed to an atmosphere containing ^{14}C, found that during the day most of the labelling was found in the organic acids of the roots, with a lower percentage found in the amino acids. On the other hand, nodules had higher labelling in amino acids, with less labelling of organic acids. However, during the night, the percentage of total ^{14}C in the organic acids of the roots decreased while it increased markedly in the nodules. Working with Pisum sativum L.

Minchin and Pate (171) observed that nitrogen fixation, respiratory output of the nodulated root, and nodule sugar level increased throughout the light period, whereas nodule soluble nitrogen level declined steadily. Several of these trends in the night period resulted in minimizing N fixation rate, sugar level and respiration, but maximized soluble nitrogen. These authors concluded that the daily changes in transpiration rate are likely to be responsible for controlling the rate of release of fixation products from nodules, whereas the opportunity during the photoperiod for new supplies of carbohydrate to form and be translocated to the nodules is selected as the primary factor allowing sugar levels to build up and fixation rates to increase as the photoperiod progresses.

In Nigeria, Ayanaba and Lawson (17), have found that nitrogen fixation in field-grown cowpeas and soybeans shows one maximum and one minimum during the light period, increasing again towards the early evening. These authors attributed the decline on rates of N fixation occurring during 12 noon and 6 pm to a deficit in the vapor pressure of the air.

Several authors have also observed that during flowering and pod filling, nitrogenase activity decreases probably because the nodules do not have a first priority on photosynthate partitioning. During this stage, plant carbohydrates preferentially move upward to the pods, thus causing a shortage of energy and reductants for N fixation and carbon skeletons for ammonia assimilation.

In the experiments conducted by Lawrie and Wheeler (145) it was observed that during the period from flowering to fruiting nitrogenase activity and accumulation of ^{14}C-photosynthates in pea nodules declined by 60%, whereas the photosynthesis of the plant doubled.

In Vetch (Vicia sativa L.), Pate (198) observed that plant flowering induced marked decreases in nodule number and nodule weight, but these effects could be temporarily arrested by removal of flower buds. This apparently was also observed by Lawrie and Wheeler (145) in their experiment, and the accumulation of photosynthate in the nodule increased nitrogenase activity.

The work conducted by Latimore, Jr., and associates (142), showed that the pods had a first call on photosynthate, and that the plants exported very little carbohydrate to the nodules during pod filling, and as a consequence symbiotic N fixation decreased. These authors also pointed to the fact that this decline in nitrogenase activity occurred during a period in which the N requirements of the plant was at its highest. To accommodate these facts Purcino (206) has proposed that her flowering winged bean plants absorbed N from the soil, by a less energy demanding pathway, i.e., direct absorption of NH_4^+ from the soil by glutamate dehydrogenase activity. The uptake of NH_4^+ by glutamate dehydrogenase independently of nitrate reductase (NR) activity, in roots of rice plants has been demonstrated by Kanamori and his associates (129).

The possibility also exists that nitrate reductase (NR) may play an important role on the host N metabolism during periods of low nitrogenase activity. The common occurrence of NR in nodules of leguminous plants was first reported by Cheniae and Evans (49). They then, indicated that, although it seemed logical to postulate that the nodule nitrate reductase system was related in some manner to the nitrogen fixation process, the presence of NR in nodules should be regarded as a system involved in nitrate respiration. More recently,

however, Vance and co-workers (265) have reported that after detopping of alfalfa plants, the decrease in nitrogenase activity and leghemoglobin content of the nodules were correlated with an increase in NR activity. The difference in NR between nodules from detopped and control plants became less evident as shoot regrowth occurred and nitrogenase activity increased in the detopped plants, although NR activity was never totally absent.

According to Broghton and collaborators (36) NR is the first measurable enzymatic activity in the nodules of Centrosema pubescens, Benth, and Vignia unguiculata L. Walp. followed by nitrogenase (C_2H_2 red) and leghemoglobin. Nitrate reductase activity in C. pubescens nodules was negatively exponentially correlated with nitrogenase activity of the same nodules, suggesting a change in metabolism of old nodules.

Apparently sucrose, fructose and glucose are the main carbohydrates donated by the host for the metabolism of the bacteroid (13, 249), although other compounds have also been identified. Sucrose is the major product of photosynthesis transported from the leaves to the nodules (171). Lawrie and Wheeler (146) have determined that maximum accumulation of ^{14}C-assimilates in nodules occurred within 90 minutes of synthesis.

The hydrolysis of sucrose in the nodules can be accomplished by inverstase. Robertson and Taylor (212) have determined the presence of an alkaline inverstase in the nodule cytoplasm of Lupinus angustifolius L. infected with Rhizobium lupin. It has also been suggested by Kidby (130) that under physiological conditions orthophosphate is the activator for invertase of Lupinus luteus L. root nodules, and in Rhizobium japonicum the

catabolism of glucose occurs by the Entner-Doudoroff pathway and operation of the Krebs cycle (130).

The Biochemistry of N Fixation and NH_3 Assimilation

In this section a brief description of the mode of action of nitrogenase and the enzymes of NH_3 assimilation into plant amino acids is presented. Despite the fact that the enzymes of ammonia assimilation are not part of the N fixation process, their presence has an influence on the mechanism of nitrogenase synthesis. For a detailed account of our present knowledge about the enzyme nitrogenase the reader is referred to recent review articles written by Newton, Corbin and McDonald (183), Burns and Hardy (39) and Eady and Smith (74).

Nitrogenase. Nitrogenase (nitric-oxide reductase-EC 1.7.99.2) (Nase) is a unique enzyme, in the sense that it acts on a triple bond (39) and to date has been found only in prokariotyic cells such as the rhizobia (101).

Figure 3 shows a schematic diagram of the relationship between nitrogenase and other nodule reactions and components according to Evans and Barber (80).

The enzyme nitrogenase is composed of two proteins, often referred to as Mo-Fe protein or Component I and an Fe protein or component II (35). The larger Mo-Fe protein contains molybdenum, iron and acid-labile sulfur, and the smaller Fe protein contains iron and acid-labile sulfur (74). Neither protein exhibit nitrogenase activity by itself,

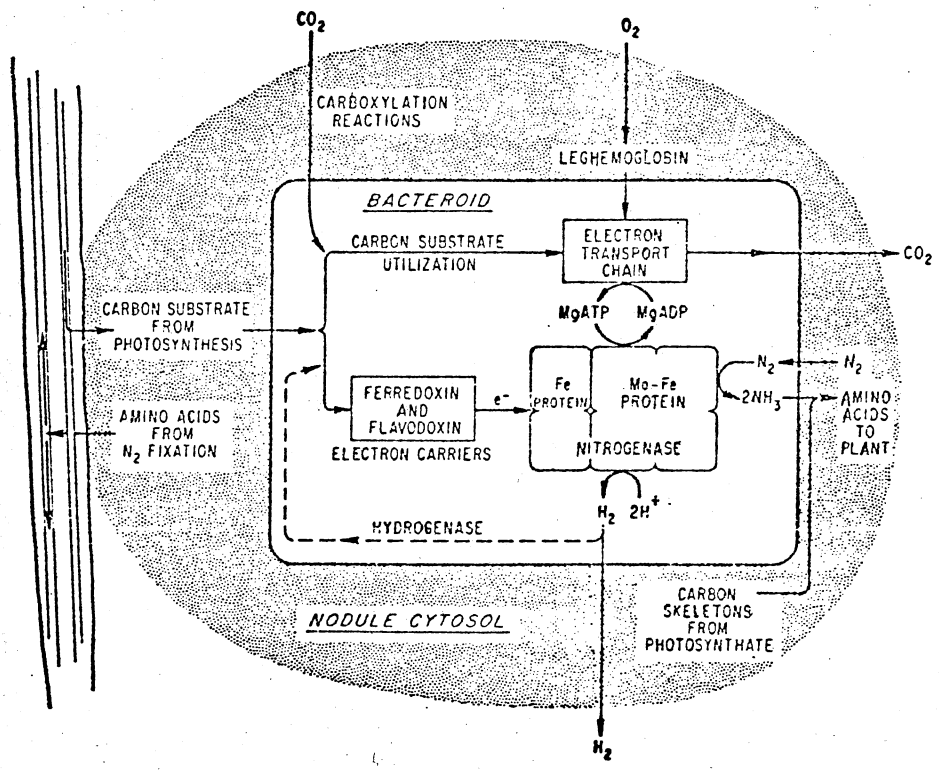


Figure 3. A Schematic Diagram of the Relationship Between Nitrogenase and Other Nodule Reaction and Components (From Evans and Barber, Ref. 80, p. 336)

but the observation that mixing of both proteins from different sources produces an active nitrogenase, indicates that these proteins from different organisms conform to a similar pattern (67).

Both proteins from several nitrogen-fixing entities have been isolated from extracts prepared by either French pressure cell treatment or by osmotic shock, purified by DEAE cellulose chromatography and crystalized.

According to Eady and Smith (74) the Mo-Fe protein, when exposed to air, becomes amorphous and is too small for x-ray analysis. However, it has been determined that component I from Clostridium pasterianum is composed of four subunits of two types with molecular weights near 50,000 and 60,000 daltons. Its amino acid composition depends on the rhizobium strain used, suggesting that the bacteria carries the genetic information for the Mo-Fe protein synthesis. Although all common amino acids are present in this protein, higher concentrations of glutamate and aspartate are observed.

The smaller Fe protein is composed of two subunits, with a dimeric molecular weight of about 55,000 daltons. Similar to the Mo-Fe protein, this protein has a higher concentration of acidic residues (about 20% are glutamate and aspartate), but generally shows an absence of tryptophan (74).

The reaction catalysed by the reductase activity of nitrogenase is exergonic, and research on the mechanism of action of nitrogenase was considerably delayed because no theoretical basis for the observed high ATP requirement could be formulated (39).

The overall reaction catalysed by nitrogenase can be represented as (147):

$$N_2 + 6H^+ + 6e^- + 12\ Mg\ ATP + 12\ H_2O \rightarrow 2NH_3 + 12\ Mg\ ADP + 12\ Pi$$

Adenosine triphosphate is an absolute requirement (67) but apparently the relationship $6ATP/6e^-$ (201) has not been universally accepted for the reduction of N_2 to $2NH_3$ (39).

It has been suggested that the function of MgATP is to lower the midpoint potential of the reduced Fe protein from -290 mV to -400 mV. During the MgATP-Fe protein binding the latter will simultaneously accept electrons which then flow to the Mo-Fe protein during catalysis. Regeneration of reducing power, occurs through the enzyme NADH-ferredoxin reductase as follows (147):

$$NADH + Fd_{ox} \rightarrow NAD^+ + Fd_{red} + H^+$$

The electron flow between the two nitrogenase proteins and the consequent reduction of atmospheric nitrogen to ammonia is graphically shown in Figure 4.

Nitrogen during reduction complexes first with iron, followed by subsequent reduction involving both the molybdenum and iron to form in sequence a metal-bound diazene, hydrazine, and ammonia, with release of ammonia (101).

The unquestionable determination of ammonia as the first stable product of N fixation was achieved only after many years of research. The suggestion that NH_3 is the first stable product was made by Aprison and colleagues (14) in 1954, but definite proof was given by Carnahan and co-workers (43) many years later in 1960, using enzyme preparations from cell free extracts. This key intermediate role of ammonia was confirmed for soybean root nodules by Bergensen (27) in 1965, and in 1966, Kennedy (131, 132) working with serradela nodules, showed that this

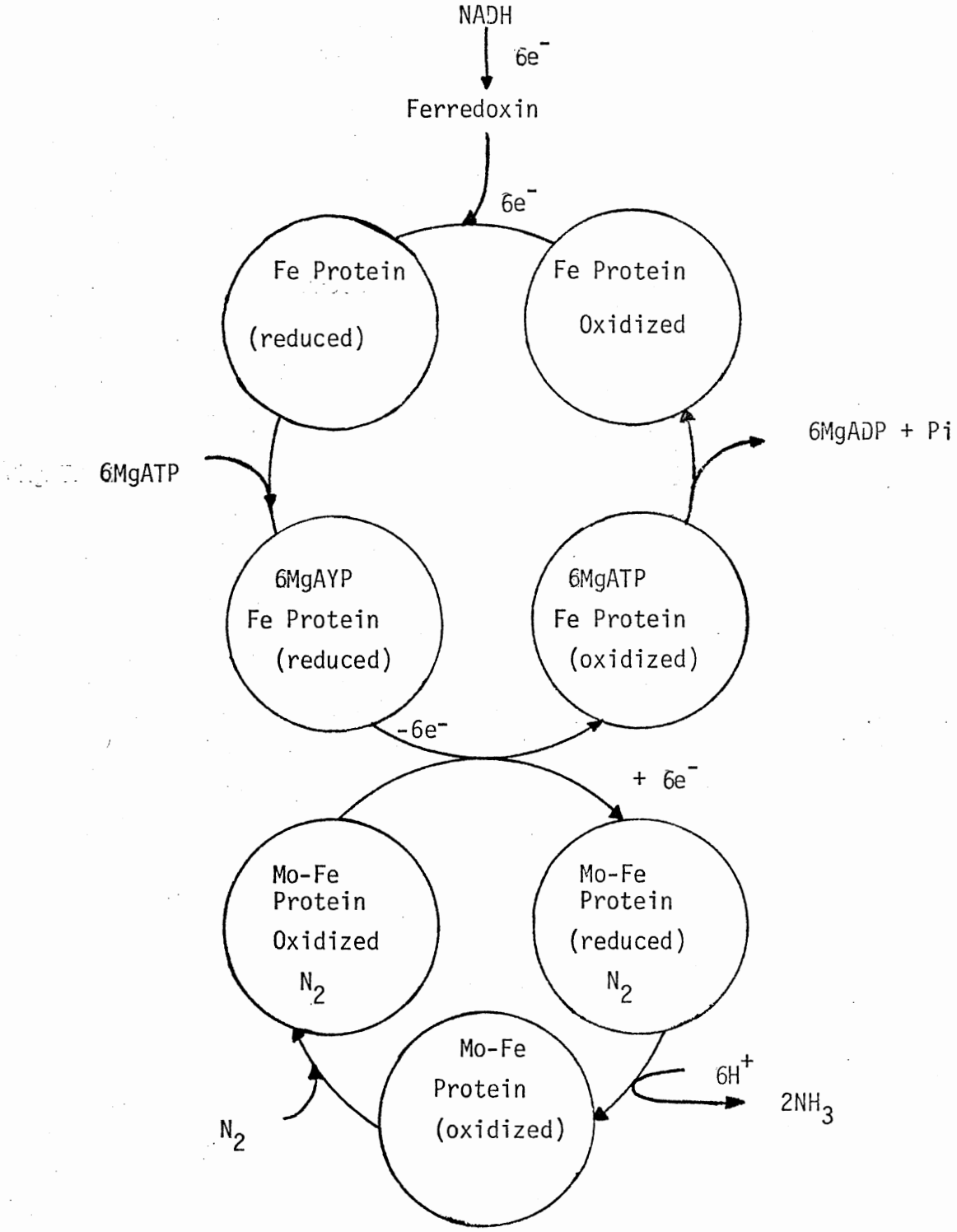


Figure 4. Schematic Representation of the Electron Flow Between the Fe Protein and the Mo-Fe Protein of Nitrogenase During Reduction of Atmospheric Nitrogen (From Fig. 25-28 of Lehninger, Ref. 147, p. 722)

newly fixed nitrogen was immediately incorporated into glutamic acid and glutamine.

Nitrogenase has the ability to reduce several other substrates, and in 1966, Dilworth (66) demonstrated that the reduction of acetylene (C_2H_2) and nitrogen by this enzyme were analogous processes, a fact that permitted Hardy and Knight (99) to develop the C_2H_2-C_2H_4 reduction assay, as a sensitive method for indirect evaluation of nitrogenase activity. In this assay the amount of C_2H_4 formed after incubation of C_2H_2 in the presence of nitrogenase is divided by the theoretical value three to express nitrogenase activity, usually in terms of μ moles C_2H_4/g fresh nod/time. However, it has often been observed that this theoretical value is not always correct (28, 158), a fact that coupled with the instantaneous nature of the measurement makes extrapolation of results for estimation of N fixation over a growing season very difficult (6).

It is also apparent that nitrogenase is capable of evolving hydrogen in the presence of ATP, a fact that has been taken to indicate that the enzyme has more than one active site (39). Shubert and Evans (233) have reported that this nitrogenase feature lowers the N fixation process efficiency between 30 and 60%, although selected rhizobium strains can diminish the energy waste.

Nitrogenase is sensitive to elevated pO_2, and oxygen is believed to inactivate it in two ways: (1) reversible uncompetitive inhibition of ATP hydrolysis and substrate, and (2) irreversible inactivation of the enzyme. To prevent this, active nodules contain large amounts of leghemoglobin which is believed to lower the pO_2 within the nodule down to the levels optimal for nitrogenase

activity (276). However, although the plant and mammalian hemoglobin are not very much different, this O_2 transport function of leghemoglobin has never been fully accepted despite the fact that no other role has ever been found for it (39).

Several early experiments (93) had shown that nodule leghemoglobin content was correlated with N fixation, but recently Nash and Schulman (181) have demonstrated that leghemoglobin content is more directly related to nodule weight and not so much to nitrogenase activity.

Burns and Hardy (39) have summarized data indicating that high level of free NH_4^+ controls the synthesis of nitrogenase in Clostridium, Azotobacter and Klebsiella. An "ammonium effect" was also observed in Rhizobium sp. by Tubb (260) causing a partial repression of nitrogenase synthesis, but he still observed nitrogenase activity in cultures growing in excess ammonium. More recently, Houwaard (115, 116) has proposed that assimilation of ammonium ions by glutamine synthetase controls the functioning of nitrogenase in the root nodules. In his experiments he observed that the decrease in the amount of nitrogenase of bacteroids brought about by excess NH_4^+ was caused by a reduced supply of energy-delivering photosynthates. These data reported by Houwaard (115, 116) are essentially similar to the model forwarded by O'Gara and Shanmugan (191, 192) in which the mechanism of NH_4^+ assimilation, especially glutamine synthetase and glutamate synthase, is responsible for the regulation of nitrogen fixation by rhizobia.

Apparently the action of glutamine synthetase on nitrogenase biosynthesis depends on its catalytic properties, which in turn is greatly influenced by its adenylylation state (30). It is generally believed that adenylylation blocks the binding of glutamine synthetase

to the nif promotor, or conversely, deadenylylation leads to binding of glutamine synthetase and subsequent activation of nif (228). Nif is the genotype designation of genus specifically required for N fixation and nif is the corresponding phenotype designation (34).

A different mechanism accounting for NH_4NO_3 inhibition of nitrogenase biosynthesis has been proposed by Bisseling and collaborators (32). According to them addition of NH_4NO_3 to the pea-Rhizobium leguminosarum growing medium decreased the amount of leghemoglobin in the nodules thus decreasing the nitrogen-fixing capacity of the symbiotic process. A repression of the nitrogenase biosynthesis by NH_4NO_3 was not observed. An interaction between pO_2 and the repressive effect of NH_4^- on nitrogenase bioxynthesis has also been reported by Bergensen and Turner (30).

As indicated in Figure 3, nitrogenase activity and N fixation are processes confined to the bacteroid, but NH_3 is then excreted into the nodule cytosol. O'Gara and Shanmugam (191) have reported that as much as 94% of the $^{15}N_2$ fixed by Rhizobium japonicum was recovered as $^{15}NH_4^+$ from the cell supernatant following alkaline diffusion. The work conducted by Brown and Dilworth (37) and by Kurz and co-workers (139) have demonstrated that enzyme activities in the bacteroids are too low to account for the utilization of ammonia produced during N fixation. The level of these enzymes within the bacteroids probably are only sufficient to perform the bacteroids' own anabolic processes.

Pathways of Ammonia Assimilation Into Plant Amino Acids

The recent discovery of glutamine (amide): 2 oxoglutarate amino

transferase oxido-reductase (NADP) in cultures of Aerobacter aerogenes growing in NH_3-limited media by Tempest and colaborators (254), with the subsequent inclusion of several other prokarioties to this list (164), prompted many investigators to reexamine the central role of glutamate dehydrogenase (GDH) (L-glutamate: $NAD(P)^+$ oxidoreductase deaminating-EC 1.4.1.3) in the pathway of ammonia assimilation into plant amino acids. The enzyme system proposed was later renamed L-glutamate: $NADP^+$ oxidoreductase (transaminating) EC 1.4.1.13) (GOGAT), and often referred to as glutamate synthase, is capable to catalyse the following reaction:

Glutamine + 2-oxoglutarate + NADPH → 2 glutamate + $NADP^+$

Because glutamine synthetase (GS) (L-glutamate: ammonia ligase (ADP-forming) EC 6.3.1.2), an ubiquitous plant enzyme can catalize the reaction:

NH_3 + glutamate + ATP ⇄ glutamine + APT + Pi + H_2O

it became apparent that the coupled action of GS-GOGAT could constitute an important pathway for utilization of fixed nitrogen.

The sum of the reactions catalysed by GS-GOGAT is (254): NH_3 + 2-oxoglutarate + ATP + NADPH → glutamate + ADP - Pi + NADP + H_2O

In this discussion the above reaction will be referred to as the GS-GOGAT pathway.

The GDH pathway of ammonia assimilation can be summarized as:

NH_3 + 2-oxoglutarate + NADPH → glutamate + H_2O + $NADP^+$

Thus, both presently known pathways for ammonia assimilation have glutamate as the end product.

Miflin and Lea (168) have reviewed the data available concerning

the operation of these pathways and pointed out that, due to their more favorable enzyme kinetics the GS-GOGAT pathway is more likely to play the major role in the assimilation of symbiotically fixed nitrogen. Glutamine synthetase has a high affinity for ammonia (Km values usually lower than 0.5 mM) a fact that coupled with the low Km values of GOGAT for glutamine (0.2 - 0.5 mM) and 2-oxoglutarate (2-7 mM) makes the operation of the GS-GOGAT system more likely than the GDH pathway, because the Km of GDH for ammonia is in the range 5-40 mM (67). However, on the other hand, the operation of the GS-GOGAT system requires the expenditure of energy in the form of ATP, a price that the cell must pay in order to be able to utilize such small concentrations of ammonia (169).

The irreversible nature of the reaction catalysed by GOGAT (250) is typical of several other byosynthetic pathways (168), whereas GDH has both the aminating and deaminating activities, with the balance controlled by the rate NAD/NADH (59). In pea mitochondria the synthesis of glutamate by GDH does not require ATP - rather ATP inhibits GDH activity (59) - and this fact will influence the plant's ability to absorb nitrogen. Energy is probably not a limiting factor for the operation of the GS-GOGAT pathway during periods of vigorous plant growth and abundant availability of photosynthate, but it has been shown that during flowering and pod filling (145) the nodules do not have a first call on carbohydrate partitioning and therefore have low levels of glucose for synthesis of ATP, a factor that can impede the coupled action of GS-GOGAT.

The low activity of the GS-GOGAT pathway during flowering and the large N requirement of the plant during this stage of growth indicates

that the plant may utilize the available N in the soil for pod filling.

In this regard Duke and Ham (71) have found that soybean plants infected with several strains of Rhizobium japonicum had low GOGAT activity during flowering, and the ones inoculated with an ineffective strain showed high levels of nodular GDH activity after addition of nitrogen. These authors concluded from their data that either GDH or GOGAT could account for total NH_3 assimilation in soybeans under appropriate physiological conditions. They further proposed that GDH and/or GOGAT activity could control synthesis, activation or inhibition of the other. A relationship such as this has been suggested by Berberich (20) to occur in Escherichia coli, where certain compounds such as cyclic AMP are capable of increasing synthesis of GDH and decrease synthesis of GOGAT, indicating that physiological processes could control levels of these enzymes in a reciprocal fashion. This mechanism apparently is in accordance with the observations that low carbohydrate availability in the nodules supresses both nitrogenase and the GS-GOGAT pathway activity (144, 206).

Utilization of ammonia by alanine dehydrogenase (ADH) EC 1.4.1.1) and aspartate dehydrogenase (AspDH) (EC 1.4.1.x) is also possible (147), but these pathways are not considered to be important and have received little attention. However, a study by Dunn and Klucas (72) has shown that, under certain conditions, ADH activity is 10-fold higher than GDH's in the bacteroid of soybean root nodules.

The reaction catalysed by ADH is:

pyruvate + NH_3 + NAD(P)H + $H^+ \rightleftharpoons$ L-alanine + $NAD(P)^+$ + H_2O and

Asp DH catalysis:

oxaloacetate + NH_3 + NAD(P)H + $H^+ \rightleftharpoons$ aspartate + $NAD(P)^+$ + H_2O

Once glutamate has been formed in the nodules, several transaminases are able to transfer its α amino group to several other keto acids forming new amino acids and regenerating alpha ketoglutaric acid (αKG) (2-oxoglutarate). Of these transaminases, glutamate-oxaloacetate transaminase (GOT) (L-aspartate: 2-oxoglutarate aminotransferase EC 2.6.1.1) and glutamate-pyruvate transaminase (GPT) (L-alanine: 2-oxoglutarate aminotransferase EC 2.6.1.2) have received the most attention (84, 96, 149, 218). They catalyse the byosynthesis of aspartate and alanine, respectively.

