Effect of Iron Application on the Growth, Biomass Production, Nodulation and Nitrogen Fixation in the Green Manure - *Sesbania rostrata*

By

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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

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Certified as bonafide research work

Signature of the Head of Department	Signature of the Dean of Faculty of Science	Signature of the Guide
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Introduction

1. INTRODUCTION

Agriculture today consumes high inputs of nitrogen (Farm Bulletin, 1993). The element nitrogen, comprising about 78 % of the earth's atmosphere (Hardarson, 1993) is one of the most important factors governing plant productivity (Becker et al., 1988). The gaseous form of atmospheric nitrogen is essentially useless to green plants. However, two groups of bacteria can change atmospheric dinitrogen into nitrates, nitrites and ammonia (Chapman and Reiss, 1994) which can be used up by plants. One group lives in soil, the other lives in roots of legumes.

The nutritional security for the evergrowing population of the country can be assured only when the availability of sufficient nutrients to the plants is also assured. To solve this critical problem, there is a need to use biofertilizers, which are called low cost inputs, renewable, pollution free (Singh and Dixit, 1994) and help in increasing the biologically fixed atmospheric nitrogen. Among the biofertilizers, nitrogen fixing bacteria (Rhizobium, Azotobacter and Azospirillum), blue green algae and Azolla are important (Hegde and Dwivedi, 1994).

Legumes in symbiosis with rhizobia are the best known nitrogen fixing systems (Tilak and Singh, 1994). They can fix upto 100 - 300 kg N/ha in one crop season

and in certain situations leave substantial nitrogen for the following crop (Venkatraman, 1988).

Organic matter management and biological nitrogen fixation are the major components of a sustainable rice agro eco-system. With the increasing costs of inorganic fertilizers and the growing concern in long-term soil fertility and environmental protection, interest in nitrogen-fixing green manure has recently increased (Ventura and Watanabe, 1993). Green manures, incorporated into the soil, undergo a process of microbial degradation which results in the conversion of organic nitrogen into plant available inorganic nitrogen (Sur et al., 1993).

Among green manures, Sesbania species are favoured by the farmers for their tolerance to stress conditions (Tiwari et al., 1995). Green manuring with Sesbania species for rice crop considerably increased organic carbon, nitrogen, phosphorus and potassium status of the soil and also enhanced the uptake of nitrogen, phosphorus, calcium, magnesium, sulphur, iron, manganese and zinc (Swarup, 1988).

Among 50 species of Sesbania, Sesbania rostrata is of special importance because of its rapid growth, high nitrogen fixing potential and tolerance for flooding (Becker et al., 1991). Especially this species has received a great deal of attention due to its unique

characteristics of stem nodulation by Azorhizobium caulinodans (Saraswathi et al., 1992). Azorhizobium caulinodans neither forms effective root nodules in other Sesbania species nor it produces stem and root nodules in any of the stem nodulating Aeschynomene species (Ladha et al., 1990). Kalidurai and Kannaiyan (1991) have shown that Sesbania rostrata produces maximum number of stem and root nodules on 45th and 60th day after sowing. In general, stem nodules have shown higher nitrogenase activity than root nodules (Dreyfus et al., 1985). Sesbania rostrata is the fastest nitrogen fixing plant known, fixing about 70 - 90 % of the total plant nitrogen in 45 - 55 days (Pareek et al., 1990).

Mineral nutrient deficiencies are a major constraint, limiting legume nitrogen fixation and yield. The mineral elements required for plant growth fall into two groups : the Macronutrients like nitrogen, phosphorus, calcium, magnesium, potassium and sulphur, and the Micronutrients like iron, chlorine, manganese, boron, zinc, copper and molybdenum (Shorrocks, 1992).

Nutrient limitations to legume production not only occur from deficiencies of the more common macronutrients such as phosphorus, potassium and suiphur, but also from micronutrients such as iron,

molybdenum and boron (O'Hara et al., 1988).

As lands are farmed more intensively, the need for micronutrients is steadily growing (Atwill, 1981). Micronutrients play a very significant role in crop growth as well as influencing nodules and nitrogen fixation in legumes (Bhanavase et al., 1993).

Iron, a micronutrient is an essential element for all organisms (Williams, 1981). It has many catalytic roles in plant growth like photosynthesis, respiration, nitrogen assimilation, synthesis of chlorophyll, reduction of nitrogen and of nitrate to ammonia and in the assimilation of ammonia by the glutamine-synthetase-glutamate synthase pathway. The activity of some enzymes like superoxide dismutases, catalase and peroxidase requires iron (Marschner, 1986 ; Raven, 1988).

Iron is an essential nutrient for the growth of both host legume and root nodule bacteria (Tang and Robson, 1992). Iron is important in nodule nitrogen fixation as it is a component of several key proteins such as nitrogenase, leghemoglobin and ferredoxin (Tang et al., 1990).

In the present study, the effect of application of iron as ferrous sulphate at varying levels (10, 20, 30 and 40 kg Fe/ha) on biomass production, nodulation and

nitrogen fixation in the green manure Sesbania rostrata has been investigated at the end of 30, 45 and 60 days after sowing. The parameters analysed were :

1. Biometric measurements
   - Root and Shoot length
   - Number of root and stem nodules
   - Plant fresh and dry weight
2. Enzyme assay
   - Glutamate dehydrogenase
   - Glutamate synthase
   - Nitrogenase activity
   - Catalase
   - Peroxidase
3. Biochemical parameters
   - Total chlorophyll
   - Allantoin
   - Amino nitrogen
   - Total nitrogen, phosphorus and potassium
   - Total iron

Review of Literature

2. REVIEW OF LITERATURE

The review of literature pertaining to the present investigation Studies on the effect of application of iron on the growth, biomass production, nodulation and nitrogen fixation in the green manure Sesbania rostrata is discussed under the following headings.

2.1 Introduction

2.2 Biofertilisers - Ecofriendly

2.3 Biological nitrogen fixation

2.3.1 Rhizobial magic

2.3.2 Legume - rhizobium symbosis - A successful performance

2.4 Green manures - substitute to chemical nitrogen fertilisers

2.5 Sesbania rostrata - A promising green manure

2.6 Sesbania rostrata - Azorhizobium caulinodans - a classical symbiosis

2.7 Mineral constraints to plant nutrition

2.8 Iron - The micronutrient

2.1 Introduction

There is a big gap between our needs and production (Farm Bulletin, 1993). The effort of achieving self-sufficiency in food production in India continues to be a challenging one. In the present day context of energy crisis, fast depletion of fossil resources and escalation of fertiliser prices, use of organics particularly green manuring has gained

importance for supplementing inorganic fertilisers in crop production. Therefore, it is essential to identify unknown nitrogen fixing leguminous plants that have green manure potential for rice (Kalidurai, 1988).

2.2 Biofertilizers - Ecofriendly

The long term future of Indian agriculture depends on the success of various efforts towards exploitation of biofertilisers which ultimately means going back to nature and reducing dependence on synthetic chemical fertilisers (Farm Bulletin, 1993). The term 'biofertiliser' means a preparation containing living micro organisms which are known to bring significant increase in biomass production and grain yield. It is a low cost input for increased output (Bhattacharya et al., 1993). Nitrogen fixing microbes constitute the predominant group of useful biofertilisers (Patronobis, 1994). The commonly used biofertilisers are Rhizobium, Azotobacter and Azospirillum (Katyal et al., 1994). The most important agronomically useful biofertilisers are the symbiotic Rhizobia which form nodules in legumes (Thomas, 1993).

2.3 Biological Nitrogen Fixation

Biological nitrogen fixation is an important process in flooded rice field ecosystem which contributes nitrogen to the rice crop. Despite the

use of fertiliser nitrogen in modern rice production, soil nitrogen and biological nitrogen fixation remain primary sources for a vast area under rice production (Kalidurai and Kannaiyan, 1992).

The need for sustained rice cropping had led to an urgent search for alternatives to chemical nitrogen fertiliser. Hence, increasing attention is being paid to biological nitrogen fixation to meet the nitrogen requirements of rice (Senaratne and Ratna Singhe, 1995). The enzymatic conversion of dinitrogen gas to ammonia is the most important source of fixed nitrogen entering the soil. The reduction of dinitrogen is catalysed by the nitrogenase enzyme system. Nitrogenase is found only in procaryotic microorganisms and eucaryotes such as plants can benefit from dinitrogen fixation only if they interact with nitrogen fixing species of microorganisms or obtain the fixed nitrogen after the death of the organism. The main agents of biological nitrogen fixation in rice fields are free living blue green algae, symbiotic blue green algae with azolla and hetero trophic bacteria in the rhizosphere and soil (Balasubramaniam, 1990).

2.3.1 Rhizobial Magic

Microorganisms have always played a vital but generally undetected role in agriculture, often working

below the soil surface to combat diseases, fix nitrogen or improve soil structure (Cook, 1991). Sustainable agriculture would be hard to acheive without rhizobia, because they abridge the gap between nitrogen in the air and the soil (Adams, 1995).

Seed inoculation with specific rhizobium have shown beneficial effects on nodulation, nitrogen fixation and yield of common pulse crops of India (Ramamoorthy et al., 1988). Competitive and efficient strains of rhizobium used for inoculation will ensure maximum nitrogen fixation (Tilak and Singh, 1994).

2.3.2 Legume - Rhizobium Symbiosis : A successful Performance

Legumes in symbiosis with rhizobia are the best known nitrogen fixing systems (Hardarson, 1993). Legumes provide a home for rhizobia. The bacteria live in structures called 'nodules'. The rhizobia induce the plant to build nodules on its roots. Inside these tiny homes, rhizobia draw nitrogen from air pores in the soil and chemically convert it to a form that the plant can absorb. The rhizobia are like miniature nitrogen factories. More than 90% of the nitrogen that they fix in nodules goes directly into the plant (Sean Adams, 1995). The rhizobium-legume association can fix upto 100 - 300 kgN/ha in one crop season (Hegde and Dwivedi, 1994).

2.4 Green Manures - Substitute to Chemical nitrogen fertilisers

Introduction of legume green manure crops in cropping systems can be economically justified if the legume has high nitrogen fixing potential (Halepyati and Sheelavantar, 1990). Green manuring is an efficient way of transferring biologically fixed nitrogen to the soil (Ghai et al., 1988). To be economically viable, a green manure should not disturb the existing crop sequence, should have minimal establishment cost and accumulate adequate nitrogen (Becker et al., 1995).

Sesbania rostrata (Brem and Obrem) and Aeschynomene afraspera (Leonard) are the two promising flood tolerant green manure species recently introduced in Asia. They grow fast, fix large amounts of nitrogen and nodulate both on stems and roots (Torres et al., 1995). Pareek et al., (1990) and Becker et al., (1990) found that 70 - 90% of total nitrogen in S. rostrata and 70% in A. afraspera was derived by biological nitrogen fixation.

The content of available nitrogen, phosphorus and potassium in soil has shown an increase with green manuring. Like the chemical properties, physical properties of the soil like bulk density and moisture

content at different soil depth have improved due to green manuring (Aktar et al., 1993). Green manuring also plays an important role in supplying major and minor nutrients in addition to increasing the nutrient and moisture holding capacities of rice soils (Joseph, 1986).

