International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **1(4),** 39-50, August **(2012)** *I. Res. J. Biological Sci.*

Sodium Dodecyl sulphate Polyacrylamide gel Electrophoresis Pattern of Horse Gram Seed Storage Proteins during Germination

Pek Geok Pang[1], Asrul Afandi[1], Rahman Shefiqur[2] and Shaha Ranajit kumar[3]*
[1]Faculty of Agro Based Industry, University Malaysia Kelantan (Jeli campus), Beg Berkunci No. 100, 17600 Jeli, Kelantan, MALAYSIA
[2]Faculty of Earth science, University Malaysia Kelantan (Jeli campus), Beg Berkunci No. 100, 17600 Jeli, Kelantan, MALAYSIA
[3]Faculty of Agro Based Industry, University Malaysia Kelantan (Jeli campus), Beg Berkunci No. 100, 17600 Jeli, Kelantan, MALAYSIA

Available online at: www.isca.in
Received 10th July 2012, revised 18th July 2012, accepted 25th July 2012

Abstract

The aim of the experiment was to study the effect of germination on the three varieties of horse gram seed storage proteins. Seeds were germinated for 24, 48, 72, 96 and 110h in a dark place. Germinated seeds were frozen at -18^{0}C for 12h to stop the germination process. Spouted seeds were freeze-dried and ground to pass through a 40 mesh sieve for analysis. Total proteins, water soluble and water insoluble proteins were estimated by standard methods. Seed storage protein profiles of three varieties germinated horse gram were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Extracted protein fractions from germinated horse gram seeds in different solvents were studied by HPLC. The highest amount of storage protein degradation was observed in all varieties after 72-80h of germination. Characterization of protein fractions by HPLC showed that albumins/globulins, prolamins and glutelins increases slightly during germination time, as germinating seeds usually accompany by interconversion and production of new compounds. Electrophoregrams for each variety were shown and the high molecular weight proteins band intensity decreases during different germination period were noted. Genetic diversity of germinated horse gram was evaluated by constructing the dendrogram for high molecular weight (HMW) and low molecular weight (LMW) gluten subunit bands. In conclusion, SDS-PAGE of germinated seed storage proteins can be economically used to assess genetic variation and relation in germplasm. The specific bands of germinated seed storage protein profiles may be used as markers for identification of the mutants/genotypes.

Keywords: Horse germ seeds, seed storage protein, germination, SDS-PAGE, HPLC.

Introduction

The crop legumes account for 27% of the world's primary agricultural crop production, with grain legumes contributing 33% of daily protein nitrogen intake of humans. Well- known grain legumes include beans, lentils, lupins, peas, and peanuts and are cultivated for their seeds, and are also known as pulses[1]. The horse gram is a legume with great nutritional potential due to its high protein content and it has been suggested as an alternative protein source to soybean in countries where the former legume is not a native crop, or in situations where soybean cannot be used due to allergic reactions or intolerances [2]. Korhonen *et al.*[3] stated that legume seeds have high protein content to develop as ingredients for health-promoting functional foods or pharmaceutical preparations. There are many potential cosmetic and technical uses for legume proteins. There are opportunities to use proteins in paper- coating treatments, adhesives (hot melt using wheat gluten) and in cosmetic sectors (haircare, skincare, bathing products). The protein fractions themselves can be modified by processing, for example to increase solubility, to optimize emulsifying and foaming capabilities, or film- forming capabilities to increase opportunities for use in biopolymer markets[4]. Seed storage proteins (SSPs) are a set of proteins that are synthesized in the developing seed at the stage when cell division is complete. The main sources of SSPs are carbon, nitrogen and sulphur which accumulates in protein bodies in the cotyledonary parenchyma cells is important for seed germination and early seedling growth until they undergo hydrolysis upon germination[5]. Analysis of seed storage proteins provide aid for identification and characterization of diversity in crop varities, cultivars and their wild varities and phylogenetic relationship of the accessions[6]. Genetic diversity of seed storage proteins has been reported for Lima bean[7], *Phaseolus vulgaris*[8] and chickpea[9]. Polymorphism in seed storage proteins has been associated with geographical origin of germplasm[10,9]. Information about genetic diversity of germplasm is a useful tool in gene bank management and helps in the establishment of core collection [11]. Cultivar identification is useful for describing a new cultivar, testing genotype purity and speeding up-distinctness uniformity stability (DUS) test for candidate cultivar[12]. The protein profiling of germplasm and use of genetic markers have been widely and effectively used to determine the taxonomic and evolutionary aspects of several crops[13].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is most economical simple and extensively used biochemical technique for analysis of genetic structure of germplasm. As seed storage proteins are largely independent of environmental fluctuation, their profiling using SDS-PAGE

technology is particularly considered as a consistent tool for economic characterization of germplasm[14,15]. The current increased interest in utilization of legume grains not only from the fact that they typically possess two to four times the quantity of protein than traditional cereal grains, but that their protein are typically of higher nutritional quality[16,17]. Germination improves the nutritive value of cereals and legumes by decrease the level of anti nutrients present in cereal and maximize the level of utilizable nutrients[18,19]. It is an important technology for improving the nutritional quality of legumes and variably affects the proximate composition of seeds[20,21]. During germination metabolic enzymes are activated and utilization or synthesis of wide range of chemical compounds occurs in seeds and results in the enhancement of nutritional quality[22]. Germinated seeds are rich in vitamins, minerals and are reported to contain important phytochemicals for disease prevention[23]. Germination is a simple biochemical enrichment tool to enhance the palatability (minerals and vitamins) result in increasing the digestibility and nutritive value[24,25].

The horse gram is a high nutritional legume and become a staple food for people living in dry and rural regions in Malaysia. It is one of the most nutritious, high in proteins, and easily-digestible. Even though, these seeds were used for centuries, less information is available regarding the biochemical changes occurring during germination. Hence, this experiment was conducted to observe the protein electrophoregrams by SDS-PAGE and HPLC study of fractionated proteins during germination.

Material and Methods

Dry seeds of horse germ (three varieties) were purchased from Indian grocery shop kluang, Johor, Malaysia. Good and mature seeds were disinfecting with 60% ethanol solution at 15 minutes. Then they were washed thoroughly and soaked in distilled water for 4 h and transferred into petri plate containing moist filter paper and about 5ml of distilled water was added in it and germinated in a seed germinator (Germany) in the dark at 20^0C, with 95% relative humidity. Only distilled water was sprayed daily during germination period. Seeds were harvested at different times of germination 24h, 48h, 72h, 96h and 110h. Germinated seeds were frozen at -18^0C for 12h to stop the germination process. Spouted seeds were freeze-dried and ground to pass through a 40 mesh sieve for analysis.

Nitrogenous Compounds of Germinated horse germs: Total nitrogen was determined according to Kjeldahl's method. Percentage crude protein was calculated as total nitrogen x 6.25. Soluble protein, insoluble and non-protein nitrogen were measured according to the methodology described by Periago and co-workers[26].

Preparation of protein samples: For extraction of soluble proteins, seeds were grounded in 50mM phosphate buffer (pH 7.8) and centrifuged in micro-centrifuge machine (Eppendorf) for 10 min at 14000 rpm. The supernatant was separated and used for protein profiling. Protein concentration of extracts was measured by dye binding assay as described by Bradford[27].

Supernatant was mixed (4:1) with cracking solution (10ml containing 1g SDS; 0.01g bromphenol blue; 2ml β-mercaptoethanol; 1.5ml 0.5M tris, pH6.8; 5g sucrose and 6.5ml water) on vortex mixer and heated in a boiling water bath for five minutes to denature the proteins.