Both of these aminotranferases contain pyridoxal 5-phosphate (PLP) as a prosthetic group whose removal from the apoenzyme is facilitated by conversion into pyridoxamine 5-phosphate (PMP) by acid pH, and by either phosphate or precipitation with ammonium sulfate (68). Prosthetic groups are essential requirements for enzyme activity, and are usually tightly bound to the apoenzyme (124).

Wong and Cossins (277) were able to isolate GOT from the mitochondria and the cytoplasmic fraction of pea cotyleclons. According to Ryan and co-workers (219) at least four electrophoretically distinct forms of aspartate aminotransferase were present in root nodules of soybeans. Two forms originated from the cytosol of the host plant, a third from the mitochondria of the host and a fourth from the Rhizobium japonicum bacteroid. Usually GOT activity, both in the nodule cytosol and bacteroid, is several fold higher than GPT (97, 206, 218).

The analysis of the bleeding xylem sap of soybeans (248) and lupin (214) has demonstrated that asparagine is the main amino acid exported from the nodules to the plant xylem, and recently Scott and colleagues (225) have reported the synthesis of this amino acid by a

cell free extract of the plant fraction of lupin nodules in the presence of ATP, aspartate, glutamine and Mg^{2+}. Ammonia could replace glutamine as the source of the amide nitrogen but a four-fold greater concentration of NH_3 gave only a rate of synthesis half that observed with glutamine. The enzyme asparagine synthetase (EC 6.3.1.1) was implicated as the catalyst for the following reactions:

Aspartate + glutamine + ATP → asparagine + glutamate + AMP + PPi.

or

Aspartate + NH_4^+ + ATP → asparagine + AMP + PPi.

Figure 5 shows the model proposed by Scott and colaborators (225) for the assimilation of the ammonia produced after nitrogen reduction in the bacteroids into asparagine by enzymes located in the plant fraction of lupin nodules.

This model accounts for most of the aspects of N assimilation described here. For its construction it was assumed that N is fixed by nitrogenase in the nodule bacteroid and then excreted into the nodule cystosol as NH_3. The cytosol GS-GOGAT couple is active, first incorporating ammonia into glutamine and then forming 2 glutamates. Aspartate appears by transamination of oxaloacetate, with glutamate being the α amino group donor. Such reaction is very likely catalysed by the glutamate-oxaloacetate transaminase as discussed before. The synthesis of asparagine by the glutamine-dependent asparagine synthetase would then be accomplished using this aspartate and a second amide nitrogen from glutamine (216).

The model is in accordance with the observation that nitrogenase, leghemoglobin, glutamine synthetase (213) and glutamate synthase (214)

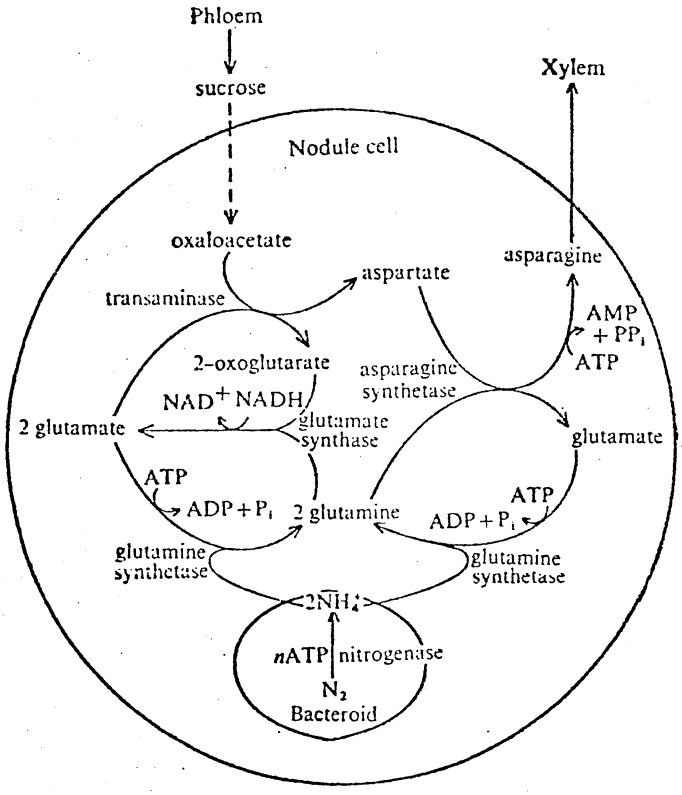


Figure 5. A Model for Incorporation of NH_4^+ into Asparagine, the Principal Nodule Amino Acid Exported to the Plant Xylem, as Proposed by Scott et al. For more details see text and ref. 225, p. 704.

are induced during development of lupin nodules, but does not show the alternate GDH pathway that is active during periods of carbohydrate shortages in the nodule.

The Dark Red Latosol

The dark red and red yellow latosols are the largest soil resources in central Brazil. These soils are generally deep, highly weathered and usually have a low natural fertility status.

Recently Lopes and Cox (154) have studied the fertility status of the surface horizon of a broad range of these latosols under cerrado vegetation and concluded that generally they are acidic, with low extractable basis, and available P and Zn. CEC values for these cerrado soils are usually extremely low, and generally related to organic matter content. High levels of Fe and high degrees of Al saturation are also common features of these soils.

Under forested conditions these latosols apparently have a better fertility status. These improved conditions have usually been associated with the calcareous origin of the parent material (5, 77).

Most latosols of Brazil fall under the order oxisol of the U. S. Soil Taxonomy system but correlation at the great group level is more difficult to obtain. They are usually classified as Acrustox or Haplustox, and the soil used in the experiments reported here is classified as a Typic Eutrustox (77).

The fertility response of this Eutrustox has been studied under field and greenhouse conditions. Field experiments using cotton (202) as the indicator crop, showed response to applications of N and P. A negative effect was also observed for application of a mixture of B, Mo, Zn and Mn.

Under greenhouse conditions contrasting responses have been observed for corn and a leguminous grain, Psophocarpus tetragonolobus. The corn experiments (204) showed a high requirement for N and K and not so much for P. In fact, P response was only obtained in the presence of N and K fertilization. A sulfur response was observed after several residual croppings. Similar to the field cotton experiments, a depressive effect was observed for Zn, B, and Mo when studied in factorial combinations in the presence and absence of the major macronutrients.

However, on the other hand, P fertilization was required for growth, nodulation and enzyme activity of P. tetragonolobus (206). Although the two P. tetragonolobus cultivars responded differently to additions of Ca, K and S, usually beneficial effects were observed. Apparently in these experiments Mg was detrimental for plant growth and N fixation.

CHAPTER III

MATERIALS AND METHODS

Seven experiments were conducted in the greenhouse and under laboratory light bank to study the effects of fertility treatments on Copada (Cratylia floribunda Benth) herbage yield, nodulation nitrogenase activity, and nodule enzyme and carbohydrate components involved with the metabolism of symbiotically fixed nitrogen.

These experiments were carried out on a siliceous, thermic, Psammentic Paleustalf (Eufaula) (94) from Oklahoma, USA, and a dark red latosol (Typic Eutrustox, isohyperthermic, fine, Kaolinitic) (77) from Minas Gerais, Brazil. For this dissertation the results obtained from four experiments conducted with the Brazilian soil will be presented and results from the Alfisol are being published elsewhere (205).

The experiments using the dark red latosol were all conducted in a greenhouse facility at the Oklahoma State University campus. To facilitate the collection of the entire nodulated root system, the pots in these experiments were filled with 100 g of actual soil thoroughly mixed with 400 g of white quartz sand. The sand grit was mainly of medium size and prior to use was washed in a 0.1 N HCl solution and then several times in distilled water for HCl removal (tested with Ag NO_3)

The chemical and particle size properties of this dark latosol are summarized in Table III.

Except where indicated all experiments were conducted in a complete randomized block design, and each treatment was replicated three times. Watering of the pots was done with distilled water and the soil moisture was maintained at approximately field capacity. Cooling and heating of the greenhouse was done when required.

In all experiments the Copada seeds were first scarified and then germinated in vermiculite. Transplant of the seedling to experimental pots was performed when the seedlings had developed one pair of true leaves. During transplanting the soil and seedlings where inoculated with 3 ml of a liquid medium containing more than 10^8 viable Rhizobium leguminosarum cells, cultured from nodules of Strophostyles sp. (203).

Preliminary Fertility Studies

Two preliminary studies were conducted to determine the most limiting nutrient factors for optimum Copada herbage yield in this latosol.

The treatment combination for these experiments are shown in Tables IV and V, and Table VI shows the nutrient source, treatment symbol and nutrient levels utilized in both experiments.

Five clippings of the top growth were obtained in each of these experiments before collection of the root system. Shoot and root dry weights were obtained for oven dried material at 105°C for 24 hours.

Effect of Plant Age on Nodulation and Nodule Physiology

For this study 72 pots were fertilized with the P + KS treatment

TABLE III

SOIL ANALYSIS OF THE 0-20 CM DEPTH LAYER OF THE DARK RED LATOSOL USED IN THE GREENHOUSE EXPERIMENTS

Properties	
pH (H_2O)	6.1
Buffer Index	6.8
CEC (NH_3EC), meq/100 g	25.4
Percent Organic Matter	3.3
Exchangeable Cations, meq/100 g	
Ca	13.8
Mg	2.5
K	0.4
Al	not detected
Available P (Bray P_1), ppm	7.5
Fe ppm	680.0
Mn ppm	208.0
Zn ppm	1.0
% Sand	24.5
% Silt	19.5
% Clay	56.0
Texture	Clay

TABLE IV

FERTILITY TREATMENTS FOR FIRST PRELIMINARY STUDY

Treatment	Symbol
1	Check
2	Ca
3	P
4	KS
5	KCl
6	NaS
7	S
8	P + KS

TABLE V

FERTILITY TREATMENTS FOR SECOND PRELIMINARY STUDY

Treatment	Symbol
1	Check
2	P
3	P + KCl
4	P + NaS
5	P + KS
6	P + KS + 1*B + 1 Mo + 1 Zn + 1 Cu
7	P + KS + 5 B + 5 Mo + 5 Zn + 5 Cu

* Numerals refer to nutrient level used.

TABLE VI

TREATMENT SYMBOL, SOURCE AND NUTRIENT LEVELS USED IN THE FIRST TWO PRELIMINARY STUDIES

Symbol	Source	Nutrient Level (ppm)
P	$CaH_4(PO_4)_2 \cdot H_2O$	200 P
KCl	KCl	400 K
KS	K_2SO_4	400 K - 328.2 S
NaS	Na_2SO_4	328.2 S
S	S flower	328.2 S
Ca	$CaCO_3$	1000 Ca
B	$Na_2B_4O_7 \cdot 2H_2O$	5 Mo
Zn	$ZnSO_4 \cdot 7H_2O$	50 Zn
Cu	$CuSO_4 \cdot 5H_2O$	50 Cu

as used in the preliminary studies and then planted with inoculated uniform Copada seedlings. Twice a week, between the ages of 24 and 67 days, six randomly selected plants were harvested. To minimize the daily short term pattern effect of variable carbohydrate supply to the nodules on N fixation, harvesting was always performed in the morning. Between 8 and 10 am, the plants were brought from the greenhouse to the Soil Microbiology Laboratory, detopped, the nodulated root system gently shaken free of soil, briefly washed for further removal of soil particles, blotted with paper towel to remove excess water, and then incubated in serum cap bottles for nitrogenase activity

(C_2H_2 reduction) determination (258). After determinations of C_2H_2 reduction, the nodules were picked from the roots, counted and weighed. Shoot and root dry weights were obtained after oven drying at 105°C for 24 hours.

For each harvesting (age) picked nodules were separated into four size classes (ST - small non-striped nodules, SL - small striped, MD - medium striped, and LG - large striped nodules) (205) and after preparation of the cell free extract (nodule cytosol fraction) the following determinations were made: αKG, GDH, GS, GOGAT, GOT, GPT, PLP, soluble protein, glucose, sucrose, and starch. A detailed description of these determinations is presented within the Enzyme Activity and Carbohydrate Determinations section at the end of this chapter.

Soil Fertility Effects on Plant Growth, Nodulation and Nodule Physiology

In this experiment the anions P and S, and the cations Ca, Mg, and K were arranged in a complete 2^5 factorial structure, giving rise to 32 possible treatment combinations. Pot preparation, seedling transplanting and inoculation were performed as before. The treatments tested and the nutrient sources and levels used appear in Table VII and VIII, respectively.

Since the P source was $NH_4H_2PO_4$ the treatment combinations that did not receive P were balanced with NH_4Cl, so that all treatments received the same amount of N as ammonium ion. The levels of Ca, Mg, and K were selected to give a base cation ratio (BCR) equal to one.

TABLE VII

TREATMENT COMBINATIONS FOR 2^5 FACTORIAL EXPERIMENT

Treatment	Symbol	Treatment	Symbol
1	0	17	PKCa
2	P	18	PKMg
3	K	19	PKS
4	Ca	20	PCaMg
5	Mg	21	PCaS
6	S	22	PMgS
7	PK	23	KCaMg
8	PCa	24	KCaS
9	PMg	25	KMgS
10	PS	26	CaMgS
11	KCa	27	PKCaMg
12	KMg	28	PKCaS
13	KS	29	PKMgS
14	CaMg	30	PCaMgS
15	CaS	31	KCaMgS
16	Mg	32	PKCaMgS

TABLE VIII

SOURCE AND NUTRIENT LEVELS FOR 2^5 FACTORIAL EXPERIMENT

Nutrient	Source	Level
Phosphorus	$NH_4H_2PO_4$	50 ppm
Sulfur	Na_2SO_4	50 ppm
Calcium	$CaCO_3$	6 meq/100 g soil
Magnesium	$MgCl_2 6H_2O$	2 meq/100 g soil
Potassium	KCl	2 meq/100 g soil

Four clippings of the top were obtained before collection of the nodulated root system. The same determinations indicated in the third experiment were also performed in this one.

Enzyme Activity and Carbohydrate Determinations

The C_2H_2-C_2H_4 reduction assay as proposed by Hardy et al. (100) was used for determination of nitrogenase activity. Because the rate C_2H_2 red/NH_3 is not known for Copada, and theoretical values may not be adequate, results are generally presented as C_2H_2 reduction but discussed as a measure of nitrogenase activity.

For the C_2H_2 reduction determination, the entire nodule-root system was placed in serum cap bottles, incubated with 0.1 atm of C_2H_2 (lab. spec. purified grade, Linde Div. Union Carbide, Inc.) at 27°C, and C_2H_4 production was detected with a Perkin-Elmer GC 3920 with

1.83 m x 3.2 mm Poropak N 80/100 column (Walters Assoc.). The ethylene standard utilized for calibration and monitoring of gas chromatography analysis was the Scott Ev. Tech. 1090 ppm $\pm$ 5% C_2H_4/N_2 (Supelco, Inc.). Results were then calculated as production of μ moles C_2H_4/g fresh nodule weight/hr and also as nitrogenase specific activity as μ moles C_2H_4/mg protein/min.

After the C_2H_2 reduction analysis, the nodules were picked from the roots, counted and weighed. All other determinations were performed in the plant fraction of the nodule tissue, where the bacteroids had been removed by centrifugation.

These cell-free nodule extracts - termed cytosol - were obtained, by slight modifications of the methods described by Grimes and Fottrell (96), and by Brown and Dillworth (37).

Samples of freshly picked nodules were placed in glass tubes at 0-5°C, and then crushed with a pestile in the buffer solution. For each gram of nodule in the glass, 10 ml of 0.1 M phosphate buffer, pH 7.41 were added before crushing. The nodule homogenate was then subjected to ultrasonication at 7.3 pulse frequency in an ice bath for 30 sec using a Polytron PT 10 OD (Kinematic GMBH, Luzern-Schwirz-imported by Brinkman Inst.), followed by refrigerated centrifugation at 12 x 10^3 g for 10 minutes. This clear, cell-free nodule extract (cytosol) was aseptically transferred to sterile culture tubes and stored at 0-5°C. Aliquots of this extract were used for the enzymes, carbohydrates, αKG, PLP's, and soluble protein analysis, as described below. Unused portions of the extract were lyophilized when all analysis were completed.

Enzyme activities determined in the nodule cytosol extract were

expressed as International Units (U), and defined as the amount of enzyme which causes transformation of 1.0 μ mole of specific substrate per minute (147) determined in 3.0 ml of reaction volume, 1 cm light path, at 27°C (218). The enzyme activity for each fertility combination was first calculated in terms of U/g fresh nodule, and enzymatic specific activity was calculated in terms of U/mg protein.

All enzymatic activity determinations except glutamine synthetase, were carried out in 80 μM phosphate buffer, pH 7.41, and the oxidation of cofactor NADH to NAD was spectrophotometricaly monitored at λ=340 nm for 14 minutes. The extinction coefficient for NADH at 340 nm was used as 6.22 cm^2/micromole.

Glutamate dehydrogenase activity was determined as described by Schmidt (232). The reaction and assay medium are summarized below:

$$\alpha KG\ (13\ \mu\ mol/ml) + NADH\ (0.14\ \mu\ mol/ml + (NH_4)_2\ SO_4\ (5\ \mu g/ml) \underset{}{\overset{GDH}{\rightleftharpoons}}$$
$$glutamate + NAD + H_2O. \qquad (1)$$

The biosynthetic activity of glutamine synthetase was determined as described by Shapiro and Stadtman (231) and the assay medium is characterized below:

glutamate (1.0 μ mol/ml) + NADH (0.14 μ mol/ml) + Phosphoenlpyruvate (6 μ mol/ml) + ATP - glutathione (68 μ mol/ml) + $(NH_4)_2\ SO_4$ (5 μg/ml)

$$\xrightarrow[\text{LDH}\ \text{PK}]{\text{GS}} \text{glutamine} + NAD + ADP + P_i \qquad (2)$$

The coupling enzymes, lactate dehydrogenase (LDH) (20.0 U/ml) and pyruvate Kinase (PK) (14.0 U/ml), were used to shift the equilibrium of the reaction towards the synthesis of glutamine. The reaction was carried out in a 0.1 M (Mg) Tris buffer solution at pH 9.0.

Glutamate synthase was assayed as described by Ryan and Fottrell (218):

glutamine (1.0%) + αKG (13 μ mol/ml) + NADH (0.14 μ mol/ml)

GOGAT

2 Glutamate + NAD. (3)

The procedure described by Bergmeyer and Bernt (21) was used for determination of the glutamate-oxaloacetate transaminase activity. The two reactions used in this procedure were:

Aspartate (33 μ mol/ml) + αKG (13 μ mol/ml) $\overset{GOT}{\rightleftharpoons}$ Oxaloacetate + glutamate (4)

Oxaloacetate was then reduced to malate, in a reaction catalysed by malic dehydrogenase (MDH) (0.5 U/ml).

Oxaloacetate + NADH (0.14 μ mol/ml) $\overset{MDH}{\rightleftharpoons}$ malate + NAD (5)

The Bergmeyer and Bernt (22) procedure was also employed for determinations of glutamate-pyruvate transminase activity:

Alamine (33 μ mol/ml) + αKG (13 μ mol/ml) $\overset{GPT}{\rightleftharpoons}$ pyruvate + glutamate (6)

Pyruvate was then reduced to lactate, using the enzyme lactic dehydrogenase (LDH) (0.5 U/ml):

Pyruvate + NADH (0.14 μ mol/ml) $\overset{LDH}{\rightleftharpoons}$ Lactate + NAD (7)

The tricarboxylic acid cycle intermediate, alpha ketoglutarate was determined as proposed by Bergmeyer and Bernt (23):

2-oxoglutarate + NADH (0.14 μ mol/ml) + $(NH_4)_2\ SO_4$ (5 μg/ml) $\overset{GDH}{\rightleftharpoons}$ glutamate + NAD + H_2O . (8)

To catalyse the above reaction 1.8 U/ml of GDH were used in the assay medium.

The pyridoxyl phosphates were assayed in a medium containing 0.2 M Na-borate buffer at pH 9.0 following the method described by

Johnson and Metzler (125). Pyridoxal-5-phosphate and pyridoxamine-5-phosophate were first excited at λ=348 nm in a Turner Model III UV fluorometer, with the emitted fluorescence being read at λ=436 nm.

The nodule cytosol water soluble protein was determined using the method described by Lowry et al. (157), using the blood serum albumin as standard.

$$\text{Copper}^{++} + \text{nodule cytosol proteins} \xrightarrow{\text{NaOH}} \text{Copper protein complexes (purple)} \quad (9)$$

Development of the purple color was monitored at λ=750 nm, and soluble protein was calculated as mg protein/g fresh nodule, and then transformed to percent protein.

Glucose levels were also determined in the phosphate buffer at pH 7.41using the procedure described by Bergmeyer and Bernt (24).

$$\text{Glucose} + H_2O + O_2 \xrightarrow[(100\ \text{PGO U/ml})]{\text{Glucose oxidase}} \text{gluconate} + H_2O_2 \quad (10)$$

$$H_2O_2 + \underset{(\text{reduced})}{\text{O-dianisidine}} \xrightarrow[(75\ \text{U/ml})]{\text{Peroxidase}} \text{oxidized dianisidine} \quad (11)$$

Oxidation of reduced dianisidine was monitored at λ=450 nm.

The determination of sucrose was carried out in 0.1 M citric acid buffer solution at pH 4.6 as described by Bergmeyer and Bernt (25). Sucrose was hydrolysed to fructose and glucose by invertase, and glucose levels were then determined by the PGO method described above.

$$\text{Sucrose} \xrightarrow[(75.0\ \text{U/ml})]{\text{Invertase}} \text{fructose} + \text{glucose} \quad (12)$$

The method of Keppler and Decker (133) was used for starch determinations, in a 0.1 M citric acid buffer solution at pH 4.8. The enzyme amyloglucosidase was used to hydrolyse starch into glucose and

the latter was then determined by the PGO method.

$$\text{Starch} \xrightarrow[(150\ U/ml)]{\text{amyloglucosidase}} \text{glucose} \qquad (13)$$

Levels of glucose, sucrose and starch were expressed as mg/g fresh nodule.

CHAPTER IV

RESULTS AND DISCUSSION

Preliminary Fertility Studies

Results obtained for total dry herbage yield (5 clippings) and root dry weight of the first two preliminary fertility studies are presented in Tables IX and X.

Table IX shows that when used singly P, S and Ca increased Copada herbage yield. The KCl treatment yield was less than the check, and apparently these four nutrients interact among themselves to influence herbage yield. The combination of K and S significantly improved the K effect, and highest dry matter production was obtained with the P + KS combination. In this experiment, less root dry matter was also produced when the soil was fertilized with KCl, although its effect was not significantly different from the check and the KS and NaS treatments. Calcium apparently favored root development in this experiment as shown by increased production of root dry matter.

It is apparent from these results that forage production can be improved by exploring the possible interaction effects among P and the other nutrients.

A first approach to this research line was made in the second preliminary study, where the P + KS effect was separated into P + KCl

TABLE IX

TOTAL DRY HERBAGE YIELD (5 CLIPPINGS) AND ROOT DRY WEIGHT OF FIRST PRELIMINARY FERTILITY STUDY

Treatment Symbol	Total Dry Herbage Yield g (5 clippings)		Root Dry Wt g	
P + KS	5.40*	a**	1.16	ab
P	4.05	b	1.02	abc
S	3.75	b	1.13	ab
NaS	3.65	bc	0.75	bcd
Ca	3.50	bc	1.27	a
KS	3.40	c	0.62	cd
Check	2.90	c	0.62	cd
KCl	2.00	d	0.50	d

*Figures are means of 3 replications.

**Figures with different letter differ significantly (P=0.05).

TABLE X

TOTAL DRY HERBAGE YIELD (5 CLIPPINGS) AND ROOT DRY WEIGHT OF SECOND PRELIMINARY FERTILITY STUDY

Treatment Symbol	Total Dry Herbage Yield g (5 clippings)		Root Dry Wt g	
P + KS	10.40*	a**	1.77	a
P + KS + 1B + 1Mo + 1Zn + 1Cu	7.80	b	1.52	ab
P + KS + 5B + 5Mo + 5Zn + 5Cu	***		-	
P + KCl	6.35	c	1.20	bc
P	3.90	d	0.86	cd
P + NaS	3.85	d	0.98	cd
Check	2.00	e	0.57	d

*Figures are means of 3 replications.

**Figures with different letter differ significantly (P=0.05).

***Seedlings did not grow, and died within several days.

and P + NaS. An attempt to find response to B, Mo, Zn, and Cu fertilization was also made. The data obtained in this experiment are summarized in Table X.

These results indicated that both dry herbage yield and the root dry matter of Copada were not improved by the micronutrient mixture. A highly toxic effect was observed for the highest level of these nutrients, killing the seedlings after transplanting to the experimental pots. These results are apparently similar to earlier observations that in this dark red latosol, crop yields are not improved by micronutrient fertilization (202, 204).

