CHANGES IN THE RNA LEVEL IN RELATION TO PROTEIN ACCUMULATION IN DEVELOPING SEEDS OF BENGAL GRAM (*Cicer arietinum* L.)

by

Monika Garg

(L-93-BS-61-M)

Thesis

Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of

Master of Science

in

Biochemistry

(Minor : Biotechnology)

Department of Biochemistry
College of Basic Sciences and Humanities
PUNJAB AGRICULTURAL UNIVERSITY
LUDHIANA-141 004

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DEDICATED

TO

MY PARENTS

CERTIFICATE- I

This is to certify that this thesis entitled,

" Changes in the RNA level in relation to protein accumulation in developing seeds of Bengal gram (*Cicer arietinum* L.)"

submitted to the Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of **Master of Science**, in the subject of Biochemistry [Minor subject: Biotechnology], is a bonafide research work carried out by **Monika Garg** (L-93-BS-61-M) under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

MAJOR ADVISOR

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CERTIFICATE-II

This is to certify that the thesis entitled, **"Changes in the RNA level in relation to protein accumulation in developing seeds of Bengal gram (*Cicer arietinum* L.)"** submitted by **Monika Garg** (L-93-BS-61-M) to the Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of **Master of Science**, in the subject of **Biochemistry** [*Minor subject: Biotechnology*], has been approved by the student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

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Monika Garg

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ABSTRACT

Electrophoretic separation of protein fractions, isolation and estimation of total RNA and mRNA at various stages of seed development in eight genotypes of Bengal gram (*Cicer arietinum* L.) have been studied with a view to improve the seed protein quality. Studies on dry matter accumulation revealed maximum increase between 32 and 46 days after flowering (DAF). Differences in the protein content and total RNA content have been observed among the seeds of various genotypes. SDS-PAGE pattern of seed proteins revealed that bands corresponding to albumins appeared earlier than bands corresponding to globulin storage proteins. Maximum increase in total RNA content in Bengal gram seeds was observed between 32 and 39 DAF. This period preceded the period of maximum protein synthesis. Agarose gel electrophoretic pattern of total RNA showed two major bands. Isolation of mRNA by oligo-(dT) cellulose chromatography revealed that the content of mRNA and its percentage in terms of total RNA decreased with seed maturity. Among the Bengal gram genotypes GGC-47 was best with respect to protein content.

Monika Garg
gnature of student

Sukhdev Singh
Signature of Major Advisor

CONTENTS

CHAPTER-I

INTRODUCTION

Legume seed storage proteins are an important source of human dietary proteins. Large quantities of storage proteins are accumulated over a short period of time during seed development in legumes. Bengal gram (*Cicer arietinum* L.) is the fifth important legume of the world, in terms of total production, after soybean, peanut, common bean and pea. The crude protein content of Bengal gram ranges from 15-30% with one of the best nutritional qualities in legume family (Sotelo *et al.*, 1987; Fernandez & Berry, 1988; Jambunathan and Singh, 1989).

Legume storage proteins are mainly salt soluble and have been divided into two major groups: the legumin (11S) and vicilin (7S) fractions (Croy *et al.*, 1982). Legumin is a hexamer (M.wt 360 kD - 400 kD) consisting of six subunit pairs each of which comprises of a 40 kD acidic polypeptide linked by disulphide bonds to a 20 kD basic polypeptide. The 7S fraction (vicilin) is more complex in being composed of many classes of subunits of M. wt ranging from approximately 12 kD to 70 kD (Gatehouse *et al.*, 1981). Studies have shown that vicilin appears before legumin in cotyledons (Wright and

Boulter, 1972; Millerd *et al.*, 1978).

Seed proteins are encoded by several nonhomologus multigene families that vary in size, organisation and chromosomal locations (Casey *et al.*, 1986).

It has been observed that there is co-relation between the period of maximum storage protein synthesis in developing seeds and mRNA accumulation. The transcriptional activity is primary determinant of changing mRNA and protein population in seeds. Further it has been reported that mRNA levels are influenced by post-transcriptional processes and the translational efficiency may be important in determining the extent of storage protein accumulation in seeds (Krochko *et al.*, 1992).

Storage protein gene expression has been studied during cotyledon development by assaying specific transcripts produced by nuclei at different stages in developing pea seeds (Evans *et al.*, 1984). Vicilin transcripts predominated at 9 and 11 days after flowering (DAF) and were similar in amount of legumin at 14 DAF whereas at 18 DAF legumin transcripts predominated. Studies on pea legumin mRNA during seed development revealed that maximum amount of mRNA was found at 9 DAF for two classes of legumin mRNA and at 24 DAF for the third class (Domoney and Casey, 1987).

Studies on soybean seed protein mRNA revealed that these mRNAs are abundant during seed maturation stage of

embryogenesis. Collectively, these mRNA's represented 50 % of embryo - mRNA mass at the mid point of maturation (Goldberg *et al.*, 1981).

Although considerable literature is available on changes in protein during seed development in *Pisum sativum*, *Vicia faba*, *Glycine max* the studies on the storage proteins of Bengal gram are scanty . Therefore studies on Bengal gram storage proteins and RNA were taken up with the following objectives.

In order to accomplish alteration in genome activity information on mRNAs encoding the storage proteins of Bengal gram is required. This information will be useful to study the relationship between various mRNAs and protein fractions. It is expected that the results of this study will be useful for evolving better quality proteins through site directed mutagenensis, transformation techniques and marker based technology. Furthermore genes for vicilin and legumin storage proteins can also be cloned and manipulated.

The following studies on Bengal gram seeds at different stages of development have been carried out.

- Isolation and separation of different protein fractions.
- Isolation of total RNA.
- Separation of poly (A^+) RNA from total RNA by oligo-(dT) column chromatography.

CHAPTER-II

REVIEW OF LITERATURE

The literature on legume seed storage proteins and RNA (ribonucleic acid) has been reviewed under the following headings:

2.1 Seed protein fractions.

2.2 RNA- fractionation and isolation.

2.3 mRNA- general considerations

2.4 cDNA cloning in legumes.

2.5 Improvement of protein quality of legume seeds through biotechnololgy.

2.1 SEED PROTEIN FRACTIONS

Seeds are one of the richest sources of plant proteins. Large quantities of proteins accumulate during legume seed development. Research on storage proteins dates as early as 1745 when Beccari extracted glutelin from wheat flour (Brohult and Sandegren, 1954).

The proteins of legume seeds have been divided into two groups on the basis of their solubilities in water and other solutions (Osborne, 1924) and sedimentation co-efficient

(Danielsson, 1949). Globulins are salt soluble whereas albumins are soluble in water. Prolamine (alcohol soluble) and glutelins soluble in acid or alkali have also been found in cereal and legume grains.

Osborne (1924) extracted the globulins from pea seeds and separated them into two components vicilin and legumin. Danielsson (1949) investigated methods for the further purification of these two proteins and found that isoelectric precipitation gave preparations with least cross contaminations. He also showed that vicilin and legumin sediment at 8.1 S and 12.6 S and have molecular weight of 186 kD and 331 kD respectively.

Proteins showing characteristics of the legumin and vicilin proteins of pea have been isolated from a number of dicot seeds (Derbyshire *et al.*, 1976). These are 11S and 7S storage proteins. 11S proteins have M.wts in the range of 300 kD-400 kD. Holoprotein is composed of acidic and basic subunits with M.wts of 25 kD-37 kD and 20 kD-23 kD respectively. Estimated M.wts for the 7S proteins vary from 140 kD to 200 kD. These proteins are composed of several subunits but their composition is not well characterized. These proteins have high levels of amide amino acids, although they often have lesser amounts of methionine and cysteine.

2.1.1 Methods for Fractionation of Seed Proteins

Seed proteins are fractionated usually by solubility

method. Pant and Tulsiani (1969) fractionated proteins from defatted seed meal by successive extraction with water (for albumins), sodium chloride solution (5% w/v) (for globulins), ethanol (75% v/v) (for prolamines) and sodium hydroxide (0.25% w/v) (for glutelins).

Osborne and Campbell (1898) for the first time separated globulins into three fractions : legumin which is insoluble in dilute salt solution but did not coagulate when heated to 95°C; vicilin which is soluble in dilute salt solution but coagulates when heated at 95°C and legumelin which had properties of both albumins and globulins and precipitated partially after a reduction in the salt concentration.

Danielsson (1949) failed to confirm the existence of legumelin and found that most effective method of separation of legumin and vicilin was isoelectric precipitation of the higher M.wt globulin (legumin) by adjusting the pH to 4.5-5.0.

Zonal isoelectric precipitation fractionation of legumins was carried out by Wright and Boulter in (1974). Globulins were dissolved in 0.2M NaCl, 0.05 M- $Na_2PO_4^-$ (pH 8.0) and applied to a column of sephadex G-50 equilibrated with 0.2M NaCl-0.05M citric acid (pH 4.7). Elution of column was performed with citrate buffer. In addition ion exchange chromatography was used for further purification.

Singh *et al*. (1981) extracted salt-soluble proteins with 0.5M NaCl in 0.01M Pi buffer (pH 7.0) by shaking in a centrifuge tube for 1 hr followed by centrifugation.

Croy *et al*. (1984) used 20 mM acetate buffer (pH 5.0) for extraction of albumin proteins followed by precipitation with $(NH_4)_2 SO_4$ at 0°C. After dissolving the precipitate in 50 mM Tris-HCl buffer pH 7.5, was dialysed against water. Albumin fraction was purified on Sephadex G-150 followed by DE-52 cellulose column chromatography. For extraction of globulins 50 mM sodium borate buffer (pH 8.0) was used. Globulins were precipitated on dialysis with 20 mM acetate buffer pH 5.0.

Gueguen *et al*. (1984) developed ion-exchange chromatography for the major proteins of pea viz. legumin and vicilin. This method was adopted to produce immunologically pure glycinin, β-conglycinin and α-conglycinin of soybean.

Singh *et al*. (1988) used isoelectric focusing method for isolation of legumin and vicilin fractions in Bengal gram. Isolated globulins were dissolved in 0.1N borate buffer (pH 8.3) containing 0.2 M sodium chloride and dialysed them against 25 mM sodium citrate buffer (pH 4.6) for precipitation of legumins.

Protein isolates were prepared by micellization and isoelectric precipitation by Paredes-Lo'pez *et al*. (1991). For the micellization procedure, NaCl (0.5 M, pH 7.0) was used to

extract proteins from defatted flour. After concentrating it to half the volume it was flocculated with water at pH 7.0 for micellization. For isoelectric precipitation proteins were extracted with alkali(0.1N NaOH)at pH 8.5 and precipitated by adding acid (0.1 NHCl) to pH 4.5.

Wolf *et al*. (1992) obtained small quantities of highly purified proteins from soybean by efficient techniques like electrophoresis, sedimentation gradient and HPLC.

Mandaokar *et al*. (1993) isolated vicilins from the globulins by extraction with 0.2M NaCl in McIlvaine buffer pH 4.7 (0.096 M Na_2HPO_4, 0.052 M citric acid) and precipitation by dialysis with cold distilled water. Vicilins were purified by zonal isoelectric precipitation using Sephadex G-50 column equilibrated with McIlvaine buffer pH 7.0. Further purification was achieved by ion-exchange chromatography on DEAE - cellulose column equilibrated with 50 mM Tris - HCl buffer (pH 8.0).

Dreau *et al*. (1994) gave the procedure for semi-quantitative purification of the soybean proteins i.e. glycinin, β-conglycinin and α-conglycinin. In the first step fractions were purified by successive precipitation of respective proteins at an appropriate isoelectric point and/or ionic strength. Then glycinin, β-conglycinin and α-conglycinin fractions were isolated by gel filtration.

2.1.2 Proteins of Bengal gram (*Cicer arietinum* L.)

The crude protein content of Bengal gram ranges from 15-30% with one of the best nutritional qualities in the legume family (Sotelo *et al*.,1987, Fernandez and Berry, 1988). Mandaokar *et al*. (1993) has emphasized that considerable literature is available on the changes in protein during seed development in *Pisum sativum, Vicia faba and Glycine max* however, information on storage proteins of Bengal gram is scanty.

Jackson *et al*. (1969) made a comparison of the globulins, vicilin and legumin prepared by isoelectric precipitation of seeds of *Pisum sativum, Vicia faba and Cicer arietinum*. The aminoacid composition, band pattern after electrophoresis upon polyacrylamide urea gels and N-terminal amino-acids were compared. The homologous proteins of the three species resembled each other more closely than do the vicilin and legumin of any one species.

Siddiqui (1984) studied chromatographic separation and physico-chemical properties of vicilin from Bengal gram seeds. Its sedimentation co-efficient was 6.72 S. Vicilin was rich in isoleucine and poor in arginine. Similar studies were performed on legumin by Siddiqui (1985). The sedimentation coefficient was 11.72 S. Legumin was rich in arginine and tyrosine and poor in histidine and threonine.

El-Sayed *et al*. (1986) isolated a homogeneous globulin from Bengal gram by ion exchange and gel permeation chromatography. It has a M.wt of 330 kD and PI of 5.8. Three subunits (20 kD, 32 kD and 56 kD M.wts) were identified by SDS-PAGE.

Singh *et al*. (1988) found in Bengal gram that seed protein content was 19.8%, with globulin constituting 62.4 % of the total seed proteins. The ratios of albumin to globulin and legumin to vicilin were 1:4 and 6:1 respectively. The proportion of basic amino acids in the albumins was low whereas it was high in globulins. The legumin fraction was superior in terms of total amino acids to those of other sources. Sulphur containing amino acids were most limiting followed by tryptophan or threonine depending on the fraction. However, the ratio of methionine to cysteine was high (2.76:1).

Paredes-Lo'pez *et al*. (1991) isolated proteins from Bengal gram flour by micellization and isoelectric precipitation techniques. Protein content in the isolates ranged from 84.8-87.8%. SDS-PAGE showed M.wt distribution between 16.6-66.4 kD for micelle and 14.9 - 84.2 kD for isoelectric proteins.

Solomko *et al*. (1991) demonstrated that protein concentrates from Bengal gram contained 14.1% total N and 88.1% crude protein.

Dhawan *et al*. (1991) analysed six Bengal gram strains for their protein content and various protein fractions. Protein content was 20.9-25.27%. Albumin, globulin, prolamine and glutelin contents were 8.39-12.31%, 53.44-60.29%, 3.12-6.84% and 19.38-24.4% respectively. Salt soluble proteins resolved into 19-23 bands where as albumin proteins into 30-34 bands on electrophoresis. The M.wt of various polypeptides were 10-91 kD.

Mandaokar *et al*. (1993) purified and characterised vicilin, one of the major storage proteins of Bengal gram during seed development. Vicilin on SDS-PAGE resolved into 5 major bands ranging in M.wt from 14 to 66 kD. More heterogenous pattern emerged on isoelectric focusing.

Dhankher and Matta (1992) studied effect of salinity on storage protein of Bengal gram seeds . Bengal gram lines grown on saline soil showed reduction in the seed protein content but four protein fractions were affected differently. Level of albumin and glutelins decreased whereas globulins exhibited an increase under saline conditions. SDS-PAGE did not show any qualitative change in polypeptides but densitometric scanning showed an increase in relative concentration of certain vicilin-like polypeptides and a decrease in legumin-like polypeptides.

2.1.3 Proteins of Pea (*Pisum sativum*)

Jackson *et al*. (1969) studied the amino acid

composition and sequence of globulins of *Pisum sativum* and found that amino acid sequence of vicilin and legumin were probably the same or very similar.

Beevers and Poulson (1972) studied the changes in the protein content of pea cotyledons from 9 to 33 days after flowering. The protein content increased gradually with a rapid period of deposition occurring between 21 and 27 days after flowering. Albumins were synthesised early in cotyledon development whereas globulin synthesis predominated with advancing maturity.

Scharpe and Parijs (1973) demonstrated in *Pisum sativum* during seed development that RNA increase between 9 and 45 days after flowering (DAF) was accompanied by synthesis of proteins between 18 and 30 DAF. The amount of protein per cell increased about 350 times from the first isolated stage to the full grown cotyledons.

Legumin and vicilin of pea seeds were serologically distinct proteins that accounted for approximately 90% of the proteins in the protein body (Thompson *et al.*,1978) Legumin has a sedimentation co-efficient of 12-13S and M.wts of 330 kD-400 kD whereas vicilin sediments at 7S and has a M.wt of 186 kD (Derbyshire *et al.* (1976). Millred and Spencer (1974) demonstrated that during development; vicilin was detectable in the cotyledons earlier than legumin.

Thompson and Schroeder (1978) demonstrated that as many as four distinct forms of legumin could be separated by agarose gel or cellulose acetate electrophoresis. These proteins were composed mainly of 20 kD and 40 kD M.wt subunits with variable amount of 18 kD, 25 kD, 27 kD and 37 kD M.wt subunits.

Thompson *et al.* (1978) isolated two or three vicilins that differed quantitatively and qualitatively in subunit composition. Each of these forms included one or more major polypeptides with a M.wt near 18 kD, 30 kD, 50 kD or 75 kD and minor amounts of 12 kD, 14 kD and 24 kD M.wt polypeptides.

It was demonstrated that a native protein containing only subunits of M.wt approximately 70 kD was separable from other 7S protein molecules under non-dissociating conditions (Casey and Sanger 1980; Croy *et al.*,1980) and this protein was named convicilin. Amino acid composition for convicilin and vicilin appear to be quite similar (Casey and Sanger,1980) although the glutamic acid, aspartic acid ratio is higher for convicilin than for vicilin. Former contained small amount of sulphur containing amino acids in contrast to vicilin which contained non (Croy et al.,1980).

Croy *et al.* (1984) isolated two closely related albumin proteins. The large protein had M.wt - 53 kD and consisted of two 25 kD M.wt subunits whereas the smaller had

M.wt 48 kD and contained two 24 kD M.wt subunits. Both proteins contained significant amounts of sulphur containing amino acids.

SDS-PAGE of pea seed protein extract showed synthesis of major legumin and vicilin polypeptides from 30 DAF onwards though very faint bands could be seen at early stage of 10 DAF (Chandna and Matta, 1994). In the germinating seeds legumin polypeptides (M.wt 66-75, 41-55 kD) and vicilin polypeptides (M.wt 54 kD) were degraded increasingly from 2nd to 6th day of seed germination.

Wenzel *et al.*(1995) demonstrated that protein bodies from pea cotyledons contained proteins tightly bound to the membrane with subunit M.wt of 26 kD/27 kD and 82 kD. The double band of M.wt 26 kD/27 kD being clearly distinct from the lectin and its precursors as judged by solubility studies.

2.1.4 Proteins of Soybean (*Glycine max*)

Analysis of soybean seed proteins (Catsimpoolas and Ekenstam, 1969) by ultracentrifugation revealed three major components of 2,7 and 11S. The 2S fraction α-conglycinin is heterogenous and contain several enzymes as well as trypsin inhibition activity. The 7S fractions β and γ-conglycinin contain a group of heterogenous storage proteins. The 11S fraction contains major storage protein glycinin.

Accumulation of different protein fractions during seed development was studied by Hill and Briedenbach (1974

a,b). These investigators found that the 2S protein predominated during early stages of development but their proportion decreased later when the 7S and 11S protein were synthesized.

Wolf and Briggs (1959) and Badley *et al*. (1975) reported that glycinin protein had a sedimentation co-efficient of 12.2-12.3S and M.wt between 300 kD-360 kD with isoelectric point of 6.4 and was formed by association of 6 acidic and 6 basic polypeptides.

Studies by Catsimpoolas and co-workers (1967, 1971) indicated that the glycinin protein consist of six nonidentical subunits. Three of these have basic isoelectric points and M.wt of 22 kD and three have acidic isoelectric points and M.wt of 37 kD.

Kitamura *et al*. (1976) showed that glycinin could be separated into 4 acidic and 4 basic subunits using alkaline and acid urea gel electrophoresis of reduced and alkylated glycinin. Basic subunits (Bx) had M.wt of 22.5 kD. Subunits B_1, B_2, B_3, B_4 were present in molar ratio of 1:1:2:2. Three of acidic subunits A_1, A_2, A_3 had M.wt of 37 kD each but the fourth A_4 had a M.wt of 45 kD and were present in molar ratio of 1:1:2:2.

Medeiros (1985) found that β-conglycinin precipitates at a pH of 4.8 and it has a polymeric structure. It contains two subunits α (72 kD) and β (52 kD).

A third class of globulin sedimenting at 8.2S has been characterized in soybean (Hu and Esen, 1982). This relatively minor component accounts for 5 to 10% of the seed protein. This globulin is tetramer of M.wt 168 kD composed of four identical subunits (M.wt 42 kD) and in contrast to 7S and 11S globulins was basic with PI 9.5 (Shotwell and Larkins, 1989). Each subunit was composed of a large polypeptide M.wt 30 kD disulphide bonded to a small polypeptide M.wt 16 kD. Large polypeptide was basic and small polypeptide acidic. The 8S globulin had higher levels of methionine and cysteine than 7S and 11S protein of soybean but lower in acidic amino acid accounting for its higher isoelectric point.

Shotwell and Larkins (1989) concluded that glycinin isolated from soybean was a hexameric complex of M.wt 360 kD and was composed of six nonidentical subunits with M.wt averaging 60 kD. The glycinin hexamer was assembled from five different subunit molecules that fall into two classes: (1) The group I glycinins (M.wt 58 kD) which had five to eight methionine residues and consisted of two members and (2) The group II glycinins (M.wt 62 kD to 69 kD) which had three methionine residues and consisted of two members.

β-conglycinin generally isolated as a trimer of M.wt 180 kD. It is made up of three major subunits of α, α' and β of M.wt 76 kD, 72 kD and 53 kD (Meinke *et al.*,1981) as well as several minor components. In contrast to glycinin these

subunits contain a single polypeptides (Shotwell and Larkins,1989) and β-conglycinin is glycosylated with about 5% sugar by weight. Koshiyama *et al.* (1981) found that α-conglycinin was a monomeric protein of 20.1 kD with PI of 4.5.

Wolf *et al.* (1992) obtained small quantities of highly purified soybean proteins by efficient techniques like electrophoresis, sedimentation gradient and HPLC.

Dreau *et al.* (1994) used isoelectric precipitation followed by gel filtration for purification of three soybean proteins- glycinin, β- conglycinin and α conglycinin. Using this procedure it was possible to prepare about 2g of purified glycinin (91%) and 1g each of β-conglycinin (100%) and α conglycinin (96%) from 1 kg of defatted soybean flour. Only β-conglycinin seemed to be highly pure as assessed by SDS-PAGE and immunoblotting.

2.1.5 Proteins of French Bean (*Phaseolus vulgaris*) and related species

Danielsson (1949) showed that an 8S globulin was the major storage protein in *P.vulgaris*. This protein was later purified by Pustazi and Watt (1970) who examined some of its association- dissociation characteristics. At neutral, alkaline or very acidic pH, the protein existed as a monomer with a M.wt of 140 kD. Between pH 3.4 and 6.6 it formed a tetramer with a M.wt of 560 kD. The protein was found to

contain mannose and glucosamine and was therefore designated glycoprotein II.

Ericson and Chrispeels (1973) characterised glucosamine containing storage proteins from *P. aureus* and showed them to be identical with legumin and vicilin, the major storage proteins of the leguminosae. Vicilin had a sedimentation constant of 8S and legumin had a sedimentation constant of 11S and were made up of four and three non-identical subunits respectively.

