BIOCHEMICAL ANALYSIS OF THE GENUS *Pisum* L. THROUGH SDS-PAGE

A dissertation submitted in the Partial Fulfillment of the Requirements for the degree of Master of Philosophy in CELL AND MOLECULAR BIOLOGY

BY

MADIHA IFTIKHAR

DEPARTMENT OF PLANT SCIENCES,
FACULTY OF BIOLOGICAL SCIENCES,
QUAID-I-AZAM UNIVERSITY
ISLAMABAD, PAKISTAN.
2009

In the name of Allah, the Most Compassionate and the Most Merciful

But ALLAH bears witness by what He has revealed to you that HE has revealed it with His knowledge, and the angels bear witness (also): and ALLAH is sufficient as a witness".

(Al-Quran)

DECLARATION

This is to certify that this dissertation entitled **"Biochemical analysis of the genus *Pisum* L. through SDS-PAGE"** submitted by Madiha Iftikhar is accepted in its present form by the Department of Plant Sciences, Quaid-i-Azam University, Pakistan, as satisfying the dissertation requirement for the degree of *M.Phil.* in Cell and Molecular Biology.

Supervisor: ----------

Dr. Abdul Samad Mumtaz

External Examiner: ----------

Chairman: ----------

Prof. Dr. Mir Ajab khan

Date: 7-3-2009 ----------

Dedicated

To

My worthy parents and sweet siblings

SPECIAL THANKS
TO
Nazish Bostan
Sidra sultan
And
Nazia Nazar

ACKNOWLEDGMENTS:

All Acclamation and Appreciation are for ALMIGHTY ALLAH, the most Magnificent and the most Merciful who is perfect in all respects and who showered his countless blessings and courtesies on me to complete this work. Praise and salutation to the city of wisdom and knowledge, Hazrat Muhammad (S.A.W) who declared it an obligatory duty for every Muslim to seek knowledge.

I deem it my utmost pleasure to extend my sincerest appreciation to Prof. Dr. Fayaz, Dean Faculty of Biological Sciences for his benign and ample support. I am thankful to Prof. Dr. Mir Ajab Khan, Chairman Deptt. of Plant Sciences, Quaid-i-Azam University, Islamabad who encouraged me to embark and achieve my goal. I am grateful to my supervisor Dr. Abdul Samad Mumtaz , whose help enabled me to get my goal through the pencil onto a piece of paper.

Acknowledging the many debts, I would particularly like to thank Asst. Prof. Dr. Tariq Mahmood who had been an ardent and a candid guide for me and encouraged me during my whole research work.

Undescribable thanks to my senior Nazish Bostan and my friend Sidra Sultan who both had been inkling for me and did every possible to help me even in cumbersome situations. Without their guidance and persistent help this dissertation would not have been possible. I am indebted to both and have no words to say.

Special thanks to my sweet friend and colleague Nazia Nazar whose care, sincere and excellent guidance eliminated my hurdles during research work. I am extremely grateful to her for supporting me in every thick and thin. Moreover, I am thankful to my senior Nadia Mubarik and my friend Samiya Rehman for providing intellectual, scientific and academic approach throughout my research work.

I am grateful to my juniors Uzma, Samina and Ayesha for their support and encouragement.

I would like to express the deepest appreciation to my colleague Waqas for his impetuous behaviour and giving this thesis a final shape.

I set my unfeigned thanks to my father Muhammad Iftikhar and my mother who vigourated me with fortitude.I pay my cordial obligation to my great father whose super

moral training gave me self confidence and my mother who always raised her hands for my zenith and success.

My heartfelt gratitude to my sisters Fozia, Bushra and Mahvish for their kind and caring attitude, patience, prayers and love during my research work. I pay special thanks to my sister Bushra who being in hostel cared for me and tolerated my behaviour during hard times in my research work. I have warm wishes for my younger brother Awais, who couldnot understand but used to ask about my research in an interesting manner. I wish to express my special gratitude to my brother in law Kamran bhai for his keen interest and guidance during my research work. Thanks to everyone who raised his hands for my success.

Madiha Iftikhar

CONTENTS:

LIST OF GELS:

LIST OF TABLES:

LIST OF UPGMA CLUSTER ANALYSIS:

ABSTRACT:

The present study was conducted with the objective of finding out protein polymorphism in order to investigate the relationship between and within worldwide and cultivated species of the genus *Pisum.*

For biochemical analysis , SDS-PAGE was performed which was efficient in detecting polymorphism and genetic variation within and among *Pisum* species. The data was analyzed using software NTSYS .The results showed a considerable degree of polymorphism. Mixed pattern of grouping in the analysis indicated close affinities of species with each other.

INTRODUCTION

INTRODUCTION:

Pisum sativum L. (commonly known as pea) is an annual herbaceous plant having both wild and cultivated forms. It is grown at higher altitudes in tropics and requires a cool, relatively humid climate (Duke, 1981; Davies, 1985). Initially the name for the legume was "pise" acquired from the Latin "*Pisum*". Pea is not frost sensitive and is widely grown in varying conditions.

P. sativum is placed within the tribe Vicieae of the family Papilionaceae and is thus closely related to *Vicia* and *Lathyrus* (Ellis and Poyser, 2002). During the 18th century Gregor Mendal in his classic work on the genetics of pea recognized 32 different types. Today about 10,000 cultivars have been recorded most from temperate areas of the world were the crop is grown best (Almgard, G., 1970).

HISTORY AND ORIGIN:

The pea has been known since Antiquity. Theophrastus in his book "Enquiry Into Plants" mentioned peas much earlier than 3000 B.C. The pea crop was known in Europe since the Prehistoric age. Peas dating from Stone Age have been discovered in lake dwellings of Switzerland (Fourmont, 1956). According to Gibault (1912), in France, peas were found in dwellings during Bronze age (1000- 2000). Recently Smartt (1990) in his book "Grain Legumes: Evolution and Genetic Resources" indicated that pea dates back 7000-6000 B.C.

Pea is one of the oldest grain legumes during the Early Neolithic (more than 80,000 years ago) in the near East (Zohary and Hopf, 1994). Pea was originally cultivated as a winter annual crop in the Mediterranean region. Mediterranean region is rich in grain legumes and the study of crop evolution began here (Smartt, 1990). It is regarded as the primary center of diversity (Ambrose, 1995). It was introduced into America soon after Columbus and thereafter spread to the temperate zones of Europe (Kay, 1979: Makasheva, 1983). Pea was taken to China in the First century and was introduced in United Kingdom in the

Middle Ages (Davies et al., 1985). Based on genetic diversity, peas probably originated in Abyssinia and Afghanistan while areas in Mediterranean colonized later. From these areas, the pea spread to other parts of Europe and Asia. (Fourmont, 1956). Erskine *et al.* (1994) mentioned that peas were rare at Jamo and Iraq (6750 B.C.), while they constituted the prevalent pulse at a Neolithic site at Erbaba in Turkey (5800-5400 B.C.). Evidence from carbonized remains indicated that pea had been cultivated with cereal crops such as wheat and barley since domestication.

At a taxonomic level there has been a recent increase in genome size within the Vicieae and this appears to be associated with the accumulation of repetitive elements rather a consequence of segmental duplication. This description is in close agreement with the model set out by Thompson and Murray (1980), but can be taken be further by a genetic analysis of the repetitive sequences that appear to have accumulated in *Pisum*.

The genome size ranges approximately 10 fold within the genera *Vicia* and *Lathyrus* indicates that within the period of time associated with speciation events, there have been large scale changes in genome size in this taxonomic group. Within *Pisum* which is represented by a large number of species, variation in genome size has been described clearly by Baranyi *et al.* (1996) who showed an approximately 10% difference between some accessions, consistent with a slightly larger genome in *P. fulvum* and *P. abysinicum* than in *P. sativum*. These observations suggest that the accumulation of the repetitive elements has been an active process during the diversification of *Pisum*.

MORPHOLOGY AND FLORAL BIOLOGY:

This annual plant ($2n = 14$) has hermaphrodite flowers and is self pollinating (Lazaro and Aguinagalde, 2006).Flowering time is from May to September and seeds ripen from July to October. (Lazaro and Aguinagalde, 2006). Pea plant is a climber and all of the species have different morphological characters (Waines, 1975) and many intermediate forms are also found at various ecological transition zones (Ben-Zen and Zohary, 1973).

Pisum is classified into six sub-species on the basis of morphology. The wild forms include *P. fulvum*, *P.humile*, *P. elatius* and *P. formosum* where as the cultivated forms

include *P. sativum* and *P. abysisnicum* (Giles, 1975). These wild species often have tall, slender and branched stems, purple or pink flowers and small pods producing a small quantity of seeds with colored coat but *P. elatius* has large pods and as many as eleven seeds.

P. fulvum is an annual decumbent, red yellow pea having different polymorphic forms (Mattia, 1977). *P. fulvum* may have two fructification types, a normal one located in the upper part of the plant, the other very peculiar with very short basal branches which push the pods slightly into the ground i.e. amphicarpy.

P. abyssinicum is a cultivar found at the highlands of Ethiopia and Egypt and has pink flowers and dark purple seeds with unique black hilum (Westphal, 1975). This cultivar is morphologically distinct from other Ethiopian cultivars by having leaves with one pair of leaflets, small red purple flowers and glossy seeds having black hilum (Westphal, 1975). *P. abysinicum* is the ancient cultivated pea. *P. formosum* is the connecting form between *Pisum* and *Lathyrus* genus. However morphologically share many characters with *P. fulvum.*

P. elatius has colored flowers, lilac-blue standards, whereas dark purple wings and maroon veiny brown seeds.

P. humile is characterized as a medium sized climbing species with dentate leaf margins and light blue flowers). *P. humile* resembles the cultivated forms. It is the progenitor of cultivated pea and shares some karyotype with it (Ambrose, 1995).