Gel Electrophoresis: SDS gel electrophoresis of extracted germinated horse gram seeds proteins were performed using 5% stacking and 12.5% separating gels according to the method of Laemmli[28] with modifications. The freeze-dried germinated horse germs were solubilised in sample buffer consisting of 50mM Tris, pH 6.8; 2% SDS; 10% glycerol; 0.1% bromophenol blue and 5% β-mercaptoethanol. The mixture was heated for 30 min at 40^0C; centrifuged (14000xg, 10 min) and the supernatants were applied to the gel. Electrophoresis was performed at a constant voltage of 150v. Gel were fixed and stained with 0.2% Coomassie Brilliant blue R-250 in methanol: acetic acid: water (5:4:1, v/v/v).

High Performance Liquid Chromatography: Analysis of protein fractions was carried out using analytical HPLC (clarity control shimadzu). Direct control of Shimadzu LC-10 series using clarity chromatography software controlled through the SCL-10AVP or CBM-20A (Lite) controllers.

Protein fraction extraction for HPLC and SDS-PAGE: 5 g of dry seeds which germinated at different interval time (0, 24, 48, 72, 96, 110 h) were grounded and subjected to consecutive extraction according to their solubility based on Wieser[29]. Albumin / globulin were extracted with buffered NaCl, (0.4 mol/L, pH 7.6), prolamin was extracted with 60 % (v/v) ethanol and glutelins was reduced with 2 mol/L urea. All extracts were centrifuged at 18000 rpm for 10 min under 4ºC and the supernatant filtered.

Protein Assay: Protein content of samples was assessed by the Biuret method on the Unico UV-2100 spectrophotometer. Bovin serum albumin was used as a standard. Different concentrations of albumin were mixed with the biuret dye reagent and read, after 30-min incubation period, at 540nm. Samples at different stages of purification were diluted appropriately and the absorbance was read and compared to the standard.

Results and Discussion

Germination: Germination is a natural biological process of all superior plants by which the seed comes out of its latency stage, once the minimal environmental conditions needs for its growth and development, such as humidity, temperature, nutrients etc are given[30]. It is known that during germination a sequence of metabolic changes such as the activation of the respiratory processes and protein synthesis are triggered. The mobilization

of seed storage proteins represents one of the most important post germinative events in the growth and development of seedling[31]. Proteolytic enzymes play central role in the biochemical mechanism of germination. The developments of acid, neutral and alkaline proteases involve in the degradation of proteins in germinating legumes. These proteases increase in the early stages of germination and decrease later[32].

According to Rodrıguez *et al*.[33], seeds germination involves mobilization of the protein reserves in cotyledons, coupled with the synthesis of new proteins, necessary for sprout's growth. Due mainly to enzyme activity in moist seeds, which is engaged in protein hydrolysis, some amino acids and peptides, can be released. Furthermore, the amino acids produced by hydrolysis of the protein reserves are not used solely in synthesizing new components, but may also utilizing as an energy source, especially in the early stages of the germination.

As a result of the germination process, horse gram seeds undergo considerable metabolic changes in their storage proteins. The content of soluble and insoluble protein nitrogen was significantly decreased by 5 days of germination and increased degradation of high molecular weight proteins during germination was accompanied with concomitant increase protein fraction with increasing germination periods [34].

Protein Estimation: Kjeldahl method is used to calculate the percentage (%) crude protein as shown in table-1. Prolonged germination process caused degradation of water soluble and water insoluble protein content as shown in figure-1 and figure-2. The content of water soluble protein was significantly decreased with increasing germination periods by 1.5-fold after 72 hour of germination compared to ungerminated seeds. For the variety of *Macrotyloma unifloru*, the raw seeds had total crude protein of 37 % and total protein content is higher in first two days of germination process and decrease gradually until 110 h. For the variety of *Macrotyloma bieense*, total crude protein of raw seeds is 33% and total protein content decrease very fast during germination. Total crude protein for *Macrotyloma axillare* is lower as compare with another two varieties. The raw seeds only have total crude protein of 22 %.

There is a recent study by Yadav S. *et al*.[35] regarding to the protein content for the seeds of the wild horse gram (IC 212722) belonging to the new species *Macrotyloma sar-garhwalensis Gaur* and *Dangwal*. Seed contains 38.37 ± 1.03 % crude proteins. More than 95 % of the total nitrogen belongs to protein nitrogen. Contribution of albumin-globulin, glutelin and residual protein fraction such as prolamins to the total seed protein accounts for 75.27 %, 17.52 % and 7.19 % respectively. Hence, the protein content estimation result obtained from *Macrotyloma unifloru, Macrotyloma bieense*, and *Macrotyloma axillare* have abundant of water insoluble protein content.

Characterization of Protein Fraction by HPLC: Albumins/ Globulins: For HPLC studies, the proteins were extracted consecutively by using three different buffers according to their solubility as demonstrated by Wieser[29]. The HPLC results of three protein contents are showed in figures-3, 4 and 5. The results showed that almost the same retention time (R_t) of particular protein fraction for each variety of samples at different germination interval time. There are two major salt soluble protein, albumin and globulin. For *Macrotyloma uniflorum*, only one highest peak can be detected and that peak can be identified as globulin. The reasons behind this speculation as there are reports which revealed that globulins are the main seed storage proteins in legume seeds[36].

Prolamins: The protein fraction grows significantly during the germination especially in the final part of the process. Germination is a simple biochemical enrichment tool to causes important changes of legume seeds figure-6 (prolamins) and figure-7 (glutelins). Germinating seeds are usually accompanied by interconversion and production of new compounds[34,37]. Another reason can be explained by the HPLC detection method, it is possible that many polypeptide fractions to be detected which it is out of expectation.

The HPLC results showed that globulin was the major salt soluble protein as what had reported in the other studies which revealed that globulins were the main seed storage proteins in legume seeds[36]. The protein fraction grows significantly during the germination especially in the final part of the process. Germinating seeds are usually accompanied by interconversion and production of new compounds[37].

The SDS–PAGE profiles of germinated horse gram seeds (figure-8, 9 and 10) are in line with those of other legume crops[38]. Protein profiles were very distributed one and that it decrease in quantity and quality from the beginning of the germination until the end of the process. It was characterized by the presence of proteins in a wide range of molecular weights. A rich profile considering the number of bands was observed in the control seeds (0 h) and the seeds at the beginning of germination (24 h). The predominant proteins of horse gram resolved into two groups of bands of 21.5-34 kDa and 52-66 kDa. In the germination process seeds composition undergoes a complex transformation. The SDS-PAGE electrophoregrams showed that there was disappearance of high molecular weight (HMW) polypeptides from 97.4 kDa until 45 kDa. It is a well-known fact that storage proteins are degraded during germination and seedling growth. During germination, there was a differential degradation of higher molecular weight proteins bands indicating that they were hydrolyzed more rapidly than are proteins of having low molecular weights (LMW). Studies with seeds of *L. sativus*, *Dolichos lablab*, *Cicer arietinum*, *Vicia faba* and also *Gossypium hirsutum* revealed various protein fractions ranging from 92 to 12 kDa with a faster degradation of high-molecular weight polypeptides during the early stages of germination from cotyledons were legumins and vicilins (globulins) family[39,38].