Derbyshire and Boulter (1976) isolated an 11S seed globulin from *Phaseolus aureus* and *P. vulgaris* by zonal isoelectric precipitation and determined the M.wts of their constituent subunits. Derbyshire *et al*. (1976) summarised the structural features of 11S and 7S proteins from a number of dicot seeds and concluded that Phaseolus seeds were an exception in that the 7S protein is major storage protein in this species.

Mcleester *et al*. (1973) and Sun and Hall (1975) separated *Phaseolus* storage globulins into two fractions G_1 and G_2 using an acidic buffer.

Soni *et al*. (1975) fractionated green gram proteins into albumins, globulins, prolamines and glutelins and compared their amino acid composition.

Hall et al. (1977) found that *Phaseolus* seeds contained 20 to 30% protein and on the average 60% of the

protein was globulin. Actual amount of G_1 and G_2 in different strains were variable and there were qualitative differences in subunit composition. There were at least two forms of the largest subunit of the G_1 protein having M.wt of 50.5 kD and 53 kD. The synthesis of either of these is controlled at a single gene locus.

Hall *et al*. (1978) also summarized the properties of G_1- the major component was composed of three subunits of M.wts of 43 kD, 47 kD and 53 kD. Each of the subunits of G_1 was glycosylated and the protein was analogous to glycoprotein II of Pustazi and Watt (1970). G_2 contained major polypeptides of 30 kD, 32 kD and 34 kD M.wt. The ratio of G_2 to G_1 was about 1:6.

Sun *et al*. (1978) analysed accumulation of G_1 and G_2 during seed development by SDS-PAGE and rocket gel immuno—electrophoresis. By analysis of total seed proteins on SDS gels it was possible to detect both G_1 and G_2 in 9 mm seeds. During the 12 day period when the seed grew from 12 to 19 mm the G_1 content increased from 0.5 to 21.5 mg per cotyledon pair. The ratio of the G_1 subunits remained unchanged during development, indicating that it was a single protein. However, there was variation in the G_2 subunits consistent with the heterogeneity of this fraction.

Duranti *et al*. (1989) analysed the seed storage proteins of Phaseolus cultivars using PAGE, SDS-PAGE,

isoelectric focusing and erythroagglutination techniques. Major storage proteins legumin and vicilin had the qualitative and quantitative differences.

2.2 RNA - ISOLATION AND FRACTIONATION

2.2.1 RNA Isolation:

Kirby (1964) developed an extraction procedure for RNA using equal volume of two immiscible liquids one aqueous and other organic. For extraction by dissociating proteins from the RNA and to inhibit nuclease activity, detergents such as SDS (Sodium dodecyl sulphate), sodium amino salicylate, sodium tri-isopropylnaphthalene sulphonate, sodium naphthalene 1, 5 disulphonate either alone or in combination were usually dissolved in aqueous phase.

Eaton and Faulkner (1972) gave method for better extraction of poly A containing mRNA and also to ensure partitioning of this RNA into the aqueous phase. The detergent solution is buffered with 10-50 mM Tris-HCl pH 8.5-9.0. The other phase consists of buffer saturated phenol containing 10% v/v m-cresol and 1% w/v 8- hydroxyquinoline or alternatively a phenol-chloroform mixture.

Beintema *et al*. (1973) found that preparation of undegraded ribonucleic acid from a number of cell types was hindered by presence of active nucleases. An extreme example of this is the rat pancreas which contains 200 μg of ribonuclease-A per gm of tissue wet weight.

Scharpe and Parijs (1973) isolated RNA from ripening cotyledons of *Pisum sativum* L. and studied the changes in the RNA level during the seed development.

Glisin *et al*. (1974) first used a solution of CsCl for removing contaminant from isolated RNA. They showed that CsCl centrifugation method was very efficient for isolating RNA free of proteins, DNA, polysacchride, and other cellular components.

Chirgwin *et al*. (1979) isolated intact RNA from rat pancreas by efficient homogenization in a 4 M solution of the potent protein denaturant guanidinium thiocyanate and 0.1M 2-mercaptoethanol to break protein disulphide bonds. The RNA was isolated free from protein by ethanol precipitation or by sedimentation through cesium chloride. Sambrook *et al*. (1989b) have described methods in detail for isolation of RNA from different tissues.

Nair and Koundal (1993) isolated RNA from the polysomal preparation by increasing detergent (SDS) and salt concentration. It was followed by deproteinization by phenol and then reextraction with equal volume Chloroform: isoamylalcohol (24:1) and precipitation with ethanol. Their studies on chickpea developmental stages revealed that total RNA yield was higher in the early stages of development and declined at the later stages.

2.2.2 RNA Fractionation

Ingle *et al*. (1965) performed sucrose density gradient centrifugation of RNA from soybean (*Glycine max*). Centrifugation was for 16 hours at 23,000 revolution min^{-1} in 20 - 95% sucrose gradient. Three fractions of 26S, 18S, 5S + tRNA were obtained.

Loening (1967) resolved high M.wt RNA on gel electrophoresis. The method offered much greater resolution than was previously possible. This method enabled Loening and Ingle (1967) to distinguish the rRNA from chloroplast and cytoplasmic ribosomes which differ slightly in molecular weight. It was later established by Loening (1969) that, for many RNA molecules the distance moved during electrophoresis is inversely proportional to the log of the M.wt.

Sobota *et al*. (1968) fractionated soybean nucleic acids by chromatography on columns of methylated albumin-kieselguhr (MAK). Nucleic acids were eluted in a linear gradient of 0.45-1.35 M KCl in potassium phosphate buffer pH 6.7. Fractions obtained were tRNA, 5SrRNA, DNA, 18S and 26S rRNA.

Vanderhoef and Key (1970) performed reverse phase chromatography of tRNA from roots of pea (*Pisum sativum*) seedlings. Separated samples of tRNA were charged with different radioactive amino acids. The samples were fractionated by reverse phase chromatography and the charged tRNAs were detected by measuring the amount of each of the

amino acid used.

Guderian *et al*. (1972) fractionated leucyl.tRNA from chloroplast, mitochondria and cytoplasm of tobacco (*Nicotiana tobaccum*) by reverse phase chromatography. Mixture for total cellular leucyl-tRNA was labelled with ^{3}H-leucine and mitochondrial and chloroplast leucyl-tRNA were labelled with ^{14}C -leucine. It was found that six leucyl - tRNA can be detected in tobacco leaves, two are present in the mitochondria and two others are confined to the chloroplast.

2.3 mRNA- GENERAL CONSIDERATIONS

2.3.1 mRNA Isolation

The fact that most of eukaryotic RNAs bear a poly (A^{+}), tail at their 3'terminal (Hadjivassiliou and Brawerman, 1966; Brawerman *et al*., 1972; Brawerman, 1974) led to the development of affinity chromatography isolation methods based on the pairing of the poly A^{+} sequence with poly U or oligo-dT bound to sepharose or cellulose.

Higgins *et al*. (1973) purified poly [A] containing RNA from polysomes isolated from leaves of mungbean (*Phaseolus aureus*). Gel electrophoresis of poly (A) containing RNA revealed that this RNA molecule of 1500 nucleotides, sufficient to code for 450 amino acids has a M.wt of 0.45 x 10^{6}.

Verma *et al*. (1974) studied sedimentation characteristics and coding properties of poly (A) containing

RNA isolated from polysomes of soybean (*Glycine max.*) root nodules. It contains polydisperse RNA but two distinct peaks sedimenting at 9S and 12S are also observed. When these RNA fractions were supplied to an *in vitro* protein synthesizing machinery they stimulated the incorporation of amino acids into haemoglobin like protein.

Grierson *et al.* (1975) isolated poly (A) containing RNA using oligo-dT cellulose column and fractionated by gel electrophoresis.

Larkins *et al.* (1976) and Burr *et al.* (1978) purified Zein mRNA and bean globulin mRNA to the level approaching homogeneity.

Poly (A^+) RNA which can direct *in vitro* biosynthesis of polypeptides in cell- free translation system has already been isolated from cotyledons of seeds of *Glycine max* L. (Beachy *et al.*, 1978) *Pisum sativum L.* (Evans *et al.*, 1979) Phaseolus vulgaris L. (Hall *et al.*, 1978) and *Vicia faba L.* var minor (Puchel *et al.*, 1979).

Roger *et al.* (1980) isolated mRNA from immature soybean seeds that bound to oligo-dT cellulose. This mRNA was fractionated by centrifugation in sucrose density gradient containing dimethyl sulfoxide. mRNAs with sedimentation values between 21S and 25S coded for the *in vitro* translation of polypeptides with electrophoretic mobilities similar to α' and α subunit of 7S seed storage protein.

Bi *et al.* (1989) isolated total RNA from the immature Beijing wild soybean seeds by LiCl precipitation followed by purification of total mRNA by Oligo-dT cellulose affinity chromatography. The mRNA thus prepared showed enough translation activity when assayed in the rabbit reticulocyte cell-free system *in vitro*.

Yadav *et al.* (1990) isolated free cytoplasmic ribonucleo-protein (RNP) and poly (A^+) ribonucleo-protein complex from post polysomal supernatant of high lysine barely mutant notch-2 embryo using sucrose density gradient and affinity chromatography on oligo-dT cellulose. The proteins of mRNP-1 and mRNP-2 were analysed by SDS-PAGE. Two major polypeptides of M.wt 41 kD, 22 kD, and a few minor proteins of M.wt 64 kD and 84 kD were found to be associated with poly (A^+) RNA in both mRNP-1 and mRNP-2.

2.3.2 mRNA Accumulation

Puchel *et al.* (1979) showed in cotyledon cells of developing field beans (*Vicia faba L.*) the RNA content per cell did not change in the elongation phase whereas globulin biosynthesis continued. This results from equilibrium between RNA synthesis and degradation. All types of RNA are synthesized until the end of globulin biosynthesis but poly (A) containing RNA was preferentially labelled during maximum globulin formation.

Evans *et al.* (1984) found in developing pea seeds that vicilin transcripts predominated at 9 and 11 DAF and were similar to amount of legumin transcripts at 14 DAF whereas at 18 DAF legumin transcripts predominated and little vicilin transcripts were observed. The storage protein synthesis correlated with previously determined mRNA level during seed development.

Domoney and Casey (1987) studied the pattern of accumulation of 3 classes of legumin mRNA from *Pisum sativum* during seed development by cell free translation and hybridization to complementary DNA. Maximum amount of mRNA was found at 19 DAF for 2 classes and 24 DAF for the third class. Domoney and Casey (1990) described novel class of vicilin gene in *Pisum* corresponding to a precursor polypeptide of M.wt 88 kD. The mRNA corresponding to this polypeptide accumulates during seed development in a pattern similar to that in convicilin.

Changes in the contents and components of DNA and RNA during senescence of mung bean cotyledons were determined and mRNA was separated from total RNA by oligo-dT cellulose column (Wen *and Liang*., 1991). Results showed that contents of both DNA and RNA decreased during senescence of mung bean cotyledons.

Nair and Koundal (1993) studied the changes in polyribosomes and RNA in developing seeds of chickpea.

Northern hybridization of heterogenous probe (Pea legumin cDNA) with mRNA coding for legumin protein at different stages of seed development was also studied. The hybridization results indicated that legumin mRNA was hardly present at initial stages (9 and 12 DAF). Legumin mRNA begin to accumulate during later stages of seed development and reaching the maximum at 18 DAF, maintain without decline for several days upto 33 DAF and thereafter showing a decline with the onset of maturity.

2.3.3 mRNA Abundance

For characterization of abundance distribution and sequence diversity of RNA population RNA/cDNA hybridization analysis is a powerful tool (Galau and Dure, 1981; Chaudhari and Hahn, 1983).

In general, plant mRNA/cDNA hybridization reaction can be resolved into three to four kinetic components representing as many as 30,000 diverse mRNA. By convention these mRNA sequences are grouped into three abundant classifications according to their cytoplasmic concentrations and sequence diversity - superabundant, abundant and rare class mRNA. These terms conceptually simplify the complex architecture of an mRNA population. In reality a typical eukaryotic mRNA population contains an almost continuous spectrum of mRNA species with unique cellular concentrations (Flytzanis *et al.*, 1982).

Superabundant mRNAs are unique to highly specialized cell types that must produce large amounts of specific polypeptides. Gene encoding superabundant mRNAs in plants include the small subunits of ribulose bisphosphate carboxylase, seed storage proteins (Goldberg *et al.*, 1981) and leg-haemoglobin. The corresponding mRNAs all represented by 5000 molecules per cell or more and are so abundant that they can be visualised as distinct bands on an ethidium-bromide stained agarose gel (Okamuro and Goldberg, 1989).

The second most prevalent class of mRNA sequence in the cell are abundant class mRNAs. They constitute 50-75% of the mRNA mass. There are nearly 200-2000 different abundant class gene sequences in the cell represented by approximately 500-2500 mRNA molecules per cell (Okumuro & Goldberg, 1989).

The last sequence to hybridize in an RNA/cDNA hybridization reaction represents rare class transcripts or the lowest detectable level gene expression in the cell. Unlike superabundant and abundant class mRNAs, rare class transcripts cannot be visualized as bands on EtBr-stained agarose gels or be detected by radio-actively labelling *in vitro* translation products on two dimensional acrylamide gels (Dure *et al.*, 1981).

2.3.4 mRNA Stability

mRNA degradation vary widely among different mRNAs. Mammalian C-fos proto-oncogene mRNA has a half life of only 8

to 30 minute. (Kruijer *et al*., 1984). In oat plants the mRNA encoding the photoreceptor phytochrome has a short half life of about 1 hour, which may be an important factor in light regulation of development (Seeley *et al*., 1992). In contrast, β-globulin mRNA in erythroid cells has a half life of greater than 24 hours which contributes to sustained and high level synthesis of the encoded protein (Ross and Pizarro, 1983).

Walling *et al*. (1986) investigated soybean seed protein gene transcription during development and concluded that both transcriptional and post-transcriptional processes regulate seed protein mRNA levels in the absence of detectable DNA methylation changes.

PABP (Poly A$^+$ binding protein) also act as determinants of mRNA stability (Baer and Korenberg, 1983). Changes in the stability might be brought out by alternation in the folding of region of mRNA near the junction of poly (A$^+$) RNA with rest of mRNA in response to specific signals.

Shyu *et al*. (1989) demonstrated for unstable mRNAs such as C-myc and C-fos mRNAs, portions of the coding regions and AU rich sequences in the 3′ untranslated region had been identified as two mRNA components conferring instability.

Thompson *et al*. (1992) found that process of rbcs mRNA degradation involves loss of poly (A$^-$) tail and discrete RNA cleavage intermediates in soybean and petunia.

Green (1993) identified 2 types of sequence elements that can substantially accelerate the decay of reporter gene transcripts in tobacco. These are repeats of the motif AUUUA and the DST sequences.

Zhang *et al*. (1993) found in bean cells treated with fungal elicitor, the transcripts of PVRRP, a gene encoding proline rich protein, decreases with time. The rapid decrease in the PRP_1, mRNA level in elicited cells is due to destabilization which is dependent on new RNA and protein synthesis.

2.4 cDNA CLONING IN LEGUMES

The structural organisation and regulation of gene expression information, is often necessary to isolate or synthesize a segment of gene as a probe for hybridization analysis. To generate specific DNA probes, cloning of double stranded cDNA is one of the approaches available. Reverse transcriptase from avian myeloblastosis virus is the most widely used enzyme (Efstratiadis and Villa-komaroff, 1979) for the synthesis of single stranded cDNA (sscDNA).

Domoney and Casey (1983) isolated a cDNA clone for convicilin a major storage protein, from a cDNA library prepared in the plasmid vector PAt 153, using poly (A^+) RNA from developing seeds of *Pisum sativum L*. The clone was identified by hybrid selection with poly (A^+) RNA, translation of selected products by antibody raised against purified

convicilin subunits. The size of mRNA coding for convicilin polypeptide has been established using this convicilin cDNA clone and was found to be appreciably larger than the mRNA coding for polypeptides of vicilin.

A cDNA was isolated that codes for α-amylase in mungbean (*Vigna radiata L.*) cotyledons, and the nucleotide sequence was determined (Koizuka *et al.*, 1990). Northern blot analysis showed the levels of α-amylase mRNA in parallel with the activity of α-amylase synthesis in cotyledons. Under the conditions where the solute leakage from cotyledons is accelerated during imbibition, a rapid increase in the amount of the α-amylase mRNA occurs.

Sharma *et al.* (1992) isolated some cDNA clones which span the full length of transcript (PV PR_3) encoding a novel pathogenesis related (PR) protein in *Phaseolus vulgaris.*

Ng *et al.* (1993) constructed a full length cDNA for canavalin by polymerase chain reaction. The nucleotide sequence coding for canavalin and the corresponding amino acid sequences were determined and shown to be homologous with those of other seed storage proteins. A recombinant protein with M.wt 47 kD was expressed and purified to 95% homogeneity. This protein exhibited the same physical, immunological and biochemical properties as native jack been canavalin.

Turley *et al.* (1994) isolated and characterized cDNA

encoding ribosomal protein L41 from cotton (*Gossypium hirusutum L.*). L41 cDNA is 449 bp structure and has a 5′ (50 bp) and 3′ (324 bp) including an 186 bp poly (A^-) tail noncoding region, along with a 75 bp open reading frame. Two overlapping sequences (residues 6-22 and 7-23) that meet the criteria of bipartite nuclear targeting sequences were identified in the deduced amino acid sequence of L-41 (Dingwell and Lasky, 1991). Three other cDNA clones coding ribosomal proteins from cotton i.e. S40, S16 and L44 had been characterized and only one (S16) lack the bipartite nuclear targeting sequence.

Watanable and Hirano (1994) determined the nucleotide sequences of the basic 7S globulin gene from soybean. cDNA encoding the basic glycoprotein named Bg had been cloned and sequenced by Kagawa and Hirano (1989). Hybridization analysis suggest that the Bg gene is present in atleast four copies per genome. Structural similarities suggest that Bg may have an insulin receptor like function.

cDNA library form cotyledon poly (A^-) RNA of wild soybean was constructed and screened with the insert of genomic clones λS312 which contains the coding sequence for the glycinin subunit precursor – $A_5A_4B_3$ (Xue *et al.*,1986) as a probe. Among the six clones isolated pWS228 and pWS242, with the longest inserts (1.8 kbp) were further analysed (Weng *et al.*, 1995). Based on sequencing analysis it was shown that

pWS228 encodes $A_5A_4B_3$ precursor and pWS242 encodes A_3B_4 precursor. Nucleotide sequence of pWS242 contains a 1551 bp open reading frame coding for 517 amino acid residues and a 40 bp 5′ end non-translated sequence and a 179 bp 3′ end non-translated region that contains two characteristic polyadenylation signals (AAUAAA) at the nucleotide positions 1669 and 1753 followed by a poly ($A^{\cdot}$) tail.

Chui and Falco (1995) identified and characterized maize gene encoding a new methionine rich zein that is distinct from but closely related to 10 kD zein gene from maize genomic DNA library. The DNA fragment sequenced is 2123 bp and contains open reading frame of 633 nucleotides. The high sulphur zein codes for a protein that is the highest in methionine content (about 30% by weight methionine and 40% total sulphur amino acid) of any currently known.

2.5 IMPROVEMENT OF PROTEIN QUALITY OF LEGUME SEEDS THROUGH BIOTECHNOLOGY

The improvement of amino acid profile is one of the important objectives in legume improvement. The biotechnological strategies for such an improvement may be:

(i) to increase the quantity of specific amino acid/acids present within a protein

(ii) to develop the lines which overproduce specific amino acids by selecting against amino acid analogue.

(iii) to modify the expression of existing genes for preferential synthesis of proteins which are relatively rich in deficient amino acids by changing the subunit composition. (Asthana *et al.*, 1994).

Site directed mutagenesis for studying the structure function relationship in proteins have given good results. [Ulmer (1983), Estelle *et al.*(1985a), Estelle *et al.* (1985b)]. Gene mutations permit virtually any modification in the amino acid sequence of the corresponding proteins, which might result in modified molecules with increased or novel biological activity.

Recombinant DNA technology is being utilized to transfer storage protein genes to other plants using Ti-plasmid. Murai *et al.* (1983) showed that phaseolin gene from *Phaseolus vulgaris* gets expressed after transfer to sunflower via Ti-plasmid vector of *Agrobacterium tumefaciens*. The RNA transcribed in sunflower cells transformed with these constructions was characterized by hybridization procedures and by translation *in vitro* of extracted RNA. The success of this experiment showed that eukaryotic genes can be expressed to yield detectable levels of proteins in alien plant cells.

Beachy *et al.* (1985) introduced a soybean gene for the α' subunit of β-conglycinin into petunia. Soybean α'-subunit accumulated in parallel with the petunia seed storage

protein and was estimated to be between 0.1 and 1% of the total seed protein. Majority of the α'-subunit polypeptides assembled into multimeric proteins of 7-8S with smaller amounts existing in 9-11 S complexes.

Legumes are uniformly limiting in methionine and cysteine because their major storage proteins, the globulins, are low in these amino acids (Gupta, 1983). One approach to increase the level of methionine is to introduce methionine residues or methionine rich peptides into non conserved regions of stored proteins. It can be achieved by making mutations in one sub unit of glycinin A_2B_1a which is well characterized (Nielson, 1984). The other approach is to transfer genes coding for methionine rich protein (MRP) from other species.

Complementary DNA (cDNA) clone for MRPs from Brazil nut (Altenbach *et al.*, 1987), sunflower seed (Lilley *et al.*, 1989) 10 kD zein protein from maize (Kirhara *et al.*, 1988) and new MRP from maize seed (Chui *and Falco,* 1995) have been isolated and sequenced. The Brazilnut MRP gene when transformed into tobacco, resulted in seed specific increase in methionine of upto 30% of transgenic plants (Altenbach *et al.*, 1989).

Third approach is to increase the level of endogenous MRP. A 10.8 kD MRP that has 12.10% methionine has been identified by Kho and de Lumen (1988) from soybean. The

level of this MRP has to be increased about 17 fold from its level of 0.6% of the total protein to bring the overall methionine content of soybean seeds to that of the FAO reference proteins.

Pederson *et al.* (1986) found that nutritional quality of soybean could be improved by introducing and expressing the gene encoding the methionine rich 15 kD zein.

Hoffman *et al.* (1987) placed maize 15 kD zein structural gene under the regulation of French bean β-phaseolin gene flanking regions and expressed in tobacco. Zein accumulation was found as high as 1.6% of the total seed protein.

In vitro mutagenesis was used to supplement the sulphur amino acid codon content of a gene encoding β-phaseolin-a *Phaseolus vulgaris* storage protein (Hoffman *et al.*, 1988). The nutritional quality of β-phaseolin was increased by the insertion of 15 amino acids, six of which were methionine. The inserted peptide was essentially a duplication of a naturally occurring sequence found in the maize 15 kD zein storage protein (Pederson *et al.*,1986). However, this modified phaseolin achieved less than 1% of the expression level of normal phaseolin in transformed seeds.

De Clercq *et al.* (1990) genetically engineered the Arabidopsis at2S1 gene. The alternations were made in a region that is highly variable among different 2S albumins in both

length and amino acid composition. This variable region was replaced with different oligonucleotides containing mainly methionine codons. The modified gene was introduced into tobacco, *Brassica napus* and *Arabidopsis* plants and directed the synthesis of new, methionine rich 2S-albumins.