As in the first experiment highest dry herbage yield was obtained by the P + KS treatment. The P + KCl treatment yielded better than P + NaS combination, possibly indicating that although singly S yields better than K, when in combination, K interacts more strongly with P than S. This prominent P effect on yield was also observed in the winged bean experiments (206) when grown in this latosol.

Effect of Plant Age on Nodulation and Nodule Physiology

Experimental results for this study are presented in Figures 6 to 21. During harvesting it was possible to distinguish three sizes and two types of nodules. Many of the small nodules (< 1 mm) (SL) were not striped and are referred to as ST. The non-striping characteristic was only observed in nodules having their largest dimension smaller than 1 mm. Because both medium sized (1 < MD < 3 mm) and large nodules (LG > 3 mm) were always striped, this characteristic can be presumed to be in some way related to nodule maturity and effectiveness.

Plants were collected when they were 24, 26, 30, 32, 37, 39, 44, 46, 54, 57, 60, and 67 days old. The dry matter data obtained indicated that little shoot and root growth was realized during the first 39 days of plant growth. However, both shoot and root dry matter production more than doubled between 39 and 46 days, and a steady rate of growth was then maintained until the end of the experiment at 67 day age.

Figure 6 shows the effect of plant age on the total weight of the freshly harvested nodules. The weight of the nodules increased ($P \leq 0.001$) linearly with age and the following linear equation was obtained to describe this trend:

$$\text{Nod wt.} = -0.584 + 0.024 \text{ day}; \; r = 0.97 \qquad (14)$$

However, although the total number of nodules (Fig. 7) was also influenced by age ($P \leq 0.05$), this trend is only poorly predicted by a linear equation:

$$\text{T NOD} = 43.61 + 0.612 \text{ day}; \; r = 0.43 \qquad (15)$$

Apparently the number of nodules smaller than 1 mm (ST + SL) (Fig. 8) increased up to around day 39, and then remained low between 44 and 67 day age. The following linear model describes this age effect ($P \leq 0.001$) on the number of small nodules:

$$\text{S NOD} = 75.59 - 1.01 \text{ day}; \; r = -0.78 \qquad (16)$$

Figure 9 shows the plant age effect on the number of nodules larger than 1 mm (MB + LG). A highly significant ($P \leq 0.001$) effect was observed, and the number of these nodules increased with age:

$$\text{MDLG} = -30.63 + 1.58 \text{ day}; \; r = 0.87 \qquad (17)$$

The nitrogenase activity of the entire root-nodule system (Fig. 10) was also affected by plant age ($P \leq 0.001$), apparently increasing on a linear fashion:

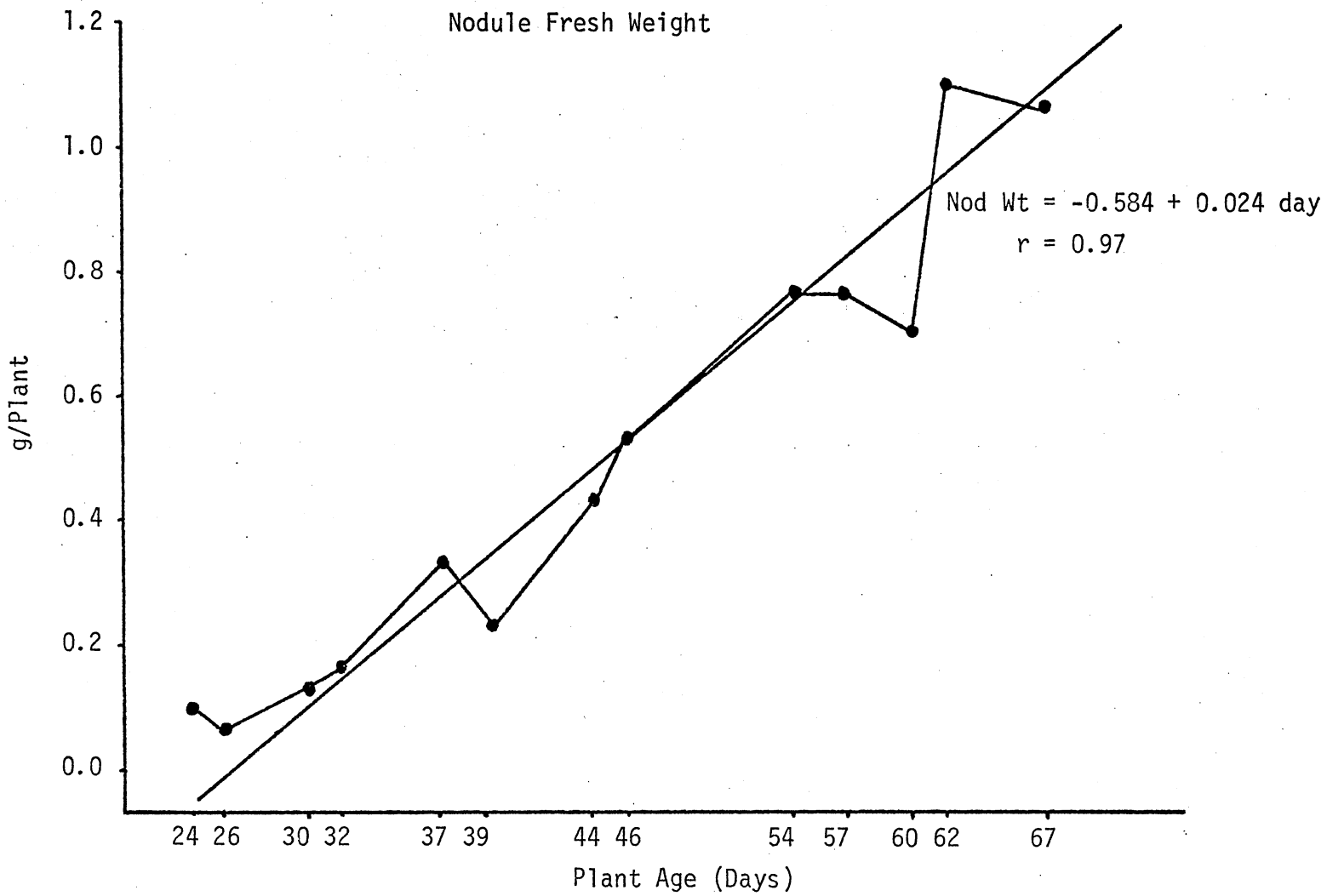


Figure 6. Effect of Plant Age on Nodule Fresh Weight of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

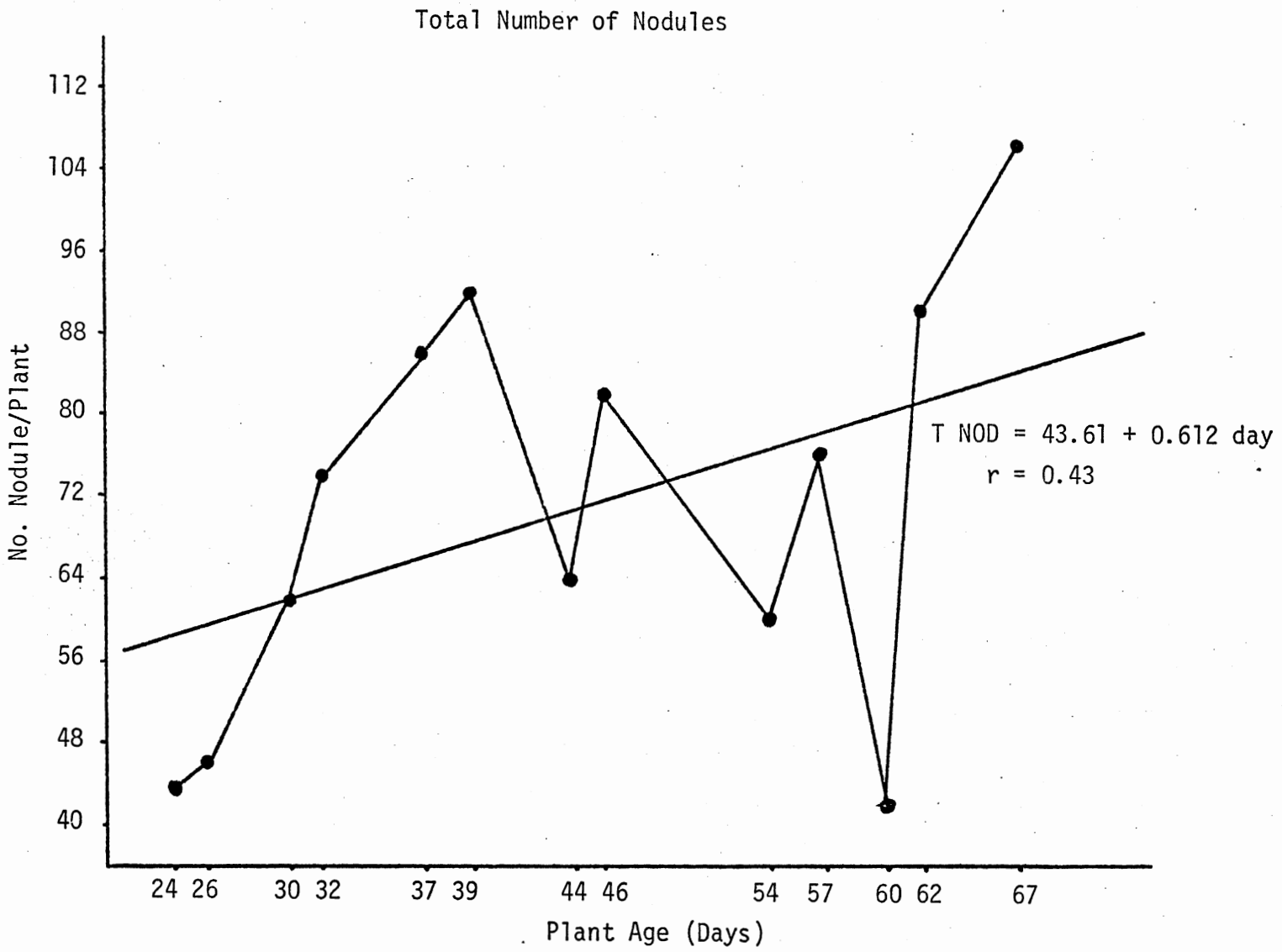


Figure 7. Effect of Plant Age on the Number of Nodules of Cratylia floribunda, Grown on a Dark Red Latosol, Brazil

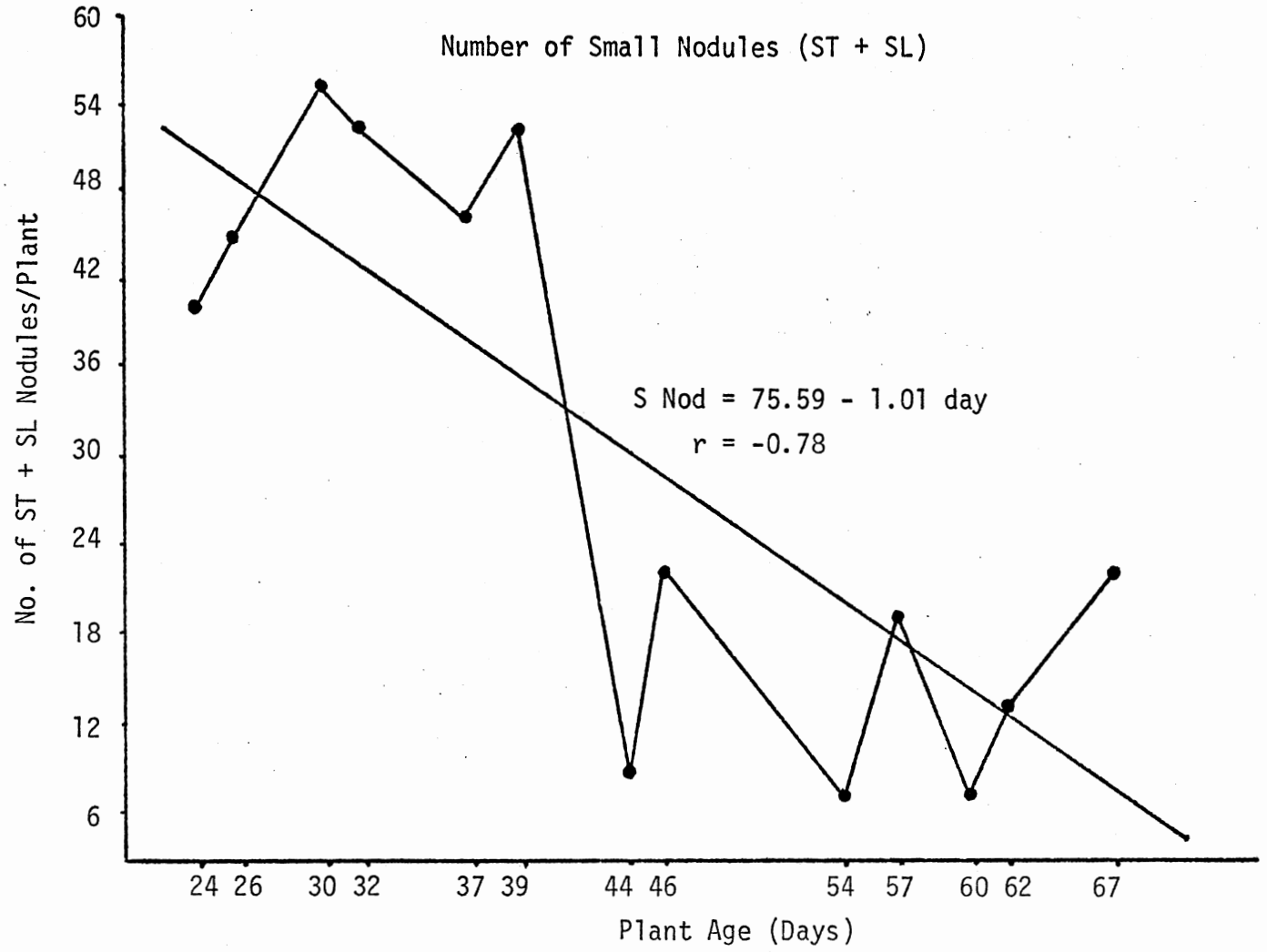


Figure 8. Effect of Plant Age on Number of Small Nodules (ST + SL) of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

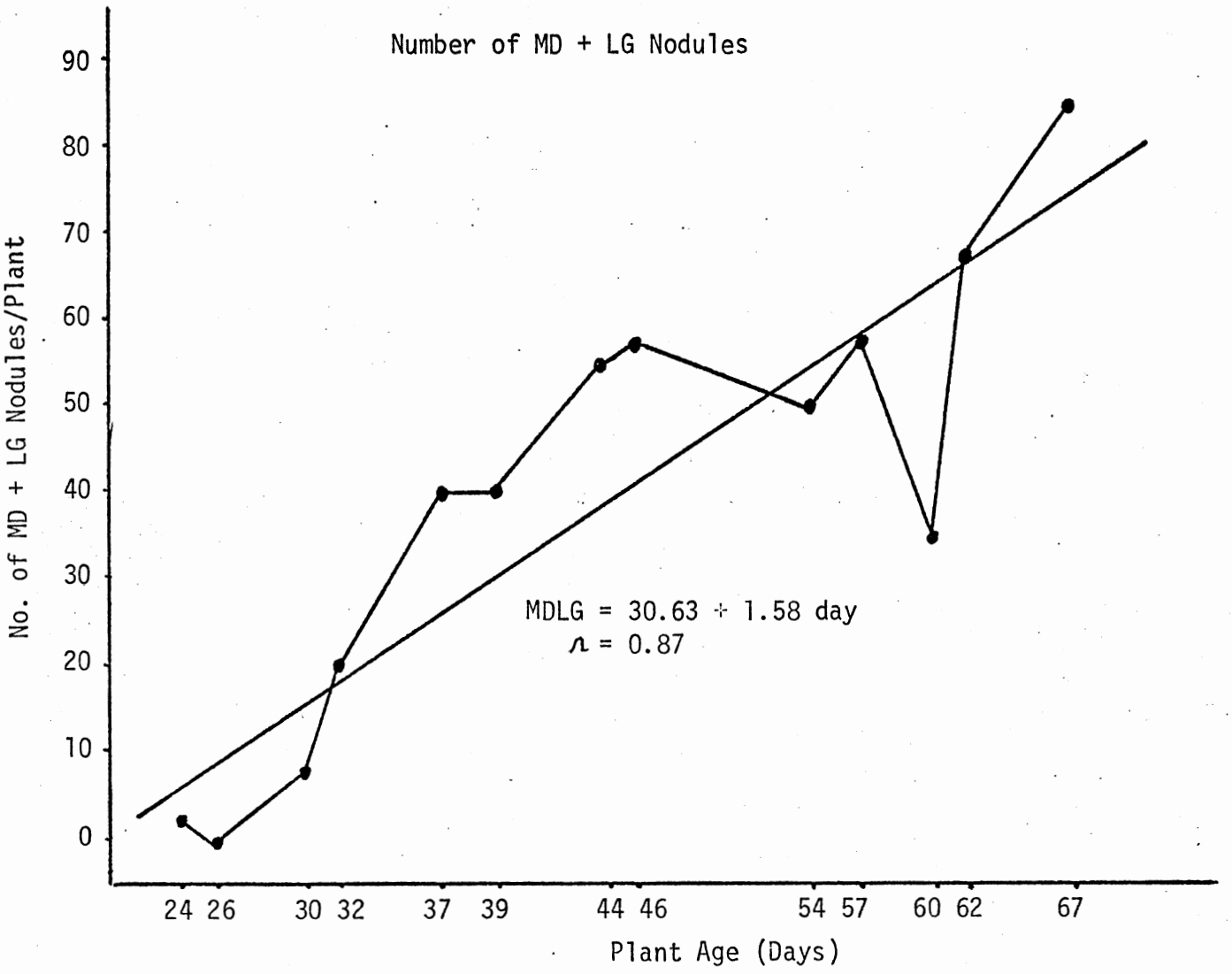


Figure 9. Effect of Plant Age on Number of Medium and Large Nodules (MD + LG) of Cratylia floribunda, Grown on a Dark Red Latosol, Brazil

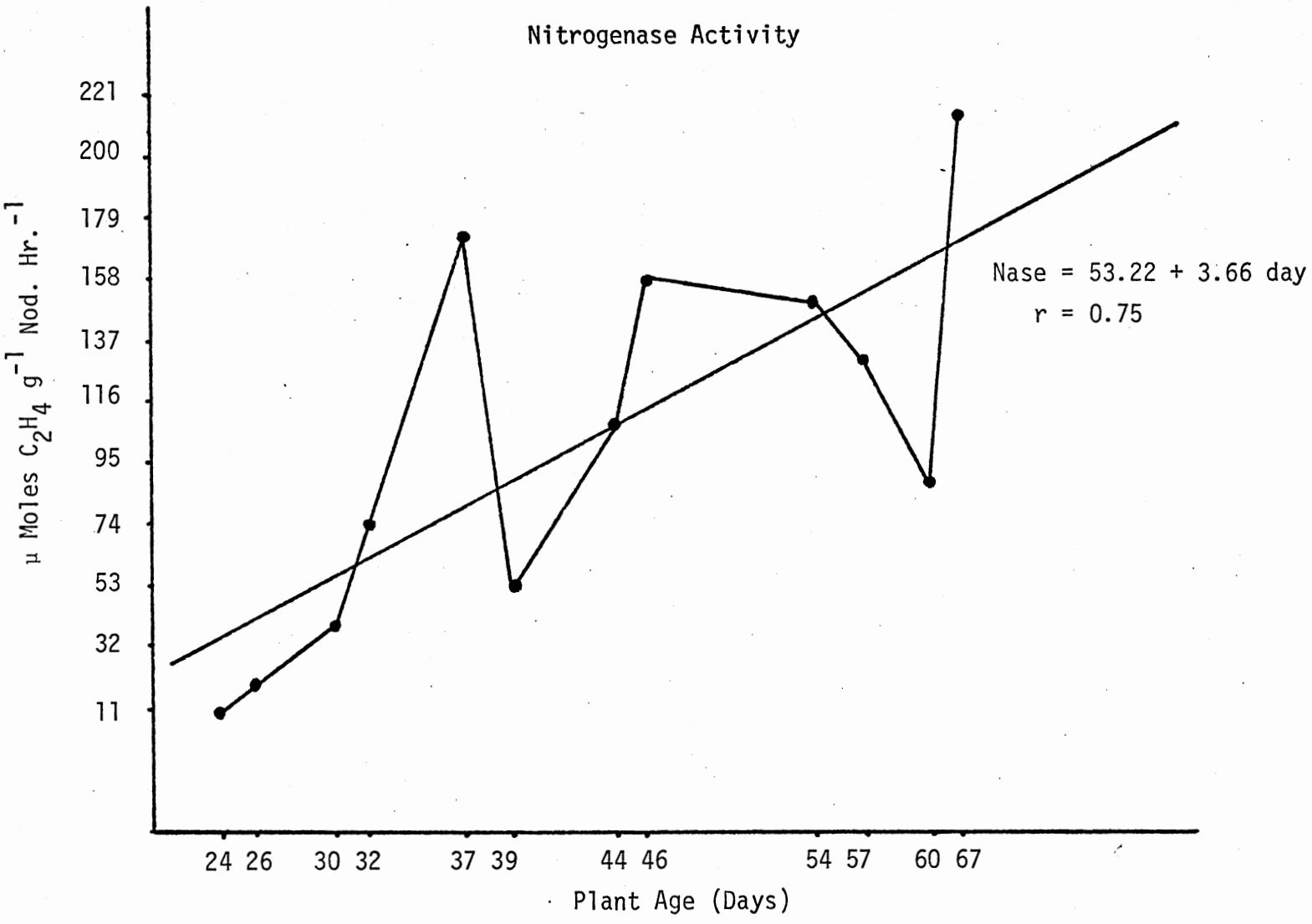


Figure 10. Effect of Plant Age on Nitrogenase Activity (C_2H_2 Red) of Cratylia floribunda Grown on a Dark Red Latosol, Brazil[2]

Nase = -53.22 + 3.66 day; r = 0.75 (18)

Using the data obtained in this experiment, six linear regression models were derived to relate nitrogenase with nodulation parameters. The following equations were obtained:

Nase = 34.94 + 151.55 NOD WT r^2=0.52*** (19)

Nase = 125.83 - 0.76 S NOD r^2=0.09** (20)

Nase = 34.95 + 1.86 MDLG r^2=0.54*** (21)

Nase = 41.26 + 0.89 T NOD r^2=0.16*** (22)

Nase = 4.07 + 139.27 NOD WT + 0.53 T NOD r^2=0.57*** (23)

Nase = 24.12 + 84.64 NOD WT + 1.13 MDLG r^2=0.62*** (24)

Although all equations were significant for $P<0.05$ or less, models (20) and (22) indicate that only 9% and 16% of the total variation in nitrogenase activity can be explained by the number of nodules smaller than 1 mm or by the total number of nodules/plant.

Equations (19) and (21) suggest that the variation in nitrogenase activity can be equally accounted for by the variations on nodule weight/plant or by the number of nodules larger than 1 mm. The coefficient of determination for these equations were r^2=0.52 and r^2=0.54 respectively.

A comparison of the coefficient of determination of equations (20) and (21) suggests that Copada nodules must have at least 1 mm in their largest dimension before they can have any significant nitrogenase activity. Thus, variations on nitrogenase activity on the pot cultures were more related to nodule growth than to the number of nodules present. Equations (23) and 24) indicate that Nase activity can be better predicted by a linear model including NOD WT and the number of nodules larger than 1 mm, than NOD WT and the total number of nodules.

Results similar to equation (24) have been reported by Dobereiner (69). In experiments carried out before the C_2H_2-C_2H_4 technique was available, she found that the total plant nitrogen in soybean and dry bean (Phaseolus vulgaris) was a function of the amount of nodule tissue formed. She plotted the log total plant N (mg/pot) x nodule weight (mg/pot) and concluded that when equations of the form

$$Y = a + bx \tag{25}$$

where derived, the slope of this line (b) was an estimation of efficiency of the symbiotic N fixation process.

Levels of alpha Ketoglutarate (Figure 11) on the nodule cytosol were influenced both by plant age and nodule size. Highest αKG levels were observed in non-striped small (ST) nodules, with the striped nodules (SL, MD, LG) having lower values. Apparently αKG levels tended to be higher at 39 days, and to reach a lowest value of 46 days.

Glutamate dehydrogenase (Figure 12) levels were affected by both plant age, nodule size and the interaction of these two factors. Although the highest observed GDH activity was in SM nodules at 46 days, high levels of activity were usually associated with MD nodules. Except for day 57, lowest GDH activities were always found in ST nodules.

Glutamine synthetase (Figure 13) activity was not influenced by plant age, but a significant effect ($P \leq 0.08$) was observed for nodule size. Highest GS activity was observed in MD nodules (8.66 U/g nod.), lowest in the ST nodules (4.10 U/g nod.) and intermediate values in the SL and LG ones (6.60 and 6.81 U/g nod. respectively).

