Effect of Molybdenum and Phosphorus On Growth, Nodulation, Biomass Production And Nitrogen Fixation in *Sesbania rastrata*

By

V. Subhashree

A THESIS SUBMITTED TO THE AVINASHILINGAM INSTITUTE FOR HOME SCIENCE AND HIGHER EDUCATION FOR WOMEN (DEEMED UNIVERSITY), COIMBATORE - 641 043

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN BIOCHEMISTRY

MAY 1997

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Certified as Bonafide Research Work.

12/5/97

Signature of the
Head of the Department.

12/5/97

Signature of the Dean
of faculty of Science

12/5/97

Signature of the Guide.

Acknowledgement

ACKNOWLEDGEMENT

The investigator expresses her gratitude to ***Dr. (Mrs.) RAJAMMAL P. DEVADAS****, M.A., M.Sc., Ph.D. (Ohio State), D.Sc.(Madras), Hon. D.H.L. (Oregon State), Hon. D.H.L. (Ohio State), Hon. D.Sc. (C.A. Agri. University, Kanpur), Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, for providing the opportunity to conduct the study.*

The author records her sincere thanks to ***Dr. (Mrs.) LAKSHMI SANTA RAJAGOPAL****, M.S(Tennessee), Ph.D. (Madras), Vice Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, for providing facilities to carry out the study.*

The author extends her deep sense of gratitude to ***Dr. (Mrs.) SAROJA PRABHAKAR****, M.A., Diploma in Education (Madras), Ph.D.(Mother Teresa), Registrar and* ***Mrs. C.V.R. INDIRA*** *M.Sc., Ph.D (Madras), Dean, Faculty of Science, Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, for facilitating the conduct of the study.*

The investigator expresses profound sense of faithfulness and thanks to ***DR.(MRS.) NIRMALA KRISHNAMURTHY****, B.Sc., (Hons.), MS (Iowa), Ph.D. (Madras), Professor and Head of the Department, Biochemistry, for her learned counsel and sustained encouragement.*

She places her sincere thanks to ***DR. SAROJA*** *S. M.Sc., M.Phil., Ph.D., (Madras), Reader, Department of Biochemistry, Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, for her constant support and valuable suggestions in embellishing this study.*

The investigator is more indebted to her guide ***MRS. SIVAGAMI SRINIVASAN****, M.Sc., M.Phil., Dip. H. Ed., Lecturer (Selection Grade), Department of Biochemistry for her inspiring guidance, esteemed advice, encouragement and titanic help rendered during the study.*

The investigator registers her thankfulness to ***DR.(MRS.)PARVATHY****, M.Sc., Ph.D., Associate Professor, Centre for Cellular and Molecular Biology, TNAU,* ***MRS. MAHESWARI****, M.Sc., Lecturer, Department of Environmental Sciences, TNAU and* ***DR. D. PURUSHOTHAMAN****, M.Sc., (Ag), Ph.D., Madras, Associate Professor of Micro Biology, TNAU, Coimbatore for the timely help offered during the study.*

The author places on record her gratitude to ***ICAR*** *for granting scholarship for the conduct of the study.*

The investigator expresses her grateful thanks to all the ***staff members*** *of the Department of Biochemistry.*

The investigator wishes to thank her ***friends*** *for their timely help. She owes the completion of this endeavour to her* ***parents and brother*** *for the inspiration and encouragement rendered during the course work.*

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Introduction

1. INTRODUCTION

Nitrogen gas constitutes 80% of earth's atmospheric air. This inexhaustible supply is not availed by plants and animals directly because of their inability to assimilate nitrogen in its free from (Rajesh, 1997). However, there are few microorganisms like Rhizobia which fixes atmospheric nitrogen and other soil microbes helps in the conversion of amide forms of nitrogen to nitrate which is easily taken up by plants (Basavaraj et al., 1997).

To meet the food demands of an increasing population, tremendous pressure is being put on land resources to fulfill their potential (Joshi et al.,1994). The economic and environmental costs of the heavy use of chemical nitrogen fertilizers have been seriously restricted (Senaratne and Ratnasingh, 1995). Leguminous green manure crops or nitrogen rich plant residues are promising alternatives to mineral fertilizers for cereal crops (Becker et al., 1994).

Rice food crops meets the food demand of 40% population of the globe (Rajkumara, 1996). Nitrogen is the nutrient most limiting rice production worldwide (De Datta, 1995). Nitrogen use efficiency is critical to increase rice productivity (Ladha, 1996). Biological Nitrogen fixation has the potential to meet the substantial portion of nitrogen required by rice plants (Pillai and Nair., 1995).

Bilogical Nitrogen fixation by biofertilisers is an important process in flooded rice field ecosystem and contribute nitrogen to the rice crop (Kalidurai and Kannaiyan, 1992). The Rhizobia involved in nodulation can influence the

percentage and amount of nitrogen fixed by the legume Rizobium symbiosis (Sprent,J.J., 1994).

Biofertilizers have an important role to play in improving the nutrient Supplies to Indian Agriclture. Among Biofertilizers for increasing nitrogen supply, Nitrogen fixing bacteria (Rhizobium, Azorhizobium, blue green algae) and for enhancing Phosphorus supply phosphorus solubilising bacteria are important (Hegde and Dwivedi, 1994). The global energy crisis and increasing fertilizer costs have necessitiated the use of supplementary nitrogen (Kandaswamy and Ramaswamy, 1996). The incorporation of nitrogen fixing green manure viz.S.rostrata in the rice fields releases nitrogen on mineralisation (Kumar et al 1992). Green manures and crop residues contain most of their nitrogen in organic form and mineralisation is a prerequisite for plant uptake (Rees et al., 1993). Green manuring can favourably influence the availability of several plant nutrients, improve the physical and chemical conditions of the soil, increases water retention and reduces leaching losses of nutrients (Rekhi and Bajwa, 1993). Green manures may serve as a direct source of nutrients (Prasad et al. 1995). Growing a green manure crop before a rice crop is therefore an attractive and efficient way of improving soil productivity (Becker et al., 1988).

Sesbania species in symbiotic association with Rhizobium have proved promising and popular in Asia as sources of biofertilizers due to their rapid growth and wide tolerance to a range of edaphological conditions (Rao and Gill, 1993). **S.rostrata**, salt tolerant species produce both root and stem

nodules (Joshua et al., 1995). **S.rostrata** can fix more than 200 kg of Nitrogen/ha in 50 days (Robertson, et al 1995). Green manuring with Sesbania spp. considerably improved organic carbon, available N,P & K status and enhanced the uptake of N,P, Ca, Mg, Fe, S, Mn and Zn by the crop (Swarup, A., 1987) With increasing cost of fertilizer nitrogen complex and interactive problems, integration of fertilizer nitrogen with low priced organics is being emphasised (Tiwari and Singh, 1995). Hence S. rostrata is more suitable as a green manure for rice crop (Balasubramani and Kannaiyan, 1993) and efforts to enhance the growth of Sesbania is therefore important.

Mineral nutrient deficiencies are a major constraint limiting legume Nitrogen fixation and yield (O'hara et al., 1988). Besides macronutrients, micronutrients are also found to enhance the legumes yield (Kishor et al. 1991). Legumes in general have a high requirement for both Molybdenum and Phosphorus in the nodules for nitrogen fixation (Rebafka et al., 1993).

Molybdenum plays an important role in the nutrition of Rhizobium and in Nitrogen fixation process (Daterao et al., 1992). Phosphorus is essential for root growth, nodule formation and seed production (Vijayakumar and Gopalakrishna, 1996). The physiological effect of phosphate on nitrogen fixation is indirect one and is presumably related to the metalolism of the host plant (Mengel, K., 1994). The most sensitive process to phosphate deficiency is the synthesis of ribonucleic acids and therefore meristematic growth which may also have am impact on the growth of nodules.

The present study was undertaken to find the effect of Molybdenum and Phosphorus both individually and in combination on the growth, nodulation and biomass production of **Sesbania rostrata.**

The plants were uprooted at the end of 30, 45 and 60 days and the following biometric and biochemical parameters were analysed. The biometric parameters studied were root length, shoot length, fresh weight, dry weight, root and shoot nodule number.

The biochemical parameters studied were chlorophyll, Allantoin, Amino Nitrogen, Total Nitrogen, Phosphorus, Potassium and Ammonia assimilating enzymes such as Glutamate dehydrogenase, Glutamate Synthase, Glutamine Synthetase, and Nitrate Reductase.

Review of Literature

2 REVIEW OF LITERATURE

The Review of literature pertaining to the present study "Effect of Phosphorus and Molybdenum on Growth, Nodulation and Biomass Production of **Sesbania rostrata**" is discussed under the following headings.

2.1 Introduction

2.2 Biofertilisers and Biological Nitrogen Fixation.

2.3 Rhizobium

2.4 Legume Rhizobium Symbiosis

2.5 Nodulation

2.6 Green Manure

2.6.a. Its potential benefits.

2.6.b. **Sesbania rostrata -Azorhizobium caulinodans** symbiosis

2.6.c. Nitrogen fixation by Green Manure in Rice fields

2.7. Enzymes of Nitrogen fixation and ammonia assimilation

2.8. Effect of Micronutrient and Macronutrient - Molybdenum and Phosphorus on the growth and nodulation of **Sesbania rostrata.**

2.1 Introduction

In Irrigated Agriculture, large Scale uses of chemical fertilisers causes problems of pollution and soil deterioration (Panda, 1997). Moreover, the increase of chemical fertiliser price associated with the excessive application of chemical fertilisers have led to the promotion of utilisation of biofertilisers (Mappaona,et al., 1995). Biofertilisers are environment friendly, low cost

agricultural input playing a significant role in improving nutrient availability to the crop plants (Tilak and Singh, 1994). The use of organic manures in combination with fertilisers is essential for sustaining soil productivity (Bindra and Thakur, 1995). Intergrated use of plant nutrients is important for supplying the crop demand, balanced fertilisation and sustainability of crop production.(Rajamani et .al .,1996)

2.2 Biofertilizer and Biological Nitrogen Fixation :

Biofertilisers in general means generation of certain plant nutrient in response to introduction of appropriate micro organisms (Katyal et al, 1994). Biofertilizers are increasingly used due to the increasing cost of inorganic fertilizers(Gomathi et al., 1996). Biofertilizers help in increasing the biologically fixed atmospheric nitrogen and enhancing native Phosphorus availability to crops. Among biofertilisers for increasing Nitrogen supply, nitrogen fixing bacteria, Rhizobium, Azotobacter, Azospirillum, blue green algae and Azolla are important (Singh and Dwivedi, 1996). Use of phosphobacteria a biofertiliser which could solubilise the fixed phosphorus in the soil through the secretion of organic acid is a low cost technology. (Ahmed.N.& Parameswaran, 1997). As biofertilisers fixes atmospheric nitrogen in the soil, it reduces application of chemical nitrogen fertiliers to the extent of 15-20 kg/ha (Majjigudda and Badigannavar, 1996).

Nitrogen is important among the 13 essential minerals required by the plants (Badigannavar and Angadi, 1997). Hence, Biofertilisers have to play a definite supplementary

and complementary role to chemical fertilisers in supplying plant nutrients in the country (Hegde and Dwivedi, 1994).

Biological Nitrogen Fixation is estimated to be about around 175 million tonnes of which close to 79% is accounted for by terrestial fixation.(Burns and Hardy.,1988). Symbiotic biological nitrogen fixation is a combined process of both host plant and rhizobia and factors that influence the growth and survival of both the host plant and microbial symbiont are important. The exchange of metabolites between the symbionts is a much more complex process.(Rajesh.,1997). Legumes applied as green manure is an important source of nitrogen in many crop production systems (Janzen and Radder, 1989).

2.3. Rhizobium

Inoculation of legumes with specific species of Rhizobium is a widely used and successful practice throughout the world .(Singh & Dwivedi, 1996) In Bangladesh, application of Rhizobium and Bradyrhizobium has been widely used to improve the production of food legume crops (IRRN,1995). Rhizobium is a genus of gram negative, aerobic bacteria which form nodule in roots of leguminous plants (Patronobis, 1994). The more recently described genus Azorhizobium (type species **A. Caulinodans** strain ORS571) constitute a group of bacteria that are capable of inducing the formation of nitrogen fixing nodules on the roots of leguminous plants (Geothals.K et al., 1992).

2.4 Legume Rhizobium Symbiosis

Legume crop is a natural mini nitrogen manufacturer

factory in the fields (Prasad,R., 1996). Rhizobium legume symbiosis could meet more than 80% nitrogen needs of the legume crop. The nitrogen fixed by bacteria not only meets all the nitrogen needs of a legume crop but also a sizeable amount is left for the succeeding crop (30 - 90 kgN/ha) . Nodules fix atmospheric nitrogen and supply the same to the crop because of the symbiotic relationship between the bacteria and the crop (Narasimhan, L., 1997). Many leguminous nitrogen fixing plants are nodulated by bacteria of the species Rhizobium (Rajesh, 1997). Leghemoglobin (LHb) represents an essential component in legume Rhizobium symbiosis, since it is required for oxygen transport to the actively respiring nitrogen fixing bacteroids in the nodule (Pawlowski et al., 1993).

2.5 Nodulation in Sesbania rostrata

When Rhizobium coated seeds are sown and given life irrigation, the bacteria will also germinate along with seeds and enter the radicle of seedling through root hairs and establish their symbiotic nature. In 15 - 20 days they produce small visual nodules on the main stem just one inch below the soil level. Each nodule will contain a number of bacteria called bacteroids. These bacteroids will contain leghemoglobin content which produce nitrogen fixation.The efficiency of Rhizobia in Biological Nitrogen Fixation depend upon selection of strains of cultures, inoculation methods and soil conditions. When proper bacteria cultures are used, the size of nodules can be increased (Narasimhan, L., 1997). Stem nodules express high rates of nitrogen fixation (Parsons et al., 1993).

2.6 Green Manure

Increase in cost of fertilisers, world wide energy crisis, rapid exhaustion of fuel and their limited availability have inspired the scientists to rely more on renewable resources through integrated nutrient supply (Thakur et al., 1995). Biological nitrogen fixation by a leguminous crop is a low cost input for rice crop (Balasubramani,et al 1992). Legumes are superior green manure crops because they fix atmospheric nitrogen (Kolar et al., 1992). The advantages of green manuring are due to greater nitrogen availability in the soil higher nitrogen recovery and an increased contribution towards the grain production of the rice (Sharma and Das, 1994). Green manuring significantly increased the content of available nitrogen in the soil (Thakur et al., 1995).

Apart from fixing nitrogen, incorporation of these green manures in farm land will accrue benefits like timber, fuel, wood, fodder, biofertilizer etc., (Rajesh, 1997). In a study by (Kalidurai and Kannaiyan, 1992) species of **Sesbania** have shown common ability to withstand water logged condition.

Among several types of leguminous green manure used **S. rostrata** is an outstanding nitrogen fixer and most acceptable to Asian farmers (Sadasivam and Lakshmi, 1989). **S. rostrata** can be successfully exploited as a green manure when stem cuttings are planted under flooded conditions (Patel et al., 1996). **S. rostrata** nodulates on both root and aerial stem (Becker and George ., 1995). The nodules in **S. rostrata** are 5 - 20 times more than most of the nodulated crop plants

(Halepyati and Sheelavantar, 1990).

S. rostrata tolerate prolonged soil flooding owing to the formation of aerenchyma on roots and the lower stem (Ladha et al., 1992b). **S. rostrata** showed a high nitrogen accumulation of which the major portion about 80% is derived from biological nitrogen fixation (Becker et al., 1995).

2.6.a. Its Potential Benefits

The significant beneficial effect have been obtained from legume green manuring of wetland rice. These effects are mainly attributed to rapid mineralisation of the green manure, allowing an increase in the uptake of nutrient by crops. (Bhardwaj and Datt, 1995). Green manuring definitely contributes sizeable amounts of plant nutrients, particularly nitrogen and brings improvement in soil physical conditions (Singh and Dwivedi,1996). (Buresh and Dedatta, 1991) reported higher efficiency of green manure than that of mineral nitrogen and (Ladha et al., 1992) reported residual effects from several years of green manure application of about 5 - 10 % compared to fertiliser nitrogen.