Table-1
Result for Protein Estimation of Three Horse gram Varieties

Varieties **Interval time (h)**	**Crude protein (%)**	**Water soluble protein (%)**	**Water insoluble protein (%)**
Macrotyloma uniflorum			
Control	37.3403	6.7573	30.583
24	36.604	6.624	29.98
48	35.828	6.574	29.254
72	29.4068	5.3958	24.011
96	26.2386	4.8146	21.424
110	24.7859	4.5479	20.238
Macrotyloma bieense			
Control	33.0814	7.1844	25.897
24	31.0669	6.7469	24.32
48	25.1818	5.4688	19.713
72	20.6726	4.4896	16.183
96	16.389	3.125	13.264
110	10.2159	1.9479	8.268
Macrotyloma axillare			
Control	21.93	7.251	14.679
24	21.4325	7.0865	14.346
48	20.8903	6.9063	13.984
72	18.907	6.651	12.256
96	17.8081	6.4271	11.381
110	14.6627	5.2917	9.371

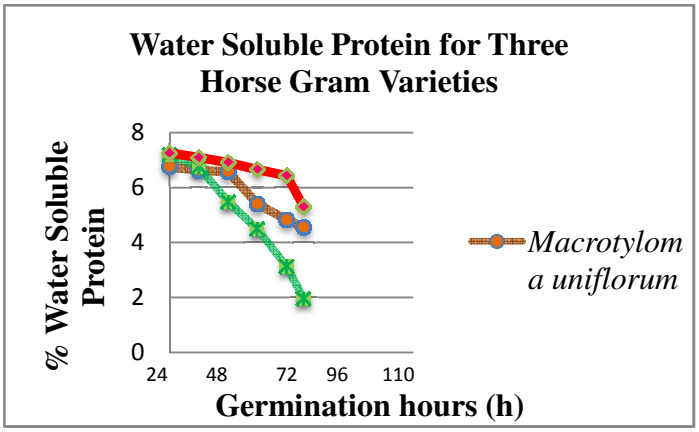

Figure-1
Concentration of water soluble protein for three horse gram varieties at different germination interval time

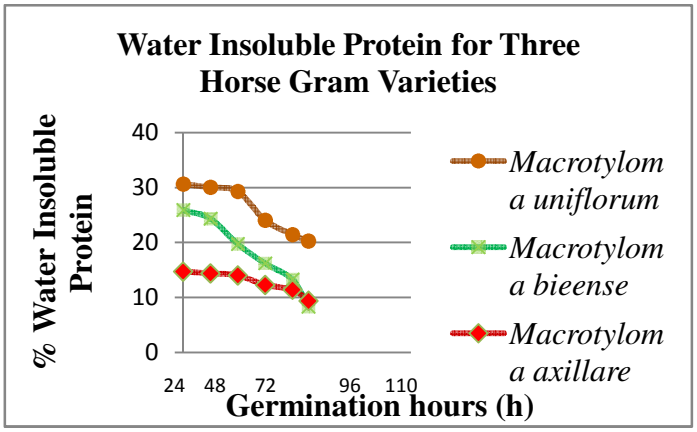

Figure-2
Water Insoluble Protein for three horse gram varieties at different germination interval time

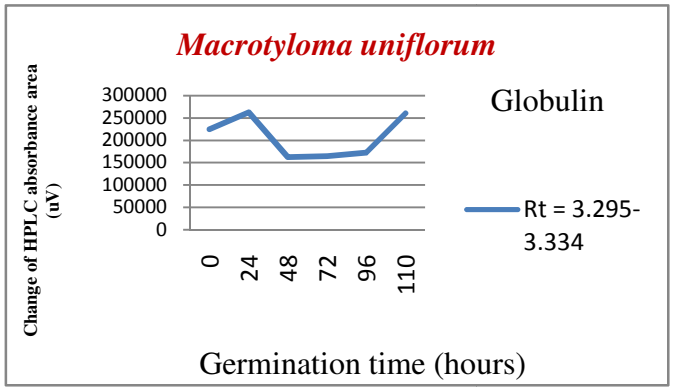

Figure-3
Change of HPLC absorbance area of *Macrotyloma uniflorum* at different germination interval time

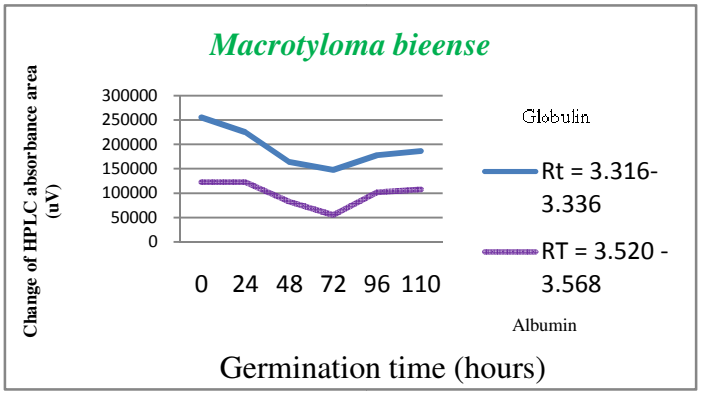

Figure-4
Change of HPLC absorbance area of *Macrotyloma bieense* at different germination interval time

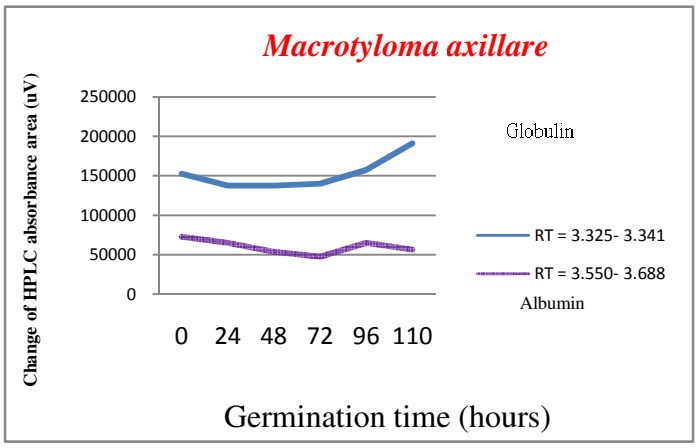

Figure-5
Change of HPLC absorbance area of *Macrotyloma axillare at* different germination interval time

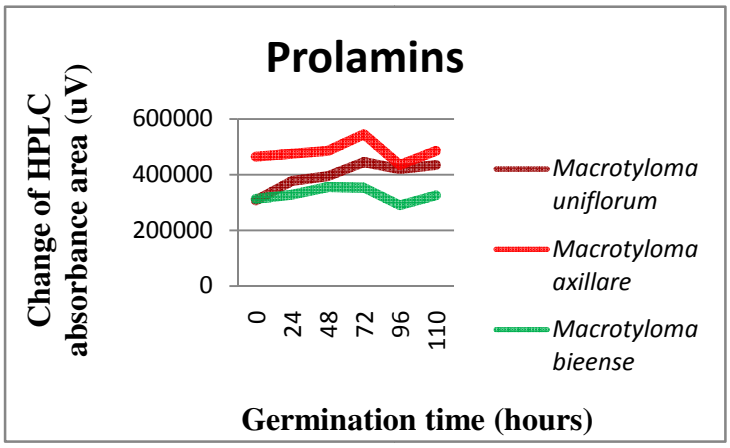

Figure-6
Change of Prolamins HPLC absorbance area of three varieties of horse gram at different germination interval time

