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Identification of lentil (*Lens culinaris* Medik.) germplasm rich in protein and amino acids for utilization in crop improvement

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Abstract

Lentil is a good source of protein, dietary fibre and essential minerals and therefore, has the potential to be used as a staple food crop for eradicating the hidden hunger. Lentil genetic resources including germplasm and wild species possess genetic variability for different agronomic and economic traits which needs to be studied. In the present study, 570 germplasm accessions of lentil were characterized and evaluated for quality traits for over five years. Protein contents were estimated for five years, while amino acid composition was assessed in seeds harvested during the year 2015-16. Significant variation for the protein content and amino acids was recorded in the germplasm. Two medium bold seeded germplasm accessions, IC208326 and IC208329 were found promising possessing high percentage of protein contents of 27.4 to 28.5 with 1.5-2.0 times higher content of total aromatic amino acids and methionine. The magnitude of heritability in broad sense for seed protein content was found moderate (31.31%) whereas for some agronomic traits like plant height, days to flowering, no. of pods per plant and 100 seed weight it was high in the range of 66% to 97%. Identification of unique/potential germplasm and the utilization for improvement of lentil is an option in conventional breeding progrmme.

Key words: Legumes, amino acids profile, characterization, protein content

Introduction

Lentil (*Lens culinaris* Medik), is one of the ancient crops that was domesticated during the Neolithic period in near East, from Jordan northward to Turkey and southeast to Iran, an area called as cradle of agriculture (Kislev and Bar-Yosef 1988). It is one among the few early domesticated crops like einkorn and emmer wheat, barley and pea (Harlan 1992). It is a self-pollinated, annual, cool season and true diploid legume (2n=2x=14) with a relatively larger genome size of 4.2 Gbp. *L. culinaris* ssp. *orientalis* (Boiss.). Lentil is grown mostly in Canada, India, Nepal, Australia, Turkey, USA and China. In India, the area under lentil cultivation in 2013-14 was 1.44 mha with production of 1.07 mt with an average yield of 742 kg/ha (Ahlawat et al. 2016).

The most serious malnutrition problem is of protein deficiency especially among children in the developing countries who cannot afford to drink milk and get meat as regular source of protein and therefore, they have to rely upon legumes as a source of protein (Amjad et al. 2006). Lentil is an inexpensive, sustainable and affordable source of nutrition being rich in protein, micronutrients and vitamins including iron, zinc, selenium, folates, and carotenoids. It is used as complete food in many parts of the world including Indian subcontinent. Although proximate composition of lentil has been reported in the literature (Dhindsa et al. 1985; Naivikul and D'Appolonia 1979; Sosulski et al. 1976), the data are not always comparable due to differences in genotypes, environments and methods of analysis. Studies on quality analysis in lentil, have reported a wide range of protein content (Hamdi et al. 2001; Wang et al. 2009; Burstin et al. 2011), essential minerals and dietary fibre (Roy et al. 2010). Differences for protein concentration in green and red lentils have been also reported (Boyle et al. 2010). The plant genetic resources (PGR) including landraces, different germplasm accessions and wild species possess high genetic variability for various nutritional and agronomic traits (Kumar et al. 2016). However, there is further

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need to explore this variability for higher seed protein content and essential amino acids. Most effective way of utilizing the PGR is to select a potential trait in the germplasm and to transfer the desired trait in to lentil cultivars. Cereal proteins are deficient in certain essential amino acids especially lysine, whereas, legumes contain adequate amounts of lysine but are deficient in sulphur containing amino acids *viz*., methionine, cystine and cysteine (Farzana and Khalil 1999; Amjad et al. 2003). Therefore, the aim of this study was to identify high protein as well as essential amino acid rich germplasm lines, which can be consumed and/or used in breeding programmes to improve quality, particularly the essential amino acids and the protein contents.