2.5 Sesbania rostrata - A promising Green Manure

Sesbania species have been recognised as promising green manure crops (Matoh et al., 1992). Profuse stem nodulation and non-inhibition by soil nitrogen are the two characteristics which probably contribute to the high nitrogen fixing potential of Sesbania rostrata (Rinaudo et al., 1983).

This plant possesses the ability to grow in flooded as well as in dry condition (Pillai and Nair, 1995). Flooded condition increased biomass production, nodulation and nitrogen fixation (Kalidurai and Kannaiyan, 1992). Root nodulation and biological nitrogen fixation of Sesbania species are reported to be either inhibited or affected by soil flooding, but stem nodules are unaffected, probably due to their position above ground plant parts (Becker and George, 1995).

It was reported that the total activities of the stem nodules were twice as high as those of the root

nodules (Saraswati et al., 1992). Upto 200 kg N/ha may be accumulated in 56 days under flooded conditions by S. rostrata incorporation (Engels et al., 1995). The important factor for utilisation of S. rostrata as green manure for rice crop is its less pithy nature of stem and undergo easy mineralisation in rice soils (Kalidurai, 1988).

2.8 Sesbania rostrata - Azorhizobium Caulinodans : A classical symbiosis

It is very important to match the right rhizobium with a particular plant (Berkum, 1995). Inoculation with appropriate strain of rhizobium increases nodulation and nitrogen fixation, but there could be difference in nitrogen fixing efficiency with different varieties (Roy et al., 1995). The azorhizobium - legume is the best known symbiotic system and is of major importance in agriculture (Dreyfus et al., 1988). The characteristic stem nodule rhizobium in S. rostrata is designated as Azorhizobium caulinodans. The cells are gram negative, small motile rods that are 0.5 to 0.6 u width and 1.5 - 2.5 u length. They are known to fix nitrogen in micro aerophilic conditions and have higher tolerance to oxygen (Gebhardt e al., 1984).

Ladha et al. (1989) showed that Azorhizobium released from stem nodules could survive and grow in flooded condition and in the rhizosphere of rice.

Adebayo *et al.* (1989) have reported that *Azorhizobium caulinodans* is present on *S. rostrata* plant leaves, flowers and non-host plant of *S. aculeata*. Legocki *et al.* (1983) found substantially higher amount of leghemoglobin in stem nodules than root nodules of *S. rostrata*.

2.7 Mineral Constraints to plant Nutrition

The level of soil fertility, in relation to plant nutrition is determined by the level of available nutrients (Amer *et al.*, 1993). The essential mineral nutrients for symbiotic legume nitrogen fixation are those required for the normal establishment and functioning of the symbiosis. Mineral nutrients may limit nitrogen fixation by affecting growth of rhizobia, infection and nodule development and nodule function as well as by affecting host plant growth (Robson, 1986).

The chemical elements like Carbon, hydrogen, oxygen, Nitrogen, phosphorus, sulphur, potassium, calcium, magnesium, iron, manganese, copper, zinc, molybdenum, boron, chlorine, nickel and cobalt are known to be essential for the legume-rhizobium symbiosis (O'Hara *et al.*, 1988).

Besides macronutrients, micronutrients are also found to enhance the growth of legumes (Kishor *et al.*,

1991). Micronutrients have a significant role on nodulation and nitrogen fixation in legumes (Robson, 1983). Like major nutrients, micronutrients are also important in boosting the agricultural production (Bhalerao et al., 1994). Micronutrient deficiencies or excesses may also affect nitrogen fixation directly through effect on function of nodules or indirectly through effects on assimilate supply from the host plant (Bhanavase et al., 1993).

2.8 Iron - The Micronutrient

As with other mineral nutrients, the symbiotic rhizobia are totally dependent upon the host plant for provision of supplies of iron, once the root infection has occurred (Dilworth and Glenn, 1984). Iron is an essential nutrient for the growth of both host legumes and root nodule bacteria.

Iron deficiency generally decreases nodule formation, leg-hemoglobin production and nitrogen activity, leading to low nitrogen concentrations in the shoots of some legumes. More iron is required for plants reliant on nitrogen fixation. The sensitive stage of nodulation to iron deficiency appears to be nodule initiation (Tang and Robson, 1992).

The multipurpose enzyme - ferric chelate reductase - was first recognised in the 1970's as a key player in

plant's transformation of iron into a form that the plant roots would absorb (Hays, 1993). Iron plays an important role in the formation of chlorophyll, which is essential for the process of photosynthesis. Iron is also associated with the enzyme mechanism that operates the respitatory system of plant cells. In addition, iron compounds have a role on cell division and growth.(Raven, 1988).

Rai et al. (1984) have reported that iron, with its interrelationship with other micronutrients is an important factor in controlling the nitrogenase make up and activity inside the nodules. According to them, extrinsic factor which limit the synthesis and functions of leg-hemoglobin, ferredoxin and active iron (Fe^{++}) inside nodules, are of great importance. Iron in plants is a constituent of humin enzyme and present in complex bond in iron porphyrin molecules (Hemantaranjan, 1988). The green leaf is a seat of various biochemical activities (Mahurkar et al., 1992). Photosynthesis and nitrogen metabolism are major physiological activities in plants. Chlorophyll plays an important role in photosynthesis. The enzymes associated with chlorophyll metabolism are catalase and peroxidase which contain iron as prosthetic group (Kadam et al., 1988).

Materials and Methods

3. MATERIALS AND METHODS

An experiment was conducted to study the effect of iron application on the growth, biomass production, nodulation and nitrogen fixation in the green manure Sesbania rostrata. The study was carried out in pot cultures in completely randomised block design with four replications for each treatment.

3.1 Preparation of soil

The soil was tested for nitrogen, phosphorus, potassium, iron content and its calcarity prior to usage. The soil was enriched by basal application of farm yard manure at the rate of 10 tonnes/ha basis. Each of the twenty pots used for the experiment was filled with 10kg of soil and treated with iron in the form of ferrous sulphate at the level of 10, 20, 30 and 40 kg of Fe/ha.

3.2 Seed Treatment

The seeds of S. rostrata, collected from TNAU were soaked in hot water for 10 min and then soaked in cold water for about 6 hrs, shade dried for 3 hours, treated with peat based inocula of Azorhizobium caulinodans with starch as an adhesive agent.

3.3 Pot Culture layout

The treatments were as follows :

T1 - *Azorhizobium* seed inoculated *S. rostrata* alone

T2 - *Azorhizobium* seed inoculated *S. rostrata* + 10 Kg of Fe/ha

T3 - *Azorhizobium* seed inoculated *S. rostrata* + 20 kg of Fe/ha

T4 - *Azorhizobium* seed inoculated *S. rostrata* + 30 kg of Fe/ha

T5 - *Azorhizobium* seed inoculated *S. rostrata* + 40 kg of Fe/ha

3.4 Foliar Spraying

Foliar inoculation with foliar spraying was given on 30th and 40th day after sowing. The stem isolates of *A. caulinodans* used for foliar spraying was cultured in Yeast Extract Mannitol (YEM) medium consisting of :

Mannitol	- 10 g	Yeast extract	- 0.5 g
K_2HPO_4	- 0.5 g	NaCl	- 0.1 g
$MgSO_4$	- 0.2 g	Distilled Water	- 1000 ml

3.5 Maintenance of Crops

Bavistin 0.05% was sprayed to the plants of *S. rostrata* on 15th day after sowing. Nuvacron was sprayed on 45th day after sowing. Flooding or 100% moisture saturation was maintained throughout the study.

3.6 Harvest Methodology

At the end of 30,45 and 60th day the plants were uprooted from the pots without any damage to the root system. The soil particles were removed and the roots were washed with gently flowing water. They were kept between filter paper folds to absorb adhering water droplets and the plants were used for the various assays carried out.

3.7 Biometric Observations

1. Fresh Weight :

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

2. Dry Weight :

The uprooted plants after recording the fresh biomass were oven 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

The plants were uprooted, soil particles were removed and the total number of stem and root nodules per plant were counted separately and recorded.

3.8 Biochemical Analysis

The biochemical constituents analysed were chlorophyll, allantoin, amino nitrogen, nitrogen, phosphorus, potassium, total iron and enzymes namely glutamate dehydrogenase, glutamate synthase, nitrogenase, catalase and peroxidase.

The details of the parameters studied, various parts of the plant used, method of analysis, the appendices in which the details of the method described and the relevant reference are given in Table - I.

3.9 Soil Analysis

The procedures for the estimation of soil nitrogen, phosphorus, potassium and iron were described in Appendix XIII, XIV, XV and XVI respectively.

3.10 Statistical Analysis

The results of the experiment were analysed and subjected to statistical scrutiny by arithmetic mean and two way analysis of variance.

Table - I

Analysis of biochemical parameters and enzyme assays

S.No	Parameters	Parts of plant analysed	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, (1973)	VI
7	Total Iron	Whole Plant dry sample	Atomic absorption spectro-photometry	Shanmugam et al.,(1994)	VII
Enzyme Assay :					
8	Glutamate synthase	Fresh roots	Spectro-photometry	Casteele et al.,(1975)	VIII
9	Glutamate dehydrogenase	Fresh roots	Spectro-photometry	Doherty, (1976)	IX
10	Nitrogenase	Fresh root & stem nodules	Gas liquid chromato-graphy	Hardy et al., (1968)	X
11	Catalase	Fresh leaves	Spectro-photometry	Luck, (1974)	XI
12	Peroxidase	Fresh leaves	Spectro-photometry	Malik and Singh, (1980)	XII

Results and Discussion

4.RESULTS AND DISCUSSION

An experiment was conducted to study the effect of Iron application on the growth, biomass production, nodulation and nitrogen fixation in the green manure Sesbania rostrata. The study was carried out in pot cultures in completely randomised block design with four replications for each treatment.

Seeds of S. rostrata were soaked in hot water for 10 minutes and then soaked in cold water for about 6 hours. The soaking of seeds in boiling water increased the percentage of germination from 4 to 78 (Sheelavantar et al., 1989). The pre soaked seeds were then shade dried for 3 hours and treated with peat based inocula of stem and root Azorhizobium caulinodans isolates. Plate-1 shows the Azorhizobium treated and untreated S. rostrata seeds. Inoculation with appropriate strain was found to induce early and repeatable nodulation (Bhagwat et al., 1988)

4.1 SOIL ANALYSIS :

The soil was analysed for the macronutrients like nitrogen, phosphorus, potassium and micronutrient - iron, its calcarity and pH. Table II depicts the same.

