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Nutraceutical characterisation of common bean (*Phaseolus vulgaris* L.) germplasm from Pakistan

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Abstract

Malnourishment is one of the major issues faced by people of mountain areas of Pakistan. Common bean, being a rich source of protein, minerals, and nutraceutical compounds, is considered as a potential food to address the malnutrition problems in these areas. Azad Jammu and Kashmir is widely distributed with the indigenous unexplored landraces of common bean. In the present work, the nutritional profiling of 36 common bean ecotypes collected from the wide range of altitude, based on protein percentage, lysine contents, antioxidants activity, and total phenolic contents was carried out. Significant protein and lysine contents were found in the range of 13 - 24.5% and 4.4 - 7.4%, respectively. The DPPH free radical assay revealed very high antioxidants capacity of 67.8 - 97.8% with significant variation in the selected common bean germplasm. Total phenolic contents were determined using Folin-Ciocalteu reagent method and ranged from 68.9 to 110.3 mg GAE/100 g. Principal Component Analysis (PCA) revealed significant variation, based on hierarchical clustering, within germplasm in all studied attributes. Two main factors were elucidated which imparted 67.36% variation based on PCA. Lysine gene was identified, and its presence was confirmed through primer's amplification. E20 and E34 were found comparatively more nutritious based on biochemical characterisation. The present work will help in selection of high nutrient ecotypes for further substantial research including breeding of nutritionally promising ecotypes for farming community of Azad Jammu and Kashmir.

Introduction

Malnutrition is considered a major problem faced by population of Pakistan due to several factors including low protein input in diet. The World Bank declared Pakistan as a country with low middle income and 5th most populated country around the globe, with only one quarter of the population having access to a balanced diet (UN, 2013; World Bank, 2015). It is estimated that approximately 58% population of the country has no access to a balanced caloric diet due to poverty, ignorance, topography, mismanagement of resources, and various other reasons. The rate of prevalence of malnourishment is higher among the people living in the mountain regions; therefore, it is imperative to focus on approaches which ensure the availability of adequate and nutritious food for mountain communities (Ministry of Health, 2011).

Common bean (*Phaseolus vulgaris* L.), being a cheap and rich source of proteins, is consumed throughout the world (Carla, 2014). The highest bean consumption regions include Latin America, Sub-Sahara Africa, and the subcontinent of India (Nyau, 2014). In Northern Pakistan and Azad Jammu and Kashmir, it is considered as a staple food after maize and wheat (Danish *et al*., 2002, Jannat *et al*., 2019). Production of an environmentally adaptable, disease resistant, high yielding, and good quality crop is the key to encounter problems such as malnutrition.

Dry beans have been suggested as the almost perfect diet as it contains low fat and high protein contents, dietary fibre, complex carbohydrates,

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minerals, and vitamins including folic acid (Celmeli *et al*., 2018). In underdeveloped and developing countries, it is considered as poor man's meat due to its rich protein contents, fibres, and other minerals. Although common bean is deficient in methionine while most cereals have sufficient levels of it, common bean contains generous amounts of essential amino acid, lysine, of which other cereals are deficient in. Therefore, if cereals and legumes are consumed at a ratio 2:1, a balanced diet can be obtained (Broughton *et al*., 2003). The average protein content in beans is approximately 24% with a reported range of 19 to 31%, and the average lysine content is approximately 464 mg/g N with values ranging from 207 to 607 mg/g N (Bressani, 1983). Meng *et al*. (2004) analysed the expression of transgenic lysine-rich protein gene from beans into wheat, and found that it was effective.

In addition to high protein and mineral contents, the presence of phytonutrients allows common bean to be categorised as a healthy food. It contains secondary metabolites such as polyphenols, isoflavonoids, lignin, and others, and its consumption has been associated with numerous health benefits including reduction of cholesterol level (beneficial against coronary heart diseases), defensive effects against cancer (breast and colon cancer, specifically), decreasing obesity and diabetes, resistance against osteoporosis and menopause, and high antioxidant capacity (Bazzano *et al*., 2001; Hangen and Bennick, 2002; Park and Yu, 2004; Heimler *et al*., 2005).