2.6.b. Sesbania rostrata Azorhizobium symbiosis

A. caulinodans is able to nodulate roots and stems of tropical legume **S. rostrata** (Geothals K et al., 1994).Dreyfus et al (1988) Characterised stem nodule Rhizobium and designated as **A. caulinodans. S. rostrara** stem isolates of **A. caulinodans** produced higher number of nodules on stem in water logged condition on 35th, 45th and 60th day after sowing (Balasubramani and Kannaiyan, 1993). **A.Caulinodans** (a diazotroph known as a specific endophyte of **S.rostrata**) was

introduced and became established inside rice Paranodules and in root tissues around tumor bases. Intracellularly estalibshed **A. Caulinodans** was found inside the cytoplasm (Weniger, 1996).

2.6.c Nitrogen fixtation by green manure in rice fields

Rice is an important staple food crop in India (Singh and Grewal, 1996). Agriculture today consumes high input of nitrogen.(Financial Express (1993) Nitrogen fixing plants are often incorporated into soil as green manure to enhance soil fertility.(Robertson and Alexander, 1994). Green manures have consistently shown positive contributions to tropical rice porductivity (Torres et al., 1995) When preceded by a green manure crop, the yield of rice increased (Kalidurai and Kannaiyan, 1990) An increase of 65% grain yield was reported due to incorporation of **S.rostrata** The nitrogen content of the plant and yield were very high in treatments having **S.rostrata** incorporation than with highest dose of nitrogen (Sivakumar and Sundaram, 1989).

Stem nodulating legumes assume importance as they can tolerate water logged conditions and also fix atmospheric nitrogen (Rajkumara, 1996). Hence also **S.rostrata** is a promising species for low land rice farming systems (Becker et al., 1991) .

2.7 Enzymes Involved in Nitrogen Fixation and Assimilation

Nitrate Reductase is a key enzyme of inorganic metabolism (Mahurkar et al., 1992). Nitrate reductase

earlier in about 20% stage of its growth (Bhattacharya and Jain, 1996).

Experimental Procedure

3. EXPERIMENTAL PROCEDURE

An experiment was conducted to study the individual and combined effect of Molybdenum and phosphorus on the growth, nodulation and Biomass pproduction in the green manure **Sesbania rostrata .**

3.1 Preparation of soil :

The soil was tested for its Nitrogen, Phosphorus, Potassium, Calcarity, pH and electrical conductivity before usage. The soil was enriched by the application of farm yard manure at the rate of 10 tonnes/ha. In all the pots, 10kg of soil was filled and treated with Phosphorus in the form of superphosphate and Molybdenum in the form of Sodium molybdate at the concentrations of 50 and 100 kg P/ha and 5 and 10kg Mo/ha according to the treatments.

3.2. Seed Treatment :

Seeds of **S. rostrata** were collected from paddy breeding station, Thondamuthur. Seeds were soaked in hot water, boiled to $70^{o}C$ for 6 hours and shade dried for three hours, treated with peat based inocula of **Azorhizobium caulinodans** with rice kanchi as an adhesive agent.

3.3 Pot Culture layout and treatments :

The study was carried out in pot cultures in completely randomised block design with four replications for each treatment.

The treatments were as follows :

T_1 - **Azorhizobium** seed inoculated **S.rostrata** alone.

T_2 - **Azorhizobium** seed inoculated **S.rostrata** and 50 kg of Phosphorus/ha.

T_3 - **Azorhizobium** seed inoculated **S.rostrata** and 5 kg of Molybdenum/ha.

T_4 - **Azorhizobium** seed inoculated **S.rostrata** and 50 kg of Phosphorus and 5kg of Molybdenum/ha.

T_5 - **Azorhizobium** seed inoculated **S.rostrata** and 100 kg of Phosphorus/ha.

T_6 - **Azorhizobium** seed inoculated **S.rostrata** and 10 kg of Molybdenum/ha.

T_7 - **Azorhizobium** seed inoculated **S.rostrata** and 100 kg of Phosphorus and 10kg of Molybdenum/ha.

3.4 Sowing and Watering:

Thirty three furrows were made in each pot and each furrow was filled with a seed. The furrows were then closed with the soil. After the germination of seeds, water logging condition was maintained.

3.5. Foliar Spraying :

Foliar inoculation was done on 15th and 30th day after sowing. The stem isolates of **A.Caulinodans** used for foliar Mannitol medium consisting of :.

Mannitol - 1.0g	Yeast extract - 0.5 g
K_2HPO_4 - 0.5 g	Nacl - 0.1 g
$MgSO_4$ - 0.2g	Distilled water - 1000 ml.

3.6. Maintenance of Crops:

Nuvacron and Bavistin (0.05%) were sprayed on 15th day

after sowing. 100% moisture saturation was maintained through out the study.

3.7. Harvest Methodology :

At the end of 30, 45 and 60th day plants were uprooted from the pots without any damage to the root system. The roots were washed gently with water to remove any soil particles adhering to them. Plants were blotted with filter paper to adsorb the water droplets. Then the plants were used for various assays.

3.8 Biometric Observations :

(1) Fresh Weight :.

The fresh plants after removing the soil particles were weighed immediately and expressed as g/plant.

(2) Dry Weight :.

The uprooted plants after recording the fresh biomass were over dried at 70°c for 36 hours and expressed as g/plant.

(3) Root and Shoot height :

The height of the root and shoot were measured from the collar region to the tip of the root and shoot respectively and expressed in cm/plant.

(4) Nodule Number :

Soil particles were removed from the uprooted plants and the total number of stem and root nodules per plant were counted and recorded.

3.9. Biochemical Analysis :

The following Biochemical constituents like Chlorophyll, Allantion, Amino Nitrogen, Nitrogen, Phosphorus, Potassium and Ammonia Assimilating enzymes namely Glutamate dehydrogenase, Glutamate Synthase Glutamine Synthetase and Nitrate reductase were analysed.

The details of the parameters were studied, various parts of the plant used, Method of Analysis, and the relevant references are given in Table - 1.

3.10. Soil Analysis :

The procedures for estimation of soil Nitrogen, Phosphorus, Potassium and Molybdenum are described in Appendix.

3.11. Satistical Analysis :

The results of the experiment were analysed and statistical test by arithmetic mean and two way analysis of variance were done.

Table - I

Analysis of Biochemical Parameters and Enzyme Assay

S.No.	Parameters	Portion of plant	Method of Analysis	Reference	Appendix Number
Biochemical Analysis :.					
1.	Chlorophyll	Fresh leaves	Spectro-photometry	Yoshida, et al., 1971	I
2.	Allantoin	Fresh leaves	Spectro-photometry	Young and Conway, 1942	II
3.	Amino Nitrogen	Fresh leaves	Spectro-photometry	Spies (1955)	III
4.	Nitrogen	Whole plant dry sample	Microkjel-dahl Method	Humphries (1956)	IV
5.	Phosphorus	Whole plant dry sample	Spectro-photometry	Jackson (1973)	V
6.	Potassium	Whole plant dry sample	Flame photometry	Jackson (1975)	VI
Enzyme Assay					
7.	Glutamate Synthase	Fresh root and shoot	Spectro-photometry	Casteele et al. (1975)	VII
8.	Glutamate Dehydrogenase	Fresh root and Shoot	Spectro-photometry	Doherty, (1976)	VIII
9.	Glutamine Synthetase	Fresh root & Shoot	Spectro-photometry	Pateman (1969)	IX
10.	Nitrate Reductase	Fresh root	Spectro-photometry	Sadasivam & Manickam (1992)	X
SOIL ANALYSIS :					
11.	Available Nitrogen	Soil	Titrimetry	Subbiah & Asija	XI
12.	Available Potassium	Soil	Flame Photometry	Stanford & English(1949)	XII
13	Available Phosphorus	Soil	Colorimetry	Olsen et al., (1954)	XIII

Results and Discussion

4. RESULTS AND DISCUSSION

The effect of phosphorus and Molybdenum supplements on Growth , nodulation, Biomass production and nitrogen fixation in **Sesbania rostrata** was studied.

The study was carried out in pot cultures in completely randomised block design with four replications for each treatment. Red soil was used for the study and it was analysed for its nitrogen, phosphorus, potassium content, calcarity and pH before it was used. Farmyard manure was added at the rate of 10tonnes/ha to enrich the soil. 10kg of soil was filled per pot. Phosphorus at the concentrations of 50 and 100kg/ha and Molybdenum at the concentrations of 5 and 10kg/ha was added to various pots both individually and in combination. The result of the study are discussed under the following headings in this chapter.

4.1 Soil Analysis.

4.2 Biometric parameters, to evaluate the effect of **Azorhizobium** and micro nutrients on growth, nodulation in **S. rostrata.**

4.2.1 Root length.

4.2.2 Shoot length.

4.2.3 Fresh weight.

4.2.4 Dry weight.

4.2.5 Nodule number.

4.3 Biochemical parameters, to evaluate the effect of biofertiliser **Azorhizobium** on the growth of **S. rostrata.**

4.3.1 Chlorophyll.

4.3.2 Allantoin.

4.3.3 Aminonitrogen.

4.3.4 Total nitrogen, phosphorus and potassium.

4.4 Ammonia assimilating enzymes.

4.4.1 Glutamate dehydrogenase.

4.4.2 Glutamate synthase.

4.4.3 Glutamine synthetase.

4.5 Nitrate reductase.

4.1 Soil Analysis :

The soil which was used for the pot culture of **S. rostrata** was analysed for various macro nutrients like nitrogen, phosphorus, potassium, calcarity and pH. Table II reveals the same.

Table II

Soil Analysis

Avaiable Nitrogen	92 Kg/ha
Available Phosphorus	6 Kg/ha
Available potassium	318 Kg/ha
Calcarity	Non calcareous
pH	7.9

The soil was found to contain very low level of phosphorus which is a macro nutrient essential for the growth of plants. Hence phosphorus was applied in low and high concentrations along with the micro nutrient,Molybdenum to evaluate the effect on the growth of the experimental plant **S. rostrata**

4.2 Biometric Parameters

The growth attributes of **S. rostrata** such as root and

shoot length fresh and dry weight and the number of nodules were recorded on 30, 45 and 60 days after sowing. Plate 1 picturises the growth of **S. rostrata** on 60th day. These indices were considered as the effect of the bio fertiliser **Azorhizobium** and the minerals on the growth of **S. rostrata.**

4.2.1 Root length

Table III

Effect of Phosphorus and Molybdenum on shoot and root length of **S. rostrata**

	Shoot length(cm)			Root length (cm)		
Treatments	Days after sowing			Days after sowing		
	30	45	60	30	45	60
T_1	14.47	40.00	86.75	10.20	13.00	19.75
T_2	17.65	43.50	105.12	14.00	15.27	20.50
T_3	18.65	45.00	101.12	10.75	14.00	19.62
T_4	16.65	43.50	108.37	15.00	18.68	24.50
T_5	16.10	42.00	119.00	13.00	19.75	20.00
T_6	16.70	38.50	109.75	12.60	20.00	20.50
T_7	15.50	36.00	115.00	10.17	18.00	19.50
	D	T	DT	D	T	DT
SED	1.191	1.820	3.152	0.306	0.471	0.816
CD(0.05)	2.405	2.367	6.363	0.622	0.951	1.648

Table III and figure 1 indicates the root length of **S. rostrata** on 30th, 45th and 60th day after sowing. From the data it was evident that the root length increased with growth period for each treatment. On 30th day, there was a significant increase for all the treatment except T_3 and T_7

FIGURE - I

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON ROOT LENGTH OF S. rostrata

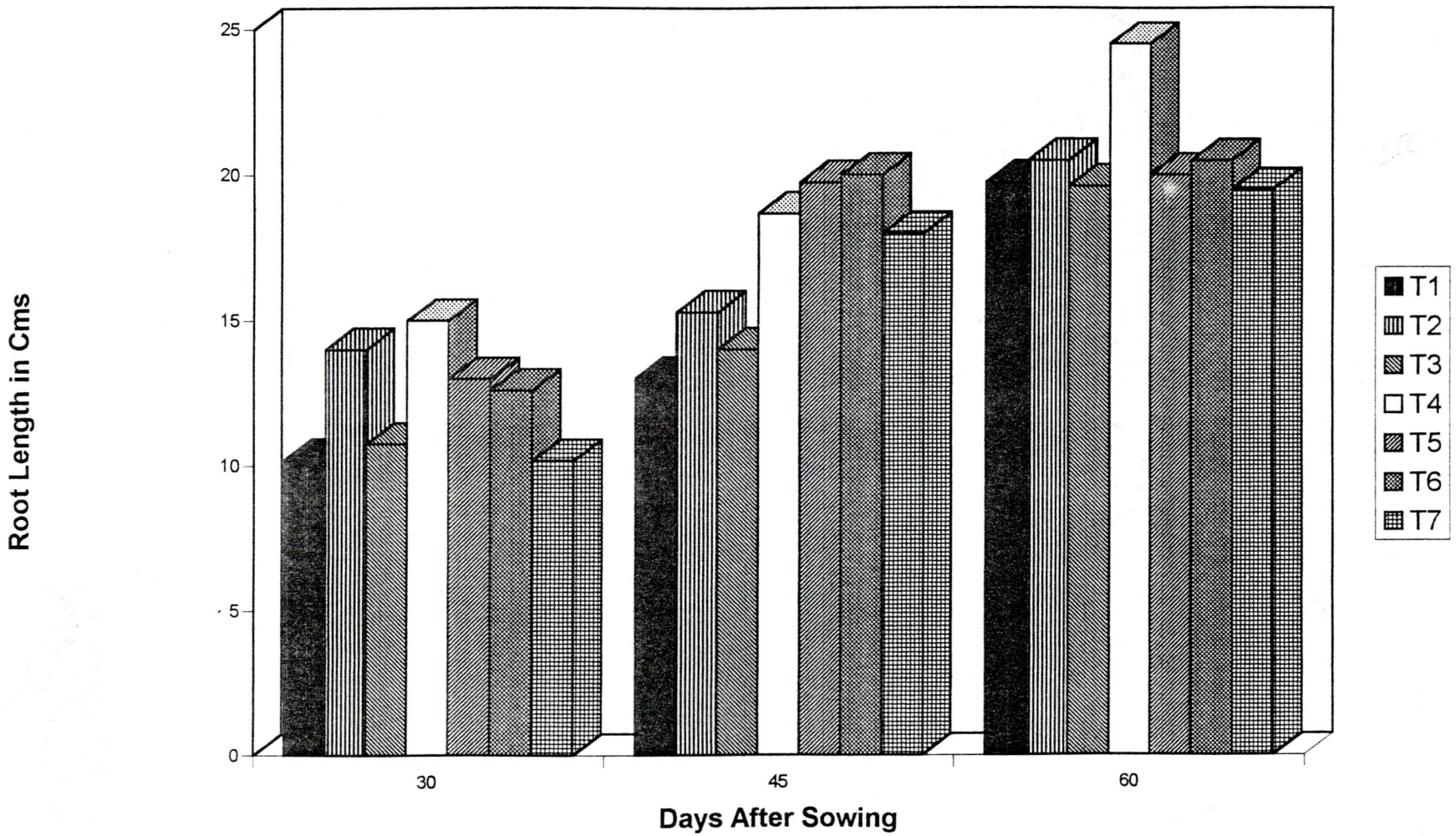


PLATE 1. GROWTH OF SESBANIA ON 60th DAY.

which registered comparable values with that of control. T_4(50 Kg P+5Kg Mo/h) was found to be superior on 30th which was followed by T_2, T_5 and T_6, where as on 45th day T_6 superseded and recorded the maximum root length which was followed by T_5, T_4, T_7, T_2 and T_3.

On 60th day T_4, registered the highest root length and it was followed by T_6 and T_2 which exhibited comparable values and T_3, T_5 and T_7 had values closer to that of control.

According to study by Das et al., (1990) Phosphorus enhances the uptake of Molybdenum and thus it is in par with the present study and T_4(50 Kg P and 5 Kg Mo/h) had recorded the maximum root length.