This soil was enriched by basal application of farm yard manure at the rate of 10 tonnes/ha basis (Deshmukh et al., 1994). Rao and Dakhore (1993) had

PLATE 1 : Azorhizobium TREATED AND UNTREATED Sesbania rostrata SEEDS

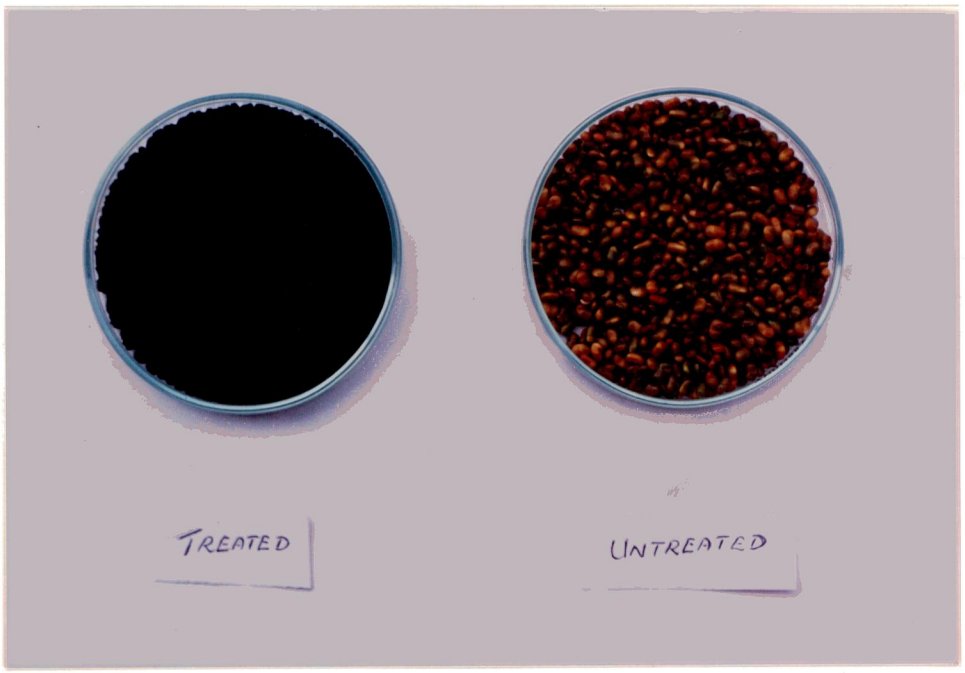


PLATE 2 : GROWTH OF Sesbania rostrata ON 60th DAY

demonstrated that farm yard manure application increased the available iron content of the soil.

TABLE - II

SOIL ANALYSIS

Available Nitrogen	289 kg/ha
Phosphorus	9 kg/ha
Potassium	129 kg/ha
Calcarity	Non calcareous
pH	6.5
Micronutrient Iron	3.23 ppm

Each of the 20 pots used for the experiment were filled with 10 kg of farm yard manure enriched soil and treated with iron in the form of ferrous sulphate at the levels of 10, 20, 30 and 40 kg of Fe/ha.

The seeds were uniformly sown and later thinned to obtain a sufficient plant density. At the end of 30, 45 and 60 days, the plants were carefully pulled out from the pots keeping the root system intact, washed and removed the water droplets by blotting between the folds of filter paper.

The whole plant was used to record the root and shoot length, fresh and dry weight. The fresh leaves were used for the estimation of chlorophyll, allantoin, amino nitrogen and the enzymes namely catalase and peroxidase. The fresh roots and nodules were used for

estimating the activity of ammonia assimilating enzymes (Glutamate dehydrogenase and glutamate synthase) and nitrogenase respectively. The plants were dried at 80°C for 24 hours. The dried plants were weighed and then powdered in a mortar with a pestle and were used for analysis of nitrogen, phosphorus, potassium and total iron. The results of the investigation are discussed under the following headings.

4.2 GROWTH ATTRIBUTES :

The growth characteristics of S. rostrata such as root and shoot length, plant fresh and dry weight, and number of nodules were recorded at three stages viz., 30, 45 and 60 days after sowing. Plate-2 indicates the growth of S. rostrata on 60th day.

4.2.1 ROOT LENGTH :

The length of the root was measured from collar region to the tip of the root and the results were presented in Table III and Fig. 1.

It was evident from the data that among the various treatments T1 (Control) registered minimum root length at all 3 stages of growth period. The maximum root length was observed in the treatment T5 which was supplied with 40 kg of Fe/ha at 30, 45 and 60 days after sowing.

Though there was a significant increase numerically, analysis of variance indicated that there was no significant increase in root length at 5 per

cent level within the treatments at all 3 stages of growth. A significant increase in root length was noticed for each treatment between 30 and 45 days. Root length increased significantly from T4 to T5 between 45 and 60 days whereas T2 and T3 did not vary significantly from the control (T1).

Thus, application of iron had influenced the root length during the growth period. Similar results were depicted by Tang et al., (1990).

TABLE - III

EFFECT OF IRON ON ROOT AND SHOOT LENGTH IN S. rostrata

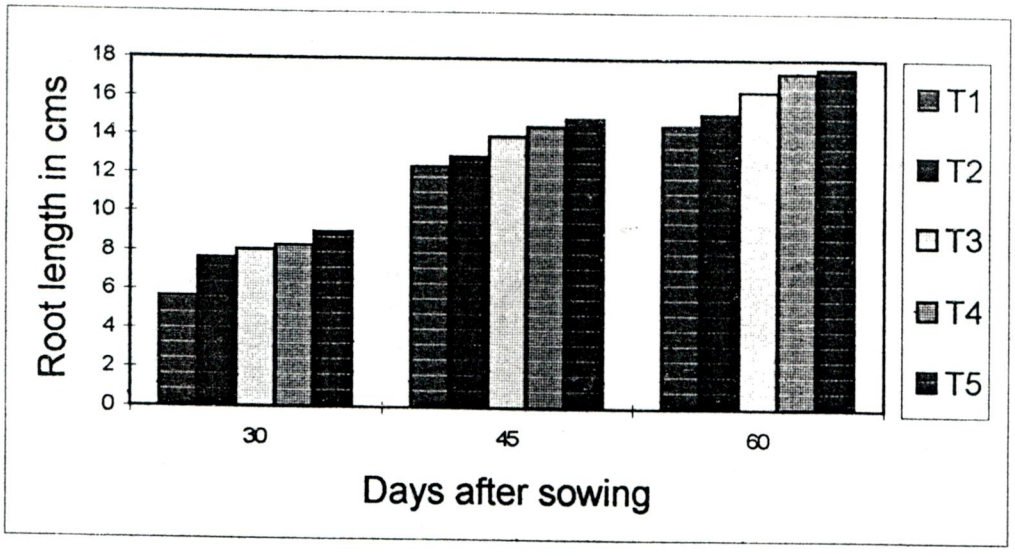
Treatments	Root Lengths(cms)			Shoot Length (cms)		
	Days after sowing			Days after sowing		
	30	45	60	30	45	60
T1	5.64	12.39	14.55	7.92	15.95	31.62
T2	7.63	12.92	15.19	8.88	18.49	33.29
T3	8.05	13.97	16.35	9.33	20.87	35.94
T4	8.27	14.50	17.30	9.77	22.06	38.37
T5	8.97	14.93	17.53	10.08	22.53	42.06
	D	T	DT	D	T	DT
SED	0.52	0.67	1.17	0.30	0.39	0.67
CD(0.05)	1.07	1.38	2.39	0.62	0.79	1.38

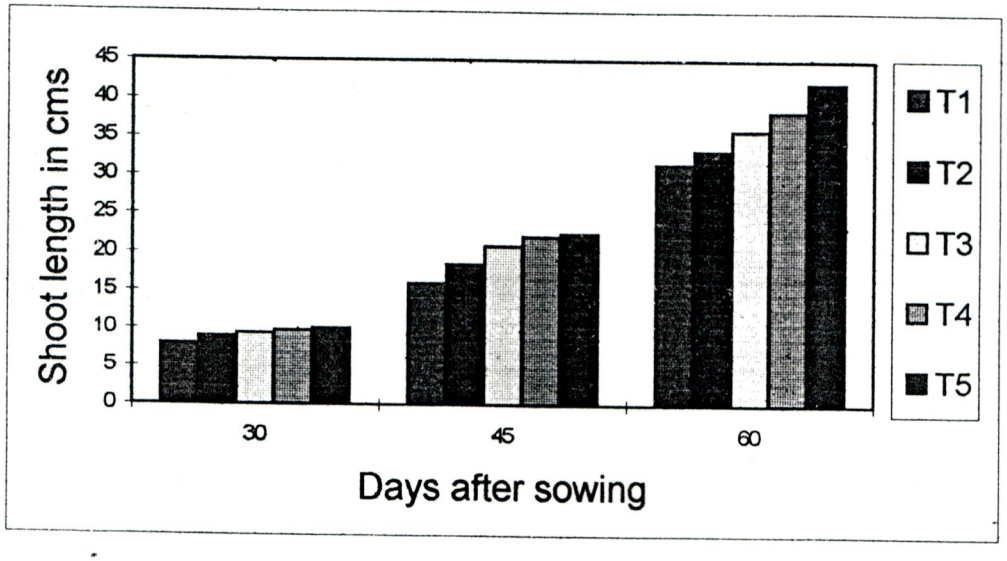
4.2.2 SHOOT LENGTH

The data in Table III and Fig - 2 depicted that there was a gradual increase in shoot length from T1 to

Figures - 1 and 2

EFFECT OF IRON APPLICATION ON ROOT AND SHOOT LENGTHS IN *S.rostrata*





T5. On 30th day, the increase was however, not significant. Nevertheless, on 45th and 60th day, the shoot length increased significantly ($P < 0.05$) as the concentration of iron was increased. The increase in shoot length of S. rostrata was found to be highly significant for each treatment when compared between days.

The increase in shoot length with increase in the levels of iron correlates well with the results of Raven (1988).

4.3 NODULE NUMBERS

4.3.1 ROOT NODULES

Table IV and Plate - 3 indicates that the number of root nodules increased in all the treatments steadily with the increase in age of the plant.

The number of root nodules increased gradually on 30th day from T1 to T5 on numerical basis. However, on 45th day the nodule number increased significantly from T1 to T3 and T4 to T5 except T3 to T4. A highly significant increase ($P < 0.05$) was noticed on 60th day from T1 to T5 as well as between the days viz 30,45 and 60 days after sowing.

Thus the present study clearly revealed that iron application had increased the root nodulation on 45th and 60th day, Similar results were reported by (O'Hara et al., 1988 and Tang et al., 1990).

4.3.2 STEM NODULES

It was evident from Table - IV that there was stem nodulation in S. rostrata on 45th and 60th day which had increased with the growth period as well as with the concentration of iron. Statistical analysis

TABLE - IV

EFFECT OF IRON ON ROOT AND STEM NODULATION IN S. rostrata

Treatments	Number of Root Nodules			Number of Stem Nodules	
	Days after sowing			Days after sowing	
	30	45	60	45	60
T1	1.00	4.78	13.00	2.33	4.75
T2	1.70	6.75	17.30	3.10	5.67
T3	2.00	8.67	21.00	4.33	7.80
T4	2.40	9.50	25.00	5.45	8.75
T5	3.00	11.00	28.75	5.75	9.50
	D	T	DT	D	T	DT
SED	0.32	0.41	0.71	2.86	0.45	0.64
CD(0.05)	0.65	0.83	1.44	0.60	0.95	1.34

predicted that the increase was found to be significant ($P < 0.05$) for the treatments T2, T3, T4 and T5 when compared to the untreated control (T1).