Common bean has very good free radical scavenging capacity in human body as it contains strong antioxidants against reactive oxygen species (ROS), which are associated with the development of several chronic and degenerative diseases (Wani *et al*., 2013; Hayat *et al*., 2014). Polyphenols, with predominant bioactive components of flavonoids and anthocyanins, are considered to contain promising antioxidant capacity and strong antimutagenic and antigenotoxic activities, which mostly occur in coloured beans (Yang *et al*., 2018). Cooked beans contained chemical components which facilitate the neutralisation of free radicals. It is evident that chemical compounds were not inactivated by heat treatment during cooking (Intriago-Ortega *et al*., 2004).

The simultaneous deterioration of nutritional quality is due to loss of diversity within crops and extinction of genetic resources, as majority of the crop genetic diversity with desirable traits remained underutilised in elite varieties. The landraces of common bean, being indigenous resource with intact taste and nutrient composition, is an important repository of quality associated alleles for breeding purposes especially for improvement of nutrition and quality attributes in *Phaseolus* (Carla, 2014).

Northern Pakistan and mountain areas of Azad Jammu and Kashmir are richly loaded with the natural resources including common bean landraces. There is a dire need for collection of such indigenous resources for allelic screening and nutraceutical profiling (Khan *et al*., 2012). Common bean crop has tremendous genetic resources with promising future in Pakistan and Azad Jammu and Kashmir (Hayat *et al*., 2014). As people of Azad Jammu and Kashmir are suffering from malnourishment and food insecurity, exploitation and utilisation of such potent crop can be significant in attaining the dietary requirements of mountain communities. On the other hand, the profiling of the diverse germplasm based on their nutritional attributes will help to select some of the ecotypes for further improvement and commercial cultivation in the region. The present work was therefore conducted for biochemical profiling of common bean germplasm based on proteins, lysine, antioxidant, and phenolic contents.

Materials and methods

Seeds of 35 landraces from different locations of Azad Kashmir and Northern areas of Pakistan were collected along with one reference variety from CIAT (International Centre for Tropical Agriculture) in 2015. Following collection, some of the seeds were dried, ground using mortar and pestle, and subjected to a range of biochemical assays including estimation of protein contents, lysine percentage, antioxidant activities, and phenolic contents.

Determination of total proteins

The ground samples of each ecotype were digested with H_2SO_4 in the presence of catalyst. All organic nitrogenous compounds were converted into ammonium sulphate ($(NH_4)_2SO_4$). The addition of strong alkali (NaOH) and boiling converted $(NH_4)_2SO_4$ to ammonium. This was distilled as NH_4OH, and this amount was determined by titrating it with known normality of H_2SO_4. The percentage of nitrogen was converted to protein by multiplying it with a nitrogenous factor, which is different for various food types. The calculation was as follows:

$$\% \text{ protein} = \%N \times \text{protein factor} \quad \text{(Eq. 1)}$$

where: protein factor for wheat flour = 5.7, others = 6.25 (FAO and WHO, 1973)

Estimation of lysine contents

Lysine is an essential amino acid limited in cereal grains; but in legumes it is presented in large amounts. Lysine was estimated using two different methods, one for analysis of lysine contents within seeds of different genotypes and other for determination of lysine genes within the genome. The lysine extraction method described by Mertz *et al*. (1974) was followed for its estimation in common bean. The basic principle involves the binding of acidic dye acriline orange G with the basic amino acid lysine and screening by a spectrophotometer (Jenway, UK) at 480 nm.

The lysine contents were calculated by dividing the readings with total protein contents to avoid the effect of protein change as follows:

Lysine contents = (Absorption of sample – Absorption of dye) / Total protein (Eq. 2)

Reagents

For buffer dye solution, 2 g of acriline orange G dye along with 15.84 g of citric acid, 2.98 g sodium biphosphate ($NaH_2PO_4.2H_2O$) and 0.3 g thymol was dissolved in 1 L of distilled water at 80°C. The buffer solution showed an absorbance of 0.65 per mL at 580 nm using a spectrophotometer (Jenway, UK).