Divakar and Reddy (1990) performed tissue culture studies in chickpea (*Cicer arietinum L.*). Multiple shoots were induced directly from the different seedling explants-hypocotyl, epitocyl, cotyledons, and shoot apices. Somatic embryogenesis and subsequent shoot development was achieved in cotyledons derived cultures. Efficient multiple shoot formation, which could be induced in a number of explants is visualized to be useful for production of somaclonal variants and genetic transformation using Ti-plasmid.

Schroeder *et al.* (1993) developed a reproducible transformation system of Pea (*Pisum sativum L.*) using explant sections from embryo axis of immature seeds. The transformed plants were resistant to herbicide basta.

CHAPTER-III

MATERIALS AND METHODS

MATERIALS

The material included in this study were:

(i) Bengal genotypes GGC-17, GGC-42, GGC-47, GGC-48 and GGC-50 developed by the Department of Genetics, PAU, Ludhiana.

(ii) Two Bengal gram genotypes PBG-1 and BG-209 developed by the Department of Plant Breeding, PAU, Ludhiana.

(iii) One Kabuli gram cultivar L-550.

3.1 RAISING OF THE CROP

Bengal gram (*Cicer arietinum L.*) for the present study was raised in experimental area of Department of Genetics, Punjab Agricultural University, Ludhiana. Kabuli gram genotype L-550 was raised in the experimental area of Department of Agronomy, PAU, Ludhiana following the recommendations given in "Package of Practices" of Punjab Agricultural University. Date of sowing of different lines of Desi Bengal gram genotypes was 18th Oct, 1994 and of Kabuli gram was 26th Oct., 1994. The distance between the lines was

30 cm and plant to plant distance was 10 cm.

3.1.1 Collection of Samples

Fully opened flowers of the uniformly growing plants were tagged at the time of flowering in order to ascertain the stage of seed development. The tagging was started on 2nd Feb., 1995. The pods of different lines of Bengal gram were collected at 18, 25, 32, 39, 46 and 53 days after flowering (DAF). Pods were kept at -70°C deep freezer at Biotechnology centre for Biochemical analysis.

METHODS

The various methods used in the present study are described under the following headings:

3.2 Determination of Dry matter

3.3 Estimation of Protein

3.4 Electrophoretic studies on the storage proteins

3.5 Estimation of total RNA content

3.6 Isolation of total RNA

3.7 Agarose gel electrophoresis of total RNA

3.8 Isolation of mRNA from total RNA by oligo-dT column chromatography

3.2 DETERMINATION OF DRY MATTER (A.O.A.C., 1965)

An accurately weighed quantity (1 gm) of seeds of each genotype at different developmental stages was taken in glass crucible and dried in an oven at 60-65°C for 48 hours. The crucible containing the dried material were put in a

desiccator and weighed for the determination of dry matter percentage in the sample.

3.3 PROTEIN ESTIMATION

3.3.1 Determination of Total Protein [Mckenzie and Wallace, 1954]

The sample containing protein was digested with concentrated H_2SO_4 in the presence of potassium sulphate and copper sulphate in the ratio of 10:1 (W/W). The mixture was steam distilled with 40% NaOH and the ammonia released was passed into boric acid solution (4 %) which was then back titrated with standard acid (N/100 HCl). The percentage of nitrogen was multiplied by factor of 6.25 to account for the protein percentage in the seed sample.

(a) Reagents

(i) Conc. H_2SO_4, specific gravity 1.84, nitrogen free.

(ii) Digestion mixture : Prepared by mixing K_2SO_4. $5H_2O$ and $CuSO_4.5H_2O$ (10:1).

(iii) 4 % Boric acid.

(iv) 40 % NaOH.

(v) 0.01N HCl.

(vi) Mixed indicator Methyl red (0.2% in 95% EtOH) - bromocresol green (0.2% in 95 % EtOH) in a ratio of 1:5.

(b) Procedure

500 mg of powdered sample of seed of different lines

was taken in a long necked 300 ml Kjeldahl digestion flasks. To this 20 ml conc. H_2SO_4 was added followed by 2.5 g of digestion mixture. The contents were heated gently until frothing ceased and then strongly heated till the solution became clear. After cooling, volume was made to 100 ml. Two ml of the digested sample was taken in microkjeldahl distillation assembly, followed by 10 ml of 40 % NaOH and allowed to distil the contents. Ten ml of 4 % boric acid and 2 drops of mixed indicator were added and then titrated against 0.01N HCl. The total quantity of HCl used for the neutralization of ammonia was noted. The crude protein content was estimated as follows:

Crude protein content (% age) = Nitrogen % x 6.25

3.3.2 Estimation of Soluble Proteins (Lowry *et al.*, 1951)

(a) Reagents:

(i) 2 % Sodium carbonate in 0.1N NaOH.

(ii) 0.5 % Copper sulphate ($CuSO_4.5H_2O$) in 1 % Sodium citrate.

(iii) Mixed the reagents (i) with reagent (ii) in the ratio of 50:1 to get reagent (iii).

(iv) Folin Ciocalteau's reagent (diluted with water in 1:1 ratio).

(b) Procedure

200 mg of seed sample was homogenised with 10 ml of 0.1 N NaOH and centrifuged at 5000 g for 5 minutes. An aliquot (0.1 ml) of extract was taken in duplicates and diluted to 1

ml with distilled water. Bovine serum albumin (BSA) samples (100- 600 μg) were also taken in 0.1 N NaOH for the standard curve. 5 ml of reagent (iii) was added to each tube. After keeping for 10 minutes, added 0.5 ml of Folin Ciocalteau's reagent. The contents were again kept for 30 min. and the absorbance was determined at 520 nm using spectronic-20-D. The concentration of protein was calculated from the standard curve made with bovine serum albumin (BSA).

3.4 FRACTIONATION OF PROTEINS

3.4.1 Extraction, fraction and purification of Bengal gram cotyledonary proteins (Basha and Beevers, 1975)

Following extraction procedure was employed

Cotyledon flour (500 mg)

Twice homogenised (0°C, 30 min) with 10 volumes of borate buffer (0.02M, pH 8.0) containing NaCl (0.5M), followed by stirring (45 min) and centrifugation (15000 g, 30 min)

Residue (Discarded)

Supernatant (Total extractable proteins).

Extract was brought to 70% saturation with $(NH_4)_2SO_4$. Kept (1h, 0°C) and centrifuged 15000 g, 30 min).

Supernatant
(Discarded)

Precipitate
Dissolved the precipitate in minimum volume of phosphate buffer (0.02M, pH 7.0) containing NaCl (0.2M) dialysed (48h) against deionized water (several changes) and centrifuged (15000 g, 30 min)

Supernatant
(Water soluble albumins)

Precipitate
(Crude globulins)
The precipitates were suspended in acetate buffer (0.02M, pH 4.6) containing NaCl (0.2M) stirred (0.5h 0°C) and centrifuged (5000g, 20 min). Repeated 6 times.

Supernatant
(Vicilin like protein)

Dialyzed against distilled water and centrifuged (5000 g, 20 min). Precipitates were dissolved in phosphate buffer (0.02M, pH 8.0) containing NaCl (0.2M).

Precipitate
(legumin like protein)

Dissolved in phosphate buffer (0.02M, pH 8.0) containing NaCl (0.2M).

3.4.2 Electrophoretic Studies on the Storage Proteins

One dimensional sodium dodecyl sulphate - Poly acrylamide gel electrophoresis (SDS-PAGE), as described by Smith and Payne (1984) was used to analyze variations in different genotypes of developing Bengal gram seeds.

Modifications

- The concentrations of acrylamide and bisacrylamide were changed.
- The pH of separating gel was reduced from 8.8 to 8.6.
- Time-period for running gel was adjusted to 14 hr at 8mA current.

Purification of Chemicals

For higher resolution chemicals of analytical grade from SISCO were purified by methods given by Hames and Rickwood (1982) as below:

(i) **Acrylamide**: Acrylamide was purified by dissolving 60 g of it in one litre of chloroform at 50°C and filtering the solution while hot, through Whatman No. 1 filter paper. The filtrate was stored at-20°C for recrystallization. The crystals were collected by filtration and then dried and stored in the refrigerator.

Bisacrylamide

It was purified by dissolving 10 g bisacrylamide in 1 litre of acetone at 50°C and filtering the solution while hot, through Whatman No. 1 filter paper. The filtrate was stored at -20°C for recrystallization. The crystals were collected by filtration and then dried and stored in the refrigerator.

Sodium dodecyl sulphate: SDS was purified by dissolving 200 g in 3 litre of boiling ethanol and filtering while hot. The filtrate was kept at 4°C for 2-3 days for recrystallization crystals were separated by filtering and dried.

Preparation of Stock Solution

(i) Acrylamide (35%) - 35 g of purified acrylamide was dissolved in glass double distilled water to 100 ml volume.

(ii) Bisacrylamide (2%)- 2g of purifed N, N'-methylene bisacrylamide was dissolved in glass double distilled water to 100 ml volume.

(iii) 10% SDS-10 g purified SDS was dissolved in distilled water to 100 ml volume.

(iv) 1M Tris (pH 6.8)-12.11 g Tris base was dissolved in 50 ml of distilled water. The pH was adjusted to 7.0 with conc. HCl and accurately with 1N HCl to 6.8. The final volume was made to 100 ml.

(v) 1M Tris (pH 8.6)-12.11 g of Tris base was dissolved in distilled water. The pH was adjusted to 8.6 with conc. HCl. There was a slight increase in pH after cooling at room temperature which was again adjusted and the final volume was made to 100 ml.

(vi) Electrophoresis buffer (pH 8.3) - 75.5 g glycine, 15g Tris and 5 g SDS were dissolved in distilled

water and final volume made to 5 litres.

(vii) 3 x sample buffer : 10 ml glycerol, 6.25 ml 1M Tris (pH 6.8), 2g SDS(purified), 20 mg pyronin-G — —(tracking dye) and 12.05 ml distilled water were mixed into a 50 ml measuring cylinder and stored as stock solution.

(viii) Staining solution or Dye - 2 g Brilliant coomassie Blue was dissolved in 95% ethanol, and final volume was made to 500 ml and stored in dark.

Extraction of Proteins

50 mg of seed samples of different Bengal gram genotypes were homogenised. Extraction solution was prepared by mixing 4 ml distilled water, 1.7 ml of 3 x sample buffer and 0.3 ml β-mercaptoethanol. The amount of extraction solution added to each tube was calculated by multiplying the factor 25 with their respective seed weight (50 mg). After adding extraction solution, the contents were mixed on vortex mixture for 2-3 minutes and then allowed to stand for 1 hour at room temperature. Then samples were boiled for 2-3 minutes, cooled to room temperature and centrifuged at 2000 rpm for 2 minutes.

Preparation of Stacking and Separating Gels

The whole electrophoresis apparatus was disinfected by dipping in 1N NaoH solution for 3-4 hours. It was washed thoroughly with tap water and then rinsed with distilled

water. The glass plates were dried and fixed in the gel casting apparatus. The sealing of the plates was done with a rubber seal around the space.

Preparation of Separating Gel

7.25 ml of 35 % acrylamide, 2.08 ml of 2 % bisacrylamide, 5.1 ml of distilled water, 9.4 ml of Tris pH 8.6, 0.25 ml of 10 % SDS, 0.94 ml of freshly prepared 1 % ammonium persulphate and 30 μl of N, N, N', N' TEMED were taken in 50 ml beaker and solution was thoroughly mixed. The solution was immediately poured into the gel mould upto the level of 1 cm below the position of sample wells.

The surface of polymerising gel was overlaid with 1-2 ml of water using micropipette. The separating gel was left to polymerize for about 15-30 minutes.

Preparation of Stacking Gel

Stacking gel was prepared by adding 1.75 ml of 35 % acrylamide, 0.75 ml of 2 % bisacrylamide, 2.5 ml of Tris buffer (pH 6.8), 8 ml of distilled water, 0.20 ml of 10 % SDS, 0.94 ml of freshly prepared 1 % APS and 40 μl of N, N, N', N' TEMED in a 50 ml beaker and solution was thoroughly mixed. The water was poured off from the top of separating gel and the surface was washed with little amount of stacking solution. The mould was filled with the solution upto the top of the plates and the comb was inserted. The stacking gel was left to polymerize. After about 1 hour the comb was carefully

removed without disturbing the wells and the wells were washed twice with the running buffer.

Sample loading : Plates with gel were placed in electrophoresis apparatus containing running buffer. The air bubbles from the lower surface of gel were removed. The upper reservoir was filled with running buffer. 50 μl of sample was loaded in each well with the help of micropipette. After loading the samples the electrodes were connected to powerpack. Current was 8 mA. Gel was allowed to run overnight for 14 hours.

Staining : After termination of electrophoresis, the glass plates were removed and carefully separated and the stacking gel was removed from separating gel by cutting interface with scalpel. The separating gel was then placed in staining solution which contained 125 ml of 10% W/V TCA, 375 ml of double distilled water, 25 ml of filtered coomassie brilliant blue dye. Gel was slowly agitated at a speed of 80 rpm for 24-48 hours on an orbital shaker. The gel was then destained with water for 1-2 days. After destaining the gels were photographed.

3.5 ESTIMATION OF TOTAL RNA CONTENT (Schneider, 1957)

Reagents:

Orcinol Reagent - 1 g of purified orcinol was dissolved, immediately before use, in 100 ml of conc. HCl containing 0.5 g of $FeCl_3$.

Procedure

Removal of acid soluble compounds

1 ml of 20 % tissue homogenate was mixed with 2.5 ml of cold 10 % TCA and centrifuged. The sediment was washed with 2.5 ml of cold 10 % TCA.

Removal of Lipoidal Compounds

The final sediment remaining after removal of acid soluble compounds was extracted twice with 5 ml of 95 % ethanol and recovered by centrifugation.

Separation of Nucleic Acids

The lipid free residue was suspended in 1.3 ml of water and 1.3 ml of 10 % TCA, and the mixture was heated for 15 minutes at 90° with occasional stirring. Proteins were left as an insoluble residue which were centrifuged off and washed with 2.5 ml of 5 % TCA.

Estimation of RNA: Two tenths mil ilritre of nucleic acid extract was diluted to 1.5 ml and heated with 1.5 ml of orcinol reagent for 20 min. in boiling water. The intensity of the green colour was determined at 660 nm.

A standard curve was prepared relating colour intensity to RNA with xylose serving as the standard.

3.6 ISOLATION OF TOTAL RNA (Sadasivam and Manikam, 1992)

Reagents:

1. Phenol (Freshly distilled).
2. Extraction buffer (pH 9.0):-

Tris- Hcl (0.1 M)	1.21 g
Nacl (0.075 M)	0.44 g
EDTA-Na_2 0.005 M	0.19 g

water to make 100 ml volume.

3. Elution buffer (pH 7.5)

0.01M Tris -	0.12 g
0.001 M EDTA- Na_2	0.04 g
10 % SDS -	0.5 ml

Water to make 100 ml volume

4. Ethanol

5. SDS 10 %

6. Ether

7. Tris 100 mM for phenol saturation (pH 8.5)

Procedure

The apparatus was thoroughly washed with teepol, rinsed with distilled H_2O and then dipped in 1N HCl for 2-3 hrs. After that apparatus was thoroughly rinsed with distilled H_2O to remove any traces of acid. All glassware including pestle-mortar were baked at 120°C for 2 days. After baking whole apparatus was dipped overnight in 0.1 % diethyl pyrocarbonate (DEPC). Next day apparatus was dried in oven and then autoclaved. Sterilized autoclaved gloves were used while carrying out the procedure.

All solutions were prepared in 0.1 % DEPC treated autoclaved H_2O.

Steps Involved

1. 2- 3 g of seeds were put in liquid nitrogen, ground to a fine powder and extracted with 10 volumes of extraction buffer.
2. Centrifuged the homogenate at 2000 g for 7 min.
3. Transferred the supernatant to a volumetric flask and stirred with 0.1 volume of 10 % SDS for 2-3 min.
4. Added an equal volume of buffered phenol (Freshly redistilled and saturated first with autoclaved double distilled water and then 100 mM Tris- pH 8.5) : Chloroform mixture (1:1).
5. Partitioned the contents by centrifuging at 2000 g for 10 minutes and collected the upper phase into a volumetric flask.
6. Shook the lower and interphase again with an equal volume of extraction buffer for 5 minute and centrifuged.
7. Combined the upper aqueous phase with the first one (step 5) and stirred with an equal volume of phenol: Chloroform (1:1) for 5 min.
8. Repeated the extraction and centrifugation steps until the interphase showed no proteins.
9. Collected the upper aqueous phase containing RNA. Dissolved in it about 250 mg NaCl and two volumes

of cooled ethanol (95 %) and left the flask overnight at - 20°C for precipitation.

10. Collected the RNA by centrifugation at 2000 g for 10 min, washed the pellet (RNA) with 70 % ethanol, ethanol: ether (1:1 v/v) and finally with ether. Dried the pellet *in vacuo* for a few min.

11. Dissolved the RNA completely in elution buffer.

Estimation of Isolated RNA

Diluted 20 μl aliquot to 2 ml with elution buffer and read the absorbance (A) at 260 nm. One A_{260} unit was assumed to be equivalent to 40 μg RNA/ml (Sadasivam and Manikam, 1992).

3.7 AGAROSE GEL ELECTROPHORESIS OF TOTAL RNA (Sambrook *et al*., 1989b).

Reagents:

i) 5 X Formaldehyde gel running buffer

- 0.1 M morphalinopropane sulfonic acid (MOPS) (pH 7.0)
- 40 mM sodium acetate
- 5 mM EDTA (pH 8.0)

ii) Sample preparation

RNA (upto 30 μg)	4.5 μl
5 X formaldehyde gel running buffer	2 μl
Formaldehyde	3.5 μl
Formamide	10.0 μl.

iii) Loading buffer -

50 % glycerol

1 mM EDTA (pH 8.0)

0.25 % bromophenol blue

0.25 % xylene cyanol FF

iv) Staining solution

0.5 μg/ml ethidiumbromide in 0.1 M ammonium acetate and 0.1M β-mercaptoethanol.

Procedure:

Prepared the gel by melting agarose in water. Cooled it by 60°C and added 5 X gel running buffer and formaldehyde to give final concentration of IX and 2.2 M respectively. Cast the gel in a chemical hood and allowed the gel to set for atleast 30 min at room temperature. Prepared the sample by mixing the given reagents in a sterile eppendorf tube. Incubated at 65°C for 15 min. and then chilled them on ice. Added 2 μl of gel loading buffer.

Loaded RNA samples to the gel. After running the samples to 5-6 cm, stained the gel for 15 min in staining solution. Destained with 0.1M ammonium acetate and 0.01 M β-mercaptoethanol and photographed the gel under UV using a polaroid film.

3.8 ISOLATION OF mRNA FROM TOTAL RNA BY OLIGO-(dT) COLUMN CHROMATOGRAPHY (Sambrook *et al.*, 1989a)

Materials

(i) Oligo-(dT) cellulose

(ii) 1 x column loading buffer

- 20 mM Tris-Hcl (pH 7.6)
- 0.5 M Nacl
- 1 mM EDTA (pH 8.0)
- 0.1 % Sodium dodecyl sulphate (SDS)

(iii) Elution buffer

- 10 mM Tris-Hcl (pH 7.6)
- 1 mM EDTA (pH 8.0)

(iv) Spectrophotometer

(v) Chromatography column

(plastic disposable syringe)

Procedure:

1) Suspended 0.25 g of oligo-(dT) cellulose in 0.1 N NaOH.
2) Poured a mixture of oligo-(dT) cellulose in a sterile 2 ml syringe.
3) Washed the column with sterile 1 X column loading buffer unitl pH of effluent is less than 8.0.
4) Dissolved the RNA in sterile water and heated the solution at 65°C for 5 minutes. Cooled the solution to room temperature quickly and added an equal volume of 2 X column loading buffer. Applied the solution to the

column and immediately began to collect the elute in a sterile tube, when all the RNA solution has entered the column, added 1 X column loading buffer and continued to collect the elute.

5) Heated the collected elute to 65°C for 5 minutes and reapplied the elute to the top of the column. Again collected the material flowing through the column.

6) Washed the column with 5-10 column volumes of 1 X column loading buffer, collected equal volume (200 μl) fractions. Read the absorbance at 260 nm of each fraction collected from the column.

7) Eluted the poly (A$^+$) from the oligo-(dT) cellulose with sterile RNase free elution buffer.

8) Collected mRNA fractions till the absorbance at 260 nm reaches zero.

CHAPTER IV

RESULTS AND DISCUSSION

The present study was carried out to understand the relationship between the pattern of accumulation of protein and RNA in various Bengal gram genotypes at different stages of seed development. The results have been discussed under the following headings:

4.1 Dry matter accumulation.

4.2 Estimation of protein content.

4.3 Electrophoretic pattern of seed proteins.

4.4 Estimation of RNA content.

4.5 Isolation and electrophoretic pattern of total RNA.

4.6 Isolation and estimation of mRNA.

4.1 DRY MATTER ACCUMULATION

The dry matter accumulation in Bengal gram developing seeds was determined with a view to understand the pattern of seed development. Fresh weight and dry matter per seed at 18, 25, 32, 39, 46 and 53 DAF of genotypes L-550, PBG-1 and BG-209 are shown in Fig. 1. Fresh weight increased upto 32 DAF for Kabuli gram (L-550) and BG-209 and upto 39 DAF for PBG-1. Rapid increase in weight of fresh seeds was observed between 18 and 32 DAF, however, an indication of initiation of

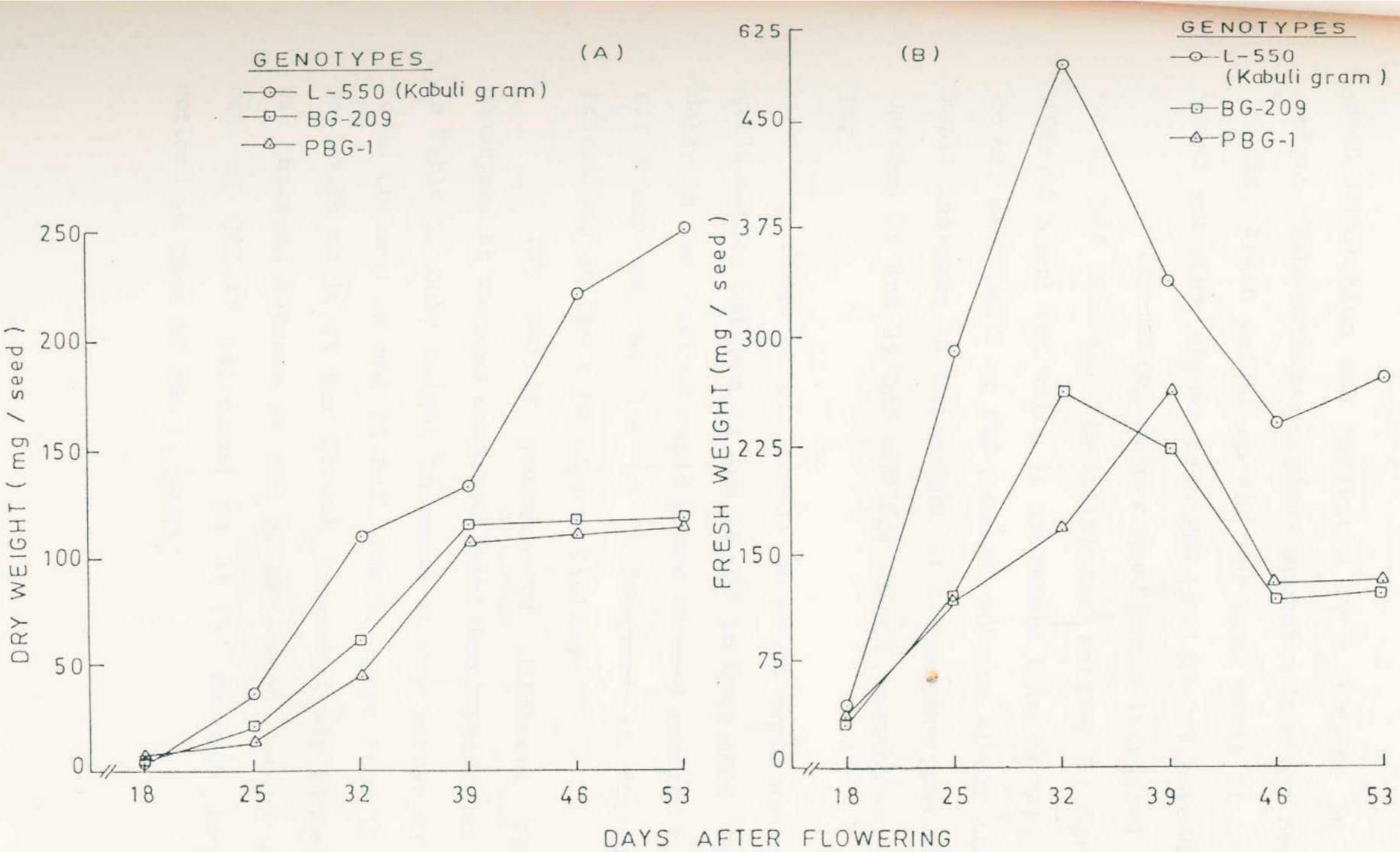


FIG.1.(A) DRY WEIGHT (mg/seed)AND (B) FRESH WEIGHT (mg / seed) OF BENGAL GRAM GENOTYPES L-550 (Kabuli gram), BG-209 AND PBG-1

seed maturation was reflected by a decline in their fresh weight. This maturation phase appeared to start between 32 and 39 DAF. Fresh weight on the per seed basis of L-550 (Kabuli gram) was much higher than PBG-1 and BG-209 beyond 18 DAF.

