ASSESSMENT OF VARIABILITY IN GRASS PEA GERMPLASM USING β-ODAP CONTENT AND SEED PROTEIN ELECTROPHORESIS

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Abstract
Grass pea (Lathyrus sativus L.) is a nutritious food and fodder crop. It can withstand unfavorable climatic conditions making it suitable for cultivation in wide range of environments. This work evaluated variations among fifty grass pea accessions collected from different geographic locations of Ethiopia for their β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) content, a neurotoxic amino acid, and assessed the diversity among them using seed storage protein profiles. The content of the antinutritional factor β-ODAP varied from 0.21% to 0.55% with a mean value of 0.36%. Six accessions showed medium level of β-ODAP (0.21-0.28%). These accessions could be potential sources for selecting and breeding grass pea verities with safe β-ODAP level. Further analysis of accessions with medium level of β-ODAP content on single plant basis might result in revealing promising lines that could be used for crop improvement. Seed protein electrophoresis resulted in 27 reproducible bands. The average gene diversity estimate (hs) was 0.192 and it ranged from 0.166 to 0.220. Cluster analysis based on genetic similarities grouped the analysed samples irrespective of their geographic origin suggesting accessions from different origins might have similar genetic background. The mean differentiation value (θ^{II} =0.09 ± 0.008) indicated that most of the total variation comes from within accession variation. The observed level of heterozygosity using seed storage proteins implicated the potential of these markers for genetic diversity assessment in Ethiopian genetic materials.

Keywords: antinutritional factor, germplasm characterization, seed storage protein profile

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1. INTRODUCTION

Grass pea (*Lathyrus sativus* L.) is a food and fodder crop tolerant to extreme environmental conditions such as drought and excessive floods; popular in areas practicing subsistence farming that have extreme weather conditions (Smartt *et al.*, 1994; Campbell, 1997). It is a good source of high quality protein and essential amino acids except tryptophan, methionine and cysteine (Pastor-Cavada, 2011). These properties make grass pea a resilient crop suitable to meet the challenges of climate change. In Ethiopia it is an important pulse crop mostly grown at an altitudinal range of 1700-2700 m altitude (Yadav and Bejiga, 2006). Presently it is cultivated on 9.6% of the total pulse production area of the country (CSA, 2016).

Despite its important qualities, consumption of grass pea for a long period as a staple diet has a negative impact on health. β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) is a free amino acid which is found in grass pea and causes cortical motor neuron disease (Spencer *et al*,. 1986), resulting in paralysis of lower limbs (neurolathyrism). Neurolathyrism is a problem not only in humans but also in animals (Hanbury, 2000). Daily dose, duration of consumption, and physical condition determine the development of lathyrism. Consumption of grass pea for 2-3 months as one-third to one-half of total dietary intake results in neurolathyrism (Barceloux, 2008). On the positive side, β-ODAP is investigated for bioactive properties that may be beneficial in the treatment of diseases such as hypoxia (Eslavath *et al*., 2016) and Alzheimer's disease (Rao, 2011).

β-ODAP is present in all tissues of the plant with variable concentration at different developmental stages of the plant (Addis and Narayan, 1994). Various types of treatments have been proposed and used (Kebede *et al*., 1995; Kuo *et al*., 2000) to reduce the neurotoxin level from the seeds; however, the

ideal solution would be to breed a grass pea variety with no β-ODAP or with β-ODAP level of the minimum possible concentration. Even though grass pea is cultivated extensively in Ethiopia, only few varieties with low-ODAP content have been released (Kumar *et al*., 2011).

Characterization and evaluation of germplasm collections greatly assists in the identification of genetic materials that could be utilized in crop improvement. Diversity assessment can be carried out based on various types of data that emanate from morphological, biochemical, nutritional and DNA based differences. Seed storage protein fractions are mixtures of components which show polymorphism both within and among genotypes of the same species (Shewry *et al*., 1995). Seed protein of grass pea constitutes about 20% of the seed dry weight, of which more than 60% is composed by globulins and 30% by albumins (Rosa *et al*., 2000). Electrophoresis of seed storage proteins is widely used to describe genetic diversity of crop germplasms. In the genus Lathyrus, this method has been used to study the diversity of seed protein classes in grass pea and related species (Przybylska *et al*., 2000), and to study genetic diversity in *L. inconspicuous* (Radwan *et al*., 2013). Variability in β-ODAP content in conserved genetic resources can also be exploited to develop low ODAP containing varieties to reduce the risk of lathyrism and identify genetic materials that can be used in crop improvement.