Procedures

Ground powder of the seeds was weighed (200 mg) into a 100 mL flask. Next, 15 mL of buffer dye solution was added to flask and agitated to equilibrate the dye with reactive group of the sample. This was performed on shaker for 30 min. The suspension was filtered and collected in a new flask and then diluted 200-folds with distilled water. The absorbance was read against the original dye solution, diluted similarly at 200-folds, at 480 nm using a spectrophotometer (Jenway, UK).

Estimation of antioxidative activity

The antioxidative potential of the seeds using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was estimated following the method reported by Yen and Duh (1994). Measurements in the measuring cuvettes were performed 30 min after addition of DPPH with four different extract concentrations i.e.: 1 mg, 2 mg, 4 mg, and 5 mg, respectively in order to give enough time for the reaction of the cellular antioxidants with DPPH. During this time, the extract solution with negative control was kept in the dark at ambient temperature. Absorbance was taken at 517 nm using a spectrophotometer (Jenway, UK), and the scavenging % was calculated as follows:

Scavenging % = [(Mean value of given concentration – Mean value of control) / Mean value of control] × 100 (Eq. 3)

Analysis for total phenolic content

The total phenolic content was analysed using the Folin-Ciocalteu method with some modifications (Ghafoor and Choi, 2009). This method depends on the reduction of Folin's reagent by phenols to a mixture of blue oxides which have a maximal absorption in the region of 765 nm. A 200 µL properly diluted sample or a standard solution of varying concentrations was mixed with 400 µL Folin-Ciocalteu reagent. Deionised water was used for both dilution and control. The solution was diluted to a total volume of 4.6 mL using deionised water and then thoroughly mixed after incubation for 10 min at room temperature. Next, 1 mL of 20% Na_2CO_3 solution was added immediately to the mixed solution and incubated for 2 h. The absorbance was read at 765 nm using a spectrophotometer (Jenway, UK). Measurements were recorded in triplicate. Gallic acid of mg/mL with 1.15 standard absorbance values was used as the standard, and the total phenolic compounds of the samples were expressed in milligram gallic acid equivalent (GAE) per 100 g (mg GAE/100 g). The calculation was as follows:

Phenolic contents (mg GAE/100 g) = Absorption value (1.15) at 765 nm × Gallic acid absorbance × 100 (Eq. 4)

Estimation of mineral element; phosphorus

Lysine gene identification

DNA Isolation and amplification with selected lysine gene primer was performed. Genomic DNA was isolated following the method of Doyle and Doyle (1987). DNA quality was checked by running the genomic DNA sample on 0.8% agarose gel. Next, lysine gene primers were used for amplification of the lysine gene from the genome of 36 common bean ecotypes (Meng *et al*., 2004); LG-Forward: CATTATGGGTGTTTTCACATATGAG and LG-Reverse: ATTGTATTCAGGATGGGCCAAAAGG.

For lysine gene amplification, PCR was performed using genomic DNA, 10× PCR buffer, $MgCl_2$, dNTP's, primers, and Taq polymerase. Amplified DNA was run on 2% agarose gel, DNA band was visualized on a UV-transilluminator and gels were photographed using gel documentation system.

Statistical analysis

Factor analysis and cluster was carried out with the help of XLSTAT 2014. Dendrogram was constructed by the Ward's method using squared Euclidian distance. Mean and standard deviation of biochemical data was used for evaluation.

Results

Hierarchical cluster

Hierarchical clustering of studied common bean ecotypes based on biochemical attributes is displayed in Figure 1 which shows significant variations. All 36 ecotypes were classified in three logical clusters at approximately 900 dissimilarity index. Cluster I comprised of two sub-clusters including 15 ecotypes in further sub-clusters. It also contained a variant "an outlier E2" among the 36 ecotypes. In cluster II, two more sub-clusters grouped seven ecotypes based on similarity. Cluster III comprised of 14 ecotypes at closer linkage distance in the map. From the tree diagram, E2 and E24 were found to be distant from each other in their ancestry pattern and greatly differed in their metabolite production as both were collected from different eco-geographic zones.