Dry matter on per seed basis increased from 18 DAF to 39 DAF for all the cultivars studied except for L-550 (Kabuli gram) for which it increased upto 53 DAF. Dry matter on per seed basis of PBG-1 and BG-209 was almost same (Fig.1). Rapid increase in the weight of these genotypes was observed between 25 and 39 DAF and for Kabuli gram between 25 and 46 DAF.

Singh *et al.* (1981) observed rapid accumulation of grain matter between 14 and 28 DAF in this seed. The apparent delay in the onset of rapid phase of seed reserve formation in our study may be due to differences in the temperature prevailing at the time of pod filling.

Dry matter percent of different Bengal gram genotypes at various stages of seed development has been given in Table-1. Only slight increase in dry matter of seeds was found between 18 and 25 DAF. The increase from 18 to 25 DAF was 15.2% to 16.4% for GGC-47. Comparatively higher increase was observed between 25 and 32 DAF, when the dry weight per cent of GGC-47 increased to 18.3%. Maximum increase was noticed in case of PBG-1 (24.9%)

Table 1 : Dry matter percentage in the seeds of Bengal gram genotypes at different stages of development

GENOTYPE	DAYS AFTER FLOWERING					
	18	25	32	39	46	53
GGC-17	13.3	14.1	18.1	40.0	93.0	94.0
GGC-42	14.2	14.6	24.3	30.2	95.0	95.1
GGC-47	15.2	16.4	18.3	38.2	95.5	95.5
GGC-48	19.3	19.6	24.0	35.4	95.6	95.5
GGC-50	16.0	19.2	27.7	43.4	95.0	95.0
BG-209	14.2	17.1	23.8	32.5	94.5	94.0
PBG-1	12.4	18.6	24.9	43.4	93.3	94.0
L-550 (Kabuli gram)	10.8	11.8	22.2	30.5	82.2	91.4

Per cent dry matter of seeds increased rapidly between 32 DAF and 46 DAF indicating maturation stage of seed development. Dry matter of GGC-47 increased from 18.3 at 32 DAF to 38.2% at 39 DAF and to 95.5% at 46 DAF stage.

After 46 DAF per cent dry matter of seeds remained almost constant, except for kabuli gram cultivar (L-550) in which it increased from 82.2 to 91.4% indicating that kabuli gram seeds take more time for complete development and drying of seeds. Moreover, per cent dry matter of kabuli gram seeds at different stages was comparatively less than desi gram cultivars.

4.2 ESTIMATION OF PROTEIN CONTENT

Alkali soluble and total protein content of different genotypes were determined at 18, 25, 32, 39, 46 and 53 DAF.

Studies on soluble proteins in fresh seeds were carried out to relate them to the level of RNA because later has very short life and can be analysed only on fresh seeds. Fig. 2 represents the soluble protein content of GGC-17, GGC-42, GGC-47 and GGC-48. Protein content increased slowly from 18 to 32 DAF and marginally from 32 to 39 DAF. Then protein content increased rapidly from 39 to 46 DAF. After that there was slight increase upto 53 DAF. Alkali soluble protein content (Table 2) increased from 2.19% at 18 DAF to 19.8% at 53 DAF for GGC-50 and 1.16% at 18 DAF to 17.9% at 53 DAF for

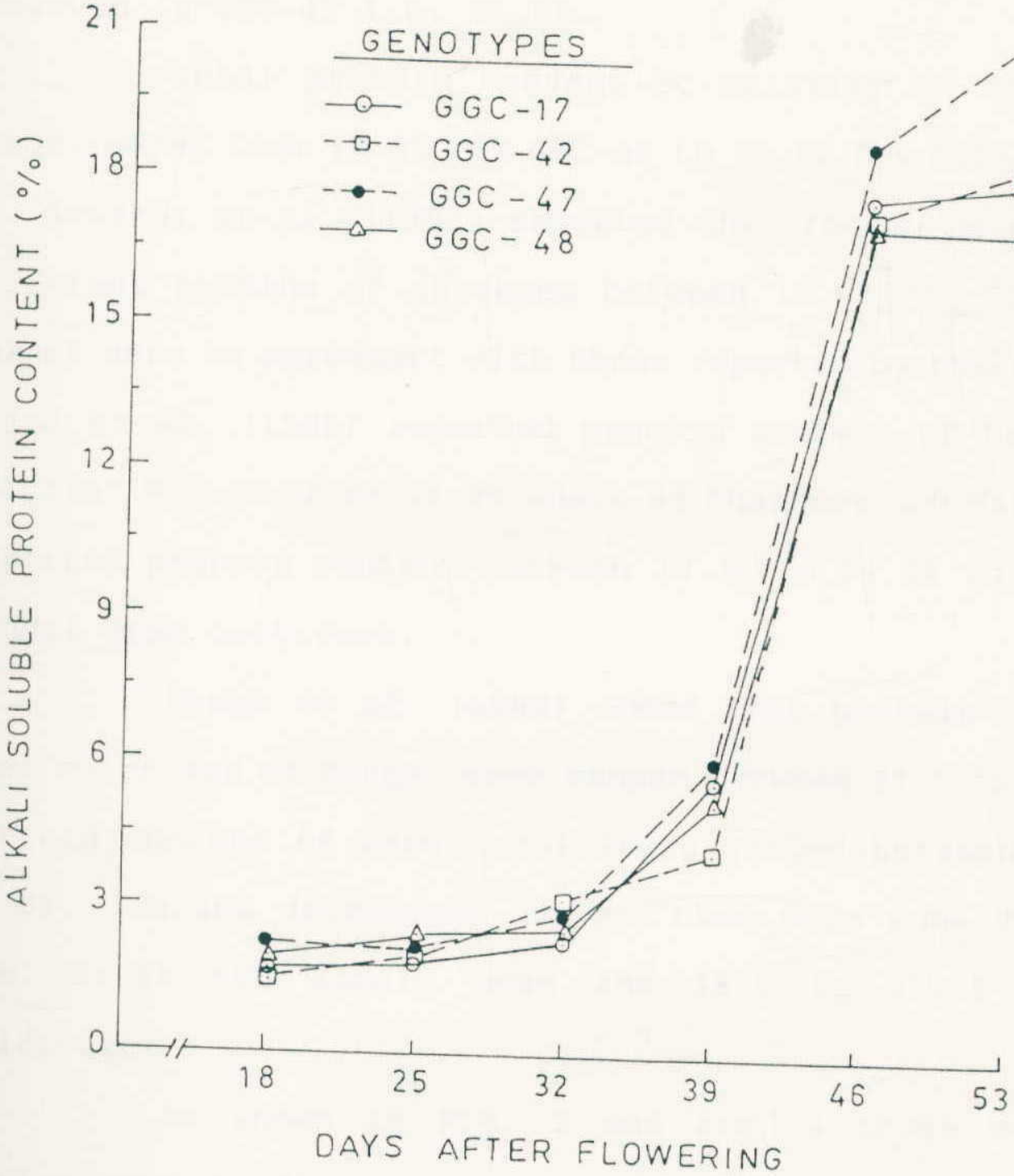


FIG.2 ALKALI SOLUBLE PROTEIN CONTENT (FRESH WEIGHT BASIS) OF BENGAL GRAM GENOTYPES AT DIFFERENT STAGES OF SEED DEVELOPMENT

Kabuli gram. Maximum alkali soluble protein at maturity is observed in GGC-47 i.e. 21.0%.

Total protein content at maturity of Bengal gram seeds ranged from 19.5% for GGC-48 to 23.5% for GGC-47 (Table-3). Awasthi *et al.* (1991) reported the protein percentage of different strains of chickpea between 15.61% to 26.65%. Our values were in agreement with those reported by these workers. Singh *et al.* (1988) reported protein content of Bengal gram cultivar H75-35 to be 19.8% where as Dhankher and Matta (1992) reported protein content between 20.1% to 24.9% in different Bengal gram cultivars.

Singh *et al.* (1980) found that protein content of desi cultivars of Bengal gram ranged between 23.7 to 26.8% and protein content of kabuli cultivars ranged between 22.0% to 27.0%. Values determined in our case were some what lower i.e. 21.6% for kabuli gram and 19.5 to 23.5% for desi cultivars.

As shown in Fig. 2 and Fig. 3 there were small differences in the protein content of different varieties. Tomar *et al.* (1973) also observed narrow differences in the protein content. Maximum increase in the protein content was observed between 39 and 46 DAF for all the cultivars.

Changes in the total protein content have been shown in Fig 4 and Fig. 5 and Table 3. At 18 DAF total protein content on dry weight basis ranged between 11.0% for kabuli

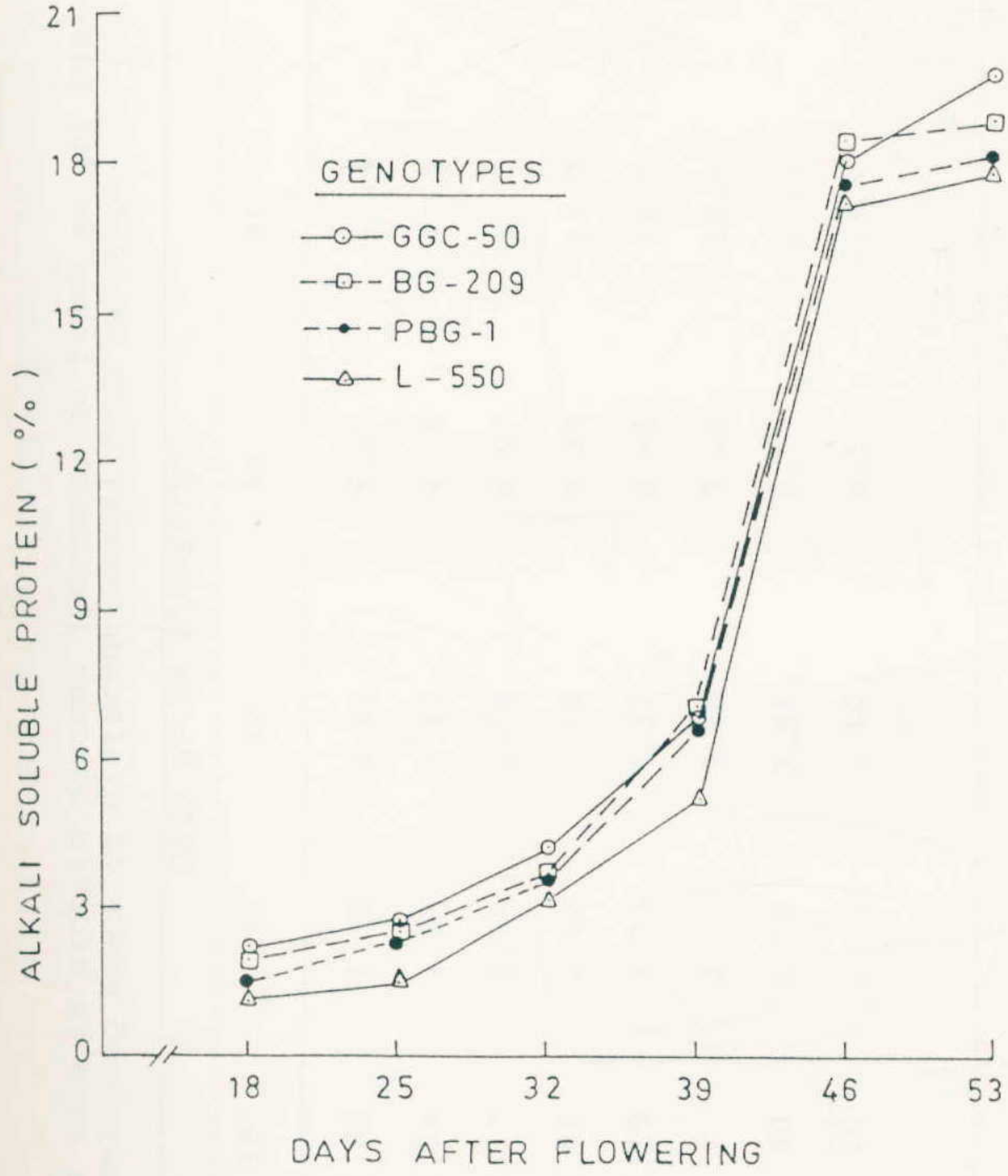


FIG. 3. ALKALI SOLUBLE PROTEIN CONTENT (FRESH WEIGHT BASIS) OF BENGAL GRAM GENOTYPES AT DIFFERENT STAGES OF SEED DEVELOPMENT

Table 2 : Alkali soluble protein content (percent) on fresh weight basis in developing seeds of different Bengal gram genotypes

GENOTYPE	DAYS AFTER FLOWERING					
	18	25	32	39	46	53
GGC-17	1.66	1.80	2.47	5.60	17.4	18.0
GGC-42	1.54	1.81	3.15	4.28	17.3	18.4
GGC-47	2.17	2.39	2.76	6.07	18.7	21.0
GGC-48	2.08	2.40	2.54	5.38	17.0	16.9
GGC-50	2.19	2.70	4.21	6.85	18.1	19.8
BG-209	1.7	2.3	3.66	7.08	18.5	18.9
PBG-1	1.50	2.39	3.56	6.59	17.6	18.2
L-550 (Kabuli gram)	1.16	1.45	3.10	4.57	15.7	17.9

Table 3 : Total protein (percentage) on dry weight basis in developing seeds of different Bengal gram genotypes

GENOTYPE	DAYS AFTER FLOWERING					
	18	25	32	39	46	53
GGC-17	12.8	13.0	13.9	14.3	19.7	20.6
GGC-42	11.1	12.5	13.2	15.2	20.6	20.9
GGC-47	14.7	14.9	15.5	17.1	23.2	23.5
GGC-48	13.2	14.1	15.2	16.2	18.8	19.5
GGC-50	14.0	14.9	15.8	17.0	22.3	23.2
BG-209	14.1	15.1	15.9	17.8	21.4	21.8
PBG-1	12.6	13.3	14.8	16.5	20.5	21.9
L-550 (Kabuli gram)	11.0	12.9	14.4	16.8	20.3	21.6

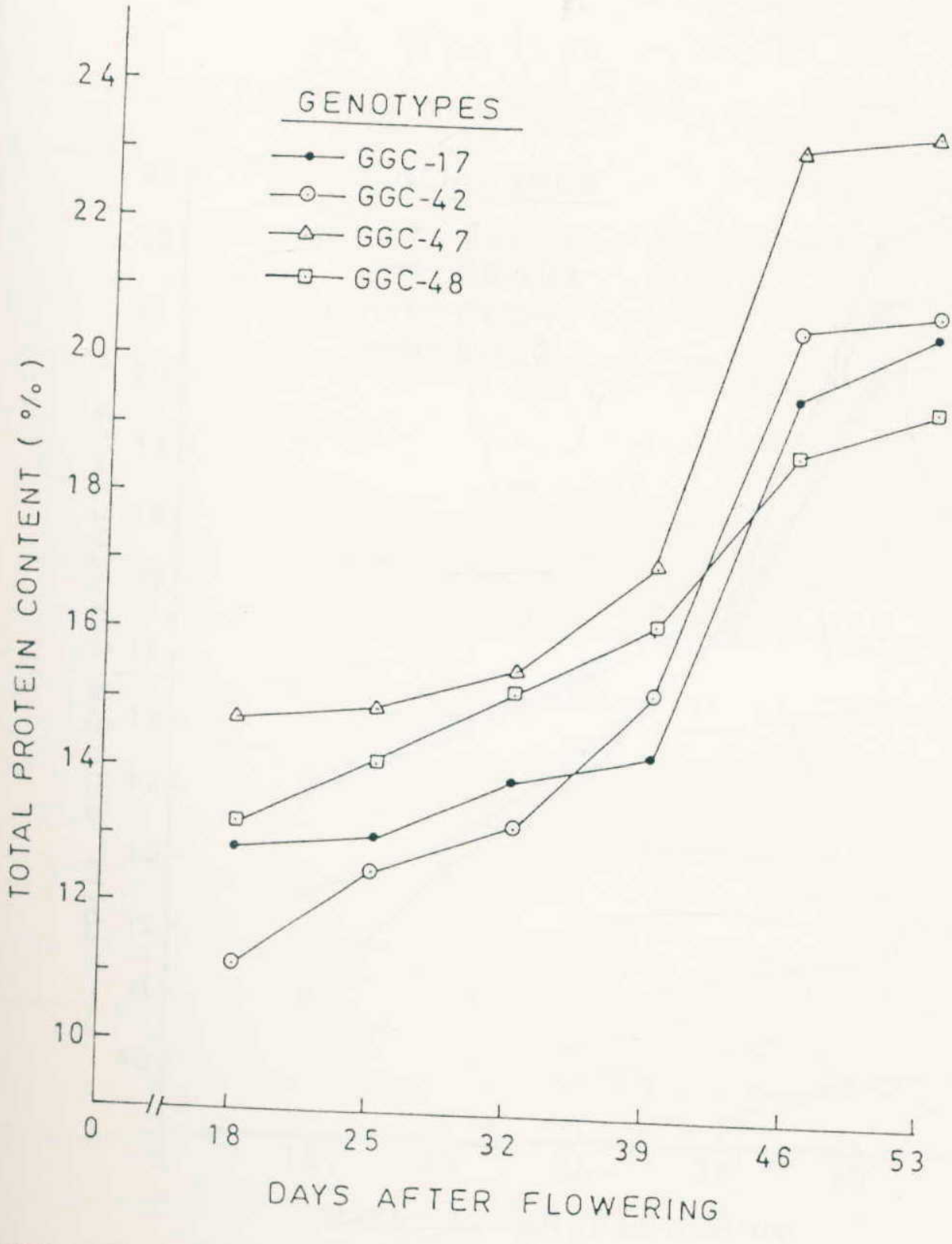


FIG. 4. TOTAL PROTEIN CONTENT (DRY WEIGHT BASIS) OF BENGAL GRAM GENOTYPES AT DIFFERENT STAGES OF SEED DEVELOPMENT

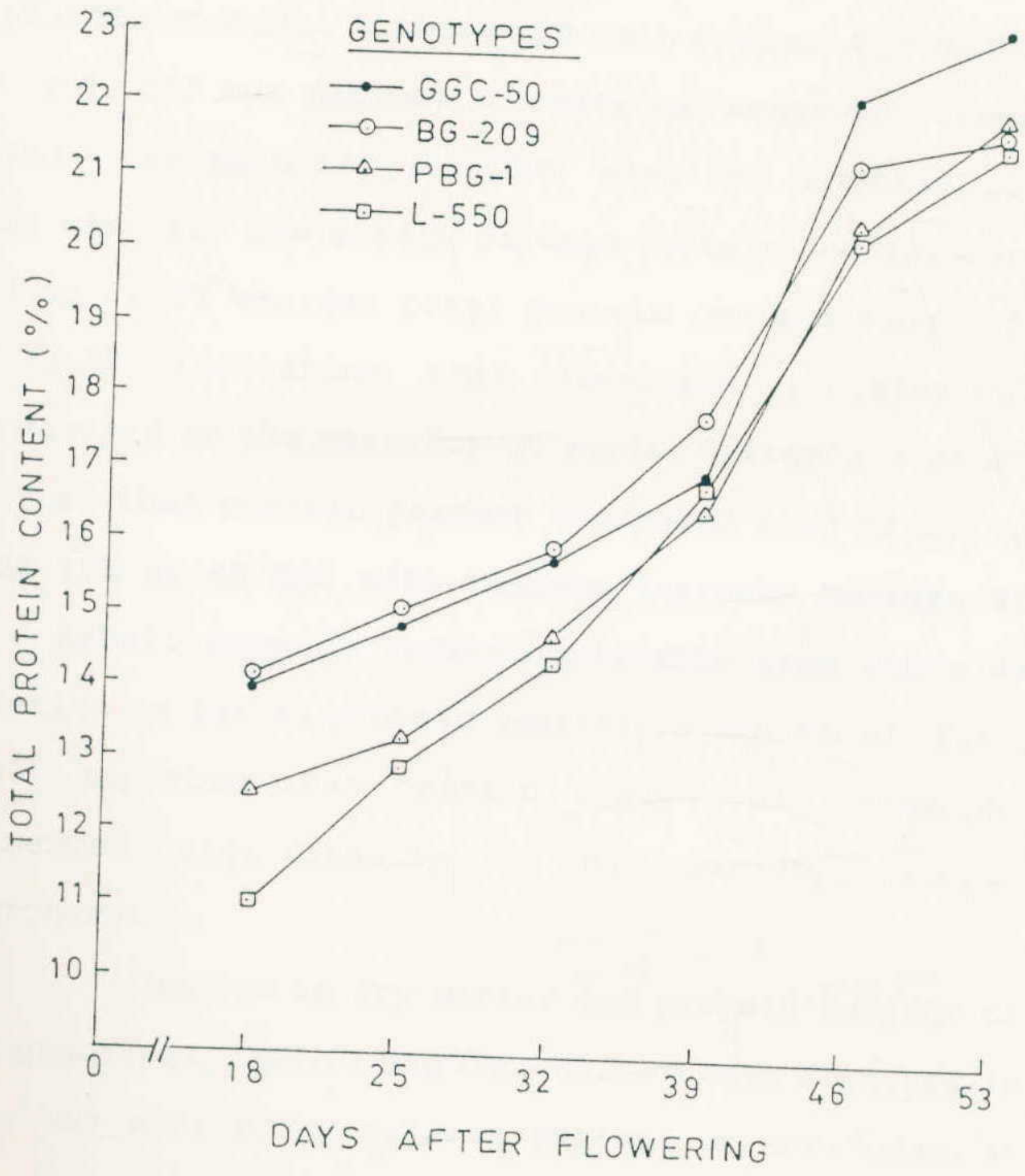


FIG.5. TOTAL PROTEIN CONTENT (DRY WEIGHT BASIS) OF BENGAL GRAM GENOTYPES AT DIFFERENT STAGES OF SEED DEVELOPMENT

gram to 14.7% for GGC-47. At 53 DAF protein content ranged from 19.5% for GGC-48 to 23.5% for GGC-47. Thus GGC-47 can be considered to be the best among the varieties studied in terms of protein content. Although protein content increased from 18 DAF to 53 DAF but maximum increase was observed between 39 and 46 DAF. At maturity (53 DAF) when the moisture percent of seeds was very low alkali soluble protein content ranged from 17.8-to 22.0% whereas total protein content ranged from 19.5 to 23.5% indicating that insoluble proteins are also synthesised at the maturity of seeds. Srivastava *et al.* (1981) reported that protein percent increased from 20.25% at 15 DAF to 26.55% at 45 DAF with maximum increase between 25 and 35 DAF. Kabuli gram is indistinguishable from other desi gram varieties as far as protein content is concerned. Singh *et al.* (1973) reported that protein content of legume seeds was influenced significantly by the variety, location and environment.