Unlike GS, glutamate synthase (Figure 14) levels were affected ($P \leq 0.001$) by plant age, but not by nodule size. GOGAT activity peaked at 30, 39 and 57 days (3.35, 3.83 and 2.88 U/g nod. respectively), with

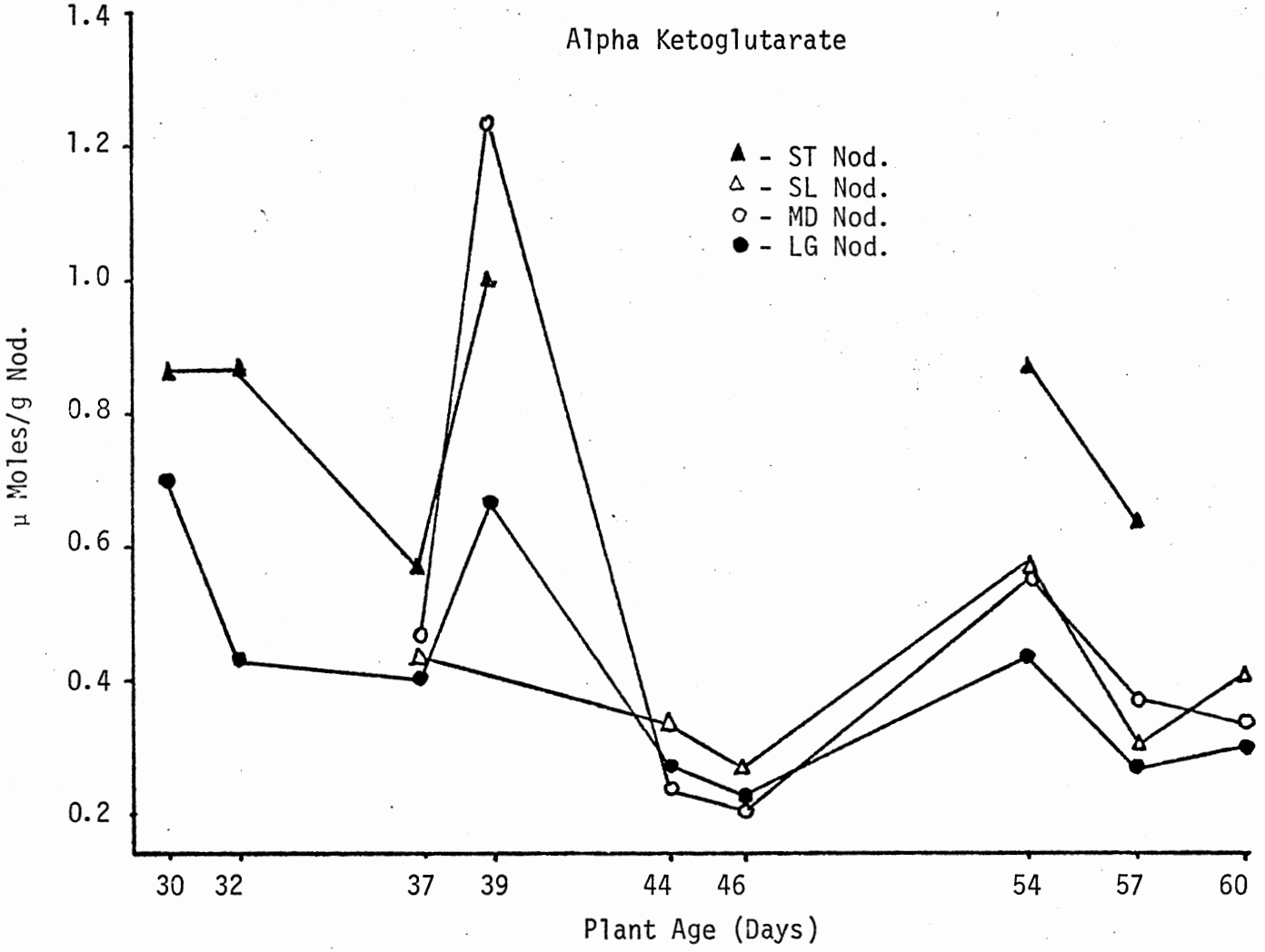


Figure 11. Effect of Plant Age and Nodule Size on Levels of Alpha Ketoglutarate (αKG) in the Nodule Cytosol of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

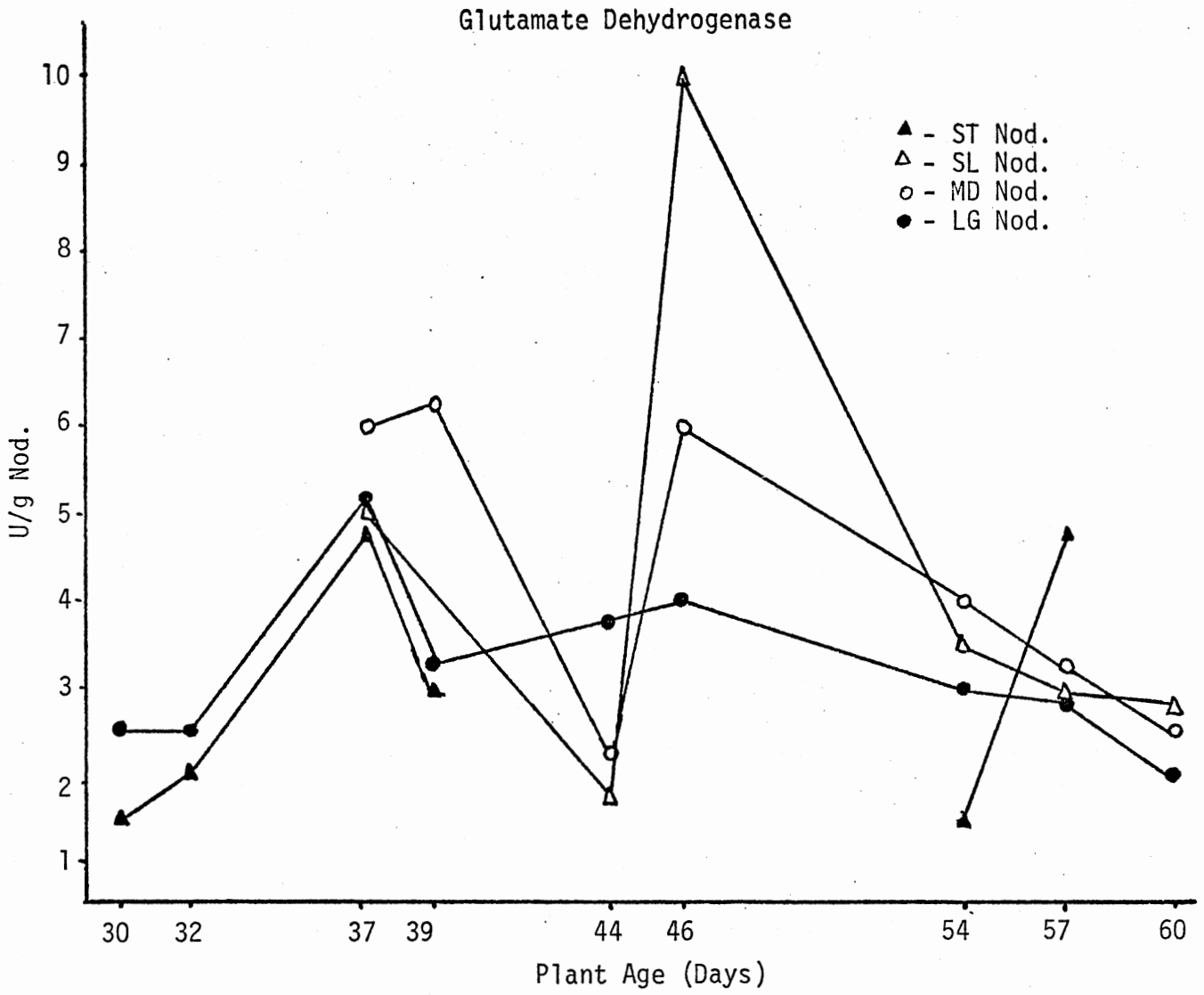


Figure 12. Effect of Plant Age and Nodule Size on Nodule Cytosol Glutamate Dehydrogenase (GDH) Activity of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

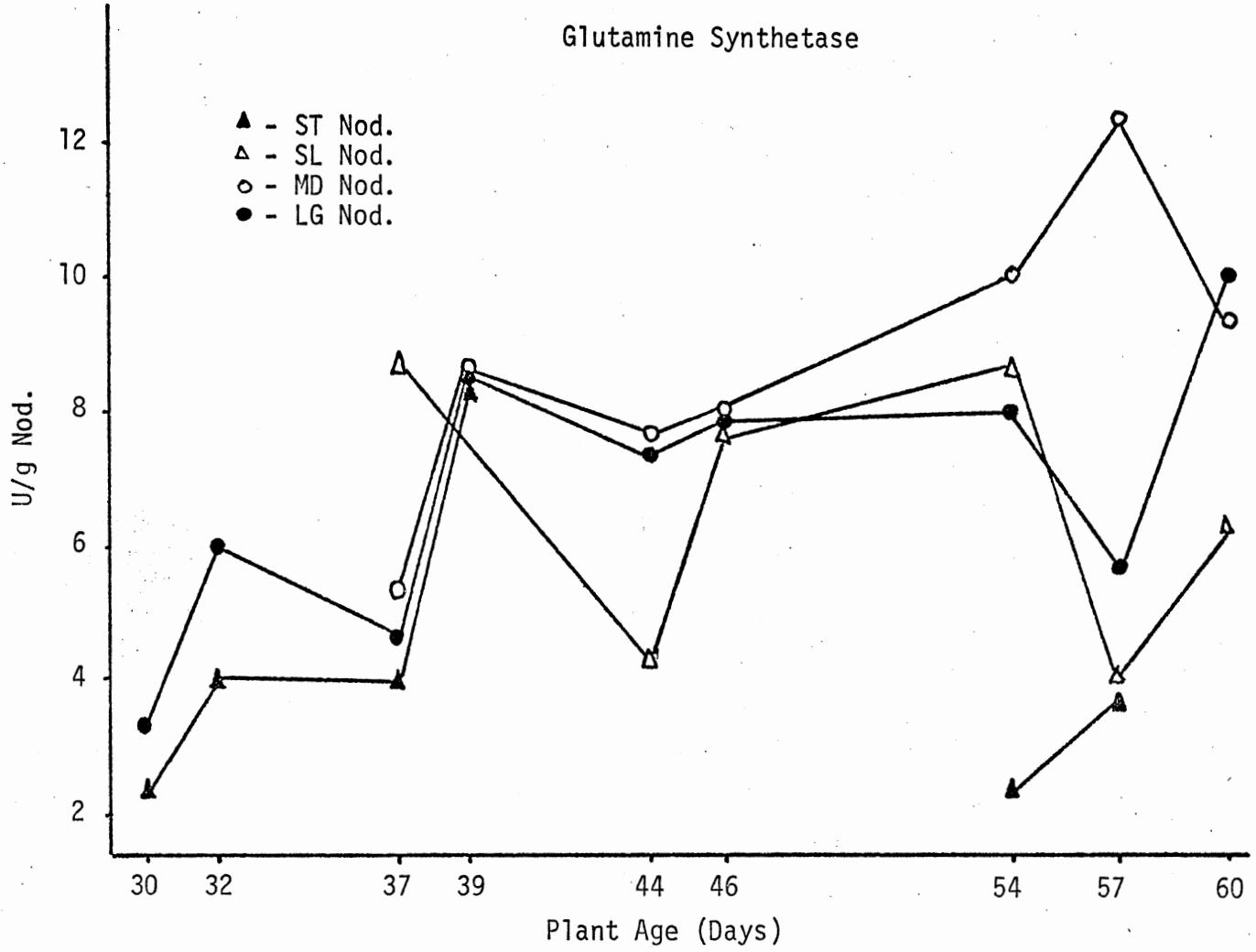


Figure 13. Effect of Plant Age and Nodule Size on Nodule Cytosol Glutamine Synthetase (GS) Activity of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

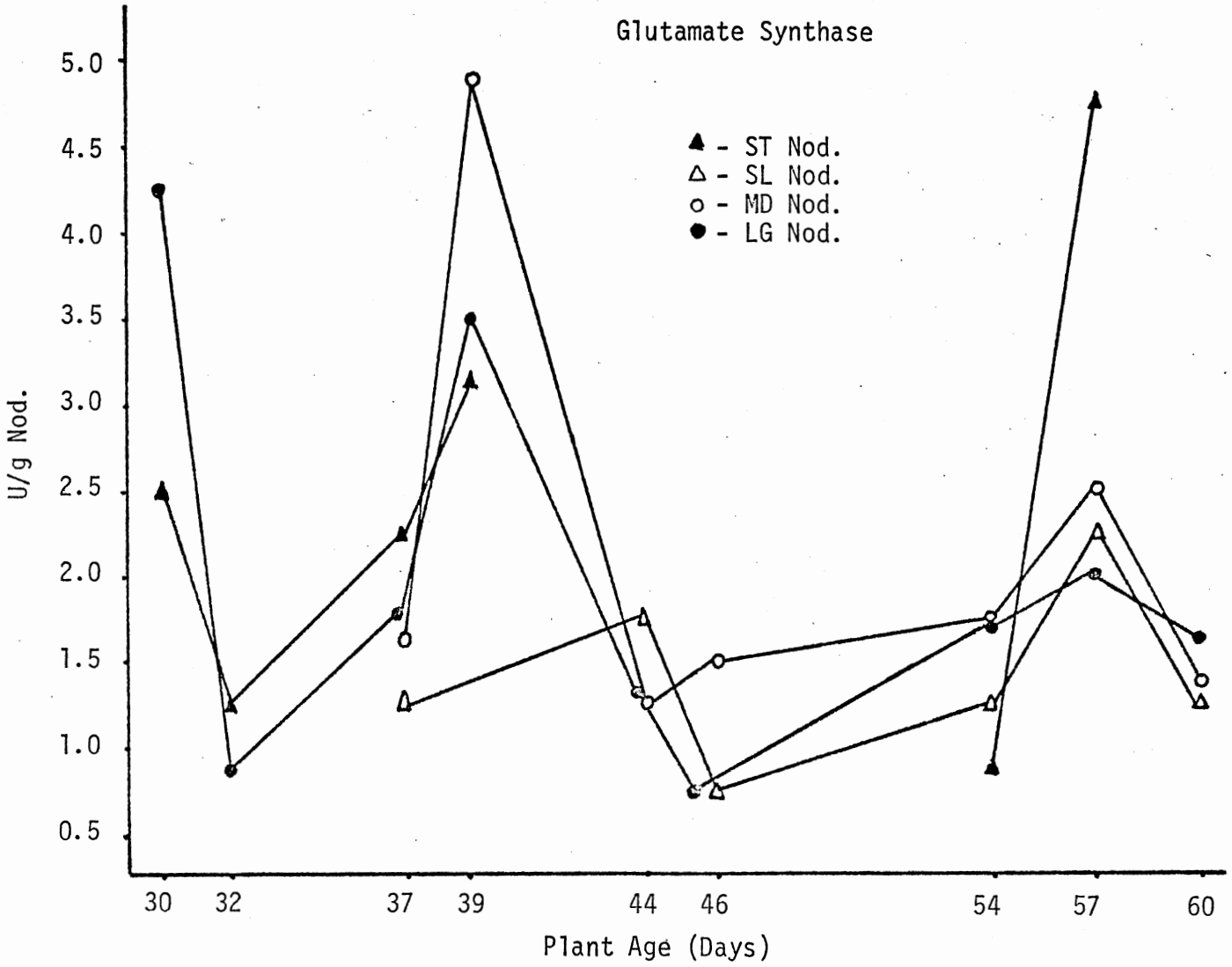


Figure 14. Effect of Plant Age and Nodule Size on Nodule Cytosol Glutamate Synthase (GOGAT) Activity of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

lower activity being observed in between.

Effects for plant age ($P \leq 0.05$) and nodule size ($P \leq 0.05$) were observed on glutamate-oxaloacetate transaminase (Figure 15) activity. GOT activity increased from 30 days (33.4 U/g nod.) to 44 days (45.7 U/g nod.) but then sharply decreased on day 46 (3.8 U/g nod.). After that an increasing trend was observed between 54 days (15.6 U/g nod.) and 60 days (27.7 U/g nod.).

With regards to nodule size, GOT activity was lowest in the ST nodules (17.7 U/g nod.), but linearly increased in the SL, MD and LG nodules (22.1, 30.8 and 33.2 U/g nod. respectively).

Figure 16 shows the data obtained for glutamate-pyruvate transaminase activity. Effects were observed for plant age ($P \leq 0.001$), nodule size ($P \leq 0.001$) and the age x size interaction ($P \leq 0.001$). For all nodule sizes, GPT activity was highest at day 39, and then in a manner very similar to GOT activity, decreased at age 44 days and remained low, until day 60.

Apparently the levels of pyridoxyl phosphates (Figure 17), essential coenzymes for GOT and GPT were not affected by either plant age or nodule size in this experiment.

Effects for plant age ($P \leq 0.1$) and nodule size ($P \leq 0.01$) were observed on the percentage of soluble protein (Figure 18) on the nodule cytosol of these Copada plants. Averaged over nodule size, protein content increased up to day 39 (2.69%) and then remained fairly unchanged from day 44 (2.13%) until day 60 (2.26%). Largest amounts of soluble protein were observed in MD nodules (2.52%) and lowest concentrations on the ST nodules (1.79%). Intermediate values were observed in the SL (2.36%) and LG (2.43%) nodules.

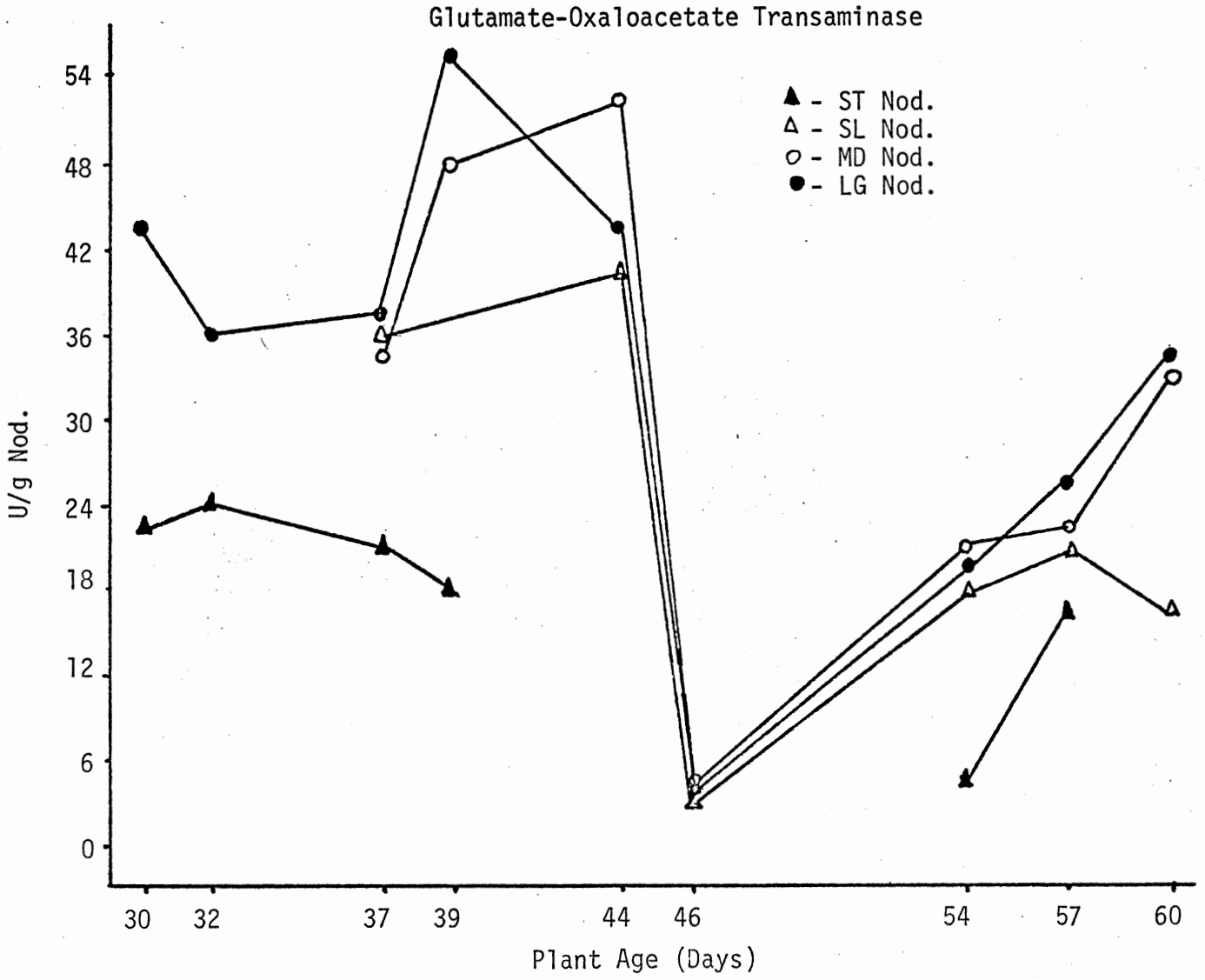


Figure 15. Effect of Plant Age and Nodule Size on Nodule Cytosol Glutamate-Oxaloacetate Transaminase (GOT) Activity of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

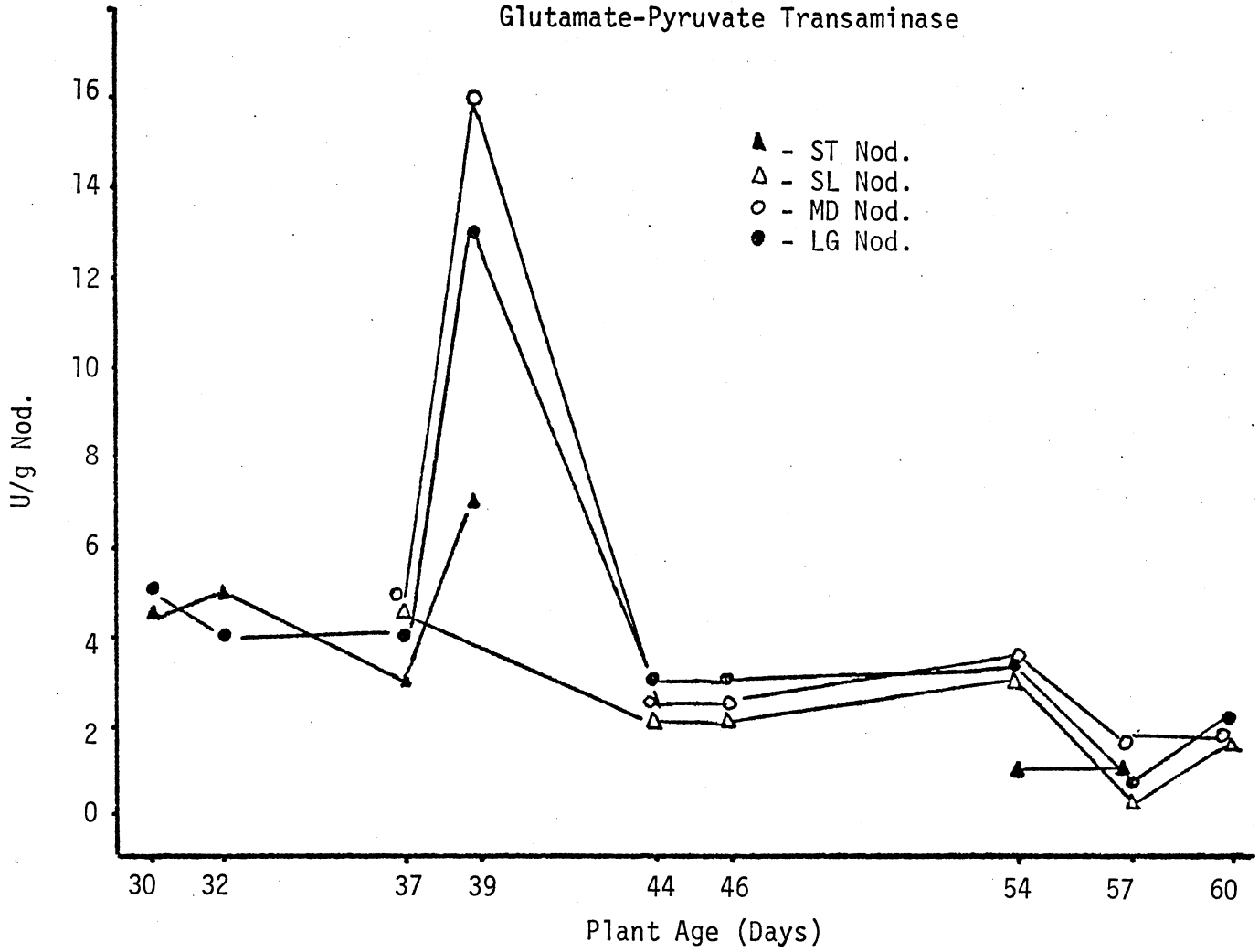


Figure 16. Effect of Plant Age and Nodule Size on Nodule Cytosol Glutamate-Pyruvate Transaminase (GPT) Activity of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