Thus the study showed that iron application increased stem nodulation. Plate - 4 and Plate - 5 indicates stem nodulation in S. rostrata.

PLATE 3 : ROOT NODULATION IN TREATED AND UNTREATED

Sesbania rostrata

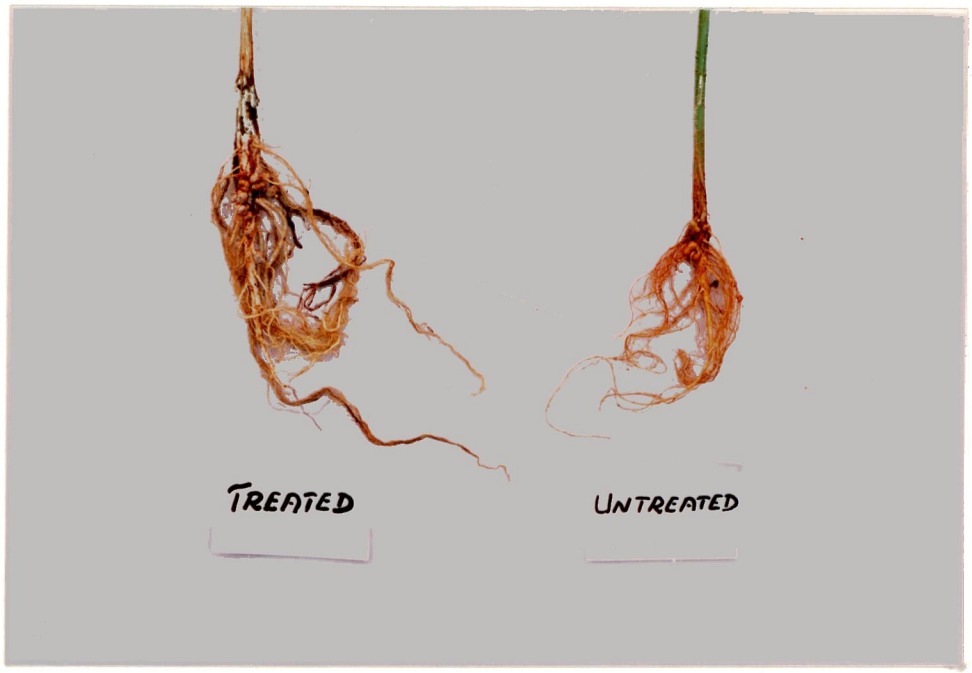


PLATE 4 : STEM NODULATION IN TREATED AND UNTREATED

Sesbania rostrata

PLATE 5 : STEM NODULATION IN *Sesbania rostrata*

4.4 BIOMASS PRODUCTION

The various levels of iron was found to have a significant influence on the plant fresh and dry weight over iron untreated plants as indicated in Table V.

4.4.1 FRESH WEIGHT

The data (Table V) revealed that the untreated control recorded the minimum and the treatment (T5) recorded the maximum fresh weight in all the three stages of growth. Among the iron treated plants, there was a steady increase in fresh weight from T2 to T5 when compared to control (T1). Statistical analysis presented that there was a significant increase ($p < 0.05$) in plant fresh weight for treatment T2 to T5 on 45th and 60th day but on 30th day only for T4 to T5 when compared to the control. A positive effect of iron application on fresh weight was reported by Tang and Robson (1992).

4.4.2 DRY WEIGHT

Table V predicts that the dry weight of S. rostrata varied considerably within the treatments and also between the 3 stages of growth period. On the 30th day, increase in biomass was not significant for the treatments T2 and T3 but for T4 and T5 there was a significant increase ($P < 0.05$) when compared to the control.

TABLE - V

EFFECT OF IRON ON BIOMASS PRODUCTION IN *S. rostrata*

Treatments	Fresh Weight (g)			Fresh Weight (g)		
	Days after sowing			Days after sowing		
	30	45	60	30	45	60
T1	0.95	2.30	5.82	0.14	0.44	0.91
T2	1.45	3.68	8.37	0.22	0.92	1.67
T3	1.62	5.13	9.71	0.28	1.28	1.94
T4	1.81	7.37	10.24	0.33	1.84	2.06
T5	2.05	9.52	12.38	0.56	2.05	2.48
	D	T	DT	D	T	DT
SED	0.16	0.21	0.36	0.05	0.06	0.11
CD(0.05)	0.33	0.43	0.74	0.10	0.13	0.23

However, on 45th and 60th day, the biomass production was significantly increased ($P < 0.05$) for the treatments T2 to T5 when compared to the control (T1).

In general, iron application had increased the biomass production with increase in iron concentration as well as with increase in growth period. This result was supported by the reports of Hemantaranjan (1988).

4.5 BIOCHEMICAL ANALYSIS

The benefit of nitrogen fixation was evaluated in terms of specific constituents of the plant.

4.5.1 CHLOROPHYLL CONTENT

The amount of chlorophyll accumulation might

indirectly denote the healthy condition of the plants. The data on total chlorophyll content was depicted in Table VI.

There was a gradual increase in the total chlorophyll content with the increase in concentration of iron as well as with the increase in the growth period. Among the treatments, T5 was found to be superior over the rest of the treatments and T1 (control) recorded the minimum chlorophyll content at all the three stages of growth. Though the above results were numerically significant, statistical analysis revealed that there was no significant difference in chlorophyll content of various treatments

TABLE - VI

EFFECT OF IRON ON BIOCHEMICAL PARAMETERS OF S. rostrata

Treatments	Chlorophyll			Allantoin			Amino Nitrogen		
	Days after sowing			Days after sowing			Days after sowing		
	30	45	60	30	45	60	30	40	60
T1	1.00	1.25	1.55	0.38	0.44	0.46	2.40	4.00	9.80
T2	1.10	1.45	1.60	0.48	0.54	0.60	3.60	5.60	12.00
T3	1.12	1.60	1.85	0.50	0.58	0.78	4.40	6.00	13.00
T4	1.23	1.65	1.95	0.56	0.64	0.82	6.00	7.20	13.80
T5	1.30	2.00	2.35	0.64	0.70	1.12	7.40	9.40	14.00
	D	T	DT	D	T	DT	D	T	DT
SED	0.06	0.07	1.12	0.02	0.03	0.05	0.08	0.11	0.18
CD(0.05)	1.07	0.15	0.25	0.05	0.06	0.10	0.17	0.22	0.38

on 30th day. Nevertheless, on 45th and 60th day, the treatments T3, T4 and T5 recorded a significant difference ($P < 0.05$) in chlorophyll content in comparision to the control (T1).

Chlorophyll, one of the best parameters to study the intensity of nitrogen uptake and this content might find better association with nitrogen uptake than other factors. Because of the higher content of total chlorophyll it was evident that iron application might trigger photosynthesis in S rostrata. It was predictable that high photosynthetic efficiency of the leaves might increase nitrogen fixation in S. rostrata.

Similar results were presented by Agarwal and Srivastava (1984) that increasing levels of iron profoundly improved total chlorophyll content in the leaves at all the 3 growth stages.

4.5.2 ALLANTOIN CONTENT

It is obvious from the data presented in Table VI that the control (T1) showed minimum allantoin content when compared to iron treated S. rostrata. Within the iron treated plants, the plants supplied with 40 kg of Fe/ha (T5) showed maximum allantoin content (0.64, 0.70 and 1.12 mg/g plant) and plants treated with 10 kg of Fe/ha (T1) showed minimum allantoin content (0.38, 0.44 and 0.46 mg/g plant) at all the three stages of growth.

The allantoin content had increased numerically during the entire growth period but statistical analysis had not shown any significant increase on 30th and 45th day, However, allantoin content had increased significantly ($P < 0.05$) on 60th day from T1 to T5 except for T4.

Thus, it was evident that iron application increased allantoin content in all 3 stages of growth period which is in accordance with the study by Sujatha (1989) that the allantoin content of Sesbania species were found to be increased with the age of the plant.

4.5.3 AMINO NITROGEN

The amino nitrogen content of iron treated and untreated S. rostrata was depicted in Table VI.

The data revealed that the amino nitrogen content was higher in iron treated than the untreated S. rostrata at all the 3 stages. There was a significant increase ($P < 0.05$) between all treatments T1 to T5 for all 3 stages of growth viz 30, 45 and 60 days except T4 to T5 on 60th day.

The amount of aminonitrogen or allantoin was an indication of increased nitrogen fixing activity. From this study, it was clear that iron application had increased nitrogen fixation and hence, aminonitrogen content of the plant. The increase in iron

application increased nitrogen fixation was supported by a number of earlier investigators (Terry et al., 1988 ; Bhanavase et al., 1993 and O'Hara, 1988).

The aminonitrogen content was found to be higher than the allantoin for all the 3 stages of growth as well as for the various treatments. Hence it can be concluded that S. rostrata is an amide transporting type of legume.

4.5.4 NITROGEN, PHOSPHORUS, POTASSIUM AND TOTAL IRON

The data on the total nitrogen, phosphorus, potassium anbd total iron content of the various levels of iron treated S. rostrata at 30, 45 and 60 days after sowing were presented in Table VII and VIII.

TOTAL NITROGEN AND PHOSPHORUS

It was evident from Table VII and Fig 3 that the total nitrogen content was found to increase with the age of the plants irrespective of the treatments. The control plants recorded less percentage of nitrogen when compared to the iron treated plants. Among the iron treated plants, T5 showed maximum nitrogen content (1.50, 2.50 and 2.83 per cent) and T2 showed the minimum nitrogen content (1.12, 1.53 and 2.07 per cent) at all the three stages of growth. Statistical analysis revealed that there was a significant increase ($p < 0.05$) in nitrogen content between the days as well as between the treatments.

Thus, the result depicted that there was an increase in percentage of nitrogen with increasing levels of iron and also with the increase in age of plant. This might be due to increased nodulation and hence nitrogen fixation of iron treated S. rostrata. A study by O'Hara et al. (1988) indicated that legume-Rhizobium symbiosis increased the nitrogen concentration in plants.

