A STUDY ON CORRELATION BETWEEN LEGHAEMOGLOBIN AND NITROGEN CONTENT IN GRAM *(Cicer arietinum* L.*)*

By

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B. Sc. (Agri.) First Class with Distn.

A Thesis submitted to the

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in Partial fulfilment of the requirements for the degree of

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in

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in

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Approved by the Advisory Committee

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Candidate's declaration

I hereby declare that this thesis or part thereof has not been submitted by me or any other person to any other University or institute for a degree or diploma.

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Date : 1st March ,1985.

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CERTIFICATE

This is to certify that the thesis entitled " A study on correlation between leghaemoglobin and nitrogen content in gram " submitted to the Faculty of Agriculture, Mahatma Phule Agricultural University, Rahuri, Dist. Ahmednagar (Maharashtra State), in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE (AGRICULTURE) in AGRICULTURAL MICROBIOLOGY, embodies the results of a piece of bona-fide research work carried out by SHRI PRABHAKAR HARIBHAU RASAL under my guidance and supervision and that no part of this thesis has been submitted for any other degree or publication.

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C E R T I F I C A T E

This is to certify that the thesis entitled " A study on correlation between leghaemoglobin and nitrogen content in gram " submitted to the Faculty of Agriculture, Mahatma Phule Agricultural University, Rahuri, Dist. Ahmednagar (Maharashtra State) in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE (AGRICULTURE) in AGRICULTURAL MICROBIOLOGY embodies the results of a piece of bona-fide research work carried out by Shri P.H.Rasal under the guidance of Dr. P.L.Patil, Agricultural Bacteriologist, College of Agriculture, Pune-411 005.

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Dated : 3.6.85

DR. N.D.PATIL

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ABSTRACT

A STUDY ON CORRELATION BETWEEN LEGHAEMOGLOBIN AND NITROGEN CONTENT IN GRAM (*Cicer arietinum* L.)

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From the present investigation it is concluded that as the presence of leghaemoglobin at the initial stage was less the nitrogen fixation was also less in all the varieties. At the flowering stage the leghaemoglobin in a nodule was more and hence the nitrogen fixation was also more. At the maturity stage due to the irriversible change of the leghaemoglobin its percentage was reduced and nitrogen fixation was also reduced at a very low level. In general, it was found that there is a close correlation between the leghaemoglobin content in nodules and nitrogen fixation by bacteria at all stages of the crop growth in all varieties of gram.

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CHAPTER 1

I N T R O D U C T I O N

1. INTRODUCTION

An importance of legumes in agriculture was known by the people since very long. Whyte *et al*. (1958) reported that legumes are being grown as economic proposition since 6000 years. Pulses form a very important source of protein in the vegetarian diet of a quite large section of Indian population. They are well-known as poor man's meat and also used as a fodder and concentrates in cattle feed. Pulses are leguminous crops and hence they enrich the soil with nitrogen through the nitrogen fixing bacteria (*Rhizobium*) produced in the root nodules and help in improving the soil. Most of the pulse crops being bushy soon cover up the land and their roots act as soil binders which help in checking soil errosion (Patil *et al*., 1949).

Gram (*Cicer arietinum* L.) is known as chick pea and is third in importance in the world as a pulse crop and yet its products play a very small part in the world trade. These products, however, have importance in local consumption. This crop is being cultivated since antiquity mainly in the semi arid temperate to warm regions of the world.

In India the main pulse producing areas are the upper basins of the Ganges and adjoining tracts of central India. It occupies 78.6 lakh hectares of the land with the production of 53.6 lakh tonnes (Anonymous, 1980).

Pulses in general, occupy an unique position in the agriculture by virtue of their high protein content and their ability to fix atmospheric nitrogen with the help of root nodule bacteria known as *Rhizobium*. Bengal gram is world's third pulse crop and fifth food legume and fifteenth grain crop (F.A.O., 1972).

In India, gram is grown on a large scale mainly in the states of Punjab, Haryana, U.P., Bihar, A.P., Rajastan, M.P., West Bengal, Mysore and Maharashtra. The area under this crop in Maharashtra is 4.27 lakh hectares with the production of 1.58 lakh tonnes (Anonymous, 1977).

Though, an inoculation of seeds with *Rhizobium* has been known since 1896 however, it is quite recent in India. It is needless to stress the need of inoculation of seeds with effective strains of *Rhizobium* for increasing the yield of crop and for nitrogen economy of the soil.

With increased awareness amongst farmers about the value of rhizobial inoculation of various legumes, studies on strain selection and specificaity becomes an important phase of research in the pulse development programme of several States. Research work to select efficient strains for mass production of inoculants is further intensified through the ICAR sponsored, " All India Coordinated Projects on pulses " with co-ordinating agencies spread all over the country. Reports from India and abroad indicated varying degree of responses of legumes to inoculation with different strains. The performance of a particular strain is influenced by the soil and agro-climatic conditions of the region as reported by Bhargau *et al.*(1976); Kumar Rao and Patil (1977) and Singh and Tilak (1977).

The formation of nodules on root of a legume and the degree of nodulation are dependent on the strain of *Rhizobium* species. Proper nodulation of a legume with an effective *Rhizobium* strain helps in increasing the yield to the extent of 15 to 20 per cent (Rai *et al.*, 1977 and Medhane and Patil, 1974).

The use of bacterial fertilizers for increasing crop production is gaining popularity, particularly the use of *Rhizobium* has become a necessity in legume crops.

When one considers the possible extension of biological nitrogen fixation for the increase of world food production, an obligate partner in this symbiosis is leghaemoglobin. The nitrogen fixation by pink nodule is more than green or black nodules so the leghaemoglobin is basic thing without which no nitrogen fixation occurs in nodules of legume crops (Swaraj and Garg, 1977).

Beneficial effects of leghaemoglobin in nitrogen fixation was reported in soybean, lupin and various other pulse crops by different workers. However, this relationship in gram is not reported so far.

Therefore, with the above consideration in view, investigations were undertaken with the following objectives :

(1) To study the comparative leghaemoglobin and nitrogen contents in nodules of gram.

(2) To study the effects of leghaemoglobin contents on dry matter yield.

(3) To study the effectiveness of nodules and their nitrogen fixation.

CHAPTER 2
REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 History

Schloesing et al., (1890) gave the technical proof of nitrogen fixation by nodule bacteria in symbiosis with legumes. Later, Nobbe and Hiltner (1893) and Deherain and Demoussy (1900) have shown that all strains of any species of *Rhizobium* were alike in ability to benefit the host plant.

When we consider the possible extension of biological nitrogen fixation for the increase of world food production an obligate partner in this symbiosis is leghaemoglobin (Lb). So far the proper understanding of leghaemoglobin properties and function have assisted in the achievement of more efficient nitrogen fixation by legumes. In addition to this, if a legume type symbiosis is to be artificially introduced into other plant families then knowledge of the site of synthesis in the nodules and the evolutionary origin of leghaemoglobin may be essential prerequisites (Appleby, 1974).

Kubo (1939) was the first who recognised the red pigment present in legume root nodules as hemoprotein. He showed that soybean and other nodules could yield a soluble haemoglobin like pigment which retained the property of reversible oxygenation and similarly concluded that this new haemoglobin is connected with oxygen respiration activity of the red nodules. Whereas, Burris and Haas (1944) stated that no reversible oxygen complex from cow pea (Vigna sinensis) leghaemoglobin and doubted its function as an oxygen carrier but this essential property of oxygenation was confirmed by Keilin and Wang (1945) for soybean leghaemoglobin and by Virtanen (1945a,b) for green pea leghaemoglobin.

In prolific series of papers summarised by Virtanen (1952), Virtanen and Colleagues elucidated many aspects of leghaemoglobin structure and function including the important relationship between leghaemoglobin content and nodule effectiveness.

2.2 Localisation

Although, haemoglobin like pigments are found in yeast (Keilin, 1953), Oshino et al., (1973) and other fungi (Keilin and Tissires, 1953), there is no well founded report of a soluble haemoglobin occurring in higher plants other than legume root nodules.

Davenport (1960) found that nitrogen fixing casuarina nodules had a high concentration of total heme content, a pigment whose absorption bands suggested the ability to form complexes equipvalent to LbO_2 and LbCO and to be oxidised and reduced in forms equivalent to ferric leghaemoglobin and ferrus leghaemoglobin.

Becking (1970) has found in the search for haemoglobin in other non-legume root nodule symbiosis including those of Alnus has been unsuccessful, although, some of these nodules do have an elevated heme content.

By microspectroscopic observations of sliced soybean nodules, Smith (1949 a), established that leghaemoglobin was confined to the soft central tissue which also contained the bacteroids, as the bacteroids are devoid of leghaemoglobin. This observation meant that leghaemoglobin can occur only in the plant cells.

Following the electron microscopic detection of membrane sacs which surround individual bacteroids or small groups of bacteroids in effective nodules. There has been a continuing controversey over the location of leghaemoglobin, solely within these sacs, solely within the plant cytoplasm, outside the sacs or in both places.

By electron microscopic examination of autoradiographs of thin fixed sections of Serradella nodules which had been grown in the presence of Iron, Dilworth and Kidby (1968) showed that most radioactive disintegration took place within the membrane sacs close to the bacteroid surface. Since leghaemoglobin accounts for a substantial part of total iron in the nodule, they concluded that this pigment was located only within the membrane sacs.

Dart and Chandler (1971) following X-ray micro-probe examination of fixed sections of several legume nodule species claimed that plant cytoplasm (outside the membrane sacs) contained most iron and sac contains the least.

Bergersen and Goodchild (1973 b) had obtained convincing results by mounting unfixed sections of soybean nodules in a modified Honda medium, staining with an oxidised diaminobenzidine (peroxidase) reagent caused the membrane sac to appear a bright red colour. This result seems to establish that leghaemoglobin does occur inside the membrane sac. Glutaraldehyde fixed diaminobenzidine stained sections of the same nodules after further staining with OsO_4 showed electron dense material both inside and outside the membrane sacs, but they were unwilling to claim this as firm evidence for location of leghaemoglobin in both the places. It located only within the membrane sacs, they calculated the effective concentration of leghaemoglobin in 36 day old soybean nodules as 1.53 mM.

Bergersen (1975) described the environment in which rhizobia fix nitrogen within the host tissue of the legumes. The bacteria surrounded by a solution containing leghaemoglobin whose location, concentration, degree of oxygenation and affinity for oxygen are important features of the environment.

2.3 Isolation

The earlier procedure involved crushing soybean or other nodules in water (Kubo, 1939) or saturated ammonium sulphate (Keilin, 1945) followed by ammonium sulphate fractionation with leghaemoglobin mostly precipitated between 0.6 and 0.8 saturation. By moving boundary electrophoresis of such preparations in unstablized liquid media at neutral or alkaline pH. Ellfolk and Virtanen (1950) recognised that soybean leghaemoglobin contains a fast electrophoretic component and a slow electropheretic component. Although, the electrophoresis of narrow bands of leghaemoglobin on filter paper (Thorogood, 1957) or in acrylamide gel (Peive et al., 1967 a) enabled the recognition and separation of multiple leghaemoglobin components and have occasionally been used for preparative work (Thorogood and Hanania, 1963). The most convenient preparative procedures involve anion exchange chromatography.