Materials and methods

A set of 570 germplasm accessions of lentil were taken from medium term storage of NBPGR, New Delhi which consisted of indigenous and exotic germplasm. The material was grown in rows of 3m each in field at Research Farm of ICAR-NBPGR, New Delhi located at 28°x352′ N, 77°x122′ E, and altitude 287.6 m during *rabi* 2000-01 and 2001-02 in Augmented Block Design (Federer 1963; Federer and Raghavarao 1975) for characterization, evaluation and quality estimation. The farm is situated in semi-arid, subtropical climate with sandy loam to loamy soil. Standard agronomic practices were practiced to raise a good crop. Observations on quantitative traits were recorded on five randomly selected plants for plant height (cm), days to 50% flowering, no. of pods per plant and 100 seed weight (g). Seed protein contents of each line were also estimated during the years. Out of the 570, thirty accessions were identified having protein content around or more than 25%. The identified 30 accessions were grown again during *rabi* seasons of 2013-14, 2014-15 and 2015-16 for validation of protein content and their characterization for agronomic traits. Standard agronomic practices and random selection of plant for taking observations were followed same as done earlier. Total protein was estimated as per AOAC official method 976.05 with some modifications in digestion of samples (AOAC 1990). One hundred mg of dried and homogenized sample was digested with sulphuric acid – selenium– anhydrous sodium sulphate – hydrogen peroxide digestion mixture in glass digestion tubes at 350°C for 45 min as per the method of Forster (1995). Nitrogen percentage in digest was estimated using Kjeltec analyzer (Model-2300) from Foss Tecator, Sweden. Factor 6.25 was used to convert nitrogen to protein. To ascertain recovery food reference material AS-FRM 14 (provided by Institute of Nutrition, Mahidol University, Thailand) and in-house QC samples were used as control. Recovery percentage of 99.7±1.4 for AS-FRM 14 and 101.5±1.2 for QC samples was obtained. All measures were recorded in triplicates and results were expressed as mean ± standard deviation. Heritability of protein content was also determined using method prescribed by Singh and Chaudhary (1985).

Amino acid profiling

HPLC based pre-column derivatization technique was used in the present study. Fluorescent active reagent, 6-aminoquinolyl-N-hydroxy succinimidyl carbamate of Millipore Corporation (Cohen and Michaud, 1993) was used for derivatization with protein hydrolysate amino acids. For sample hydrolysis, 10 mg of homogenised sample flour was taken in a clean 6X50mm sample tube. Tubes were placed in a reaction vial containing 200 µl of constant boiling HCl (6 N) and a crystal of phenol, placed to the bottom of the reaction vial. Samples were hydrolyzed in an oven at 112°C to116°C for 20 to 24 hrs. After hydrolysis, excess HCl was removed and tubes were dried under vacuum. For samples derivatization, vacuum dried samples were dissolved in 750 µl of 20mM HCl solution. To 20 µl of this protein hydrolysate amino acid solution, 20 µl of AccQ·Fluor™ reagent (waters Part no. WAT052880) and 60 µl of AccQ·Fluor™ Borate buffer (waters Part no. WAT052880) was added, vortex thoroughly and mixture was heated for 10 minutes at 55°C.

Separation of amino acids on HPLC

The HPLC system consisted of two pumps (Waters 515), auto sampler (Waters), column (Waters AccQ·Tag™ of length 3.9 X 150 mm) and fluorescence detector (Waters 2475) were used. Derivatized sample was eluted through column using a gradient of 10% solution of AccQ·Tag™ concentrate (Part no. WAT052890) mobile phases A and 60% HPLC grade Acetonitrile mobile phase B. Gradient was set as: initially A=100%, 2 min=98%, 15 min=93%, 19min=90%, 32 to 37min=67% followed by wash with 100% eluent B for 13 min. and re-equilibration for 10 min at 100% by eluent A. Waters Empower software was used to control the operation and peak integration. Detection of individual amino acid derivative was made by fluorescence detector with excitation at 250 nm and emission at 395nm with band width 18 nm. The polarity of the detector was kept positive with gain 10 and sampling rate 1.

Statistical analysis

Phenotypic coefficient of variation, genotypic coefficient of variation and heritability (Broad Sense) for the traits under study were estimated by the software SPAR 2.0 software. The data, based on three replicates, were subjected to analysis of variance by complete block design (Gomez and Gomez 1984). Standard deviation of each individual nutrient of each accession and mean were computed and variations among accessions were evaluated by least significance difference (LSD) at the 5% level of probability (P=0.05).