The aim of this study is hence to assess variation among some accessions of grass pea for β-ODAP content and evaluate the suitability of seed storage protein for genetic diversity assessment in the Ethiopian grass pea collection.

2. MATERIALS AND METHODS

Plant material

A total of Fifty *L. sativus* accessions collected from different regions in Ethiopia Shewa (11), Bale (4), Tigray (10), Gonder (5), Gojam (11), Welo (5), Arsi (3) and Haraghe (1), and obtained from the Ethiopian Biodiversity Institute were used in the present study (Table 1).

β-ODAP content analysis

Analysis for β-ODAP content was done using modified Rao's method (Rao *et al*., 1964). Grass pea flour (100 mg) was soaked in 10 ml of ethanol (60 % (v/v)) and subjected to five hours of extraction. The suspension was then centrifuged at 9000 rpm (Sigma 2K15) twice and 75 µl of the supernatant was mixed with 92 µl of distilled water and 0.33 ml of 3N potassium hydroxide in test tubes. The samples were kept in a hot water bath (98ºC) for 30 min for hydrolysis. Subsequently, the total volume was brought to 1 ml by adding 0.5 ml distilled water. To detect β-ODAP, 2 ml OPT reagent (100 mg of *O*-phthaldialdehyde, 1 ml of ethanol (95%), 0.2 ml β-mercapthoethanol and 99 ml of potassium tetraborate buffer (0.5 M, pH 9.9)) was added to the solution and kept at room temperature for 30 minutes. The absorbance was measured using spectrophotometer (Cary 50 Scan, Varian) set at λ = 420 nm. Measurements were made in seven replicates for each accession and a control sample with known β-ODAP concentration was included with each assay.

Seed protein extraction and profiling

For diversity assessment using seed storage protein, each accession was represented by 10 individual seeds. Total seed protein was extracted by grinding each seed into fine powder using mortar and pestle and protein was extracted from 0.2 gm of the seed powder by adding 400 µl extraction buffer (6% 1 M Tris-HCl (pH 6.8), 0.069 M SDS, 0.87 M sucrose, 5% β-mercaptoetanol and bromophnenol blue as a tracking dye). The tubes were mixed well by vortexing and kept at 4 ºC until use. Before electrophoresis, the crude homogenate was centrifuged at 10000 rpm (Hermle z233 M) for 5 min. The extracted protein samples were collected as supernatant and heated in a water bath (98ºC) for 5 min to denature the proteins. Protein profiling of extracted samples was analyzed through SDS-PAGE (Lammeli, 1970) using 10%

polyacrylamide gel. Prestained protein ladder (M4038, Sigma) with molecular weight of 6–205 kDa was used as standard. Electrophoresis was carried out at constant voltage (100 V) until the tracking dye reaches the bottom of the separating gel. The gels were then stained with staining solution (40% ethanol, 10% acetic acid, containing 0.1% (w/v) Commassie brilliant blue R-250) for overnight. Destaining of gels was carried out using 40% ethanol and 10% acetic acid.

Data analysis

For β-ODAP analysis, the mean of replicated results from spectrophotometer measurement were compared against a standard curve to determine the level of β-ODAP in each accession. β-ODAP for accessions pooled by region of collection was also estimated. Significance of variability observed in the different accessions and regions was checked using analysis of variance (ANOVA) in Excel. Electrophoretic protein profiles was scored for the presence (1) or absence (0) of bands and used to determine the level of variation. The resulting binary data matrix for the 500 entries was used to perform genetic diversity measures. PopGene Version 1.32 (Yeh *et al*., 1999) was used to calculate the percentage of polymorphic bands (P). Gene diversity was analysed by Bayesian estimate of gene diversity, *hs*, and genetic differentiation was determined using θ^{II}, a statistics similar to Nei's Gst, using Hickory Version 1.1 (Holsinger and Lewis, 2006). The Dice (1945) similarity coefficient was used to calculate the pairwise similarity matrix of the accessions. Cluster analysis using UPGMA (Unweighted Pair Group Method with Arithmetic mean) was performed based on the similarity matrix using NTSYS-pc program (Rohlf, 2000).