The two forms of cultivated pea, the field pea (*P. arvense* L.) and the garden pea *(P. sativum* L.) are sometimes regarded as separate species. There is very little justification for this since they can be crossed readily and are quite inter-fertile whereas *P. humile, P. elatius and P. sativum* form a single species complex .It seems probable that the garden pea was derived by selection from the field pea (Smartt, 1976). Based on analyses of morphology, cytogenetics and hybrid performance, Ben-Ze'ey and Zohary (1973) concluded that *P. fulvum* is a fully divergent species, whereas *P. humile, P. elatius* and *P. sativum* form a single species complex.

Other researchers showed that *Pisum* is monospecific, suggesting that the differences observed for *P. fulvum* relative to the other pea taxa are more a matter of degree than the basis for a distinct species (Wellensiek, 1925; Hedrick, 1928; Lamprecht,

1966; Blixt, 1974). Marx (1977) and Palmer *et al.* (1985) agree with the conclusion that *P.humile* is the wild progenitor of the cultivated pea. Recently, Hoey *et al.* (1996) have confirmed this origin through a phylogenetic analysis based on certain morphological characters and RAPD markers

CHEMISTRY:

Green peas (*Pisum sativum* L.) have a nutritionally favourable composition with respect to macronutrients. They have a low fat content, are high in fiber and protein (National Food Administration, 2002) and they contain starch with a low glycemic index (Foster, Powell and miller, 1995).Among micronutrients, peas have high contents of ascorbic acid, beta carotene, thiamine and riboflavin and are rich in iron. (National Food Administration, 2002).The green pea contains 6.7% protein, 0.5% oil and 13.9% carbohydrates. (Sehirali, 1988).

The protein concentration of peas range from 15.5-39.7% (Davies *et al.*, 1985; Bressani and Elias, 1988). "Fresh green peas contain per 100 g: 44 calories, 75.6% water, 6.2 g protein, 0.4 g fat, 16.9 g carbohydrate, 2.4 g crude fiber, 0.9 g ash while dried peas contain: 10.9% water, 22.9% protein, 1.4% fat, 60.7% carbohydrate, 1.4% crude fiber, and 2.7% ash" (Duke, 1981; Hulse, 1994).Pea protein has a high lysine content, making the crop superior nutritionally compared with other legumes. The largest chemical component in peas as in other legumes is carbohydrate (CHO) which constitutes about 56.6% of seed weight (Bressani and Elias, 1988). The most abundant pea carbohydrate is starch, 36.9-48.6%, while amylose is about 34% of seed weight in peas (Bressani and Elias, 1988).Methionine and cystine are the main limiting amino acids.

The proteins present in legume seeds can be broadly classified into metabolic proteins, which are involved in normal cellular activities, and storage proteins which are synthesized during seed development. Of the storage proteins, globulin constitutes a major proportion of the legume seed proteins and its limitations in human and monogastric nutrition are well known (Millerd, 1975).The amino acid composition of food crops can be altered, either by varying the relative proportions of embryo and endosperm, or by changing the relative proportions of metabolic and storage proteins. (Pant, et al., 1974).

Starch is the main component of the pea seed, but occurs in several forms .Smooth-seeded varieties have round starch granules, whereas most of those varieties with wrinkled-seed have composite granules. This character is controlled by a single gene R-r. Protein contents ranging from 26 to 33% occur in wrinkled-seeded varieties and from 23 to 31% in smooth-seeded varieties. This difference is due to variations in the synthesis of starch, with smooth-seeded group having a higher starch and amylopectin content than wrinkled seeded types (Cousin, 1992).

IMPORTANCE:

GENETICAL IMPORTANCE:

Pea has been the object of many genetical studies. It has thus been used as a model organism in early experimentations in genetics, particularly by the pioneer geneticist Reginald Punnett. Pea is highly suitable as a genetic subject because of its ability to self-pollinate and its easily observed Mendelian traits such as color, height and petal form. Many genetical principles were discovered or confirmed in pea. It was used by Punnett in early studies of Genetic Linkage.Gregor Mendel is today recognized as the "Father of Modern Genetics" for his work with the cross breeding of pea plant (*Pisum sativum*) with different characteristics, and sweet pea has also been used in a similar way.

AGRICULTURAL IMPORTANCE:

According to Smartt (1990), peas are widely grown for hay, pasturage and silage production either alone or mixed with cereals. Due to their high protein content peas are grown as a forage crop. It is currently ranked as the world's second most important pulse (FAO, 2005) and is important as staple protein food.

Consequently, many characters have to be improved in order to satisfy the developing needs of growers, processors and feed manufacturers. The nutritional and the functional quality of pea (*Pisum sativum* L.) seed depends primarily on their protein content and composition. For breeding as well as for industrial uses, efficient methods for analyzing protein composition are of great importance. Most agriculturally important crop species exist as a number of genetically distinct, but related, varieties. These varieties are either

indigenous, locally adapted ecotypes (landraces) or, increasingly commonly in the modern world, cultivated varieties (cultivars), actively produced via the efforts of plant breeders. The ability to discriminate between and identify varieties of crops is crucial to the seed and related industries.

AS FOOD:

It is currently ranked as the world's second most important pulse (FAO, 2005). Pea (*Pisum sativum*) is an important legume which is widely cultivated in Europe. Because of its high protein contents and high productivity in temperate European climate conditions, pea belongs to the most effective protein sources for food and feed purposes.

Peas have been grown as an important source of human and animal food for many centuries. Several thousand varieties exist throughout the world. According to Ali, S.M., 1981, peas can be classified into the following categories:

- Field peas, providing forage for animal feed;
- Green peas, from which pods are harvested for human consumption as a fresh vegetable;
- Vining peas, for canning or freezing;
- dried peas, partly for human consumption but mostly for animal feed. Pea (*Pisum sativum*) is one of the most common food plants in turkey grown for fresh consumption and raw material of canned food industry.

TRADITIONAL MEDICINAL USES:

"Seeds are thought to cause dysentery when eaten raw. In Spain, flour is considered emollient and resolvent, applied as a cataplasm. It has been reported that seeds contain trypsin and chymotrypsin which could be used as contraceptive, ecbolic, fungi static and spermicide"(Duke, 1981). Smartt (1990) reported that there are no significant amounts of toxicity or anti-metabolites in peas.

DIVERSITY ANALYSIS OF PEA:

Genetic diversity refers to the variation at the level of individual gene and provides a mechanism for the plants to adapt in ever changing environment. Genetic diversity can be assessed by:

(1) Molecular markers as RAPD, RFLP and ISSR markers that play a significant role in finding phylogenetic relationships among different varieties.

(2) Biochemical methods such as seed protein electrophoresis have also helped to investigate variation among accessions. One very simple way of detecting seed protein Polymorphisms is to analyze "total" seed protein extracts by SDS-PAGE. This can be extremely effective and has been used for variety discrimination and identification of plants.

The potential of molecular techniques and protein markers (represented particularly by isoenymes and seed storage proteins) for cultivar identification and phylogenetic studies has not been completely explored. The usefulness of protein markers represented by seed storage proteins e.g. level of polymorphism and molecular basis of polymorphism is very limited. Despite the increasing significance of DNA markers in cultivar identification and phylogenetic studies, the use of isozymes in combination with morphological traits may be still very useful and important. Among biochemical techniques SDS-PAGE is widely used due to its simplicity and effectiveness for describing the genetic structure of crop germplasm (Murphy et al., 1990; Javaid et al., 2004; Anwar et al., 2003.). The analysis of storage protein variation in pea has proved to be a useful tool not only for diversity studies but also to optimize variation in germplasm collections. SDS-PAGE can be used as a promising tool for distinguishing cultivars of particular crop species (Jha and Ohri, 1996).The purpose of this study was to investigate the level of genetic diversity in pea germplasm used in the national crop improvement efforts in Pakistan by using SDS-PAGE analysis.

LITERATURE REVIEW

LITERATURE REVIEW:

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein folding, post-translational modifications and other factors). Several qualitative and quantitative biochemical and molecular markers loci encoding storage proteins, isozymes or restriction length polymorphism are currently available for measuring variation between closely related germplasm (Baranger *et al.*, 2004).

SDS- PAGE is a particularly reliable method for assessment of genetic diversity among highly resistant and susceptible genotypes because storage proteins are largely independent of environmental fluctuations (Gepts, 1989). Screening' of a broad range of *Pisum sativum* accessions from different geographical origin was conducted by SDS-PAGE . The lowest protein content was found in the accessions from Ethiopia and highest in accessions from Italy. Significant variations in European accessions were found whereas the Asian accessions showed the narrowest range of variations in protein content. However no relationship between seed protein banding and disease status was observed (Gepts. P. 1989).

SDS- PAGE is the most widely used analytical method to resolve components of protein mixture. It is almost obligatory to access the purity of protein through an electrophoretic method. SDS- PAGE exploits differences in molecular size to resolve proteins differing by as little as 1% in the electrophoretic mobility through the gel matrix (Jha, S.S. and Ohri, 1996).

Proteins or enzymes are the final product of gene expression. Although DNA stores the genetic information, it is the protein that determines shape, structure and function of a cell and tissues of an organism. Enzymes, which are usually protein in nature, control the

expression of genes and development of an organism. Overall protein constitutes half of the dry weight of the cell. A number of molecular biology studies started from specific protein that determines a particular phenotype of an organism. DNA / protein interaction is used in gel retardation and footprinting, screening of cDNA library as probe and immunoblotting. (Davis, 1986).

SDS binds in a ratio of approximately 1.4 g SDS per 1.0 g protein (although binding ratios can vary from 1.1-2.2 g SDS/g protein), giving an approximately uniform mass: charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. A tracking dye may be added to the protein solution to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of seed storage proteins has proven a simple and effective method for distinguishing among cultivars of the largely cross-fertilized pasture grasses and legumes despite their high innate genetic variability (Ferguson and Grab 1986, Gardinar and Forde 1988). Similar techniques have been used very extensively for cultivar identification in breeding crops (Cooke, 1995) but to a lesser extent for the differentiation of cultivars of out breeding species (Gilliland 1989, Sammour 1999). Seed protein electrophoresis has also become a useful tool in evolutionary studies to determine species relationships (Smartt 1990, Przyzylska *et al.* 1999). The seed protein profiles reflect genetic affinities within a taxon and even between different biological entities (Mahmoud *et al.*2006, Vaughan and Denford 1968).