Results

Protein content

Thirty accessions were initially selected from a total of 570 germplasm accession, which were characterized and evaluated for validation of protein content. Protein content for majority of accessions ranged between 18 to 23%. Accessions with very low seed yield and inconsistent protein content were rejected and only four germplasm IC208326 (Collector No. SD 23/3, Uttar Pradesh), IC208329 (Collector No. SD 23/7, Uttar Pradesh), IC208351 (Collector No. SD 23/170, Uttar Pradesh) and IC201700 (Collector No. N-1047, Uttar Pradesh) accessions were identified possessing high protein contents of >25% (Table 1) with high value for desirable agronomic traits (Table 2). These four germplasm accessions were again subjected to protein estimation, which ranged from 25.2 to 28.6%, whereas in the check variety Precoz the contents ranged between 21.3 to 23.3%. Among four of these identified germplasm, two accessions, *viz*., IC 208326 and IC 208329 were the best germplasm for consistently displaying high protein content ranging from 27.4 to 28.5%. The protein content recorded in the accessions, IC208326 and IC208329 was 27% during 2000-01 and 2001-02, which was validated (28%) during 2013-2014, 2014-15, 2015-

Table 1. Total protein content and amino acid profile of selected lentil accessions

Year	Traits	IC201700	IC208329	IC208326	IC208351	Precoz
2015-16	Protein (%)	25.2 ± 0.56	28.5 ± 0.39	27.6 ± 0.54	23.7 ± 0.36	21.3 ±0.24
2014-15		26.3 ± 0.38	28.4 ± 0.62	27.7 ± 0.76	25.5 ± 0.18	23.3 ±0.18
2013-14		25.9 ± 0.34	28.6 ± 0.06	28.06 ± 0.14	25.6 ± 0.63	22.8 ±0.16
2001-02		26.2 ± 0.43	27.4 ± 0.53	27.7 ± 0.37	25.2 ± 0.56	21.4 ±0.21
2000-01		26.0 ± 0.64	27.5 ± 0.44	27.4 ±0.48	24.3 ± 0.18	22.6 ±0.17
Amino acids (g/100 g protein)-2015-16	Aspartic acid	9.24 ± 0.87	6.36 ± 0.45	8.22 ± 0.82	10.1 ± 0.63	12.4 ±0.36
	Serine	4.99 ± 0.38	6.28 ± 0.33	6.34 ± 0.30	5.39 ± 0.02	5.56 ±0.12
	Glutamic acid	10.4 ±0.76	6.12 ± 0.49	8.73 ± 0.80	13.6 ± 0.40	16.4 ±0.39
	Glycine	9.4 ± 0.28	10.4 ± 0.19	9.01 ± 0.02	10.2 ± 0.92	6.37 ±0.43
	Histidine	6.67 ± 0.78	9.15 ± 0.35	7.08 ± 0.07	1.21 ± 0.94	2.20 ±0.09
	Arginine	11.0 ± 0.81	12.1 ± 0.33	9.89 ± 0.76	13.8 ± 0.78	8.47 ±0.51
	Threonine	1.04 ± 0.37	1.18 ± 0.39	0.65 ± 0.07	1.69 ± 0.24	3.66 ±0.16
	Alanine	5.92 ± 0.24	5.17 ± 0.34	5.87 ± 0.02	8.35 ± 0.31	5.99 ±0.21
	Proline	4.85 ± 0.25	4.64 ± 0.05	4.99 ± 0.33	4.48 ± 0.18	5.22 ±0.08
	Cystine	1.62 ± 0.56	1.03 ± 0.15	0.96 ± 0.37	1.08 ± 0.11	1.25 ±0.06
	Tyrosine	4.86 ± 0.82	**7.15 ± 0.41**	**6.97 ± 0.44**	2.71 ± 0.09	2.93 ±0.23
	Valine	4.39 ±0.07	4.57 ± 0.15	4.56 ± 0.21	4.28 ± 0.17	4.84 ±0.21
	Methionine	1.16 ± 0.13	**2.23 ± 0.17**	1.5 ± 0.05	1.59 ± 0.01	1.01 ±0.18
	Lysine	7.21 ± 0.42	5.81 ± 0.45	7.11 ± 0.61	**9.59 ± 0.38**	6.47 ±0.32
	Isoleucine	3.21 ± 0.16	3.42 ± 0.07	3.34 ± 0.15	2.2 ± 0.28	4.24 ±0.22
	Leucine	6.98 ± 0.15	7.17 ± 0.03	7.23 ± 0.06	5.89 ± 0.29	7.72 ±0.13
	Phenylalanine	7.02 ± 0.49	**7.28 ± 0.30**	**7.55 ± 0.29**	3.84 ± 0.48	5.32 ±0.21