3. RESULTS AND DISCUSSION

β-ODAP content among the analysed accessions varied from 0.21% (accession 207497) to 0.55% (accession 236700) with a mean value of 0.36%. Six accessions were identified as having medium (0.2% - 0.29%) β-ODAP content. The majority of the accessions (88%) showed high level (> 0.29%) of β-ODAP content and a value between 0.3 and 0.39% was registered for most of the samples (Figure 1). The overall result revealed the presence of significant variation ($P < 0.05$) in β-ODAP content among accessions. Region-wise analyses also showed significant ($P < 0.05$) variation for β-ODAP level among regions. The highest mean value was recorded for accessions from Gonder region (0.452%) and the lowest for accessions from Arsi region (0.297 %). Urga *et al*. (2005) had also indicated the presence of high variability in β-ODAP content in Ethiopian grass pea. None of the accessions in our study were identified as having β-ODAP level of low/safe range (< 0.2%) but six accessions (238955, 207497, 215246, 46050, 215706, and 231325) showed medium level content which varied from 0.21% to 0.28%. In an earlier study, grass pea accessions of Asian and Ethiopia origin were reported to show high β-ODAP content in dry seeds compared to samples of other origins (Abd El Moneim *et al*., 2001).

In this study, β-ODAP concentration was determined on accession basis (sample from different single plants and pods), but for breeding purposes it is recommended to analyse β-ODAP level in seeds from a single pod. It has been reported that single plants selected from different accessions of Ethiopian grass pea showed low level (up to 0.149%) of β-ODAP content (Tadesse and Bekele, 2003). The availability of low β-ODAP cultivars reduces the need for pre-treatment of grass pea to make it usable for animal feed (Hanbury, 2000) and guarantee safe human consumption. There are animal feeding studies that were able to quantify safe feeding levels in the diet of animal feed resulting in no lathyrism effects (Chowdhury *et al*., 2005) which shows that with adequate information on the level of antinutritional factors, grass pea can be consumed without ill effect. The accessions with medium ODAP level identified in our study could be potential source for breeding grass pea verities with safe β-ODAP level.

On the basis of the relative mobility of seed proteins on the gel, 27 reproducible bands were detected of which 88% were polymorphic (Figure 2). The percentage of polymorphic bands in the accessions ranged from 25% to 54.17%, and averaged 42.08%. The gene diversity estimates (hs) within accessions ranged from 0.166 to 0.220 with mean 0.192 (Table 1).

The highest and lowest diversity were observed for accession 212742 from Gondar region and accession 46015 from Gojam region respectively. The average level of gene diversity (0.192) found using storage proteins is comparable to results obtained using isozyme markers in Ethiopian grass pea (Tadesse and Bekele, 2001) and grass pea collected from the Sudan-Ethiopia geographic region (Chowdhury and Silinkard, 2000) which shows that seed storage proteins revealed comparable level of diversity with that of isozymes.