The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide (Sammour, 1999).

Electrophoresis separation is nearly always carried out in gels (or in solid supports such as paper) rather than in free solution for two reasons. Firstly gels suppress connective currents produced by small temperature gradients, a requirement for effective separation. Secondly gels serve as molecular sieves that enhance separation. Molecules that are small compared with the pores in the gel readily move through the gel, whereas molecules much larger than the pores are almost immobile. Intermediate-size molecules move through the gel with various degrees of facility (Loris *et al.*, 1998.

Polyacrylamide gels are choice supporting media for elelectrophoresis because they are chemically inert and are readily formed by the polymerization of Acrylamide. Moreover, their pore sizes can be controlled by choosing various concentrations of Acrylamide and ethylenebisacrylamide (a cross-linking reagent) at the time of polymerization.

The proportion of each type of protein varies from species to species. For instance, the seeds of cereals such as wheat, barley, maize or rye contain high levels of prolamin-type proteins; whereas in other cereals (oats, rice) higher levels of globulins are found. Leguminous seeds (e.g. beans, peas, lentils) also have a large proportion of their proteins in the form of globulins. In plants, they have been detected from a large number of taxonomic groups. Despite the impressive studies with seed lectins,the physiological role of these proteins is still controversial. Lectins are proteins widely distributed nature, forming a highly heterogeneous group carbohydrate-binding proteins (Loris *et al.*, 1998).

Przybylska *et al.*, 1983 carried out a comparative study of seed proteins in order to find out phylogenetic relationships and variations in genus *Pisum*. They reported that electrophoretic analysis of legumin fraction was useful in detecting variation among distant forms. As it had demonstrated the distinctness of *P. fulvum* and *P. abysinicum* to some extent but it has not efficiently discriminate between *P. sativum*, *P. humile* and *P. elatius*.

An investigation by Przybylska in 1981on isozyme variation was carried out on 180 *Pisum* forms. They found rare phenotype (a) in P. abysinicum. Phenotype 'c' was common in more or less all accessions. They have reported that isozyme analysis was able to identify the *Pisum* genetic resources. *Pisum* is a young genus because of lack of

seed protein differentiation and low number of biological species within genus (Lu et al., 1951).

On the basis of geographical origin, Przybylska (1999) and Della (2002) studied genotype specificity, genetic diversity and intraspecific variation in *Lathyrus sativus* by means of seed storage proteins. Among 18 grass pea accessions collected from different geographical regions, no correlations were found between protein content and seed weight indicating genetic independence. Experiments showed that the SDS page of albumins and globulins of different grass pea even of the same geographical origin have variations in number, width and intensity of bands. A high genetic variability among the accessions of the same region was found by multivariate analysis concluding that geographical origin does not influence specific seed protein content and its polymorphism.

The interspecific and intraspecific variations in genus Lathyrus was inverstigated by Maberly *et al.*, 1997 through SDS- PAGE technique. In this study total 9 taxa belonging to 4 different sections were studied. The analysis revealed that seeds of Lathyrus are very rich in storage proteins with a large number of stable bands in the electrophoregram under reducing conditions.

Legumin, the major storage protein of *Pisum sativum* L., has been described as a hexameric protein of between 360,000 and 400,000 MW. Each monomer consists of a 40,000 MW subunit and a 20,000 MW subunit linked by disulphide bonds (Croy *et al.* 1979). Both sizes of subunit are heterogeneous by charge and molecular weight in legumin isolated from mature seeds (Gatehouse, J. A., 1984). Ellis *et al* in 1979 reported the in vitro synthesis of vicilin using polysomes from developing *Pisum sativum* but were unable to show the specific synthesis of legumin 40 and 20,000 subunits. Historically legume seed protein has been grouped into two major fractions, albumins and globulins (Gueguen and Barbot, 1988). The later comprise the storage proteins of which there are two major families termed legumin and vicilin, in pea. Each family is made up of combinations from about 10 similar gene products (Hulse, J. H., 1994). The albumin fraction includes all other seeds proteins extractable by aqueous solutions; hence this fraction represents many hundred different protein types of which only few are abundant.

Together the albumin and globulin fractions make up approximately 90% of pea protein, the remainder being cell wall and cell membrane bound proteins. Amounts of albumin , legumin and vicilin were determined using a recently developed HPLC- based system (Makasheva, R.Kh.,1983).

The fact that proteins are direct products of gene translation and transcription makes them ideally suited for plant variety identification purposes. Analysis of protein composition can be considered to be an analysis of gene expression and a comparison of the composition of a particular set of proteins becomes a comparison of the genetic differences between individuals. Since all varieties are different from one another, and the differences must be at least in some part genetically based, protein composition forms an ideal means of variety discrimination. In plants, there are many proteins that are highly polymorphic, particularly the seed storage proteins but also many other seed and vegetative proteins and enzymes. Also, there are many types of electrophoresis method that can be used to separate plant proteins (R. J. Cooke.*et al.*, 1990).

For breeding as well as for industrial uses, efficient methods for analyzing protein composition are of great importance.Inorder to check the variability in protein composition of pea seeds, a procedure combining fast protein liquid chromatography and statistical analysis was developed by Baniel *et al.*, in 1998. Chromatographic conditions optimized the resolution and repeatability of peak areas and peak retention times sufficiently for statistical analysis. The analytical procedure was a practical compromise between the time of chromatographic separation and the repeatability of the fractionation. The method utilizes the variability among pea cultivars and the repeatability of their chromatographic profiles and can characterize seed protein composition with sufficient precision to distinguish cultivars.

A phylogenetic study was conducted among 148 *Pisum* accessions mostly from Western Europe including both primitive germplasm and cultivated types by using 121 protein and PCR-based markers. 148 *Pisum* accessions were divided into eight groups. The molecular data was found consistent with geographical origin of known cultivated types. The study revealed a significant loss of alleles in the course of recent Western European feed pea breeding. This study showed that the intensive selection for yield in feed combining peas has lead to decrease in the genetic diversity among cultivated varieties. (Baranger *et al.*,

(2004).

SDS-PAGE was used to analyze the Hordein polypeptide patterns of Brazilian barley varieties (*Hordeum vulgare* L.) and of two native species of *Hordeum* from Southern Brazil (*H .euclaston* Steud. and *H. stenostachys* Godr.). Forty different Hordein polypeptide bands with molecular weights ranging from 30 to 94 kDa were found in the seeds of the three species studied. There was a greater similarity between the native species than with *H. vulgare*, although *H. stenostachys* was slightly closer to the cultivated species than *H. euclaston.*

Variety discrimination in pea (*P. sativum*) by Peter et al., in 2008 was carried out by biochemical, molecular and morphological markers. Twelve morphological characters enabled discrimination of the varieties. Investigated varieties were reported to be genetically similar, showing narrow genetic base. They found no significant correlation between molecular markers and the classical morphology description while isozyme gave very little information for variety discrimination (Peter *et al.*, 2008).)

The proportion of each type of protein varies from species to species. For instance, the seeds of cereals such as wheat, barley, maize or rye contain high levels of prolamin-type proteins; whereas in other cereals (oats, rice) higher levels of globulins are found. Leguminous seeds (e.g. beans, peas, lentils) also have a large proportion of their proteins in the form of globulins.

Gel electrophoretic investigations were made on the seed albumins of several members of the family Papilionaceae. Relationships were found with taxa of a lower order i.e. between mutants, varieties and subspecies. More distantly related ones, for example species of the same genus or species of different genera, did not show similarities. Thus, it was concluded that the albumin banding pattern is only suitable for studying phylogenetic and taxonomic problems if the material under investigation is not too distantly relate (Palmer, J. D., 1974).

Experiments to check the protein quality of non edible legumes were performed by Bressani in 1972.The seed flour of 28 pea species were separated into fractions such as albumin, globulin, prolamine, glutelin, and non protein nitrogen. Fractionation and amino acid composition of bean cotyledon protein revealed that the alkali-soluble fraction had the highest proportion of methionine (Bateson, W., 1906). Information on the distribution

of seed protein fractions and amino acids of pea is still not sufficient.

Total protein patterns were studied in the course of development of pea somatic embryos using simple protocol of direct regeneration from shoot apical meristems on auxin supplemented medium. Protein content and total protein spectra (SDS-PAGE) of somatic embryos in particular developmental stages were analysed in *Pisum sativum, P. arvense, P. elatius* and *P. jomardi*. Major storage proteins typical for pea seed (globulins legumin, vicilin, convicilin and their subunits) were detected in somatic embryos . In general, the biosynthesis of storage proteins in somatic embryos was lower as compared to mature dry seed and the banding pattern of pea proteins showed much diversity.

Variation in the storage proteins of peas in relation to sulphur amino acid content was studied by D. Roy Davies in 1976. A cellulose acetate Electrophoretic Technique was used to characterize the proteins present in pea seeds in order to find the genetic diversity and to determine the proportions of proteins present which are enriched in sulphur amino acids. Eight *Pisum* varieties examined were shown to differ in their proportions of the various proteins. When the proteins were separated and estimated quantitatively and also characterized by their sub-unit composition, it was shown that cellulose acetate electrophoresis of crude protein extracts or of whole globulins did not give the degree of resolution required and thus this method was not sufficient to find out the genetic diversity among *Pisum* accessions.

On the basis of chloroplast variation, a phylogenetic analysis of twenty two wild and cultivated accessions of genus *Pisum* was conducted. This study proved that cultivated pea was domesticated from Northern *P. humile*. The presence of one accession of garden pea near *P. elatius* and Southern *P. humile* indicated the secondary hybridization period of garden pea (Palmer *et al.*, (1984).

A phylogenetic analysis of seventeen wild and cultivated taxa of *Pisum* was carried out using morphological, allozyme and RAPD markers (Hoey *et al.*, 1995). Close relationship was found between wild species and cultivars of pea that was later supported by systematic studies.