Changes in dry matter and protein content of Bengal gram genotypes GGC-17 and GGC-50 have been depicted in Fig.6. There was similarity between pattern of accumulation of dry matter and protein content. Both dry matter and protein content increased most rapidly between 39 and 46 DAF. Although dry matter was almost constant between 46 and 53 DAF protein content increased slightly at this stage of seed development.

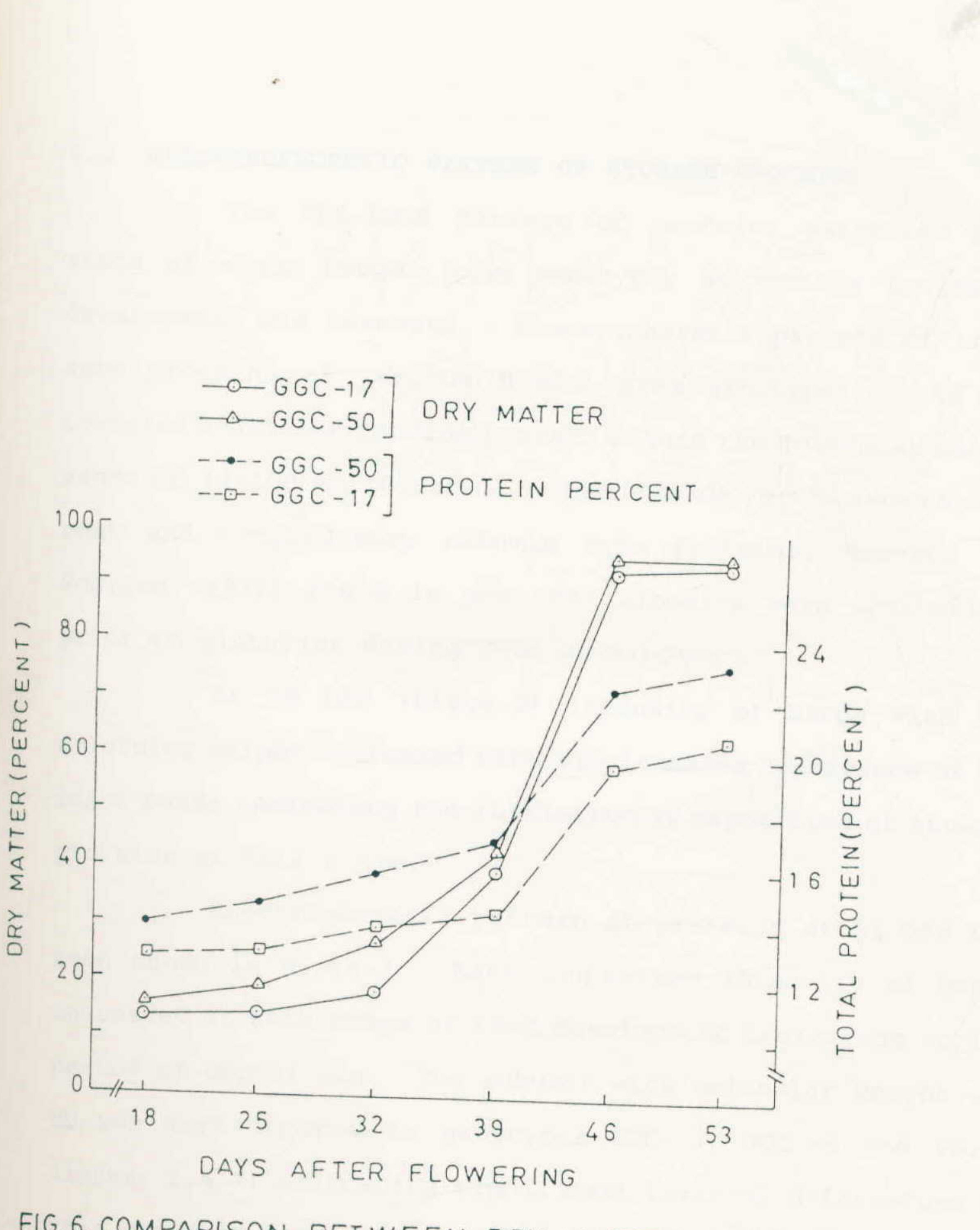


FIG. 6. COMPARISON BETWEEN DRY MATTER AND PROTEIN CONTENT OF BENGAL GRAM GENOTYPES GGC-17 AND GGC-50

4.3 ELECETROPHORETIC PATTERN OF STORAGE PROTEINS

The SDS-PAGE pattern of proteins extracted from seeds of eight Bengal gram genotypes at various stages of development was assessed. Electrophoretic pattern of total seed proteins of various Bengal gram genotypes at 18 DAF revealed a similar banding pattern within the molecular weight range of 14-100 kD (Plate-1). These bands may be due to seed coat and cotyledonary albumin type proteins. Beevers and Poulson (1972) found in pea that albumins were synthesized prior to globulins during seed development.

At 25 DAF (Plate-2) intensity of bands with low molecular weight increased with simultaneous appearance of two major bands indicating the initiation of deposition of storage proteins at this stage.

Electrophoretic pattern of proteins at 32 DAF has been shown in Plate-3. Both number and intensity of bands increased at this stage of seed development indicating active period of deposition. The subunit with molecular weight ≈45 kD was more intense in genotypes GGC-17, GGC-48 and PBG-1 (lanes- 1,4,7) indicating significant varietal differences at this stage of seed development.

Plate-4, represents the electrophoretic pattern of seed proteins at 39 DAF. Bands with M.wt. range 20-70 kD pertaining to legumin and vicilin subunits became more intense. It was seen that 39 DAF an additional band with M.wt

Plate 1 SDS-PAGE pattern of seed proteins of different Bengal gram genotypes at 18 DAF.

Lane-1 GGC-17; Lane-2 GGC-42; Lane-3 GGC-47
Lane-4 GGC-48; Lane-5 GGC-50; Lane-6 BG-209
Lane-7 PBG-1; Lane-8 L-550

Plate 2 SDS-PAGE pattern of seed proteins of different Bengal gram genotypes at 25 DAF.

Lane-1 GGC-17; Lane-2 GGC-42; Lane-3 GGC-47
Lane-2 GGC-48; Lane-5 GGC-50; Lane-6 BG-209
Lane-3 PBG-1; Lane-7 L-550

Plate 1

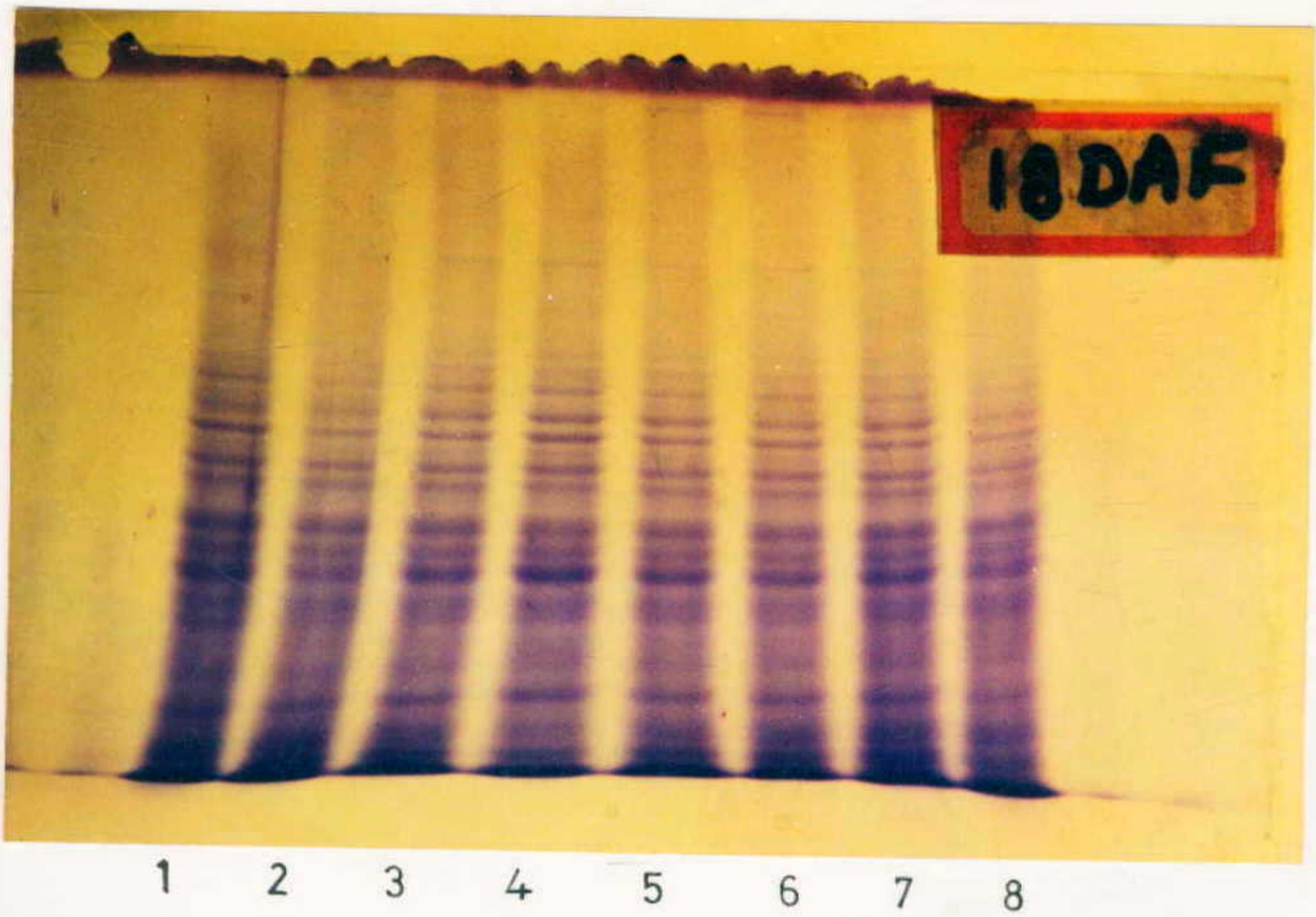


Plate 2

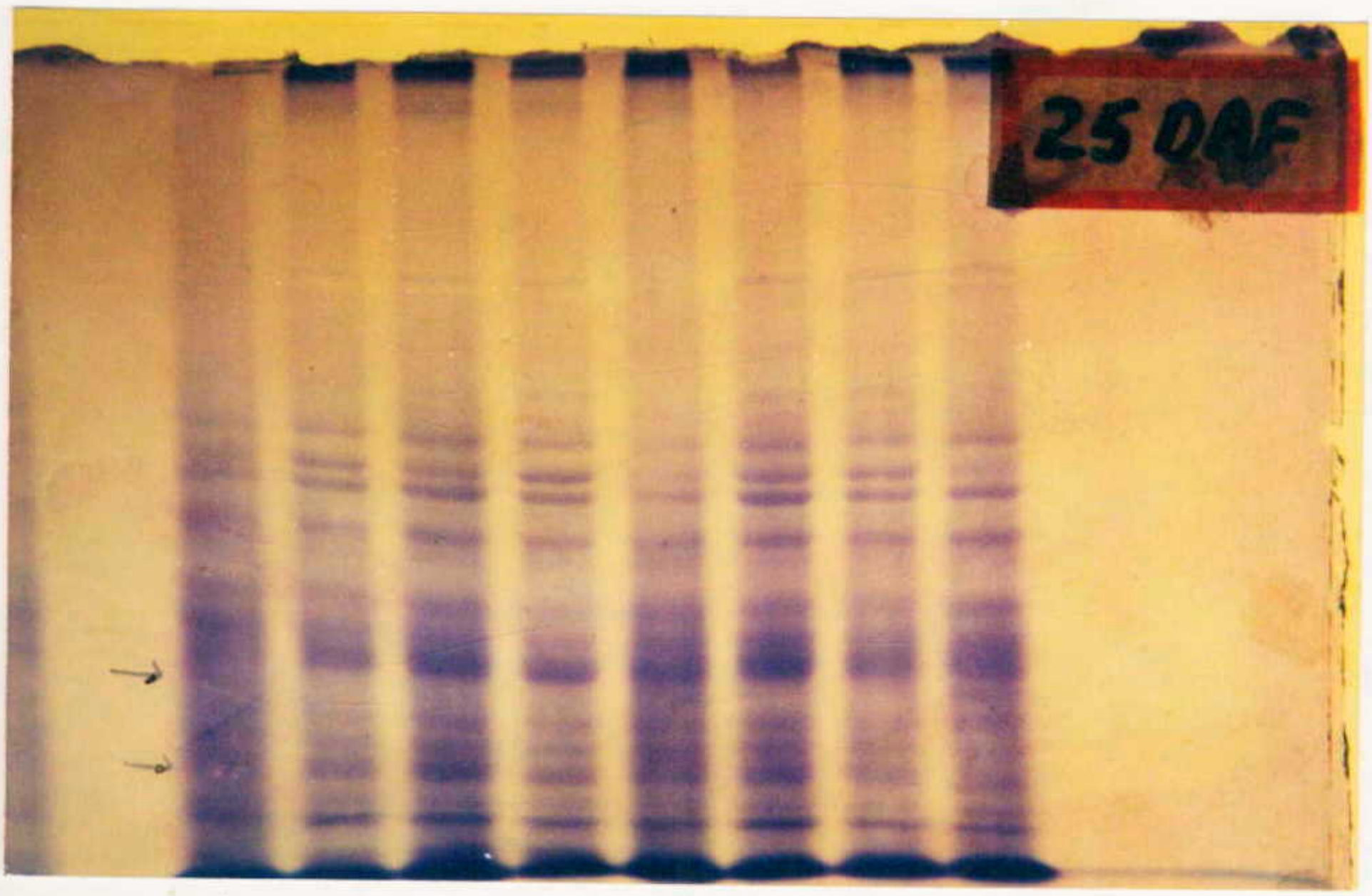


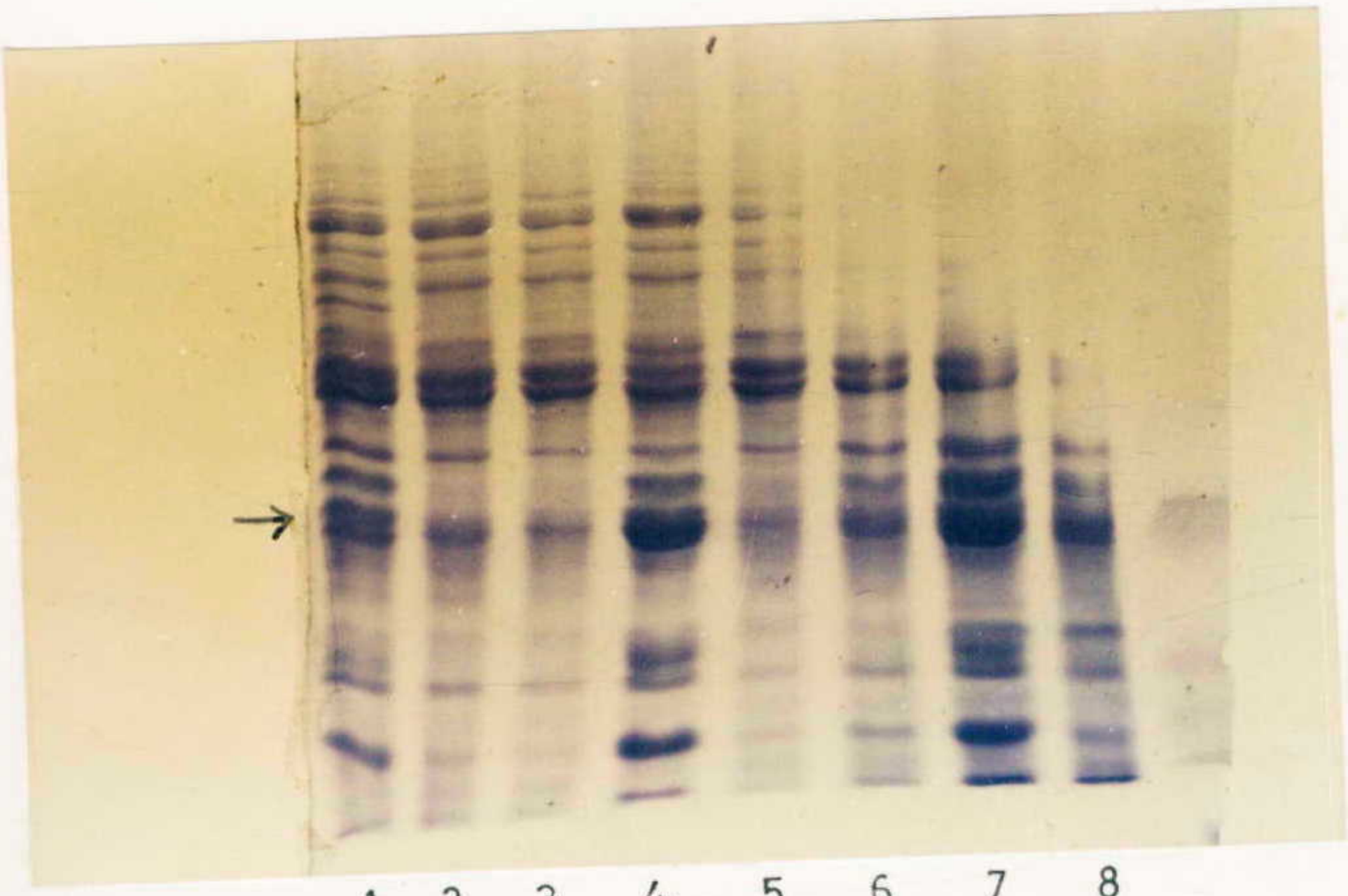
Plate 3 SDS-PAGE pattern of seed proteins of different Bengal gram genotypes at 32 DAF.

Lane-1 GGC-17; Lane-2 GGC-42; Lane-3 GGC-47
Lane-2 GGC-48; Lane-5 GGC-50; Lane-6 BG-209
Lane-3 PBG-1; Lane-7 L-550

Plate 4 SDS-PAGE pattern of seed proteins of different Bengal gram genotypes at 39 DAF.

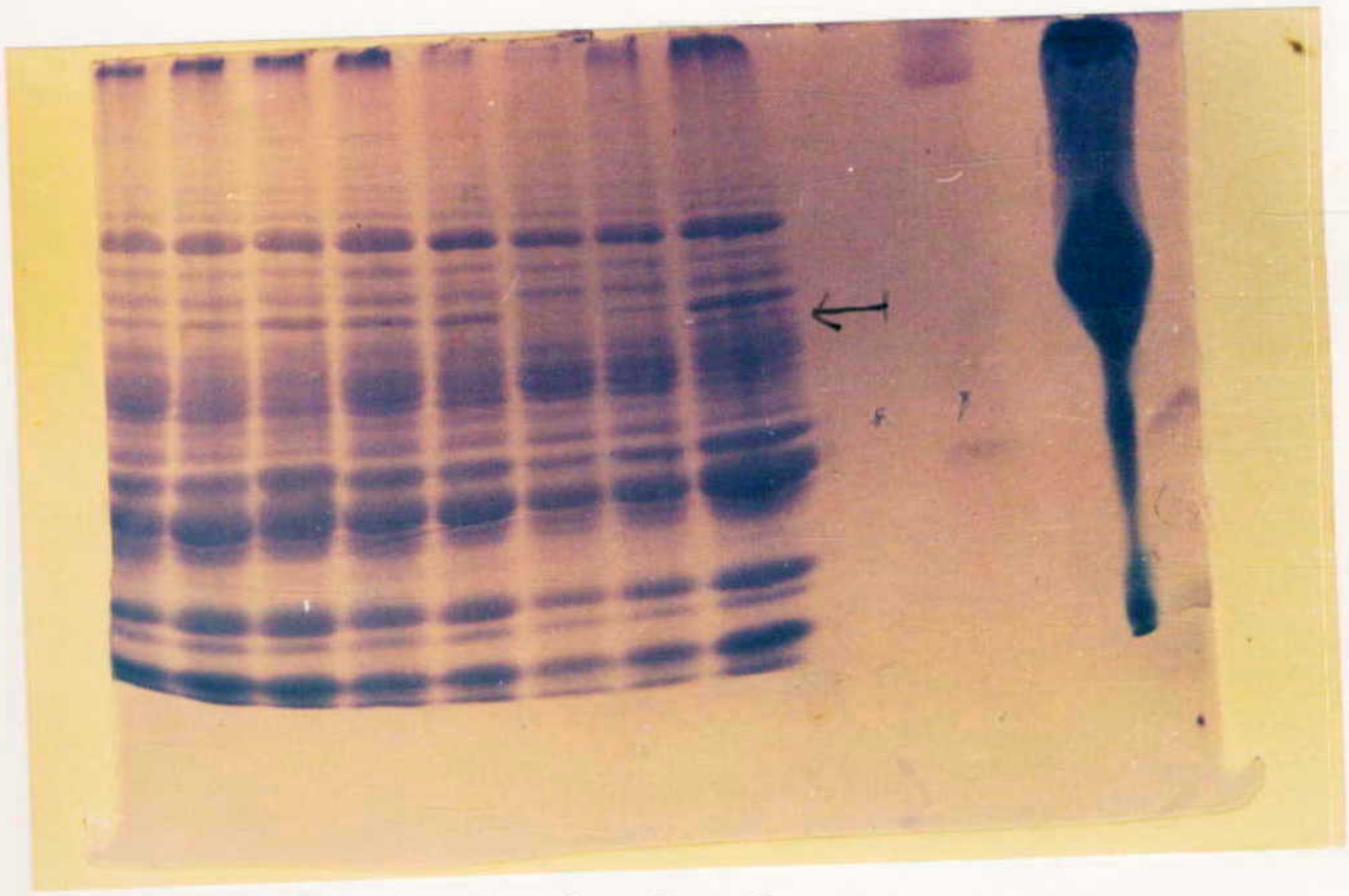
Lane-1 GGC-17; Lane-2 GGC-42; Lane-3 GGC-47
Lane-2 GGC-48; Lane-5 GGC-50; Lane-6 BG-209
Lane-3 PBG-1; Lane-7 L-550

Plate 3



1 2 3 4 5 6 7 8

Plate 4



1 2 3 4 5 6 7 8

≈66 kD (lane-8) appears in the Kabuli gram which missing in desi cultivars.