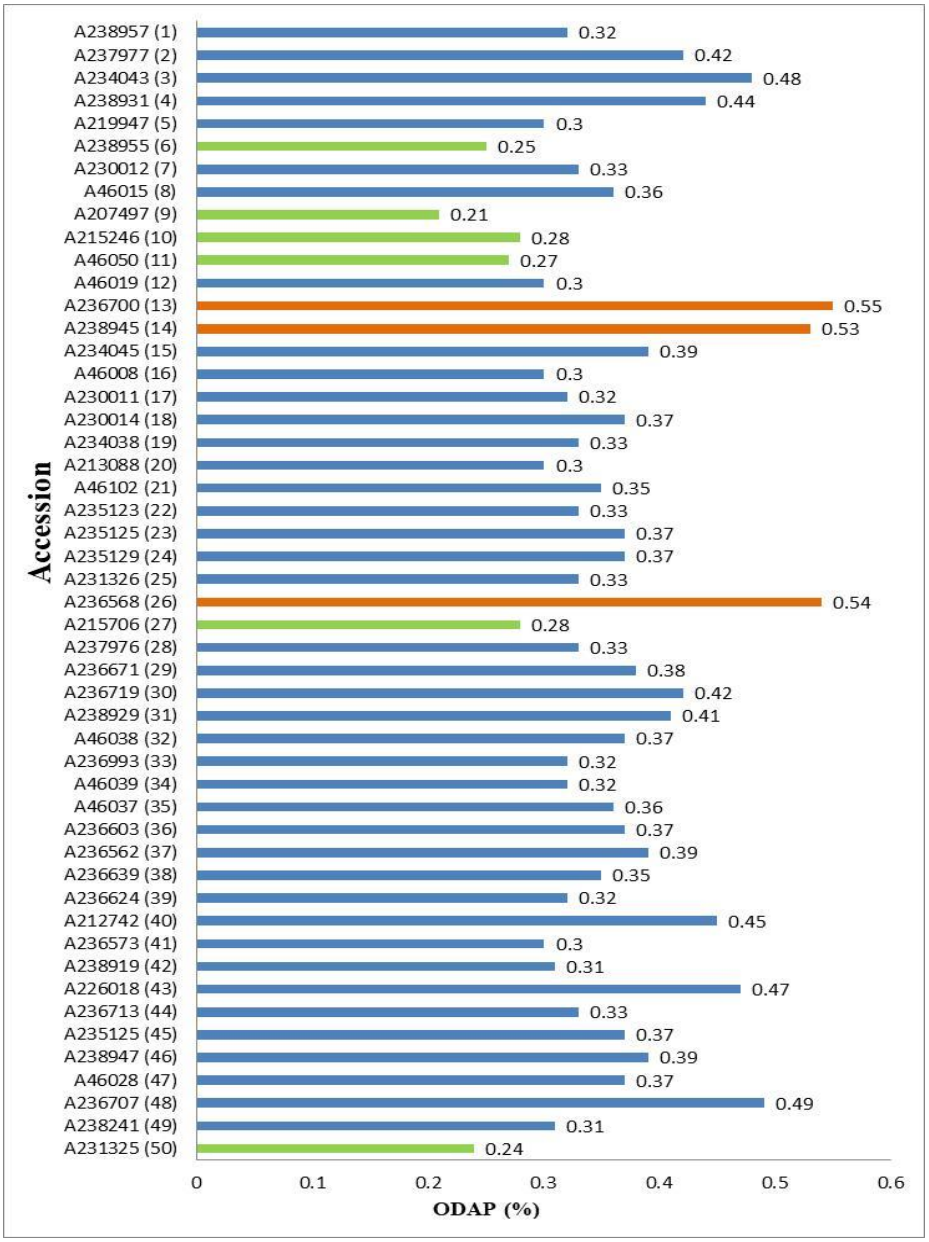

Figure 1. β-ODAP concentration in seed extracts of 50 grass pea accessions.

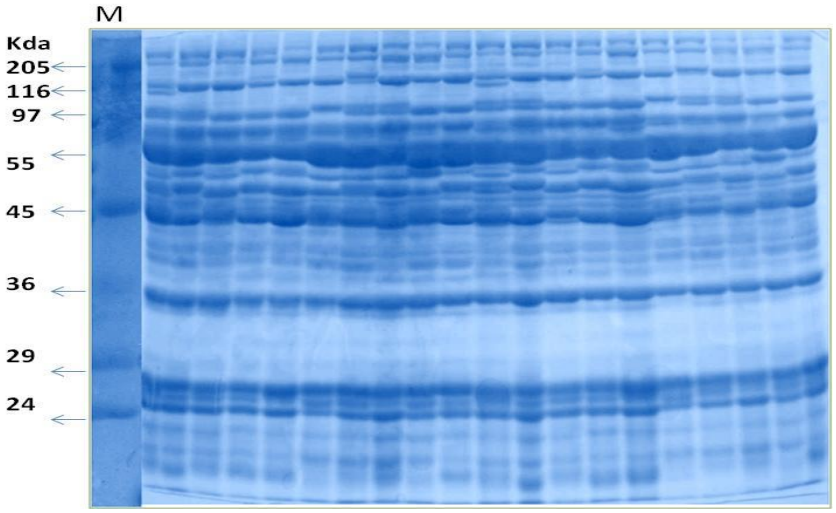

Figure 2. Seed storage profile of an accession of grass pea using SDS-PAGE

Table 1. List of analysed accessions with collection origin, percentage of polymorphic bands (%P) and gene diversity (*hs*)