Cultivar identification of commercial pea *(P. sativum L.)* was done by utilizing isozyme variation (Punnett, R. C., 1923). He found less polymorphism by all enzyme

systems among the accessions except estrase (EST). *P sativum* displayed some unique isozyme patterns. Estrase (EST) was found much informative for cultivar identification.

A study on the Ethiopian pea was conducted by Buttler et al., in 2003). They reported that *P. abysisnicum* is separate specie than *P. elatius*. However protein analysis showed that it might be a hybrid between *P. fulvum* and *P. elatius*. Seed characters were found similar to *P. sativum* complex. The origin of Ethiopian pea is still unknown.

Kay, D. 1979 carried out a comparison of yield and its components in field pea (*P. arvense* L.) lines. They reported that tall plants showed better yield and the protein content in all the lines of field pea showed little protein diversity.

Variety discrimination in pea (*P. sativum*) was carried out by biochemical, molecular and morphological markers. No significant correlation was found between molecular markers and the classical morphology whereas twelve morphological characters enabled discrimination of pea varieties .Isozyme analysis gave very little information for variety discrimination(Punnett, R.C., 1923).

In a comparison among AFLP (Amplified fragment length polymorphism), RFLP (Restriction fragment length polymorphism) and RAPD (Random amplified polymorphic DNA), analysis of 14 soybean cultivars by Lu et al., 1951, it was concluded that AFLP generated more polymorphic bands per primer or probe than other molecular biology techniques. RAPD produced a polymorphic band with only 35% of the primers tested and more than 50% of the RFLP probes failed to distinguish among polymorphic bands.

Protein compositions for six Pisum varieties were conducted by Hydroxyapatite Chromatography. Under appropriate conditions three peaks were eluted which were shown by SDS Polyacrylamide Gel Electrophoresis to correspond to albumin, legumin and vicilin respectively. This technique provided results of a high degree of accuracy.

AIMS AND OBJECTIVES:

The present study has following aims and objectives:

(1) To investigate genetic diversity among cultivated accessions of pea especially grown in Pakistan and compare them with wild peas.

(2) Other important aspect of investigation in polyacrylamide gel electrophoresis is to establish genetic similarity index on the basis of bands.

(3) Selection and characterization of genetic material for the quantitative and qualitative improvement of seed storage proteins.

MATERIAL AND METHODS

MATERIALS AND METHODS:
PLANT MATERIAL:

In the present study, 35 *Pisum* accessions (table 1) including both worldwide and cultivated forms were evaluated for genetic diversity. Seeds were obtained from John Innes center (JIC) as well as from National Agriculture Research centre (NARC). Detailed information about their origin and identification are given in table1 and table 2 respectively.

TABLE 1:

SERIAL NO.	ACCESSION NO.	COUNTRY	IDENTIFICATION
1	JI0045	Georgia	*Pisum transcaucasicum*
2	JI0049	Afghanistan	*Pisum sativum*
3	JI0145		*Pisum sativum*
4	JI0156	Sudan	*Pisum sativum*
5	JI0185	Sudan	*Pisum sativum*
6	JI0189	Sudan	*Pisum sativum*
7	JI0241	Israel	*Pisum humile*
8	JI1846	Greece	*Pisum*
9	JI1854	Israel	*Pisum humile*
10	JI2201	Russia	*Pisum elatius*
11	JI2473		*Pisum*
12	009015	Pakistan	*Pisum sativum*
13	009021	Pakistan	*Pisum sativum*
14	009022	Pakistan	*Pisum sativum*
15	009023	Pakistan	*Pisum sativum*
16	009028	Pakistan	*Pisum sativum*
17	009030	Pakistan	*Pisum sativum*
18	009032	Pakistan	*Pisum sativum*
19	009033	Pakistan	*Pisum sativum*
20	009035	Pakistan	*Pisum sativum*
21.	009036	Pakistan	*Pisum sativum*
22.	009051	Pakistan	*Pisum sativum*
23.	009052	Pakistan	*Pisum sativum*
24.	009053	Pakistan	*Pisum sativum*
25.	009054	Pakistan	*Pisum sativum*

26.	009057	Pakistan	*Pisum sativum*
27.	018292	Pakistan	*Pisum sativum*
28	018299	Pakistan	*Pisum sativum*
29	018300	Pakistan	*Pisum sativum*
30	018301	Pakistan	*Pisum sativum*
31	018302	Pakistan	*Pisum sativum*
32	018303	Pakistan	*Pisum sativum*
33	018351	Pakistan	*Pisum sativum*

SOLUTIONS FOR SDS PAGE: Solutions for SDS PAGE were prepared following Laemmli's Protocol.

PROTEIN EXTRACTION BUFFER:

REAGENTS	AMOUNTS
Tris	0.302g
SDS	1.25g
Urea	15g
Beta mercaptoethanol	0.25ml
Distilled water	35ml
Conc.HCl	Adjusted to pH 8
Bromophenol blue	0.1g

PREPARATION OF PROTEIN EXTRACTION BUFFER:

Protein extraction buffer was prepared by dissolving 0.302g of Tris, 1.25g of sodium dodecyl sulfate, 5ml of glycerol and 15g of urea in about 35ml of distilled water using a magnetic stirrer. About 0.25ml of beta mercaptoetanol and 0.1g of bromophenol blue were added and the volume was made up to 50ml by distilled water. The pH 8 was obtained by adding con. HCl .The buffer was stored at 4 C for further use.

PROCEDURE FOR SEED PROTEIN PROTEIN EXTRACTION:

0.02g crushed and grounded seed flour wad placed in 1.5ml appendorf. To extract proteins, the flour was mixed with 800µl of protein extraction buffer and mixed well with vortex and centrifuged @ 13,000rpm for 15minutes at room temperature. The supernatant

was transferred to another microtube and the palette was discarded. The prepared sample was preserved at 20°C.

1.5M TRIS HCl:

REAGENTS	AMOUNTS
Tris	18.15g
SDS	0.2g
Distilled water	100ml
Conc.HCl	Adjusted to pH 8

METHOD OF PREPARATION:

1.5M Tris HCl was prepared by dissolving 18.15g of Tris and 0.2g of SDS in about 70ml of distilled water using a magnetic stirrer. Thereafter pH 8.8 was adjusted by conc. HCl.Volume was made up to 100ml by distilled water. The solution was stored in refrigerator for further use.

0.5M TRIS HCl:

REAGENTS	AMOUNTS
Tris	6g
SDS	0.2g
Distilled water	100ml
Conc.HCl	Adjusted to pH 8

METHOD OF PREPARATION:

0.5Mtris HCl was prepared by dissolving 6g of Tris and 0.2 g of SDS in 60ml water by using a magnetic stirrer. Thereafter conc. HCl was added to adjust pH 8.8.volume was made up to 100ml by adding distilled water. The solution was stored in refrigerator for further use.

10% SDS:

REAGENTS	AMOUNT
SDS	1g
Distilled water	10ml

METHOD OF PREPARATION:

10% SDS was prepared by dissolving 1g of SDS in 10 ml of distilled water by using a magnetic stirrer. The solution was stored in refrigerator for further use.

10% AMMONIUM PER SULFATE SOLUTION:

REAGENTS	AMOUNT
Ammonium per sulfate	0.1g
Distilled water	1ml

METHOD OF PREPARATION:

10% ammonium per sulfate solution was prepared by dissolving 0.1g Ammonium Per Sulfate in 1ml-distilled water. Fresh solution was used each time for electrophoresis.

STAINING SOLUTION:

REAGENTS	AMOUNTS
Methanol	220ml
Acetic acid	30ml
Distilled water	250ml
Coomassie brilliant blue(CBB)	1.15g

METHOD OF PREPARATION:

In 500ml beaker, 220ml methanol, 30ml acetic acid and 1.15g Coomassie Brilliant Blue was added and stirred for 15 minutes by means of magnetic stirrer. The final volume was made upto 500ml by adding distilled water.

DESTAINING SOLUTION:

REAGENTS	AMOUNTS
Methanol	90ml
Acetic acid	20ml
Distilled water	Total volume up to 200ml

METHOD OF PREPARATION:

Destaining solution was prepared by mixing 90ml methanol, 20ml acetic acid and 200ml distilled water in a beaker.

ELECTRODE BUFFER SOLUTION:

REAGENTS	AMOUNT
Tris	6g
Glycine	28.8g
SDS	2.50g
Distilled water	Total volume upto 2 litres

METHOD OF PREPARATION:

28.8g of glycine, 6g Tris and 2.50g SDS was dissolved in 100ml distilled water using a magnetic stirrer. The volume was made upto 2 litres by adding distilled water.

PREPARATION OF GEL:

Following types of gels were prepared:

SEPARATING GEL:

REAGENTS	AMOUNTS
Acrylamide	16ml
Distilled water	13.4ml
1.5M Tris HCl	10ml
SDS	0.4ml
APS	200µl
TEMED	20µl

METHOD OF PREPARATION:

For the preparation of separating gel, 16ml Acrylamide, 10ml of 1.5M Tris HCl, 13.4ml distilled water, 0.4ml SDS and APS was added in a beaker. Thereafter TEMED of about 20µl was added to the solution.

12 % STACKING GEL:

REAGENTS	AMOUNTS
Acrylamide	1.3ml
Distilled water	6ml
0.5M Tris HCl	2.5ml
10% SDS	100ml
10% APS	50µl
TEMED	10µl

METHOD OF PREPARATION:

For the preparation of 4.5% of stacking gel,1.3ml of Acrylamide,6ml distilled water,2.5ml 0.5M Tris HCl , 10%sds and 10%APS was added.10µl TEMED was added at the end and the solution was shaken well.

ELECTROPHORESIS:

The two glass plates were placed on each other while sealing with spacers and were clamped with clips. The separating gel was poured into the space between a set of glass plates upto 2cm from the top.