Electrophoretic pattern of seed proteins at 46 DAF (Plate-5) and at 53 DAF (Plate 6) showed that maximum accumulation of storage proteins has attained as indicated by increased band intensities.

Plate-7 shows changes in total protein subunits of genotype PBG-1 and L-550 at different stages of seed development. It showed similar banding pattern with more sharp and intensified bands in L-550 Kabuli gram towards later stages of seed development.

At maturity (Plate 8), the banding pattern of genotypes PBG-1 and L-550, total proteins showed the appearance of subunits with M.wt range 12-120 kD (lanes-2,3). Albumin (lanes-6,7) showed bands with M.wt range of 24-100 kD with a prominent subunit of M.wt 90 kD.

Globulins resolved into 9 major subunits (lanes-4,5). Within globulins the subunits with M.wt about 14, 25, 48 and 64 kD corresponded to legumin (lanes-8,9) and of M.wt around 12, 20, 35, 55 and 90 kD corresponded to vicilin (lanes -10,11) respectively. Bands corresponding to legumin were more intense as compared to vicilin at maturity (plates- 5,6) indicating that legumin is major globulin in Bengal gram. Singh *et al.*(1988) also observed similar results and showed that legumin and vicilin are in 6:1 ratio in Bengal gram.

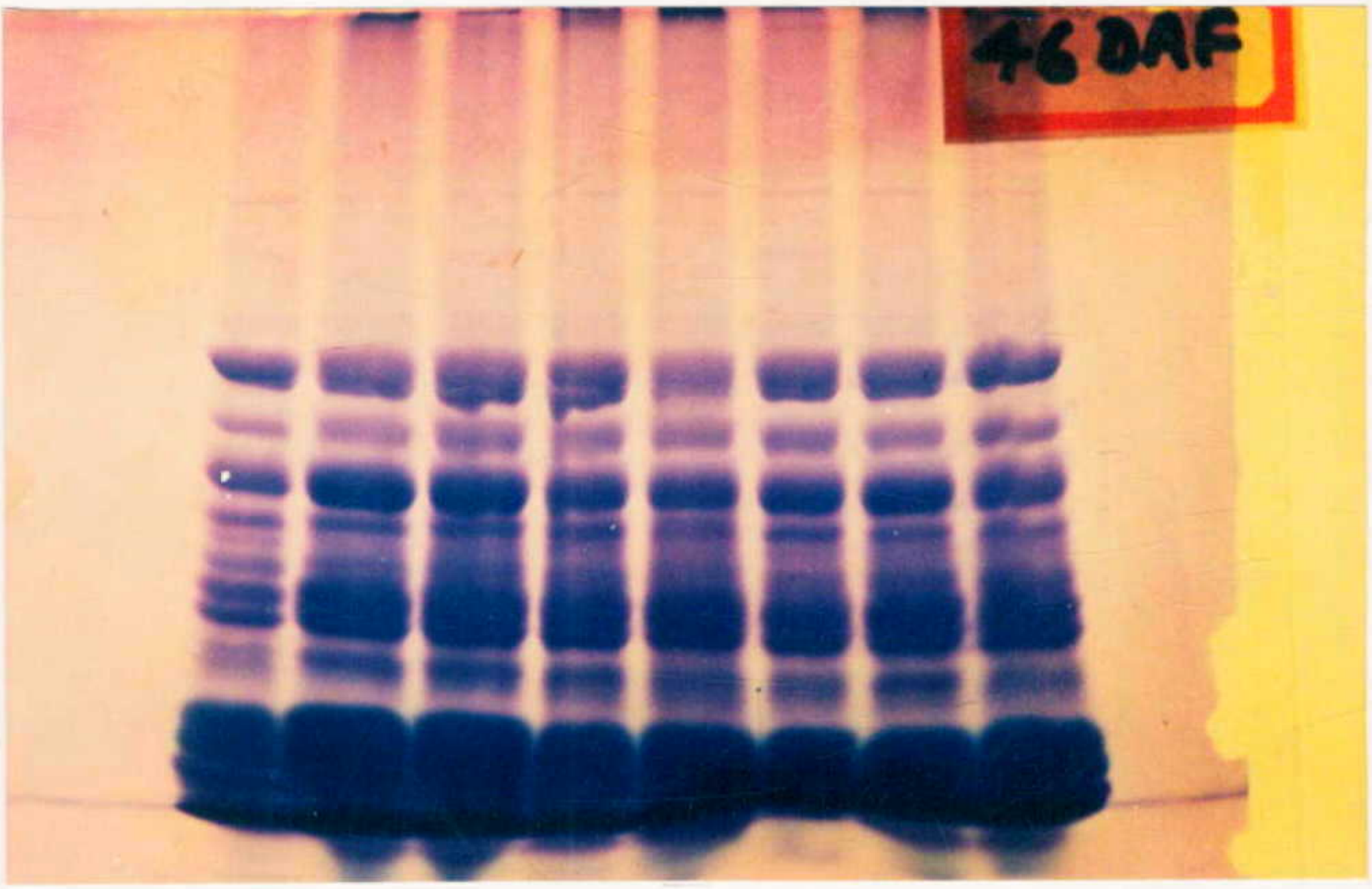
Plate 5 SDS-PAGE pattern of seed proteins of different Bengal gram genotypes at 46 DAF.

Lane-1 GGC-17; Lane-2 GGC-42; Lane-3 GGC-47
Lane-2 GGC-48; Lane-5 GGC-50; Lane-6 BG-209
Lane-3 PBG-1; Lane-7 L-550

Plate 6 SDS-PAGE pattern of seed proteins of different Bengal gram genotypes at 53 DAF.

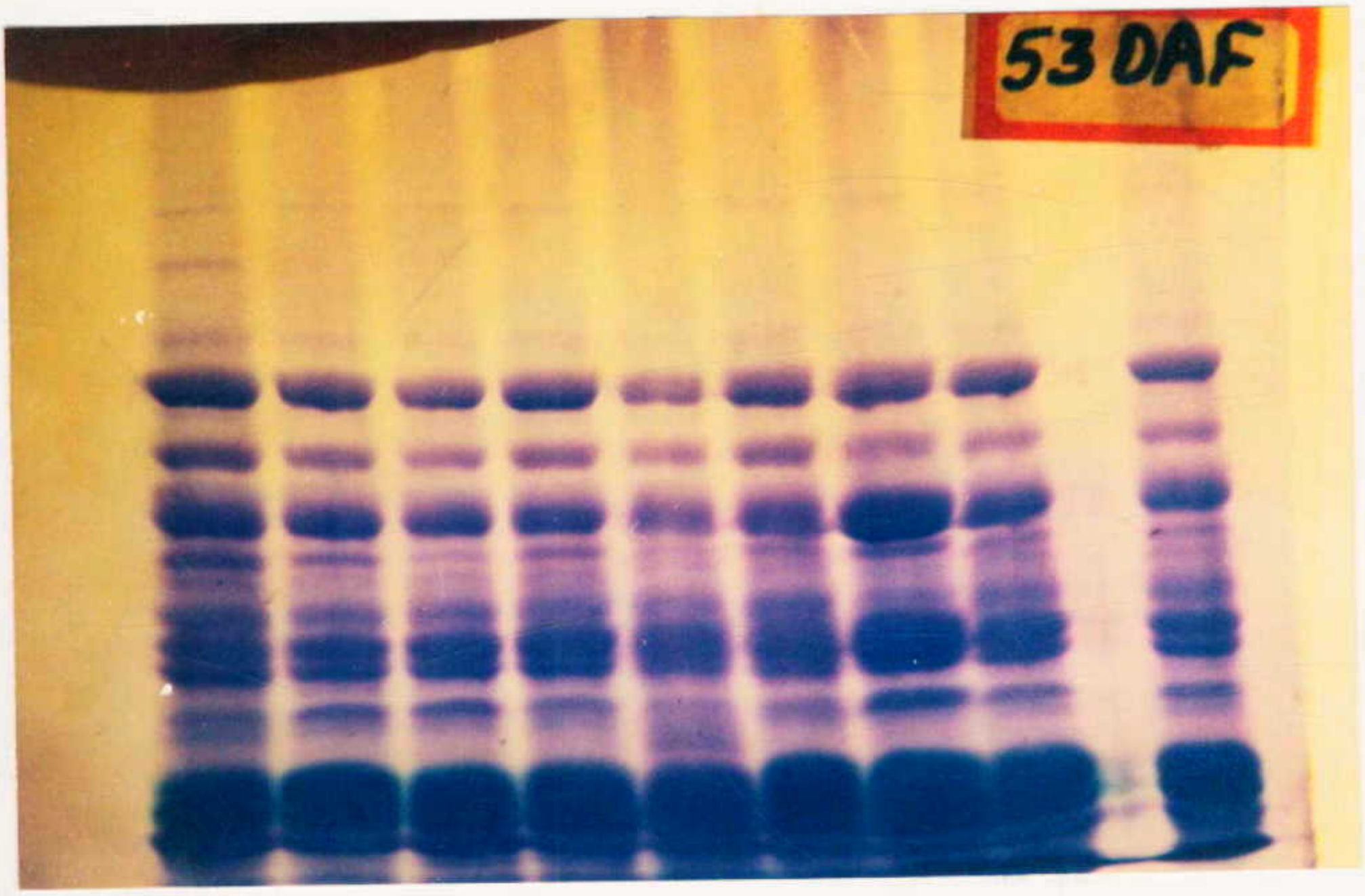
Lane-1 GGC-17; Lane-2 GGC-42; Lane-3 GGC-47
Lane-2 GGC-48; Lane-5 GGC-50; Lane-6 BG-209
Lane-3 PBG-1; Lane-7 L-550

Plate 5



1 2 3 4 5 6 7 8

Plate 6



1 2 3 4 5 6 7 8

Plate 7 SDS-PAGE pattern of seed proteins of Bengal gram genotypes PBG-1 and L-550 at different stages of development.

Lanes 1, 3, 5, 7, 9 and 11 represent seed proteins of PBG-1 at 18, 25, 32, 39, 46 and 53 DAF.

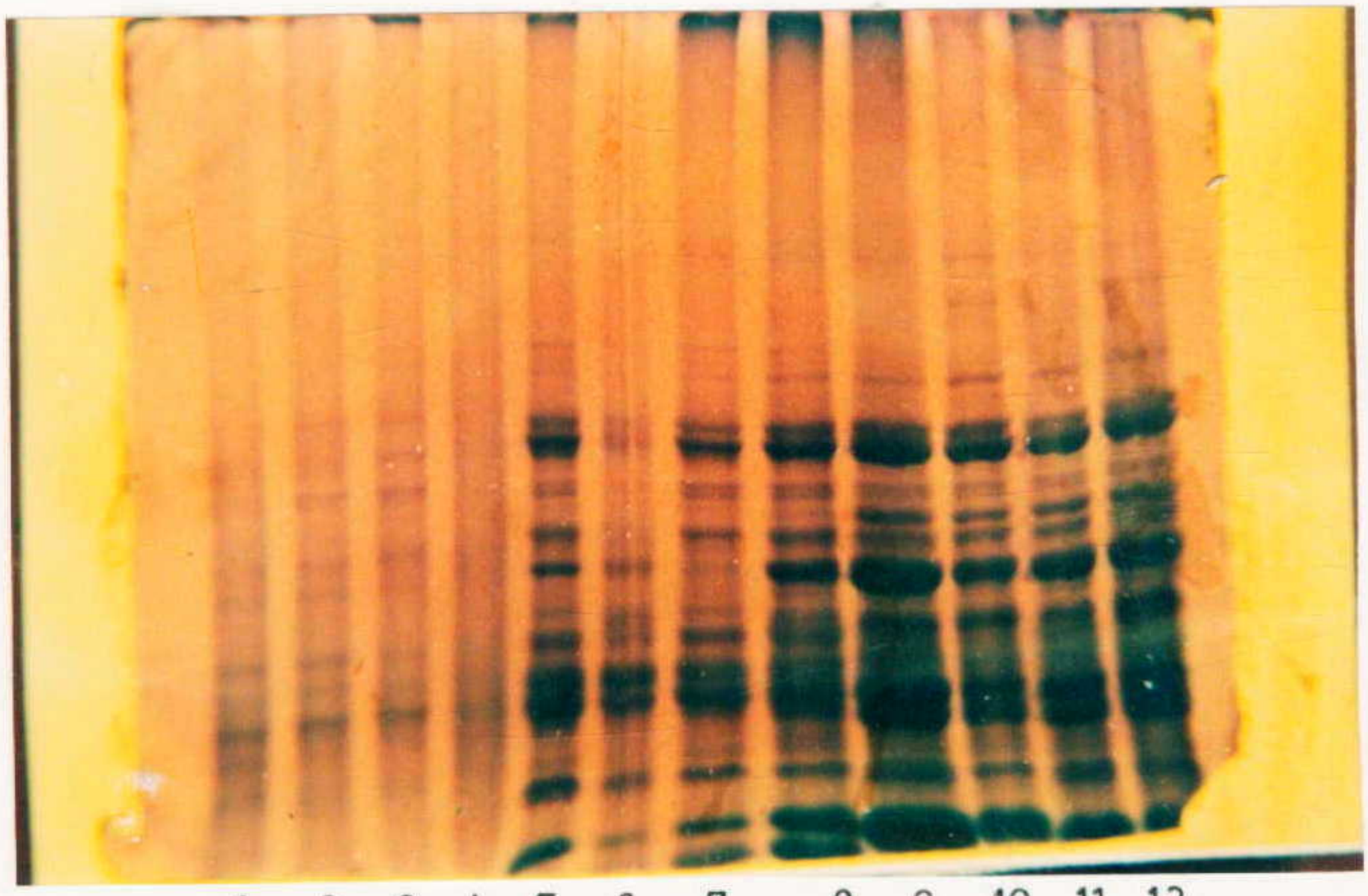
Lanes 2, 4, 6, 8, 10 and 12 represent seed proteins of L-550 at 18, 25, 32, 39, 46 and 53 DAF.

Plate 8 SDS-PAGE pattern of different protein fractions of Bengal gram genotypes PBG-1 & L-550 at maturity.

Lanes 1 and Lane-12 represent markers

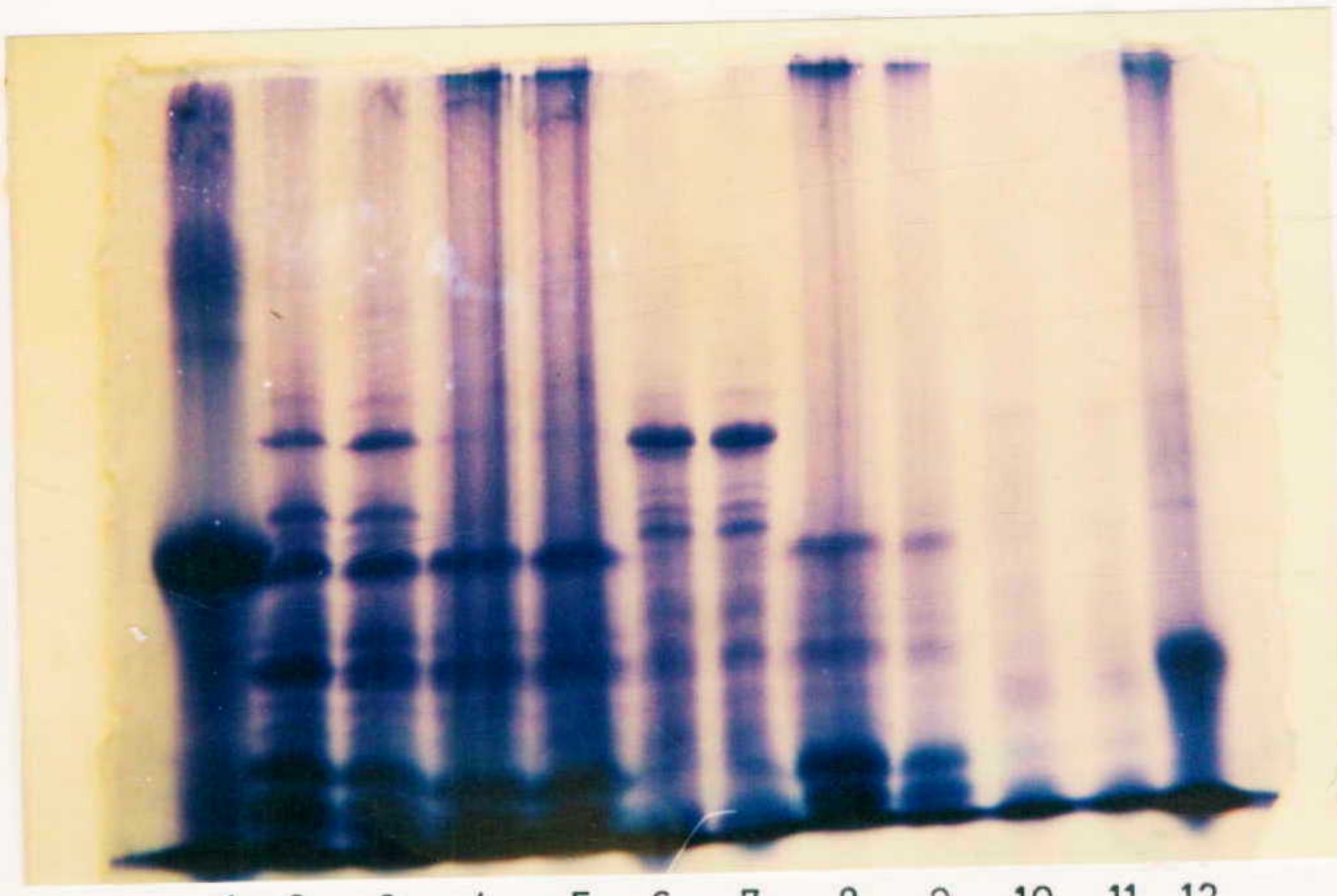
PBG-1	Lane-2 Total protein;	Lane-4 Globulin
	Lane-6 Albumin;	Lane-8 Legumin
	Lane-10 Vicilin;	
L-550	Lane-3 Total protein;	Lane-5 Globulin
	Lane-7 Albumin;	Lane-9 Legumin
	Lane-11 Vicilin;	

Plate 7



1 2 3 4 5 6 7 8 9 10 11 12

Plate 8



1 2 3 4 5 6 7 8 9 10 11 12

These results are different from that reported in mungbean in which legumin to vicilin ratio is 1:2 i.e., vicilin is major component (Sital and Narang, 1994).

4.4 ESTIMATION OF RNA CONTENT

The seeds of different Bengal gram genotypes at 18, 25, 32, 39, 46 and 53 DAF were analysed for total RNA using orcinol reagent (Schneider, 1957). The total RNA content showed differences among genotypes indicating genetic variability.

The level of RNA increased gradually from 18 DAF to 46 DAF for all the genotypes except GGC-17 and GGC-47 in which RNA content increased upto 39 DAF. Later on RNA content decreased slightly upto 53 DAF which is in accordance with findings of Goldberg *et al.* (1981) who found that transcription activity of ribosomes increased during early part of seed development and declined later on.

Fig. 7 depicts the changes in the RNA content of Bengal gram genotypes GGC-17, GGC-42, GGC-47 and GGC-48 at different stages of seed development. There are only small differences in the RNA content among these varieties between 18 and 25 DAF. Maximum RNA content was observed at 46 DAF for GGC-48.

RNA content in genotypes GGC-50, BG-209, PBG-1 and L-550 is presented in Fig.8. There were considerable variations in the RNA content among these genotypes.