Ref No.	Accession no.	Origin	%P	*hs*	Ref No.	Accession no.	Origin	%P	*hs*
1	238957	Shewa	41.67	0.182	26	236568	Shewa	45.83	0.210
2	237977	Bale	50.00	0.200	27	215706	Welo	45.83	0.186
3	234043	Tigray	41.67	0.190	28	237976	Bale	41.67	0.184
4	238931	Gonder	33.33	0.183	29	236671	Gojam	45.83	0.212
5	219947	Tigray	41.67	0.203	30	236719	Gojam	37.50	0.187
6	238955	Shewa	41.67	0.209	31	238929	Gonder	45.83	0.195
7	230012	Bale	50.00	0.193	32	46038	Welo	29.17	0.181
8	46015	Gojam	25.00	0.166	33	236993	Shewa	41.67	0.184
9	207497	Tigray	54.17	0.199	34	46039	Harerge	45.83	0.180
10	215246	Welo	41.67	0.183	35	46037	Welo	29.17	0.179
11	46050	Gojam	41.67	0.188	36	236603	Shewa	33.33	0.182
12	46019	Shewa	45.83	0.215	37	236562	Shewa	37.50	0.201
13	236700	Gojam	45.83	0.186	38	236639	Shewa	37.50	0.195
14	238945	Gojam	50.00	0.200	39	236624	Shewa	41.67	0.214
15	234045	Tigray	41.67	0.179	40	212742	Gonder	50.00	0.220
16	46008	Shewa	45.83	0.192	41	236573	Shewa	45.83	0.210
17	230011	Arsi	33.33	0.180	42	238919	Gojam	50.00	0.211
18	230014	Bale	33.33	0.170	43	226018	Gonder	45.83	0.173
19	234038	Tigray	45.83	0.196	44	236713	Gojam	37.50	0.183
20	213088	Welo	45.83	0.183	45	235125	Tigray	37.50	0.210
21	46102	Gojam	37.50	0.178	46	238947	Gojam	54.17	0.194
22	235123	Tigray	45.83	0.218	47	46028	Gojam	37.50	0.186
23	235125	Tigray	37.50	0.199	48	236707	Gonder	41.67	0.212
24	235129	Tigray	37.50	0.189	49	238241	Tigray	50.00	0.170
25	231326	Arsi	50.00	0.209	50	231325	Arsi	37.50	0.169
Over all mean								42.08	0.192 ± 0.0034

Seed storage proteins showed low gene diversity when compared with results of EST-SSR markers (H=0.477) obtained on Ethiopian grass pea (Shiferaw *et al.*, 2012) but the result found in the present study differs from the findings of Lioi *et al.* (2011) who reported the absence of polymorphism in grass pea landraces from Italy using seed protein markers.

The mean differentiation value (θ^{II}) is 0.09±0.008 and it showed that only nine percent of the total variation comes from between accession variation. Previous findings also reported high within population variation in Ethiopian grass pea using morphological and biochemical markers (Tadesse and Bekele, 2003). The genetic similarity of the 50 accessions based on Dice coefficient of similarity (1945) ranged from 0.77 to 0.90 (Figure 3). Accession 8 from Gojam and 32 from Welo regions were the most similar (GS=0.90) followed by accession 35 and 43 from Welo and Gondar regions respectively (GS=0.895). Accession 45 from Tigray and 46 from Gojam regions were the most distant pair (GS =0.781). At 0.824 coefficient, the analyzed accessions could be divided in to two main groups each containing comparable number of accessions 24 (Group I) and 26 (Group II). Accessions were grouped irrespective of their origin indicating that accessions that have similar genetic background are distributed among different regions. Three of the accessions with medium level of ODAP content (6, 11, and 9) were grouped close to each other. Similarly, accessions with lower ODAP values were grouped together in a study conducted on a different species of Lathyrus (Sammour *et al*., 2007).