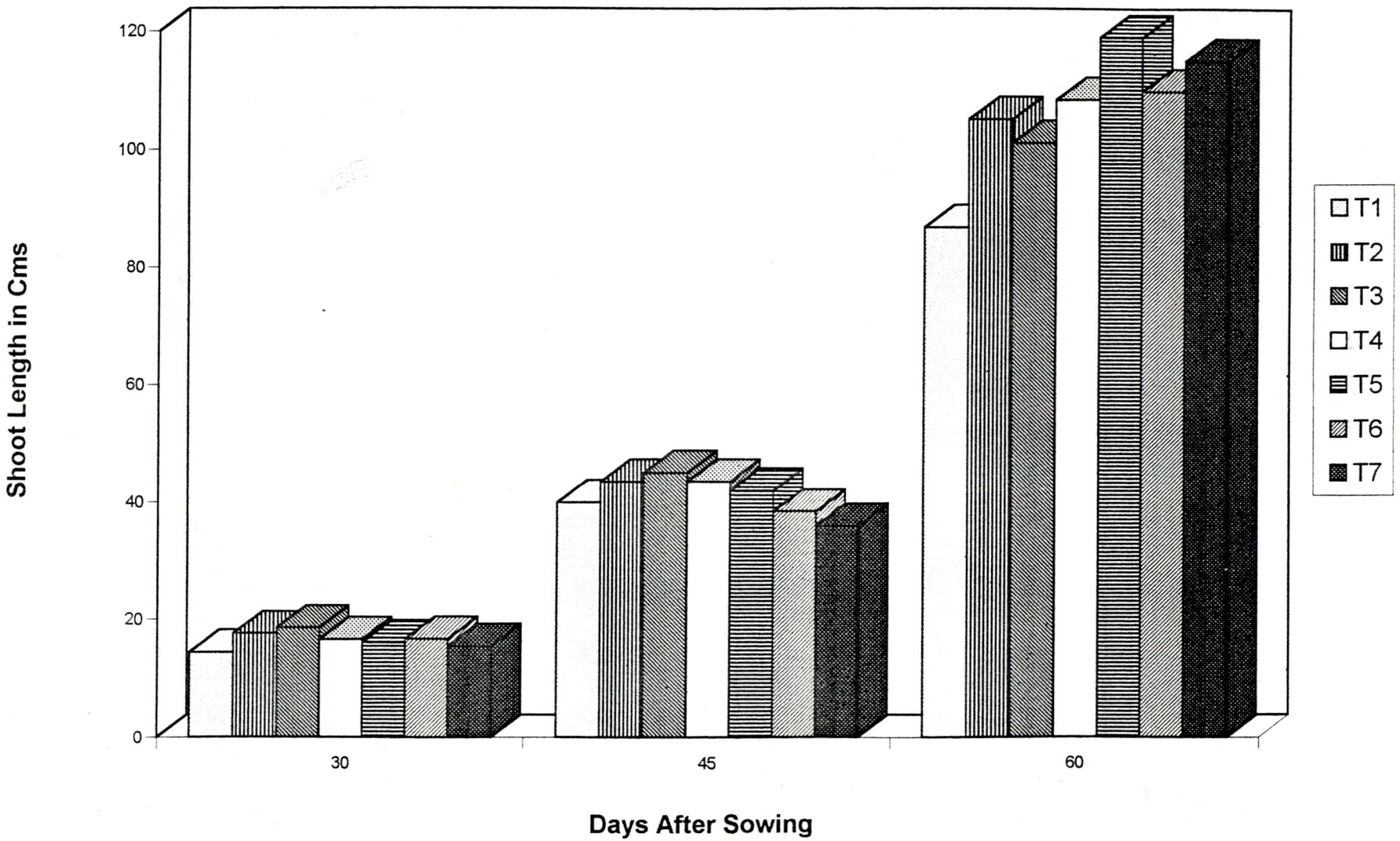
4.2.2 Shoot Length

The effect of phosphorus and Molybdenum on the shoot length of **S. rostrata** on 30th, 45th and 60th day was depicted in Table III.and Fig.2. On 30th day, only T_2 and T_3 were found to register statistically significant increase in shoot length when compared to control (T_1) whereas all the other treatments were recorded non significant increase. The treatments T_4, T_5 and T_6 recorded comparable values and T_3 (5 Kg Mo/h) registered the maximum shoot length both on 30th and 45th day whereas on 60th day, T_5 had registered the maximum shoot length.

A significant ($P < 0.05$) increase in the shoot length of **S. rostrata** was seen only on 60th day of growth for all the treatments when compared to the control (T_1) (100 Kg P/h). Eventhough the individual effect of Molybdenum (5 Kg Mo/h) ie., T_3 had recorded the maximum shoot length during the

FIGURE - 2

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON SHOOT LENGTH OF S. rostrata



growth period, the treatment T_5 (100 Kg P/h) was found to be superior on 6oth day. A study by Yawalkar et al., (1967) suggested that the trace elements like Molybdenum if added to the soil, helps to improve the growth of the green manure crop and also in a study of Engels et al., (1995) the addition of liming nutrient phosphorus increased the growth of **S. rostrata** considerably through the symbiotic effect of **Azorhizobium caulinodans** with the plant **S. rostrata.**

4.2.3 Fresh Weight :

From Table IV, it was evident that the application of micronutrient such as Molybdenum and Macronutrient, phosphorus had improved the fresh weight of **S. rostrata** plants with the increasing days of growth. On 30th day, all the treatments registered nonsignificant increase in fresh weight when compared to the control except T_7, which had shown lesser value than the control (T_1). Among the treatments, T_5 (100 Kg P/h) exhibited the maximum fresh weight on 30th day but on 45th day and 60th day T_4 (50 Kg P+ 5 Kg Mo/h) registered the maximum fresh weight (8.83 g and 47.50 g). Only T_2 and T_4 were found to record ($P < 0.05$) a statistically significant ($P < 0.05$) increase in fresh weight on 60th day. Kolar et al., (1993) also reported that fresh weight and dry matter accumulation of **Sesbania** species increased with age and highest biomass accumulation was recorded on 60th day.

Table IV

Effect of Phosphorus and Molybdenum on Fresh and Dry Weight of **S. rostrata**

	Fresh Weight (g)			Dry Weight (g)		
Treatments	Days after sowing			Days after sowing		
	30	45	60	30	45	60
T_1	0.980	7.440	32.00	0.400	3.310	8.20
T_2	0.990	8.190	37.00	0.500	3.350	11.39
T_3	1.070	7.820	30.00	0.660	2.960	9.03
T_4	1.390	8.830	47.50	0.370	3.030	13.63
T_5	1.410	6.570	31.58	0.340	2.260	7.11
T_6	1.350	7.690	23.35	0.470	3.400	11.33
T_7	0.920	8.530	30.52	0.390	2.710	12.38
	D	T	DT	D	T	DT
SED	0.269	0.411	0.712	0.224	0.342	0.593
CD(0.05)	0.543	0.830	1.439	0.452	0.691	1.198

4.2.4 Dry weight :

Table IV picturises the dry weight of S. rostrata for various stages of growth. A statistically significant increase in dry weight was recorded only on the 60th day for all the treatments except T_5 when compared to the control, whereas on 30th day only T_2, T_3 and T_6 exhibited higher values and on 45th day, T_2 and T_6 showed higher values when compared to the control. Eventhough T_3 was found to be superior on 30th day, the trend was reversed on 60th day where T_4 was found to be superior recording the highest dry weight (13.63

g/plant) when compared to the other treatments. This clearly portrays that phosphorus and Molybdenum at low concentration (50 Kg P + 5 Kg Mo/h) had a cumulative effect on the dry weight of the plants.

Halepyati and Sheelavantar (1991) also suggested that phosphorus mainly influenced the root growth, nodulation, nodule mass and nitrogen fixation thereby affecting dry matter production. Thus the application of phosphorus to leguminous green manure crops has been shown to stimulate nitrogen fixation and biomass production by the **Azorhizobium** symbiotic effect with the plant **S. rostrata.**

4.2.5 Nodule Number :

Table V presents the number of stem and root nodules of **S. rostrata** on 30th, 45th and 60th day of growth plate 2 picturises the nodulation in **S. rostrata.** The nodule number of both stem and root had increased many fold for all the treatments steadily with the age of the plant. This was in agreement with the study of Alazard and Duloux (1987), that the stem nodules appeared after 45 days of inoculation and were numerous and sometimes continuous. All the treatments exhibited comparable values of root and stem nodules on 30th day whereas on 45th day T_7 exhibited maximum number of stem nodules which was followed by T_6, T_4 and T_3. The treatments T_2 and T_5 recorded comparable number of nodules to that of control.

PLATE 2. STEM NODULATION IN TREATED AND UNTREATED S.rostrata

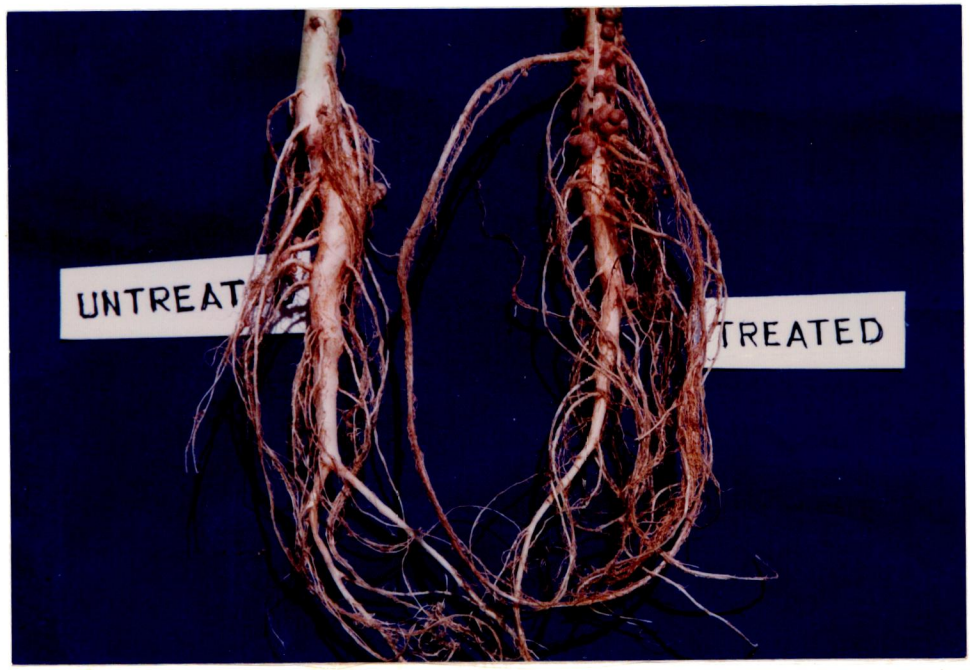


PLATE 3. ROOT NODULATION IN TREATED AND UNTREATED S. rostrata

Table V

Effect of Phosphorus and Molybdenum on nodule number of **S. rostrata**

Treatments	Stem nodule Days after sowing			Root nodule Days after sowing		
	30	45	60	30	45	60
T_1	3	28	33	10	19	25
T_2	4	25	106	13	25	33
T_3	5	36	39	14	15	30
T_4	4	48	51	11	30	60
T_5	2	27	48	9	35	45
T_6	4	49	60	9	43	30
T_7	4	51	56	8	1	15
	D	T	DT	D	T	DT
SED	2.341	3.576	6.159	2.141	3.270	5.665
CD(0.05)	4.722	7.220	12.505	4.332	6.602	11.435

On 60th day, all the treatments had shown significant increase in stem nodules when compared to the control, but the treatment T_2 (100 Kg P/h) was found to be superior (106 stem nodules) when compared to the other treatments.

The treatments T_6 and T_4 recorded the maximum number of root nodules on 45th and 60th day respectively. When compared to the other treatments T_3 and T_6 recorded similar number of root nodules on 60th day. Thus it was obvious that the combined appliation of phosphorus and Molybdenum facilitated and increased the nodulation of **S. rostrata.** Thus the efficacy of green manure could be increased

considerably. with either Molybdenum or phosphorus to improve nodulation and thus be helpful to the succeeding crops in the .sequence was reported by Bhaskar and Shivashankar (1992). In a study reported by Aggarwal and Kumar, (1993) Phosphorus application promotes nodulation in legumes which has intimate link with nitrogen fixation.

4.3 Biochemical Parameters

4.3.1 Chlorophyll :

The data in table VI and Fig. 3 represents the chlorophyll content of **S. rostrata** on the 30th, 45th and 60th day after sowing. There was a statistically significant increase in the chlorophyll content of all the treatments when compared to the control as well as between the days, except T_4 and T_6 on 60th day.

Among the treatments T_5 exhibited the highest chlorophyll content on 30th day which was statistically significant over the other treatments and was followed by T_4, T_3, T_6, T_2 and T_7. On 45th day, as well as on 60th day, T_2 recorded the highest chlorophyll content and was followed by T_3, T_4, T_7, T_6 and T_5 on 45th day and by T_7, T_5, T_3, T_4 and T_6 on 60th day. Thus it was clear that the inclusion of phosphorus and Molydenum had an additive effect on the chlorophyll content of the plants. Photosynthesis and nitrogen metabolism are major physiological activities in plants and the chlorophyll plays an important role in photosyntesis as reported by Kadam et al., (1988) and Spiller (1981) said that photosynthesis is the ultimate source of energy (ATP) for nitrogen fixation. According to Kalidurai and Kannaiyan, (1992), the total chlorophyll content was

FIGURE - 3

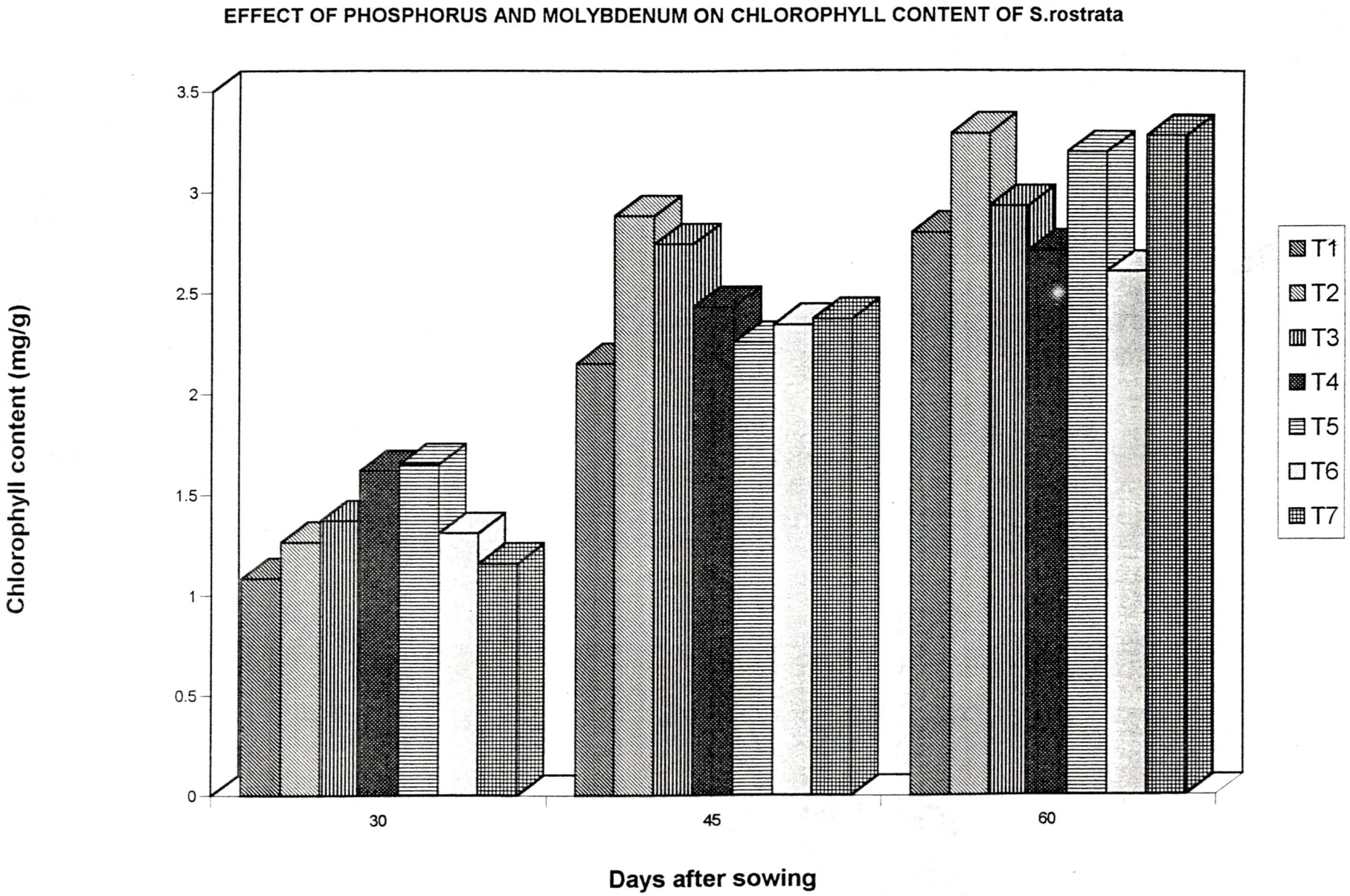

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON CHLOROPHYLL CONTENT OF S.rostrata
Chlorophyll content (mg/g)
3.5
3
2.5
2
1.5
1
0.5
0
30
45
60
Days after sowing
T1
T2
T3
T4
T5
T6
T7