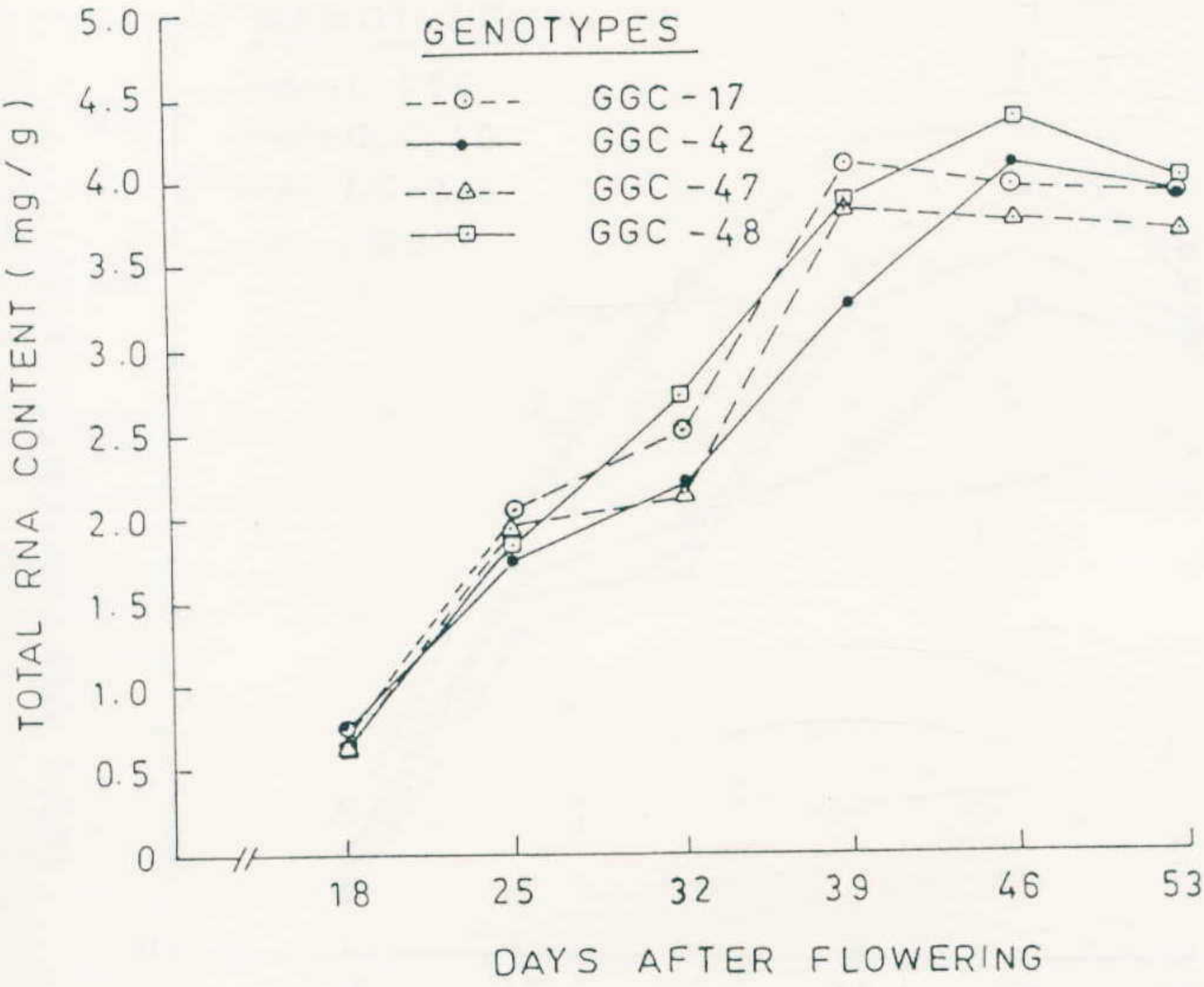


FIG. 7. RNA CONTENT (mg / g) OF BENGAL GRAM GENOTYPES AT VARIOUS STAGES OF SEED DEVELOPMENT

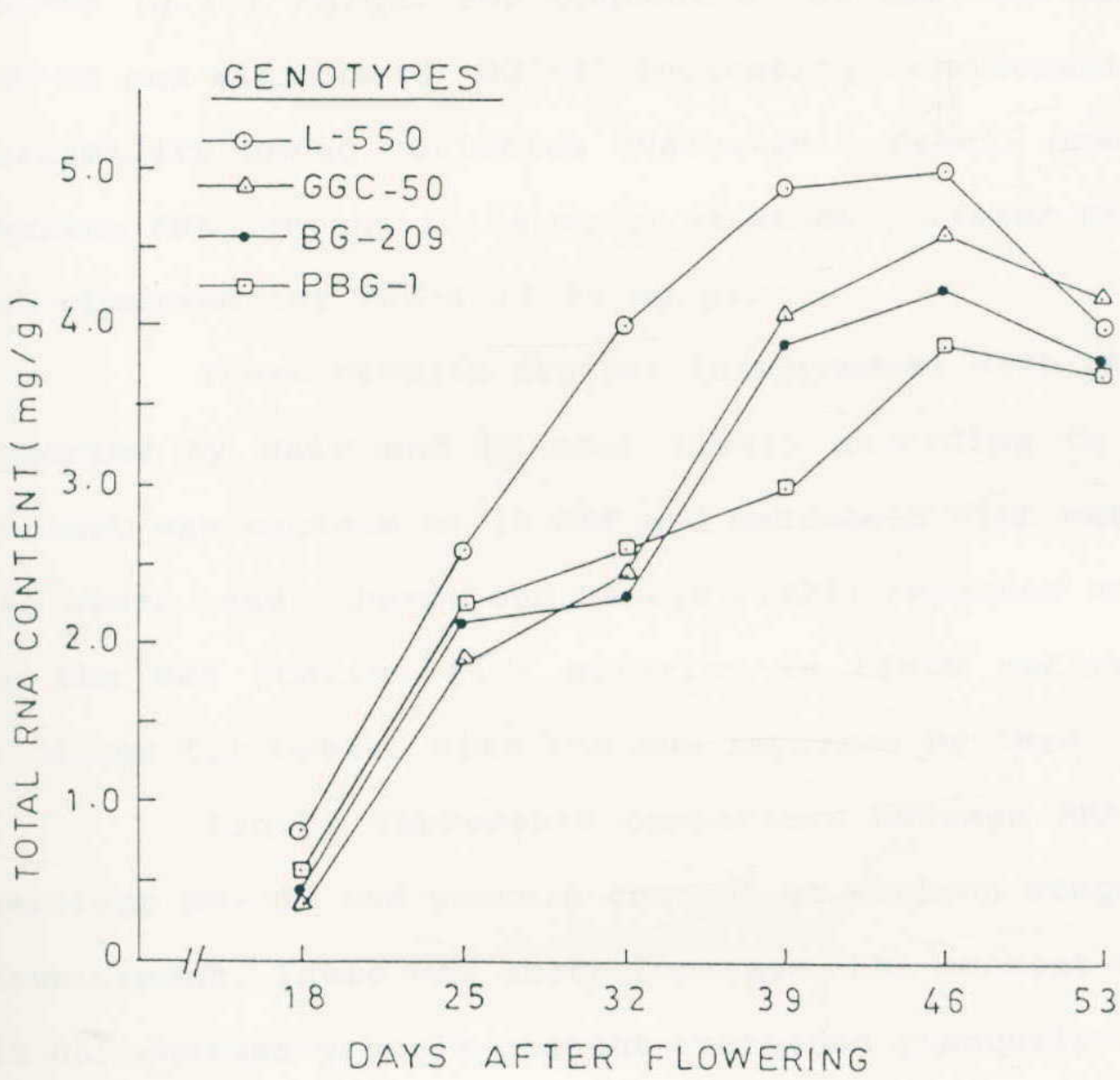


FIG.8. RNA CONTENT (mg/g) OF BENGAL GRAM GENOTYPES AT VARIOUS STAGES OF SEED DEVELOPMENT

Table 4 shows the RNA content of all the genotypes. RNA content of Kabuli gram was higher than all the cultivars from 18 to 46 DAF and decreased at 53 DAF. Among the desigram genotypes studied GGC-42 had highest RNA content (0.735 mg/g) initially (18DAF) and it was found to be lowest in case of GGC-50 (0.351 mg/g). RNA content at 53 DAF was maximum for GGC-50 and minimum for GGC-47 indicating considerable genetic variability among varieties evaluated. Kabuli gram has the maximum RNA content (5.04 mg/g) at 46 DAF, lowest RNA content was observed for PBG-1 (3.90 mg/g).

These results are not in agreement with the results reported by Nair and Koundal (1993) according to them RNA content was maximum at 18 DAF and decreased with maturity. On the other hand Scharpe and Parijs (1973) reported an increase in the RNA content till maturity in *Pisum sativum L.* Our findings fit better with the one reported by them.

Fig. 9 represents comparison between RNA level of genotype BG-209 and protein content at various stages of seed development. There was sharp increase RNA content from 18 to 25 DAF whereas protein content increased gradually from 18 to 32 DAF. Maximum increase in RNA level was found between 32 and 39 DAF whereas protein content increased maximally from 39 to 46 DAF indicating that protein synthesis follows RNA accumulation.

Table 4 : Total RNA content (mg/g) on fresh weight basis in developing seeds of different Bengal gram genotypes

GENOTYPE	DAYS AFTER FLOWERING					
	18	25	32	39	46	53
GGC-17	0.715	2.08	2.53	4.09	3.96	3.90
GGC-42	0.735	1.75	2.21	3.25	4.09	3.90
GGC-47	0.650	1.95	2.14	3.84	3.77	3.68
GGC-48	0.680	1.85	2.73	3.90	4.42	3.98
GGC-50	0.351	1.88	2.45	4.10	4.61	4.20
BG-209	0.455	2.16	2.36	3.90	4.29	3.81
PBG-1	0.550	2.25	2.34	2.99	3.90	3.70
L-550 (Kabuli gram)	0.890	2.60	4.09	4.87	5.04	4.00

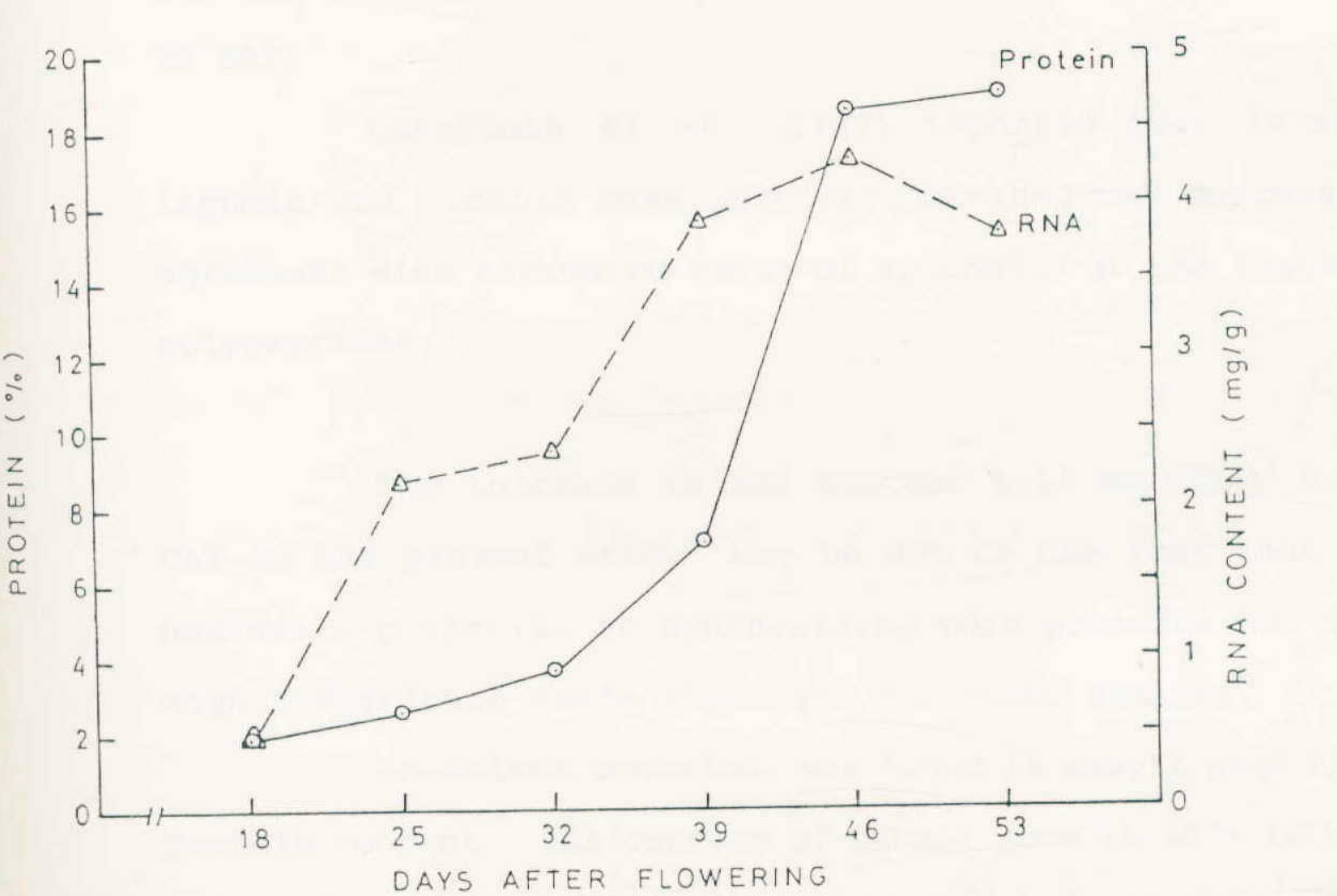


FIG. 9. COMPARISON BETWEEN SOLUBLE PROTEIN CONTENT AND TOTAL RNA CONTENT ON FRESH WEIGHT BASIS OF BENGAL GRAM GENOTYPE (B G-209).

RNA content increased slightly from 39 to 46 DAF and then decreased at 53 DAF. Scharpe and Parijs (1973) found that RNA synthesis took place most intensively from 15 to 24 DAF and was followed by synthesis of proteins between 18 and 30 DAF.

Gatehouse *et al*. (1982) reported that levels of legumin and vicilin mRNA species increased and decreased in agreement with estimated rates of synthesis of the respective polypeptides.

The increase in RNA content till maturity i.e. 46 DAF in the present study may be due to the fact that seeds had still potential of synthesising more proteins but due to high temperature seeds ripen and desiccate earlier.

Anomalous behaviour was found in kabuli gram RNA and protein content. RNA content of kabuli gram (L-550) is higher than other varieties between 18 and 46 DAF, protein content was almost similar to that found in other varieties indicating that protein content was not only determined by RNA level rather some other factor was also involved.

4.5 ISOLATION AND ELECTROPHORETIC SEPARATION OF TOTAL RNA

4.5.1 Isolation of Total RNA

The total RNA was isolated by phenol-chloroform extraction and then estimated spectrophotometrically. Five genotypes GGC-48, GGC-50, BG-209, PBG-1 and L-550 (Kabuli

gram) were chosen for these studies. Differences in RNA content were observed indicating genetic variability.

The RNA content of PBG-1 increased gradually from 0.287 mg/g at 18 DAF to 0.314 mg/g at 32 DAF. Thereafter sharp increase was observed at 46 DAF (0.784 mg/g) (Table-5).

In case of kabuli gram RNA content increased from 0.380 mg/g at 18 DAF to 0.500 mg/g at 32 DAF with a further increase to 0.920 mg/g at 46 DAF.

Fig. 10 represents comparison between RNA content of PBG-1 and L-550 (kabuli gram) at different stages of seed development. L-550 has higher RNA content than PBG-1.

Nair and Koundal (1993) observed total RNA content of Bengal gram seeds ranged between 0.650 mg/g to 1.20 mg/g. These values are higher than the values obtained in present study.

Table 6 reveals isolated RNA content in developing seeds of Bengal gram genotype BG-209. RNA content increased from 18 to 46 DAF. Then RNA content decreased slightly at 53 DAF.

Comparison between estimated RNA content and isolated RNA content of BG-209 at various stages of seed development indicated that there were large differences in the estimated and isolated RNA content (Fig.11). The reason may be that some other components also give colour with orcinol reagent. The other reason may be incomplete isolation of RNA

Table 5 : Isolated RNA content (mg/g) on Fresh weight basis in developing seeds of Bengal gram genotypes

GENOTYPE	DAYS AFTER FLOWERING		
	18	32	46
GGC-48	0.298	0.338	0.800
GGC-50	0.260	0.320	0.806
PBG-1	0.287	0.314	0.784
L-550 (Kabuli gram)	0.380	0.500	0.920

Table 6 : Isolated RNA content (mg/g) on Fresh weight basis in developing seeds of Bengal gram genotype BG-209

DAYS AFTER FLOWERING	RNA content (mg/g fresh seed)
18	0.266
25	0.506
39	0.626
46	0.693
53	0.666

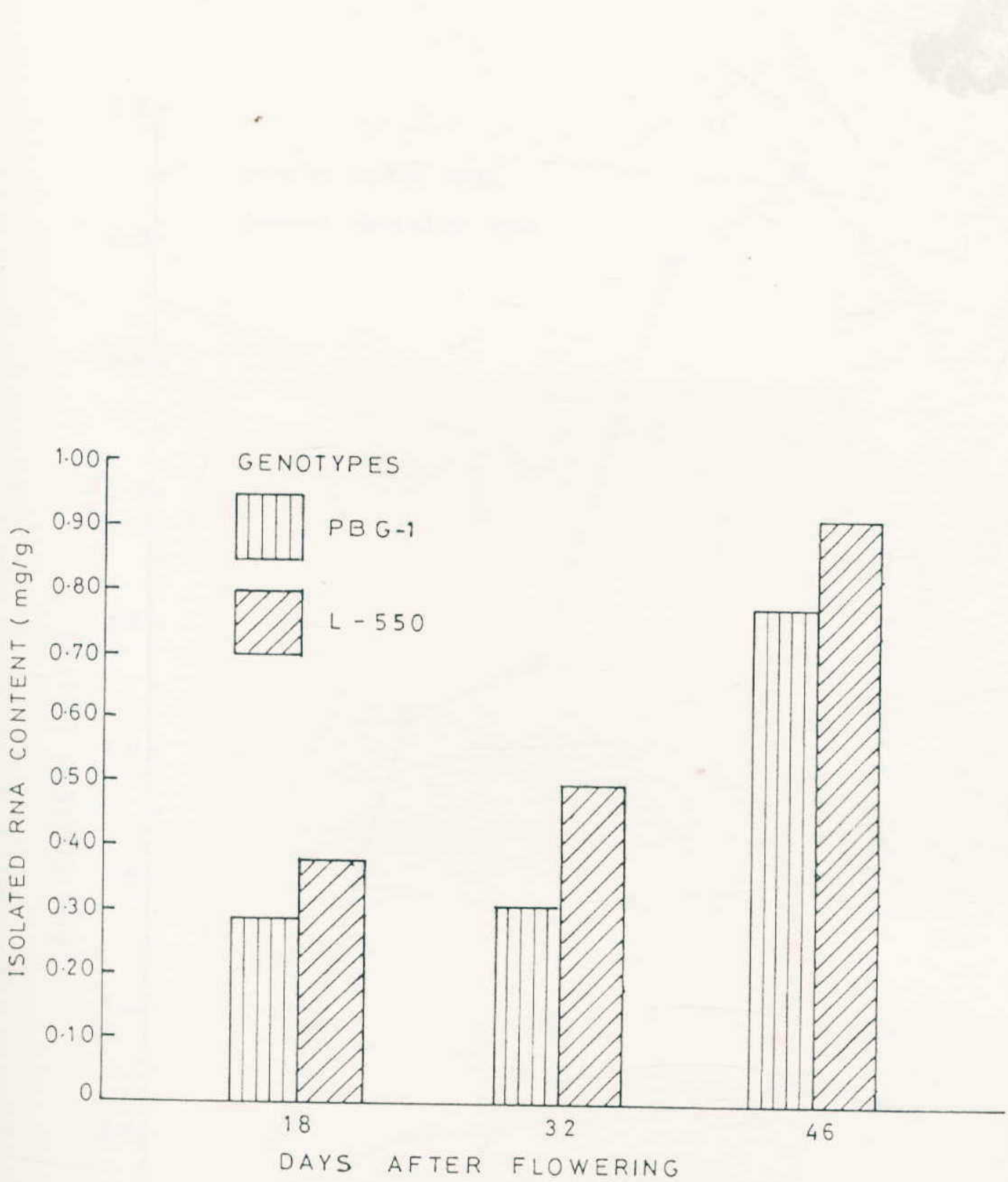


FIG.10. COMPARISON BETWEEN ISOLATED RNA CONTENT OF PBG-1 AND L-550 (KABULI GRAM) VARIETIES OF BENGAL GRAM SEEDS AT DIFFERENT STAGES OF DEVELOPMENT.

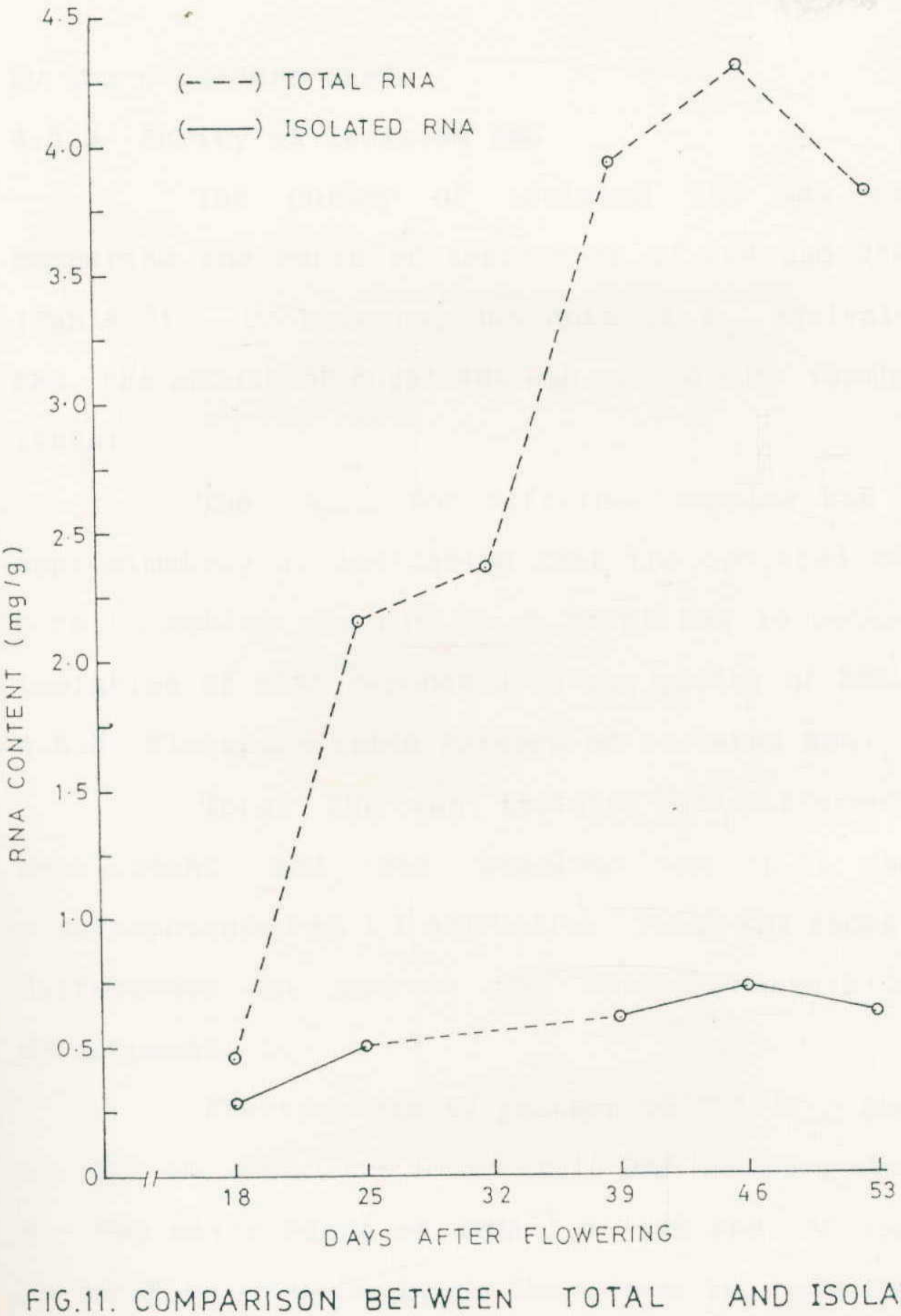


FIG.11. COMPARISON BETWEEN TOTAL AND ISOLATED RNA CONTENT (mg/g) OF BENGAL GRAM GENOTYPE (BG-209) AT VARIOUS STAGES OF SEED DEVELOPMENT.

by the procedure used.

4.5.2 Purity of Isolated RNA

The purity of isolated RNA was estimated by comparing the ratio of absorbance of 260 and 280 nm ($A_{260/280}$) (Table-7). Considering one unit of A_{260} equivalent to 40μg RNA, the amount of total RNA was worked out. (Sambrook *et al.*, 1989a).

The $A_{260/280}$ for different samples was found to be approximately 2, indicating that the isolated RNA was quite pure. Checking the purity of total RNA is necessary as the isolation of mRNA depends upon the purity of RNA isolated.

4.5.3 Electrophoretic Pattern of Isolated RNA

Total RNA was isolated at different stages of development and was resolved on 1.3% agarose gel electrophoresed in 1 X gel buffer. Total RNA shows qualitative differences on agarose gel electrophoresis during seed development.

Electrophoretic pattern of RNA from genotypes GGC-48, GGC-50, PBG-1 and L-550 at 18 DAF has been shown in Plate-9. Two major bands of rRNA i.e. 18S and 28S could be seen. In addition to major bands there were two more bands that are comparatively less clear, these may be due to 5S and 5.8S rRNA. Sambrook *et al.* (1989a) reported that total RNA contains 80-85% rRNA (28S, 18S, 5S) and 15-20% tRNA. Amount of mRNA is very less.