Ellfolk (1960 a) by chromatography of autoxidised ferric leghaemoglobin on DEAE cellulose in 0.01 μ sodium acetate (pH. 5.2) with stepwise increments of sodium chloride, achieved the separation of the following soybean leghaemoglobin components in order of elution.

Ferric Lba a major fraction equivalent to the slow electrophoretic component of isoelectric point 4.7 ferric Lbb a major component ferric Lbc the second major fraction equivalent to the fast electrophoretic component of isoelectric point 4.4. Ferric Lbd is a second minor electrophoretic component. The homogeneous component ferric Lba and Lbe are crystalizable at pH 5 (Ellfolk, 1960 a), but this hazardous procedure offers no further gain in purity.

Appleby (1969 a) achieved a somewhat better resolution of soybean leghaemoglobin components on DEAE Sephadex (Pharmacia) and Dilworth (1969) achieved the best resolution of lupin leghaemoglobin components on DEAE cellulose by using stepwise addition of sodium acetate (pH 5.2) rather than sodium chloride to the elution buffer.

Leghaemoglobin is less readily autoxidizable above pH 7 (Thorogood, 1957), but the present author's experience is that chromatographic separation is less discriminating at alkaline pH because of higher ionic strength buffers needed for elution, for chromatographic isolation of native LbO_2 components. Melik-Sarkisyan et al. (1969) separated the components of lupin at pH 6.3.

Efforts have been made to prevent autoxidation of native ferrus leghaemoglobin or LbO_2 by extracting nodules in the presence of capron (Melik-Sarkisyan et al., 1970) or under inert gas (Abel and Bauer, 1962). Even so the most stable preparations of LbO_2 are obtained by deliberate oxidation of ammonium sulphate fractioned leghaemoglobin to ferric leghaemoglobin with two to five fold excess of potassium ferricynide at pH 7 and preliminary chromatography on G-75 sephadex (phqrmacia) at pH 9. This step also gets rid of nicotinic acid a natural hemachrome forming ligand which has extra ordinary high affinity for ferric or ferrous leghaemoglobin at pH 5.2 (Appleby and Daniel, 1973 a), chromatography on DE 52 Cellulose (Whatman) beginning at a 0.01 m sodium acetate (pH 5.2) with increasing acetate gradient produces a clean separation of soybean ferric leghaemoglobin a (Lba), Lbb, Lbc and Lbd components with none of the pink ferric hemochromes which contaminated earlier preparations (e.g. Ellfolk, 1960 a, Appleby, 1969 a). If metal free glass distilled water is used for all buffers the purified ferric leghaemoglobin components may be converted to very stable LbO_2 by anaerabic addition of dithionite at pH 7 following by rapid chromatography on small column of G-15 sephadex (phqrmacia) in air equilibrated buffer at pH 7-8 (Wittenberg et al., 1973 b). In a somewhat similar procedure, Emamura et al.,(1972) deliberatedly oxidised their crude leghaemoglobin in the presence of cyanide chromatographed it as the very stable complex ferric Lbc finally converting the pure components to leghaemoglobin oxide.

Good separation of the leghaemoglobin in microcomponents was achieved and the components were purified using simple material and equipments (Pelve et al.,1973).

Appleby and Nicola (1975) had described the improved procedure for isolation of five leghaemoglobin components from the nodules of soybean plants. After a preliminary oxidation with ferricyanide and endogenous nicotinate at pH 8.2 the ferrileghaemoglobin is separated by DEAE cellulose chromatography using gradient elution with acetate buffer (pH 5.2).

Lentovaara and Ellfolk (1975) had purified leghaemoglobin components 'a' and 'b' from root nodules of _Phesolus vulgaris_. Homogenity of Lba was demonstrated by polyacrylamide gel disc.

Kuranova (1976) has filtered through sephadex G-25, the leghaemoglobin in extract of lupin nodules was absorbed by DEAE cellulose equilibrated with 0.01 M ammonium acetate (pH 7.6) and eluted by stepwise increase in concentration of this buffer. Three components of leghaemoglobin were obtained with N-terminal sequences. Lentovaara and Perttila (1978) showed the site specificity of bile pigment formation from leghaemoglobin which can be tentatively explained by specific differences in the amino acid sequences at those regions of the polypeptide chain that are in the vicinity of the appropriate methenbridge.

2.4 Properties

Dilworth (1969) showed that there is a risk of over stating the number of components if nodule extracts are subjected to chromatographic analysis without recognition of the possibility that any one component may appear in atleast three places if present as ferric leghaemoglobin ferric Lb nicotinate and ferrous LbO_2 forms.

Thorogood (1957) from the most reliable investigations proved that soybean leghaemoglobin contains four components, serredella contains two to three components (Dilworth, 1969), Snakebean contains two components (Broughton and Dilworth, 1971).

The most useful information is given by Ellfolk and Siever (1971, 1972), whose work has determined the amino acid sequence of soybean Lba, Hunt (1972) who has compared the sequence alignment of soybean Lba with those of many other haemoglobins and myoglobins. Ellfolk and Siever (1971) showed many other structural similarities between soybean Lba and human haemoglobin chains by visual comparison of sequences. Nicola et al., (1975) showed that the association between haem and protein is much weaker in leghaemoglobin than in myoglobin.

Melik-Sarkisyan and Kretovich (1975) showed that cells of the nodules contained legoglobin characterised by two main components each one of which consists of a polypeptide chain and one heme group. In relation to special composition legoglobin was near to haemoglobin and myoglobin and belonged to the group of acid proteins.

Falk _et al_, (1959) showed that there is something else than heme present in crude leghaemoglobin.

Broughton _et al_., (1972) have also shown that protoheme IX is the prosthetic group of lupin and serradella leghaemoglobins. Similarly, Ellfolk and Sivers (1965) concluded that both the propionic acid side chains of protoheme IX were involved in the specific leghaemoglobin structure but only one was essential to the heme protein recombination reaction.

The pH 6.4 optical spectra of unligated ferric Lb ferrus Lb and LbO_2 (Appleby, 1969 b) and of other Lb derivatives (Sternberg and Virtanen, 1952; Peive _et al_., 1972 b) and their close family resemblance to the corresponding spectra of vertebrate hemoglobin and myoglobin.

Peive _et al_.,(1972 b) stated that the leghaemoglobin structure is more flexible than the myoglobin structure. Antonin and Brunori, 1971 confirmed the place of leghaemoglobin in the haemoglobin family but certain of these reactions give specific information on leghaemoglobin structure and possible close to its biological reactivity.

The reduction of ferric leghaemoglobin (Henderson and Appleby, 1972), the binding of acetate (Ellfolk, 1961 b) and of nicotinate (Appleby _et al_.,1973 a) are all protein dependent reactions but no heme linked ionizable group could be detected by the titration of ligand free ferric

Lb between pH 7 and 5.3 (Ellfolk, 1961 b). If proton binding occurs it must be at sites which have no effect on the heme environment in the absence of an exogenous ligand or electron.

The simplest way to detect the presence of ferric leghaemgglobin in nodule slices or extracts is to add a large excess up to 0.1 M of sodium fluoride (Kubo, 1939; Keilin and Smith, 1947) which cause the displacement of other ligands with formation of the diagnostic ferric LbF complex.

Appleby (1969 c) showed that leghaemoglobin when extracted from soybean nodules at pH 5.6 was mostly autoxidizable and appeared as a ferric hemochrome structure. Keilin and Wang (1945) were the first to recognise the extremely high affinity of ferrous leghaemoglobin for oxygen and carbon monoxide.

The kinetic measurements were particularly important because they showed the high oxygen affinity of LbO_2 to be due to a combination of the fastest oxygen on constant yet recorded for a haemoglobin and a moderately fast oxygen off constant (Wittenberg _et al._,1972). These rapid rate constants mean that LbO_2 is ideally suited for the process of facilitated diffusion (Wittenberg, 1970) which is now proposed as the principal function of leghaemoglobin.

Bergerson and Turner (1975) showed the effects of purified oxyleghaemoglobin when added to suspensions of bacteroids, prepared anaerobically, leghaemoglobin allowed maximum rate of oxygen uptake to continue to a much lower range of concentrations of free & dissolved 02 than in the absence of the protein. This effect was diminished when the leghaemoglobin concentration was less than about 50 µ m. Nitrogenase activity at a given oxygen concentration was not increased by raising the leghaemoglobin concentration ~~was not increased by raising the leghaemoglobin concentra-~~ ~~tion~~ above about 50 µm. Rates of nitrogenase activity were invariably greater during periods when the discharge of oxygen from oxyleghaemoglobin was occurring at the maximum rate than when similar maximum or uptake rates were being supported by higher concentration of free dissolved oxygen.

A new experimental system has been devised in which the gas phase was eliminated. Nitrogenase activity of soy-bean bacteroid suspension was measured during experiments in which oxygen concentration in solution was measured electrically or by monitoring spectrophotometrically the degree of oxygenation of added leghaemoglobin (Bergersen and Turner, 1974). Leghaemoglobin allowed high rates of oxygen consumption to continue to much lower concentrations of free dissolved oxygen than was possible in its absence. Nitrogenase activity was invariably greater at low concen-trations of free oxygen when there was a net discharge of oxygen from oxyhaemoglobin than when similar rates of oxygen consumption were being supported by much higher concentrations of dissolved oxygen in the absence of the carrier. The effects were relatively insensitive to leghaemoglobin concentration compared with results from shaken experiments with a gas phase present when myoglobin was substituted for leghaemoglobin nitrogenase activity was also stimulated during net discharge of oxygen from this carrier but the effect was not as great and it occurred at higher concentration of free dissolved oxygen.

Melik-Sarkisyan et al., (1976) showed the haemoprotein leghaemoglobin possesses an exceptionally high affinity for oxygen. It is believed that this protein provides oxygen for the bacterioids during the respiration of which the ATP necessary for nitrogen fixation is synthesised. They also showed that ferric leghaemoglobin as well as ferrous oxyleghaemoglobin could stimulate bacteroid oxygen uptake and nitrogenase activity. The bacteroids seoreats a reductase enzyme which can reduce ferric leghaemoglobin to ferrous leghaemoglobin. NADH or NADPH serve as electron donor for this reduction.

Bergersen and Turner (1975) showed high affinity oxidase system which was most active when dissolved oxygen was between 0.01 and 0.1 micromoles and also affinity system active at concentrations g/mm oxygen and above were shown to be present. The high affinity path way produced up to 5 times greater bacteroid ATP concentrations than the other system in the absence of leghaemoglobin.

