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Evaluation of wild *Rhynchosia minima* (L.) DC. through morphometric and biochemical markers

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Abstract

Aim of the study, to explore the intra-specific diversity among the genotypes of specie *Rhynchosia minima*. The methods to study genetic variation are morphological and SDS-PAGE. Total 13 morphological characters was studied which include both qualitative and quantitative traits. Significant level of variation was observed in four qualitative traits, two types of pod shape was found (70.8%) curved and (20.1%) oval. In case of seed color three colors were observed (54.1%) grey, (37.5%) dark grey and (8.3%) light grey. Leaf color and seed shape showed no variation. Descriptive statistics done for nine quantitative traits in which seed per pod showed maximum coefficient of variance CV (173.66%) followed by petiole length (49.62%), biomass per plant (46.21%) and pod per plant (45.65%). While minimum coefficient of variance showed by leaf width (25.62%) followed by internode length (29.48%), leaf length (30.80%) and pod length (34/68%). Comparative correlation was also done in which petiole length show strongly negative correlation with internode length and biomass per plant. One way cluster analysis for nine quantitative traits also done which separated the genotypes into different cluster on the basis of genetic similarity and dissimilarity. SDS-PAGE was carried out on 12% gel electrophoresis which revealed 10 reproducible bands. High level of variation was observed in band B-1 (0.54%) followed by B-2 (0.54%), B-3 (0.46%) and B-5 (0.42%), similarly low level of variation was found in B-7 (0.25%) followed by B-4 (0.29%) and B-8 (0.33%).The entire bands loci show polymorphism. report addressing genetic variability in *R. minima*.

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Introduction

Rhynchosia minima are a wild species belonging to the genus *Rhynchosia* of family leguminosae (ILDIS/CHCD, 1994). The genus *Rhynchosia* consist of 230 species distributed in tropical and subtropical hemisphere (Grear, 1978). The word "Rhync" refer to its nose-or snout shaped flowers while the "minim" indicates its very small pod, flowers and seed. *Rhynchosia* is a diploid species having 2n=22 number of chromosomes (Dundas, 1990). *Rhynchosia minima* are annual, perennial weed, herbaceous plant found in every continent (Lopez, 2012). *Rhynchosia minima* known by various names in different countries such as Least Snout bean and Turvel in India Sri Lanka and United States (Kirtikar and basu, 1999). Their Stems are lean and 80 to 120 cm long. Leaves are trifoliate having rhomboid leaflets which is 0.5 to 3 cm wide and 0.5 to 3 cm long. The texture of leaves are velvet or glabrescent. *A Rhyncosia minimum has* raceme inflorescence with 6 – 12 flowers. The floral part consists of 3 to 4 mm long calyx with five acuminate lobes. Corolla is 1 cm long and yellow in color. Pods are two seeded with short beaks. Mature pod color is black and often 1 to 1.5 cm long and 0.4 to 0.6 cm wide. The seeds are 3 mm long having a short hilum. The color of seeds range from brown or black and grey to tan (FAQ, 2006). In the investigation of genetic diversity of plants, morphological characterization have dynamic role but some time it show several drawbacks as they are easily effected by environmental factors (Nisar *et al.*, 2016). While molecular method such as biochemical evaluation at protein level and DNA based practice has many advantages over the classical morphology in genetic diversity (Nidiaye *et al.*, 2012). Among all the biochemical measures, molecular study of DNA markers is too expensive as compared to biochemical evaluation at protein level (Win *et al.*, 2011). Sodium dodecyl sulphate (SDS-PAGE) considers the most consistent, simple and inexpensive method that is independent of the environmental changeability (Wadood *et al.,* 2016). Over the last two decades considerable attention has been focused on the use of SDS-PAGE for assessment of genetic divergence, documentation and regular finding the species of plant. In a number of crop plant species protein profiling of seed and markers are widely use characterized cultivated verities and determined taxonomic interaction such as *Lima bean* (Lioi *et al.*, 1999) *Chickpea* (Ghafoor *et al.*, 2003**:** Nisar *et al.*, 2007), *Pisum sativa* (Nisar *et al.*, 2009) *Lens culinaris* (Wadood *et al.*, 2016**;** Sultana *et al.,* 2006) *V. unguiculata* (Win *et al.*, 2011). The revealing and analysis of variant in the electrophoresis sample of protein storage seed is a valuable way for establish associations with the plant accession contained by the species. Protein is the primary product of genes they give a precious mean of mark inherited variety, the variation in protein composition is a reflection of the genotypic variation (Sammour 1991**;** Gepts *et al.*, 1986). Pakistan is considering the region of the center of diversity of *R. minima* based on biochemical description. *R. minima* are widespread throughout the country and hold important adaptation, but there is no record is present on the diversity of *R. minima*. The aim of the present study is to estimate the level of genetic variation of 24 germplasm of *R. minima,* on the basis of SDS-PAGE and morphological assessment present in Pakistan.