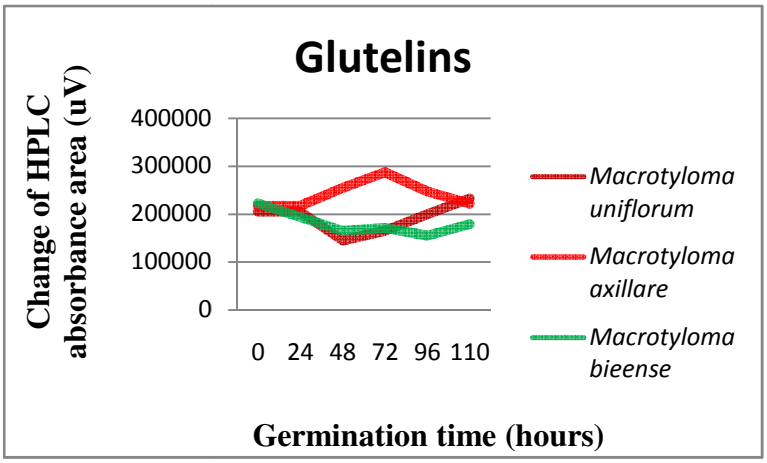

Figure-7
Change of Glutelins HPLC absorbance area of three varieties of horse gram at different germination interval time

Below show three electrophoregrams of the proteins extracted in phosphate buffer during germination at various interval time and standard protein markers (M):

Characterization of HPLC Protein Fractions by SDS-PAGE: Composition changes of three proteins which were albumin/ globulins, prolamins and glutelins for HPLC have also been further studied in SDS- PAGE. Studies with seeds of *L. sativus*, *Dolichos lablab*, *Cicer arietinum*, *Vicia faba* and also *Gossypium hirsutum* revealed various protein fractions ranging from 92 to 12 kDa with a faster degradation of high-molecular weight proteins[38-41]. Savelkoul *et al.*[42] suggested that fast-degrading high-molecular-weight polypeptides during the early stages of germination from cotyledons were legumins and vicilins (globulins) family. SDS-PAGE profiles showed that legumin-like proteins became less stained in the longer germination period, which was normal because of the transformations suffered by the proteins. The horse gram seeds contain albumin and globulin storage proteins; act as amino acid reserves which were mobilized to nourish the seedling. As a result, there was a decrease in intensity of high molecular weight polypeptides of globulins (figure 11, 12 and 13). For *Macrotyloma bieense,* germinating seeds which extracted with salt buffer, revealed that even though some degradation starts at 24 h, marked differences were visible after 48h (figure 11, 17 and 17). At 72 h, the amount of the 55-59 kDa globulins as well as other proteins of low molecular weight band declined in the intensity rapidly with the concomitant appearance of a 40 kDa protein. Germination of three horse grams varieties have been investigated as a means of the proteins tend to decrease, more evidently in the extraction of prolamins with ethanol (figure-12, 15 and 18). Most of the proteins at 45 kDa in size disappeared. A streak of proteins below 31 kDa position was observed and maintained thereafter. Legumes do contain a small percentage of alcohol soluble protein has been often reported. As a result of the germination process, metabolic enzymes, such as proteinases, were activated and seeds undergone considerable metabolic changes in their storage proteins. For horse gram seeds, proteolysis occurs and reached maximum after third day (72 h) of seed germination. It could be explained by the hydrolysis of the storage proteins to be utilized as food by the growing embryo. It can be assumed that the 20–30 kDa polypeptide group and the polypeptide fractions with lower molecular weights obtained by means of SDS-PAGE, represent the products of proteolytic degradation of the seed storage proteins. Increased degradation of high molecular weight proteins during germination was accompanied with concomitant increase in the amount of a 23 kDa protein band (figure 13, 16 and 19). Using SDS-PAGE to study changes in germinating lentil proteins, Hsu and co-workers[43] observed a progressive decrease in large protein subunits and formation of small subunits as germination progressed. They found more changes in proteins for lentils than pea and faba bean. The significantly changes in protein content of seeds were observed in the final part of germination could be related to protein hydrolysis, synthesis and re-arrangement. This was explained mainly through the initial alteration of the seeds component proportion (the rundown of the seeds reserve), then through the *de novo* synthesis. The results of this study were supported by other reports showing selective protein band disappearance and appearance[44,45]. Ahmed *et al.*,[45] suggested that protein disappearance represents degradation of reserve proteins, while new proteins appearing at specific times during germination and seedling development have stage- specific developmental functions.

Electrophoregram patterns for three varieties of horse gram samples at different interval time with standard protein markers (M) are shown below:

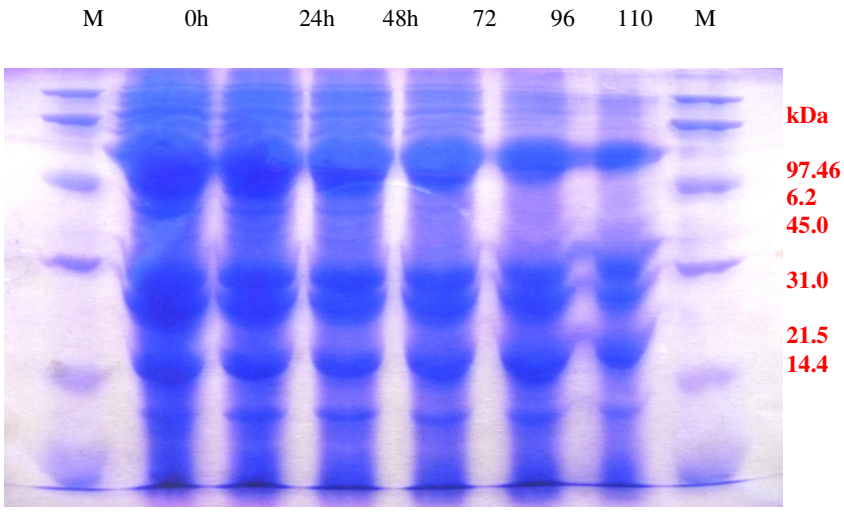

Figure-8
Macrotyloma uniflorum

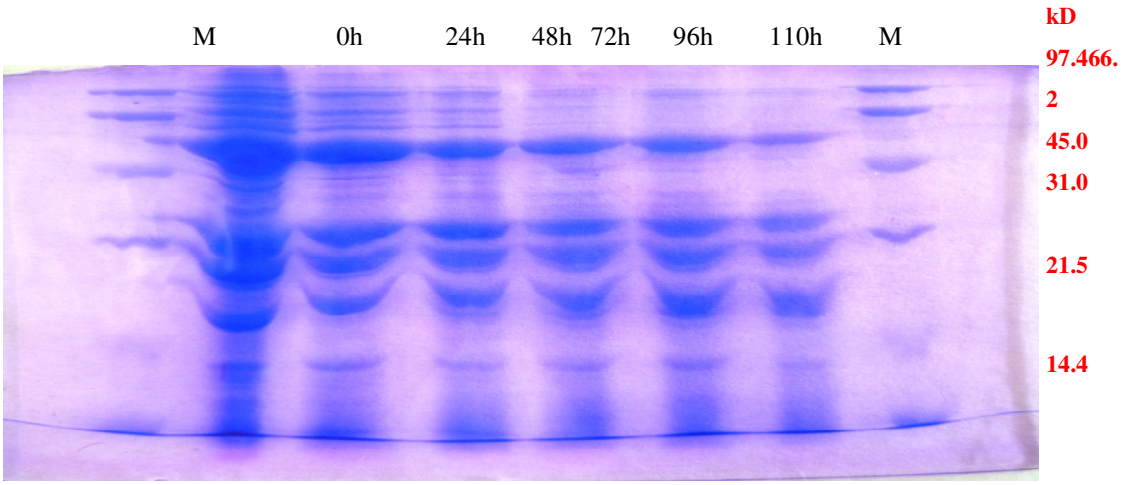

Figure-9
Macrotyloma bieense

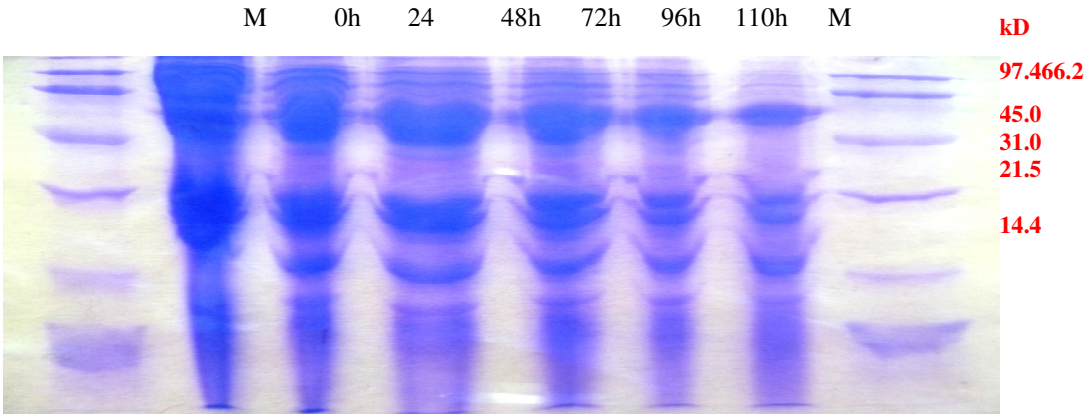

Figure-10
Macrotyloma axillare

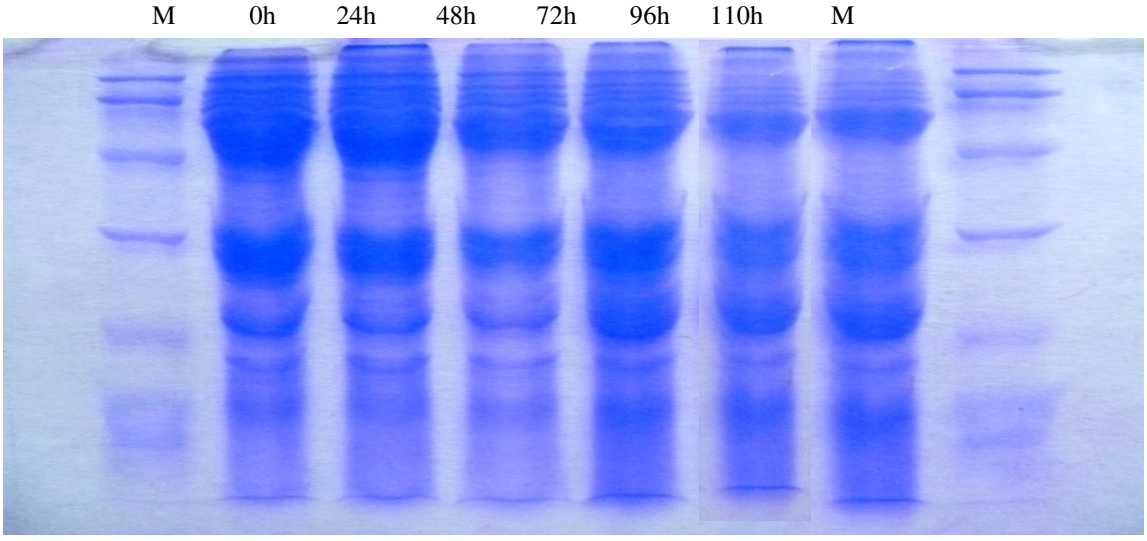

Figure-11
Extraction of albumins/ globulins with buffered salt solution (NaCl)