Since leghaemoglobin content and nitrogen fixing ability of legume nodules are closely correlated it is not surprising that attempts have been made (Bauer, 1960) to discover whether this hemeprotein is directly concerned with nitrogen gas absorption, transport or activation in relation to the reduction process to ammonia. The evidence obtained is mostly unimpressive. From the masses of thermodynamic data provided by Mortimer and Bauer (1960) , it is possible to calculate that under their experimental conditions about 100 atm. of nitrogen gas would have been necessary for 50 per cent formation of a ferric LbN_2 complex and about 30-60 atm. for 50 per cent formation of ferrous LbN_2. Later in an attempt to repeat the isolation of a leghaemoglobin N_2 complex reported by Appleby (1969c) could obtain only a physical mixture of leghaemoglobin and cytochrome C, which apparently had the spectronic properties of the so called nitrogen complex. However, Ewing and Ionescu (1972) reviewed the suggestion that leghaemoglobin might be an ancillary nitrogen carrier.

Coventry and Dilworth (1976) found out when bacteroid protein synthesis was inhibited, leghaemoglobin and soluble plant protein syntehsis were unaffected. This result is consistent with leghaemoglobin being synthesised on plant ribosomes rather than on bacteriod ribosomes. The leghaemoglobin had an apparent half life of 18 days when tested with in CO_2 and is a stable protein in N fixing yellow lupin nodules.

Swaraj and Garg (1977) showed the heme containing protein of red nodules were absent in green non-functioning nodules. It was concluded that greening is accompanied by destruction of heme prophyrin ring as well as denaturation of the protein. Genetic diversity in the number of proteins constituting leghaemoglobin was confirmed.

Bergersen (1976) showed that haemoglobin of nodules was positively correlated with their volumes of bacteroid containing central tissue. The haemoglobin content per unit nodule volume may, therefore, be considered as an index of the volume of active bacteroid tissue in nodules. Since in legumes the maximum heme content of nodule is attained shortly prior to host flowering time when nodules attain their peak activity in N-fixation the heme contain per unit volume of nodules at this time may be used in comparing the relative potential of different legumes in symbiosis.

Iyer (1976) stated that among the legumes he examined _Dolichas lablab_ which showed a higher heme content per unit nodule volume than other species and thus, appears to have a greater potential in nodular activity.

Swaraj and Garg (1977) showed that total and heme protein content of leghaemoglobin were the highest in the red nodule and reduced with the onset of nodules senesence. In the fully senescent green nodules the total proteins decreased to a very low level, while the heme proteins were almost absent.

Johnson *et al.*,(1978) reported that their proton magnetic resonance study suggests that the extremely high oxygen affinity of leghaemoglobin is due to a very mobile distal histidine.

2.6 *Function*

The various proposals for leghaemoglobin involvement in symbiotic nitrogen fixation have included functions as an electron donor to nitrogenase bound to plant cell membranes (Bergersen, 1960) , as electron acceptor in the transformation of N_2 to NH_2OH (Virtanen and Laine, 1948) , or as nitrogenase itself with ferrous LbN_2H_2 as intermediate species (Hanstein *et al.*, 1967).

Bergersen *et al.*, (1973) showed that leghaemoglobin does not have a direct role in the N_2 fixation system but may act by facilitating the diffusion of oxygen into the nodule tissue under the low oxygen existing in the dense tissue. Bergersen and Turner (1975) found t out the presence of leghaemoglobin allowed maximum rate of oxygen uptake to continue to a much lower range of concentration.

Differences in acetylene reduction by different cultivars of soybean inoculated with the same strains of *R. japonicum* were demonstrated under three different sets of growth conditions indicating that rhizobia did not have complete control of the nitrogenase system of the plant (Davidson, 1973). He further reported that in experiments in which the four soybean cultivars were inoculated each with six rhizobia strains, cultivar had a significant effect on nodule content of leghaemoglobin and hematin as well as nodule weight and nitrogen fixation. Green nodules taken from soybeans, actively fixed nitrogen but at a lower rate than red nodules.

Appleby *et al.*,(1975) stated that when the isolated rhizobial bacteroids from soybean root nodules were remixed with purified leghaemoglobin from the same nodules a spectacular increase occurred in the efficiency of nitrogenase activity relative to respiration rate. It has been shown that an approximately linear relationships exists between ATP/ADP ratio and nitrogenase activity. They further showed that an oxyleghaemoglobin delivered oxygentensions, the efficiency of *R. japonicum* soybean bacteroid, oxidative phosphorylation is increased.

Wittenberg and Appleby (1975) found out that when oxyleghaemoglobin added to well stirred suspensions of bacteroids isolated from soybean root nodules enhances the rate of oxygen consumption and acetylenereduction to ethylene, a measure of the activity of the enzyme complex nitrogenase.

In the absence of oxyleghaemoglobin suspensions of bacteroids maintained a substantial oxygen uptake which is largely ineffective in supporting nitrogenase activity. The addition of oxyleghaemoglobin brings about an increment of oxygen consumption and promotes nitrogenease activity which increased roughly ten fold. The ratio of the increase in oxygen consumption to the increase of acetylene reduction is corresponding. So the result is that in the absence of leghaemoglobin the ineffective oxygen uptake maintains a very low oxygen pressure at the bacteroid surface. In the presence of leghaemoglobin it appears that the operation of leghaemoglobin facilitated oxygen diffusion increases nitrogenase activity.

The mutual relationship among weight of nodules and leghaemoglobin content, bacteroid number, plant dry matter and total plant nitrogen of six cultivars of soybean was examined by Jain and Riwari (1975) at two stages of growth. The correlation among all these factors were positive and mostly significant. Nash and Schulman (1978) showed as lower rate of increase of leghaemoglobin content, compared with that of nitrogenase activity which results in a progressive decline in the ratio of flowering.

The leghaemoglobin content represented 40 per cent of the total soluble protein in the nodules. Nitrogenase activity was induced in free living rhizobia in the absence of detectable leghaemoglobin. They further observed that the soybean root nodules at different ages in an atmosphere of carbon-monoxide revealed no oxidase-leghaemoglobin. It was concluded that the decline of nitrogenase activity in older root nodules could not be attributed to the accumulation of oxidized leghaemoglobin.

Melik-Sarkisyan (1976) confirmed that the oxyleghaemoglobin, oxymyoglobin, oxyhemoglobin stimulate the nitrogen fixation rate and oxygen uptake of lupine bacteroids, unlike the results obtained with soybean bacteroids, he further found that the ferric protein also stimulated nitrogen fixation and oxygen uptake. They consider this result due to an enzymic reductase system of lupin bacteroids following inhibition studies, the author concluded that bacteroids do not contain a special oxidase which reacts with oxyleghaemolglobin.

Fuchsman et al.,(1976) also showed that leghaemoglobin components have a singal biochemical role in nitrogen fixation.

Chahal and Hewari (1977) examined relationship between leghaemolglobin content and strains efficiency in Vigna aureus. Bisseling et al., (1978) showed that the Ammonium nitrate decreased the amount of leghaemoglobin in the nodules and there was a quantitative correlation between the leghaemoglobin content and the nitrogen fixing capacity of the nodules.

Kudryavtseva and Borodenko (1978) have shown that the leghaemoglobin of lupin which was salted out in the range of 55-90 per cent saturation by ammonium sulphate can be separated into components LbI and LbII by ion exchange chromatography on DEAE cellulose.

Appleby and Bergersen (1980) carried out an experiment on the use of leghaemoglobin and topics covered are preparation and properties, spectrophotometry use of leghaemoglobin in experiment with a gas phase and leghaemoglobin in no gas phase experiment and showed effect of leghaemoglobin.

Publication about the properties in vivo location, functions and in vitro use of leghaemoglobin are respiration and nitrogenase activity in Rhizobium japonicum bacteroids is described by Bergersen (1980). Uheda and Syono (1982) showed disc-gel electrophoresis of the leghaemoglobin isolated from pea root nodules revealed 2 major and 3 minor components. The ratio of the 2 major components decreased with increasing age and was higher in distal than in proximal regions of the nodules. Their experimental result suggest that the leghaemoglobin components participate in more effective nitrogen fixation by controlling oxygen transport to bacteroids.

It will be seen from above review that chemistry, localisation, isolation, properties and function of leghaemoglobin in different leguminous crop was studied in India and abroad. However, the relationship between leghaemoglobin and nitrogen content in nodules of different cultivars of gram was not yet studied in Maharashtra. Therefore, the present investigations were undertaken in order to throw some light on above points. The results obtained are reported in following chapters.

CHAPTER 3
MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present investigations were undertaken during 1981-82 in the Department of Plant Pathology and Agricultural Microbiology, Mahatma Phule Agricultural University, Rahuri.

In Maharashtra, generally, gram is grown on the medium to heavy soils under receding moisture conditions in *rabi* season i.e. after the harvest of *kharif* crops. The optimum date of sowing is mid of October but many times if *kharif* crop is delayed sowing is done even up to first fortnight of December.

3.1 Source of seed material :

The seeds of the gram cv. *viz.*, PG-2, PG-5, N-31, N-59 and *chafa* were selected on the basis of phenotypic diversion and differences in yield, growth habit etc. For the purity of such seed, which was maintained by the University Pulse Breeder, was selected. Seeds were selected true to type, bold and having high germination percentage and less of inert matter which were not damaged by pests and diseases and with full maturity. Such seeds were obtained from the Pulse Breeder in different container to avoid the mixture of two or more varieties.

3.2 Source of *Rhizobium* culture :

As the nitrogen is essential for the plant growth and production, it is essential to supply it artificially to the plant. An application of *Rhizobium* culture to the legume seed which fixes atmospheric nitrogen symbiotically in root nodules which ultimately used by a plant for its growth. For that purpose an effective strain of *Rhizobium* which was freshly prepared having more number of effective bacteria per gram of culture and stored in good condition in perforated polythine bags. Such type of culture was prepared by the Agricultural Bacteriologist, College of Agriculture, Pune-5. So far the purity and effectiveness of *Rhizobium* culture, it was obtained from the above source.

3.3 Experimental details :

The pot culture experiment was conducted in the glass-house at the Central Campus, Rahuri in the Department of Plant Pathology and Agricultural Microbiology. In the glass-house all the environmental conditions were made favourable for the healthy plant growth, such as humidity, temperature, irrigation, airation, sunlight etc.

3.4 Selection of pots :

Pots selected were plastic ones having equal size of all the pots of nine inches diameter and height about one foot, because nodules confined to the 0-15 cm depth (Sheldrake and Saxena). They were perforated at bottom to drain off the excess water. These pots were washed with clean water and made free from dirt and chemical materials adhere to them.

As the chick pea is grown mostly on a medium to heavy black soil for high production and hence the soil was collected from such fields. After removing bolders and course particles it was kept on clean floor. Then well decomposed organic manure was added to the soil and then soil and organic manure were mixed well so as to get equal composition of soil and organic manure to each pot. Pots were then filled in with the above soil mixture in an equal quantity in each pot. Pots were filled in such a way that small bolders were placed first at the bottom on the hole to avoid the soil loss and then soil mixture was filled in up to the height of one inch below the top edge of pot.