Table VI

Effect of Phosphorus and Molybdenum on Chlorophyll, Allantoin and Amino Nitrogen content of **S. rostrata**

Treatments	Chlorophyll(mg/g)			Allantoin(mg/g)			Amino Nitrogen(mg/g)		
	Days after sowing			Days after sowing			Days after sowing		
	30	45	60	30	45	60	30	45	60
T_1	1.084	2.147	2.800	0.297	0.552	0.582	1.405	2.184	3.750
T_2	1.263	2.880	3.291	0.325	0.470	0.660	2.321	3.499	7.702
T_3	1.370	2.742	2.932	0.287	0.392	0.867	1.804	3.367	6.882
T_4	1.618	2.428	2.711	0.307	0.487	0.660	1.775	4.577	6.238
T_5	1.652	2.256	3.198	0.400	0.890	0.947	3.501	7.138	8.045
T_6	1.309	2.343	2.604	0.367	0.710	0.662	2.150	5.791	11.01
T_7	1.157	2.372	3.281	0.460	0.480	0.593	2.940	6.840	9.620
	D	T	DT	D	T	DT	D	T	DT
SED	0.028	0.043	0.075	0.030	0.046	0.079	0.028	0.043	0.075
CD(0.05)	0.057	0.088	0.152	0.060	0.092	0.060	0.057	0.088	0.152

higher in **S. rostrata** raised under water logged condition.

4.3.2 ALLANTOIN:

Tabel VI and Fig. 4 portrays the allantoin content of **S.rostrata** for various stages of growth. On 30th day, a statiscally significant increase in allantoin content was exhibited only by the treatments T_5, T_6, T_7 and T_2 where as T_4 registered comparable value with that of control. T_7 (100 Kg P and 10 Kg Mo/h) recorded the highest allantoin level on 30th day where as on 45th and 60th day T_5 (100 Kg P/h) showed the maximum allantoin content. Except T_5 and T_6 all other treatments were non significant on 45th day.

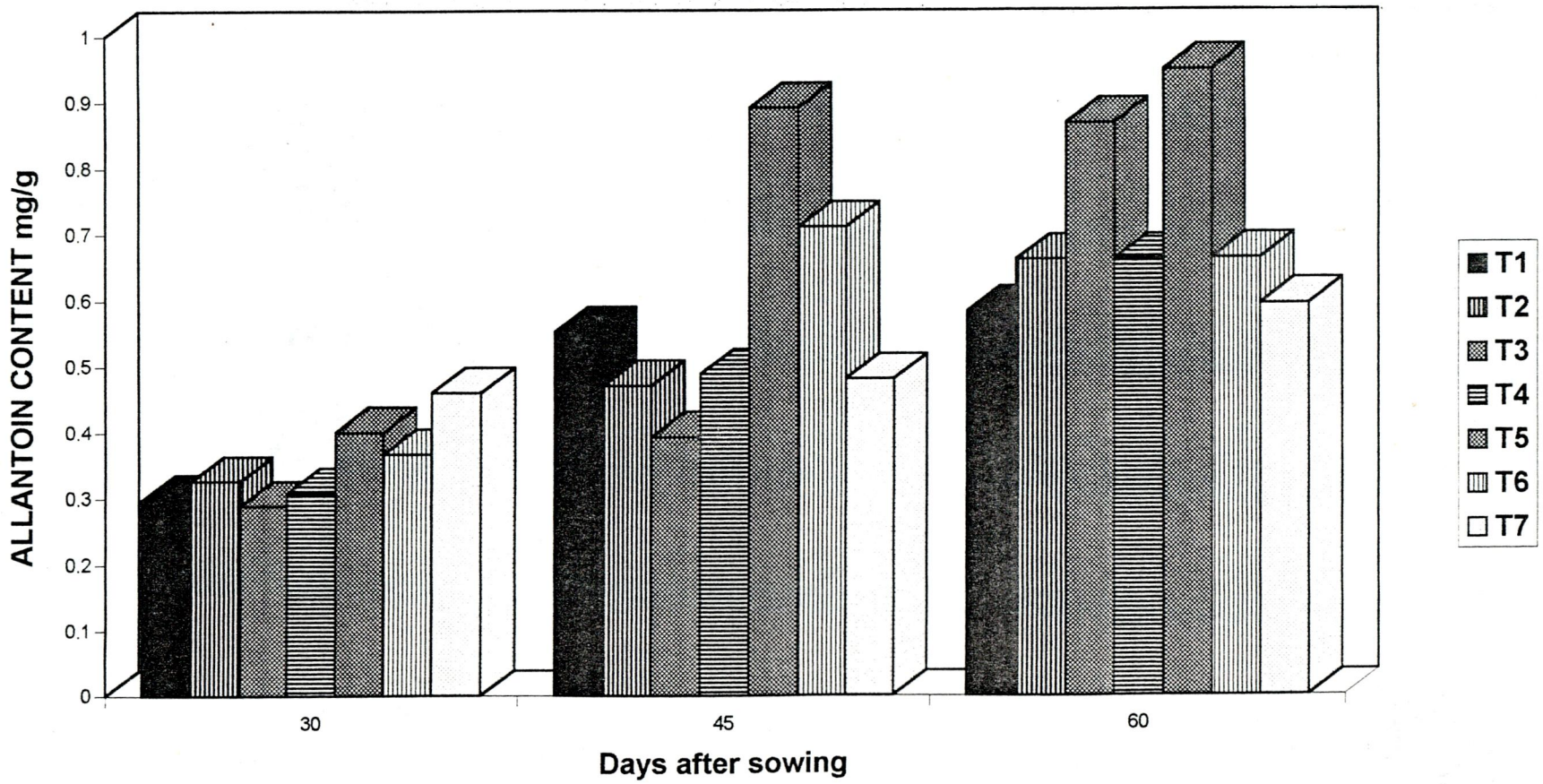
On 60th day, T_5 had recorded the highest allantoin content which was followed by T_3. The treatments T_2, T_4 and T_6 had comparable allantoin level and T_7 exhibited comparable value with that of the control. Thus there was an increase in allantoin content with increase in days after sowing and this was in agreement with the study of Sukanya (1996) that the allantoin content in **Sesbania rostrata** increased with the age of the plant.

4.3.3 AMINO NITROGEN

Table VI and Fig. 5 depicts the amino nitrogen content of **S.rostrata** on 30th, 45th and 60th day after sowing. Phosphorus and Molybdenum individually and in combinaition had enhanced the amino nitrogen content of all the plants for all the three stages of growth when compared to the control. However a statistically significant difference ($P < 0.05$) was exhibited only by the treatments T_2, T_5 and T_7 on 30th day, where as all the treatments recorded a statistically

FIGURE-4

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON ALLANTOIN CONTENT OF S.rostrata



significant ($P < 0.05$) difference in the amino nitrogen content on 45th and 60th day of growth. Among the treatments T_5 was found to be superior on 30th and 45th day of growth recording the maximum amino nitrogen content (3.501 and 7.138 mg/g/plant) and T_6 was found to exhibit the maximum amino nitrogen content (11.014 mg/g/plant) on 60th day of growth. According to Sadasivam and Lakshmi (1992), Amino nitrogen content was higher in all the three stages of growth when compared to allantoin nitrogen. The increase in amino nitrogen content in leaves after the formation of stem nodules indicates that the fixed nitrogen is assimilated and transported in the form of amino nitrogen.

4.3.4 TOTAL NITROGEN, PHOSPHORUS AND POTASSIUM :

The nitrogen content of **S.rostrata** on various stages of growth period was illustrated in table VII and Fig. 6. It was clear from the table that the nitrogen content of the Phosphorus and Molybdenum treated plants were higher through out the growth period when compared to the control (T_1). Among the treatments, T_4 registered a higher nitrogen content on 30th and 60th day of growth were as T_6 on 45th day. Thus Molybdenum in higher concentration (10 Kg/h) enhances the nitrogen content of **S.rostrata** but the same effect was brought about at lower concentration when Molybdenum was applied with phosphorus.

According to Das et al., (1990) Phosphorus application significantly increased the Molybdenum uptake by green gram. At the lower rates of applied Phosphorus **S.rostrata** accumulated substantially more nitrogen that other species.

FIGURE-5

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON AMINO NITROGEN CONTENT OF S.rostrata

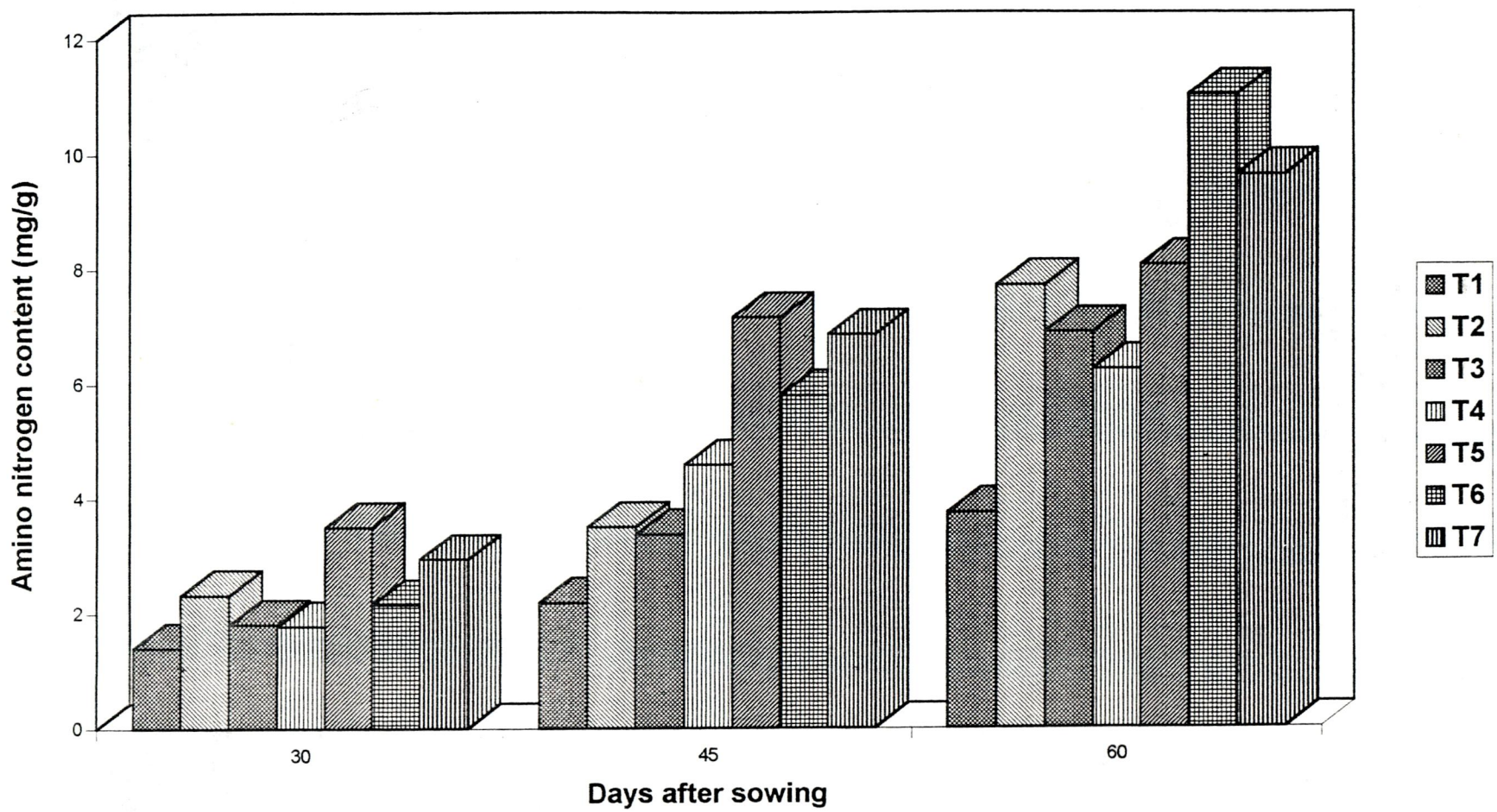


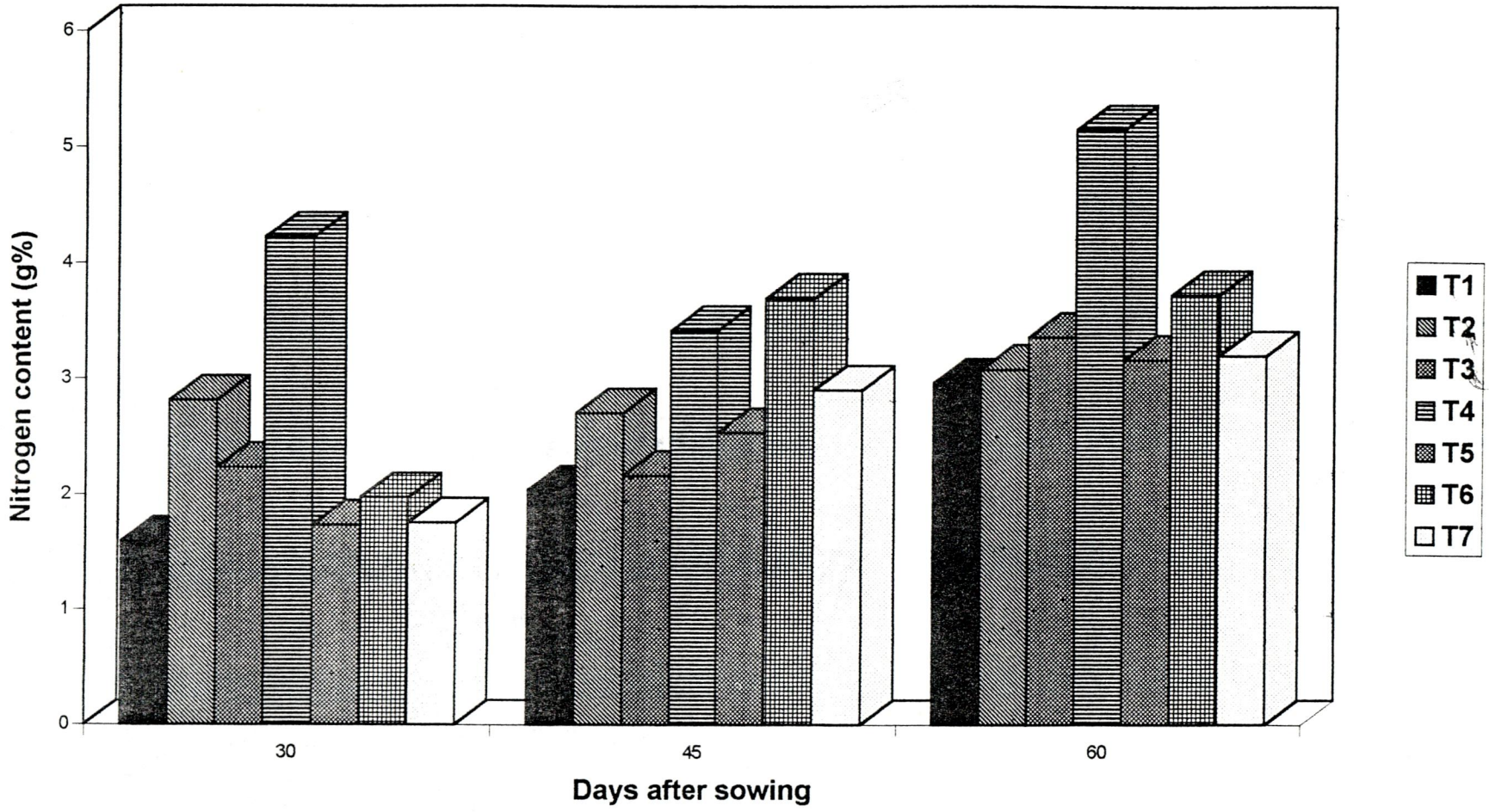
Table VII

Effect of Phosphorus and Molybdenum on Nitrogen, Phosphorus and Potassium content of **S. rostrata**