TABLE - VII

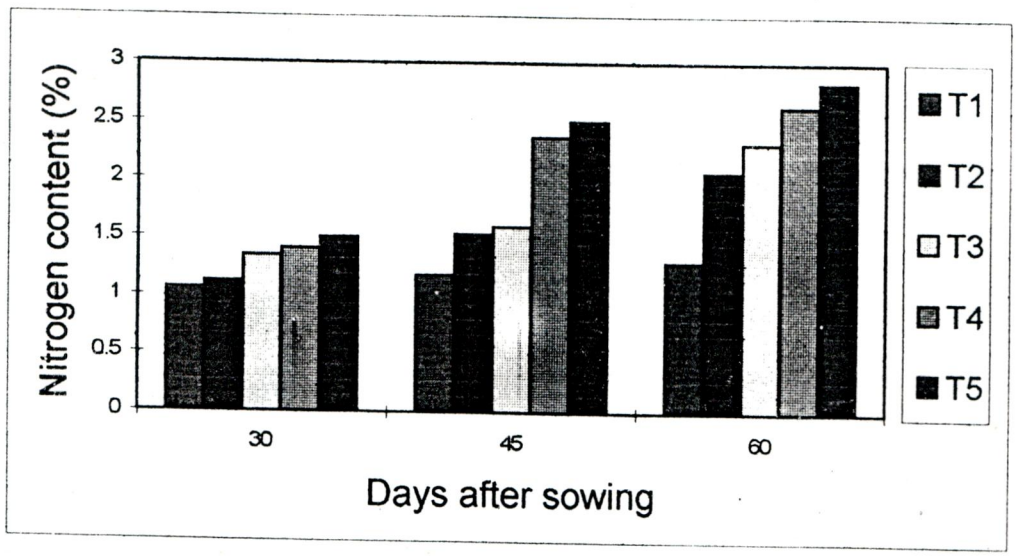
EFFECT OF IRON ON NITROGEN AND PHOSPHORUS CONTENT OF S. rostrata

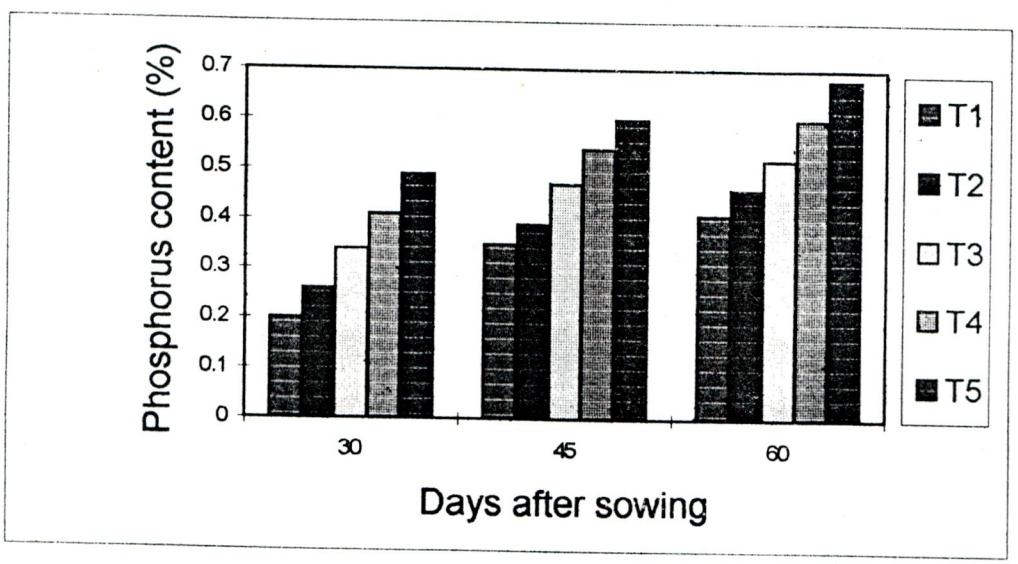
Treatments	Nitrogen (%)			Phosphorus (%)		
	Days after sowing			Days after sowing		
	30	45	60	30	45	60
T1	1.06	1.18	1.29	0.20	0.35	0.41
T2	1.12	1.53	2.07	0.26	0.39	0.46
T3	1.34	1.59	2.31	0.34	0.47	0.52
T4	1.40	2.36	2.63	0.41	0.54	0.60
T5	1.50	2.50	2.83	0.49	0.60	0.68
	D	T	DT	D	T	DT
SED	0.06	0.07	0.13	0.02	0.02	0.03
CD(0.05)	0.12	0.15	0.26	0.03	0.04	0.07

As depicted in Table VII and Fig 4 that for all the three stages of growth, maximum phosphorus content was found in T5 that had received iron at the level of 40 kg Fe/ha and the minimum was recorded in T1 (control) which did not receive any iron. There was a

Figures 3 and 4

EFFECT OF IRON APPLICATION ON NITROGEN AND PHOSPHORUS CONTENT IN *S. rostrata*





significant increase ($P < 0.05$) in phosphorus content for the iron treated plants (T2, T3, T4 AND T5) when compared to the control on all the 3 stages of growth. The phosphorus content also increased significantly with the age of the plants. Similar reports on the effect of iron on phosphhorus content was reported by Nagar and Awad in 1987.

POTASSIUM

Table VIII and Fig : 5 clearly depicts that the application of iron had influenced the potassium content in S. rostrata. T5 recorded the maximum potassium content (3.00, 3.54 and 3.89 per cent) on 30, 45 and 60 days after sowing and T1 recorded the minimum content of potassium (1.95, 2,12 and 2.31 per cent).

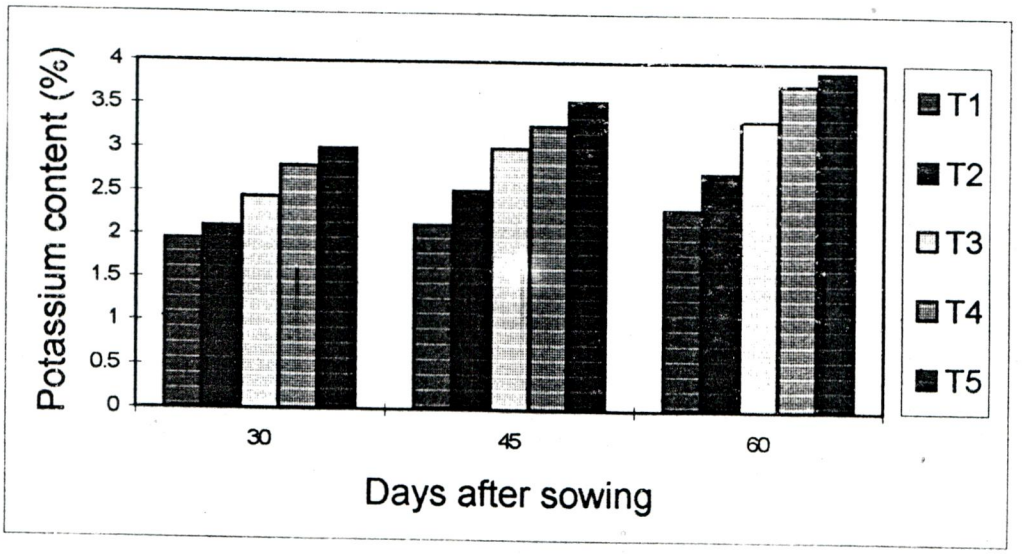
TABLE - VIII

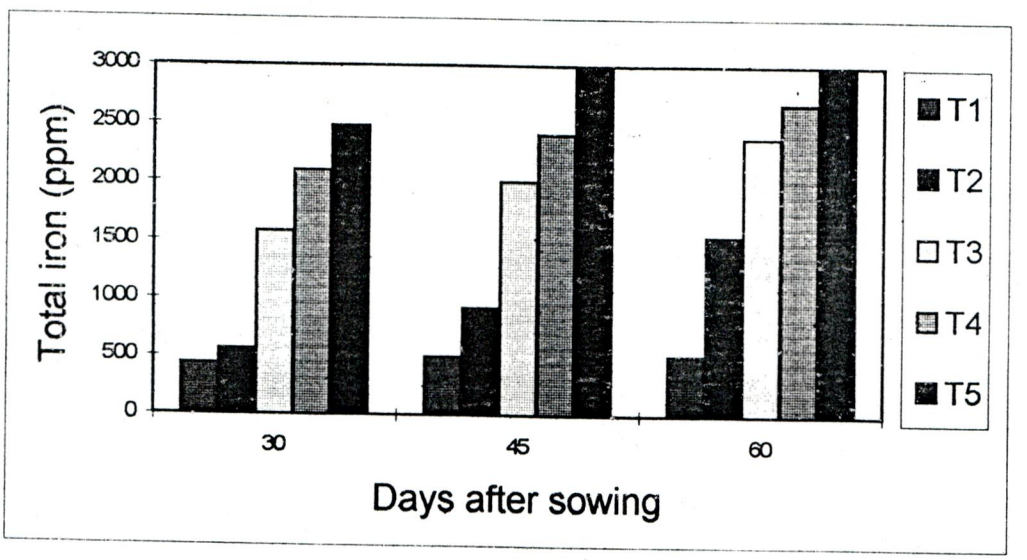
EFFECT OF IRON ON POTASSIUM AND TOTAL IRON CONTENT IN S. rostrata

Treatments	potassium(%)			Total Iron (ppm)		
	Days after sowing			Days after sowing		
	30	45	60	30	45	60
T1	1.95	2.12	2.31	433	496	513
T2	2.10	2.52	2.73	557	926	1540
T3	2.44	3.01	3.33	1575	2002	2376
T4	2.80	3.27	3.75	2094	2406	2672
T5	3.00	3.54	3.89	2477	2970	2990
	D	T	DT	D	T	DT
SED	0.02	0.03	0.05	3.23	4.17	7.23
CD(0.05)	0.04	0.05	0.09	6.60	8.52	14.76

Figures 5 and 6

EFFECT OF IRON APPLICATION ON POTASSIUM AND TOTAL IRON CONTENT IN *S. rostrata*





Among the iron treated plants T2 recorded the minimum content of potassium (2.10, 2.52 and 2.73 per cent). Analysis of variance indicated that the potassium content increased significantly ($P < 0.05$) between treatment T1 to T5 as well as between the days.

This interesting increase in potassium content in S. rostrata with increase in the levels of Iron (10 to 40 kg Fe/ha) correlates well with the same such results from the work of Pande et al., (1993).

TOTAL IRON

Table VIII and FIg 6 depicted the total iron content of iron treated and untreated S. rostrata at the end of 30,45 and 60 days after sowing. From the data it was evident that the total iron content washigher in iron treated plants (T2 to T5) than untreated (T1) plants. Among the treatments, T5 was found to be superior over the rest of the treatments and T2 recorded minimum iron content.

Greater availability of iron by way of ferrous sulphate application had resulted in greater nutrient absorption. A study by Terry et al (1988) also indicated that iron content of plants was consistently higher in the plants which had received iron.

4.6 AMMONIA ASSIMILATING ENZYMES

4.6.1 GLUTAMATE DEHYDROGENASE

The activity of glutamate dehydrogenase estimated

in fresh root samples of various treatments was given in Table IX and Fig 7.