3.5 Preparation of seeds for sowing :

A slurry was prepared of *Rhizobium* culture by adding water. Seeds from the bags were then taken out of the required quantity and were mixed with slurry already prepared so as to ensure the continuous coating of *Rhizobium* culture on each seed. This was repeated for other varieties. Each time, the slurry was prepared freshly in order to avoid mortality of bacteria. Inoculated seeds then dried on the clean floor in shade separately.

3.6 <u>Sowing</u> :

Sowing was done immediately after inoculation. Eight seeds were sown in each pot at an inch depth and well spaced. This was repeated for remaining treatments of varieties. Eight pots of each variety were prepared and sown as above. Pots were then kept separately from each other and they were labelled immediately to avoid the confusion. After labelling they were watered well so as to moisten the soil. Excess water was drained off through pours provided at the bottom of the pot. The germination started after 4th day of sowing and it was completed on 7th day.

Fifteen days after germination thinning was done by removing 2 plants from each pot and allowing the healthy and vigorous 6 plants which were well spaced from each other for observations.

3.7 <u>Observations to be recorded</u> :

Main object of this investigation was to estimate the leghaemoglobin content of nodules of gram plants. For these observations minimum weight of nodule required was 1 g so as to get that much weight of nodules first observation was recorded on 40th day of sowing. Plants were firstly removed carefully from pots by inverting it in water and after washing all soil particles and other sticking materials to the root system was removed. Nodules were then separated by counting them as effective, ineffective, nodules on tap roots and nodules on latteral roots. After separation of nodules, they were collected in a small butter paper bag labelled and kept in the hot air oven at 60°C temperature for 48 hours for drying.

Nodules from other plants of same variety were removed and one gram nodule material was weighed. This quantity was used for the estimation of leghaemoglobin each time.

By the same procedure observations on nodule, its number, effectiveness, ineffectiveness and its position were recorded for the other varieties e too.

Plants, of which nodules were removed, were preserved in a paper bag, labelled and kept in the hot air oven for drying at 60°C temperature for 48 hours.

After complete drying, dry matter of nodules was weighed and used for estimation of total nitrogen. This material was ground for fine powder, by grinding machine, weighed as the total weight of the dry matter and 1 g of such dry matter was used for further study. In the process of grinding to avoid the contamination from dry matter of other varieties after grinding one plant, the machine was brushed before grinding of another plant or variety.

In this way the dry matter was ground and 1 g sample of each variety was taken separately on a paper and used for nitrogen distillation.

3.7 (a) <u>Observations on leghaemoglobin content</u> :

<u>Procedure</u> : Nodules from freshly uprooted plants were removed and taken for an estimation. Nodules were washed by clean water to remove the dirt and dust particles sticking to the nodules. Nodules were then kept on blotting paper to remove the excess water. These fresh nodules were then weighed to exact 1 g on the chemical balance. They were then transferred to a clean mortar. Two spoonful of fresh sodium hyposulphide was added to the mortar containing nodules, with the help of a spatula. The nodules were properly crushed with the sodium hyposulphide to a fine paste. To this 4 ml fresh cold pyridine was added in which leghaemoglobin gets dissolved. This paste was transferred to the centrifugal tube which was previously washed and cleaned. Again 4 ml fresh cold pyridine were added to the mortar to wash the mortar and pestle and that washings were then transferred to the same centrifugal tube. Then, 2 ml pyridine were added to the centrifugal tube to make the exact volume of total solution. The contents of the tube were properly mixed in order to

dissolve all the leghaemoglobin in pyridine. Then the centrifugal tube was pluged with plastic plug, the tube was then kept in the centrifugal machine. This procedure was repeated for remaining varieties and labelled properly.

The centrifugal machine was run at 2000 rpm for 30 minutes. This much time is sufficient for settling the course particles and suspended materials to get settled at the bottom of the tube. Tubes were then taken out and kept in the tube holders.

The spectronic-20 was adjusted to the wave length of 550nm and blank reading was taken. The Spectronic-20 reading tube was removed and washed by the clean water and then outer surface of it was wipped out by the blotting paper. The clear reddish suspension from centrigugal tube was then transferred to the Spectronic-20 reading tube upto the sufficient height. The tube was then placed in its place and density of leghaemoglobin was recorded in terms of transmission percentage. From this transmission percentage an optical density was obtained which indicates its relative density of the solution i.e. leghaemoglobin reading from all the 5 varieties at 4 different stages were recorded in the tabular form.

3.7 (b) Total N_2 estimation from nodules and plants

The nitrogen content in nodules and dry matter at 4 stages of five varieties of gram was estimated by the microkjeldahl method of nitrogen distillation separately so as to avoid the contamination. After getting readings by titration, nitrogen percentage was calculated by the following formula -

$$\frac{(\text{Sample reading} - \text{Blank reading}) \times \text{Normality of HCl} \times 14 \times \text{Volume made after digestion} \times 100}{\text{Aliquot digest taken} \times \text{Weight of sample taken} \times 1000}$$

In this way the nitrogen percentage of respective variety at different stages was calculated.

3.8 Statistical analysis :

After getting values of nitrogen and leghaemoglobin content, it was necessary to correlate the date whether these two values correlate, for this, statistical method used was Karlpearson's simple correlation coefficient. In this method 'r' values were calculated as follows :

$$r = \frac{\sum xy}{\sqrt{\sum x^2 \cdot \sum y^2}}$$

$$R\,xy = \frac{\sum x_i y_i - \frac{(\sum x_i)\,(\sum y_i)}{n}}{\left[\sum x_i^2 - \frac{(\sum x_i)^2}{n}\right]\left[\sum y_i^2 - \frac{(\sum y_i)^2}{n}\right]}$$

After calculating this 'r' value i.e. correlation coefficient test of significance was calculated to see whether two data were significantly correlated or not and that was calculated as

$$t_{cal} = \frac{r\sqrt{(n-2)}}{\sqrt{1-r^2}}$$

Then 't' table value was seen at 5 % level of significance and 3 degrees of freedom. When it was less than 't' calculated value of the correlation then correlation is said to be significantly correlated and when 't' table value is more than 't' calculated value then it was said that data are not correlated with each other.

CHAPTER 4

EXPERIMENTAL RESULTS

4. EXPERIMENTAL RESULTS

The glass-house experiment to study the correlation between leghaemoglobin and nitrogen content in five varieties of gram (*Cicer arietinum*, L.) at different growth stages was conducted during *rabi* season of 1980-81. Observations regarding nodulation pattern, leghaemoglobin and nitrogen content of nodules and plant at different growth stages were recorded and are presented in this chapter.

The first observation for total number and colour of nodules of each variety was recorded on 40th day. A differentiation of nodules and other observations *viz.*, nodule number and colour was subsequently recorded in all the varieties under study on 60th, 80th and 100th day of sowing.

4.1 Nodulation pattern :

In general, all the varieties under study showed different number of nodules at different growth stages. The data presented in Table 1 indicate that the maximum nodules under the glass-house conditions was obtained in Phule G-5 variety. However, in the case of this variety, a slight decrease in number of nodules was observed after 60 days of sowing, while in case of Phule G-2 an increase in number of nodules was observed on 60th day after sowing

Table 1 : Number of nodules/plant at different growth stages of gram

Variety	Phule G-2			Phule G-5			N-31			N-59			chaffa		
sta-/days ge	Effective	Ineffective	Total *	Effective	Ineffective	Total	Effective	Ineffective	Total	Effective	Ineffective	Total	Effective	Ineffective	Total
40	7.20 (90)	0.80	8.00	8.20 (90)	1.00	9.20	6.80 (88)	1.00	7.80	6.80 (84)	1.20	7.50	8.00 (87)	1.00	9.00
60	16.40 (93)	1.40	17.80	16.20 (94)	1.20	17.40	16.20 (90)	2.20	18.40	15.0 (87)	2.40	17.40	12.40 (84)	2.40	14.80
80	15.80 (87)	2.40	18.20	13.20 (83)	2.80	16.00	12.00 (75)	4.00	16.00	13.60 (83)	2.90	16.40	9.00 (72)	3.60	12.60
100	5.20 (56)	4.20	9.40	7.40 (63)	4.40	11.80	6.60 (51)	6.40	13.00	4.20 (54)	3.60	7.80	3.00 (40)	4.50	7.50

* Mean of 5 plants.

Figures in a bracket are in percentage.

and thereafter nodule number was decreased. Similarly, in case of Phule G-5 the maximum number of nodules was observed on 60th day of sowing and thereafter it was decreased.

In N-59 variety an increase in nodulation up to 60 days was observed and thereafter nodule number was decreased. The variety chaffa showed an increased nodules even after 60 days from sowing but a peak point was in between 40 to 60 days.

4.2 Nodule effectiveness :

Observations recorded in respect of number of pink and black nodules are given in Table 1 indicate that the number of black nodules was increased from 60th day in all the varieties but an increase in black nodules was more at 80 and 100 days, whereas less number of black nodules were observed at 40 days of sowing. The highest number of total nodules was recorded at the second observation i.e. at 60th day of sowing except in PG-5 in which the highest nodules were noted on 80th day of the sowing of which the black nodules were maximum. The highest number of nodules was observed in N-31, PG-2, PG-5 and N-59 but chaffa variety produced less number of nodules as compared to other varieties at the second stage.

The peak period of pink nodules was observed at 60 days of sowing except in *chaffa* in which pink nodule percentage decreased eventhough, number of nodules increased from 9.60 to 14.80 in numbers.

At the third stage of an observation, it was found that the per cent pink and total number of nodules were decreased in all the varieties except PG-2, in which nodules were increased in number from 17.80 to 18.20 per plant, but red pigmented nodule percentage was decreased from 93 to 87.

At this stage a decrease in pink or total nodules was not more in varieties *viz.*, PG-2, PG-5, N-31 and N-59 but it was more in *chaffa* eventhough total number of nodules was not fluctuating much.

At the fourth stage of observation i.e. at 100 days of sowing it was found that the total number of nodules in all the five varieties were decreased tremendously and pink nodule percentage was also decreased considerably with increased number of black nodules. While decrease in pink nodule percentage was found to be the maximum in *chaffa* variety as compared to other varieties. The total nodules were also least in the same variety. It was further observed that total number of nodules were more in N-31 followed by PG-5, PG-2 and N-59 whereas the per cent of pink nodules was more in PG-5 followed by PG-2, N-59 and N-31. In general,

Plate 1 : Nodulation in gram 40 days after sowing.

Plate 2 : Nodulation in gram 60 days after sowing.

1=PG-2, 2= PG-5, 3= N-31, 4= N-59, 5= <u>chaffa</u>.

Plate 3 : Nodulation in gram 80 days after sowing.