A small amount of distilled water was added on separating gel gently. In about 30 minutes the gel was completely polymerized .after polymerization the isopropanol solution was decanted and gel surface was rinsed with distilled water to remove monomers of Acrylamide .stacking gel was poured on the separating gel. The comb was placed on the stacking gel and left for about 20minutes until the gel got polymerized. The comb and the bottom spacers were also removed and the wells were formed. The electrode buffer was put into the bottom and upper pools of the apparatus and gel plates were placed in the apparatus. Extreme precautions were taken to prevent water bubbles to be formed at the bottom of the gel. The samples were centrifuged at 13,000rpm for about 5minutes.15µl of the supernatant of each sample was loaded into each well. In one well, protein molecular weight marker was also added. The gel was run at 80Ma for 7 hours.

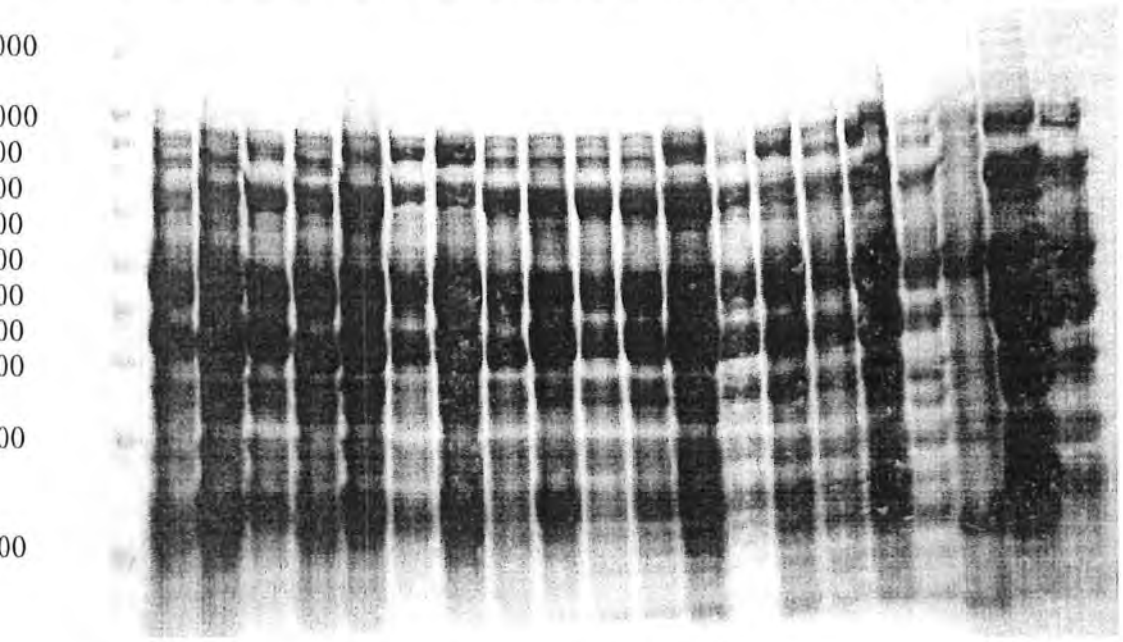
STAINING AND DESTAINING OF GEL:

The set of gel plates were removed by means of spatula and the stacking gel was removed. The separating gel was put into the staining solution for overnight and was shaken gently. The staining solution was replaced by destaining solution and was shaken gently until the bands become cleared. After destaining, the gel was preserved in water and sealed with plastic bag without any diminutioin in the intensity of staining.20% glycerol was added into the water to avoid the gel from swelling.

RESULTS

RESULTS: Electrophorogram showing the pattern of seed storage proteins

FIGURE 1:



In figure1,1 represents protein marker,2:JI-284,3:JI-185,4:2385,5:189,6:85,7:241,8:2201,9:9015,10:pk;21,11:18300,12:9030,13:pk1,14:9033, 15:18301,16:18303,17:9057,18:18299,19:9052,20:1846,21:9054.

The details of accessions are given in table 1 of Material and Methods.

TABLE 1:

lo.	Accessions	205kDa	116kDa Da	97kDa	84kDa	66kDa	55kDa	45kDa	36kDa	29kDa	24kDa	20kDa
	JI-284	0	1	1	1	1	1	0	1	1	1	1
	JI-185	0	1	1	1	1	1	0	1	1	1	1
	JI-2385	0	1	1	1	1	1	1	1	1	1	0
	JI-189	0	1	1	1	1	1	1	1	1	0	1
	JI-85	0	1	1	1	1	1	1	1	1	1	1
	JI-241	0	1	1	1	1	1	1	1	1	0	0
	JI-2201	0	1	1	1	1	1	1	1	1	1	1
	9015	0	1	1	1	1	1	1	1	1	1	1
	18304	0	1	1	1	1	1	0	1	1	1	1
	18300	0	1	1	1	1	1	0	1	1	1	1
	9030	0	1	1	1	1	1	1	1	1	1	1
	18305	0	1	1	1	1	1	1	1	1	1	0
	9033	0	1	1	1	1	1	1	1	1	1	1
	18301	0	1	1	1	1	1	1	1	1	1	1
	18303	0	1	1	1	1	1	0	0	1	0	1
	9057	0	1	1	1	1	1	1	0	1	1	1
	18299	0	1	1	1	1	1	0	1	1	0	1
	9052	1	0	1	0	0	1	0	0	1	0	1
	JI-1846	1	1	1	1	1	1	1	1	1	1	1
	9054	0	1	0	1	0	1	0	1	1	0	0

Data Analysis:

The data obtained from SDS-PAGE was scored for the presence (1) and absence (0) of the bands and entered in a binary data matrix. Based on the results of electrophoretic band spectra, similarity index was calculated for all possible pair of electrophoregrams. The similarity matrix thus generated was converted to a dissimilarity matrix and used to construct the dendrogram by the unweighted pair

group average method (UPGMA). The data was analyzed using NTSYS computer software.

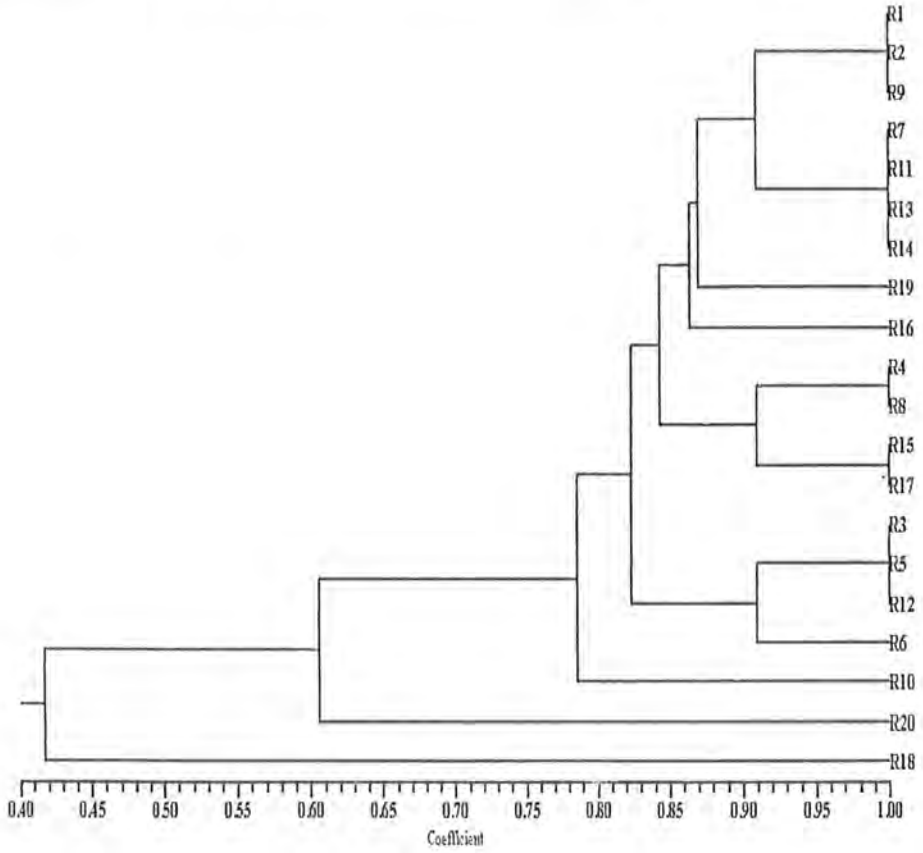
In total, 165 unique polypeptide bands were noted in figure 1.The maximum number of bands in any one taxon was 11 recorded in *Pisum sativum*(JI-1846) of worldwide variety and the minimum number was recorded in two cultivated varieties of *Pisum sativum*(9052,9054).

On the basis of results obtained by SDS –PAGE, proteins of molecular weight 205kDa were absent in all the exceptions in figure 1 except one cultivated (*Pisum sativum*) and one wild accession (JI;1846).

Proteins of molecular weight 116kDa showed little polymorphism and were present in all accessions except cultivated 18300 and 9052(*Pisum sativum*).

Polymorphism shown by proteins of molecular weight 97kDa was 1%.97kDa band was absent in 9054 (*Pisum sativum*).