Each column contains mean and S.D. of mean

16 (ICAR-NBPGR Annual Report 2015-16). Some of the important morphological traits of agronomic importance associated with seed yield *viz.,* plant height, days to 50% flowering , days to 80% maturity, no. of pods/plant, seed weight etc. were also compared with the check Precoz. These accessions showed significantly higher mean values (Fig. 1) for different traits with good agronomic background during five years 2000-01, 2001-02, 2013-14, 2014-15 and 2015-16 (Table 2). Based on five years data, seed protein content was found moderately heritable (31.31%),

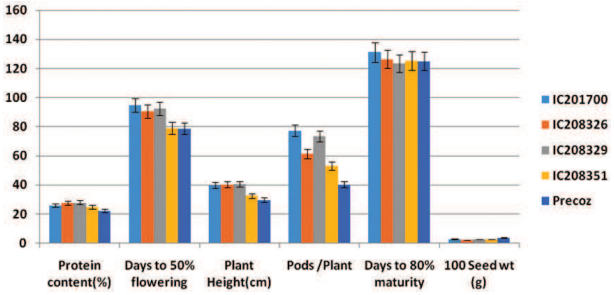

Fig. 1. Variation among mean performance of genotypes in comparison to check Precoz

Table 2. High protein lentil germplasm with mean values of some important agronomic traits

Accessions	Year	Days to 50% flowering	Plant height (cm)	Pods/plant (No.)	Days to 80% maturity	100 seed wt. (g)
IC201700	2000-01	94.00	32.80	95.60	129.00	2.87
	2001-02	97.00	44.80	77.20	129.00	2.70
	2013-14	88.00	43.86	79.15	131.00	2.65
	2014-15	99.50	42.90	66.50	135.00	2.92
	2015-16	95.00	36.46	59.20	134.00	2.70
	Mean	94.70+1.92	40.16+2.35	75.53+6.19	131.60+1.25	2.77+0.05
IC208326	2000-01	90.00	41.60	79.80	127.00	2.32
	2001-02	94.00	44.40	60.60	127.00	2.25
	2013-14	90.00	39.50	59.50	126.00	2.18
	2014-15	95.00	38.50	66.00	133.00	2.10
	2015-16	83.00	34.24	32.40	118.00	2.22
	Mean	90.40+2.11	39.65+1.69	59.66+7.71	126.20+2.39	2.21+0.04
IC208329	2000-01	88.00	41.20	102.40	125.00	2.60
	2001-02	103.00	37.67	55.40	125.00	2.65
	2013-14	85.00	43.32	58.80	120.00	2.71
	2014-15	92.00	40.73	72.20	123.00	2.69
	2015-16	92.00	40.70	72.20	123.00	2.74
	Mean	92.00+3.05	40.72+0.90	72.20+8.29	123.20+0.92	2.68+0.02
IC208351	2000-01	82.00	34.50	62.00	124.00	2.65
	2001-02	80.00	32.00	58.00	128.00	2.50
	2013-14	78.00	36.72	64.05	127.00	2.60
	2014-15	76.00	30.85	47.85	126.00	2.50
	2015-16	77.00	28.40	26.18	121.00	2.60
	Mean	78.60+1.08	32.49+1.44	51.62+6.94	125.20+1.24	2.57+0.03
Precoz	2000-01	85.00	25.24	57.25	122.00	3.90
	2001-02	78.00	32.73	30.38	131.00	3.78
	2013-14	75.00	31.82	35.70	123.00	3.50
	2014-15	76.00	30.85	47.85	126.00	3.60
	2015-16	77.00	28.40	26.18	121.00	3.80
	Mean	78.20+1.77	29.81+1.35	39.47+5.74	124.60+1.80	3.72+0.07

whereas the agronomic traits, namely, plant height, days to 50% flowering, number of pods per plant and seed weight were highly heritable (0.660 to 0.971), except days to 80% maturity (0.308) (Table 3).