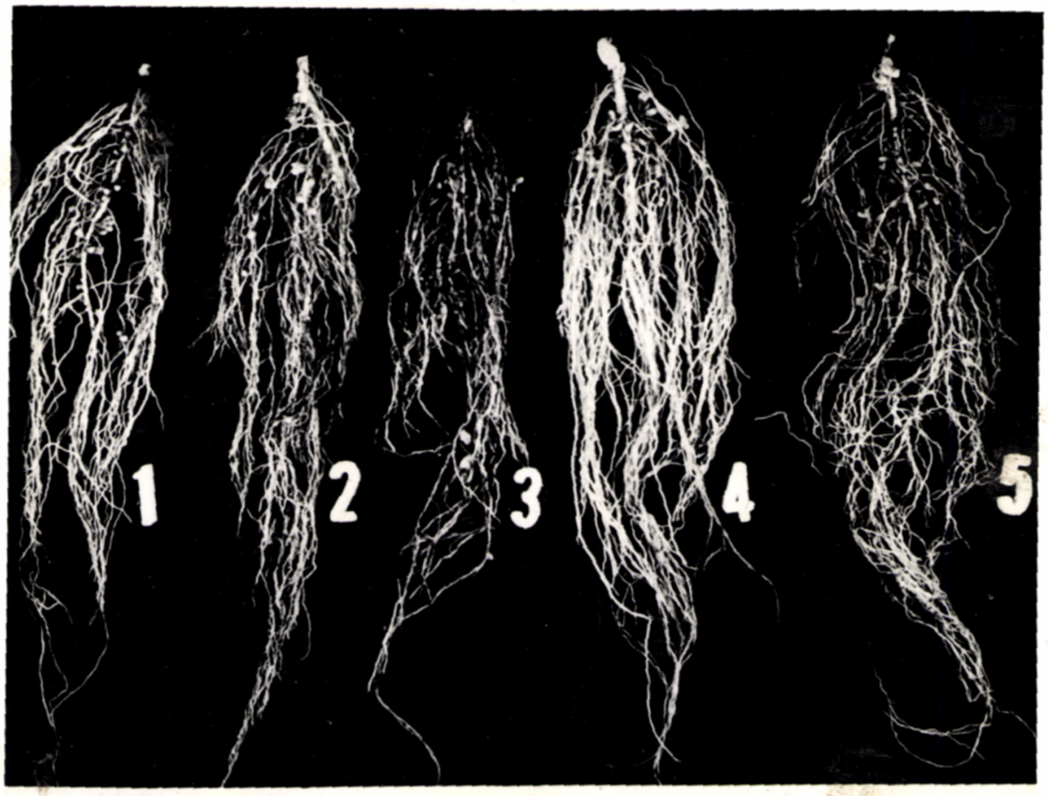


Plate 4 : Nodulation in gram 100 days after sowing.

it was revealed from Table 1 that total number of nodules were the maximum on 60th day from sowing in PG-5, N-31, N-59 and *chaffa* varieties except PG-2 variety in which maximum nodules were found 80th day of sowing.

An increase in total number of nodules from 40 to 60 days was two-fold in all varieties except *chaffa*. After 60 days, total nodules were decreased except in PG-2 and it was continued during 80 to 100 days in all the varieties. In PG-2 variety the maximum nodules were on 80th day which was slightly increased from60 to 80 days and decreased thereafter.

The per cent pink or effective nodules increased from 40 to 60 days after sowing in all varieties except in *chaffa*. So the peak point of nodule activity was noted around 60 days in PG-2, NG-5, N-31, and N-59 and in case of *chaffa*, it was between 40 and 60 days.

After 60 days, pink nodule percentage sas found to be decreased slowly during flowering and pod filling stages. It was also found that the decrease in percentage from 80 to 100 days was more than 60 to 80 days in all the varieties. Besides, it was found that in all the varieties at initial stage i.e. at 40 days of sowing black or ineffective nodules were less in number but the number was increased thereafter. In that also an increase was slower from 40 to 60 days but it was more after 60 days in all varieties and was found to be highest in between 80 to 100 days after sowing.

4.3 Leghaemoglobin and Nitrogen content :

The results regarding the leghaemoglobin content and nitrogen content in the nodules at different stages of various gram varieties are presented in Table 2. Results reveal that the leghaeglobin content increased with the age of a plant and it was continued up to the flowering stage.

Similarly, nitrogen percentage in nodules was found to be increased with the age of a plant up to 60 days i.e. up to the maximum flowering stage.

In the variety PG-2, the leghaemoglobin content at 40 days i.e. at first observation stage was 0.17 O.D. and thereafter it was increased up to 0.31 O.D. at the age of 60 days i.e. at the second stage of observation. Therefore, the maximum leghaemoglobin content was found between 40 to 60 days and thereafter it was decreased up to 0.20 O.D. at 80 days and 0.11 O.D. at 100 days observations i.e. at 3rd and 4th stage of observations.

It was further observed from the Table 2 that nitrogen perentage in a nodule goes on increasing with the age of a plant from 1.68 to 2.10% at 40 days and 60 days, respectively. In general, it was revealed that the maximum nitrogen content was at flowering stage (60 days) and thereafter it was declined. From results of Phule G-2 variety it was found that with an increase in

Table 2 : Leghaemoglobin and nitrogen content (per cent) of nodules at different growth stages in some varieties of gram (optical density per gram)

Varieties	Phule G-2		Phule G-5		N-31		N-59		chaffa	
Stage/days	Lb	N	LB	N	Lb	N	Lb	N	Lb	N
40	0.17	1.68	0.23	1.96	0.17	1.96	0.15	1.54	0.16	1.68
60	0.31	2.10	0.36	2.80	0.28	2.66	0.29	2.38	0.21	2.66
80	0.20	1.82	0.27	2.20	0.26	2.38	0.26	2.10	0.19	2.00
100	0.11	1.20	0.14	1.50	0.11	1.90	0.12	1.69	0.13	1.48
r- value	0.96		0.99		0.97		0.95		0.94	
+ calculated	4.83		9.96		5.60		4.32		3.79	
't' table value	2.92		2.92		2.92		2.92		2.92	

Lb = Leghaemoglobin (optical density/g).

N = Nitrogen %

leghaemoglobin in nodules nitrogen content, was also increased up to the age of 60 days and thereafter the leghaemoglobin as well as nitrogen content was decreased. Hence, these two factors are interdepenuent and correlation was found to be significant at five per cent level of significance.

In variety Phule G-5 leghaemoglobin and nitrogen content in nodules was found to be significantly correlated with each other which indicated by an increase in leghaemoglobin content from 0.23 to 0.36 O.D. at 40 and 60 days and nitrogen from 1.96 to 2.80 per cent. Thereafter the leghaemoglobin content was decreased from 0.36 to 0.27 O.D. and 0.14 at 80 and 100 days, respectively and nitrogen content was also decreased from 2.8 to 2.2 and 1.5 per cent at 80 and 100 days, respectively. The maximum leghaemoglobin and nitrogen content in this variety was found to be at 60 days i.e. at maximum flowering stage, of which leghaemoglobin was found to be very active in fixing atmospheric nitrogen to the maximum extent in nodules, thereafter the leghaemoglobin as well as nitrogen content was slowly decreased with the age of a plant.

From the results of N-31 variety, it was found that at the first observation stage the leghaemoglobin content was 0.17 O.D. and thereafter it was increased up to 60 days (0.22 O.D.). By the same way nitrogen content was also

Fig. 1 : Leghaemoglobin, Nitrogen and Dry matter content of gram Cv. PG-2 as influenced by days.

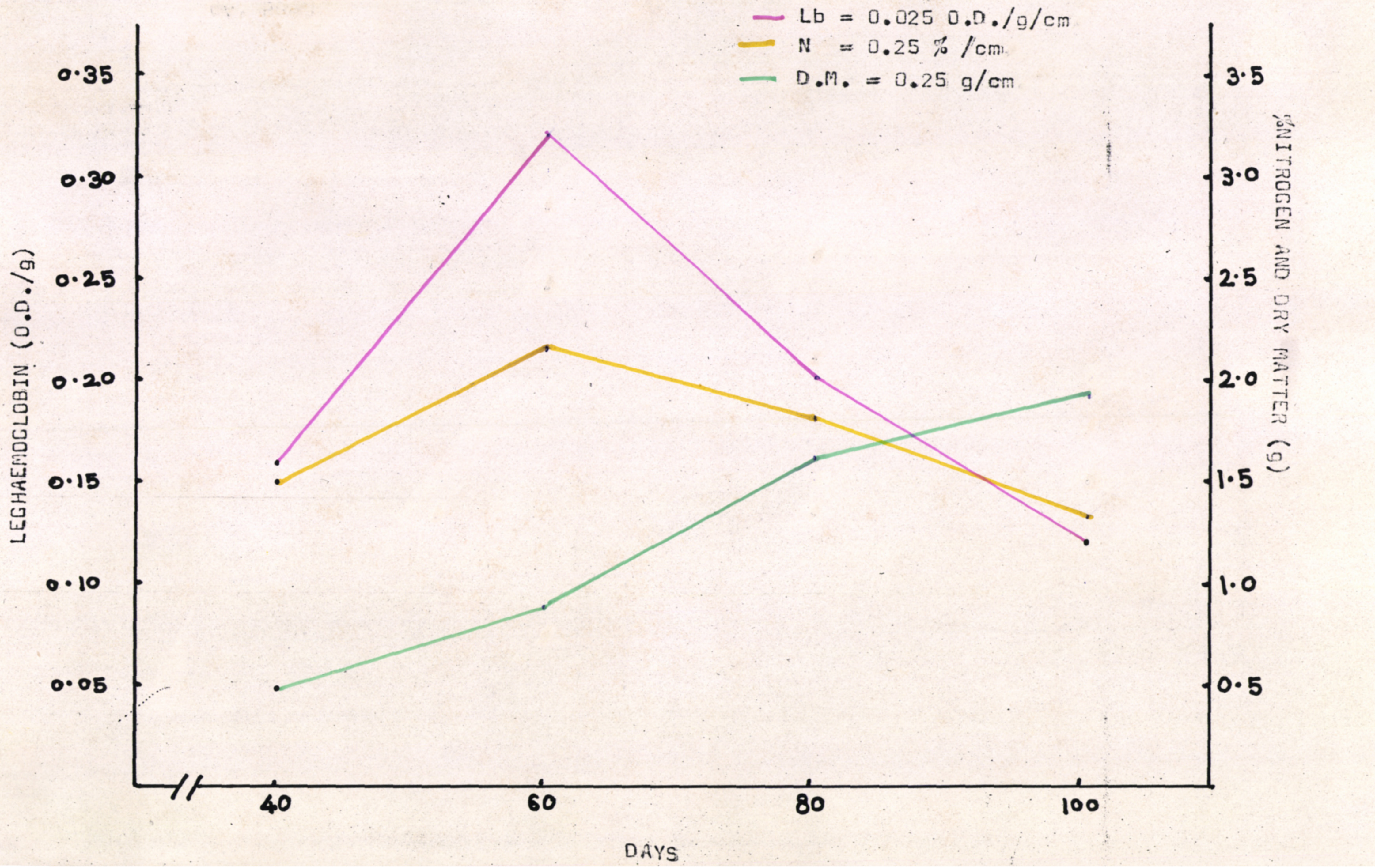


Fig.2 : Leghaemoglobin, Nitrogen and Dry matter content of gram Cv. PG-5 as influenced by days.