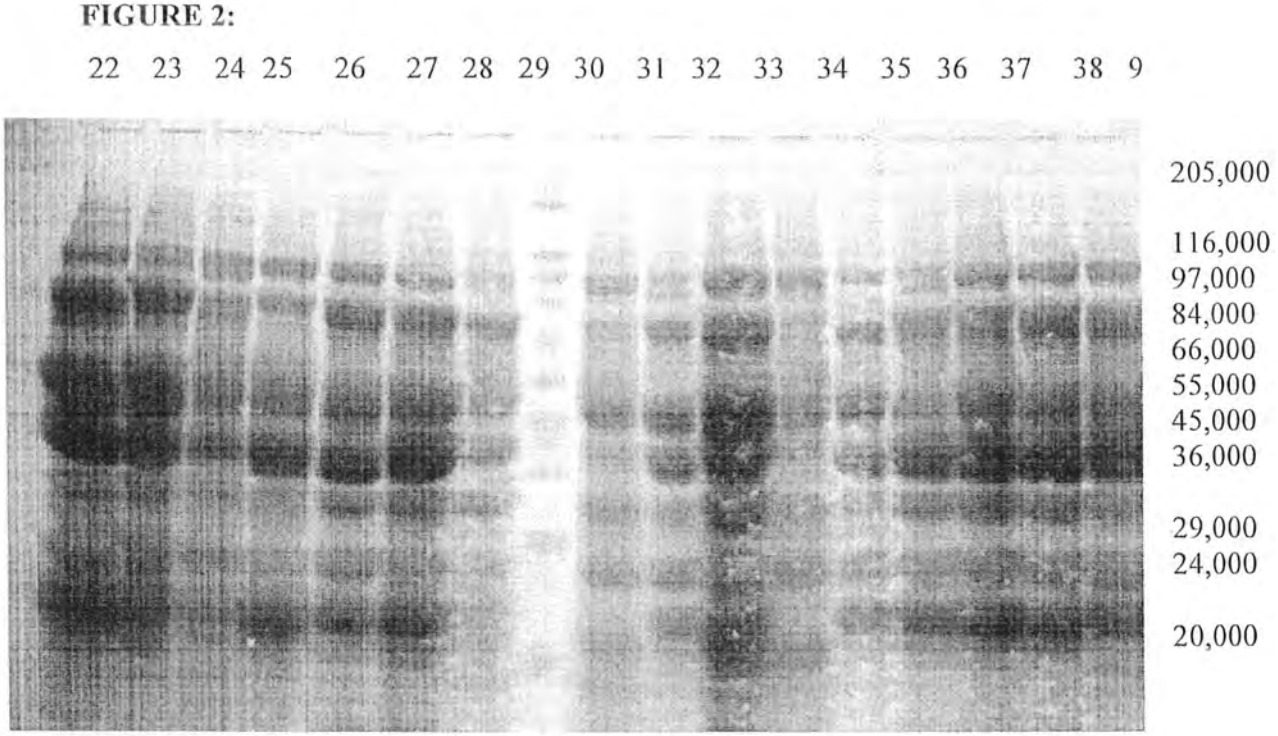
Proteins of molecular weight 66kDa and 45kDa showed a considerable degree of polymorphism whereas monomorphism was recorded in proteins having molecular weights 66kDa and 29kDa.



Dendogram of *Pisum* accessions constructed using UPGMA method based on similarity matrix.

In figure 2 ,R18 representing cultivated *Pisum sativum* (9052) showed distinct behaviour. main group was divided into R20 representing cultivated *Pisum sativum* (9054).main group was further sub –divided into a number of sub-groups.R10 representing cultivated *Pisum sativum* (18300)and R20 showed a divergent behaviour .Sub group a included R3 representing JI-2385.R5 representing JI-85.Both of these showed 100% similarity.R18 showed 42% similarity with the main group. R 20 showed 60% with the main group. 9052 showed an extremely unique pattern.

FIGURE 2:



In figure 2, 2,22 represents 1854,23:JI-156,24:JI-49,25:JI;45,26:JI-2473,27:JI-196,28:1006,29:18302,30:JI-18351,31: JI-156,JI;2473,9053156,24:JI-49,25:JI-45,26:JI-2473,27:JI196,28:JI-18292,29:9051,30:JI-254,31:9035,32:9053,33:9036,34:18308,35:18309.

TABLE 2:

No.	Accessions	205kDa	116kDa	97kDa	84kDa	66kDa	55kDa	45kDa	36kDa	29kDa	24kDa	20k
	JI-1854	1	1	1	0	1	0	1	1	0	0	1
	JI-156	1	1	1	0	1	0	1	1	1	1	1
	JI-49	1	1	1	0	1	0	1	1	1	1	1
	JI-45	1	1	1	0	1	0	1	1	1	0	1
	JI-2473	1	1	1	0	1	0	1	1	1	1	1
	JI-196	0	1	1	0	1	0	1	1	1	0	1
	JI-1006	0	1	1	0	1	0	1	1	1	1	1
	18302	1	1	1	0	1	0	1	1	1	1	1
	18351	0	1	1	0	1	0	1	1	1	1	1
	18292	1	1	1	0	0	0	1	1	1	1	1
	9051	1	1	1	0	1	0	1	1	1	1	0
	JI-254	1	1	1	0	1	0	1	1	1	1	1
	9035	1	0	1	0	1	0	1	1	1	1	1
	9053	1	0	1	0	1	0	1	1	1	1	1
	9036	1	0	1	0	1	0	1	1	1	0	1
	18304	1	0	1	0	1	0	1	1	1	1	1
	18305	1	0	1	0	1	0	1	1	1	0	1

In total, 139 unique polypeptide bands were noted in figure 2.the maximum number of bands in any one taxon was 9 recorded in JI-156 while the minimum number of bands were recorded in JI-18292,54.

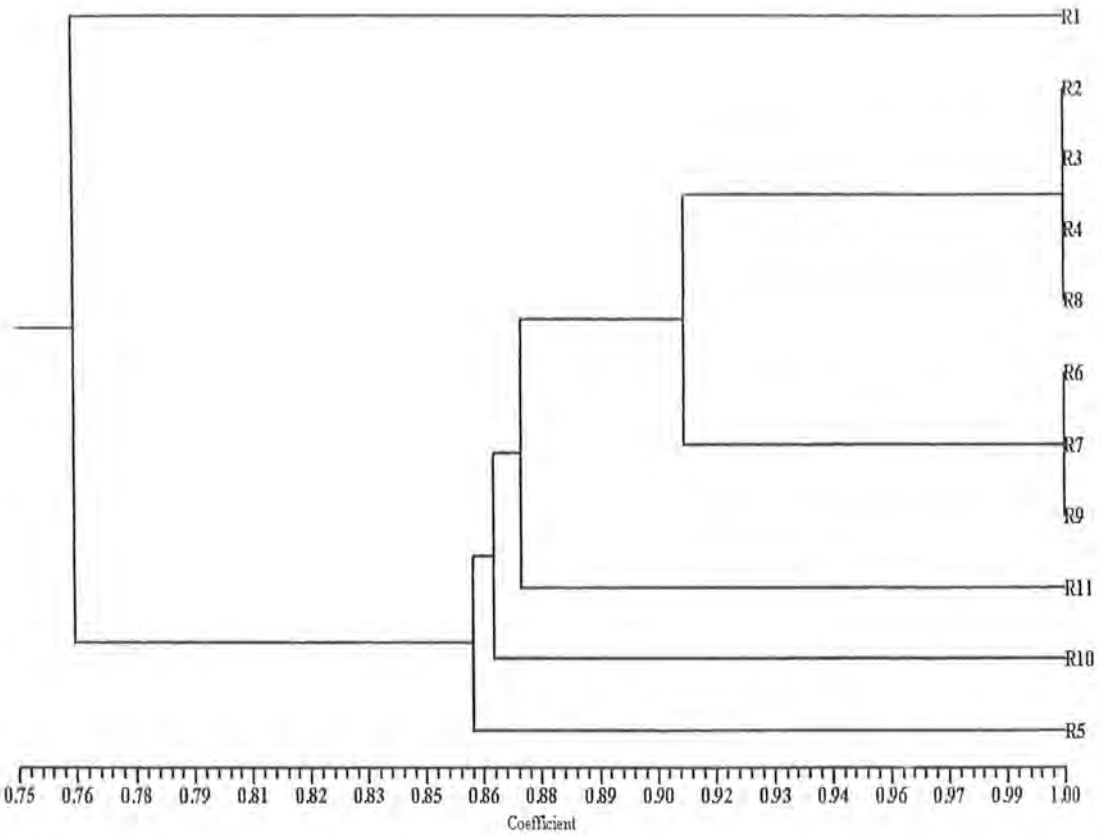
On the basis of results obtained by SDS –PAGE,proteins of molecular weight 205kDa were present in all the accessions except JI-2473,JI-196 and 18351.both JI-2473 and JI 196 seem to have link with eachother.

Proteins of molecular weight 116kDa showed little polymorphism and were absent in 9035,9053,9036,18304 and 18305 which are all cultivated *Pisum sativum* varieties and share similar characters.

84kDa and 55kDa bands were absent in all the accessions.97kDa,36kDa and 29kDa bands showed no polymorphism at all.

Polymorphism shown by proteins of molecular weight 97kDa was 1%.97kDa band was absent in 9054(*Pisum sativum*).

Proteins of molecular weight 66kDa and 45kDa showed a considerable degree of polymorphism whereas monomorphism was recorded in proteins having molecular weights 66kDa and 29kDa.



Dendrogram *Pisum* accessions constructed using UPGMA method based on similarity matrix.

In figure 2, the main group was divided into R5,R10,R11.The main group was further sub divided into 2 sub groups at 87%.

At 76% similarity UPGMA splits all the individuals of pea into a single major group except 1854 which lies outside this group.R5 representing JI-2473 showed distinct behaviour and showed similarity with the main group at 86% UPGMA cluster analysis.R10 representing 18292 showed similarity with the main group at 87% UPGMA cluster analysis.R11 representing cultivated *Pisum sativum* (9051).the main group is further sub-divided into 2 sub groups i.e. sub group a and sub group b .Sub group a is further divided into R6 representing JI-196,JI-1006 and cultivated *Pisum sativum* (18351).JI-196,JI-1006 and 18351 showed 100% similarity but shared 91% similarity with sub group b which includedR2 representing JI -156,R3 representing JI-49,R4 representing JI 45 andR8 representing 18302.all of these showed 100% similarity .group

a and group b showed similarity with each other at about 91% . At about 86 similarity, 2 groups or clusters were revealed in the analysis.R5 showed R6 similarity with the major group.R5 shows distinct behaviour from the major group. .R10 shows R7 similarity with the sub groups a and b.R6, R7, R 9 are 100 similar to eachother. They form a single sub group.R2, R3, R4, R8 are 100% similar to eachother, lie in sub b.sub a and b are similar to eachother at 91.R11 shows 87% with sub a and,b.