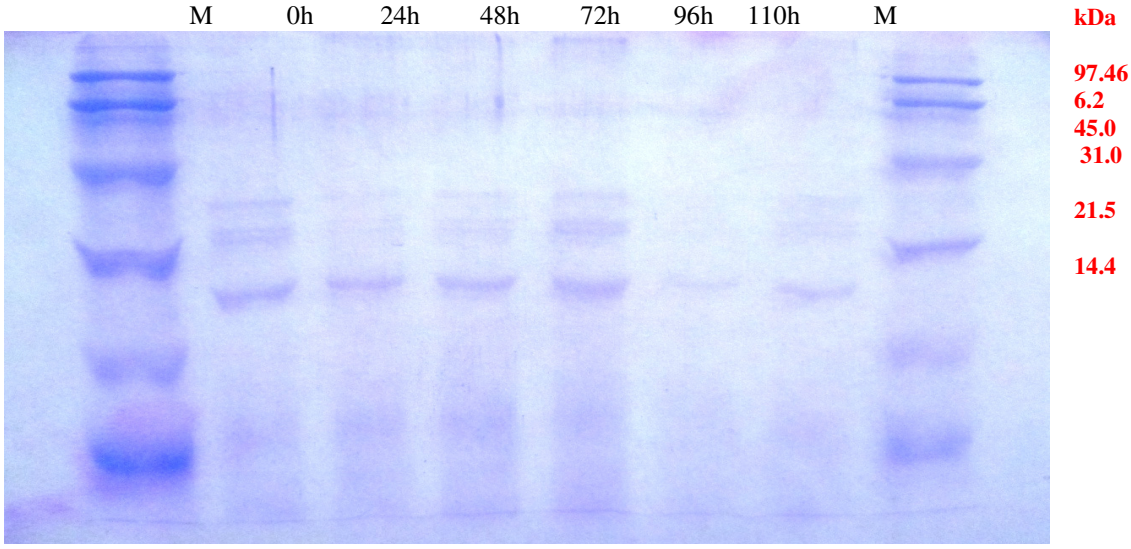

Figure-12
Extraction of prolamins with 60% (*v/v*) ethanol

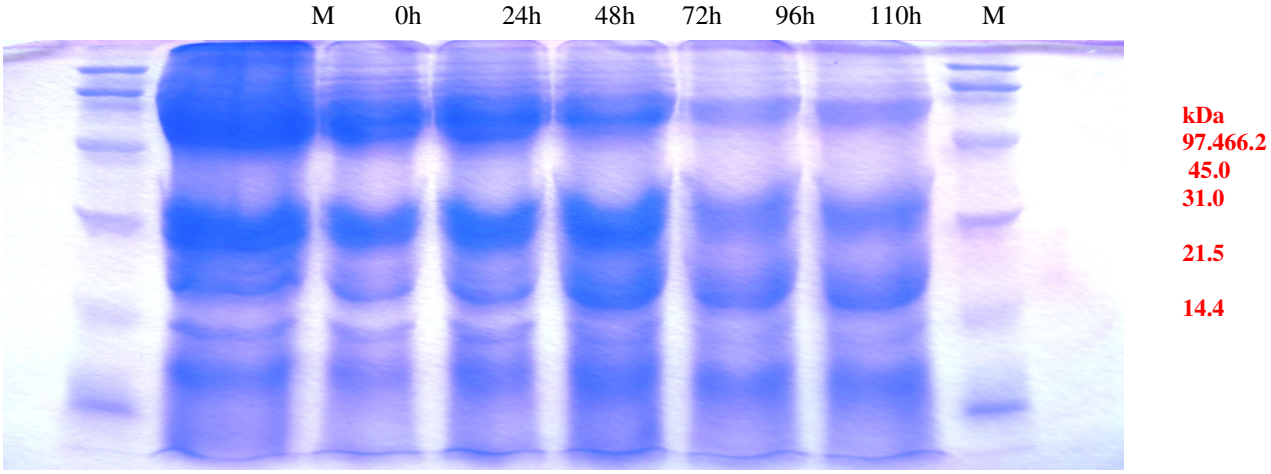

Figure-13
Reduction of glutelins with 2mol/ L urea

Macrotyloma bieense

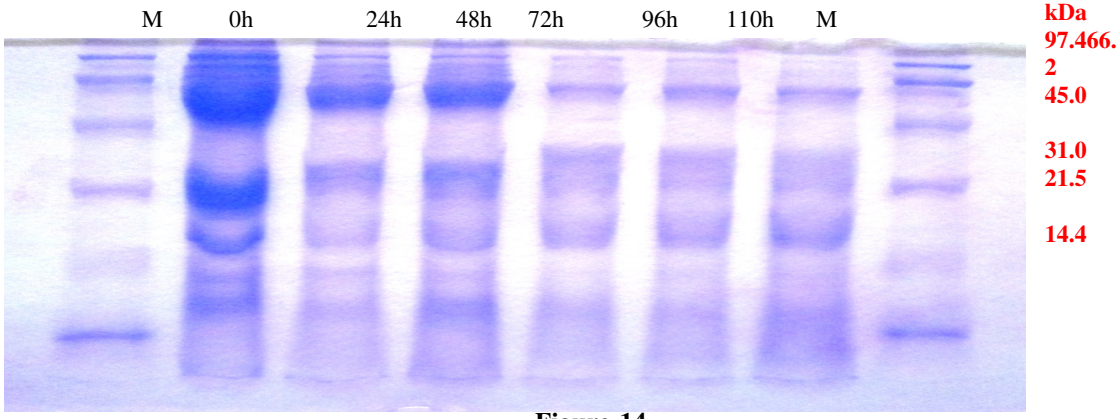

Figure-14
Extraction of albumins/ globulins with buffered salt solution (NaCl)

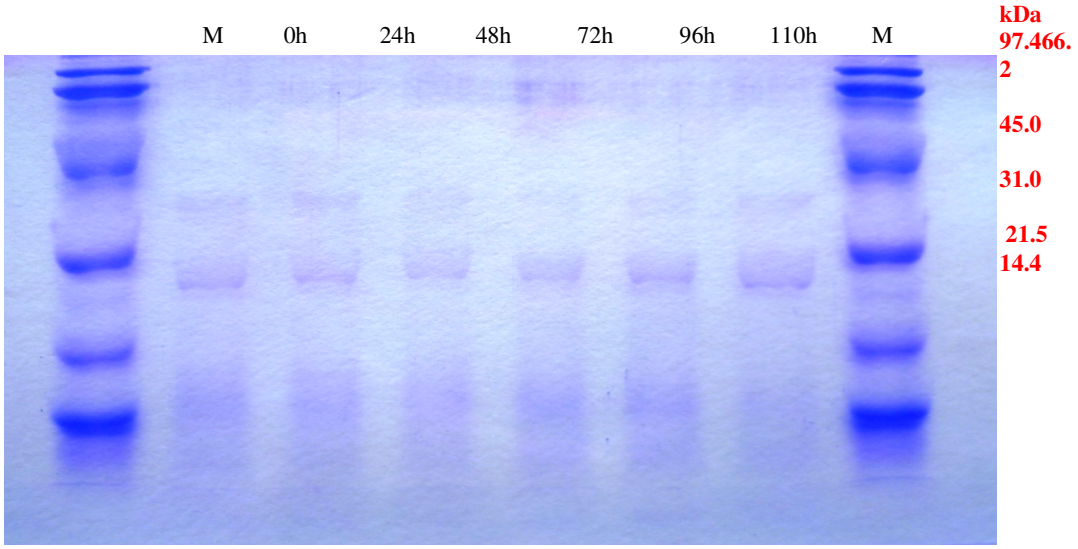

Figure-15
Extraction of prolamins with 60% (*v/v*) ethanol

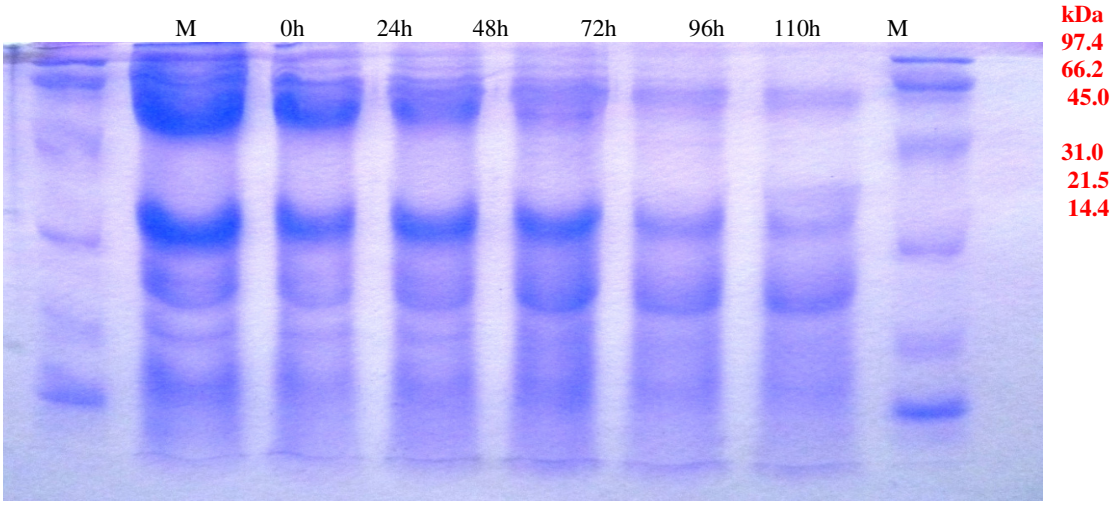

Figure-16
Reduction of glutelins with 2mol/ L urea

Macrotyloma axillare

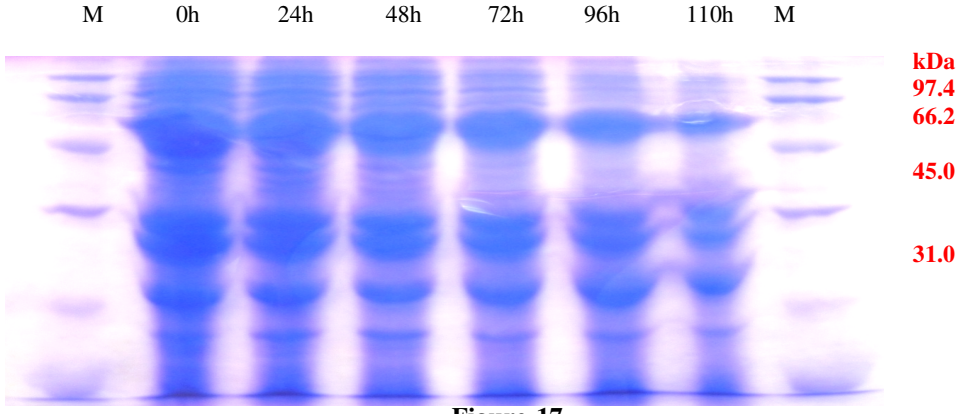

Figure-17
Extraction of albumins/ globulins with buffered salt solution (NaCl)