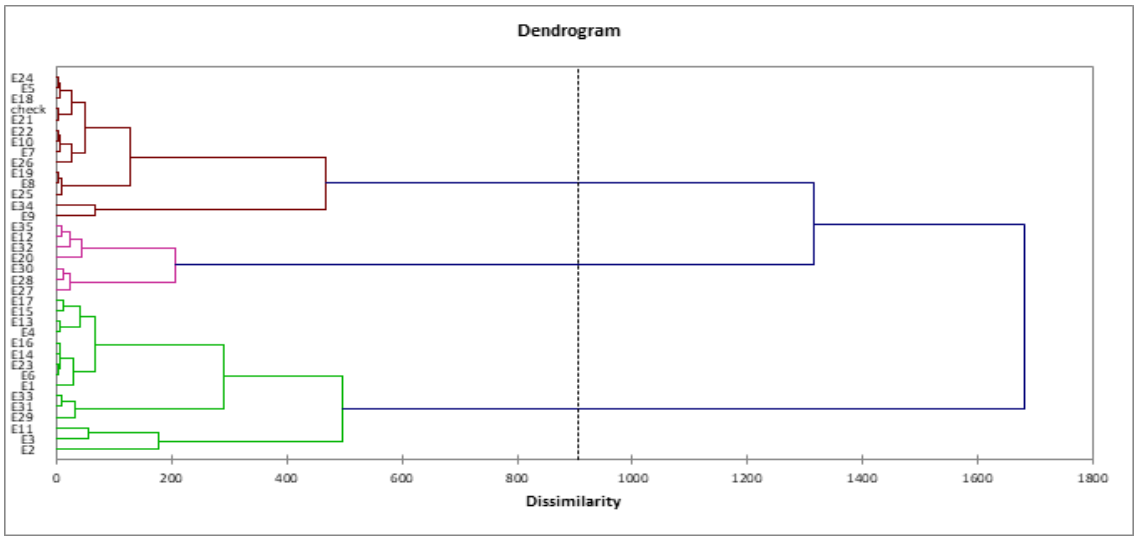

Figure 1. Dendrogram based on average linkage distance for biochemical attributes of 36 common bean ecotypes.