Treatments	Nitrogen(g%)			Phosphorus(g%)			Potassium(g%)		
	Days after sowing			Days after sowing			Days after sowing		
	30	45	60	30	45	60	30	45	60
T_1	1.590	2.030	2.960	0.050	0.190	0.430	2.140	2.750	3.400
T_2	2.810	2.700	3.080	0.180	0.210	0.510	3.390	2.870	4.000
T_3	2.230	2.160	3.360	0.150	0.250	0.510	3.280	2.940	3.950
T_4	4.220	3.410	6.150	0.080	0.470	0.480	2.090	3.090	3.690
T_5	1.730	2.530	3.160	0.120	0.290	0.450	2.340	3.560	3.710
T_6	1.970	3.690	3.720	0.370	0.210	0.460	2.300	3.120	4.080
T_7	1.750	2.900	3.200	0.130	0.330	0.556	2.880	3.590	3.960
	D	T	DT	D	T	DT	D	T	DT
SED	0.214	0.327	0.566	0.039	0.060	0.104	0.214	0.326	0.56
CD(0.05)	0.432	0.660	1.143	0.079	0.121	0.210	0.432	0.659	1.14

FIGURE-6

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON NITROGEN CONTENT OF S.rostrata



Therefore it was obvious that addition of Phosphorus at the concentration of T_2 (50 Kg P/h) and T_6 (10 Kg Mo/h) enhanced the nitrogen content of the plants. George et al., (1994) suggested that **S.rostrata** fixed greater amounts of nitrogen as a consequence of its total nitrogen requirement and due to its greater capacity of biological nitrogen fixation from nodulation.

Phosphorus :

Table VII and Fig. 7 represented the phosphorus content of **S. rostrata** on various stages of growth. Except T_4 and T_5 all the treatments exhibited statistically significant ($P < 0.05$) increase in phosphorus content of **S. rostrata** on 30th day among which T_6 was found to record the maximum (1.97 g %) which was followed by T_2, T_3 and T_7.

On 45th day only T_4 and T_7 showed statistically significant difference ($P < 0.05$) among which T_4 registered the maximum content and T_7 registered a higher Phosphorus content on 60th day when compared to all the other treatments. In a study presented by Yoshida et al., (1995) Phosphorus fertilization markedly increased the Phosphorus and nitrogen concentration in plants. Das et al., (1990) had suggested in his study that Phosphorus uptake by green gram was significantly increased with increasing levels of Phosphorus and Molybdenum and this study was found to be at par with the present study.

Potassium :

Table VII and Fig. 8 picturises the potassium content of S. rostrata on 30th, 45th and 60th day after sowing. The

FIGURE - 7

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON PHOSPHORUS CONTENT OF S.rostrata

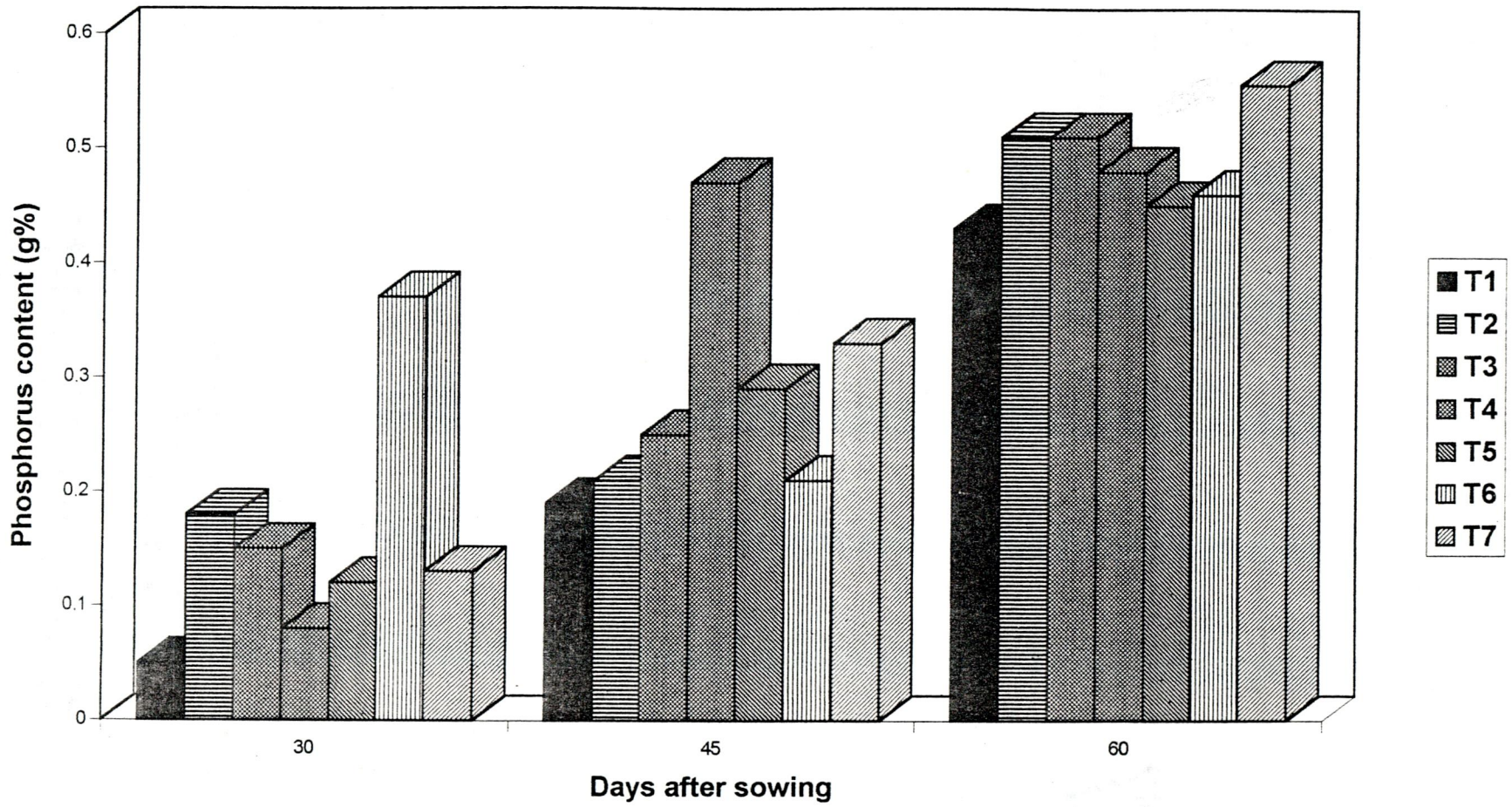
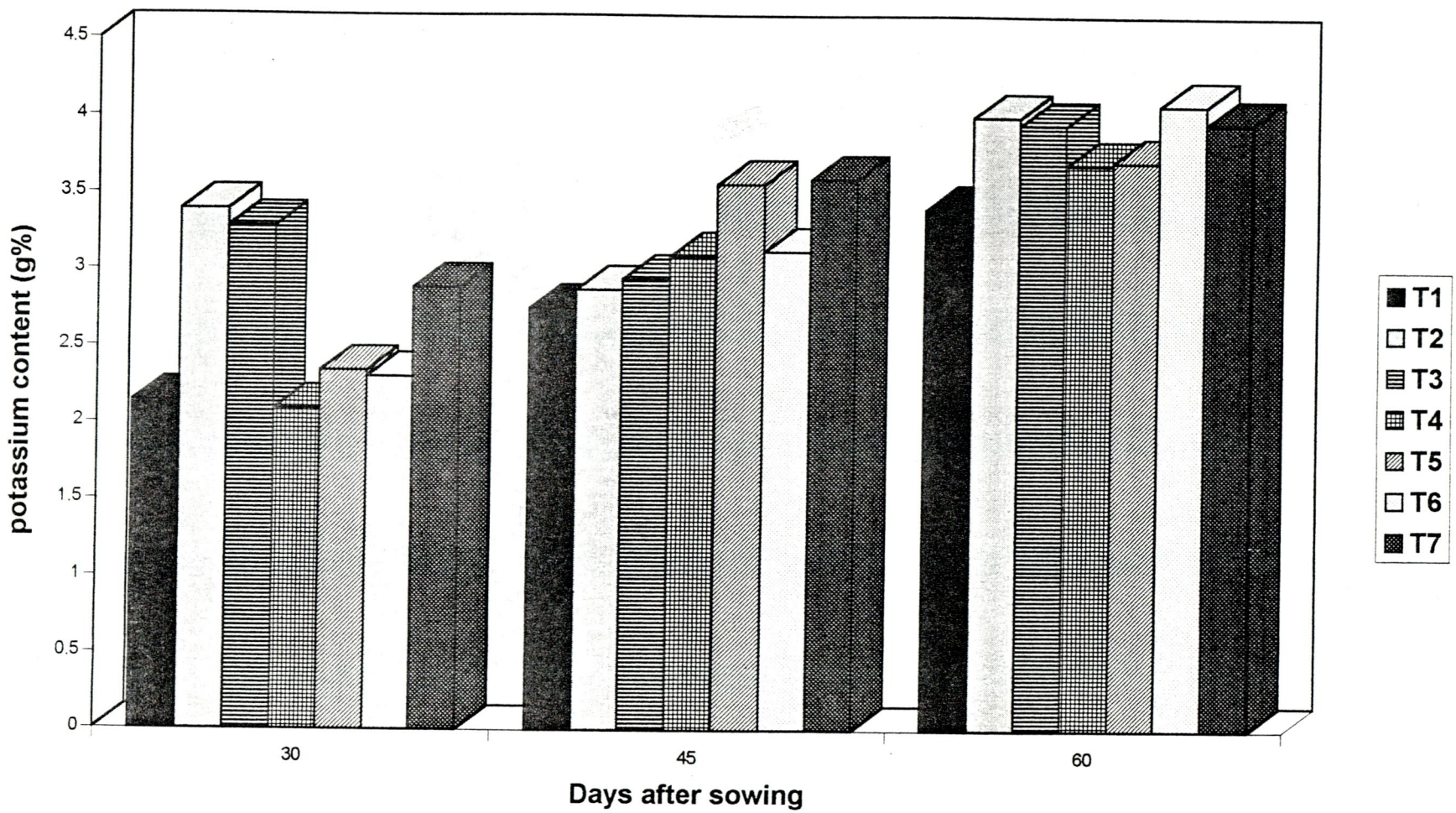


FIGURE-8

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON POTASSIUM CONTENT OF S.rostrata



treatments T_2 and T_3 registered a higher level of Potassium on 30th day when compared to all other treatments and also only these two treatments had shown a statistically significant increase ($P < 0.05$) in Potassium level when compared to the control. On 45th day, T_5 and T_7 alone exhibited a significant difference in Potassium level whereas on 60th day, only T_6 had registered a significant difference in Potassium content when compared to the control (T_1). Thus maxmimum concentration of Molybdenum (10 Kg Mo/h) in T_6 had favoured the Potassium uptake on 60th day. The above study was in accordance with the study of Vijayalakshmi, (1996) who stated that application of Molybdenum had a positive effect on Potassium uptake.

4.4 Ammonia Assimilating Enzymes

4.4.1 Glutamate dehydrogenase

Table VIII and Fig. 9 represents the Glutamate dehydrogenase activity of S. rostrata on 30th, 45th and 60th day of growth. T_5 (100 Kg P/h) recorded the maximum glutamate dehydrogenase activity in root in all the three stages of growth and only T_5 and T_6 were found to be statistically significant ($P < 0.05$) in all the other treatments were found to be nonsignificant on 30th day whereas on 45th and 60th day all the treatments except T_7 on 45th day were recorded a statistically significant increase in enzyme activity when compared to the untreated control (T_1).

Table VIII

Effect of Phosphorus and Molybdenum on Glutamate Dehydrogenase Activity of **S. rostrata**

	Glutamate Dehydrogenase*					
	Root			Shoot		
Treatments	Days after sowing			Days after sowing		
	30	45	60	30	45	60
T_1	8.800	10.99	12.18	1.207	1.130	0.660
T_2	10.090	14.72	16.48	3.330	2.500	1.215
T_3	9.110	13.93	15.95	2.120	1.599	1.080
T_4	8.133	15.63	18.52	4.440	3.630	2.109
T_5	15.500	19.88	21.63	6.530	5.070	3.255
T_6	13.700	17.30	17.79	5.260	4.280	2.020
T_7	9.260	11.60	17.84	3.700	2.370	0.180
	D	T	DT	D	T	DT
SED	0.459	0.702	1.216	2.341	3.576	6.195
CD(0.05)	0.927	1.417	2.454	4.726	7.220	12.505

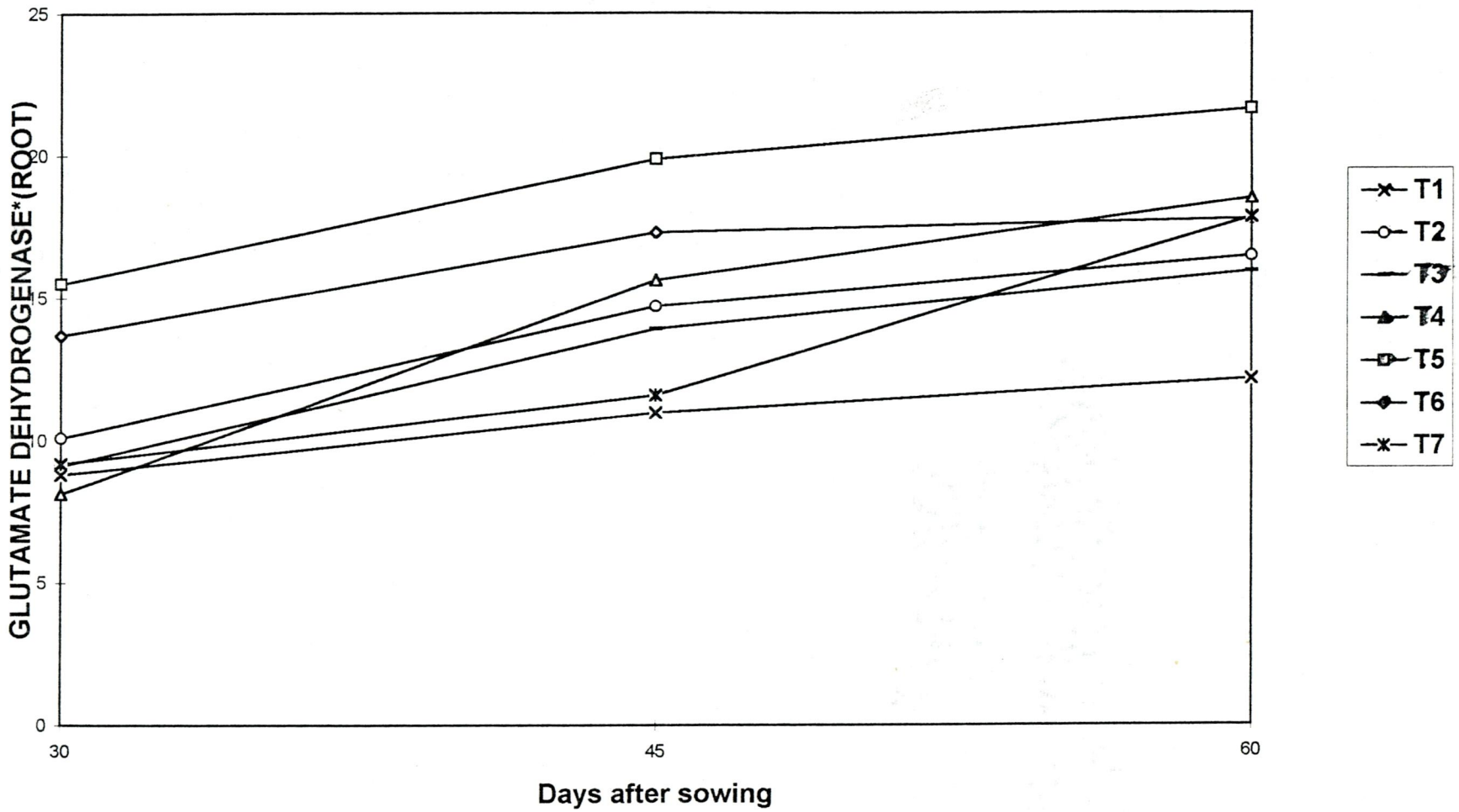
* - Nanomoles of NADH oxidised per minute per mg protein

However, a decrease in glutamate dehydrogenase activity was noticed in shoot with increasing growth period though there was an increase within the treatments. The glutamate dehydrogenase activity of root was found to be greater than that of shoot for all the three stages of growth as well as all the treatments.