Table 3. Estimation of phenotypic coefficient of variation, genotypic coefficient of variation and heritability (Broad sense)

Trait	Phenotypic coefficient of variation	Genotypic coefficient of variation	Heritability (Broad sense)
Protein content (%)	2.486	1.391	0.313
Days to 50% flowering	9.960	8.734	0.769
Plant Height (cm)	16.245	13.483	0.689
No. of pods/plant	27.879	22.664	0.661
Days to 80% maturity	3.542	1.964	0.308
100 Seed wt (g)	20.251	19.957	0.971

Amino acid profile

Significant (P<0.05) variation existed in the individual amino acid contents of four different high protein germplasm accession of lentil namely, IC208326, IC208329, IC208351 and IC201700. Accessions, IC208326 and IC208329 possessed 1.5-2.0 times higher content of total aromatic amino acids (phenylalanine and tyrosine) as compared with other two accessions and check variety Precoz (Table 1). The methionine, the most limiting amino acid was 1.5 times higher of Precoz in accessions, IC208326 and IC2088351 and 2.0 times more in IC208329. In the case of IC208351, slightly less protein content was recorded, which varied from 23.7 to 25.6%. However, higher contents of arginine, aspartic acid, glutamic acid, glycine, alanine and lysine were recorded in these accessions. In Precoz, protein content ranged from 21.3 to 22.8%, which indicated that the accessions, IC 208326 and IC 208329 were not only superior in protein content but also showed favourable amino acid composition. These two accessions are almost similar to each other but for one amino acid i.e. methionine and IC 208329 is more rich.

Correlation between seed protein content and agronomic traits

On the basis of three years (2013-14, 2014-15 and 2015-16) data, seed protein content showed significantly positive correlation ($P < 0.05$) with days to 50% flowering, plant height and no. of pods per plant. However, the protein content was negatively correlated with days to 80% maturity and also displayed significantly negative correlation ($P < 0.05$) with 100 seed weight. Similar results were observed based on two years (2000-01 and 2001-02) which indicated that even if the experiment are conducted after a long gap the trend in correlations is not changed (Tables 4A and B).

Discussion

Proteins are essential for all living beings. After water, protein contributes towards the largest proportion of our body. They are involved in almost all biological functions in living beings (Furst, 2009) and several physiological mechanisms (Uauy et al. 2015). Pulses among plant sources are generally rich in protein. Results of the present studies are in accordance with findings of earlier workers (Khaleel et al. 1994; Jood et al. 1998; Raghuvanshi et.al. 1994) who highlighted lentil as a good source of protein. Recently, Kumar et al. (2016) also analysed protein content in 72 diverse lentil accessions and reported a wide range 10.5 to 23.7 % with an average of 18.7 % in varieties/breeding lines and 14.5 to 27.1 % with an average of 22.4 % in landraces of Mediterranean origin. Heritability is a value that expresses the degree of correspondence between phenotypic and genotypic variance, which may predict outcome of selection. Heritability of a particular trait depends on method of measurement, environment of measurement and plant samples analysed. In the present study, protein content was found moderately heritable (31.31%). Rathi et al. (2002) reported similar observations in lentil with moderate heritability of protein which ranged between 40.2-59.3%. Heritability of protein content in corn kernel has been reported in excess of 50% (http://passel.unl.edu 2018). Protein quality is determined based on the composition of amino acids, more importantly the essential amino acids which cannot be synthesized by human beings.