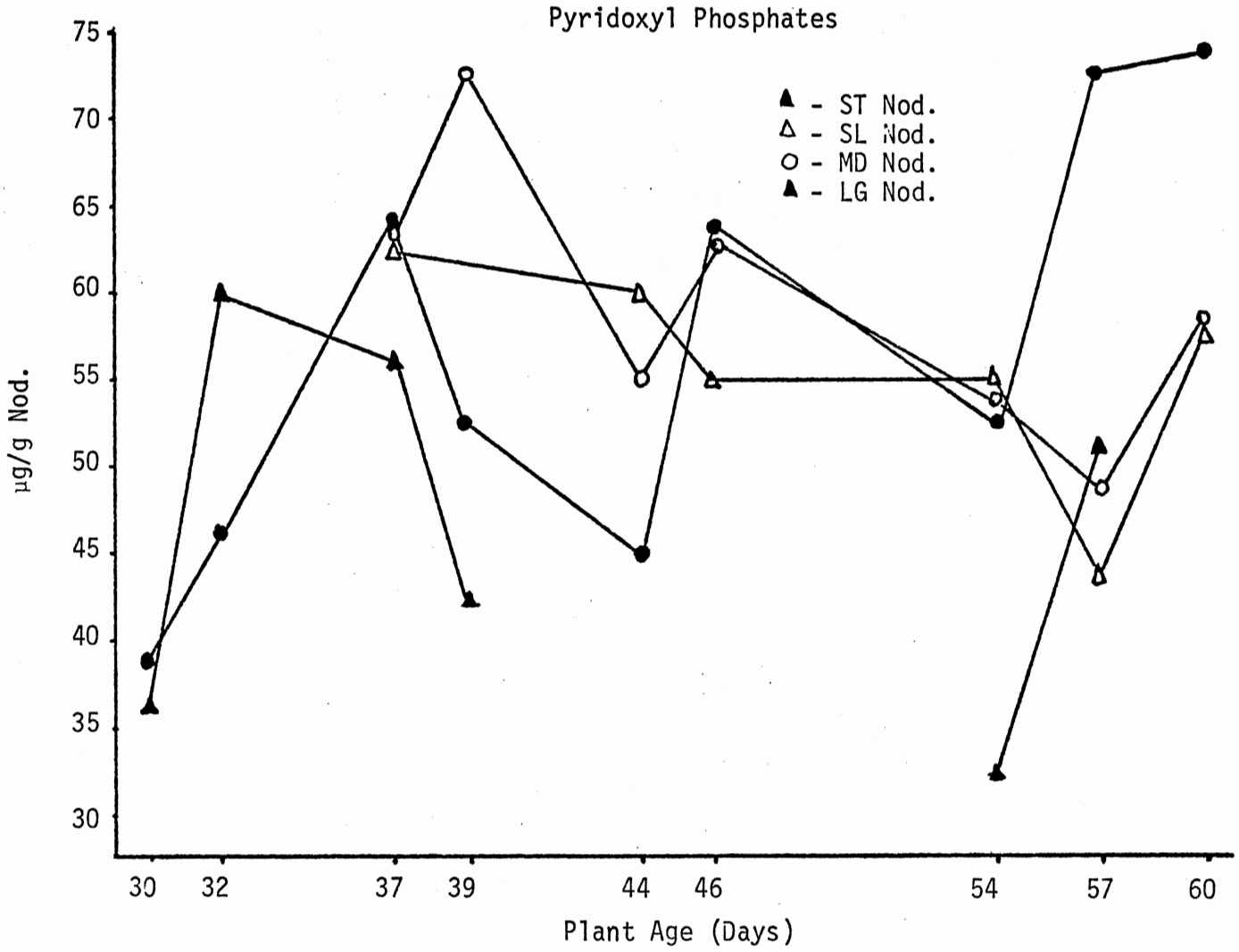


Figure 17. Effect of Plant Age and Nodule Size on Nodule Cytosol Pyridoxyl Phosphates (PLP's) Levels of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

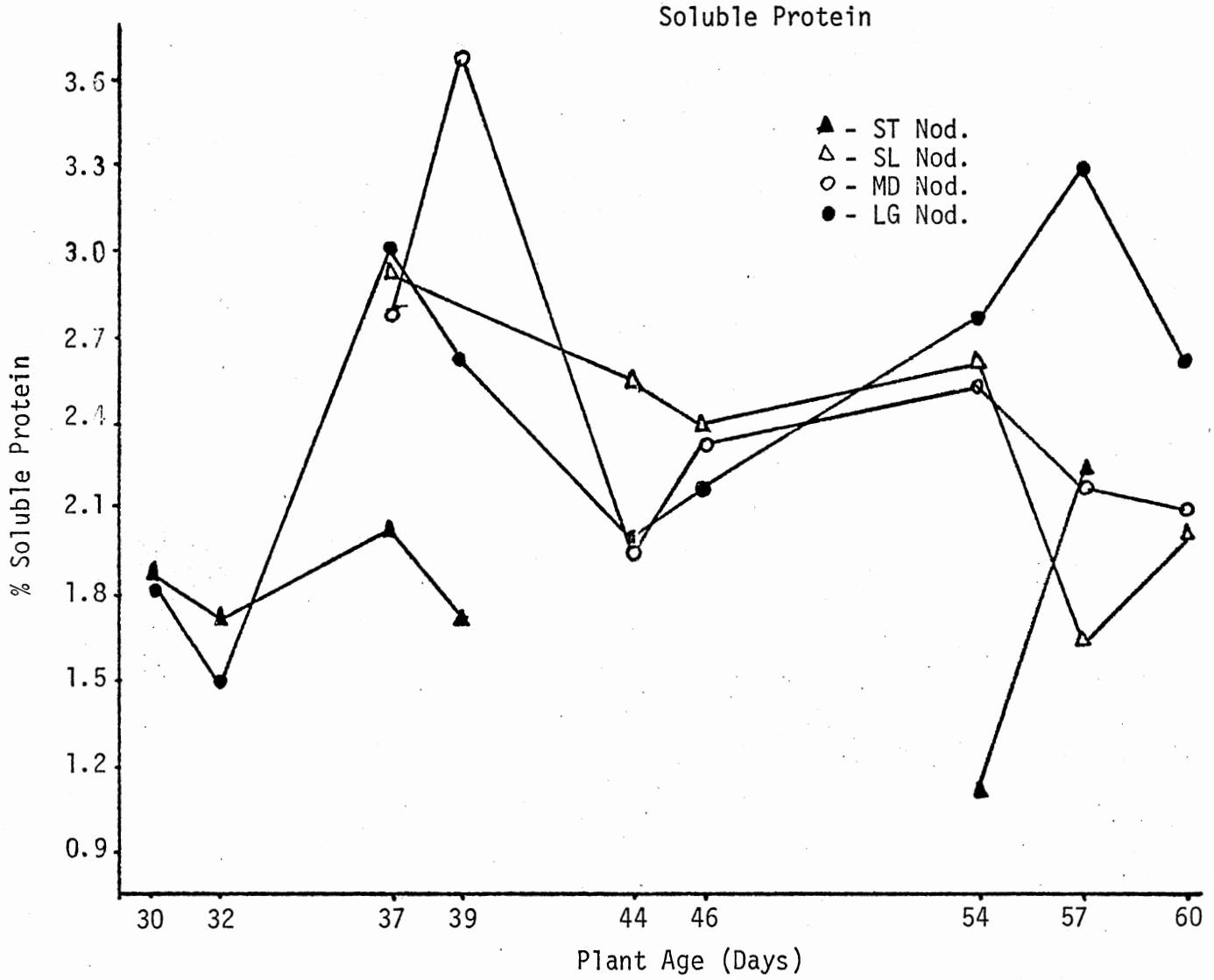


Figure 18. Effect of Plant Age and Nodule Size on Nodule Cytosol Soluble Protein (Prot.) Levels of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

Plant age ($P \leq 0.001$), nodule size ($P \leq 0.001$), and the age x size interaction ($P \leq 0.001$) influenced the glucose (Fig. 19) content of the nodules in this experiment. For the MD and LG nodules, glucose content tended to increase on day 39 and then steadily decrease to lower levels. A decreasing trend, without any intermediate peak, was also observed for the ST and SL nodules, although for the ST nodules glucose levels increased at day 57 to a level similar to that of day 32.

Figure 20 shows that increased ($P \leq 0.001$) sucrose content was detected within the ST nodules, but not much difference was observed among the SL, MD and LG nodule sizes. Although, the glucose content of the ST nodules showed a sharp peak at day 39, no plant age effect was observed on the sucrose content of the nodules. The age x size interaction was significant for $P \leq 0.01$.

In this experiment, the trends observed for nodule size and plant age on nodule starch content (Figure 21) were very similar. Significant effects were observed for nodule size ($P \leq 0.01$) and the interaction of age x size ($P \leq 0.05$). Apparently the ST nodules had a higher starch concentration than the SL, MD and LG nodules.

The data obtained in this experiment points to a marked difference on the physiological characteristics of the nodule sizes studied. The small, non-striped nodules, usually contained larger amounts of products related to photosynthesis, such as αKG, sucrose, and starch, but not glucose. The linear models derived to study nitrogenase activity within Copada nodules, suggested that these small nodules were not actively fixing atmospheric nitrogen and are perhaps a sink for photosynthesis assimilates.

Nodule growth was accompanied by induction of enzymes capable of

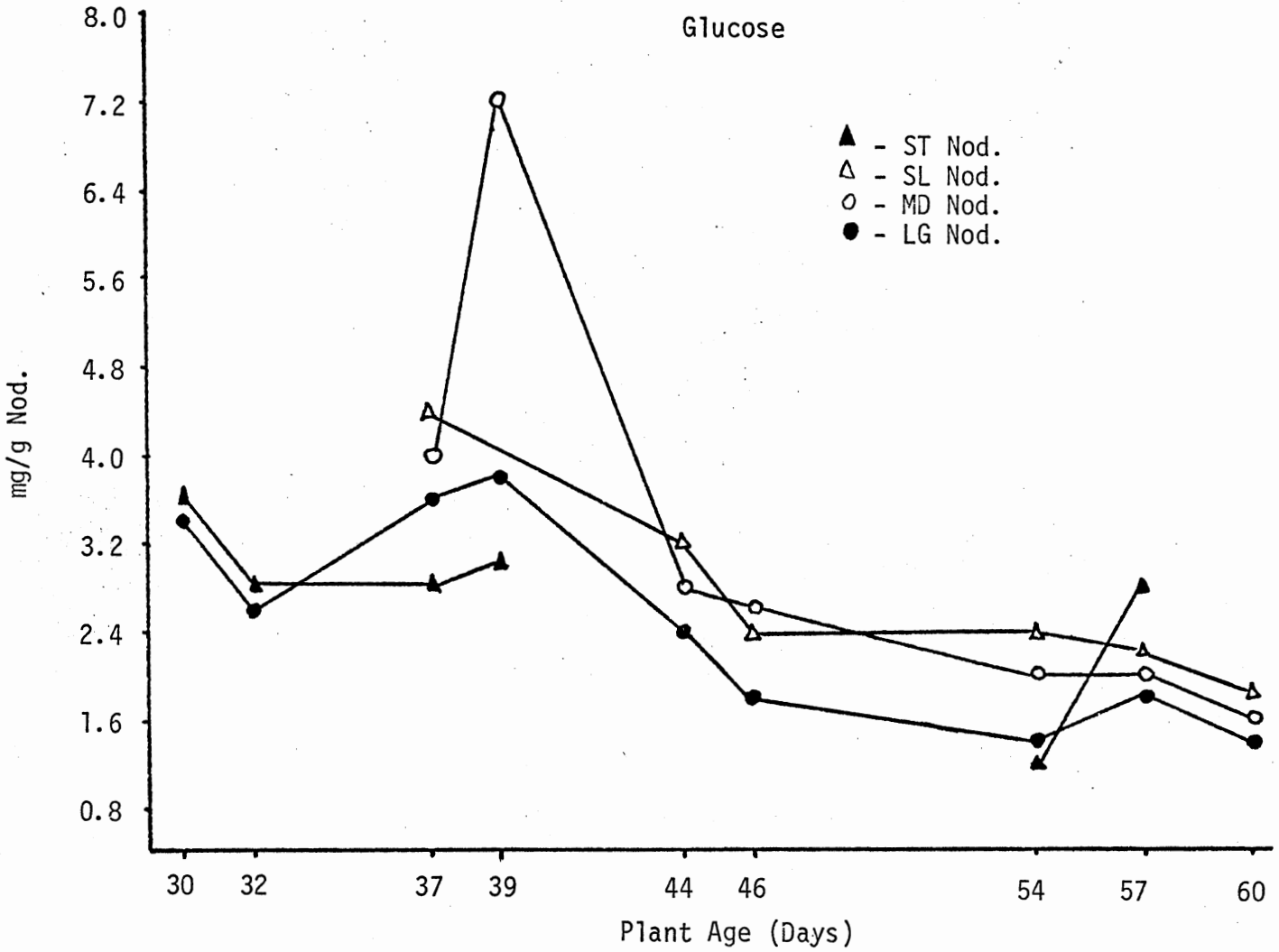


Figure 19. Effect of Plant Age and Nodule Size on Nodule Cytosol Glucose Levels of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

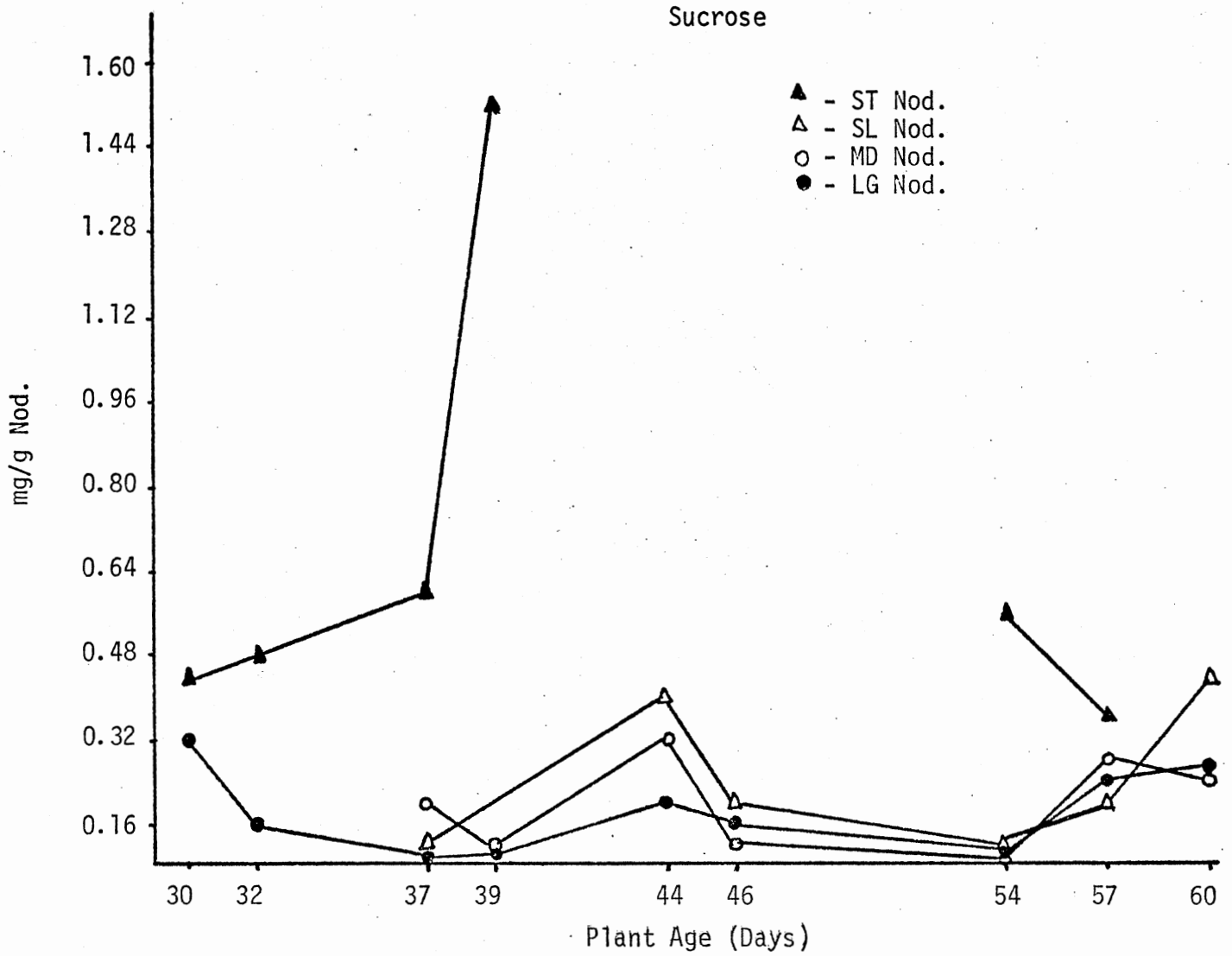


Figure 20. Effect of Plant Age and Nodule Size on Nodule Cytosol Sucrose Levels of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

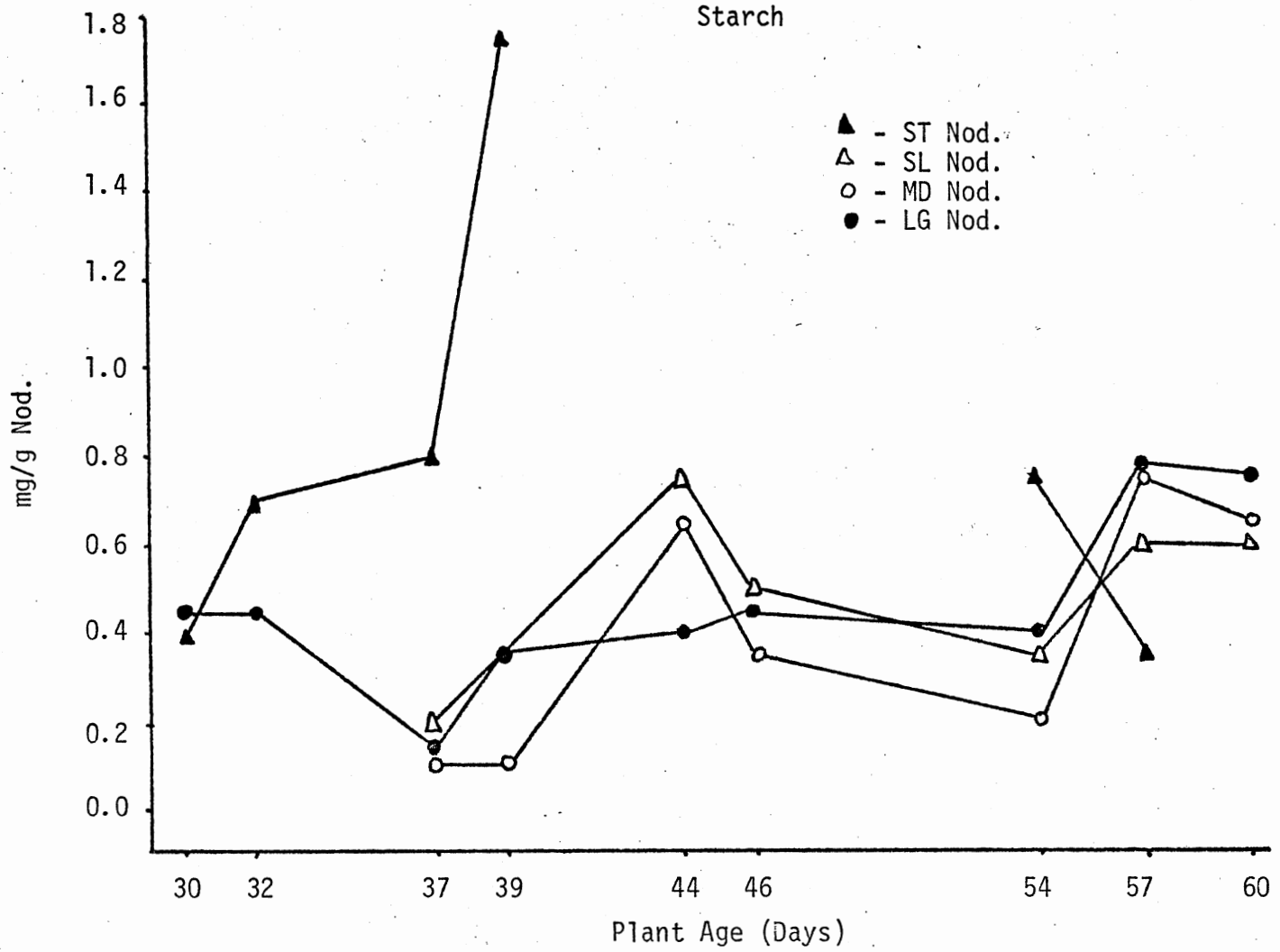


Figure 21. Effect of Plant Age and Nodule Size on Nodule Cytosol Starch Levels of Cratylia floribunda Grown on a Dark Red Latosol, Brazil

synthesizing glutamate, aspartate and alanine, and concomitant with this increased enzymatic activity, higher protein levels were also detected in more mature nodules.

Glutamate-pyruvate transaminase activity is usually about the same as the rate of substrate formation by either GDH or the GS-GOGAT pathway, but the high activity level of GOT is not equaled by either pathway of glutamate biosynthesis. These results are difficult to interprete because GOT has a low Km for its substrates and is found in both the cytoplasmic fraction and the mitochondria of plant cells (277).

Soil Fertility Effects on Plant Growth, Nodulation and Nodule Physiology

This experiment was conducted using a factorial arrangement of the macronutrients P, S, Ca, Mg and K giving rise to 32 possible nutrient combinations. Factorial experiments, as used here, are useful experimental techniques were little is known concerning the optimum level of nutrient factors, or even which ones are important. In this experiment, the objective of using a factorial design was to obtain a broad picture of the effects of each nutrient, and to select the ones that would be more likely to give responses under field conditions. Further experimentation would require the use of increasing levels of the nutrients deemed important in this first factorial experiment for determination of the optimum levels.

Fertility effects for this study will be discussed separately for each parameter with emphasis on the main effects of P, S, Ca, Mg, and K.

To determine if the main effect of each nutrient had a significant effect on the parameters studied, the following hypothesis were tested:

$$HO \quad \Delta \text{ effect} = 0$$

$$HA \quad \Delta \text{ effect} \neq 0$$

The F statistical test obtained from the analysis of variance tables were used as basis for evaluations of HO and HA. The hypothesis HO indicates that the nutrient effect was not significantly different from zero, whereas the alternate hypothesis HA suggests that the nutrient effect was significantly different from zero.

The Δ effect was defined as the difference between the mean of the 48 pot cultures treated with a particular nutrient and the mean of the pot cultures that did not receive that nutrient. Thus, the five possible Δ effects for each parameter can be represented as:

$$\Delta \text{ effect} = (\overline{P}, \overline{S}, \overline{Ca}, \overline{Mg}, \text{ or } \overline{K})_1 - (\overline{P}, \overline{S}, \overline{Ca}, \overline{Mg} \text{ or } \overline{K})_0$$

Because the Δ effect for each nutrient was calculated by averaging the effect of each nutrient within all possible combinations of the other nutrients, this difference indicates the effect obtained was due to the specific nutrient and not to the other nutrient effects. For example, the $\overline{P}_1 - \overline{P}_0$ effect was obtained without S, Ca, Mg, and K influence. Since the preliminary studies did not show beneficial effects for the micronutrients, it was assured that they would not influence the results obtained.

If the statistical results indicated that there was no evidence to reject HO, it was assumed that the effect of that particular nutrient was nil. A summary of the results obtained in these experiments are shown in Tables XI to XXXVI. The interaction effects with significance levels are shown at the bottom of each table.

Shoot Dry Weight

Results obtained for the yields of dry herbage produced in the four clippings are presented in Tables XI to XIV.

Apparently phosphorus was the most limiting nutrient for plant growth, and the effect of its application was highly beneficial ($P \leq 0.001$) for the four clippings. Magnesium gave beneficial results in the last three clippings ($P \leq 0.05$), and sulfur was beneficial for increased herbage yields in the first two cuts ($P \leq 0.05$ and 0.001, respectively). Potassium did not influence shoot growth during the first clipping, but significantly increased yields during the second ($P \leq 0.01$) and third ($P \leq 0.001$) clippings.

On the other hand, a yield depressing effect was observed for Ca during the first two clippings ($P \leq 0.001$ and 0.1 respectively).

Root Dry Weight

The yield of root dry weight (Table XV) was essentially similar to the pattern of dry herbage production in the first clipping. Increased root growth was observed in plants fertilized with both phosphorus ($P \leq 0.001$) and magnesium ($P \leq 0.1$), but a depressive effect was noted for calcium ($P \leq 0.1$). Apparently sulfur and potassium did not influence root growth in this experiment.

Fresh Nodule Weight

Results for fertility effects on fresh nodule weight are presented in Table XVI. Increased weight was observed for plants that were fertilized with phosphorus ($P \leq 0.001$), and no effect was observed for the other nutrients studied.