Vargas et al., (1988) stated that glutamate dehydrogenase activity increased in the roots while there was a decrease in activity in leaves and stem and it was in par with the present

FIGURE-9

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON GLUTAMATE DEHYDROGENASE ACTIVITY OF S.rostrata



* Nanomoles of NADH oxidised per minute/mg protein

study. Pandey and Venubabu (1988) also suggested that there was a significant role played by NADH dependent glutamate dehydrogenase during initial stages of nitrogen metabolism which are involved in assimilation of ammonium.

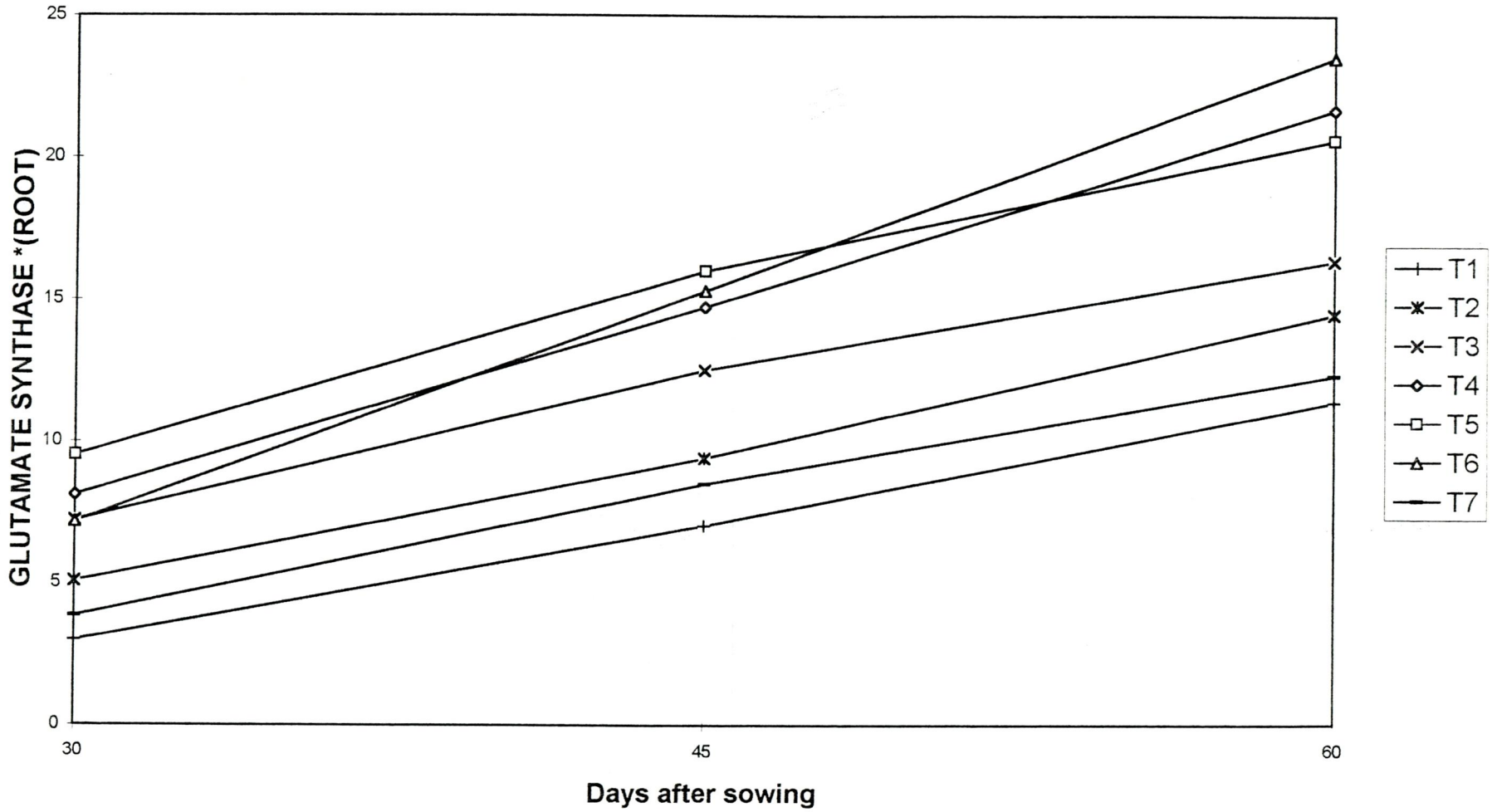
4.4.2 Glutamate Synthase

The Glutamate Synthase activity of **S.rostrata** on 30th, 45th, 60th day of growth is illustrated in Table IX and Fig.10. The glutamate synthase activity of root as well as shoot was found to be maximum for the treatment T_5 30th and 45th day and the trend was changed for the 60th day where, T_6 registered the maximum activity. T_7 registered comparable value with that of control on 30th day in root indicating that the combination of Molybdenum and Phosphorus in high concentration (10 Kg Mo/h and 100 Kg P/h) had little effect on glutamate synthase activity initially but with the increasing growth period they were found to enhance the enzyme activity significantly.

The glutamate synthase activity in root was found to be twice that of shoot indicating that the ammonia assimilation occurs predominantly in the root. This was in agreement with the study of Yamaya et al., (1995) that the level of NADH dependent glutamate synthase activity increased more than 10 fold in root but not in shoot.

FIGURE-10

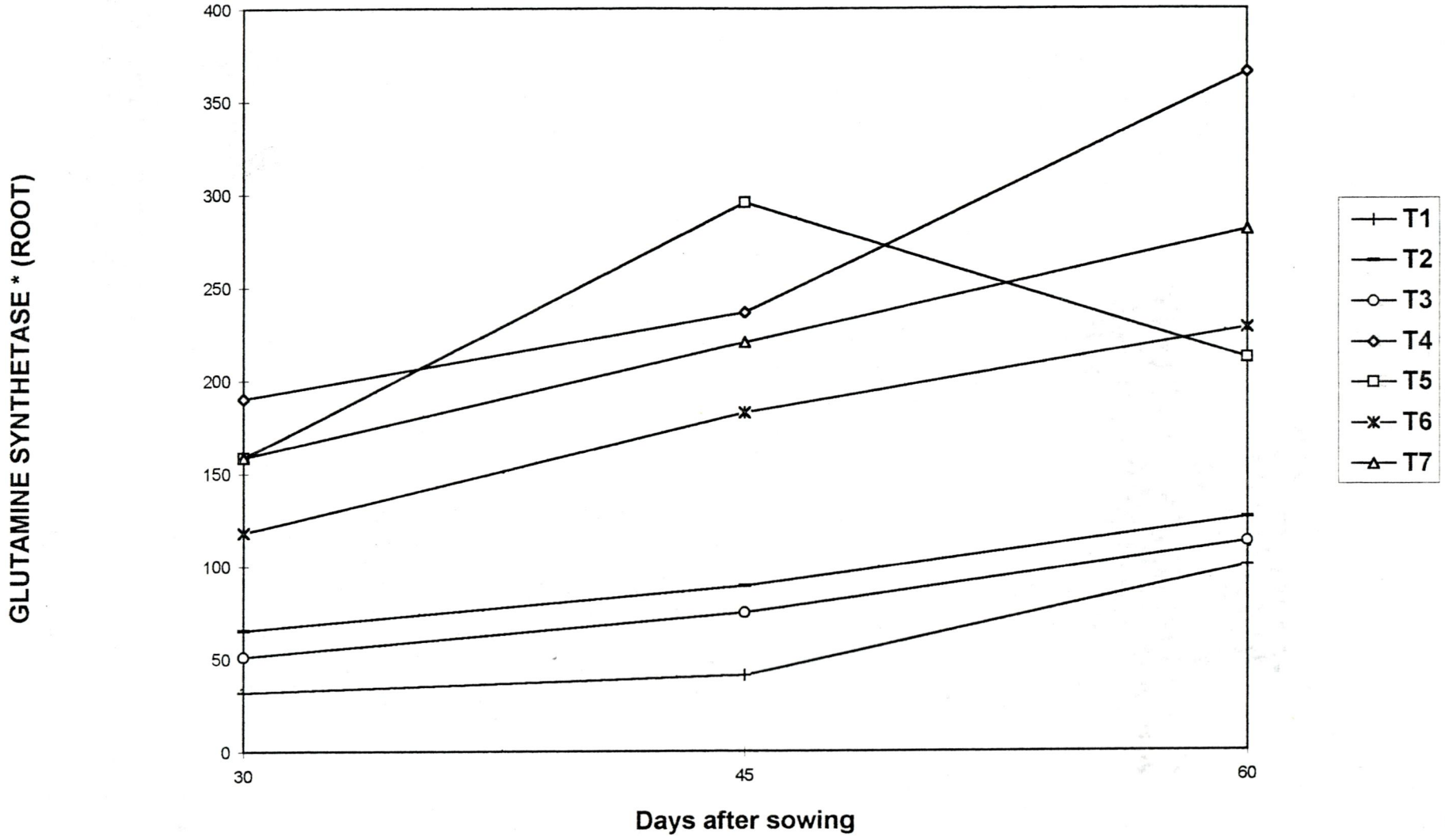
EFFECT OF PHOSPHORUS AND MOLYDBENUM ON GLUTAMATE SYNTHASE ACTIVITY OF S.rostara



* Nanomoles of NADH oxidised per minute/mg protein

FIGURE - 11

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON GLUTAMINE SYNTHETASE ACTIVITY OF S.rostrata



* Nanomoles of Glutamyl Hydroxamate produced per min per mg protein

period and the root samples exhibited higher activity as compared to shoot and this was in agreement with the study of Yamaya et al., (1995) who stated that glutamine produced in the reaction catalysed by glutamine synthetase is transported to shoots via the xylem as glutamine and hence its activity in roots is higher than in shoots.

Table X

Effect of Phosphorus and Molybdenum on Glutamine Synthetase Activity of **S. rostrata**

	Glutamine Synthetase*					
	Root			Shoot		
Treatments	Days after sowing			Days after sowing		
	30	45	60	30	45	60
T_1	31.55	40.93	100.06	28.50	68.00	103.85
T_2	65.34	88.86	125.82	34.73	76.70	114.62
T_3	51.10	74.35	112.68	67.92	84.02	130.32
T_4	190.40	236.40	365.60	57.52	78.36	175.56
T_5	158.66	295.60	211.77	37.57	100.11	148.00
T_6	117.97	182.50	228.13	30.62	95.00	160.13
T_7	158.50	220.26	280.73	55.33	83.00	135.48
	D	T	DT	D	T	DT
SED	1.648	2.518	4.361	1.200	1.834	3.176
CD(0.05)	3.327	5.082	8.803	2.423	3.702	6.412

* Nanomoles of glutamyl hydroxamate produced per minute per mg protein

In shoot except T_6 on 30th day which had registered a comparable value with that of control, all other treatments registered a statistically significant increase in activity

with the increasing growth period. T_3 exhibited maximum activity on 30th day which was followed by T_4, T_7 and T_5. On 45th day T_5 (100 Kg P/h) was found to be superior where as on 60th day, T_4 (50 Kg P + 5 Kg Mo/h) registered the maximum enzyme activity when compared to other treatments.

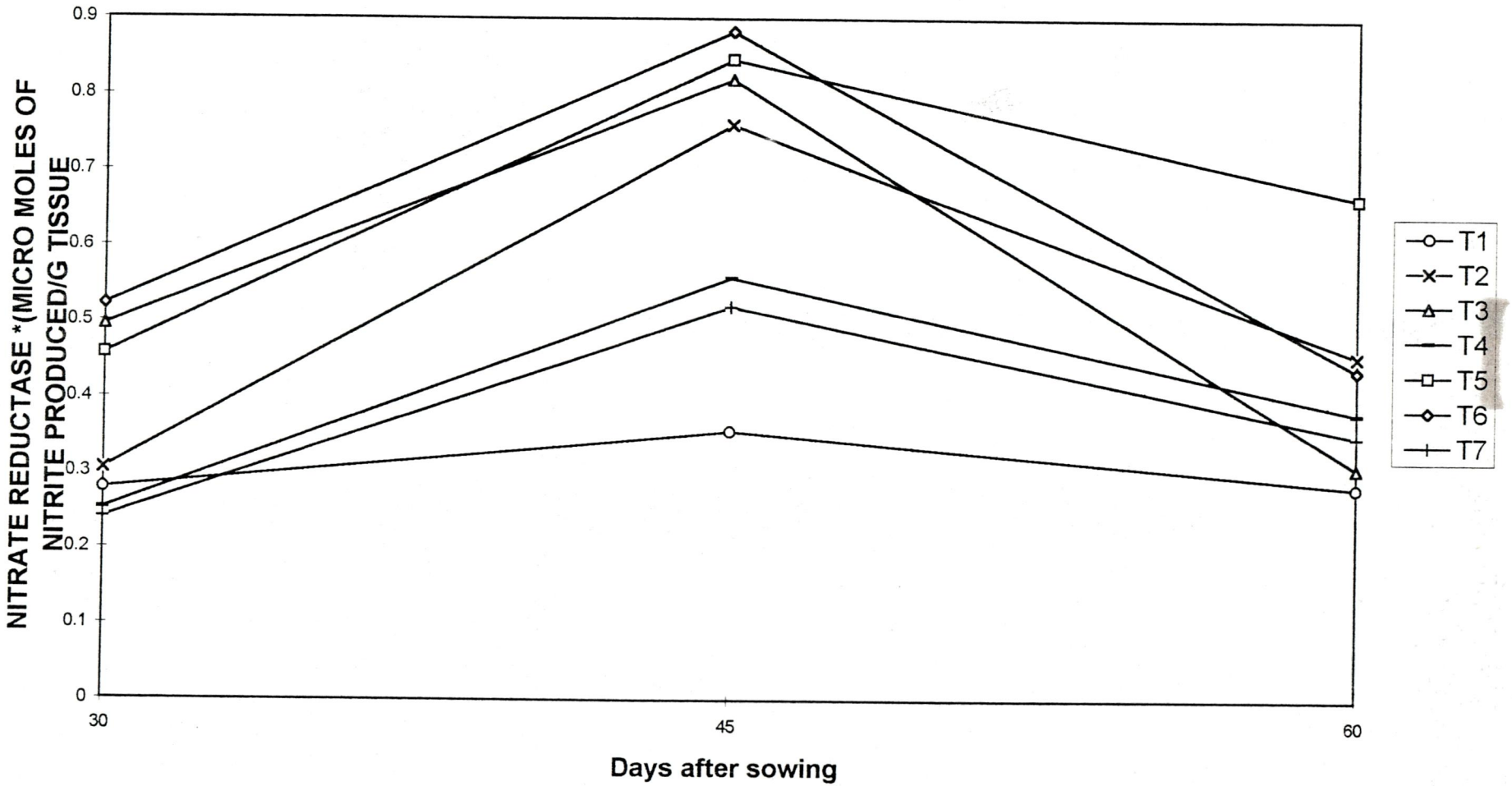
According to a report by Vargas et al., (1988) though the shoot exhibited lower glutamine synthetase activity when compared to root, glutamine synthetase activity increased in leaves with time. From the present study it was evident that the glutamine synthetase exhibited higher activity when compared to glutamate synthase and glutamate dehydrogenase and hence it plays an important role in ammonia assimilation.