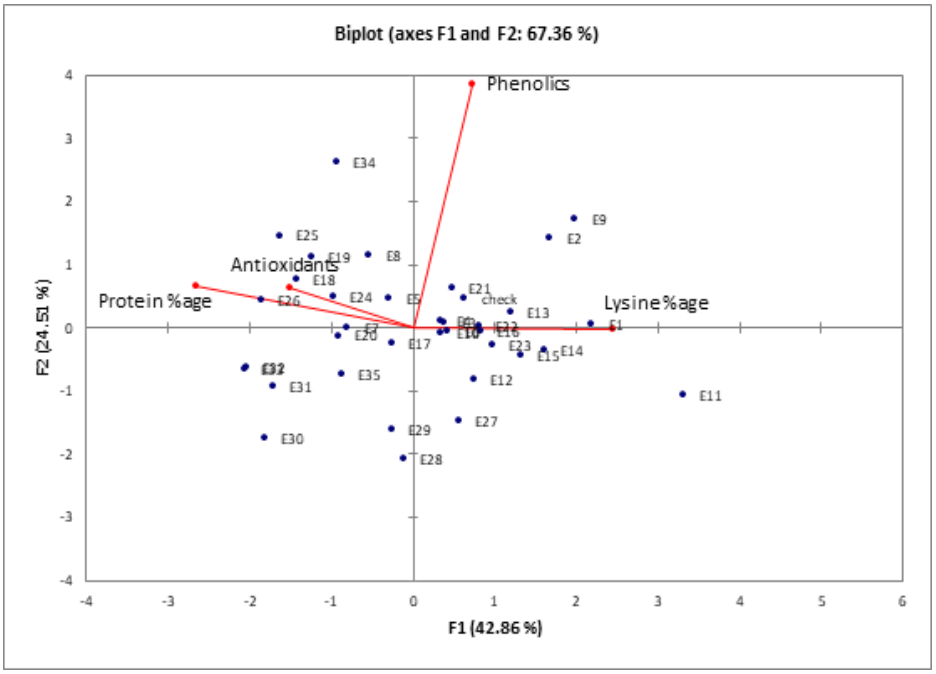

Figure 2. A biplot revealing the variability contribution of the 36 common bean ecotypes and the studied biochemical attributes.

Factor loadings

Based on Principal Component Analysis (PCA), two main components / factors with Eigen value > 1 were extracted. The factor loadings for four biochemical attributes of common bean in two main factors are presented in Figure 2. A biplot diagram shows two major factors, F1 and F2, with 42.86% and 24.51% variability, respectively, imparting 67.36 of the total variability. The Figure shows the correlation pattern of studied attributes (variables) with the distribution of the ecotypes in the elucidated factors for the contribution of variability. All ecotypes were distributed in two major factors randomly, thus showing level of variability in the studied variables. Protein and lysine contents imparted more variability in F1, while phenolics contents showed greater diversity in studied ecotypes in F2.

Protein percentage

The mean values of protein percentage with standard deviation of 36 common bean ecotypes are displayed in Table 1. These ranged from 13.0 ± 0.66 to 24.5 ± 0.40 %. Protein constituents showed a greater degree of variation among the 36 common bean ecotypes. E11 contained minimum contents of proteins in their seeds while E26 contained the maximum. The most prevailing protein % values among the 36 ecotypes were 18 to 22%. Ecotypes E4, E13, E14, E15, E16, E21, E22, and E27 had similar range of protein contents as found in the reference variety.

Lysine contents

The lysine contents of 36 common bean ecotypes are shown in Table 1. Lysine is an indispensable amino acid which cannot be synthesised in humans' and animals' body. Its intake can be accomplished by eating meats and legumes. Common beans are one of the legumes containing ample amount of essential amino acid, lysine, in their seeds. In the present work, the lysine contents of the common beans were found in the range of 4.4 ± 0.91 - 7.4 ± 0.56% of total protein. The maximum lysine content was present in E9 followed by E15, while E34 had minimum value.

Antioxidant activity

For antioxidant activity assessment, different sample concentrations were used in the experiment ranging from 1 mg to 5 mg. Any food showing higher inhibition activity at lower sample concentration is considered as an excellent source of naturally occurring antioxidants. Figure 2 clearly reveals that at maximum sample concentration (5 mg), common beans ecotypes exhibited 67.8 - 97.8% inhibition of free radical DPPH. Ecotype E2 exhibited minimum value while E20 followed by E25 and E34 exhibited maximum values.

Phenolic contents

The total phenolic contents of the 36 common bean ecotypes in mg GAE/100 g are shown in Table 1. Common beans are rich in phenolic contents which contribute to the antioxidant potential. Phenolic

Table 1. Protein, lysine and phenolic contents in 36 common bean ecotypes.