TABLE XI

EFFECT OF SOIL FERTILITY COMBINATIONS ON SHOOT DRY WEIGHT (1ST CLIPPING) OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL

Trt	g/pot	Trt	g/pot	Trt	g/pot	Trt	g/pot
0	0.71	Mg	0.82	S	0.95	MgS	0.88
K	0.78	KMg	0.77	KS	0.99	KMgS	0.89
Ca	0.65	CaMg	0.77	CaS	0.66	CaMgS	0.79
KCa	0.54	KCaMg	0.74	KCaS	0.75	KCaMgS	0.80
P	1.51	PMg	1.52	PS	1.29	PMgS	1.54
PK	0.95	PKMg	1.78	PKS	1.56	PKMgS	1.63
PCa	1.40	PCaMg	1.18	PCaS	1.43	PCaMgS	1.40
PKCa	1.32	PKCaMg	1.23	PKCaS	1.26	PKCaMgS	1.25

Element Main Effect

	P	S	Ca	Mg	K
Without	0.78	1.04	1.16	1.04	1.09
With	1.39	1.13	1.01	1.12	1.08
Δ Effect	0.61***	0.09*	-0.15***	0.08*	-0.01^{ns}

ns = not significant, *, *** significant at $P \leq 0.05$ and $P \leq 0.001$ respectively for HO: Δ effect = 0.

The interactions Ca x Mg x P, Mg x K x S, Ca x P x K x S, and Ca x Mg X P x K x S, were significant at $P \leq 0.05$.

TABLE XII

EFFECT OF SOIL FERTILITY COMBINATIONS ON SHOOT DRY WEIGHT (2ND CLIPPING) OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL

Trt	g/pot	Trt	g/pot	Trt	g/pot	Trt	g/pot
0	0.18	Mg	0.30	S	0.38	MgS	0.30
K	0.32	KMg	0.25	KS	0.59	KMgS	0.32
Ca	0.22	CaMg	0.33	CaS	0.25	CaMgS	0.22
KCa	0.27	KCaMg	0.30	KCaS	0.22	KCaMgS	0.37
P	1.92	PMg	1.40	PS	1.13	PMgS	1.62
PK	1.13	PKMg	2.43	PKS	2.10	PKMgS	2.25
PCa	1.40	PCaMg	1.37	PCaS	2.10	PCaMgS	1.75
PKCa	1.33	PKCaMg	1.52	PKCaS	1.53	PKCaMgS	2.20

Element Main Effect

	P	S	Ca	Mg	K
Without	0.30	0.92	1.04	0.94	0.93
With	1.70	1.08	0.96	1.05	1.07
Δ Effect	1.40***	0.16***	-0.08#	0.11*	0.14**

ns = not significant, #, *, **, ***, significant at $P \leq 0.1$, 0.05, 0.01, and 0.001 respectively for H: Δ effect = 0.

The interactions P x S, Ca x P x K, Ca x K x S, Ca x P x K x S, and Mg x P x K x S were significant at $P \leq 0.05$; Mg x P, Ca x K, Mg x K, K x S, and Ca x P x S were significant at $P \leq 0.01$; and Mg x P x K, Ca x Mg x K x S, and Ca x Mg x P x K x S, were significant at $P \leq 0.001$.

TABLE XIII

EFFECT OF SOIL FERTILITY COMBINATIONS ON SHOOT DRY WEIGHT (3RD CLIPPING) OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL

Trt	g/pot	Trt	g/pot	Trt	g/pot	Trt	g/pot
0	0.18	Mg	0.18	S	0.24	MgS	0.29
K	0.13	KMg	0.15	KS	0.23	KMgS	0.21
Ca	0.19	CaMg	0.30	CaS	0.30	CaMgS	0.23
KCa	0.35	KCaMg	0.22	KCaS	0.30	KCaMgS	0.33
P	0.85	PMg	1.35	PS	1.30	PMgS	1.39
PK	1.85	PKMg	1.95	PKS	1.93	PKMgS	1.58
PCa	1.57	PCaMg	1.41	PCaS	1.05	PCaMgS	1.57
PKCa	1.44	PKCaMg	2.01	PKCaS	1.51	PKCaMgS	2.34

Element Main Effect

	P	S	Ca	Mg	K
Without	0.24	0.88	0.86	0.84	0.75
With	1.54	0.90	0.92	0.95	1.03
Δ Effect	1.30***	0.02^{ns}	0.06^{ns}	0.09*	0.28***

ns = not significant, *, *** significant at $P \leq 0.05$ and 0.001 respectively for HO: Δ effect = 0.

The interactions Mg x P, Ca x P x K x S, and Ca x Mg x P x S were significant at $P \leq 0.05$; Ca x Mg x K, Ca x K x S, and Ca x Mg x P x K were significant at $P \leq 0.01$; and P x K was significant at $P \leq 0.001$.

TABLE XIV

EFFECT OF SOIL FERTILITY COMBINATIONS ON SHOOT DRY WEIGHT (4TH CLIPPING) OF *CRATYLIA FLORIBUNDA* DARK RED LATOSOL, BRAZIL

Trt	g/pot	Trt	g/pot	Trt	g/pot	Trt	g/pot
0	0.50	Mg	0.62	S	0.50	MgS	0.64
K	0.67	KMg	0.39	KS	0.59	KMgS	0.43
Ca	0.67	CaMg	0.56	CaS	0.49	CaMgS	0.31
KCa	0.82	KCaMg	0.82	KCaS	0.32	KCaMgS	0.60
P	0.79	PMg	0.99	PS	1.05	PMgS	1.25
PK	0.68	PKMg	1.52	PKS	1.23	PKMgS	0.67
PCa	1.00	PCaMg	0.88	PCaS	0.93	PCaMgS	1.00
PKCa	0.64	PKCaMg	0.81	PKCaS	1.00	PKCaMgS	0.97

Element Main Effect

	P	S	Ca	Mg	K
Without	0.58	0.77	0.78	0.74	0.76
With	0.96	0.75	0.74	0.78	0.76
Δ Effect	0.38***	-0.02^{ns}	-0.04^{ns}	0.04^{ns}	0.00^{ns}

ns = not significant, *** significant at $P \leq 0.001$ for HO: Δ effect = 0.

The interactions Ca x P, Ca x Mg x K, Ca x Mg x S, Ca x P x S, Mg x P x S, Ca x Mg x P x S, Ca x Mg x P x S, Ca x P x K x S, and Mg x P x K x S were significant at $P \leq 0.05$; and P x S and Mg x P x S were significant at $P \leq 0.01$.

TABLE XV

EFFECT OF SOIL FERTILITY COMBINATIONS ON THE ROOT DRY WEIGHT OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL

Trt	g/pot	Trt	g/pot	Trt	g/pot	Trt	g/pot
0	0.46	Mg	0.37	S	0.44	MgS	0.52
K	0.41	KMg	0.56	KS	0.37	KMgS	0.34
Ca	0.39	CaMg	0.40	CaS	0.70	CaMgS	0.60
KCa	0.40	KCaMg	0.44	KCaS	0.65	KCaMgS	0.78
P	0.72	PMg	1.88	PS	1.17	PMgS	1.29
PK	0.99	PKMg	1.60	PKS	1.64	PKMgS	0.76
PCa	1.27	PCaMg	0.98	PCaS	0.78	PCaMgS	1.11
PKCa	0.84	PKCaMg	1.07	PKCaS	0.94	PKCaMgS	0.85

Element Main Effect

	P	S	Ca	Mg	K
Without	0.49	0.80	0.85	0.76	0.76
With	1.12	0.81	0.76	0.85	0.76
Δ Effect	0.63***	0.01^{ns}	-0.09#	0.09#	0.00^{ns}

ns = not significant, #, *** significant at $P \leq 0.1$ and 0.001 respectively for HO: Δ effect = 0.

The interactions P x S, Mg x P x K, Mg x K x S, Ca x Mg x K, and Ca x Mg x P x K were significant for $P<0.05$; Mg x S and Mg x P x S were significant for $P \leq 0.01$; and Ca x P, Ca x Mg x S, and Ca x Mg x P x S were significant for $P \leq 0.001$.

TABLE XVI

EFFECT OF SOIL FERTILITY COMBINATIONS ON THE WEIGHT OF FRESH NODULES OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL

Trt	g/pot	Trt	g/pot	Trt	g/pot	Trt	g/pot
0	0.13	Mg	0.14	S	0.15	MgS	0.12
K	0.15	KMg	0.15	KS	0.16	KMgS	0.12
Ca	0.16	CaMg	0.16	CaS	0.10	CaMgS	0.12
KCa	0.18	KCaMg	0.10	KCaS	0.14	KCaMgS	0.17
P	0.36	PMg	0.48	PS	0.54	PMgS	0.38
PK	0.35	PKMg	0.44	PKS	0.38	PKMgS	0.36
PCa	0.28	PCaMg	0.36	PCaS	0.20	PCaMgS	0.36
PKCa	0.23	PKCaMg	0.26	PKCaS	0.35	PKCaMgS	0.30

Element Main Effect

	P	S	Ca	Mg	K
Without	0.14	0.25	0.27	0.24	0.25
With	0.35	0.25	0.22	0.25	0.24
Δ Effect	0.21***	0.00^{ns}	-0.05^{ns}	0.01^{ns}	-0.01^{ns}

ns = not significant, *** significant at $P \leq 0.001$ for HO: Δ effect = 0.

Number of Nodules

Table XVII shows the results of fertility effects on the number of nodules. Except for a large beneficial effect for phosphorus ($P \leq 0.001$) none of the other nutrients had any significant effect on this parameter.

Apparently the results obtained indicate that only phosphorus was capable of increasing both the fresh weight and the number of Copada nodules after the 4 clippings.

Nitrogenase Activity

Results for evaluation of nitrogenase activity by the C_2H_2-C_2H_4 technique are presented in Tables XVIII to XX. When the acetylene reduction by nitrogenase activity was expressed as μM C_2H_4/pot/hr a highly significant effect was observed for phosphorus ($P \leq 0.001$). However, if the amount of ethylene produced was expressed in terms of μM C_2H_4/g fresh nod/hr, the phosphorus effect was no longer significant. Moreover, when the amount of reduced acetylene was expressed as μM C_2H_4/mg protein/hr the phosphorus effect became highly negative ($P \leq 0.01$).

Results from Tables XVI, XVII and XXXIII indicate that phosphorus also increased nodule fresh weight, nodule number and nodule cytosol soluble protein respectively, and these factors must be considered when interpreting results of nitrogenase activity. Apparently higher nitrogenase activity was detected in phosphorus fertilized pots because these had plants with increased number and weight of nodule, and not because this nutrient increased the amount of enzyme present in individual nodules. In fact, these data indicate that accumulation of

TABLE XVII

EFFECT OF SOIL FERTILITY COMBINATIONS ON THE NUMBER OF NODULES OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL

Trt	$\bar{x}$	Trt	$\bar{x}$	Trt	$\bar{x}$	Trt′	$\bar{x}$
0	29.7	Mg	25.0	S	33.3	MgS	41.0
K	39.0	KMg	35.7	KS	-	KMgS	32.0
Ca	55.0	CaMg	37.7	CaS	-	CaMgS	27.0
KCa	48.3	KCaMg	32.3	KCaS	28.7	KCaMgS	46.0
P	93.0	PMg	81.0	PS	122.0	PMgS	112.0
PK	38.0	PKMg	86.3	PKS	63.0	PKMgS	38.7
PCa	9.5	PCaMg	87.7	PCaS	43.7	PCaMgS	68.3
PKCa	60.7	PKCaMg	29.7	PKCaS	62.7	PKCaMgS	56.0

Element Main Effect

	P	S	Ca	Mg	K
Without	36.5	49.0	57.4	53.3	57.8
With	67.0	55.3	47.0	51.0	46.7
Δ Effect	30.5***	-6.3^{ns}	-10.4^{ns}	-2.3^{ns}	-11.1^{ns}

ns = not significant, *** significant at $P \leq 0.001$ for HO: Δ effect = 0.

The interactions P x K, P x Ca x K, P x Ca x Mg x K and S x Ca x Mg x K were significant at $P \leq 0.05$; P x Ca was significant at $P \leq 0.01$.

TABLE XVIII

EFFECT OF SOIL FERTILITY COMBINATIONS ON NITROGENASE (μ MOLES C_2H_4/POT/hr) ACTIVITY LEVELS OF CRATYLIA FLORIBUNDA, DARK RED LATOSOL, BRAZIL

Trt	μM C_2H_4/ pot/hr	Trt	μM C_2H_4/ pot/hr	Trt	μM C_2H_4/ pot/hr	Trt	μM C_2H_4/ pot/hr
0	13.00	Mg	-	S	-	MgS	23.00
K	15.00	KMg	13.00	KS	15.00	KMgS	-
Ca	13.67	CaMg	9.00	CaS	4.00	CaMgS	20.00
KCa	12.67	KCaMg	20.00	KCaS	19.00	KCaMgS	24.50
P	21.00	PMg	52.67	PS	49.00	PMgS	26.67
PK	24.67	PKMg	45.67	PKS	36.67	PKMgS	47.00
PCa	49.00	PCaMg	51.33	PCaS	49.00	PCaMgS	66.00
PKCa	46.33	PKCaMg	39.50	PKCaS	57.67	PKCaMgS	54.00

Element Main Effect

	P	S	Ca	Mg	K
Without	16.05	31.22	32.50	31.86	37.63
With	44.35	39.46	36.49	38.61	32.00
Δ Effect	28.3***	8.24^{ns}	3.99^{ns}	6.75^{ns}	-5.63^{ns}

ns = not significant, *** significant at $P \leq 0.001$ for HO: Δ effect = 0.

TABLE XIX

EFFECT OF SOIL FERTILITY COMBINATIONS ON NITROGENASE (μ MOLES C_2H_4/g NOD/hr) ACTIVITY LEVELS OF CRATYLIA FLORIBUNDA, DARK RED LATOSOL, BRAZIL

Trt	μM C_2H_4/g nod/hr	Trt	μM C_2H_4/g nod/hr	Trt	μM C_2H_4/g nod/hr	Trt	μM C_2H_4/g nod/hr
0	61.50	Mg	-	S	-	MgS	119.30
K	33.75	KMg	46.00	KS	51.10	KMgS	-
Ca	59.30	CaMg	40.80	CaS	20.10	CaMgS	91.20
KCa	44.07	KCaMg	143.97	KCaS	90.05	KCaMgS	55.35
P	55.77	PMg	102.06	PS	65.20	PMgS	67.37
PK	95.40	PKMg	105.70	PKS	77.87	PKMgS	36.80
PCa	85.30	PCaMg	99.40	PCaS	193.07	PCaMgS	124.30
PKCa	177.43	PKCaMg	78.40	PKCaS	148.90	PKCaMgS	65.80

Element Main Effect

	P	S	Ca	Mg	K
Without	69.56	89.40	75.73	92.36	91.19
With	104.25	96.61	105.21	92.71	93.78
Δ Effect	34.69^{ns}	7.21^{ns}	29.48^{ns}	0.35^{ns}	2.59^{ns}

ns = not significant.

TABLE XX

EFFECT OF SOIL FERTILITY COMBINATIONS ON NITROGENASE (μ MOLES C_2H_4/mg PROT/min) OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL

Trt	μM C_2H_4/mg prot/min	Trt	μM C_2H_4/mg prot/min	Trt	μM C_2H_4/mg prot/min	Trt	μM C_2H_4/mg prot/min
0	0.176	Mg	-	S	-	MgS	1.807
K	0.068	KMg	0.079	KS	0.080	KMgS	-
Ca	0.158	CaMg	0.142	CaS	0.031	CaMgS	-
KCa	0.835	KCaMg	0.154	KCaS	0.150	KCaMgS	0.603
P	0.182	PMg	0.149	PS	0.119	PMgS	0.147
PK	0.114	PKMg	0.099	PKS	0.102	PKMgS	0.130
PCa	0.150	PCaMg	0.130	PCaS	0.379	PCaMgS	
PKCa	0.247	PKCaMg	0.152	PKCaS	0.134	PKCaMgS	0.233

Element Main Effect

	P	S	Ca	Mg	K
Without	0.364	0.201	0.212	0.206	2.231
With	0.157	0.279	0.249	0.270	0.235
Δ Effect	-0.207**	0.078^{ns}	0.037^{ns}	0.064^{ns}	0.004^{ns}

ns = not significant, ** significant at $P \leq 0.01$ for HO: Δ effect = 0.

The interactions Ca x K, P x Ca x Mg, and P x S x K were significant at $P \leq 0.05$; and Mg x S, P x S x Ca, P x S x Mg, P x Ca x K, and P x Mg x K were significant at $P \leq 0.01$.

protein in the nodules did not influence the amount of enzyme present, i.e., the protein accumulated did not have enzymatic activity. These results are in agreement with the data obtained in the third experiment, where it was demonstrated that nitrogenase activity was related to the weight and number of nodules larger than 1 mm.

Alpha Ketoglutarate Levels

Results obtained for measurement of alpha ketoglutarate levels in the nodule cytosol of Copada plants are presented in Table XXI. Increased αKG levels were observed when the plants were fertilized with calcium ($P \leq 0.01$), but a negative effect was noted for plants fertilized with magnesium ($P \leq 0.1$).

Glutamate Dehydrogenase Activity

The results for glutamate dehydrogenase activity are summarized in Tables XXII and XXIII. As for nitrogenase, fertility effects varied according to the way the activity of the enzyme was expressed.

An increased GDH activity ($P \leq 0.05$) was observed in phosphorus fertilized plants when results were presented as U/g nod, but this effect was negative ($P \leq 0.01$) if enzymatic activity was expressed as U/mg protein. However, in both cases a negative effect was observed for magnesium fertilization, and apparently this effect was independent of any Mg effect on protein, and nodule weight. A negative effect for K ($P \leq 0.1$) was observed in the specific activity of GDH, but higher specific activity was observed in the S fertilized pots ($P \leq 0.1$).

TABLE XXI

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL ALPHA KETOGLUTARATE (αKG) LEVELS OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL

Trt	μM/g nod	Trt	μM/g nod	Trt	μM/g nod	Trt	μM/g nod
0	0.60	Mg	0.09	S	0.37	MgS	0.52
K	0.43	KMg	0.66	KS	0.33	KMgS	0.72
Ca	0.94	CaMg	0.24	CaS	0.45	CaMgS	0.65
KCa	0.56	KCaMg	0.89	KCaS	1.07	KCaMgS	0.37
P	0.31	PMg	0.57	PS	0.50	PMgS	0.48
PK	0.35	PKMg	0.98	PKS	0.38	PKMgS	0.06
PCa	1.43	PCaMg	1.03	PCaS	1.54	PCaMgS	0.68
PKCa	1.37	PKCaMg	0.33	PKCaS	0.54	PKCaMgS	0.11

Element Main Effect

	P	S	Ca	Mg	K
Without	0.56	0.67	0.46	0.70	0.65
With	0.67	0.55	0.76	0.52	0.57
Δ Effect	0.11^{ns}	-0.12^{ns}	0.30**	-0.18#	-0.08^{ns}

ns = not significant, #, ** significant at $P \leq 0.1$ and 0.01 respectively for HO: Δ effect = 0.

The interactions P x K and Ca x Mg were significant at $P \leq 0.05$.

TABLE XXII

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMATE DEHYDROGENASE (GDH) ACTIVITY OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL, AS U/g FRESH NOD

Trt	U/g nod	Trt	U/g nod	Trt	U/g nod	Trt	U/g nod
0	0.75	Mg	0.65	S	1.40	MgS	2.05
K	1.10	KMg	1.35	KS	4.00	KMgS	0.55
Ca	1.15	CaMg	2.80	CaS	1.15	CaMgS	1.40
KCa	1.05	KCaMg	2.80	KCaS	3.70	KCaMgS	0.75
P	1.00	PMg	2.20	PS	4.10	PMgS	2.40
PK	2.00	PKMg	3.05	PKS	2.15	PKMgS	1.25
PCa	2.50	PCaMg	2.35	PCaS	5.25	PCaMgS	1.70
PKCa	5.05	PKCaMg	0.95	PKCaS	1.45	PKCaMgS	1.15

Element Main Effect

	P	S	Ca	Mg	K
Without	1.67	1.92	1.88	2.36	2.05
With	2.41	2.15	2.20	1.71	2.02
Δ Effect	0.74*	0.23^{ns}	0.32^{ns}	-0.65*	-0.03^{ns}

ns = not significant, * significant at $P \leq 0.05$ for HO: Δ effect = 0.

The interactions Mg x S, Ca x Mg x P, P x K x S, and Mg x P x K x S were significant for $P \leq 0.05$.

TABLE XXIII

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMATE DEHYDROGENASE (GDH) ACTIVITY OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL, AS U/mg PROTEIN (SPECIFIC ACTIVITY)

Trt	U/mg prot	Trt	U/mg prot	Trt	U/mg prot	Trt	U/mg prot
0	0.211	Mg	0.213	S	4.172	MgS	0.495
K	0.551	KMg	0.181	KS	0.945	KMgS	0.489
Ca	0.159	CaMg	1.340	CaS	1.888	CaMgS	0.188
KCa	0.720	KCaMg	1.540	KCaS	0.359	KCaMgS	0.240
P	0.117	PMg	0.509	PS	0.466	PMgS	0.237
PK	0.229	PKMg	0.150	PKS	0.227	PKMgS	0.417
PCa	0.242	PCaMg	0.184	PCaS	0.169	PCaMgS	0.101
PKCa	0.402	PKCaMg	0.098	PKCaS	0.136	PKCaMgS	0.219

Element Main Effect

	P	S	Ca	Mg	K
Without	0.769	0.343	0.601	0.695	0.684
With	0.251	0.688	0.420	0.326	0.347
Δ Effect	-0.518**	0.345#	-0.181^{ns}	-0.369*	-0.337#

ns = not significant, #, *, ** significant at $P \leq 0.1$, 0.05, and 0.01 respectively for HO: Δ effect = 0.

The interaction Mg x S and P x S x Mg were significant at $P \leq 0.05$; and S x Mg x K was significant at $P \leq 0.01$.

Glutamine Synthetase Activity

Results for glutamine synthetase activity are presented in Tables XXIV and XXV. Both GS specific activity and activity/g nodule were depressed in Mg fertilized pots ($P \leq 0.05$), and again, contrasting results were obtained for P. Phosphorus significantly increased GS activity as U/g nod ($P \leq 0.01$) but negative results were obtained for U/mg prot ($P \leq 0.01$), probably due to increased soluble protein content within the nodules of P fertilized plants, without GS activity.

Glutamate Synthase Activity

Results for GOGAT activity levels are shown in Tables XXVI and XXVII. Phosphorus and Ca had highly beneficial ($P \leq 0.001$) effects on glutamate synthase activity when expressed as U/g nod, but a negative effect was noted for Mg. However, when GOGAT activity was calculated to specific activity, the Ca and Mg effects were nonsignificant and P gave negative results ($P \leq 0.05$).

Glutamate-Oxaloacetate Transaminase Activity

The data obtained for GOT activity levels are summarized in Tables XXVIII and XXIX. Results indicated that a beneficial P effect was achieved when GOT activity was expressed as U/g nod ($P \leq 0.001$) but a depressive effect was observed on the GOT specific activity ($P \leq 0.05$). Whereas it can be suggested that this negative P effect on GOT specific activity was caused by an increase of nodule protein without GOT activity, the deleterious effect observed for Mg appears to be real.

TABLE XXIV

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMINE SYNTHETASE (GS) ACTIVITY OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL, AS U/g FRESH NOD

Trt	U/g nod	Trt	U/g nod	Trt	U/g nod	Trt	U/g nod
0	1.95	Mg	1.00	S	1.65	MgS	1.80
K	3.40	KMg	1.30	KS	2.35	KMgS	0.85
Ca	1.50	CaMg	1.45	CaS	1.75	CaMgS	1.70
KCa	2.80	KCaMg	4.00	KCaS	2.40	KCaMgS	1.80
P	2.50	PMg	2.20	PS	3.40	PMgS	2.20
PK	4.20	PKMg	3.90	PKS	2.65	PKMgS	0.90
PCa	3.50	PCaMg	1.80	PCaS	3.10	PCaMgS	5.60
PKCa	3.20	PKCaMg	0.70	PKCaS	3.10	PKCaMgS	1.70

Element Main Effect

	P	S	Ca	Mg	K
Without	1.98	2.46	2.27	2.72	2.32
With	2.79	2.31	2.51	2.06	2.45
Δ Effect	0.81**	-0.15^{ns}	0.24^{ns}	-0.66*	0.13^{ns}

ns = not significant, *, ** significant at $P \leq 0.05$ and $P \leq 0.01$ respectively for HO: Δ effect = 0.

The interactions P x K, K x S, Ca x P x K and Ca x Mg x P x S were significant for $P \leq 0.05$.