TABLE - IX

EFFECT OF IRON ON THE ACTIVITY OF AMMONIA ASSIMILATING ENZYMES AND NITROGENASE IN S. rostrata

Treatments	Glutamate dehydrogenase*			Glutamate Synthase*			Nitrogenase #
	Days after sowing			Days after sowing			Days after sowing
	30	45	60	30	45	60	60
T1	9.83	13.97	14.14	1.78	15.57	34.55	20.48
T2	10.77	16.04	18.81	2.81	14.13	34.43	30.72
T3	12.48	15.38	17.81	4.13	21.13	36.15	72.28
T4	14.11	17.32	20.16	7.39	23.86	36.56	115.20
T5	15.35	21.34	25.91	9.43	30.23	37.71	184.32
	D	T	DT	D	T	DT	T
SED	0.30	0.38	0.66	0.18	0.24	0.42	0.61
CD(0.05)	0.60	0.78	1.35	0.39	0.50	0.86	1.41

* Nanomoles of NADH oxidised / min / mg protein

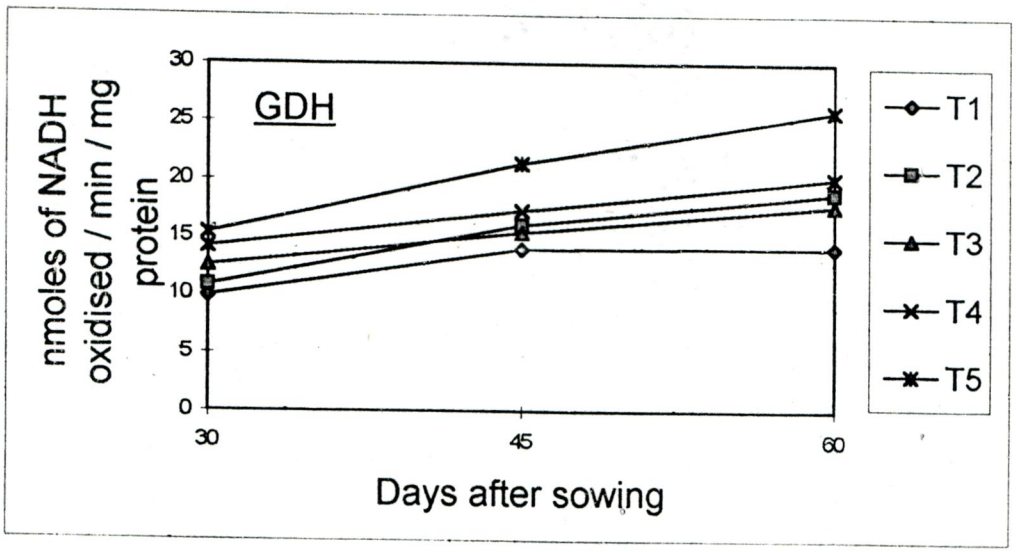
\# Nanomoles of ethylene produced / hr /g dry weight of nodules

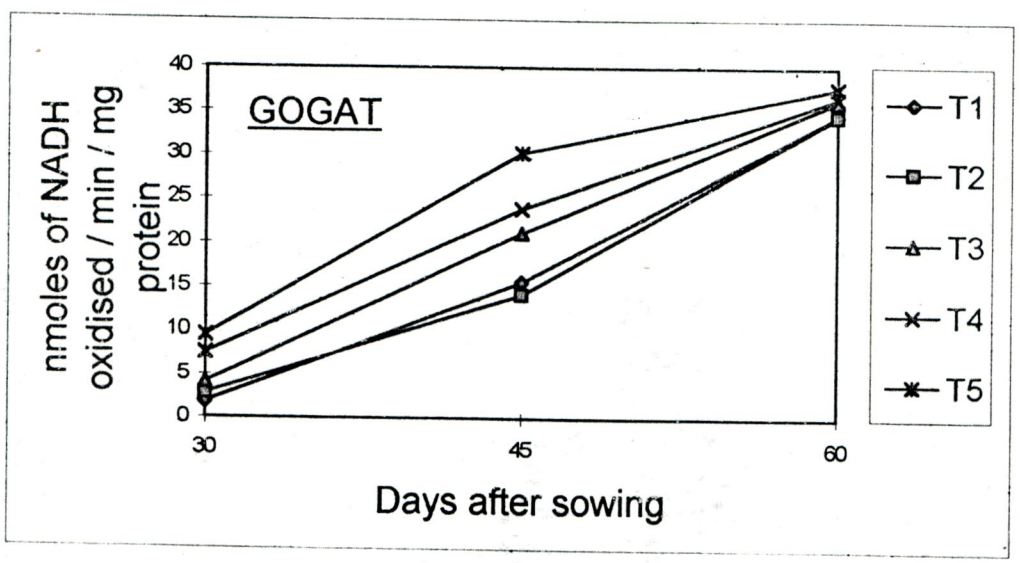
The data clearly showed that on 30, 45 and 60 days after sowing the maximum enzyme activity was found in the treatment that received 40 kg of Fe/ha and the minimum activity in control (T1). There was a significant increase ($p < 0.05$) in the activity of the enzyme with the growth period for each treatment.

Thus the present study showed that glutamate

Figures 7 and 8

EFFECT OF IRON APPLICATION ON THE ACTIVITY OF AMMONIA ASSIMILATING ENZYMES IN *S. rostrata*





dehydrogenase activity was increased on 45th and 60th day in all iron treated plants of S. rostrata, thereby influencing nitrogen assimilation in plants.

4.6.2 GLUTAMATE SYNTHASE

The application of iron at various levels had influenced glutamate synthase activity when compared to untreated control as revealed by Table IX and Fig : 8.

T5 registered a significantly higher activity on 30th and 45th day but the same was not found on 60th day. Statistical analysis confirmed that there was a highly significant ($p < 0.05$) increase in enzyme activity for each treatment on all the 3 stages of growth. Martin et al., (1994) have proposed that NADH dependent GDH and GOGAT are both involved in ammonia assimilation. The increase in activity of these two enzymes might result in increase in the amount of available nitrogen to plants.

4.7 NITROGENASE

The activity of nitrogenase was estimated in the root and stem nodules of S. rostrata at the end of 60th day after sowing and the results were presented in Table IX and Fig : 9.

It was obvious from the data that the activity of the enzyme was higher in the iron treated plants when compared to the iron untreated control plants. Among the iron treated plants, T5 recorded the maximum and T2

the minimum enzyme activity. There was a drastic increase in the activity of enzyme from T2 to T5. The enzyme activity between treatments showed highly significant increase ($p < 0.05$).

The increase in nitrogenase activity between various treatments on 60th day might be due to the increase in number of nodules. Thus iron application might had an impact on nodule growth and hence on nitrogenase activity. Similar results were reported by Tang and Robson (1990), Hemantaranjan (1988), Bhanavase and Patil (1993).

4.8 OXIDATIVE ENZYMES

4.8.1 CATALASE

It was evident from table X and Fig - 10 that the various levels of iron had influenced a steady increase in the activity of catalase within the treatments as well as between the days during all the 3 stages of growth. Maximum catalase activity was recorded for T5 and minimum for T1 on 30, 45 and 60 days after sowing. A highly significant increase ($p < 0.05$) in the catalase activity was observed from T1 to T5 as well as between days.

TABLE - X

EFFECT OF IRON ON THE ACTIVITY OF CATALASE AND PEROXIDASE IN S. rostrata

Treatments	Catalase ($X 10^4$ units/g tissue)			Peroxidase (units / litre)		
	Days after sowing			Days after sowing		
	30	45	60	30	45	60
T1	0.037	0.085	0.094	124.06	206.25	306.67
T2	0.061	0.106	0.170	144.74	317.30	515.63
T3	0.071	0.121	0.189	165.00	412.50	687.50
T4	0.094	0.142	0.213	181.32	550.00	916.67
T5	0.142	0.170	0.283	236.00	634.62	1269.23
	D	T	DT	D	T	DT
SED	0.001	0.001	0.001	0.19	0.25	0.44
CD(0.05)	0.001	0.001	0.002	0.40	0.52	0.89

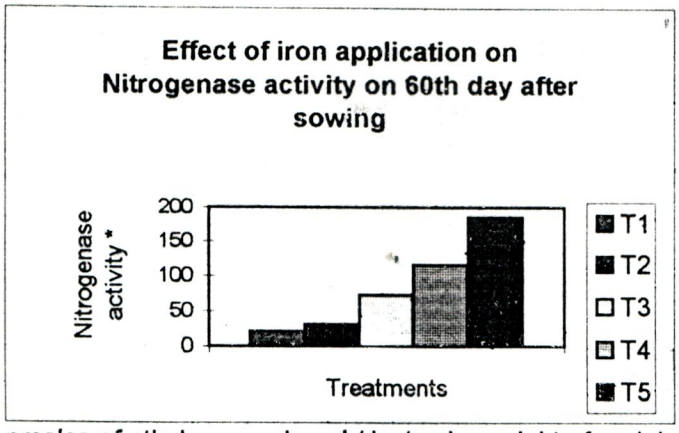
* Micromole Hydrogen peroxide degraded / minute / g leaf tissue

** Micromole guaiacol dehydrogenated / minute / g leaf tissue

4.8.2 PEROXIDASE

The data presented in Table - X and Fig : 11 obviously depicts that the control (T1) showed minimum peroxidase activity on all the 3 stages of growth when compared to iron treated S. rostrata. The peroxidase activity increased significantly from T2 to T5 ($p < 0.05$) on 30, 45 and 60 days after sowing. The

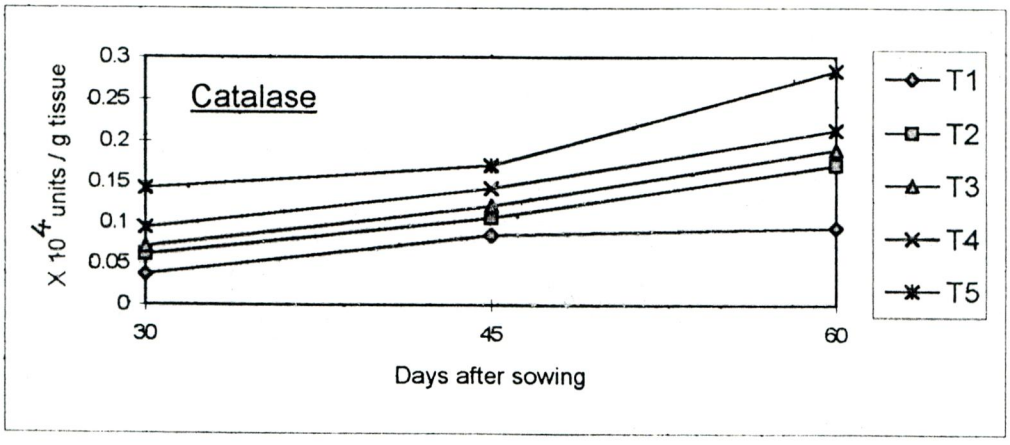
Figure - 9

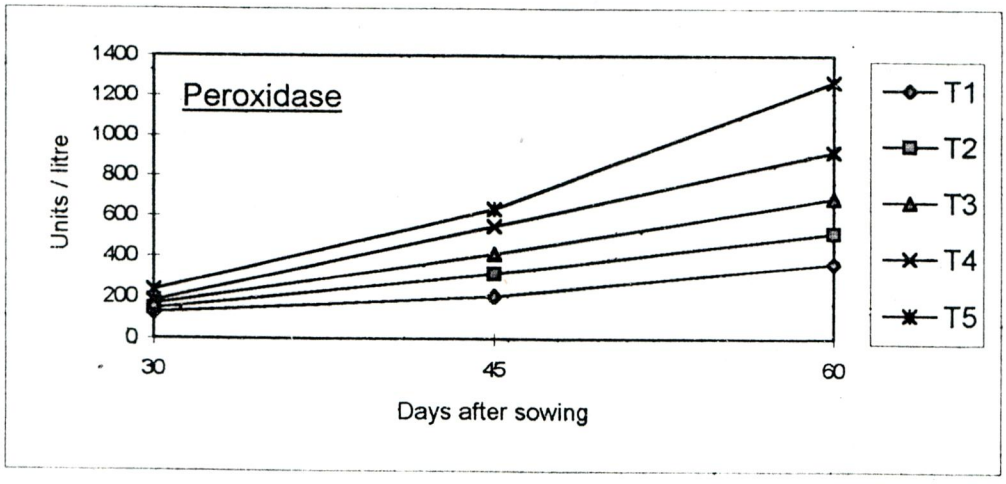


* - nmoles of ethylene produced / hr / g dry weight of nodules

Figures 10 and 11

EFFECT OF IRON APPLICATION ON THE ACTIVITIES OF CATALASE AND PEROXIDASE IN *S. rostrata*





increase in peroxidase activity might be due to the iron present in sufficient quantity that had helped in the formation of the heme of peroxidase and also the increase in activity of enzyme responsible for the incorporation of iron into the porphyrin.