Different amino acid profiles have been observed in different pulse crops because of difference among the genotypes and species, geographical locations and environmental factors. In Lentil, sulphur containing methionine is a major limiting amino acid followed by aromatic amino acids (Jaffé 1949; Bhatty 1988; Longwah et al. 2017). Amino acids diversity results into variability in the function and structure of different

Table 4A and B. Correlation coefficient among traits under study

4A. Based on two years (2000-01 and 2001-02) data

	Protein content (%)	Days to 50% flowering	Plant height (cm)	Pods/ plant	Days to 80% maturity	100-seed wt (g)
Protein content (%)	1.000					
Days to 50% flowering	0.510*	1.000				
Plant height (cm)	0.453*	0.350	1.000			
No. of pods/plant	–0.086	0.445*	0.130	1.000		
Days to 80% maturity	–0.419*	–0.709**	–0.485*	–0.125	1.000	
100 Seed wt (g)	0.373	0.404*	–0.469*	0.049	–0.222	1.000

4B. Based on three years (2013-14, 2014-15 and 2015-16) data

	Protein content (%)	Days to 50% flowering	Plant height (cm)	Pods/ plant	Days to 80% maturity	100-seed wt (g)
Protein content (%)	1.000					
Days to 50% flowering	0.769**	1.000				
Plant height (cm)	0.699*	0.873**	1.000			
No. of pods/plant	0.597*	0.384	0.575*	1.000		
Days to 80% maturity	–0.443*	–0.424*	–0.373	–0.162	1.000	
100 Seed wt (g)	–0.099	0.436*	0.392	0.003	–0.155	1.000

*Significant at 0 .05 level; **Significant at 0 .01 level

body proteins. The amino acids which cannot be synthesized by the body, are considered as essential constituents of the diet and required for maintenance of health and overall growth (Uaariyapanichkul et al. 2018). Human beings require nine essential amino acids, namely, lysine, histidine, leucine, isoleucine, valine, methionine, threonine, tryptophan, phenylalanine in the diet. Other than this, they also respond to arginine and possibly proline availability as well, during the initial stages of fast growth (Mello 2003). Essential amino acids (EAAs) are key parameters in food quality assessment (Tessari et al. 2016). Among plant protein sources, methionine and lysine are two major limiting amino acids (Gill, 2003). Methionine is involved in initiation of protein synthesis (Mello, 2003) while lysine plays important biological roles mostly related to its ε amino group and these roles are carried out through protein enzymes and hormones synthesisas well as in calcium absorption (Bhagavan, Chung-Eun 2011 and 2015). Some of the non-essential amino acids e.g., glutamate, arginine and glutamine also play important roles in regulating gene expression, cell signalling (Manta-Vogli 2018).

Therefore, the identified lines in the present study having higher protein and are rich in amino acids *viz.,* methionine, arginine, tyrosine etc. can be used directly for consumption purposes as well as utilized in lentil improvement programme for the development of cultivars with value added products after purification of proteins or amino acids. Earlier also, pulses have been exploited in various supplements owing to their rich profile of essential amino acids and supports the development of value added foods (Agarwal, 2017). Similar to the findings obtained in the present study, Hussan et al. (2018) also reported positive correlation between seed protein content and days to 50% flowering in lentil. Painkra et al. (2018) found significantly positive correlation between protein content and days to 50% flowering, maturity and plant height in soyabean. Tahir et al. (2011) reported significant negative correlation between protein content and 1000 seed weight in lentil. The lentil germplasm accessions, IC208326 and IC208329 have been identified as promising genetic resource for high protein content and higher content of methionine and aromatic amino acids, which are considered most limiting amino acids in lentil. In view of quantitative nature of protein

inheritance, systematic efforts are required to develop lentil cultivars with higher yield combined with high protein content and rich in amino acids utilizing these resources as donors.

Authors' contribution

Conceptualization of research (NKG); Designing of the experiments (NKG, RB); Contribution of experimental materials (NKG); Execution of field/lab experiments and data collection (NKG, RB, KT, BR); Analysis of data and interpretation (RB, SY, PS); Preparation of manuscript (NKG, RB).

Declaration

The authors declare no conflict of interest.

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