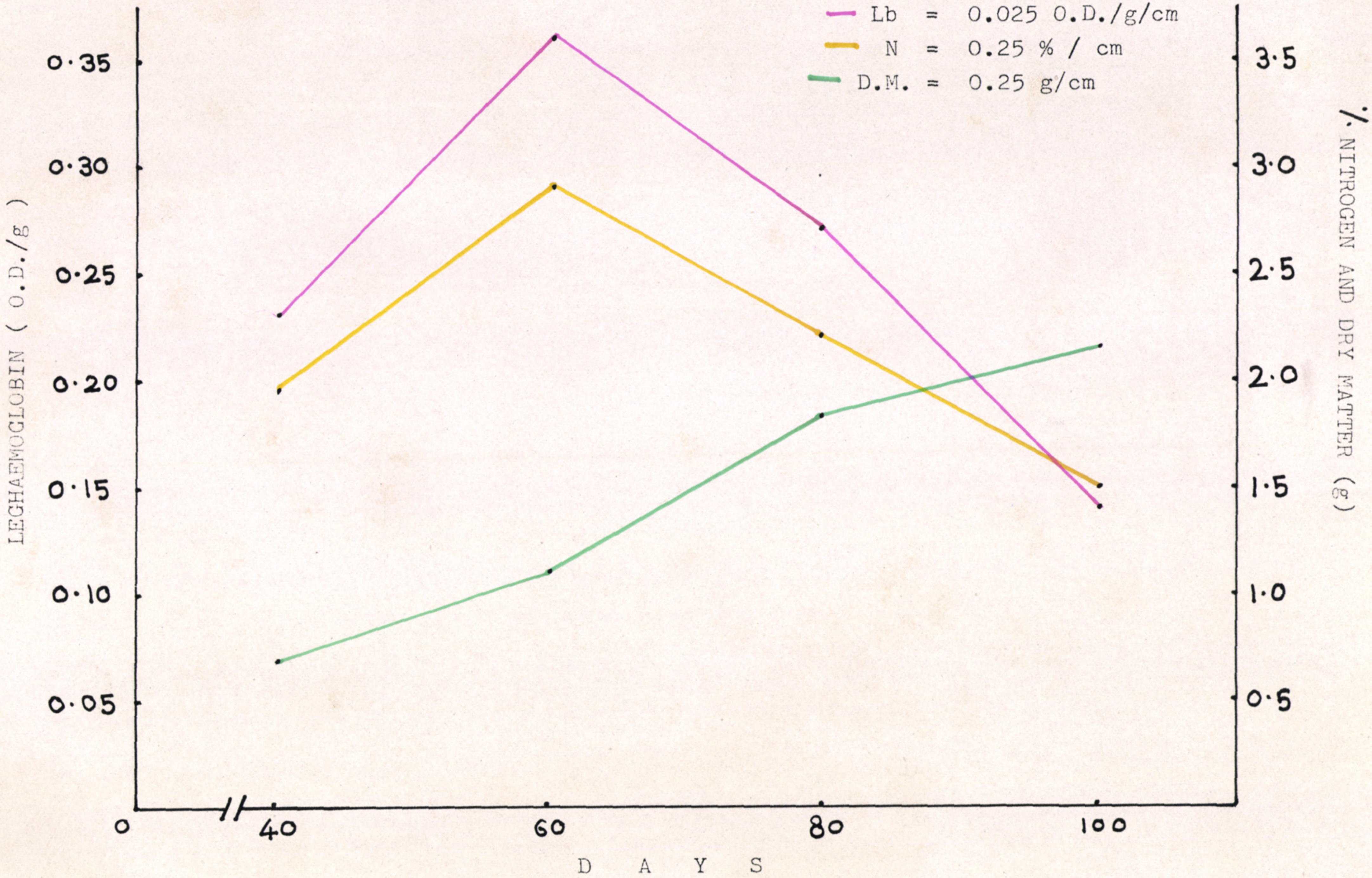


Fig.3 : Leghaemoglobin, Nitrogen and Dry matter content of gram Cv. N-31 as influenced by days.

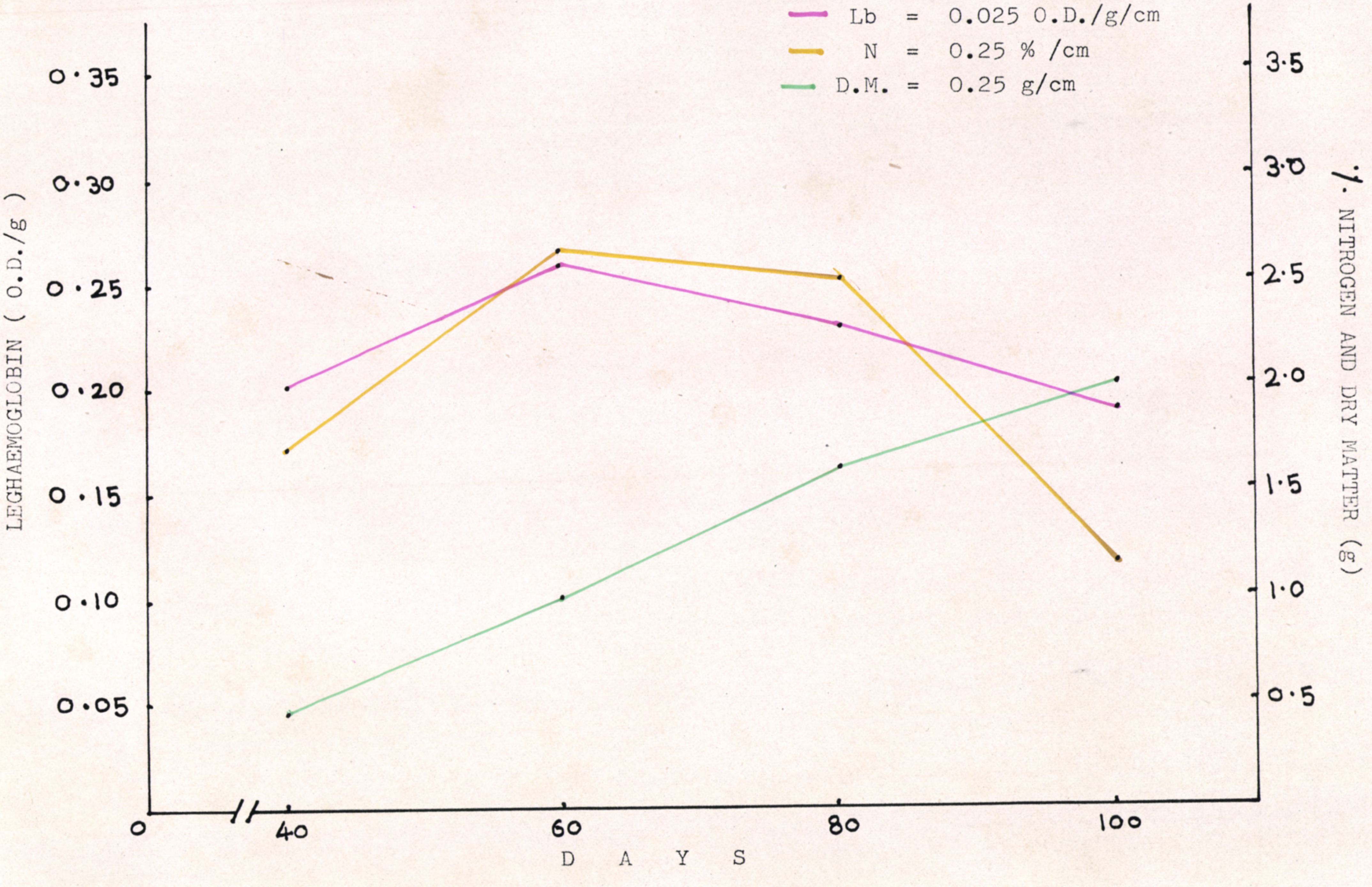


Fig.4 : Leghaemoglobin, Nitrogen and Dry matter content of gram Cv. N-59 as influenced by days.