Thus, application of iron at varying levels had evidently, increased the catalase and peroxidase activity, since iron is the prosthetic group of these enzymes. Catalase and peroxidase protects the plant from toxic products particulary peroxides.

This interesting increase in the catalase and peroxidase activity in the leaves with the increase in the levels of iron (10 to 40 kg of Fe/ha) correlates well with the studies of Agarwal and Srivastava (1984) and Kadam et al., (1988).

In general, it is clear that iron application had infulenced growth, nodulation, biomass production and nitrogen fixation in the green manure S. rostrata.

Summary and Conclusion

5. SUMMARY AND CONCLUSION

The present investigation was carried out to study the effect of iron on growth, biomass production nodulation and nitrogen fixation in S. rostrata. The experiment was carried out in pot cultures in completely randomized block design. The twenty pots used for the experiment were filled with 10 kgs of farm yard manure enriched soil and treated with iron in the form of ferrous sulphate at the levels of 10, 20, 30 and 40 kg of Fe/ha.

The seeds of S. rostrata were soaked in hot water for 10 minutes and then soaked in cold water for 6 hours, shade dried for 3 hours and were treated with peat based inocula of A. caulinodans. The treatments were as follows :

T1 - Azorhizobium seed inoculated S. rostrata alone

T2 - Azorhizobium seed inoculated S. rostrata + 10 Kg of Fe/ha

T3 - Azorhizobium seed inoculated S. rostrata + 20 kg of Fe/ha

T4 - Azorhizobium seed inoculated S. rostrata + 30 kg of Fe/ha

T5 - Azorhizobium seed inoculated S. rostrata + 40 kg of Fe/ha

The seeds were uniformly sown. At the end of 30, 45 and 60 days, the plant were carefully uprooted and its growth and nitrogen fixing efficiency was evaluated by studying.

a) the growth attributes such as root and shoot length, plant fresh and dry weight, root and stem nodulation.

b) the biochemical constituents like total chlorophyll, allantoin, aminonitrogen, total NPK. and total iron.

c) assay of the activity of ammonia assimilating enzymes (Glutamate dehydrogenase and glutamate synthase), Nitrogenase and the oxidative enzymes - catalase and peroxidase.

The results obtained from the study were summarised as below :

1. The root and shoot length of iron treated S. rostrata was found to be higher than the untreated plants. Among the treatments, T5 recorded the maximum shoot length (10.08, 22.53, 42.06 cms) and root length (8.97, 14.93 and 17.53 cms) and minimum by T2 on 30, 45 and 60 days after sowing. The increase in shoot length from T2 to T6 was statistically significant at 5 per cent level on 45 and 60 days after sowing and also between the three different stages of growth.

2. The number of root and stem nodules increased in all the treatments with the increasing age of the plant. The maximum and minimum number of root and stem nodules were recorded by T5 and T1 respectively on all the three stages of growth statistical analysis revealed that there was a significant increase in the number of root nodules on 60th day. Nevertheless, there was a numerical increase in root nodules on 30th and 45th day. However, the increase in stem nodules was found to be significant on 45th and 60th day.

3. The fresh and dry weight of the plants increased significantly with the age of the plants irrespective of iron treatments. The maximum fresh and dry weight (12.38 and 2.48 gs) were recorded by T5 on the 60th day Thus T5 showed superior results over the rest of the treatment on 30th as well as 45th day. The minimum fresh and dry weight was recorded for T1 on 60th day and the same trend was noticed on 30th and 45th day. Among the iron treated plants the increase in fresh and dry weight was found to be significant ($p < 0.05$) from T2 to T5 on 45th and 60th day when compared within the treatments as well as between the three different stages of growth.

The study clearly indicated that iron application had increased the growth, nodulation and biomass production in S. rostrata.

4. The chlorophyll assessment proved to be a better index of all events involved in nitrogen fixation. The chlorophyll content of iron treated S. rostrata was found to be higher than untreated control on 30,45 and 60 days after sowing. Statistical analysis revealed that there was a significant difference at 5 per cent level in the chlorophyll content of the treatment T3,T4 and T5, when compared to the control (T1) on 45th and 60th day.

Chlorophyll is one of the best parameters to study the intensity of nitrogen uptake and this content may find better association with nitrogen uptake than other factors. Because of the higher content of total chlorophyll it was evident that iron application might trigger photosynthesis in S. rostrata.

5. The results of allantoin and aminonitrogen estimation clearly indicated that on 30, 45 and 60 days after sowing maximum content was found in the treatment supplied with 40 kg Fe/ha followed by T4, T3 and T2 respectively. T1 recorded the minimum amount of both allantoin and aminonitrogen on all the 3 stages of growth. Between treatments, there was a significant increase at 5 per cent level in allantoin content on 60th day and aminonitrogen content on 30, 45 and 60 days after sowing.

The atmospheric nitrogen gets fixed in the nodule

and they are subsequently transformed and transported to other parts of the plants. Though allantoin and aminonitrogen contents showed an increase with the increase in the level of iron at every stage, the aminonitrogen content was found to be higher than allantoin content. So it can be concluded that nitrogen fixation had been influenced by iron application and the fixed nitrogen was transported as amide form rather than ureide form.

6. The total nitrogen, phosphorus, potassium and total iron contents of the various iron treated and untreated plants increased with the increase in the age of the plant. However, at every stage the maximum nutrient content was found in T5 and minimum in T2 among the iron treated plants. This clearly depicted that increasing the levels of iron application (10 to 40 kg Fe/ha) actually increased the nutrient content of the plant.

The study thus, revealed an increase in all the biochemical constituents which were influenced by iron treatment, which inturn results in efficient nitrogen fixation in iron treated S. rostrata.

7. Estimation of activities of ammonia assimilating enzymes such as glutamate dehydrogenase and glutamate synthase was found to be higher in iron treated plants

on 30, 45 and 60 days after sowing. Within the iron treated plants, minimum activity was recorded for T2 and maximum for T5 at all the 3 stages of growth, indicating that increasing the levels of iron would increase the activity of ammonia assimilating enzymes and hence nitrogen fixation.

8. Estimation of nitrogenase activity revealed that it was higher in iron treated plants when compared to untreated control plants. Within iron treated plants, there was a steady increase in activity from T2 to T5 at the end of 60th day. Statistical analysis revealed that there was a drastic increase in the activity of nitrogenase when compared between the treatments.

9. The oxidative enzyme namely Catalase and peroxidase increased significantly when compared between the treatments T2 to T5 as well as between the 3 stages of growth viz. 30, 45 and 60 days after sowing. As iron is the prosthetic group of catalase and peroxidase the increasing concentration of iron had actually increased the catalase and peroxidase activities.

A summation of the results obtained from this investigation had shown the superiority of iron treated plants over untreated plants. Thus, it was concluded that increasing the levels of iron had improved the growth, biomass production, nodulation and nitrogen fixation in stem-nodulating S. rostrata which had been

used a potential green manure in rice fields.

Future Prospects

Micronutrients, though required in small amount, they play an establishing role in plant growth particularly nodulating plants. Iron application had influenced the efficiency of nitrogen fixation by the stem nodulating S. rostrata. Further studies can also be carried out applying other micronutrients namely, Molybdenum and Zinc, which plays an important role in plant nutrition. The combined effect of micronutrients may also play an significant role in growth on nodulation of the plants. Hence the combined application of iron, molybdenum and zinc, plant growth can be considered for future studies.

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Appendices

APPENDIX - I

ESTIMATON 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. Reagent Acetone 80% Method

Cut 1.0 g 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 Whatman No. 42 filter paper. Added sufficient quantity of 80 per cent acetone and repeated the extraction. Transferred the contents from the mortar to the Buncher funnel and washed the brei with acetone until it became colourless. Pooled the filtrates 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.

Calculation

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)
b = Volume of the extract in ml
w = fresh weight of the sample

APPENDIX - II

ESTIMATION OF ALLANTOIN
(Young and Conway, 1942)

Principle

Allantoin 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 ferricyanide produces a red colour which is measured at 520 nm.

Reagents

1) 0.05 M Phosphate buffer

2) 0.5 N Sodium hydroxide

3) 0.65 N hydrochloric acid

4) phenyl hydrazine hydrochloride

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 0.5N sodium hydroxide and the tubes were placed in a vigourously boiling water bath for 7 minutes. On removal it is immersed in water bath at room temperature. Then to all tubes 0.5 ml of 0.65 N hydrochloric acid was added. The tubes were shaken and placed in a boiling water bath for exactly 2 minutes. The tubes were plunged into an ice bath and chilled for 20 minutes. To all the tubes 0.5 ml of phenylhydrazine hydrochloride was added, when the tubes were in the bath. On removal added 2.0 ml of 1ON hydrochloric acid also prechilled in ice and 0.5 ml of potassium ferricyanide solution. The contents were well mixed and the allantoin content of each sample tube was measured at 520 nm in a colorimeter.

APPENDIX - III

ESTIMATION OF AMINONITROGEN
(Spies, 1955)

The leaves of the plant sample were extracted with 0.05 M phosphate buffer (pH 7.5). 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 contents 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 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 0.5 g 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. Kept at the delivery end 10 ml of 2% boric acid with mixed indicator in a 100 ml beaker. 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.

Calculations

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

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 four 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 5ml 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.

Calculations

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

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 solution. From this a series 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 dissloved 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 TOTAL IRON
(Shanmugam et al., 1994)

Preparation of Triple acid

Nitric, sulphuric and perchloric acid in the ratio of 9:2:1 are mixed to get a triple acid mixture.

Preparation of standard curve

1g of Iron pure metal (99.99% pure) in 20 ml of 1:1 Hydrochloric acid and dilute to 1 litre gives 1000 ppm or µg/ml. If ferrous sulphate $7H_2O$ powder were used, then 4.4 g of $FeSO_4.7H_2O$ powder in 1 litre gives 1000 ppm of Fe.

Procedure

Weighed 1g of plant sample in a 250 ml corning conical flask and 12 ml of triple acid mixture was added to that and kept for overnight. Next day it was digested for 1 hour until white colour solution is obtained. After the digestion was over, the content was made upto 50 or 100 ml as per the requirement with double distilled water. The content was transferred to a corning injection vials and fed into the Atomic absorption spectrophotometer.