TABLE XXV

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMINE SYNTHETASE (GS) ACTIVITY OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL, AS U/mg PROTEIN (SPECIFIC ACTIVITY)

Trt	U/mg prot	Trt	U/mg prot	Trt	U/mg prot	Trt	U/mg prot
0	0.427	Mg	0.344	S	5.222	MgS	0.670
K	1.933	KMg	0.194	KS	0.789	KMgS	0.632
Ca	0.203	CaMg	1.561	CaS	1.295	CaMgS	0.219
KCa	2.040	KCaMg	0.221	KCaS	0.240	KCaMgS	0.270
P	0.266	PMg	0.571	PS	0.394	PMgS	0.211
PK	0.328	PKMg	0.187	PKS	0.278	PKMgS	0.347
PCa	0.320	PCaMg	0.150	PCaS	0.184	PCaMgS	0.349
PKCa	0.237	PKCaMg	0.080	PKCaS	0.291	PKCaMgS	0.327

Element Main Effect

	P	S	Ca	Mg	K
Without	1.017	0.556	0.800	0.905	0.794
With	0.284	0.750	0.501	0.396	0.516
Δ Effect	-0.733**	0.194^{ns}	-0.299^{ns}	-0.509*	-0.278^{ns}

ns = not significant, *, ** significant at $P \leq 0.05$ and 0.01 respectively for HO: Δ effect = 0.

The interactions P x Mg was significant at $P \leq 0.05$; and S x Mg x K and P x S x Mg x K were significant at $P \leq 0.01$.

TABLE XXVI

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMATE SYNTHASE (GOGAT) ACTIVITY OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL, AS U/g FRESH NOD

Trt	U/g nod	Trt	U/g nod	Trt	U/g nod	Trt	U/g nod
0	1.15	Mg	0.50	S	0.70	MgS	1.15
K	1.25	KMg	1.15	KS	1.00	KMgS	0.60
Ca	1.30	CaMg	0.80	CaS	1.15	CaMgS	1.05
KCa	1.25	KCaMg	2.60	KCaS	3.20	KCaMgS	1.00
P	1.55	PMg	2.00	PS	1.35	PMgS	1.80
PK	1.15	PKMg	2.95	PKS	1.75	PKMgS	0.65
PCa	1.95	PCaMg	1.75	PCaS	6.40	PCaMgS	1.50
PKCa	3.60	PKCaMg	1.40	PKCaS	1.95	PKCaMgS	1.30

Element Main Effect

	P	S	Ca	Mg	K
Without	1.24	1.65	1.29	1.92	1.63
With	2.07	1.66	2.01	1.39	1.68
Δ Effect	0.83***	0.01^{ns}	0.72***	-0.53**	0.05^{ns}

ns = not significant, **, *** significant at $P \leq 0.01$ and 0.001 respectively for HO: Δ effect = 0.

The interactions Ca x S, Ca x Mg x P, P x K x S, Ca x Mg x K x S, and Ca x P x K x S were significant at $P \leq 0.05$; P x K, Mg x S, K x S, and Mg x P x K x S, were significant at $P \leq 0.01$; and Ca x Mg and Ca x Mg x P x K x S were significant at $P \leq 0.001$.

TABLE XXVII

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMATE SYNTHASE (GOGAT) ACTIVITY OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL, AS U/mg PROTEIN (SPECIFIC ACTIVITY)

Trt	U/mg prot	Trt	U/mg prot	Trt	U/mg prot	Trt	U/mg prot
0	0.249	Mg	0.166	S	3.312	MgS	0.321
K	0.655	KMg	0.172	KS	0.236	KMgS	0.584
Ca	0.180	CaMg	0.922	CaS	1.726	CaMgS	0.151
KCa	0.827	KCaMg	0.143	KCaS	0.317	KCaMgS	0.270
P	0.171	PMg	0.472	PS	0.156	PMgS	0.186
PK	0.150	PKMg	0.138	PKS	0.187	PKMgS	0.215
PCa	0.169	PCaMg	0.137	PCaS	0.408	PCaMgS	0.095
PKCa	0.284	PKCaMg	0.150	PKCaS	0.194	PKCaMgS	0.251

Element Main Effect

	P	S	Ca	Mg	K
Without	0.651	0.310	0.473	0.585	0.569
With	0.207	0.555	0.385	0.273	0.297
Δ Effect	-0.444*	0.245^{ns}	-0.088^{ns}	-0.312^{ns}	-0.272^{ns}

ns = not significant, * significant at $P \leq 0.05$ for HO: Δ effect = 0.

The interaction S x Mg x K was significant for $P \leq 0.05$.

TABLE XXVIII

EFFECTS OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMATE-OXALOACETATE TRANSAMINASE (GOT) ACTIVITY OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL, AS U/g FRESH NOD

Trt	U/g nod	Trt	U/g nod	Trt	U/g nod	Trt	U/g nod
0	4.20	Mg	2.25	S	2.55	MgS	9.20
K	5.90	KMg	3.95	KS	7.20	KMgS	1.40
Ca	8.50	CaMg	2.70	CaS	7.30	CaMgS	9.70
KCa	3.60	KCaMg	10.60	KCaS	8.40	KCaMgS	5.00
P	10.20	PMg	19.20	PS	7.40	PMgS	12.75
PK	10.80	PKMg	19.90	PKS	10.45	PKMgS	1.70
PCa	13.20	PCaMg	14.20	PCaS	18.40	PCaMgS	15.40
PKCa	22.20	PKCaMg	5.60	PKCaS	9.70	PKCaMgS	2.80

Element Main Effect

	P	S	Ca	Mg	K
Without	5.78	9.81	8.07	9.38	9.82
With	12.12	8.08	9.83	8.52	8.07
Δ Effect	6.34***	-1.73^{ns}	1.76^{ns}	0.86^{ns}	-1.75^{ns}

ns = not significant, *** significant at $P \leq 0.001$ for HO: Δ effect = 0.

The interaction P x S was significant at $P \leq 0.05$.

TABLE XXIX

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMATE-OXALOACETATE TRANSAMINASE (GOT) ACTIVITY OF CRATYLIC FLORIBUNDA, DARK RED LATOSOL, BRAZIL, AS U/mg PROT (SPECIFIC ACTIVITY)

Trt	U/mg prot	Trt	U/mg prot	Trt	U/mg prot	Trt	U/mg prot
0	0.948	Mg	0.736	S	8.516	MgS	2.407
K	3.064	KMg	0.653	KS	1.571	KMgS	1.022
Ca	1.148	CaMg	2.409	CaS	5.207	CaMgS	1.240
KCa	2.289	KCaMg	0.582	KCaS	0.833	KCaMgS	0.939
P	1.101	PMg	1.900	PS	0.848	PMgS	1.215
PK	0.804	PKMg	0.420	PKS	1.133	PKMgS	0.597
PCa	1.182	PCaMg	1.147	PCaS	0.920	PCaMgS	0.913
PKCa	1.300	PKCaMg	0.585	PKCaS	0.915	PKCaMgS	0.518

Element Main Effect

	P	S	Ca	Mg	K
Without	2.291	1.281	1.926	1.980	2.006
With	1.012	2.046	1.377	1.323	1.329
Δ Effect	-1.279*	0.765^{ns}	-0.549^{ns}	-0.657#	-0.677^{ns}

ns = not significant, #, * significant at $P \leq 0.1$ and 0.05 respectively for HO: Δ effect = 0.

The interactions S x Mg x K and P x S x Mg x K were significant at $P \leq 0.05$.

Glutamate-Pyruvate Transaminase Activity

A summary of the data collected for GPT activity levels is presented in Tables XXX and XXXI. When expressed as U/g nod, GPT activity was increased for P fertilization ($P \leq 0.001$) and decreased by S addition to the soil ($P \leq 0.1$). However, as was the case for the other enzymes, when enzymatic activity was expressed as specific activity, the P effect was negative ($P \leq 0.05$) and S was no longer influential. In common with several of the other enzymes, the Mg effect was depressive ($P \leq 0.1$) if GPT activity was expressed as U/mg protein.

Pyridoxyl Phosphates Levels

The effects of the fertility combinations on nodule cytosol pyridoxyl phosphates are summarized in Table XXXII. The data obtained indicated that calcium was the only nutrient significantly ($P \leq 0.05$) increasing levels of PLP's on Copada nodules.

Soluble Protein Levels

The results obtained for the effect of the fertility treatments on the levels of water soluble nodule cytosol protein are shown in Table XXXIII. Increased amounts of protein were observed in nodules of plants fertilized with Ca ($P \leq 0.05$), and protein levels almost double in the nodules of P fertilized plants.

Apparently this accumulated soluble protein in the nodule cytosol of the Copada plants did not have any enzymatic activity, a fact that contributed to give depressed results when Nase, GDH, GS, GOGAT, GOT

TABLE XXX

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMATE-PYRUVATE TRANSAMINASE (GPT) ACTIVITY OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL, AS U/g FRESH NOD

Trt	U/g nod	Trt	U/g nod	Trt	U/g nod	Trt	U/g nod
0	0.90	Mg	0.55	S	0.70	MgS	2.25
K	2.30	KMg	1.15	KS	1.50	KMgS	0.95
Ca	2.05	CaMg	0.80	CaS	1.95	CaMgS	1.50
KCa	1.40	KCaMg	2.40	KCaS	1.75	KCaMgS	1.10
P	4.70	PMg	3.35	PS	2.35	PMgS	2.00
PK	2.35	PKMg	5.70	PKS	3.20	PKMgS	0.65
PCa	3.00	PCaMg	2.05	PCaS	3.65	PCaMgS	3.65
PKCa	3.80	PKCaMg	2.65	PKCaS	2.90	PKCaMgS	1.55

Element Main Effect

	P	S	Ca	Mg	K
Without	1.45	2.45	2.16	2.41	2.22
With	2.97	1.98	2.26	2.02	2.20
Δ Effect	1.52***	-0.47#	0.10^{ns}	-0.39^{ns}	-0.02^{ns}

ns = not significant, #, *** significant at $P \leq 0.1$ and 0.001 respectively for HO: Δ effect = 0.

The interactions K x S, Ca x P x S, Ca x Mg x P x S, and Ca x Mg x P x K were significant at $P \leq 0.05$; and S x Mg x K was significant at $P \leq 0.01$.

TABLE XXXI

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUTAMATE-PYRUVATE TRANSAMINASE (GPT) ACTIVITY OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL, AS U/mg PROTEIN (SPECIFIC ACTIVITY)

Trt	U/mg Prot	Trt	U/mg prot	Trt	U/mg prot	Trt	U/mg prot
0	0.216	Mg	0.185	S	2.290	MgS	0.757
K	1.830	KMg	0.167	KS	0.405	KMgS	0.790
Ca	0.282	CaMg	0.754	CaS	2.124	CaMgS	0.193
KCa	0.930	KCaMg	0.133	KCaS	0.174	KCaMgS	0.302
P	0.500	PMg	0.722	PS	0.273	PMgS	0.231
PK	0.248	PKMg	0.266	PKS	0.347	PKMgS	0.215
PCa	0.278	PCaMg	0.158	PCaS	0.161	PCaMgS	0.227
PKCa	0.243	PKCaMg	0.283	PKCaS	0.283	PKCaMgS	0.295

Element Main Effect

	P	S	Ca	Mg	K
Without	0.720	0.444	0.590	0.664	0.598
With	0.298	0.580	0.429	0.355	0.426
Δ Effect	-0.422*	0.136^{ns}	-0.161^{ns}	-0.309#	-0.172^{ns}

ns = not significant, #, * significant at $P \leq 0.1$ and 0.05 respectively for HO: Δ effect = 0.

The interactions P x S x K, S x Mg x K and P x S x Mg x K were significant at $P \leq 0.05$.

TABLE XXXII

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL PYRIDOXYL PHOSPHATES (PLP'S) LEVELS OF CRATYLIA FLORIBUNDA, DARK RED LATOSOL, BRAZIL

Trt	µg/g nod	Trt	µg/g nod	Trt	µg/g nod	Trt	µg/g nod
0	15.25	Mg	8.04	S	8.50	MgS	18.75
K	13.00	KMg	13.06	KS	25.00	KMgS	4.50
Ca	22.25	CaMg	11.75	CaS	20.25	CaMgS	11.50
KCa	24.50	KCaMg	44.25	KCaS	25.25	KCaMgS	18.75
P	19.50	PMg	24.25	PS	13.00	PMgS	18.25
PK	13.00	PKMg	36.50	PKS	19.75	PKMgS	5.65
PCa	32.75	PCaMg	2.175	PCaS	26.50	PCaMgS	29.25
PKCa	24.25	PKCaMg	13.65	PKCaS	26.00	PKCaMgS	7.50

Element Main Effect

	P	S	Ca	Mg	K
Without	17.78	21.10	15.99	20.54	18.84
With	20.72	17.40	22.50	17.96	19.66
Δ Effect	2.94^{ns}	-3.70^{ns}	6.51*	-2.58^{ns}	0.82^{ns}

ns = not significant, * significant at $P \leq 0.05$ for HO: Δ effect = 0.

The interactions P x K, Ca x P x K, and Ca x Mg x P x K were significant at $P \leq 0.05$; and Mg x K x S was significant at $P \leq 0.01$.

TABLE XXXIII

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL PROTEIN (PROT) LEVELS OF CRATYLIA FLORIBUNDA, DARK RED LATOSOL, BRAZIL

Trt	%	Trt	%	Trt	%	Trt	%
0	0.42	Mg	0.29	S	0.58	MgS	0.53
K	0.56	KMg	0.71	KS	0.63	KMgS	0.12
Ca	0.74	CaMg	0.27	CaS	0.56	CaMgS	0.72
KCa	0.61	KCaMg	1.82	KCaS	1.00	KCaMgS	0.83
P	0.90	PMg	0.60	PS	0.87	PMgS	1.00
PK	1.13	PKMg	2.29	PKS	0.93	PKMgS	0.30
PCa	1.18	PCaMg	1.24	PCaS	1.46	PCaMgS	1.71
PKCa	2.15	PKCaMg	0.89	PKCaS	1.18	PKCaMgS	0.53

Element Main Effect

	P	S	Ca	Mg	K
Without	0.62	0.99	0.71	0.90	0.78
With	1.15	0.78	1.06	0.87	0.99
Δ Effect	0.53***	-0.21^{ns}	0.35*	-0.03^{ns}	0.21^{ns}

ns = not significant, *, *** significant at $P \leq 0.05$ and 0.001 respectively for HO: Δ effect = 0.

The interaction K x S, Mg x K x S, and Ca x Mg x P x K were significant at $P \leq 0.05$.

and GPT activities were expressed as U/mg protein. Thus, care must be exercised when interpreting these results since this dileterious P effect on nodule enzymatic specific activity may not be real.

Glucose Levels

Table XXXIV shows the influence of the fertility combinations on nodule cytosol glucose levels of the Copada plants. Increased levels were observed in nodules of plants fertilized with P ($P \leq 0.1$) and K ($P \leq 0.05$), but less glucose was detected in nodule of plants fertilized with both S ($P \leq 0.001$) and Ca ($P \leq 0.1$).

Sucrose Levels

Sucrose levels in the Copada nodule cytosol were significantly depressed ($P \leq 0.001$) when the soil was fertilized with calcium. These results are summarized in Table XXXV.

Starch Levels

Table XXXVI shows the results obtained for the effect of the fertility combinations on levels of nodule starch. Whereas more starch was detected in the nodule of plants fertilized with Ca ($P \leq 0.05$), smaller amounts of this carbohydrate were present in nodules of plants fertilized with both S ($P \leq 0.1$) and Mg ($P \leq 0.05$).

Relationship Among Enzymes and Carbohydrates of Copada Nodules

A description of the association among the several parameters determined in the nodule cytosol of *Cratylia floribunda* is shown in

TABLE XXXIV

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL GLUCOSE LEVELS OF CRATYLIA FLORIBUNDA, DARK RED LATOSOL, BRAZIL

Trt	mg/g nod	Trt	mg/g nod	Trt	mg/g nod	Trt	mg/g nod
0	1.25	Mg	1.26	S	1.16	MgS	0.80
K	1.38	KMg	0.84	KS	0.80	KMgS	0.55
Ca	1.37	CaMg	1.22	CaS	0.90	CaMgS	0.78
KCa	1.23	KCaMg	0.83	KCaS	0.91	KCaMgS	0.64
P	1.75	PMg	1.05	PS	0.61	PMgS	1.11
PK	1.00	PKMg	1.67	PKS	1.24	PKMgS	1.26
PCa	0.75	PCaMg	1.90	PCaS	0.90	PCaMgS	1.17
PKCa	0.54	PKCaMg	0.95	PKCaS	0.97	PKCaMgS	0.82

Element Main Effect

	P	S	Ca	Mg	K
Without	0.99	1.19	1.10	1.05	1.12
With	1.10	0.92	0.99	1.05	0.98
Δ Effect	0.11#	-0.27***	-0.11#	0.00^{ns}	0.14*

ns = not significant, #, *, *** significant at $P \leq 0.1$, 0.05 and 0.001 respectively for HO: Δ effect = 0.

The interactions Mg x P, Ca x P x K, Mg x P x K x S, and Ca x Mg x P x K x S were significant at $P \leq 0.05$.

TABLE XXXV

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL SUCROSE LEVELS OF *CRATYLIA FLORIBUNDA*, DARK RED LATOSOL, BRAZIL

Trt	mg/g nod	Trt	mg/g nod	Trt	mg/g nod	Trt	mg/g nod
0	0.07	Mg	0.53	S	0.05	MgS	0.30
K	0.12	KMg	0.20	KS	0.30	KMgS	0.10
Ca	0.10	CaMg	0.10	CaS	0.20	CaMgS	0.24
KCa	0.06	KCaMg	-	KCaS	-	KCaMgS	0.08
P	0.08	PMg	0.20	PS	0.13	PMgS	0.20
PK	0.25	PKMg	0.50	PKS	0.27	PKMgS	0.10
PCa	0.10	PCaMg	0.16	PCaS	0.20	PCaMgS	0.10
PKCa	0.24	PKCaMg	0.02	PKCaS	0.14	PKCaMgS	0.05

Element Main Effect

	P	S	Ca	Mg	K
Without	0.17	0.16	0.20	0.15	0.16
With	0.16	0.16	0.12	0.18	0.16
Δ Effect	0.01^{ns}	0.00^{ns}	-0.08***	0.03^{ns}	0.00^{ns}

ns = not significant, *** significant at $P \leq 0.001$ for HO: Δ effect = 0.

The interactions Mg x S, P x S, P x K, Ca x Mg x K, Ca x Mg x S, and Ca x P x S were significant at $P \leq 0.05$; Ca x Mg, Ca x S, Mg x P x K and P x K x S were significant at $P \leq 0.01$; and Mg x K was significant at $P \leq 0.001$.

TABLE XXXVI

EFFECT OF SOIL FERTILITY COMBINATIONS ON NODULE CYTOSOL STARCH LEVELS OF CRATYLIA FLORIBUNDA, DARK RED LATOSOL, BRAZIL

Trt	mg/g nod	Trt	mg/g nod	Trt	mg/g nod	Trt	mg/g nod
0	0.62	Mg	0.54	S	0.45	MgS	0.65
K	0.47	KMg	0.23	KS	0.50	KMgS	0.50
Ca	0.46	CaMg	0.50	CaS	0.35	CaMgS	0.54
KCa	0.59	KCaMg	0.33	KCaS	0.21	KCaMgS	0.48
P	0.60	PMg	0.50	PS	0.42	PMgS	0.47
PK	0.80	PKMg	0.29	PKS	0.62	PKMgS	0.61
PCa	0.45	PCaMg	0.67	PCaS	0.52	PCaMgS	0.32
PKCa	0.89	PKCaMg	0.47	PKCaS	0.57	PKCaMgS	0.07

Element Main Effect

	P	S	Ca	Mg	K
Without	0.47	0.52	0.52	0.52	0.52
With	0.51	0.46	0.45	0.45	0.47
Δ Effect	0.04^{ns}	-0.06#	0.07*	-0.07*	-0.04^{ns}

ns = not significant, #, * significant at $P \leq 0.1$ and 0.05 respectively for HO: Δ effect = 0.

The interactions Ca x S, Mg x S and Ca x K x S were significant at $P \leq 0.05$; and Mg x K was significant at $P \leq 0.01$.

Table XXXVII. This table shows the correlation coefficients (r) for the data obtained in the fertility study as well as the level of significance as P>|r| under HO: r = 0. These r values are a measure of the degree of association between two variables, for example Nase and GDH.

An examination of these correlation coefficients indicates that there was a close relationship among the specific activities of all enzymes. Nitrogenase activity was significantly associated with both GDH and GOGAT activities, thus suggesting that the glutamate and glutamine pathways are in some way involved in the assimilation of the fixed N into plant amino acids, under the conditions this experiment was carried out. The highest correlation (r=0.96) was obtained between the activities of GDH and GOGAT, indicating that the GDH and GS-GOGAT pathways of NH_3 assimilation were operating at the same time. However, to accommodate this finding it must be assumed that these two pathways are compartmentalized within different organelles in the nodule, because it has been previously demonstrated that substrate concentration or allosteric inhibition prevents that both pathways be active at the same time (211, 254). Since higher GDH activity is comfined to the mitochondria (33) and in soybean nodules, the cytosol accounts for more than 90% of the total GS activity (163) these two nodule components might be suggested to be the sites of these two pathways. To study this possibility would require that sonication of nodule homogenates not be performed, since the disrupting energy delivered by the 7.3 pulse used during the preparation of the crude cell-free extract was sufficient to burst up the membranes of all cell organelles and cause enzyme leakage into the protoplasm. However,

TABLE XXXVII

CORRELATION COEFFICIENTS FOR ENZYME SPECIFIC ACTIVITY AND CARBOHYDRATE COMPONENTS OF CRATYLIA FLORIBUNDA NODULE CYTOSOL

	Nase	GDH	GS	GOGAT	GOT	GPT	αk	PROT	PLP's	Glocose	Sucrose	Starch
Nase		0.568 ***	0.690 ***	0.670 ***	0.632 ***	0.766 ***	-0.290 *	-0.432 **	-0.381 **	-0.250 #	-0.242 ns	-0.018 ns
GDH			0.900 ***	0.960 ***	0.938 ***	0.820 ***	-0.168 ns	-0.400 **	-0.307 **	-0.048 ns	-0.230 ns	-0.105 ns
GS				0.914 ***	0.907 ***	0.834 ***	-0.184 ns	-0.413 ***	-0.321 **	-0.012 ns	-0.257 #	-0.062 ns
GOGAT					0.948 ***	0.845 ***	-0.122 ns	-0.356 **	-0.285 *	-0.039 ns	-0.269 #	-0.115 ns
GOT						0.915 ***	-0.123 ns	-0.372 **	-0.260 *	-0.006 ns	-0.130 ns	-0.063 ns
GPT							-0.231 #	-0.443 ***	-0.359 **	-0.047 ns	-0.194 ns	-0.104 ns
αKG								0.651 ***	0.628 ***	0.000 ns	-0.048 ns	0.021 ns
PROT									0.775 ***	0.074 ns	0.248 #	-0.078 ns
PLP's										0.163 ns	0.151 ns	-0.016 ns
Glucose											0.021 ns	0.250 *
Sucrose												0.198 ns
Starch												

these organelles can be separated by mechanical desruption of the nodule with subsequent differential centrifugation.

The data from Table XXXVII also indicate that the glutamate formed in the nodules of Copada by either one of the NH_3 assimilatory pathways can be used in transamination reactions. High correlation coefficients were observed between these and GOT and GPT activities. Furthermore, the specific activity of these two transaminases varies in a parallel trend.

Negative correlations were consistently observed between soluble protein levels and specific enzymatic activities. This possibly indicates that accumulation of protein within Copada nodules does not lead to increased enzymatic activity. As discussed earlier, significant levels of soluble protein accumulated in nodules of plants fertilized with P, thus falsely indicating that this nutrient had negative effects on enzyme specific activity.

The negative association between Nase and αKG is consistent with the idea that this is the process of NH_3 assimilation. These results agree with the fact that, as nitrogenase activity increases levels of αKG descend, for during the process of reductive amination of this keto acid by GDH or GOGAT, the available alpha ketoglutarate substrate is continuously transformed into glutamate. A significant positive correlation was observed between protein and this keto acid.

Surprisingly, levels of pyridoxyl phosphates were negatively correlated with all enzyme activities, including the GOT and GPT transaminases, for which they are thought to be essential cofactors (66, 124). However, a highly significant and positive relationship was observed between PLP's levels and alpha ketoglutarate and soluble

protein contents.