Ecotype	Protein (%)	Phenolics (mg GAE/100 g)	Lysine (%)	Ecotype	Protein (%)	Phenolics (mg GAE/100 g)	Lysine (%)
E1	15.4 ± 0.32	93.2 ± 0.33	6.6 ± 0.26	E19	21.7 ± 0.95	95.6 ± 0.24	5.1 ± 1.10
E2	20.0 ± 0.26	104.8 ± 0.32	6.6 ± 0.13	E20	20.3 ± 0.46	84.9 ± 0.40	5.5 ± 0.21
E3	19.3 ± 0.44	92.8 ± 0.80	4.6 ± 0.61	E21	18.9 ± 0.38	93.9 ± 0.35	6.3± 0.26
E4	18.6 ± 0.31	90.6 ± 0.60	5.5 ± 0.33	E22	18.6 ± 0.40	88.6 ± 0.65	6.9 ± 0.81
E5	21.0 ± 0.36	90.7 ± 0.10	6.3 ± 0.14	E23	19.6 ± 0.46	87.5 ± 0.70	6.7 ± 0.52
E6	20.3 ± 0.47	89.2 ± 0.60	6.0 ± 0.11	E24	22.4 ± 0.59	90 ± 0.22	5.9 ± 0.63
E7	20.2 ± 0.61	87.5 ± 0.33	4.9 ± 0.80	E25	23.8 ± 0.56	97.2 ± 0.81	5.5 ± 0.47
E8	21.7 ± 0.21	96.3 ± 0.50	6.1 ± 0.67	E26	24.5 ± 0.40	88.8 ± 0.64	5.1 ± 0.10
E9	17.5 ± 0.38	105.7 ± 0.42	7.4 ± 0.56	E27	18.6 ± 0.70	74.6 ± 0.36	7.1 ± 0.80
E10	19.6 ± 0.36	87 ± 0.20	6.7 ± 0.01	E28	19.3 ± 0.55	68.9 ± 0.91	6.4 ± 0.67
E11	13.0 ± 0.66	85.9 ± 0.80	7.0 ± 1.00	E29	20.0 ± 0.26	74.5 ± 1.20	5.5 ± 0.35
E12	16.8 ± 0.85	81.8 ± 0.6	6.3 ± 0.70	E30	21.4 ± 0.36	69.6 ± 0.50	4.9 ± 0.56
E13	18.6 ± 0.36	91.5 ± 1.00	7.1 ± 0.41	E31	23.8 ± 0.31	78.6 ± 0.61	4.6 ± 0.41
E14	18.2 ± 0.56	87.4 ± 0.22	7.2 ± 0.58	E32	21.7 ± 1.01	80.9 ± 0.47	3.7 ± 0.46
E15	18.6 ± 0.44	85.7 ± 0.72	7.3 ± 0.35	E33	22.4 ± 0.38	81.8 ± 0.68	3.3 ± 0.17
E16	18.6 ± 0.66	90.0 ± 0.65	6.0 ± 0.18	E34	20.0 ± 0.17	110.3 ± 0.11	4.4 ± 0.91
E17	22.8 ± 0.91	85.0 ± 0.10	6.6 ± 0.36	E35	20.3 ± 0.57	80.2 ± 0.32	5.3 ± 0.44
E18	22.1 ± 0.36	92.2 ± 0.41	5.1 ± 0.21	Check	18.8 ± 0.85	92.4 ± 0.39	6.6 ± 0.12

Data are means ± standard deviations of triplicates (n = 3).