4. CONCLUSIONS

The observed level of heterozygosity showed the potential application of seed storage proteins for genetic diversity assessment in Ethiopian grass pea. The analysis for β-ODAP also showed the presence of variability for this antinutritional factor in different accessions. Further analysis of samples with medium level of β-ODAP containing accessions on single plant basis might result in revealing promising samples that could be used for the purpose of breeding. The presence of diversity and selection of germplasms with the desired traits helps to improve the crop and its nutrition, enabling the utilization of this resilient crop in many regions.

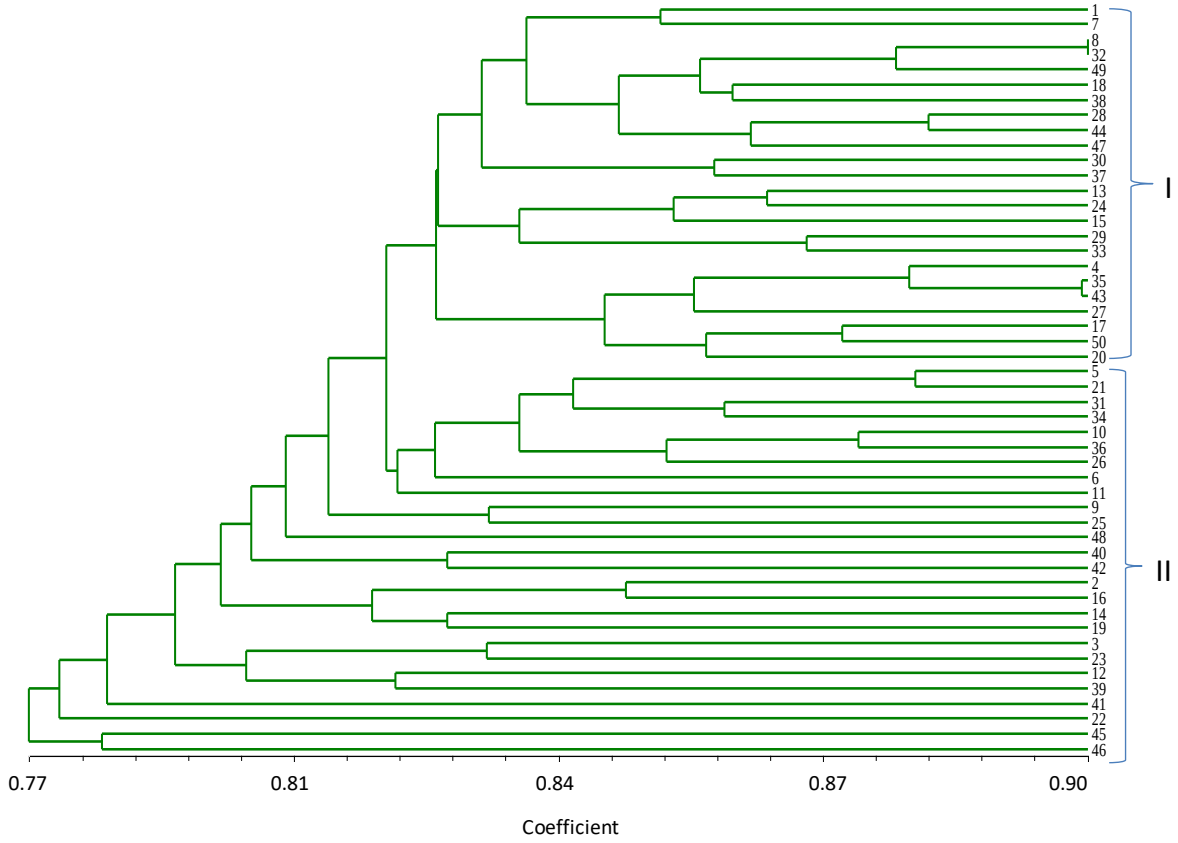

Figure 3. Dendrogram generated by UPGMA cluster analysis of 50 grass pea accessions based on data from seed storage proteins.

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