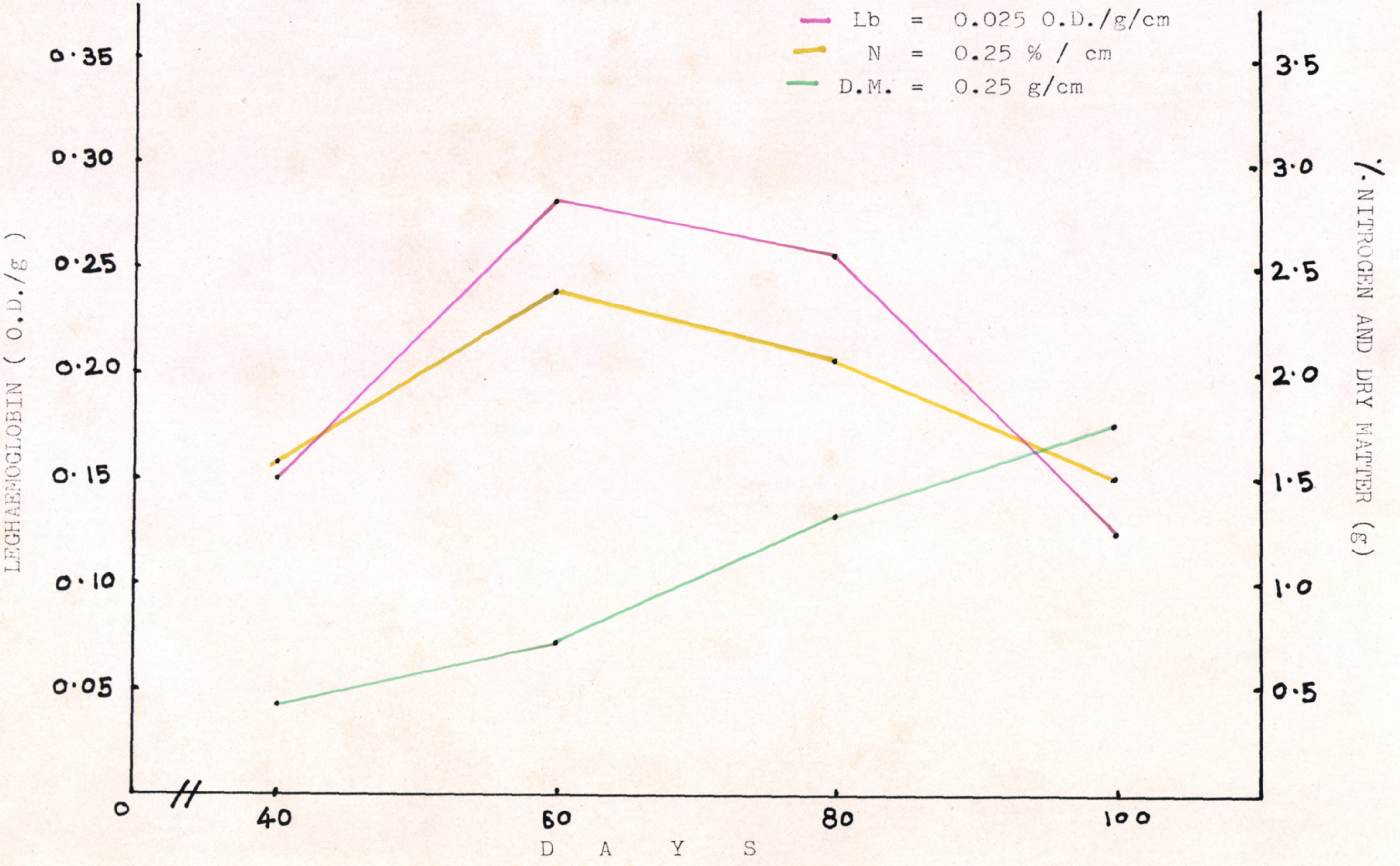
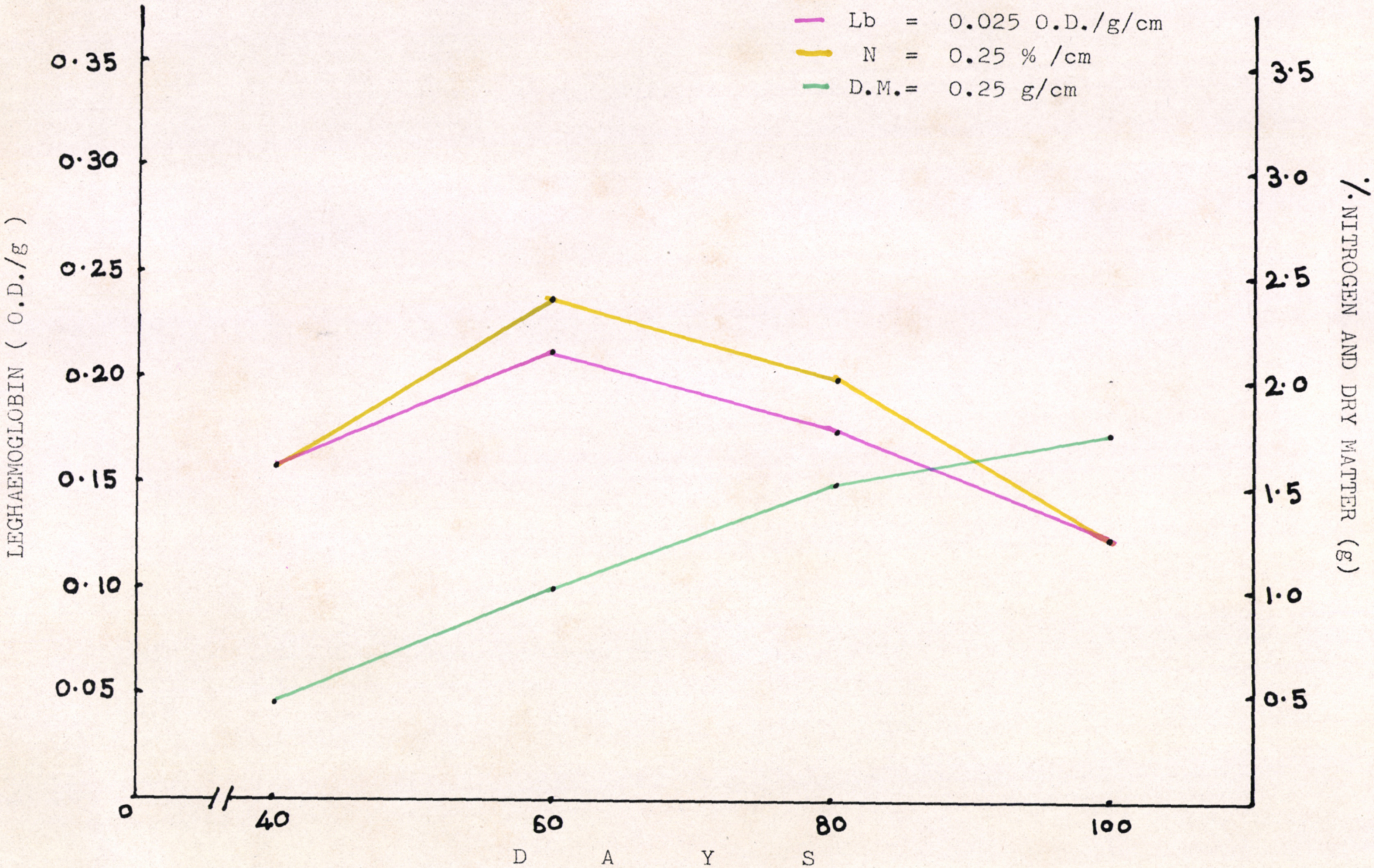


Fig.5 : Leghaemoglobin, Nitrogen and Dry matter content of gram Cv. Chaffa as influenced by days.



found to be increased from 1.96 to 2.66 per cent at 60 days or maximum flowering stage. The leghaemoglobin as well as nitrogen content was decreased from 0.26 to 0.11 and 2.38 to 1.90 O.D.,respectively at 80 days and 100 days stage of observations. However, it was further noted that the leghaemoglobin and nitrogen content during 60 to 80 days i.e. at the second and third observation stage a decrease was very slight. This was attributed towards the maximum flowering occurs during this period and also pod formation and pod filling takes place. The correlation between these two ingradients at four stages was worked out and found to be highly significant at five per cent level of significance in the variety.

The correlation between leghaemoglobin and nitrogen content in nodules of gram variety N-59 at four different growth stages was found to be significant at five per cent level of significance, in which leghaemoglobin and nitrogen content at 40, 60, 80 and 100 days was 0.15, 0.29, 0.26 and 0.12 and 1.54, 2.38, 2.10 and 1.60 per cent, respectively which indicates that the leghaemoglobin increased with an increase in age of a plant and it was maximum at 60 days i.e. at the second stage of observations at which maximum flowering took place and thereafter it was decreased. Similar results were obtained with the cvs. N-31, PG-5 and PG-2.

However, results with *chaffa* variety indicated that it being a short duration variety content of leghaemoglobin and nitrogen was less as compared to other varieties at first observation (40 days) stage. Both of these ingradients increased up to 0.21 O.D. of leghaemoglobin and 2.66 per cent of nitrogen at 60 days which was found to be the maximum and thereafter both the ingradients were decreased. The correlation between leghaemoglobin and nitrogen content at four growth stages i.e. 40, 60, 80 and 100 days of all five gram varieties was found to be significant at five per cent level of significance.

In general, it was found that the leghaemoglobin was highly correlated with nitrogen content at various growth stages of gram varieties. With an increase in the leghaemoglobin in all the five varieties from 40 to 60 days nitrogen percentage was also found to be increased correspondingly. The highest amount of leghaemoglobin was found to be present in the variety PG-5 followed by PG-2, N-31, N-59 and *chaffa* at 60 days observation i.e. at the maximum flowering stage. Similarly, nitrogen percentage was found to be highest in the same variety at the same growth stage. A decrease in leghaemoglobin and nitrogen content started from 60 days in all varieties but it was less in long duration varieties i.e. PG-2, PG-5, N-31 and N-59 and more in short duration variety like *chaffa* in 60 to 80 days period of growth but thereafter a considerable reduction in the leghaemoglobin and nitrogen content was found in all the varieties.

From the results presented in Table 2, it was found that a significant correlation of leghaemoglobin and nitrogen content was the highest in variety Phule G-5 followed by N-31, PG-2, N-59 and chaffa at all the four stages of observations.

From Table 1 and Table 2, it was further revealed that more the percentage of effective nodules more was the leghaemoglobin and nitrogen content in nodules of all the varieties. As the black nodule percentage increased from 60 days age of plants, the leghaemoglobin as well as nitrogen content decreased because of the pink or effective nodule percentage was found to be decreased from 60 days onwards and it was found to be very low at 100 days observation at this stage very low percentage of pink nodules was noticed.

4.4 Dry matter and Nitrogen :

The results recorded in Table 3 regarding dry matter weight and nitrogen content of gram at the different growth stages were found to be increasing from the first stage of observation to the second stage of observation i.e. at 60 days of gram. The dry matter weight of all varieties at all stages of growth was increasing but an increase in

Table 3. Dry matter and nitrogen content of gram

Variety	Phule G-2		Phule G-5		N-31		N-59		chaffa	
Stage/days	Weight (g)	N %	Weight (g)	N %	Weight (g)	N %	Weight (g)	N %	Weight (g)	N %
40	0.38*	1.00	0.37	1.25	0.40	1.20	0.42	0.90	0.35	1.12
60	0.80	1.22	0.96	1.40	0.97	1.32	0.73	1.15	0.81	1.15
80	1.60	1.10	1.88	1.30	1.75	1.26	1.33	1.10	1.56	1.00
100	1.95	0.95	2.15	1.10	2.10	1.05	1.75	0.85	1.65	0.89

* Mean of 5 plants.

dry weight from the first stage to the second stage was two-fold or at very fast rate but thereafter an increase was very low in all the varieties.

The highest dry matter weight was found to be at the 4th stage of observation i.e. at 100 days after sowing in the variety PG-5 (2.15 g/plant) followed by N-31, PG-2, N-59 and chaffa.

The total nitrogen in dry matter was increased from the first stage to second stage in all the varieties i.e. at 60 days observation. However, the increase was rather low and thereafter it was decreased. The low per cent nitrogen was found at the last stage of observation i.e. 100 days age of plants. The maximum percentage of nitrogen was present in the variety PG-5 at 60 days age (1.40 per cent) followed by N-31, PG-2, N-59 and chaffa.

The results regarding dry matter weight and nitrogen content are presented in Table 3 which reveal that the nitrogen content in plants was maximum in all varieties at 60 days. The rate of dry matter increase was also found to be maximum up to 60 days of a plant in all the varieties and thereafter it was decreased.

4.5 Leghaemoglobin and Dry matter :

The results in respect of the leghaemoglobin content of nodules and dry matter of a plant at various stages are

Table 4 : Leghaemoglobin content of nodules and dry matter of gram in different varieties.

Variety	Phule G-2		Phule G-5		N-31		N-59		chaffa	
stage/days	Lb OD/g	DM (g)	Lb OD/g	DM (g)	Lb OD/g	DM (g)	Lb OD/g	DM (g)	Lb OD/g	DM (g)
40	0.17*	0.38*	0.23	0.37	0.17	0.40	0.15	0.42	0.16	0.35
60	0.31	0.80	0.36	0.96	0.28	0.97	0.29	0.73	0.21	0.81
80	0.20	1.60	0.27	1.88	0.26	1.75	0.26	1.33	0.19	1.56
100	0.11	1.95	0.14	2.15	0.11	2.10	0.12	1.75	0.12	1.65

Lb = Leghaemoglobin; OD = Optical density

* = Mean of five plants.

presented in Table 4 which indicate that the rate of increase of dry matter at an initial stage was rather higher than at a latter stage. An increase of dry matter from 40 to 60 days was two-fold because of the highest activeness of leghaemoglobin which fixes more amount of nitrogen and enhanced the growth of the plant and produced more of vegetative growth.