These results are difficult to interpret, and are further complicated by the finding that when GOT and GPT activities were expressed as U/g nodule, the following linear regression equation was obtained:

$$PLP's = 8.36 + 0.95\ GOT + 1.10\ GPT \qquad (26)$$

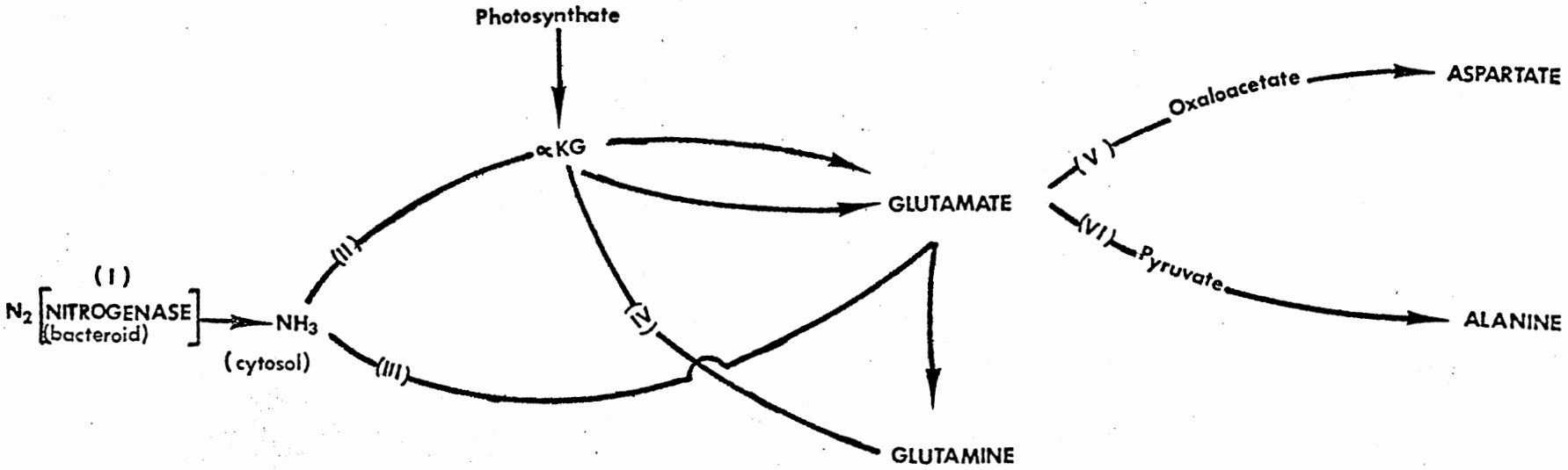
The model is significant for P=0.0001 and the coefficient of determination r^2=0.46. This equation suggests that 46% of the variation on pyridoxyl phosphate level can be accounted for by changes in the GOT and GPT activities, provided enzyme activity was calculated to U/g nod. Because in this model both β_2 and β_3 are positive, levels of nodule PLP's are expected to rise with increased GOT and GPT activities.

Apparently levels of carbohydrate within the nodules of Copada, after the four clippings were not strongly correlated with enzymatic activity. A slightly significant negative association was observed between Nase and glucose, and between sucrose and the GS and GOGAT activities. These results probably indicate that increased activity of these enzymes speeded up the use of these carbohydrates for energy production.

Figure 22 shows the two enzymatic pathways proposed for N assimilation within the nodules of Cratylia floribunda.

According to this model nitrogenase is confined to the nodule bacteroid, and the fixed N is released as NH_3 into the cytosol where it can be incorporated into plant amino acids, by the several enzyme systems present. This diagram shows both the GDH and GS-GOGAT pathways, the transaminases GOT and GPT, and stresses the central key role of αKG for operation of these ammination pathways.

As reviewed in Chapter II the GS-GOGAT pathway has a low Km for its



I. Nitrogenase (Nase) (EC 1.7.99.2)
II. Glutamate dehydrogenase (GDH) - (L-glutamate:$NAD(P)^+$ oxidoreductase deaminating EC, 1.4.1.3)
III. Glutamine synthetase (GS) (L-glutamate: ammonia ligase (ADP), EC 6.3.1.2)
IV. Glutamate Synthetase (GOGAT) (L-glutamate: $NAD(P)^+$ oxidoreductase (transaminating)
V. Glutamate-oxaloacetate transaminase (GOT)(L-apartate:2-oxoglutarate aminotransfrase, EC 2.6.1.1)
VI. Glutamate-pyruvate transaminase (GPT)(L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2)
αKG. alpha ketoglutarate (2-oxoglutarate)

Figure 22. A Schematic Composite of Enzymatic Pathways Proposed for N Assimilation Within Copada Nodules

substrates and can utilize low levels of NH_3, whereas the high Km of GDH for ammonia, requires higher concentration of this substrate before glutamate dehydrogenase can play any important role in the assimilation of symbiotically fixed nitrogen. Because high levels of NH_3 is believed to inhibit Nase activity, the ammonia "scavenging" ability of the GS-GOGAT pathways suggests that these are the enzymes involved in the utilization of symbiotically fixed N.

However, the correlation coefficients from Table XXXVII indicate that both pathways have the potential to assimilate the newly fixed N in the Copada nodules. Further support to this can be obtained from Table XXXVIII. This table was obtained by eliminating the fertility effects on the specific activity of the enzymes determined in the fourth experiment. The enzymatic activity of nitrogenase was calculated by dividing μM C_2H_4/mg protein/min by the theoretical value three as indicated by the following relationship:

$$N_2 \xrightarrow{6e^-} 2NH_3 \qquad (27)$$

$$C_2H_2 \xrightarrow{2e^-} C_2H_4 \qquad (28)$$

Thus the electron ratio between equation (27) and (28) is $6e^-/2e^- = 3$, i.e., for each 3 μM C_2H_4 formed per mg protein/min, 1 μM NH_3/mg protein/min will be reduced.

The level of alpha ketoglutarate was also expressed as μM αKG/mg protein, so that equimolar comparisons can be made.

Some important conclusions can be drawn from the data in Table XXXVIII.

Firstly, it indicates that the activities of the NH_3 assimilatory pathways are several fold higher than that of nitrogenase, thus they

TABLE XXXVIII

SPECIFIC ACTIVITIES OF THE ENZYMATIC PATHWAYS OF NH_3 ASSIMILATION ON CRATYLIA FLORIBUNDA NODULES AFTER POOLING OF FERTILITY EFFECTS - 4TH EXPERIMENT

Enzyme	Specific Activities
Nase	0.0779 μM NH_3/mg prot/min
GS	0.6505 μM glutamine/mg prot/min
GOGAT	0.4291 μM glutamate/mg prot/min
GDH	0.5103 μM glutamate/mg prot/min
GOT	1.5988 μM aspartate/mg prot/min
GPT	0.5096 μM alanine/mg prot/min
Alpha Ketoglutarate	0.0689 μM αKG/mg prot

both can account for NH_3 incorporation into amino acids. The activities of GDH, GS, GOGAT and GPT are within the same range, but a three-fold increase in activity was observed for GOT. The combined activity of GDH and GOGAT can account for the synthesis of only half of the glutamate being utilized by GOT and GPT. This observation apparently suggest that another source other than GDH and GOGAT was providing glutamate for transamination reactions.

The high specific activities of these enzymes in relation to nitrogenase, also indicates that these enzymes are not specific for assimilation of symbiotically fixed N, and are in fact involved in other nodule physiological processes.

A second, and perhaps more important indication of these data, is that the process of symbiotic N fixation in the Copada nodules, after the three clippings, was not limited by either nitrogenase or the activity of the NH_3 assimilatory pathways. Instead, levels of alpha ketoglutarate appear to be the limiting step for increased levels of N fixation, since in an equimolar basis the level of alpha ketoglurate can account for the utilization of only 88.5% of the ammonia formed by nitrogenase activity.

Under these conditions a possible way to increase nitrogenase activity would be to bypass the αKG shortage by increasing activity levels of enzymes, such as aspartate and alanine dehydrogenases, which can catalize the reductive amination of alpha keto acids other than alpha ketoglutarate, namely oxaloacetic acid and pyruvic acid.

Linear regression models were used in the 4th experiment to distinguish between the GDH and GS-GOGAT pathways as to which would account for the largest variation of either GOT or GPT when Nase and αKG were used as independent companion variables in the models.

To test for nonlinearity, a plot of residual was generated in the computer. Apparently, deviations from nonlinearity were not detected, and if present, are not likely to be strong.

The equations were generated using enzyme activity expressed as U/mg protein and best models were selected on r^2 improvement and the value of the standard deviation associated with the mean of the dependent variable.

The best models fitted for GOT were:

$$GOT = 4.658 + 0.015\ Nase + 1.485\ \alpha KG + 1.308\ GDH \qquad (29)$$

$$r^2=0.52 \quad s=5.707$$

$$GOT = 5.111 + 0.008\ Nase + 0.776\ \alpha KG + 0.201\ GS + 1.672\ GOGAT \quad (30)$$

$r^2=0.60 \quad s=5.291$

$$GOT = 4.505 + 0.008\ Nase + 0.955\ \alpha KG + 0.168\ GS + 1.392\ GOGAT + 0.472\ GDH \quad (31)$$

$r^2=0.61 \quad S=5.292$

An analysis of the r^2 and s values indicates the variations on GOT activity was associated with both GDH and GS-GOGAT pathways. However the GS-GOGAT can account for about 8% more of the variations in GOT, and equation (30) was not significantly improved when GDH was introduced as another independent variable.

Despite the fact the F values associated with the models were significant for P=0.0001 no more than 61% of the variation on GOT was explained by the models. This gives some support to the observation made earlier that a third source of glutamate, besides the GDH and GOGAT pathways might be present in the Copada nodules, and could account for some of the GOT activity.

Similar models were tested for GPT and the ones with highest r^2 and lowest s are listed below:

$$GPT = 1.570 + 2.007\ Nase - 0.620\ \alpha KG + 0.387\ GDH \quad (32)$$

$r^2=0.68 \quad s=1.72$

$$GPT = 1.539 + 0.004\ Nase - 0.804\ \alpha KG + 0.149\ GS + 0.516\ GOGAT \quad (33)$$

$r^2=0.83 \quad s=1.26$

$$GPT = 1.557 + 0.004\ Nase - 0.810\ \alpha KG + 0.150\ GS + 0.524\ GOGAT - 0.014\ GDH \quad (34)$$

$r^2=0.83 \quad s=1.28$

Similar to the GOT models, these equations indicate that both GDH and GS-GOGAT pathways can account for some of the variations in GPT

activity. However, in this case the GPT activity was more associated with glutamate levels derived from the GS-GOGAT pathway than the GDH.

Although caution must be exercised during the interpretations of these equations, they indicate that both GDH and GS-GOGAT pathways were present and active in the nodule of the Copada plants used in these studies, thus corroborating the data on Tables XXXVII and XXXVIII. However, these data also indicate that usually more precise determinations of the activities of the transaminases, GOT and GPT, can be made by the models containing the enzymes GS and GOGAT, without an advantage being observed for fitting GDH as an independent factor in these equations.

CHAPTER V

SUMMARY AND CONCLUSION

The principal objective of this study was to evaluate the effect of fertility treatments on the regrowth vigor, nodulation, nitrogenase, nodule enzymes and carbohydrate components of Copada (Cratylia floribunda, Benth) when cultivated in a dark red latosol, from Brazil.

Copada is a underutilized forage leguminous plant indigenous to large areas of Brazil, that has recently received attention as a potential grazing plant with desirable drought tolerance and high regrowth vigor.

The dark red latosol (Typic Eutrustox, isohyperthermic, fine, kaolinitic), used in this study is an important soil resource in northern Minas Gerais, where Copada is an indigenous species. Presently within the collection site area, this latosol has been used mostly for extensive beef cattle raising and production of some cash crops.

A secondary objective of this study was to determine pathways for assimilation of symbiotically fixed nitrogen into plant amino acids.

Four greenhouse experiments are reported in this monograph. In all cases the experiments were carried out in randomized complete block design, with 3 replications, and experimental pots were filled with a mixture consisting of 100 g of soil (100% passed sieve 20) and 400 g of medium particle size quartz sand. Before use, the sand was washed

once with a 0.1 NHCl solution and several times with distilled water for HCl removal, which was tested by $AgNO_3$ for absence of Cl^- ion.

For all experiments, seedlings were first germinated in a vermiculite germinator, and then transplanted to experimental pots when they had one pair of true leaves. During transplanting the seedlings and the soil were inoculated with 3 ml of a liquid medium containing viable cells of Rhizobium leguminosarum isolated from nodules of Strophostyles sp.

Two preliminary fertility studies were carried out to determine the principal limiting factors for optimum Copada herbage production. Data from the first experiment indicated beneficial effects for P and S. A depressive effect was observed for KCl, but apparently K and S interact with P to produce higher yields. Less root dry matter was obtained in the KCl treatment, but a highly beneficial effect was observed for Ca.

In the second preliminary study the beneficial effect of the P + KS treatment observed in the first experiment was divided into P + NaS and P + KCl, and the plant response to a mixture of B, Mo, Zn and Cu was also studied. Results obtained indicated a negative effect for the levels of the micronutrient mixture, in both the dry herbage yield and root dry matter of Copada. Death of the seedlings was observed at the highest level of micronutrient application. The P + KCl treatment yielded better than the P + NaS, possibly indicating that KCl interacts more strongly than NaS with P to produce better yields. However, as in the first preliminary study highest herbage production was observed in the P + KS treatment. This two preliminary studies pointed to the prominent role of P as an important factor for increased

production of Copada forage in this dark red latosol.

In a third experiment the effect of plant age on nodulation and nodule physiology was studied. In this experiment all pots were fertilized with the P + KS treatment used in the preliminary fertility studies. Six plants were harvested at 24, 26, 30, 32, 37, 39, 44, 46, 57, 60, 62 and 67 days of age. Four nodule types were recognized in these plants: small nonstriped (ST) (<1 mm), small striped (SL) (<1 mm), medium striped (1 mm <MD<3 mm) and large striped nodules (LG) (>3 mm). Plant growth was slow until day 39, but herbage dry yield doubled between 39 and 46 days, with a steady rate of growth being maintained thereafter.

The effect of plant age and nodule size were studied on the following parameters: nodule fresh weight, total number of nodules, number of small nodules (<1 mm), number of medium and large nodules (>1 mm), nitrogenase (Nase) activity (C_2H_2 reduction) (EC 1.7.99.2), alpha ketoglutarate (αKG) (2-oxoglutarate), glutamate dehydrogenase (GDH) (L-glutamate: NAD (P)$^+$ oxidoreductase deaminating, EC 1.4.1.3), glutamine synthetase (GS) (L-glutamate: ammonia ligase (ADP), EC 6.3.1.2), glutamate synthase (GOGAT) (L-glutamate: NAD (P)$^+$ oxidoreductase (transaminating) EC 1.4.1.13), glutamate-oxaloacetate transaminase (GOT) (L: aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1), glutamate-pyruvate transaminase (GPT) (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2), pyridoxyl phosphates (PLP's), soluble protein (Prot), glucose, sucrose and starch. Except for nitrogenase, all enzymatic activity, αKG, soluble protein, PLP's, and carbohydrate levels were determined in the nodule cytosol. Bacteroid-free nodule extracts were obtained using ultra-sonication and high speed centrifugation procedures.

Results obtained indicated that fresh nodule weight linearly increased with plant age. Despite the fact that the total number of nodules per plant also increased with plant age, the increase in the fresh nodule weight/plant was more directly related to nodule growth. Plants usually had a high number of small nodules (ST + SL) up to day 39, but a decrease in their number was observed after day 44 with a linear increase being observed in the number of nodules larger than 1 mm (MD + LG). Nitrogenase activity ($\mu M\ C_2H_4$/g nod/hr) linearly increased with plant age, but little activity was detected in the small nodules. Apparently Copada nodules must be at least 1 mm in diameter before they can have significant levels of nitrogenase activity. Highest nitrogenase activity was observed in the striped MD and LG nodules, and compared to total fresh nodule weight/plant, total number of nodules/plant was a poor indicator of nitrogenase activity. Best prediction of Nase activity was obtained by linear regression models including total fresh nodule weight and number of nodules larger than 1 mm as independent variables.

Both plant age and nodule size influenced levels of alpha ketoglutarate. Highest αKG levels were observed in the nonstriped (ST) nodules with the striped ones (SL, MD, LG) having lower values. Levels also tended to be higher at 39 days and reach a lowest value at 46 day.

Usually highest glutamate dehydrogenase activity was associated with MD nodules. Except for day 57 lowest GDH activity was always found in the ST nodules.

Highest glutamine synthetase was found in the MD nodules and lowest in the ST ones. No plant age effect was observed in the activity

of this enzyme.

Unlike GS, levels of glutamate synthase were affected by plant age but not by nodule size. GOGAT activity peaks were observed at 30, 39 and 57 days.

A significant effect for age and nodule size was observed in nodule cytosol levels of glutamate-oxaloacetate transaminase activity. GOT activity tended to increase up to day 44 and then sharply decreased when the plants were 46 days old. However for all striped nodule sizes activity linearly increased afterwards. With regards to nodule size, GOT activity was lower in the small nonstriped nodules, and on the striped ones, activity increased linearly with size.

Glutamate-pyruvate transaminase activity for all nodule sizes was highest at day 39 and, sharply decreased at day 46 and then remained low thereafter.

Levels of the coenzymes pyridoxyl phosphates in this study were not affected either by plant age or by nodule size.

Levels of soluble protein increased up to day 39 and then remained constant until termination of the experiment. Medium size nodules had highest protein concentration, and lowest values were found in the nonstriped small nodules.

For the MD and LG nodules glucose content peaked at day 39 and then steadily decreased to lower levels. In the nodules smaller than 1 mm, glucose levels tended to decrease with plant age but the ST nodules had similar contents of this carbohydrates at days 32 and 57.

Increased sucrose levels were found in the small nonstriped nodules, and little difference was apparent in sucrose content of the striped nodules. The levels of this carbohydrate within the Copada

nodules were not influenced by plant age.

Small nonstriped nodules also had higher starch levels than the striped ones, and similar to the results obtained for sucrose levels, the amount of starch in these nodules was not influenced by plant age.

Apparently the data collected in this experiment indicate a marked difference of the physiological characteristics of the nodule sizes studied. Usually the nonstriped nodules contained larger amounts of αKG, sucrose and starch, and this may indicate large sinks for photosynthate assimilates. The nodulation sequence for these plants was biphasic, i.e., nodules were set in the roots of the Copada plants until day 39; only nodule growth occurred between days 39 and 54, and then apparently after a period of intense top growth, new nonstriped nodules developed again, probably on the new root growth.

The process of nodule growth was accompanied by induction of higher nodule enzymatic activity, and more mature nodules contained increased amounts of soluble protein.

Similar activity levels were observed for GDH and GOGAT, thus indicating that both GDH and GS-GOGAT pathways for NH_3 assimilation into plant amino acids were present in these Copada nodules.

A fourth experiment was conducted to study the effects of soil fertility treatments on plant growth, nodulation, and on the several nodule physiological characteristics studied on the third experiment.

Fertility treatments consisted of a 2^5 complete factorial using 50 ppm of both P and S, 6 meq/100 g soil of Ca, and 2 meq/100 g of soil of Mg and K, making a base cation ratio equal one.

$$BCR = \frac{K}{\sqrt{\frac{Ca + Mg}{2}}} = 1$$

A summary of the analyse of variance obtained in this experiment appears on Table XXXIX. Stars represent significancy levels for the Δ effect of a given nutrient and the minus sign denotes significantly negative effects. Δ effect was defined as the difference between the mean of the 48 pot cultures with a particular nutrient and the mean of the pot cultures that did not receive that nutrient.

Results obtained strongly indicated that P was the most influential plant nutrient with the parameters studied. It had a large and long lasting beneficial effect on plant regrowth, root dry weight, and nodule weight and number. It also significantly increased nodule soluble protein and glucose levels, but had no influence on pyridoxyl phosphates, sucrose, and starch.

On the other hand, however, contrasting results were observed on the nodule cytosol enzymatic activities. Apparently increased nitrogenase activities were observed in pots fertilized with P (μM C_2H_4/pot/hr), but nonsignificant results were found when results were expressed as μM C_2H_4/g fresh nod/hr, and a negative effect was noted if nitrogenase activity was calculated to enzyme specific activity (μM C_2H_4/mg protein/min).

However, an explanation for thesecontrasting findings can be proposed if the effect of P on nodule weight, nodule number and soluble protein is taken into consideration.

Increased measurement of nitrogenase activity was obtained within P fertilized pots because P also increased the number of nodules exhibiting nitrogenase activity and the negative effect obtained

when activity was expressed as μM C_2H_4/mg protein/min, indicates that the protein accumulation on the nodule cytosol of the P fertilized plants did not have nitrogenase activity.

Similar interpretation can be offered for the P effect on the activity of glutamate dehydrogenase, glutamine synthetase, glutamate synthase, glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase. In all cases P increased enzymatic activity if results were expressed as U/g nodule, but negative effects were noted if activity was expressed as U/mg protein. These observations strongly suggest that the protein accumulated in these nodules did not have GDH, GS, GOGAT, GOT or GPT activity.

It can be concluded then, that P is capable of increasing plant enzymatic activity on a nodule fresh weight basis, but is not capable of increasing the nodule enzymatic specific activity.

The results obtained for Mg are difficult to interpret. This nutrient had beneficial effects on the herbage yields of the first three clippings and increased root growth, but apparently had a significantly negative effect on the activity of the enzymes of NH_3 assimilation. On the other hand no effect was observed on nodulation, nitrogenase activity, pyridoxyl phosphates, nodule soluble protein, glucose and sucrose. However, nodule cytosol of Mg fertilized plants had significantly less alpha ketoglutarate and starch.

Because these nodules had depressed levels of nitrogen utilization, these results tend to indicate that the increased herbage yields obtained in the Mg fertilized plants were not related to the process of symbiotic nitrogen fixation.

Sulfur and potassium also increased herbage production for some

clippings but little effect was observed for these nutrients on the nodule physiological characteristics.

Sulfur increased GDH specific activity, and depressed GPT activity and levels of the carbohydrates glucose and starch, whereas potassium had a negative effect on the specific activity of GDH but favored higher levels of glucose.

Despite the fact that Ca fertilized plants had nodules with increased GOGAT activity and higher levels of αKG, PLP's, soluble protein and starch, this nutrient depressed herbage production on the first two clippings. Moreover, fertilization with this nutrient also depressed root growth and glucose and sucrose levels in the nodules

The analyses of correlation for the enzymatic specific activities indicate that nitrogenase activity was significantly associated with activity of the enzymes of ammonia assimilation, and with the transaminases GOT and GPT. The highest correlation coefficient observed was between the activities of GDH and GOGAT ($r=0.96$), thus indicating that these two pathways for glutamate synthesis follow parallel trends. However, as it has been suggested that substrate concentration and allosteric inhibition prevents both enzymes systems from being active at the same time some kind of compartmentalization of these pathways must be visualized.

When experimental results were averaged over all fertility treatments and equimolar concentration comparisons were made, it became apparent that both GDH and GS-GOGAT pathways of NH_3 incorporation into amino acids, had activities about 6-7 fold higher than NH_3 synthesis. Moreover, the rate of glutamate synthesis by the combined action of these two assimilatory pathways could account for only half of the

TABLE XXXIX

STATISTICAL F TEST SIGNIFICANCY LEVEL FOR SOIL FERTILITY TREATMENT EFFECTS ON REGROWTH, NODULATION AND NODULE PHYSIOLOGICAL CHARACTERISTICS OF CRATYLIA FLORIBUNDA, DARK RED LATOSOL, BRAZIL

Parameters	P	S	Ca	Mg	K
1st clip - g/pot	***	*	-***	*	
2nd clip - g/pot	***	***	-#	*	**
3rd clip - g/pot	***			*	***
4th clip - g/pot	***				
Root dry wt - g/pot	***		-#	#	
Nod wt - g/pot	***				
No. of nod	***				
μM C_2H_4/pot/hr	***				
μM C_2H_4/g nod/hr					
μM C_2H_4/mg prot/min	-***				
αKG - μM/g nod			**	-#	
GDH - U/g nod	*			-*	
GDH - U/mg prot	-**	#		-*	-#
GS - U/g nod	**			-*	
GS - U/mg prot	-***			-*	
GOGAT - U/g nod	***		***	-**	
GOGAT - U/mg prot	-*				
GOT - U/g nod	***				
GOT - U/mg prot	-*			-#	
GPT - U/g nod	***	-#			
GPT - U/mg prot	-*			-#	
PLP's - μg/g nod			*		
Prot - %	***		**		
Glucose - mg/g nod	#	-***	-#		*
Sucrose - mg/g nod			-***		
Starch - mg/g nod		-#	*	-*	

#, *, **, *** significant for P≤0.1, 0.05, 0.01 and 0.001 respectively. Minus sign indicates depressive effect.

glutamate being used in transamination reactions. Thus, the NH_3 incorporation pathways did not constitute a limiting step on the process of symbiotic N fixation for these Copada plants.

In fact, the data obtained indicated that a shortage of photosynthate assimilates within the nodules apparently controls the efficiency of the symbiotic process.

Levels of the TCA cycle intermediate alpha ketoglutarate, were not sufficient to account for the utilization of the ammonia formed by nitrogenase activity. Increased activity of alanine dehydrogenase and aspartate dehydrogenase can bypass the shortage of αKG, but the activity of these enzymes would still be dependent on the availability of pyruvic acid and oxaloacetic acid, which are also products of carbohydrate oxidation.

Linear regression models were developed using the data from the 4th experiment to distinguish between the importance of the GDH and the GS-GOGAT pathways with regards to assimilation of symbiotically fixed nitrogen. An analysis of the models obtained indicates that fixed N was assimilated into amino acids by both pathways but more of the variation on GOT and GPT levels were associated with the GS-GOGAT enzyme system.

Because of the low nitrogenase activity in relation to the other enzymatic activities it is possible that these Copada plants also utilized soil N to supply their full nitrogen requirements.

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