APPENDIX - VIII

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

Principle

Glutamate synthase was assayed spectrophotometrically by recording the rate of NADPH or NADH as indicated by a change in absorpance 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)

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

Calculations

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 - IX

ESTIMATION OF GLUTAMATE DEHYDROGENASE (Doherty, 1970)

Principle

Glutamate dehydrogenase was assayed by following the oxidation of the reduced co-enzyme 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 1.0 M (pH 7.8 and 7.0)

- 2-oxo-glutarate 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
- Enzyme extract : Extracted 1g of the plant material with 5 m l of 10 mM phosphate buffer pH 7.5 containing 1 mM disodium EDTA, 1mM 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. Proceed for the assay of NADH-dependent glutamate dehydrogenase as per the composition of assay given in table :

Reagent	NADH assay / ml
Potassium phosphate buffer	1.0
2-oxo glutarate	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°C for 15-30 minutes
4. Record the change in absorbance at 340 nm.

Calculations

The amount of NADH oxidised was calculated from the molar extinction coefficient. 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 - X

ESTIMATION OF NITROGENASE
(Hardy et al., 1968)

Principle :

Acetylene is reduced to ethylene by nitrogenase. The ethylene produced is measured in a gas chromatograph (GLC) and the activity is expressed as nmole ethylene produced per unit time per gram dry nodules.

Materials

- Gas chromatograph with flame ionization detector (FID)
- Air-tight syringes
- Conical flasks (100 ml) with small mouth to fit serum caps
- Acetylene gas
- Ethylene gas
- GLC operating conditions
- Carrier gas - Nitrogen/Helium/Argon with a flow rate 30 to 45 ml gas for detector - Hydrogen and Air Coulmn - Propak N, R, T and Q or silica gel

	Propak	Silica gel
Oven/column temperature	60°C	150°C
Injector temperature	65°C	160°C
Detector temperature	85°C	175°C
Retention time for ethylene (min)	1.3	1.5
Retention time for acetylene (min)	1.8	3.0

Procedure

1. Removed the plants from the soil without disturbing the root nodules
2. Excised the root with nodules
3. Placed the root system into a 100 ml conical flask
4. Sealed the flask with rubber septum (serum cap).
5. Removed 10 ml of air from the flask with a tight syringe.
6. Injected 10 ml acetylene into the flask.
7. Incubated for 30 - 60 minutes at room temperature
8. Removed 0.5 to 1 ml gas mixture from the flask with an air tight syringe.
9. Injected the gas mixture into a preconditioned GLC.
10. Looked for the acetylene and ethylene peaks and measured the ethylene peak height.
11. At the end of the experiment, detach the nodules and take the dry weight.
12. For standard, injected 10 µg (Z) of pure ethylene into a 100 ml sealed conical flask to satisfy identical assay conditions. Removed 0.5 to 1 ml (same volume as of the sample) and injected into the GLC and measured ethylene peak height.

Calculations

1. Standard amount of ethylene (E) in umol

$$= \frac{0.0446 \times Z\ \mu l}{\text{peak height in mm} \times \text{attenuation}}$$

2. Amount of ethylene produced in µmol in the sample

$$= E \times \text{peak height of sample ethylene} \times \text{attenuation in mm}$$

3. Activity of nitrogenase

= nmol of µmol ethylene per unit sample per unit time (unit sample may be g dry weight nodules or mg dry weight cell or mg protein)

APPENDIX - XI

ESTIMATION OF CATALASE
(Luck, 1974)

Principle :

The uv absorption of hydrogen peroxide solution can be easily measured between 230 and 250 nm. On decomposition of hydrogen peroxide by catalase, the absorption increases with time. The enzyme activity could be arrived at from this decrease. But this method is applicable only with enzyme solutions which do not absorb strongly at 230 - 250 nm.

Reagents :

1. Phosphate buffer - 0.067 M (pH 7.0)

2. H_2O_2 - PO_4 buffer : Diluted 0.16 ml of H_2O_2 (10% w/v) to 100 ml with phosphate buffer. Prepared freshly. The absorbance of the solution should be about 0.5 at 240 nm with a 1 cm light path.

3. Enzyme extract : Homogenised the plant tissue in a blender with M/150 phosphate buffer (assay buffer diluted 10 times) at 1-4 °C and centrifuged. Stirred the sediment with cold phosphate buffer. Allowed to stand in the cold with occasional shaking and then repeated the extraction once or twice. The extraction should not take longer than 24 hrs. Used the combined supernatants for the assay. The catalase activity could change considerably on storage of the tissue. In comparitive studies, therefore always same conditions of extraction, storage and temperature were used.

Procedure :

Wave length - 240 nm

Final volume - 3.0 ml approximately

Room temperature

Pipetted imto the experimental cuvette - 3 ml H_2O_2-PO_4 buffer. Mixed in 0.01 - 0.04 ml sample with a glass or plastic rod flattened at one end. Noted the time Δ t required for a decrease in absorbance from 0.45 to 0.4. This value was used for calculations. If t was greater than 60 sec, repeated the measurements with a more concentrated solution of the sample.

The experimental solution was read against a control cuvette containing enzyme solution as in the experimental cuvette but containing H_2O_2-free PO_4 buffer (M/15).

Calculation :

1 g tissue homogenised in a total volume 20 ml, diluted 1.0 ml to 10 volume with water and taken 0.01 ml for assay. The absorbance at 240 nm decreased from 0.45 to 0.4 in 13.9 sec.

$$\frac{17}{13.9} = 1.22 \text{ units in the assay mixture or}$$

$$\frac{1.22 \times 10}{0.01} = 1220 \text{ units / ml extract}$$

i.e., 2.44×10^4 units / g tissue

APPENDIX - XII

ESTIMATION OF PEROXIDASE
(Malik and Singh, 1980)

Principle :

Guaiacol is used as substrate for theassay of peroxidase.

$$\text{Guaiacol} + H_2O_2 \xrightarrow{\text{POD}} \text{oxidised guaiacol} + 2H_2O$$

The resulting oxidised (dehydrated) guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product is a measure of peroxidase activity and can be assayed spectrophotometrically at 436 nm.

Reagents :

1. Phosphate buffer 0.1 M (pH 7.0)

2. Guaiacol solution 20 mM : Dissolved 240 mg guaiacol in water and made upto 100 ml. It could be stored frozen for many months.

3. H_2O_2 solution (0.042% = 12.3 mM) : Diluted 0.14 ml of 30 % H_2O_2 to 100 ml with water. The extinction of this solution should be 0.485 at 240 nm. Prepared freshly.

4. Enzyme extract : Extracted 1 g of fresh plant tissue in 3 ml of 0.1 M phosphate buffer, pH 7.0 by grinding with a precooled mortar and pestle. Centriuged the homogenate at 18,000 g at 5^{o} for 15 min. Used the supernatant as enzyme source within 2 - 4 hrs. Stored on ice till the assay was carried out.

Procedure :

1. Pipetted out 3 ml buffer solution, 0.05 ml guaiacol solution, 0.1 ml enzyme extract and 0.03 ml hydrogen peroxide solution in a cuvette.

2. Mixed well, placed the cuvette in the spectrophotometer.

3. Waited till the absorbance has increased by 0.05. Started a stop-watch and noted the time required in minutes (Δ t) to increase the assay by 0.1.

Calculation :

Since the extinction coefficient of guaiacol dehydrogenation product at 436 nm under the specified conditions is 6.39 per micromole, the enzyme activity per litre of extract was calculated as below :

$$\text{Enzyme activity Units / litre} = \frac{3.18 \times 0.1 \times 1000}{6.39 \times 1 \times \Delta t \times 0.1}$$

$$= \frac{500}{\Delta t}$$

APPENDIX - XIII

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.

Reagents :

1. 0.32 % potassium permanganate

2. 2.5% sodium hydroxide

3. Double (mixed) indicator : Bromocresol green (0.5g and methyl red (0.1 g) were dissolved in 100 ml of ethyl alcohol.

4. Standard sulphuric acid (N/50)

5. 2% Boric acid

Procedure :

Weighed 20g 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 0.32 % potassium permanganate solution and 100 ml of 2.5% sodium hydroxide solution. Distilled the contents at a steady rate collecting the liberated ammonia in a 500 ml 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 ammonia collected against the standard acid (N/50) and from the titre value (a) calculated available nitrogen content of the soil.

Calculation :

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

APPENDIX - XIV

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. 0.5 M 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. 0.291 g of antimony potassium tartrate was dissolved in 100 ml of distilled water. Both these solutions were added to 100 ml of approximately 5N sulphuric acid. This solution was mixed thoroughly and was made upto 2 litres with distilled water.

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

Procedure :

Weighed 5g of soil and transferred to a 100 ml 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 clear 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 25 ml. Waited for 10 minutes for the colour to develop and thereafter measured the intensity of the blue colour in a photoelectric colorimeter using a red filter (660 nm 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 - XV

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 :

$$K + clay + NH_4^+ \longrightarrow NH_4^{+\ +}\ clay + K^+ + excess\ NH_4^+$$

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.

Reagents :

1. Neutral normal ammonium acetate

2. Standard potassium solution

Procedure :

Weighed 5.0 g 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 100g of soil was calculated.

APPENDIX - XVI

ESTIMATION OF IRON
DTPA method (Shanmugham et al., 1994)

Principle :

Chelating agents combine with free metal ions in solution forming soluble complexes and thereby reduce the activities of the free metal ions in solution. Therefore more metal ions desorb from the soil surface or labile solid phases to replenish the free metal ions in solution.

Reagents :

The diethylene triamine penta acetic acid (DTPA) extracting solution was prepared to contain 0.005 M DTPA, 0.01 M $CaCl_2$, 0.1M TEA and pH adjusted to 7.3. To prepare one litre of this solution dissolved 1.967 g of DTPA, 1.47 g $CaCl_2.2H_2O$, 13.3 ml of reagent grade TEA in approximately 20 ml water. Allowed sufficient time for DTPA to dissolve and diluted to approxiamtely 900 ml. Adjusted the pH to 7.3 $\pm$ 0.05 with 1:1 HCl.

Preparation of standard solution :

1 g of Fe pure metal in 20 ml of 1:1 Hcl and diluted to 1 litre gives 1000 ppm or ug/ml. If $FeSO_4.7H_2O$ was used then, 4.4 g of $FeSO_4.7H_2O$ in 1

litre gives 1000 ppm of Fe.

Procedure :

10 g of air dried soil was placed in a 50 ml polyethylene bottle and 20 ml of the DTPA extracting solution was added. The bottle was tightly closed with a stopper and shaken on a horizontal shaker for 2 hrs. Then the suspension was filtered by gravity through Whatman No.42 filter paper. The filtrate was analysed for iron using Atomic Absorption Spectrophotometer. Used the standard solutions to prepare a calibration curve. Obtained the observation in ppm of element in the sample solution from the calibration curve or on direct read out.

Calculation :

Weight of soil taken	=	10 g
Volume of DTPA extract added	=	20 ml
Dilution	=	2 times
Concentration of element in the sample solution as read from the standard curve	=	S ppm
Concentration of element in the blank solution	=	T ppm
Concentration of element in soil	=	(S-T) X 2 ppm