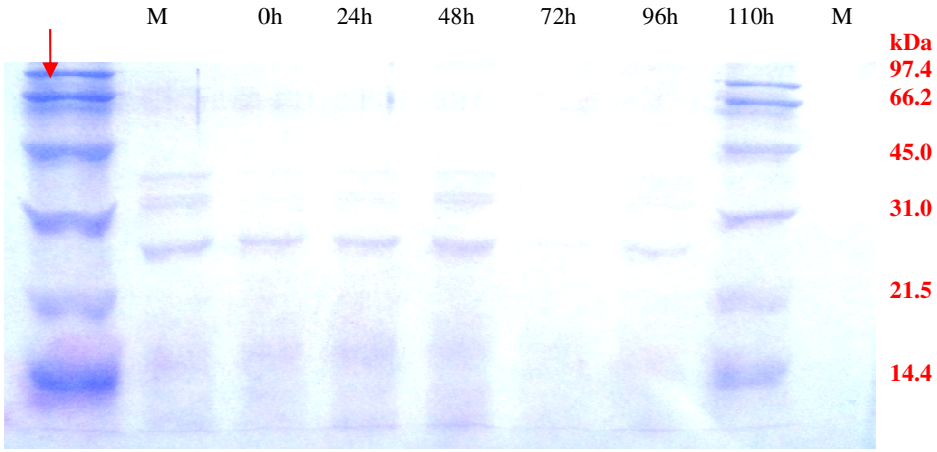

Figure-18
Extraction of prolamins with 60% (*v/v*) ethanol

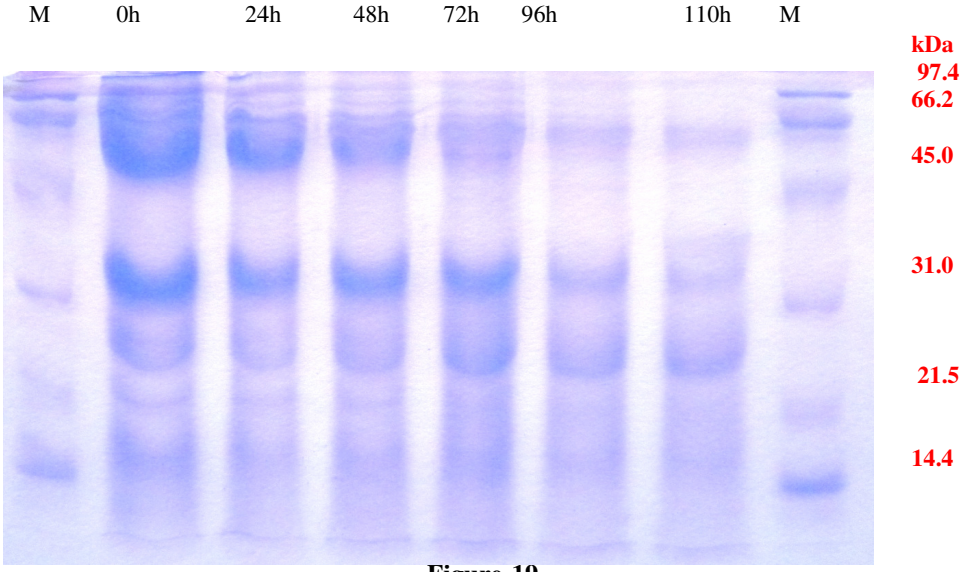

Figure-19
Reduction of glutelins with 2mol/ L urea

Conclusion

In conclusion, electrophoresis (SDS-PAGE) of seed storage proteins can be economically used to assess genetic variation and relation in germplasm and also to differentiate mutants from their parent genotype. Moreover seed storage protein based marker can be used for identification of genotype in future. It is suggested that genotypes with similar banding patterns should be further characterized by 2D electrophoresis. Major seed storage proteins used during germination is water soluble albumins and globulins. Electrophoretic study of total seed proteins of horse grams revealed that the predominant proteins are present as two groups of broad bands, ranges from 28-34 kDa and 52-66 kDa respectively. SDS-PAGE of seed storage protein fractions of three varieties of horse gram during germination showed different degrees of protein band degradation. The number and quantity of proteins with high molecular mass decrease gradually during germination. HPLC chromatograms of protein fractions for three varieties of horse gram during germination suggest differences in terms of number of peaks and peak intensities of the albumin/ globulin, prolamin and glutelin fractions. These seed storage proteins also decreases and increases during germination due to breakdown or formation of new molecules.

References

1. Graham T.A. and Gunning B.E.S., Localization of legumin and vicilin in bean cotyledon cells using fluorescent antibodies, *Nature*, **228,** 81-82 **(2003)**
2. Davidsson L., Dimitriou T., Walczyk T. and Hurrel R.F., Iron absorption from experimental infant formulas based on pea (*Pisum sativum*)-protein isolate: the effect of phytic acid and ascorbic acid, *British Journal of Nutrition,* **85,** 59-63 **(2001)**

3. Korhonen H. and Pihlanto A., Food-derived bioactive peptides–Opportunities for designing future foods, *Current Pharmaceutical Design*, **9,** 1297-1308 **(2003)**

4. Clark J.H. and Deswarte F.E.I., *Introduction to chemicals from biomass,* UK, Wiley **(2009)**

5. Li Q., Wang B.C., Xu Y. and Zhu Y.X., Systematic studies of 12S seed storage protein accumulation and degradation patterns during *Arabidopsis* seed maturation and early seedling germination stages, *J. of Biochem and Molecular Bio,* **(40),** 373-381 **(2006)**

6. Nisar M., Ghafoor A., Khan M.R., Ahmad H., Qureshi A.S. and Ali H., Genetic diversity and geographic relationship among local and exotic chickpea germplasm, *Pak. J. Bot*., **39,** 1575-1581 **(2007)**

7. Lioi L., Sparvoli F. and Bollini R., Variation and genomic polymorphism of lectin-related protein in Lima Bean (*Phaseolus lunatus L.*) seed, *Genetic Resources and Crops Evaluation*, **46,** 157-182 **(1999)**

8. Ferreira J.J., Lvarrer E.A., Roca M.A and Giraldez R., Determination of the out crossing rate of phaseolus vulgaris L., using seed protein markers, *Euphytica,* **113,** 259-263 **(2000)**

9. Ghafoor A., Gulbaaz F.N., Afzal M., Ashraf M. and Arshad M., Inter-relationship between SDS-PAGE markers and agronomic traits in chickpea *(Cicer arietinum L.), Pak. J. Bot*., **35,** 613-624 **(2003)**

10. Satija D.R., Adrash B., Gupta S.K. and Bala A., Genetic diversity in relation to protein and protein fractions in chiclpea *(Cicer arietinum L.), Crop Improvement,* **29,** 122-135 **(2002)**

11. Ghafoor A. and Ahmad Z., Diversity of agronomi traits and total seed protein in black germ vigna mungo(L) hepper, *Acta Biologia cracoviensia series Botanica*, **47,** 69-75 **(2005)**

12. Chowdhury M.A., Vandenberg B. and Warkentin T. Cultivar identification and genetic relationship among selected breeding lines and cultivars in chickpea *(Cicer arietinum L.), Euphytica*, **127,** 317-325 **(2002)**

13. Ghafoor A., Ahmad Z., Qureshi A.S. and Bashir M., Genetic relationship in Vigna mungo (L.) Hepper and V. Radiate (L.) R. Wilczek based on morphological traits and SDS-PAGE, *Euphytica*, **123,** 367-378 **(2002)**