4.5 Nitrate Reductase

Table XI and Fig. 12 indicates the nitrate reductase activity of S. rostrata on 30th, 45th and 60th day after sowing. T_6 (10 Kg Mo/h) recorded the maximum activity on 30th day which was followed by T_3 and T_5 and they exhibited statistically significant increase in activity compared to other treatments. On 45th day also T_6 registered the highest Nitrate Reductase activity which was followed by T_5, T_3, T_2, T_4 and T_7. An increase in enzyme activity was noticed till 45th day and then there was a decline in activity for all the treatments on 60th day. This was in agreement with the study by Mahadkar and Saraf (1991). Vyas et al., (1995) reported that the nitrate reduction catalysed by nitrate reductase is a key step in the overall process of nitrate assimilation.

FIGURE - 12

EFFECT OF PHOSPHORUS AND MOLYBDENUM ON NITRATE REDUCTASE ACTIVITY OF S.rostrata



* Micromoles of Nitrite per gm fresh tissue

37a

Table XI

Effect of Phosphorus and Molybdenum on Nitrate Reductase Activity of **S. rostrata**

Treatments	Nitrate Reductase*		
	Root		
	Days after sowing		
	30	45	60
T_1	0.279	0.355	0.282
T_2	0.305	0.760	0.456
T_3	0.495	0.820	0.308
T_4	0.252	0.558	0.380
T_5	0.457	0.846	0.664
T_6	0.522	0.883	0.437
T_7	0.240	0.520	0.350
	D	T	DT
SED	0.017	0.027	0.047
CD(0.05)	0.036	0.055	0.096

* micromoles of nitrite per gm tissue

Thus it can be concluded that the plants treated with Molybdenum alone exhibited a maximum nitrate reductase activity whereas the combined application of molybdenum and Phosphorus both in high and low concentrations had little effect on nitrate reductase activity.

Summary and Conclusion

5. SUMMARY AND CONCLUSION

The present study was initiated to investigate the effect of Molybdenum and Phosphorus on growth, nodulation, biomass production and nitrogen fixation in **S.rostrata.** The study was carried out in pot Cultures in completely randomised block design with four replications for each treatment, 10 Kg of soil was filled in all the pots along with farmyard Manure. Molybdenum was added at the concentration of 5 and 10 Kg/h and Phosphorus was added at the concentration of 50 and 100 Kg/h both individually and in combination to various pots.

Before the seeds were sown, **S. rostrata** seeds were soaked in peat based inocula of **Azorhizobium Caulinodans** for 3 hours. The treatments were as follows:

T_1 - **Azorhizobium** inoculated **S.rostrata** alone

T_2 - **Azorhizobium** inoculated **S.rostrata** +50Kg of Phosphorus/h

T_3 - **Azorhizobium** inoculated **S.rostrata** + 5Kg of Molybdenum/h

T_4 - **Azorhizobium** inoculated **S.rostrata** +50Kg of Phosphorus/h and 5 Kg of Molybdenum/h.

T_5 - **Azorhizobium** inoculated **S.rostrata** +100Kg of Phosphorus/h

T_6 - **Azorhizobium** inoculated **S.rostrata** +10Kg of Molybdenum/h

T_7 - **Azorhizobium** inoculated **S.rostrata** +100Kg of Phosphorus and 10 Kg of Molybdenum/h.

At the end of 30th, 45th and 60th days plants were uprooted gently, and used for the following biometric and biochemical analysis.

(a) Biometric parameters, root and shoot length, root and shoot nodule number, fresh and dry weight.

(b) Biochemical parameters, total chlorophyll allantoin, aminonitrogen, total nitrogen, phosphorus and potassium.

(c) Assay of ammonia assimilating enzymes (glutamate dehydrogenase, glutamate synthase,glutamine synthetase) and nitrate reductase.

The results of the study were summarised as follows :

(1) Among the treatments T_4 recorded maximum root length on 30th and 60th day (18.68cm and 24.50cm) but on 45th T_6 recorded maximum root length (20.50 cm) T_3 had recorded comparable values with that of control on 30th and 60th day. T_3 had registered maximum shoot length on 30th and 45th day (18.65 and 45 cm) which was superseded by T_5 on 60th day (119.00 cm)

(2) The fresh and dry weights were found to increase with increasing growth period. T_4 recorded the maximum fresh and dry weight on 60th day and on 45th day, T_4 registered maximum fresh weight where as in the case of dry weight T_6 exhibited the maximum value. On 30th day T_5 (1.410 gm) encountered the maximum fresh weight and T_3 (0.660 gm) recorded highest dry weight.

(3) Sesbania plants treated with 5Kg Mo/h (T_3) had shown a maximum number of root and shoot nodules on 30th day where as it was superseded by T_4 (50Kg P+5Kg Mo/h) for root nodule number on 45th and 60th day. T_7 (100Kg P+10Kg Mo/h) recorded maximum number of stem nodules on 45th day, but T_2 (50Kg P/h) registered the higest number on the 60th day.

(4) The micronutrient Molybdenum and the macro nutrient Phosphorus had influenced the chlorophyll content of all the experimental Sesbania plants compared to control. On 30th day T_5 (100 Kg P/h) (1.652 mg/g) registered a significant increase in chlorophyll content than all the other treatments, but on 45th and 60th day, T_2 (50Kg P/h) (2.880 mg/g and 3.291 mg/g) exhibited the maximum chlorophyll content. Photosynthesis and nitrogen metabolism are major physiological activities in plants and chlorophyll plays an important role in photosynthesis and it is the ultimate source of energy for nitrogen fixation. Thus the plants treated with phosphorus at low and high concentrations recorded maximum chlorophyll level.

(5) The content of allantoin and aminonitrogen were found to increase with the growth period. On 30th day, T_7 was found to record the maximum allantoin content, but on 45th and 60th day, it was superceeded by T_5 (100Kg P/h). In case of aminonitrogen content on 30th and 45th day, T_5 was found to be superior (3.501 and 7.138 mg/g) and was followed by T_7 and T_2 where as on 60th day T_6 (11.016 mg/g) had registered the maximum aminonitrogen content. Aminonitrogen content was higher in all the treatments at all the stages of growth when compared with allantoin content. The increase in aminonitrogen content reveals that nitrogen is assimilated and transported in the form of aminonitrogen than ureide nitrogen.

(6) Total nitrogen content increased with the increasing growth period for all the treatments. Among the

treatments T_4 (50Kg P and 5Kg Mo/h) had registered the higest nitrogen content on 30th and 60th day of growth (4.22 gm % and 6.15 gm %) where as on 45th day, T_6 recorded a maximum value.

On 30th day T_6 exhibited maximum phosphorus content (1.97 g %) but it was replaced by T_4 (5Kg Mo + 50Kg P/h) on 45th day and by T_7 (100Kg P and 10Kg Mo/h) on 60th day.

Potassium content significantly increased with increasing age of the plant. On 30th day T_2 (50 Kg P/h) recorded a maximum potassium content where as on 45th day it was superceeded by T_7 (10 Kg Mo + 100Kg P/h) and on 60th day T_6 (10 Kg Mo/h) registered the maximum potassium level.

(7) The enzyme activity of glutamate dehydrogenase was found to increase with growth period, where as in shoots the activity decreased. T_5 (100 Kg P/h) recorded the maximum glutamate dehydrogennase activity for all the three stages of growth in root and the activity in root was found to be greater than that of shoot.

Glutamate synthase activity, was at its maximum on 30th and 45th day in case of T_5(100 Kg P/h) where as on 60th day, T_6 (10 Kg Mo/h) improved over T_5.

The glutamine synthetase activity was found to be maximum on 30th and 60th day in T_4 (50 Kg P + 5 Kg Mo/h) but on 45th day, T_5 (100 Kg P/h) recorded the higest enzyme activity. Glutamine synthetase exhibited higher activity when compared to other ammonia assimilating enzymes glutamate dehydrogenase and glutamate synthase in experimental plants.

Experimintal Sesbania plants treated with 5 Kg Mo/h (T_3), 100 Kg P/h (T_5) and 10 Kg Mo/h (T_6) exhibited a statistically significant increase in their nitrate reductase activity when compared to control on 30th day but on 45th day all the treatments recorded a significant increase and T_6 recorded the maximum enzyme activity on 30th and 45th day. (0.052 micro molar and 0.883 micro molar).

From the results summarised above, we can conclude that the treatment T_5 with phosphorus at a concentration of 100 Kg P/h was found to be superior among all the treatment which was followed by T_4 (50 Kg P and 5 Kg Mo/h). This superior quality of T_4 might be due to the synergistic effect of phosphorus and Molybdenum on nitrogen fixation. But Molybdenum and phosphorus at higher doses did not fair well in comparision to T_4. This might be due to the antagonistic effect of phosphorus and Molydenum at higher concentrations. The Molydenum at higher dose, T_6 (10 Kg Mo /h) also enhanced the growth of **S. rostrata** which was found to be closer to T_4. The responsiveness of the plants to various doses of Phosphorus and Molydenum added individually and in combination was found to be different.

Thus the supplementation of Phosphorus and Molybdenum in judicious levels seemed to invigorate the growth, nodulation and nitrogen fixation by **Azorhizobium** in **S.rostrata**, which inturn can be used as a green manure to enhance the nitrogen nutrition in rice fields. By this procedure, we can increase the rice production to meet the requirement of our country.

Future Prospects :

(1) Other micronutrients like Zinc and Manganese individually and combined effect of Zinc, Manganese and Iron can be tried.

(2) Still lesser doses of Molybdenum and phosphorus can be tried.

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Appendices

APPENDIX - I

ESTIMATION OF CHLOROPHYLL
(Yoshida et al., 1971)

Principle :

Chlorophyll is extracted in 80 per cent acetone and the absorption at 663 nm and 645 nm were read in a spectrophotometer. Using the absorption coefficients, the amount of chlorophyll was calculated.

Reagents :

Acetone 80%

Procedure :

Cut 1.0g fresh leaves into small pieces and homogenised in a mortar and pestle with excess acetone. Decanted and filtered the supernatant on a Buchner funnel through Whatmann No. 42 filter paper. Added sufficient quantity of 80 per cent acetone and repeated the extraction. Transferred the contents from the mortar to the Buchner funnel and washed the brei with acetone until it became colourless. Pooled the fltrates and made up the volume to 100 ml in a volumetric flask. Transferred 5.0 ml of the extract into a 50 ml volumetric flask and diluted by making up the volume with 80 per cent acetone. Measured the absorbance at 645 and 663 nm for the determination of chlorophyll a and b and total chlorophyll.

The chlorophyll content can be calculated on a fresh weight basis employing the following formulae :

$$\text{Total chlorophyll (mg/g)} = \frac{20.2(A645)+8.02(A663)}{a \times 1000 \times w} \times V$$

where

a = length of path light in cell (1 cm)
v = volume of the extract in ml
w = fresh weight of the sample

APPENDIX - II

ESTIMATION OF ALLANTOIN
(Young and Conway, 1942)

Principle :

Allontoin in the presence of alkali forms allantoic acid. In turn allantoic acid in acidic medium forms glyoxylic acid and urea. Glyoxylic acid reacts with phenyl hydrazone. This hydrazone on reaction with potassium feericyanide produces a red colour which is measured at 520 nm.

Reagents :

1) 0.05 M, phosphate buffer, pH 7.5
2) 0.5N sodium hydroxide
3) 0.65N hydrochloric acid
4) Phenyl hydrazine hydrochloride, 0.33%
5) 10N hydrochloric acid
6) 1.67 % potassium ferricyanide

Procedure :

10 g of the leaves was taken in a glass homogeniser. The sample was homogenised with 10 ml of 0.05 M phosphate buffer of pH 7.5 and 0.05 g of polyclor AT. The tube was immersed in boiling water bath. The clear supernatant got by the centrifugation of the sample at 10,000 rpm for 5 minutes was assayed for allantoin.

0.5 ml of the supernatant was diluted to 2.5 ml with distilled water. To that added 0.5 ml of 0.5 N sodium hydroxide and the tubes were placed in a vigourously boiling water bath for 7 minutes. On removal it was immersed in water bath at room temperature. Then to all tubes 0.5 ml of 0.65 N hydrochloric acid and 0.5 N of phenylhydrazine hydrochloride solution were added. The tubes were shaken well and placed in a boiling water bath for exactly 2 minutes. Removed and plunged the tubes in an ice bath and chilled for 20 minutes. On removal, added 2.0 ml of already chilled 10 N hydrochloric acid and 0.5 ml of potassium ferricyanide solution. The contents were mixed thoroughly and tha allantoin content of each sample tube was measured at 520 nm in a spectrophotometer.

APPENDIX - III

ESTIMATION OF AMINONITROGEN
(Spies, 1955)

The leaves of the plant sample were extracted with 0.05 M phosphate buffer (pH 7.5). To 0.1 ml of plant extract, 0.9 ml of distilled water was added followed by 5 ml of Ninhydrin reagent.

Ninhydrin Reagent :

Ninhydrin reagent was prepared by mixing the following constituents, A,B and C in the ratio of 5:12:2.

A. 1% Ninhydrin in 0.3 M Citrate buffer (pH 5.5)
B. Pure glycerol
C. 0.5 M Citrate buffer

The content were shaken vigourously and heated in a boiling water bath for 12 minutes. The tubes were cooled under tap water to room temperature and the absorbance was recorded at 570 nm against reagent blank. Glycine was used to prepare the standard curve and the amount of aminonitrogen present in the given sample was estimated. The results were expressed as mg/g of the plant sample.

APPENDIX - IV

ESTIMATION OF NITROGEN
Microkjeldahl Method
(Humphries, 1956)

Principle :

Total Nitrogen is the sum of ammonia nitrogen and organic nitrogen. This does not include nitrite nitrogen and nitrate nitrogen. Nitrogen of organic matter is converted to ammonium sulphate when treated with sulphuric acid. An excess of alkali is then added to liberate ammonia and distilled. The distillate is titrated with standard sulphuric acid after absorption in boric acid solution.

Reagents :

1. Diacid :
   4 : 1 ratio of concentrated sulphuric acid and concentrated perchloric acid

2. Mixed indicator :

   Dissolved 0.5 g of bromocresol green and 1g of methyl red in 100 ml ethyl alcohol.

3. 2% Boric acid

4. 40% Sodium hydroxide

5. N/70 Sulphuric acid

Procedure :

Ground the dried plant sample and made it to a fine powder. Took 0.5g of sample in a microkjeldahl flask and added 12 ml of diacid. Digested the sample over a heated sand bath. Made up the volume to 100 ml with distilled water. Pipetted out 10 ml aliquot into a microkjeldahl distillation apparatus.

Added 10 ml of 40% sodium hydroxide into the microkjeldahl distillation apparatus and steamed the distillate until a blue colour was reached. After distillation titrated against N/70 sulphuric acid until red colour was got.

Calculation :

$$\text{Nitrogen Content}(\%) = \frac{0.00028 \times \text{Titre value} \times 100}{10} \times \frac{100}{0.5}$$

APPENDIX - V

ESTIMATION OF PHOSPHORUS
(Jackson, 1973)

Principle :

Phosphorus reacts with ammonium molybdate and ammonium metavanadate in acidic medium to give a yellow coloured product. The intensity of the yellow colour is directly related to the concentration of phosphorus and is read at 420 nm in a spectrophotometer.

Reagents :

1. Barteu reagent :

Solution A : 25 g of ammonium molybdate was dissolved in 400 ml of warm water.

Solution B : 1.25 g of ammonium metavanadate was dissolved in 300 ml of boiling water.

Solution A was added to solution B and the volume is made upto 1000 ml.

2. Triple acid mixture : Concentrated nitric acid - perchloric acid - concentrated sulphuric acid (3:2:1).

Procedure :

5.0 g of the finely powdered sample was taken in a 100 ml kjeldahl flask. Added 25 ml of the triple acid mixture and heated for few hours. Cooled and made up the solution to 100 ml with distilled water.