P. fulvum (JI-2473) retained its distinctness sharing only very few morphological characters with other accessions.

DISCUSSION

DISCUSSION:

The Molecular Marker is a useful tool for studying genetic diversity among crop species and resolving cultivar identities. Studying genetic diversity is of enormous use in identifying different cultivars suitable for different ecologies .Among biochemical techniques SDS-PAGE is widely used due to its simplicity and effectiveness for describing the genetic structure of crop germplasm. (Murphy *et al.*, 1990; Javaid *et al.*, 2004 . The analysis of storage protein variation in wheat has proved to be a useful tool not only for diversity studies but also to optimize variation in germplasm collections. SDS-PAGE can be used as a promising tool for distinguishing cultivars of particular crop species (Jha and Ohri, 1996).

Seed protein pattern obtained by electrophoresis has been successfully used to resolve taxonomic and evolutionary problems of several plants (Ladizinsky, 1997). It can also be used as a promising tool for distinguishing cultivars of particular crop species (Gardiners, 1988). However few studies indicated that cultivar identifications were not possible with SDS-PAGE method as electrophoresis pattern of proteins were similar among cultivars (Rosa, M. J., 2000).in case where proteins fail to detect differences to identify the particular species, 2D electrophoresis is suggested. Seed protein polymorphism may serve as genetic markers for the plant germplasm management because they can be quite polymorphic (Ahmad and Slinkard, 1992).

Pisum and its various species and subspecies cannot be markedly distinguished (Lu *et al.*1996).however hybridization studies suggest that *Pisum fulvum* is isolated and is therefore a fully divergent species (Benze'ev and Zohary, 1973). Furthermore, relationships based on AFLP also clearly show that genetic diversity is evenly distributed within the genus which is consistent with the present work (Hoey et al., 1996).

All these *Pisum* taxa are not much differentiated from each other and are thus closely related .the present results agree with earlier studies where no consistent differences were found between seed protein patterns of cultivated and wild taxa (Waines, 1975).

It has been shown that *Pisum fulvum* is homogenous in its genome size. On the other hand *P. abyssinicum, P. humile* and *P. elatius* vary widely as compared to *Pisum sativum* which itself shows a constant genome size (Baranyi *et al.*1996). These results are

consistent with the present work. This shows that there are populational differences among the wild taxa and only those taxa which are closest to *Pisum sativum* in their genome size should be considered as its closest relatives (Baranyi *et al.*1996). Therefore future studies on seed protein analysis should include wider range of samples of wild as well as cultivated taxa belonging to different geographical regions to bring out a coherent picture of the relationship among these taxa and settle the taxonomic status of various entities in the genus *Pisum*. The extensive genetic variability commonly associated with pea as the Classical Mendelian organism is the basis for its multiplicity of forms. Systematists have often interpreted this variation by collectively recognizing many distinct pea species in a variety of different relationships (Gepts, P and Bliss, F.A., 1985).

The cladistic analysis performed by Davies in 1974 showed that *Pisum fulum* is clearly the most distinct of the pea taxa while *P. humile, P. elatius P. sativum* form a monophyletic group which is also inconsistence with the present study.

Comparing molecular and morphological characterizations, several authors have indicated that molecular markers allow more precise discrimination than morphological traits as shown in maize (Zohary, D. & M. Hopf, 1973).

Variety discrimination in pea (*P. sativum*) was carried out by biochemical, molecular and morphological markers. Twelve morphological characters enabled discrimination of the varieties. Investigated varieties were reported to be genetically similar, showing narrow genetic base. They found no significant correlation between molecular markers and the classical morphology description while isozyme gave very little information for variety discrimination (Ambrose, M.J 1998).These results are in consistence with the present work.

An investigation on isozyme variation was carried out on 180 *Pisum* forms. They found rare phenotype (a) in P. abysinicum. Phenotype 'c' was common in more or less all accessions. They have reported that isozyme analysis was able to identify the *Pisum* genetic resources (Parsysz and Przybylska., 1881).

Przybylska *et al.*, 1983 carried out a comparative study of seed proteins in order to find out phylogenetic relationships and variations in genus *Pisum*. They reported that electrophoretic analysis of legumin fraction was useful in detecting variation among distant forms. As it had demonstrated the distinctness of *P. fulvum* and *P. abysinicum* to

some extent but it has not efficiently discriminate between *P. sativum*, *P. humile* and *P. elatius*. The present results support this idea.

Since pea is a largely self pollinating species, within accession variation was expected to be low (Symkal, 2006).the present work supports this idea. Isozyme based methods proved to be superior in terms of data acquisition seed and polymorphism, providing high resolution in a single analysis. The simplicity and high information value of isozyme analysis is hampered by the necessity of using fresh and specific tissue and the need of reference samples. Currently developed pea marker systems might serve for germplasm management and genetic diversity studies, as well as a tool for rapid variety identification within a given breeding company (Ford, 2002).

Recent diversity analysis in pea has focused either on biosystematic studies within the *Pisum* genus (Hoey *et al*, 1996) or on assessment of different molecular markers to investigate the genetic diversity within *Pisum* as in the present study. However all these studies have focused either on small number of accessions or on specific group of pea genotypes.

Since all varieties are different from one another, and the differences must be at least in some part genetically based, protein composition forms an ideal means of variety discrimination. In plants, there are many proteins that are highly polymorphic, particularly the seed storage proteins but also many other seed and vegetative proteins and enzymes. Also, there are many types of electrophoresis method that can be used to separate plant proteins. Hence it should not be altogether surprising that gel electrophoresis should be so useful for variety identification work.

As a complementary approach, biochemical analysis of isozyme markers proves its diagnostic potential in (Baranyi *et al*.1996) pea, but a limited degree of polymorphism and potential sensitivity to environmental and developmental variation prevented its broad application.

New developments continue to be made. For instance, Weber, K.,Pringle in 1977 reported the use of commercially available horizontal gels and buffer kits for the analysis of barley *Hordein*, which clearly could be useful in some of the more routine laboratory testing situations. There are, however, many other electrophoretic methods and polymorphic protein systems in plants that can be exploited for identification purposes.

The problems were standardization of methodology and gel scoring, cataloguing of profiles and the need to assess uniformity all apply, in varying degrees, to the techniques discussed in the remainder of this article.

Electrophoresis techniques are continually developing and it can be predicted with reasonable confidence that their application to plant variety testing will evolve and expand over the next few years. The use of capillary electrophoresis for the identification of varieties and the examination of DNA profiling techniques of various kinds will surely increase in future. However, gel electrophoresis of proteins and enzymes in its several forms still has much to offer in this particular field and it is possible to discern various trends for the future of its use.

One such trend is the move towards smaller, thinner and, usually, precast gels. It is now possible to buy ready-made gels in a range of formats, for all of the types of gel electrophoresis that one would normally wish to carry out for variety identification purposes. According to Wrigley *et al.*, this phenomenon is liable to have its largest impact in the areas of IEF and 2D-electrophoresis, offering simplicity, convenience, high resolution and, crucially, a guaranteed quality of gel (Javid, A., A. Ghafoor and R. Anwar, 2004)

Due to modern breeding it has been observed that genetic diversity in pea has been increasingly narrowed. Narrow genetic diversity is problematic in breeding for adaptation to biotic stresses, like diseases, and abiotic stresses such as drought and salt. Therefore, it is necessary to investigate the genetic diversity in pea germplasm in order to broaden the genetic variation in future pea breeding. Knowledge of germplasm diversity and protein polymorphism has a significant impact on crop improvement.

REFERENCES

REFERENCES:

- Akhtar, M. 2001. Phylogenetic relationships among Vigna species based on agronomic and biochemical analysis. M. Phil. Thesis, Department of Biological Science Quaid-I-Azam University, Islamabad, Pakistan. 99 pp.
- Ali, S. M., 1983. Pathotypes of 'black spot' complex pathogens of field peas, selection and inheritance of resistance in pea lines. Adelaide, Australia
- Almgard, G., 1970.Cultivar distinction by means of isozyme technique in *Pisum* Newslet 2:9.
- Badr, A. 1995. Eletrophoretic studies of seed proteins in relation to chromosomal criteria and relationship of some taxa of Trifolium. Taxon., 44: 183-191.
- Baranger A, et al., (2004) Genetic diversity within *Pisum sativum* using protein- and PCR-based markers. Theor Appl Genet 108:1309–1321.
- Baranger, A., Aubert, G., Arnau, G., Lainé, A.L., Deniot, G.,Potier, J. et al. 2004. Genetic diversity within Pisum sativum us ing protein- and PCR-based markers. Theor. Appl. Genet. 108:1309–1321.
- Bateson, W., Saunders, E.R. and Punnett, R.C., 1906. Experimental studies in the physiology of heredity. Reports to the Evolution Committee, Royal Society of London:
- Ben-Ze'ev, N. and Zohary, D., 1973. Species relationships in the genus. Pisum. Israel J. Bot., 22: 73-91.
- Blixt, S., 1974. The pea. In: ed. R.C. King, Handbook of Genetics, Vol.2. Plenum Press, New York, pp. 181-221.
- Breeding and Genetic resource. (Eds.): A.H.D. Brown, M.T. Clegg, A.L. Kahler and B.S.
- Bressani, R. and L.G. Elias., 1988. Seed quality and nutritional goals in pea, lentil, faba bean and chickpea breeding. p. 381-404. In: R.J. Summerfield (ed.), World Crops: Cool Season Food Legumes. Kluwer Academic Publishers, Dordrecht, The Netherlands Contributions to the genetics of Pisum. Genetica 12: 321-440.