14. Javid A.A., Ghafoor and Anwar R., Seed storage protein electrophoresis in groundnut for evaluating genetic diversity, *Pak. J. Bot*., **36,** 87-96 **(2004)**

15. Iqbal S.H., Ghafoor A. and Ayub N. Relationship between SDS-PAGE markers and Ascochyta blight in chickpea, *Pak. J. Bot.,* **37,** 87-96 **(2005)**

16. Adams M.R., Golden D.L., Franke A.A., Potter S.M., Smith H.S. and Anthony M.S., Dietary soy β-conglycinin (7 S globulin) inhibits atherosclerosis in mice, *Journal Nutrition*, **134,** 511–516 **(2004)**

17. Dogan D., Khawar K.M. and Özcan S., Agrobacterium mediated tumor and hairy root formation from different explants of lentils derived from young seedlings, *Int. J. Agric. Biol.,* **7,** 1019–1025 **(2005)**

18. Mohamed M.E., Amro B.H., Mashier A.S. and Elfadil E.B. Effect of processing followed by fermentation on antinutritional factors content of pearl millet *(Pennisetum glaucum L.)* cultivars, *Pak. J. Nutr*., **6,** 463-467 **(2007)**

19. Inyang C.U. and Zakari U.M., Effect of germination and fermentation of pearl millet on proximate, chemical and sensory properties of instant Fura-Nigerian cereal food, *Pak. J. Nutr*. **7,** 9-12 **(2008)**

20. Gulewicz P.C., Martinez-Villaluenga, Frias J., Ciesiolka D., Gulewicz K. and Vidal-Valverde C., Effect of germination on the protein fraction composition of different lupin seeds, *Food Chem.,* **107,** 830-844 **(2008)**

21. Onwuka C.F., Ikewuchi C.C., Ikewuchi C.J. and Ayalogu O.E. Investigation on the effect of germination on the proximate composition of African Yam beam and fluted pumpkin, *J. Applied Sci. Environ. Manage.,* **13,** 59-61 **(2009)**

22. Taraseviciene Z., Danikenko H., Jariene E., Paulauskiene A. and Gajewski M. Changes in some chemical components during germination of broccoli seeds, *Not. Bot. Hortic. Agrobot. Cluj*., **37,** 173-176 **(2009)**

23. Fernandez-Orozco R., Piskula M.K., Zielinski H., Kozlowska H., Frias J. and Vidal-Valverde C., Germination as a process to improve the antioxidant capacity of Lupinus angustifolius L. Var. Zapaton, *Eur. Food. Res. Technol*., **223,** 495-502 **(2006)**

24. Sulieman M.A., ElTyeb M.M., Abbass M.A., Ibrahim E.E.A., Babiker E.E. and ElTinay A.H., Changes in chemical composition, phytate, phytase activity and minerals extractability of sprouted lentil cultivars, *J.Biol.Sci*., **7,** 776-780 **(2007)**

25. Ramakrishna V., Rani P.J. and Rao P.R. Anti-nutritional factors during germination in Indian bean *(Dolichos lablab L.)* seeds, *World J. Dairy Food Sci*., **1,** 6-11 **(2006)**

26. Periago M.J., Ros G., Martinze C. and Rincon F., Variation of non-protein nitrogen in six Spanish legumes according to the extraction methor used, *Food Research International*, **29(5-6),** 489-484 **(1996)**

27. Bradford M.A., A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principale of dye binding, *Anal. Biochem*., **72,** 248-254 **(1976)**

28. Laemmli U.K., Clevage of structural proteins during assembly of the head of bacteriophage T4, *Nature*, **227,** 680-685 **(1970)**

29. Wieser H., Koehler P., Hartmann G., and Rychlik M., Degradation of storage proteins in different cereals by peptidases induced by germination, *Lichtenbergstrasse 4, D- 85748 Garching* **(1998**)

30. Sangronis E. and Machado C.J., Influence of germination on the nutritional quality of Phaseolus vulgaris and Cajanus cajan LWT, *Journal of Science and Technology*, **40,** 116-120 **(2007)**

31. Sangronis E., Rodriguez M., Cava R. and Torres A., Protein quality of germinated Phaseolus vulgaris, *European Food Research and Technology*, **222,** 144-148 **(2006)**

32. Ramakrishna V. and Ramakrishna Rao P., Purification of acidic protease from the cotyledons of germinating Indian bean (Dolichos lablab L. Var lignosus) seeds, *African Journal of Biotechnology,* **4(7),** 703-707 **(2005)**

33. Rodriguez C., Frias J., Vidal-Valverde C. and Hernandez A., Correlation between some nitrogen fractions, lysine, histidine, tyrosine, and ornithine contents during the germination of peas, beans, and lentils, *Food Chem.***, 108,** 245-252 **(2008)**

34. Wanasundara P.K.J.P.D., Shahidi F. and Brosnan M.E., Changes in flax (*Linum usitatissmum*) seed nitrogenous compounds during germination, *Food Chem.,* **65,** 289–295 **(1999)**

35. Yadav Sangita, Negi K.S. and Mandal S., Protein and oil rich wild horsegram, *Genetic Resources and Crop Evolution,* **51(6),** 629-633 **(2004)**

36. Lqari H., Pedroche J., Giro´n-Calle J., Vioque J. and Milla´n F., Purification and partial characterization of storage proteins in *Lupinus angustifolius* seeds, *Grasasy Aceites*, **55,** 364–369 **(2004)**

37. Ahmed A.N., Javed I.H., Akram M. and Akhtar S., Germination scenario of barley genotypes to chloride and sulphate salinities of sodium, *International Journal Agriculture Biology*, **5,** 258–261 **(2003)**

38. Ramakrishna V., Mobilization of albumins and globulins during germination ofIndian bean (*Dolichos lablab L. var. lignosus*) seeds, *Acta Botanica Croatica*, **66,** 135–142 **(2007)**

39. Muntz K., Belozersky M.A., Dunaevsky Y.E., Schlereth A., Tiedemann J., Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth, *J. Exp. Bot.*, **52,** 1741–1752 **(2001)**

40. Chandna M., Sood A. and Matta N.K., Electrophoretic studies on seed proteins of the genus Lathyrus, *Journal of Plant Biochemistry and Biotechnology*, **4,** 121–124 **(1995)**

41. Vigil E.L. and Fang T.K., Comparative biochemical and morphological changes in imbibed cotton seed hypocotyls and radicles in situ and in vivo protein breakdown and elongation growth, *Seed Science Research***, 5,** 41–51 **(1995)**

42. Savelkoul FHMG, Boer H., Tamminga S., Schepers A.J. and Elburg L., In vitro enzymatic hydrolysis of protein and protein pattern change of soya and faba beans during germination, *Plant Food and Human Nutrition*, **42,** 275–284 **(1992)**

43. Hsu D.L., Leung H.K., Morad M.M., Finney P.L. and Leung C.T., Effect of germination on electrophoretic, functional and bread baking properties of yellow pea, lentil and faba bean protein isolates **(1982)**

44. Mitsuhashi W., Oaks A., Development of endopeptidase activities in maize (*Zea maysL.*), *Plant Physiology*, **104**, 401–407 **(1994)**

45. Ahmed F.A.R., Abdel-Rahim EAM, Abdel-Fatah O.M., Erdmann V.A. and Lippmann C., The changes of protein patterns during one week of germination of some legume seeds and roots, *Food Chemistry,* **52,** 433–437 **(1995)**