1. From the digested extract pipetted out 5 ml of the aliquot into a 25 ml volumetric flask.
2. Introduced a bit of red litmus paper
3. Neutralised with ammonia solution until litmus paper turned blue.
4. Again identified it with concentrated nitric acid until litmus paper turned red.
5. Added 5 ml of Barteu reagent
6. Made up the volume to 25 ml with distilled water
7. After 30 minutes the intensity of yellow colour developed was read at 420 nm in a colorimeter
8. Using the phosphorus standard curve, the phosphorus concentration in the sample was read in ppm.

Calculation :

$$\text{Phosphorus concent (\%)} = \frac{P \text{ Concentration in ppm} \times 25 \times 100}{10^6} \times \frac{}{5} \times \frac{100}{0.5}$$

Preparation of standard curve :

Analytical grade of potassium dihydrogen phosphate was dried at 40°C and 0.2195 g was dissolved in 400 ml of distilled

water. 25 ml of 7N sulphuric acid was added and volume was made upto 100 ml. This is 50 ppm of phosphorus. Then 100 ml of this 50 ppm stock solution was diluted to 1000 ml to get 5 ppm of phosphorus standards ranging from 0.01 ppm to 1.0 ppm were prepared. Finally 5.0 ml of this solution was pipetted out into a 25 ml volumetric flask. 5,0 ml of Barteu reagent was added and volume was made upto 25 ml. Intensity of the colour of each standard was measured on the colorimeter and a standard curve was constructed using meter readings and concentrations.

APPENDIX - VI

ESTIMATION OF POTASSIUM
(Jackson, 1973)

Principle :

In flame photometry, the solution under test is passed under carefully controlled condition as a very fine spray in the air supply to a burner. In the flame, the solution evaporates and the salt dissociates to give neutral atoms. A very small proportion of these move into a higher energy state. When these excited atoms fall back to the ground state, the light emitted of characteristic wavelength is measured. Potassium is estimated at 770 nm.

Reagents :

1. Triple acid Mixture

   Concentrated nitric acid - perchloric acid - concentrated sulphuric acid (3 : 2 : 1)

Procedure :

1. 5.0 ml of the acid extract was pipetted out into a vial and the transmission was read in flame photometer.

2. Using a potassium standard curve the concentration of potassium was calculated.

$$\text{Potassium content}(\%) = \frac{\text{K concentration in ppm}}{10^6} \times \frac{100}{5} \times \frac{100}{0.5}$$

Potassium Standard Curve :

1.907 g of Analar grade potassium chloride was dissolved in 100 ml distilled water to get 1000 ppm of potassium. 100 ml of 1000 ppm of potassium was diluted to 1 litre to get 100 ppm of potassium. From this a series of potassium standards ranging from 0 - 100 ppm were prepared and percentage transmission was read on a flame photometer to construct a standard curve.

APPENDIX - VII

ESTIMATION OF GLUTAMATE SYNTHASE
(Casteele et al., 1975)

Principle :

Glutamate synthase was assayed spectrophoto-metrically by recording the rate of NADPH or NADH as indicated by a change in absorbance at 340 nm following the addition of enzyme extract.

Reagents :

- Tris HCl buffer 50 MM pH 7.6
  Prepared the following reagents in Tris HCl buffer 50 MM pH 7.6
- Glutamine, 5 mM (36.5 mg / 10 ml)
- 2 - oxoglutarate 5 mM (36.5 mg / 10 ml)
- NADH, 0.25 mM (10 mg / ml)

Enzyme Extract :

Extracted 1 g of the plant material with 5 ml of 10 mM phosphate buffer pH 7.5 containing 1 mM disodium EDTA, 1 mM dithioerythritol and 1% polyvinyl pyrolidine (PVP) and centrifuged at 10,000 g for 30 minutes at 4 °C, collected the supernatant and used it for the enzyme assay.

Procedure :

1. Prepared reaction mixture as per the table.

Reagent	ml to be added
Glutamine	1.0
2-oxoglutarate	1.0
NADH	1.0
Enzyme Extract	0.2
Buffer	1.8

2. Omitted 2-oxoglutarate in the blank, instead add 1 ml of buffer.

3. Incubated for 15 - 30 min at 37 °C

4. Recorded the change in absorbance at 340 nm.

Calculation :

The amount of NADH oxidised was calculated from the molar extinction co-efficient. Activities were expressed as nmole NADH oxidised per min / mg protein.

$$\text{Nanomole of NADH oxidised / min / mg protein} = \frac{A_{340} \times \text{Volume of assay Solution} \times 1000}{6.22 \times \text{Time of incubation(min)} \times \text{mg protein in enzyme extract used}}$$

APPENDIX - VIII

ESTIMATION OF GLUTAMATE DEHYDROGENASE
(Doherty, 1970)

Principle :

Glutamate dehydrogenase was assayed by following the oxidation of the reduced coenzyme NADH or NADPH. These reduced co-enzyme absorb light at 340 nm, which in most biological systems is uniquely uncluttered with interfering absorption by other compounds. Thus, even in crude enzyme extracts the absorption of NADH at 340 nm was easily detected.

Reagents :

- Potassium phosphate buffer 0.1 M (pH 7.0)
- 2-oxoglutarate 0.1 M - Dissolved 14.6 g in one litre distilled water
- Ammonium chloride (1.0 M) - 53.5 g in one litre distilled water
- NADH 10 mg / ml

Procedure :

1. Proceed the assay of NADH - dependent glutamate dehydronase as per the composition of assay given in table :

Reagent	NADH assay / ml
Potassium phosphate buffer	1.0
2-oxoglutarate	0.3
Ammonium Chloride	0.5
NADH	0.12
Enzyme Extract	0.2
Water	8.0

2. Add 0.3 ml of water in the blank instead of 2-oxoglutarate

3. Incubate the reaction mixture at $37^{o}C$ for 15 - 30 minutes

4. Record the change in absorbance at 340 nm

Calculation :

The amount of NADH oxidised was calculated from the molar extinction co-efficient. Activities were expressed as nanomole NADH oxidised per min / mg protein.

$$\text{Nanomole of NADH oxidised / min / mg protein} = \frac{A_{340} \times \text{Volume of assay Solution} \times 1000}{6.22 \times \text{Time of incubation(min)} \times \text{mg protein in enzyme extract used}}$$

APPENDIX - IX

ESTIMATION OF GLUTAMINE SYNTHETASE
(Pateman, 1969)

Principle :

The activity of the enzyme is measured by estimating the production of inorganic phosphate. Glutamine synthetase also catalyses the γ-glutamyl transfer reaction.

$$\text{Glutamine} + \text{Hydroxylamine} \xrightarrow[\text{Arsenate}]{\text{ADP, } Mn^{++}} \text{Glultamyl hydroxamate}$$

Hence, it can be assayed by measuring the production of γ-gultamyl hydroxeimate. The latter method is described below. The γ-glutamyl hydroxamate is made is react with ferric chloride to produce brown color in acidic medium

Activity in the transferase assay generally gives rates several times greater than those obtained in the synthetase assay(measuring Pi) and hence, this method is often used for measurement of Glutamine Synthetase in relatively crude preparations.

When the activity is measured in the presence of Mn^{++} it represents total glutamine synthetase activity (adenylated and unadenylated forms). The biologically active unadenylated form may be measured by inhibiting the adenylated form by the addition of 60mM Mg^{++}

Reagents :

Prepared the folllowing reagents in 20mM Tris-HCl buffer(pH 8.0)

- L- Glutamine 0.2 M (700mg / 21ml)
- Sodium Arsenate 20mM (500mg / 10ml) (Disodium hydrogen Arsenate)
- Manganese chloride 3mM (83mg / 10ml).
- Hydroxylamine 50mM (278mg / 10ml)
- Adenosine diphosphate 1mM (40mg / 10ml)
- Ferric chloride reagent :
  Dissolved 10g trichloro acetic acid and 8g ferric chloride in 250ml of 0.5N hydrochloric acid.
- Enzyme extract :

  Extracted 1g of plant material in 5ml of 50mM imidazole-acetate buffer pH containing 0.5mM EDTA, 1mM dithioerythritol, 2mM MnCl2 and 20% glycerol at $4^{o}C$. Centrifuged at 10,000g for 30Min. If purification is required precipitated the enzyme with $(NH_4)_2\ SO_4$ at 60% saturation. Resuspended the precipitate in extraction

buffer. Desalted over sephadex G25

Procedure :

1. Pipetted out the reagents(ml) as mentioned in the order below.

Glutamine	2.0
Sodium arsenate	0.5
$MnCl_2$	0.3
Hydroxylamine	0.5
ADP	0.5
Enzyme extract	0.2

2. To set a blank, added 2ml 20mM Tris-HCl buffer instead of glutamine.

3. Incubate the reaction mixture for 30min at 37^{o}.

4. Stopped the reaction by adding 1ml ferric chloride reagent.

5. Measured the brown colour developed at 540mM.

6. Prepared a range of standards containing 100 - 500 ug r - glutamyl hydroxamate in 4 ml buffer solution and developed the colour by adding 1 ml ferric chloride reagent.

Calculation :

Found out the amount of γ - glutamyl hydroxamate formed in the reaction using standard graph. Expressed the enzyme activity as nanomole γ-glutamyl hydroxamate formed per min/ mg protein.

APPENDIX - X

ESTIMATION OF NITRATE REDUCTASE
(Sadasivam and Manickam, 1992)

Principle :

Nitrate reductase is capable of utilising the reduced form of pyridine nucleotides, flavins or benzyl viologen as electron donors for reduction of nitrate to nitrite. NADH - dependent nitrate reductase is most prevalent in plants. Hence, nitrate reductase activity in plants can be measured by following the oxidation of NAD(P)H at 540 nm. However, Nitrate reductase activity is commonly measured by colorimetric determination of nitrite produced.

Reagents :

- 0.1 M potassium phosphate buffer (pH 7.5)
- 0.1 M potassium Nitrate
- 2 mM NADH
- 1 % (W/v) sulphanilamide
- 0.02% N-(1-naphthyl) Ethylenediamine Dihydrocholoride
- 0.01 M Potassium Nitrite Standard solution

Dissolved 851 g pure potassium nitrite in 100 ml water in a standard flask. Diluted 10 ml of this solution to 100 ml and used as working standard solution.

Enzyme extract :

Homogenized a weighed quantity of the plant material in a known volume of medium (6 ml for 1 g fresh tissue) containing 1 mM EDTA, 1 - 25 mM cysteine and 25 mM potassium phosphate adjusted toa final pH 8.8 with kOH. Filtered through four layers of cheese cloth and centrifuged for 15 minutes at 30,000 g. Decanted the supernatant through glass wool and used for assays. Extracted under ice-cold conditions.

Procedure :

1. Pipetted out 0.5 ml phosphate buffer (pH 7.4) in a test tube.
2. Added 0.2 ml potassium nitrate solution, 0.4 ml NADH solution and 0.7 ml water
3. Initialised the reaction by the addition of 0.2 ml enzyme extract. A control was set up in the same way but with water instead of enzyme extract.
4. Incubated at $30^{o}C$ for 15 min
5. Terminated the reaction by the rapid addition of 1 ml sulphanilamide followed by 1 ml naphthyl ethylene diamine

reagent.

6. Waited for 30 minutes

7. Measured the absorbance at 540 nm

8. Prepared a standard graph with potassium nitrite. Pipetted out different known aliquots of potassium nitrite standard solution into a series of test tubes and made up the volume in each tube to 2 ml by adding water. Proceeded from 5 to 7.

Calculation :

Activity was expressed as micromole nitrite produced per min per mg protein (or per g fresh tissue).

APPENDIX - XI

ESTIMATION OF AVAILABLE NITROGEN IN SOILS
Alkaline potassium permanganate method
(Subbiah and Asija, 1956)

Principle :

The amount of soil nitrogen released by the potassium permanganate oxidation of part of soil organic matter is estimated by distillation with sodium hydroxide. The distillate is collected in boric acid containing double (mixed) indicator and titrated against standard sulphuric acid.

Reagent :

1. 0.32 % potassium permanganate.
2. 2.5 % sodium hydroxide.
3. Double (mixed) indicator : Bromocresol green (0.5 g and methyl red (0.1g) were dissolved in 100ml of ethyl alchohol
4. Standard sulphuric acid (N/50)
5. 2% Boric acid

Procedure :

Weighed 20 g of soil and transferred to a distillation flask. Added 20 ml of distilled water and 1.0 ml of liquid paraffin (to control frothing). Added a few glass beads (with holes) to prevent bumping and then added 100 ml of 2.32 % potassium permanganate solution and 100 ml of 2.5 % sodium hydroxide solution. Distilled the contents of a steady rate collecting the liberated ammonium in a 500ml ice tumbler containing 20 ml of Boric acid with double (mixed) indicator. Continued the distillation. For about 30 minutes until 100 ml of distillate was collected in the beaker. Titrated the ammonium collected against the standard acid (N/50) and from the titre value (a) calculated available nitrogen content of the soil.

Calculation :

$$\text{Amount of nitrogen present in 100g of soil} = \frac{0.00028a}{20} \times 100 \text{ g}$$

APPENDIX - XII

ESTIMATION OF AVAILABLE PHOSPHORUS IN SOIL
Colorimetric method (Olsen et al., 1954)

Principle :

This method of extraction of available soil phosphorus was suited for calcareous and alkaline soils. The CO_3^{2-} ions from $NaHCO_3$ will react with Ca^{2+} and $CaCO_3$ in the solution. The amount of phosphorus extracted was determined colorimetrically.

Reagents :

1. 0.5N Sodium Bicarbonate (pH adjusted to 8.5)

2. Activated Carbon (made phosphorus free by washing with 0.5 M sodium bicarbonate)

3. Reagent A: 12 g of ammonium molybdate was dissolved in 250 ml of distilled water. 0.2918 of antimony patassium tartarate was dissolved in 100 ml of distilled water. Both the solution were added to 100 ml approximately 5 N sulphuric acid. This solution was mixed thoroughly and made upto 2 liters with distilled water.

4. Reagent B: 1.056g of ascorbic acid was dissolved in 200ml of reagent A. This solution was prepared freshly as and when required.

Procedure :

Weighed 5g of soil and transferred to a 100ml polyethylene shaking bottle (carried out a blank side by side). Added a pinch of activated carbon (to make the extracted solution colourless). Added 50 ml of 0.5 M $NaHCO_3$ and shook in a reciprocating mechanical shaker for 30 minutes. Filtered through Whatman No. 40 filter paper, collecting the filtrate in a clean dry beaker or test tube. (The filtrate should be clean and colourless). Pipetted out 5.0 ml of the filtrate in a 25 ml volumetric flask to about 20 ml with distilled water. Added 4.0 ml of Reagent B and made up the volume to 25ml. Waited for 10 minutes for the colour to develop and there after measured the intensity of the blue colour in a photoelectric colorimeter using a red filter (660nm wave length) and adjusting the meter to 100 per cent transmittance with the blank. From the standard curve for phosphorus, found out the concentration of phosphorus (ppm) in the solution against the per cent transmittance observed in the above step. The result was expressed in gram per cent.

APPENDIX - XIII

ESTIMATION OF AVAILABLE POTASSIUM IN SOIL
Flame Photometric Method (Stanford and English, 1949)

Principle :

The potassium (K^+) ions in the exchange sites are replaced with ammonium (NH_4^+) ions and leached from the soil. The reaction may be illustrated as follows.

$$K + clay + NH_4^+ \text{ --------> } NH_4^{++} clay + K^+ + \text{excess } NH_4^+$$

The reaction goes to the right because of an excess of NH_4^+ ions as shown by NH_4^+ also being present on the right side of the equation. The K^+ ions in solution is then determined with the flame photometer.

Reagent :

1. Neutral normal ammonium acetate.
2. Standard potassium solution.

Procedure :

Weighed 5.0 g of soil and transferred it to a 100 ml polythene shaking bottle. Added 25 ml of neutral normal ammonium acetate and shook in a mechanical reciprocating shaker for 5 minutes. Filtered through dry Whatman No. 40 filter paper collecting the filtrate in a dry test tube. Measured the amount of potassium in the filtrate in the flame photometer.

From the concentration of potassium read in the standard curve corresponding to the flame photometer reading, the amount of available potassium in 100 g of soil was calculated.