Table 7 : Purity of isolated RNA samples in developing seeds of different Bengal gram genotypes

GENOTYPES	18 DAF**			32 DAF			46 DAF		
	A* at 260nm	A* at 280nm	A* 260/280nm	A* at 260nm	A* at 280nm	A* 260/280nm	A* at 260nm	A* at 280nm	A* 260/280nm
GGC-48	0.279	0.141	1.97	0.316	0.167	1.89	0.750	0.385	1.95
GGC-50	0.243	0.126	1.92	0.300	0.152	1.98	0.755	0.372	2.03
PBG-1	0.269	0.135	2.00	0.294	0.153	1.92	0.735	0.383	1.92
L-550	0.356	0.170	2.10	0.468	0.232	2.02	0.862	0.466	1.85

BG-209

DAF	A* at 260 nm	A* at 280 nm	A* 260/280
18	0.249	0.131	1.90
25	0.474	0.243	1.95
39	0.589	0.295	2.00
46	0.650	0.295	2.20
53	0.624	0.317	1.97

A* = Absorbance
DAF** = Days after flowering

Plate 9 Agarose gel electrophoretic pattern of RNA isolated from four genotypes of Bengal gram at 18 DAF

Lanes 1-4 represent genotypes GGC-48, GGC-50, PBG-1 and L-550

Plate 9

Plate-10 shows the total RNA isolated from GGC-48, GGC-50, PBG-1 and Kabuli gram (L-550) at 32 DAF. Bands of 18S and 28S are quite clear at this stage. Additional bands seen at 18 DAF stage are absent at this stage.

Total RNA isolated from BG- 209 at 6 different stages of seed development has been shown in Plate 11. At 18 DAF no band was seen and only mRNA smear was present indicating that quantity of total RNA at this stage was too low. At 25 DAF bands due to 18S and 28S RNA became quite clear indicating rapid increase in RNA content from 18 to 25 DAF and that rRNA fractions form major part at this stage. Bands were quite intense at 39 DAF. Band intensity increased at 46 DAF and 53 DAF but clarity of bands at 53 DAF was comparatively less. tRNA was seen as a band at the end of the run.

4.6 ISOLATION AND ESTIMATION OF mRNA

Messenger RNA was isolated from the total RNA of BG-209 at three stages of seed development (18, 39 and 53 DAF). RNA isolated from 3g of seeds was loaded on the oligo-(dT) cellulose column and different fractions of 200 μl each were collected. Content of rRNA+tRNA fractions was higher initially and then decreased (Table-8) indicating that 2 ml of buffer was sufficient for eluting rRNA+tRNA fractions.

mRNA content was lower in fraction-I and it increased in fraction-II and then decreased (Table-8), so that 1 ml elution buffer is sufficient for getting concentrated

Plate 10 Agarose gel electrophoretic pattern of RNA isolated from four genotypes of Bengal gram at 32 DAF.

Lanes 1-4 represent genotypes GGC-48, GGC-50, PBG-1 and L-550

Plate 10

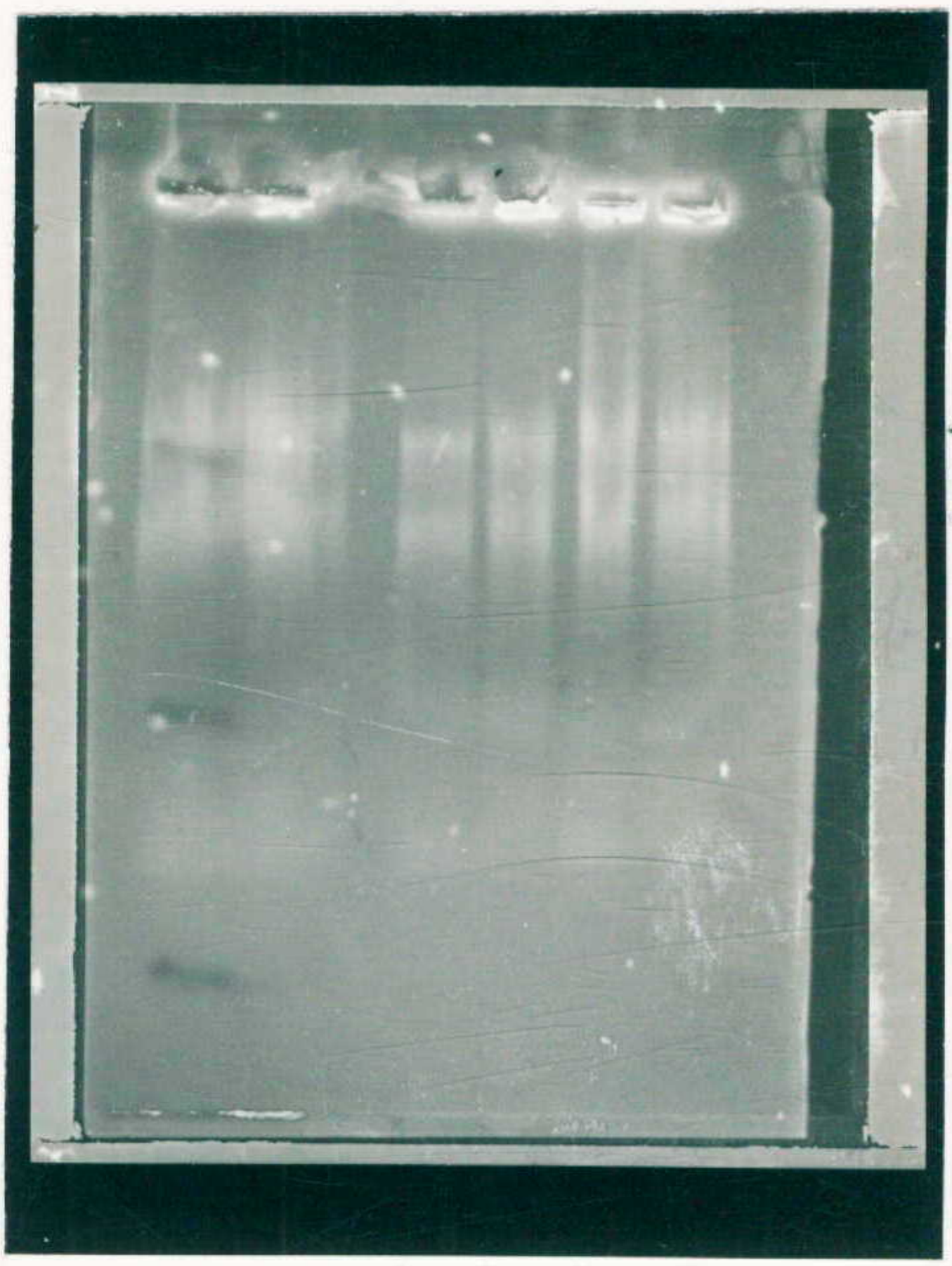
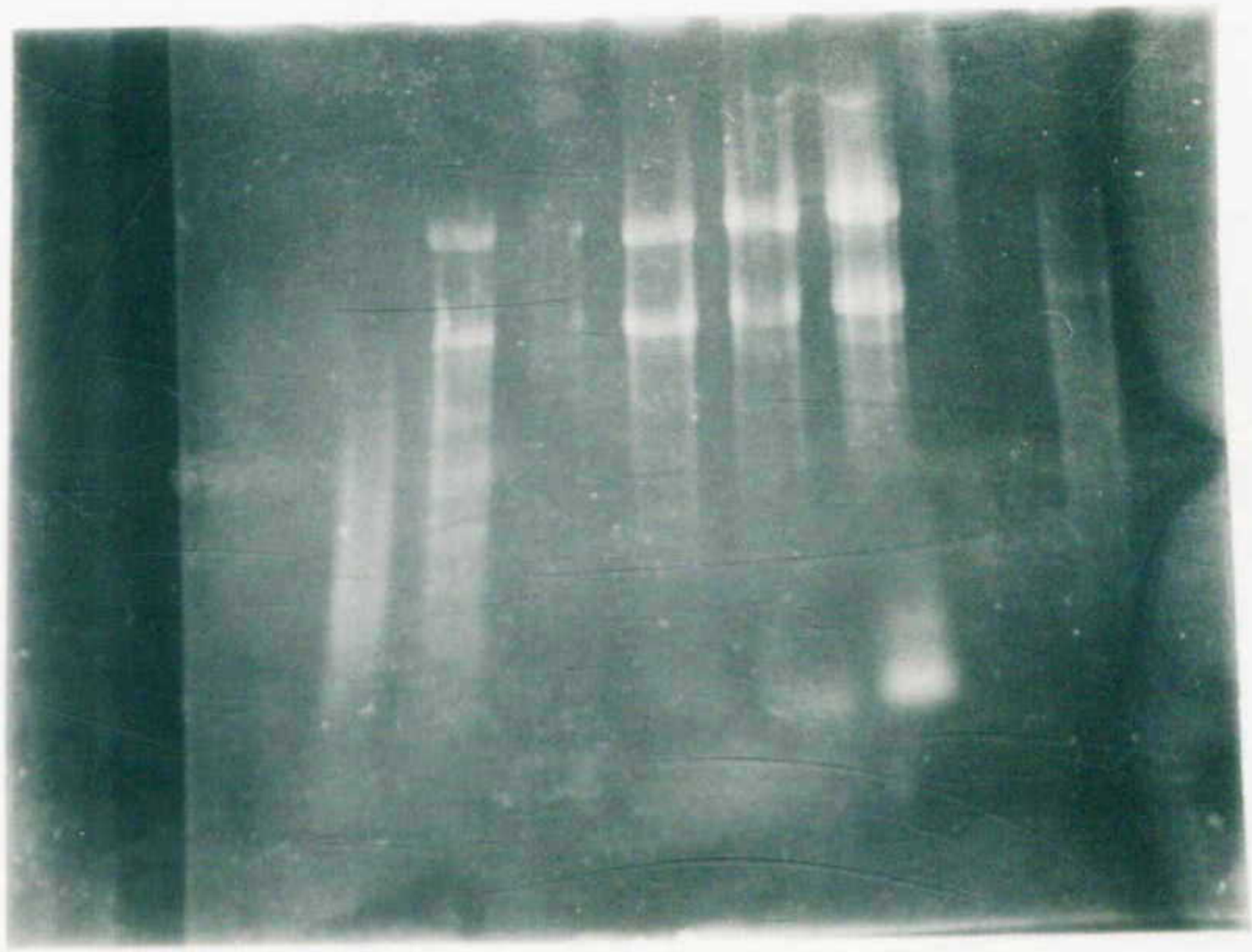


Plate 11 Agarose gel electrophoretic pattern of RNA isolated from Bengal gram genotype BG-209.

Lanes 1-6 represent isolated RNA at 18, 25, 32, 39, 46 and 53 DAF respectively.

Plate 11



1 2 3 4 5 6

mRNA. Efficiency of the column was approximately 88% as was indicated by the fact that we did not get amount of fractions equal to the loaded RNA.

The amount of mRNA was 6.10 μg/g at 18 DAF, increased to 13.2 μg/g at 39 DAF and then decreased to 12.7 μg/g at 53 DAF (Table-9). Nair and Koundal (1993) also reported similar results but the amount of mRNA was lower in our case which may be due to degradation of some mRNA by the activity of ribonucleases. Further total RNA content in our case was also lower. Davies and Larkins (1973) have also reported a decline in polysomal mRNA during the later part of seed development in pea.

Since value of mRNA was quite higher even at maturity, it indicated that mRNA was not quickly degraded even at maturity. Gatehouse *et al.* (1982) also reported that storage protein mRNA species are relatively long lived. Table 9 shows the percent of mRNA that could be isolated from total RNA. The percentage of poly (A$^+$) mRNA decreased from 2.29 to 1.91 towards maturity. High percentage of mRNA at 18 DAF might be an indication that whole of cell machinery is involved in the synthesis of proteins.

Comparison between RNA content and mRNA content indicates that there was slight increase in total RNA from 39 to 53 DAF, while mRNA decreased from 39 to 53 DAF (Fig.12). Evans *et al.* (1984) also found that non mRNA species (i.e.

Table 8 : Separation of different RNA fractions of BG-209 by oligo-(dT) cellulose column chromatography at different stages of seed development

FRACTIONS	DAYS AFTER FLOWERING		
	18 Loaded RNA = 800 μg	39 Loaded RNA = 1880 μg	53 Loaded RNA = 2000 μg
[rRNA + tRNA FRACTIONS]			
	(μg)	(μg)	(μg)
Fraction 1	400	1000	1256
Fraction 2	100	408	365
Fraction 3	91.2	88.0	76.6
Fraction 4	69.6	30.4	5.6
Fraction 5	13.6	8.0	4.8
Fraction 6	13.6	0.0	3.2
TOTAL	688.0	1534.4	1711.2
[mRNA FRACTIONS]			
Fraction 1	5.10	1.60	8.40
Fraction 2	8.80	29.4	23.4
Fraction 3	3.60	7.20	6.00
Fraction 4	0.80	1.60	0.45
TOTAL	18.3	39.8	38.25

Table 9 : Percentage of messenger RNA in total RNA from seeds of BG-209 at different stages of development

Days after flowering	Total RNA mg/g seed	mRNA µg/g seed	* mRNA as percent of total RNA
18	0.266	6.10	2.29
39	0.626	13.2	2.11
53	0.666	12.7	1.91

* mRNA content x 100/Total

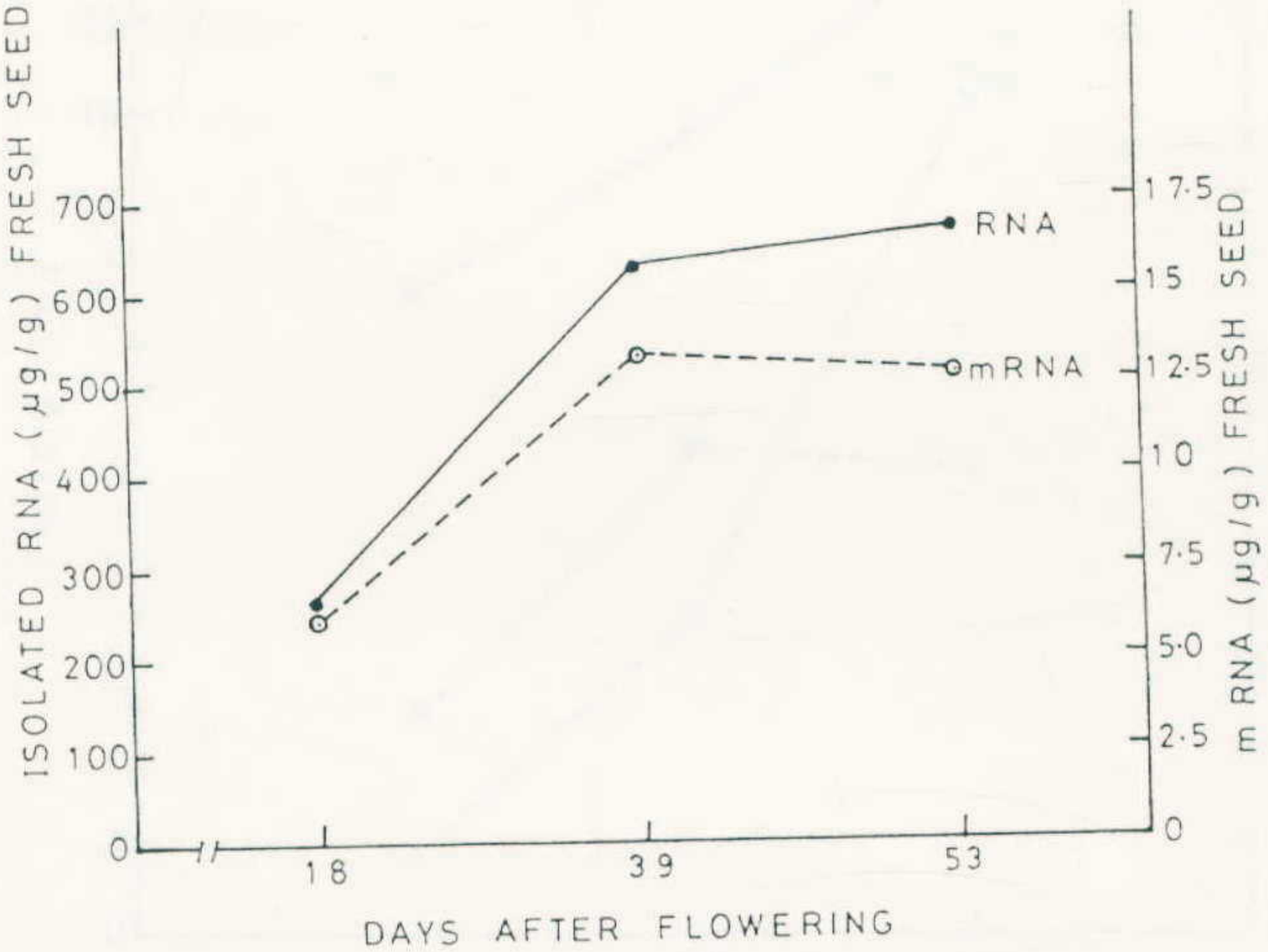


FIG.12. RNA AND mRNA CONTENT IN DEVELOPING SEEDS OF BENGAL GRAM GENOTYPE (BG-209).

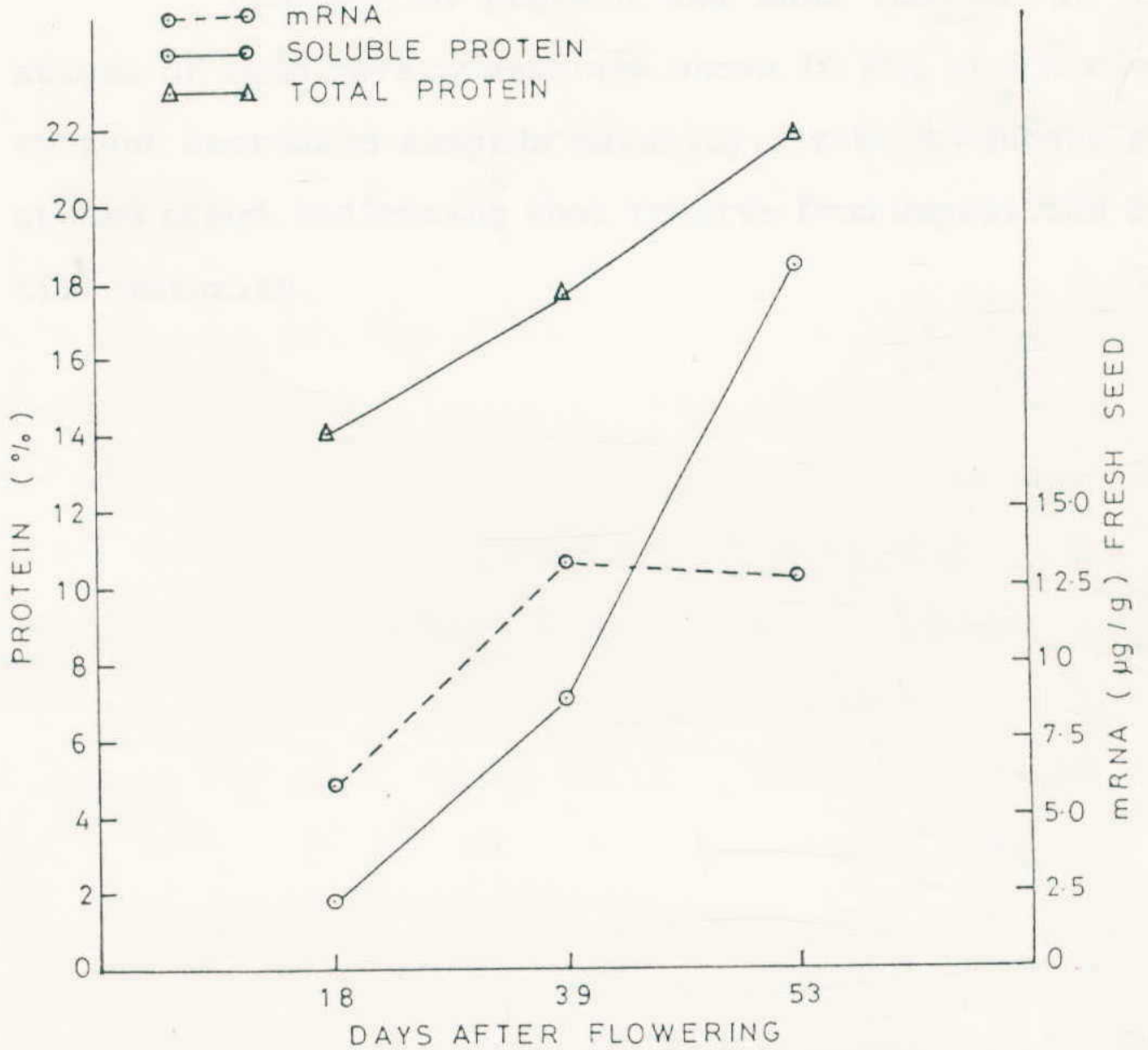


FIG.13. PROTEIN AND mRNA CONTENT IN DEVELOPING SEEDS OF BENGAL GRAM GENOTYPE (BG-209).

rRNA+tRNA) form the majority of transcripts at maturity of pea seeds.

Changes in protein and mRNA content at different stages of seed development are shown in Fig 13. Although mRNA content decreased towards maturity, protein content showed an upward trend, indicating that reserve food deposition continues till maturity.

CHAPTER V

SUMMARY

The present study was aimed at understanding the relationship between protein synthesis and RNA accumulation in developing Bengal gram seeds and to identify varietal differences. To achieve this objective the pattern of protein synthesis and accumulation, isolation, estimation and electrophoretic separation of total RNA and isolation of mRNA have been investigated in eight genotypes of Bengal gram. The summary of results obtained and main conclusions are as follows:

1) Percent dry matter of Bengal gram seeds increased from 18 to 53 DAF with maximum increase between 32-46 DAF.

2) Total protein content of Bengal gram genotypes ranged between 11.1 to 23.5% on dry weight basis from initial seed setting to maturity. Among all the genotypes studied GGC-47 was found to be the best in terms of protein content.

3) Although electrophoretic pattern of seed proteins of different genotypes was almost similar at initial and final stages of seed development, intervarietal differences were observed at 32 and

39 DAF. Bands corresponding to legumin were more intense at maturity of seeds, whereas bands corresponding to vicilin subfractions were comparatively less intense indicating that legumin in major globulin of Bengal gram. Bands corresponding to albumin fractions were visible from early stages of seed development.

4) Total RNA content of Bengal gram seeds estimated by orcinol method (Schneider, 1957) showed differences among genotypes indicating genetic variability. The level of RNA increased gradually from 18 DAF to 46 DAF for all the genotypes except GGC-17 and GGC-47 in which RNA content increased upto 39 DAF. Later on RNA content decreased slightly upto 53 DAF.

5) Maximum increase in RNA level was found between 32 and 39 DAF, whereas protein content increased maximally from 39 to 46 DAF indicating that protein synthesis follows RNA accumulation.

6) Content of isolated RNA was maximum at 46 DAF for all the genotypes studied. L-550 had maximum and BG-209 had minimum RNA content. The isolated RNA was found to be quite pure as the A260/280 ratio was close to 2.

7) Electrophoretic pattern of RNA from Bengal gram seeds at different stages of development showed two

major bands corresponding to rRNA. Bands were less clear initially (18 DAF) but band intensity increased later on. mRNA was seen in the form of a smear.

8) The amount of mRNA in BG-209 was 6.10 μg/g at 18 DAF, increased to 13.2 μg/g at 39 DAF and then decreased to 12.7 μg/g at 53 DAF. The percentage of mRNA in total RNA was 2.29 at 18 DAF and decreased to 1.91 at maturity (53 DAF).

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* Original not seen.