- Cooke, R. J., 1995. Variety identification of crop plants. In: Skerrit, J. H, Appels, R (ed), New diagnostics in crop science. Biotechnology in agriculture 13, 33–63. CAB International, Wallingford, UK.
- Cousin, R., 1983. Breeding for yield and protein content in pea.In: ed. R. Thompson and R. Casey, Perspectives for Peas and Lupins as Protein Crops. Martinus Nijhoff, The Hague, The Netherlands. pp. 146-164.
- Croft, A. M., Pang, E. C. K., Taylor, P. W. J., 1999: Molecular analysis of Lathyrus sativus L. (grasspea) and related Lathyrus species. Euphytica 107, 167–176.
- Crop Plants. Longman, New York, pp. 172-174.
- Das, S. and K.K. Mukarjee. 1995. Comparative study on seed proteins of Ipomoea. Seed Science Technol., 23: 501-509.
- Davies ,A.J.S., 1958. A cytogenetic study in the genus Lathyrus. Ph.D. Thesis Manchester Univ., 239 p.
- Davies, D. R., 1974 Peas. pp. 172-174. In: Evolution of Crop Plants, Edited by N. W. Simmonds.
- Davies, D.R., 1974. Peas. In: ed. N.W. Simmonds, Evolution of
- Davies, D.R., G.J. Berry, M.C. Heath, and T.C.K. Dawkins., 1985. Pea (Pisum sativum L.). p. 147-198. In: R.J. Summerfield and EH Roberts, (eds.), Williams Collins Sons and Co. Ltd, London, UK.
- Duke, J. A., 1981. Handbook of legumes of world economic importance. Plenum, New York.
- Duke, J.A. 1981. Hand book of legumes of world economic importance. Plenum Press, New York. p. 199-265.
- Ellis ,T. H ., Poyser, S. J., Knox, M.R., Vershinin, A.V., Ambrose, M.J (1998) Polymorphism of insertion sites of *Ty1-copia* class retrotransposons and its use for linkage and diversity analysis in pea. *Mol. Gen. Genet.* **260**: 9–19.
- Erskine,W., 1997. Lessons for breeders from landraces of lentil. Euphytica 93, 107–112.
- FAO, 1970. Amino acid contents of foods and biological data on proteins. , Food Policy and Food Science Service, Nutrition Division, FAO, Rome.

- Ferguson, M., 2000. Lens spp: Conserved resources, priorities and future prospects. In: Linking research and marketing opportunities for pulses in the 21st century (R. Knight, ed.). Kluwer Acad. Publ. The Netherlands, pp. 613-620.
- Ferguson, J. M., Grabe, D. F., 1986: Identification of cultivars of perennial ryegrass by SDS-PAGE of seed proteins. Crop Sci. 26, 170–176.
- Ferguson, M. E., Robertson, L. D., Ford-Lloyd, B. V., Newbury, H. J., Maxted, N., 1998: Contrasting genetic variation amongst lentil landraces from different geographic origins. Euphytica 102, 265–273.
- Food and Agriculture Organization of the United Nations. 1994 Production Year Book. Rome, Italy.
- Gepts, P. 1989. Genetic diversity of seed storage proteins in plants. In: Plant Population Genetics,
- Gepts, P.& Bliss, F.A (1985). F1 hybrid weakness in the common bean; differential geographic origin suggests two gene pools in cultivated bean germplasm. J. Hered 76: 447–450.
- Ghafoor, A., Ahmad,Z., Qureshi, A.S and Bashir, M (2002). Genetic relationship in *Vigna mungo* (L.) Hepper and *V. radiata* (L.) R. Wileczek based on morphological traits and SDS-PAGE. *Euphytica,* 123: 378-2002.
- Gibanlt, G., 1912. Legumineuses, Pois. In: Histoire des l_6gumes.Librairie Agricole, pp. 314-322.
- Gritton, E.T. 1980. Field Pea. Hybridization of Crop Plants. p. 347-356. In: W.R. Fehr and H.H. Hadley (eds.), American Society of Agronomy, Inc., and Crop Science Society of America, Inc., Wisconsin, USA.
- Hoey et al., (1996), Hoey,B .K, Crowe, K.R, Jones, V.M and Polans,N.O: A phylogenetic analysis of *Pisum* based on morphological characters, allozyme and RAPD markers, *Theoretical and Applied Genetics* **92** (1996), pp. 92–100.
- Hoey, B.K., Crowe, K.R., Jones, V.M. and Polans, N.O., 1996. A phylogenetic analysis of Pisum based on morphological characters, and allozyme and RAPD markers. Theor. Appl. Genet; 92: 92-100.

- Hoey, B.K., K.R. Crowe, V.M. Jones and N.O. Polans, 1996. A phylogenetic analysis of Pisum based on morphological characters, and allozyme and RAPD markers.Theor Appl Genet,92: 92–100.
- Hulse, J.H., 1994. Nature, composition and utilization of food legumes. In: Muehlbauer FJ, Kaiser WJ (ed) Expanding the production and use of cool season food legumes. Kluwer Academic Publishers, Dordrecht, pp 77–97
  Isozyme electrophoresis. In: Hillis DH, Moritz C {eds.,} *Molecular systematic*
- Javid, A., A. Ghafoor and R. Anwar. 2002. Evaluation of local and exotic pea Pisum- sativum germplasm for vegetable and dry grain traits. Pak. J. Bot., 34(4): 419-427.
- Javid, A., A. Ghafoor and R. Anwar. 2004. Seed storage protein electrophoresis in groundnut for Electrophoretical evidence of variation in populations of the fodder legume (Chamaecytisus proliferus) from Canary Islands. Bioch. Syst Ecol., 23: 53-63.
- Jha, S.S. and Ohri, 1996. Phylogenetic relationship of Cajanus cajan (L) Millsp. (Pigeon pea) and its wild relatives based on seed protein profiles.Grace 43: 275-281.
- Kay, D. 1979. Food legumes. Tropical Products Institute (TPI). TPI Crop and Product Digest No. 3, p. 26-47. UK.
- Ladizinsky, G., 1997. A new species of Lens from Southwest Turkey. Bot J Lin Soc 123, 257-260.
- Ladizinsky,G., and Hymowitz,T.,1979. Seed electrophoresis in taxonomic and evolutionary studies.Theor. Appl. Genet.54: 145-151.
- Laemmli, U. K., 1970. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature. 227(259): 680-5.
- Loris et al., R. Loris, T. Hamelryck, J. Bouckaert and L. Wyns, 1998.Legume lectin structure, Biochimica Et Biophysica Acta 1383), pp. 9–36.
- Lu, J., Knox, M.R., Ambrose, M.J., Brown, J.K.M.,1951.Comparitive analysis of genetic diversity in pea assessed by RFLP- and PCR based methods.Theor.appl.Genet. 93:1103-1111, 1996.
- Mabberly,D.J.,1997.The Plant Book. Cambidge University Press,pp:320-323.

- Makasheva, R.Kh.,1983. The Pea. 267p. Oxonian Press Pvt. Ltd., New Delhi, India.
- Murphy, G., and Kavanagh, T. A. (1988) Nucleic Acids Res. 16,5198
- Murphy, R.W., J. W. Sities., D. G. Buth and C. H. Haufler. 1990. Protein 1: 45-126.
- Murphy, R.W.,Sites, J.W., Buth , D.G and Haufler,C H., 1990. Protein isozymes eletrophoresis. In: *Molecular systematic.* D.H. Hillis and C. Moritz (Eds): *Sinnauer Association, Sunderland*, MA pp. 45-126.
- Palmer, J. D., and W. F. Thompson, 981b Clone banks of the mung bean, pea and
- Pant, R., Nair, C.R., Singh, K.S. and Koshti, G.S., 1974. Amino acid composition of some wild legumes. Current Science 43, pp. 235–239
- Przybylska, J., E. Pagowska and J. George, 1989. Isoenzyme variation in the genus Pisum. VI. Further electrophoretic analysis of different enzyme systems. Genetica Polonica 30: 129–138.
- Przybylska, J., S. Blixt, H. Parzysz and Z. Zimniak-Przybylska, 1982. Isoenzyme variation in the genus Pisum. I. Electrophoretic patterns of several enzyme systems. Genetica Polonica, 23:103–121.
- Przybylska, J.,1986. Identification and classification of the Pisum genetic resources with the use of electrophoretic protein analysis. Seeds Sci Technol, 529–543.
- Punnett, R.C., 1923. Linkage in the sweet pea (Lathyrus odoratus). Journal of Genetics 13: 101–123
- R.J. Cooke,1989 Plant Varieties and Seeds.
- Rabbani, M.A., Qureshi, A.A., Afzal., M, Anwar., and S. Komatsu. 2001. Characterization of mustard [*Brassica junncea* (L.) Cezern. & Cross] germplasm by SDS-PAGE of total seed protein. *Pakistan Journal of Botany*, 33(3): 173-179.
- Rao, R., M. Del Vahlio, M. Paino D,Urzo and L.M. Monti. 1992. Identification of Vigna spp.,through specific seed storage polypeptides. Euphytica, 62: 39-43.
- Rosa M. J., Ferreira R. B., 2000. Storage proteins from Lathyrus sativus seeds. J. Agric. Food Chem. 48, 5432–5439.

- Sammour, R. H., 1988: Flax seed proteins: Comparison by various PAGE-techniques in slabs. J Agron. Crop Sci. 160, 271–276.
- Sammour, R. H., 1999: SDS-PAGE analysis of the seed protein of some Trifolium taxa.Plant Var. Seeds 12, 11–21.
- Sanchez-Yelamo, M.D., M.C. Espenjo-Ibanez, J. Francisco-Ortega and A. Santos-Guerra. 1995.
- Scopes, R.K.1994. "Protein Purification : Principles and Practice",3RD ED.,Springer Verlag,New York.
- Sing, U. and Jambunathan, R., 1982. Distribution of seed protein fractions and amino acids in different anatomical parts of chickpea (Cicer arietinum L.) and pigeonpea (Cajanus cajan L.). Qualitas Plantarum Plant Foods for Human Nutrition 31, pp. 347–354.
- Smartt, J., 1976. Tropical Pulses. Longman, London, 348 pp.
- Smartt, J., 1990, Grain Legumes: Evolution and genetic resources. Cambridge University Press, Cambridge.
- USA Dry Pea and Lentil Council. 1996. USA dry peas, lentils and chickpeas, the standard for quality. Directory of US suppliers and industry information. Moscow, USA.
- Weber, K.,Pringle, J.R., and Osborn, M. 1971 Meth.Enzymol.26, 3-27
- Zohary, D. & M. Hopf, 1973. Domestication of pulses in the Old World. Science 182: 887–894.