After 60 days observation the pod formation started, an energy stored in the plant body gets declined as well as leghaemoglobin was also declined and ultimately nitrogen fixation was affected as well as dry matter production also gets reduced considerably. The rate of vegetative production at the first to second stage was increased at faster rate than the second to third and third to fourth stage. The maximum dry matter production was observed in the variety Phule G-5 (2.15 g) because of the presence of more amount of leghaemoglobin in nodules of the variety in its whole growth period followed by N-31 (2.10 g), PG-2 (1.95 g), N-59 (1.75) and chaffa (.1.65 g).

Although, the leghaemoglobin content was more in PG-2 at the second stage than N-31 but the total leghaemoglobin in the overall growth period was less than N-31 and hence, the production of dry matter was more in N-31 than in PG-2 and same case was found in the variety N-59.

An increased nitrogen content in the nodules of gram variety was found to be increased from 40th day of sowing to maximum flowering stage, thus, the maximum nitrogen content in nodules was observed at the flowering stage and decreased thereafter. This decrease in nitrogen content of nodules at the advance stages of a plant was attributed towards their utilisation for protein synthesis in the seed.

CHAPTER 5

D I S C U S S I O N

5. DISCUSSION

5.1 The present investigation was undertaken to study the following objects :

(i) A study on the nodulation pattern, effective and ineffectiveness of the nodules in some promising varieties of chickpea.

(ii) A study on the comparative leghaemoglobin and nitrogen content at different growth stages of the crop.

(iii) A study on the effects of the leghaemoglobin content on nitrogen fixation in nodules at different growth stages.

(iv) A study on the relationship of leghaemoglobin and the total dry matter of plants.

(v) A study on the leghaemoglobin content and its effect on the nitrogen content in a plant body.

5.2 Nodulation :

The results obtained regarding the number of pink and black nodules (Table 1) reveal that a larger percentage of white, pink and black nodules was observed at 40 days after sowing, 60 days or at the flowering stage and maturity stage, respectively. In general, the maximum number of nodules were observed at the flowering stage (i.e. at 60 days of sowing) and decreased as the plant aged. In early stages of the growth, an assimilation takes place with very less amount of dissimilation. However, after flowering stage whatever nutrients absorbed and synthesised by a plant, were utilised for the pod formation. Similarly, whatever nutrients available in nodules, get diverted towards the pod formation. Therefore, nodules get disintegrated as a plant goes to maturity and then they become black and non-functional. These results are in conformity with the results of Bhag Mal and Yadav (1972) ; Sahu (1973) ; Pawar and Khuspe (1976) ; Singh (1967) and Patil (1978) who also reported that in general, the total number of nodules increased even up to post flowering stage (pod formation stage) but decreased as the plant matures. In the present investigation, the maximum number of pink nodules (94 per cent) were observed in the variety PG-5 followed by N-31, PG-2, N-59 and chaffa. Similar results were also reported by Patil (1978) and Ghag (1981).

It has been well established by Kubo (1939), Virtanen et al. (1947) and number of other workers that functional root nodules contained a red pigment of haemoglobin nature named leghaemoglobin. With the aging of the nodules the leghaemoglobin gets irriversible converted to the black non-functional forms in all the varieties under study. These results are in conformity of Swaraj and Garg (1977), who stated that in cowpea, the presence of leghaemoglobin was more at the flowering stage which gave red colour to the nodules. After flowering it was converted to a non-functional form i.e. black form. Therefore, the ineffective nodule percentage at later age gets increased.

5.3 Leghaemoglobin and Nitrogen content :

The results regarding the presence of leghaemoglobin and nitrogen content in nodules presented in Table 2 show that at the initial stage i.e. at the 40 days observation stage, the presence of leghaemoglobin was less. This was attributed towards the less number of pink nodules and more number of white nodules which resulted into less nitrogen fixation.

At 60 day observation i.e. at the maximum flowering stage, pink nodules were more in number and the content of leghaemoglobin was also increased in all varieties. Besides, the nitrogen percentage was also increased during this stage.

When pod formation starts the leghaemoglobin content gets decreased, results into the reduction in the nitrogen fixation. These results confirm the findings of experiments of Rigaud and Puppo (1977). They reported that leghaemoglobin enhances the nitrogen fixation in soybean. They further stated that nitrogen fixation decreased with the decrease in leghaemoglobin content in pea. Similarly, Swaraj and Garg (1977) reported that a high amount of leghaemoglobin and nitrogen was present in nodules at flowering and decreased with maturity in cowpea. Devidson (1973) ; M@lik sarkisyan et al.(1976) started that nitrogen fixation is stimulated due to the presence of leghaemoglobin in lupin. Jain and R@wari (1975) also showed relationship between weight of nodules, their leghaemoglobin content and nitrogen content of soybean.

As the leghaemoglobin is a main factor for the nitrogen fixation, at flowering stage, it increased to its maximum since at this stage the maximum leghaemoglobin was present. It was also found that at this stage the maximum per cent of pink nodules in all the varieties was noticed after which nodules get disintegrated. As the carbohydrates content is reduced, the leghaemoglobin gets irriversible converted into black non-functional form which resulted into the reduction of nitrogen too.

The leghaemoglobin and nitrogen content of nodules was highly correlated in the variety PG-5. This variety also showed the maximum amount of leghaemoglobin at the flowering stage as compared to other cultivars. This was attributed towards the presence of high amount of nitrogen besides, this variety formed the maximum number of pink nodules at this stage. More or less similar results were recorded in remaining varieties.

5.4 Dry matter and Nitrogen content :

The results regarding dry matter weight and nitrogen content (Table 3) show that the nitrogen content of all the varieties was maximum at 60 days of crop age (flowering stage). So also the dry matter increase was found to be maximum. These results are in conformity of those reported by Bhide *et al.* (1961) who established a correlation between the dry matter weight of plants and their nitrogen content. Patil (1979) studied effects of commercial *Rhizobium* inoculants under different levels of nitrogen on nitrogen content of shoot and root dry matter of plants at various growth stages and found out a higher percentage of nitrogen in dry matter at maximum flowering stage. Ionescu (1958); Fedrov and Niste (1961); Patil (1978) and Ghag (1981) who observed that the nigrogen content of a plant was maximum at the flowering stage as compared to other stages of the growth in Vetch, pea and gram, respectively.

After the maximum flowering stage, the pod formation starts and nitrogen in a plant body started accmulating in pods instead of vegetative production. Hence, the nitrogen in a plant body declines to a very low levels at this stage. The variety PG-5 produced a maximum dry matter followed by N-31, PG-2, N-59 and *chaffa*. Similar results were reported by Deshmukh (1980); Patil (1980); Patil (1978) and Ghag (1981).

The nitrogen is meant for the growth and production of a plant. At the initial stage or early stage, nitrogen is utilised for the vegetative growth of a plant and gets saturated in a plant body itself. At a later stage it is utilised for the pod formation and gets concentrated in pods only. So also due to more vegetative growth at the initial stage carbohydrates synthesis was more. These carbohydrates were utilised by the bacteria which were present in nodules. Because of getting carbohydrate as a source of food in ample quantity, bacteria fix atmospheric nitrogen in higher quantities which create balance in nutrient status of a plant for its satisfactory growth and production. Hence, the rate of dry matter production increased up to the flowering stage after which the nitrogen is used for pod formation and irriversible change of leghaemoglobin in nodules create imbalance of nutrients in a plant body and decrease the rate of dry matter production proportionately.

5.5 <u>Nitrogen utilisation</u> :

The increased nitrogen content in the nodules of gram, was found to be increased from 40th day of sowing to the maximum flowering stage. Thus, the maximum nitrogen content in nodules was observed at the flowering stage and decreased thereafter. This decrease in nitrogen content of nodules at the advanced stages of a plant was attributed towards their utilisation for protein synthesis in the seed. These results are, therefore, in agreement with those reported by Ionescu (1978) ; Federou and Niste (1961) ; Linta (1963) ; Hari Shankar and Kushwaha (1971) ; Sahu (1973) ; Patil and Moniz (1974) ; Habish Ishag (1974) ; Singh and Saxena (1978) and Patil (1978) who also observed a differential response in different varieties of the crops they studied.

5.6 Nodules and Nitrogen :

A close relationship between the total number of nodules and nitrogen content of plant at 40 days, flowering stage, pod formation and maturity stage of the plant was observed. In other words, higher the number of nodules more was the nitrogen content of the plant at a respective stage. The variety PG-5 formed nodules of bigger size and pink colour as compared to other varieties in the present investigation. These results are, therefore, in conformity of those reported by Virtanen *et al.* (1947) ; Ionescu (1958) ; Linta (1963) ; Sahu (1973) and Khurana *et al.* (1978) who also reported a close and positive correlation between the total number of nodules and nitrogen content of a plant. It is further stated that the maximum nodules and nitrogen content were noticed in the variety PG-5 at 60 day i.e. at the maximum flowering stage, whereas it was least in the variety *chaffa*.

CHAPTER 6

S U M M A R Y

6. SUMMARY

The present investigation was undertaken to study the nodulation pattern, leghaemoglobin and nitrogen content and its correlation with the age of a plant in some promising varieties of gram.

Results reveal that more number of nodules were noticed in PG-5 variety at the 60 day growth stage followed by N-31, PG-2, N-59 and *chaffa*. At the initial stage of the growth effective nodules were more as compared to ineffective nodules. The maximum per cent of effective nodules was found in a variety PG-5 at the flowering stage, followed by N-31, PG-2, N-59 and *chaffa*. In the variety PG-5, a nodule size was also big as compared to other varieties at the flowering stage. At the initial stage, black nodules were less in all varieties but it was increased as a plant reaches to its maturity. It was further noted that the total dry weight of nodules per plant was also more in the variety PG-5 than other varieties at the flowering stage. After the flowering stage a maturity of grain starts and due to diversion of nutrients, nodules get disintegrated. Hence, a maximum nodule weight was found at the flowering stage in the variety PG-5.

As the presence of leghaemoglobin at the initial stage was less the nitrogen fixation was also less in all the varieties. At the flowering stage the leghaemoglobin in a nodule was more and hence the nitrogen fixation was also more. At the maturity stage due to the irriversible change of the leghaemoglobin its percentage was reduced and nitrogen fixation was also reduced at a very low level. In general, it was found that there is a close correlation between the leghaemoglobin content in nodules and nitrogen fixation by bacteria at all stages of the crop growth in all varieties of gram.

From the initial stage up to the flowering stage, the leghaemoglobin was increasing and it was in an active form due to which vegetative growth rate was higher than later stages. Due to more vegetative growth carbohydrate formation (synthesis) was more which creates balance in the nutritional ratio in a plant body for growth.

As leghaemoglobin content was more at the flowering stage the nitrogen fixation was also more of which very little amount of nitrogen was utilised for the growth of a plant. Therefore, more amount of nitrogen was present in a plant body before fruiting. After flowering a pod formation starts and nitrogen in a plant body gets accumulated in pods.

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