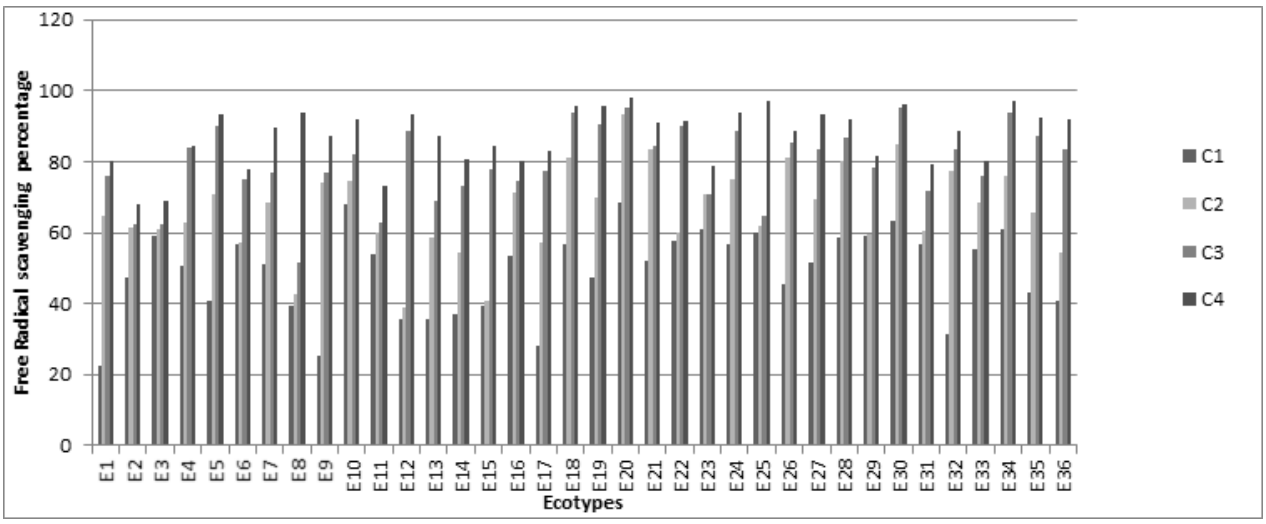

Figure 3. Free radicals scavenging percentage of 36 common bean ecotypes. C1: 1 mg, C2: 2 mg, C3: 4 mg, C4: 5 mg.

Figure 4. Amplified lysine gene primer with genomic DNA of common beans.

contents of the common beans were found in the range of 68.9 ± 0.91 to 110.3 ± 0.11 mg GAE/100 g. Ecotype E34 followed by E9 exhibited maximum values while E28 ehibited minimum values.

Lysine gene primer amplification

The present work reported a maximum of 7.4% lysine content in common bean landraces. Being a rich source of lysine, common bean germplasm was tested for lysine gene identification. Genomic DNA was isolated and amplified using lysine gene primer LG forward and reverse in PCR experiment. Under an optimised PCR conditions, the primer was used to identify the lysine gene among common bean accessions. The lysine gene was found present in the genome of all experimented genotypes. Figure 4 displays a clear profile of the genetic pattern of 36 bean ecotypes, and the amplification of the gene with the primer was identified in all common bean ecotypes. The presence of lysine gene in all ecotypes confirmed the biochemical results of lysine contents in the bean seeds. The diversity in lysine contents may be due to the expression proportion of the respective gene in the genome.

Discussion

Common bean constitutes the main source of cheaper protein for the population of many countries of the world. Its nutritional value is, therefore, of great importance due to the high protein percentage and higher percentage of essential amino acids, lysine, and secondary metabolites in their seeds.

The results for protein percentage (13 – 24.5%) among the common bean ecotypes studied were comparable with the work of Bressani (1983) who reported a range of 19 - 31% protein in common bean. He also discovered the variation in seed proteins of common bean similar to the outcome of the present work. The protein profile showed that the studied local bean ecotypes are excellent sources of protein for the people of mountain areas. The lysine contents obtained in the present work agree with the results reported by Baudoin and Maquet (1999).

It is important to note that there was a significant diversity in protein percentage and lysine contents, antioxidant activity, and phenolic contents among 36 common bean ecotypes which were collected from different eco-geographic localities of Azad Kashmir and Northern areas of Pakistan. Even the ecotypes

collected from the same area showed difference in their metabolite production, revealing diversity in the genetic makeup of all collected ecotypes. Similar diversity was observed by Alexy *et al*. (2009) in protein contents of common bean varieties. He reported that this diversity in protein contents is triggered by both environment and genetic factors.

The present work also revealed that the environmental and genetic interaction, including geographic pattern, soil conditions, existing climate, fertilisation doses, and type of the landrace are significantly influential in the protein diversity profile. Similarly, earlier report on the diversity of common bean protein percentage (20 – 27%) revealed by Corte *et al*. (2003) is also in accordance with the values obtained in the present work. The protein profile along with the lysine concentration of studied ecotypes are also comparable with the nutritional value reported by Baudoin and Maquet (1999).

The expression of the lysine gene product is high in common beans. Therefore, it could be incorporated into the lysine deficient cereals to improve cereal protein contents. The present work highlighted the potential for future research on lysine gene transformation and mineral fortification in cereals.

Common beans were found rich in antioxidants including phenolic contents. The free radical scavenging percentage profile of 36 common bean ecotypes is shown in Figure 3. In the present study, free radical DPPH was used to analyse the scavenging capacity of the bean flour. The DPPH radical has been widely used to test the ability of compounds such as free-radical scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts and foods (Irina *et al*., 2012). Evaluation of phenolic content using Folin-Ciocalteu's reagent and DPPH radical trapping assays has also been reported on other crops (Sim *et al*., 2010; Tajoddin *et al*., 2013).

Xu *et al*. (2007) confirmed that common bean is a powerful antioxidant as also demonstrated in the present work. A diverse range of antioxidant contents (1 – 82%) was observed by Biswas *et al*. (2012) in kidney bean varieties by using different solvents and temperatures. More antioxidants were found in the seed coat as compared to the cotyledons which depicted impact of seed coat colour on the free radical scavenging activity (Chávez-Mendoza *et al*., 2019). Luthria and Pastor-Corrales (2006) estimated the phenolic acids in different classes of common beans from 19.1 – 48.3 mg/100 g, whereas Tajoddin *et al*. (2013) reported up to 310 – 340 mg/100 g in whole seeds of *Phaseolus aureus* L. The phenolic contents in the present work are not consistent with the aforementioned reports. This may be due to the types of germplasm analysed in those studies, or the procedure used to evaluate the phenolic contents. Similar reasons for differences in the antioxidants and phenolics were observed by Pritchard *et al*. (1973) and Cerning *et al*. (1975) who reported different cultivars and analytical procedures were responsible for the observed variation. Golam *et al*. (2011) reported a great deal of variation in antioxidant as well as in phenolic contents from 17.09 – 36.96% and 5.87 – 14.14 mg GAE/ 100 g, respectively.

In addition to diversity characterisation of common bean ecotypes based on biochemical attributes, the present work also revealed a strong correlation between the antioxidant efficiency (AE) and total phenolic contents (TPC). As phenolic contents were found more contributing towards the antioxidant activity (Rashed *et al*., 2018), its increasing value increases the free radical trapping percentage. Most of the ecotypes of common beans analysed in the present work showed synchronisation in the free radicals scavenging capacity and total phenolic contents with only minor variations observed. The variations may be due to amount of other antioxidant compounds than phenolics, since phenolics are not solely responsible for antioxidant activity. Similar correlation was found by Golam *et al*. (2011) and Biswas *et al*. (2012) in the antioxidant efficiency and total phenolic contents in common beans.

The nutrient profile of common bean ecotypes confirmed its role as a promising source of protein, and essential amino acid, lysine. Similarly, common beans were found as a rich source of secondary metabolites including phenolics with potential antioxidant capacity. In this regard, Deshpande (1992) proposed to initiate a substantial research on nutritional related aspects of legumes and its improvement for provision of a sustainable protein source to the malnourished population.

Conclusion

Ecotypes E26, E9, E20, and E34 were found to be more nutritious based on protein contents, lysine contents, antioxidant activity, and total phenolics. The nutritional profile of common bean which includes rich-protein contents, essential amino acids, and promising antioxidant properties makes it an excellent diet for the malnourished population of mountain areas of Pakistan. However, a range of diversity in the germplasm infers variety of nutrients stored in the seeds. The nutritional profile revealed in the present work will open opportunities to select the best ecotypes for developing more nutritious

common bean cultivar(s) for mountain communities. The present work will also help in identifying high nutrient ecotype(s) for further research including breeding of promising ecotypes. Promoting local food to meet the problems such as malnutrition, its nutritional screening, and improvement is of great significance especially in the scenario when the growing population is facing serious issues of food insecurity. Common bean is a potent source of essential amino acid, lysine. The lysine-producing gene can be isolated from the common bean genome for further transformation in other cereal crops.

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