Material and methods

The research work was carried out both in field and in laboratory. The methods and procedures for each experiment are given below.

Experimental plant

To investigate the genetic divergence among the genotypes of *Rhynchosia minima* for both morphometric and biochemical SDS-PAGE analysis be carried out. Total 24 samples of *Rhynchosia minima* plant was collected from different regions of Dir lower, Khyber Pakhtun Khwa Pakistan in the year of 2018. Mature seed of 24 genotypes were chosen from every sample of specie. Then these seeds were grind of into fine powder for SDS-PAGE to extract seed storage protein.

Morphological characterization

Both qualitative and quantitative traits were reported

in the present research. 4 Qualitative characters including leaf color, seed color, seed shape and pod shape. The 9 measured Quantitative characters including petiole length, leaf length, leaf width, no of pod per plant, no of seed per pod, no of branches per plant, pod length, internode length, and plant height. Character mean of each quantitative trait was also found after measuring three different samples.

Protein profiling

For estimation the level of genetic divergence SDS-PAGE was used. From each sample a single seed was grind up through pestle martin into fin powder then take 0.001g powder in each appendorf tube and add, About 400 µl of protein extraction buffer (PEB) with a composition of 0.5 M Tris-HCL, 0.2% SDS, 5 M Urea, 1% B-mercapto ethanol under 7-pH. The appendorf tube containing PEB and seed fine powder (PEB-FP), then Coomassie Brilliant Blue (CBB) was added to the appendorf tube as tracking dye to see the movement of PEB-FP on the separation, was vortexes thoroughly to homogenize the mixture. The homogenate samples were centrifuged at 14,000 rpm for 40 minutes under room temperature. The electrophoresis process was carried out using 12% polyacrylamide gel (composition of resolution gel: 3.0M Tris-HCl pH 9.0, 0.4% SDS and staking gel 0.4 M Tris-HCl pH 7.0, 0.4% SDS). The electrode buffer containing 0.025 M Tris, 129 M Glycine and 0.125% SDS was added to the tank Electrophoresis. In the same way, 12 µl from each samples were fill through micropipette in to each well of gel. Then the plate fit through cliff, and keep in electrophoresis tank add electrode buffer run at 120V until the blue line reached at the bottom of gel plates. The gels were then removed from glass plate and stained and distained through shaker for data score of protein finding. The morphological and protein data was analyzed through cluster plotting using software's SPSS and PC-ORD. Principal Component Analysis (PCA) was conducted by using PC-ORD.

Results and discussion

Qualitative traits

A significant level of variation was observed in Qualitative traits which include pod shape, seed shape, leaf color and seed color. In the present research, three types of seed color were found i.e. grey, dark grey and light grey (Table 1). In case of pod shape, two types were found i.e. (70.8%) curved and (29.1 %) oval. seed color was of three types, 13 genotypes had grey (54.1%), 9 had dark grey (37.5%) and 2 showed light grey (8.3%) show (Table 1).

It was found that all the genotypes have oval shape seed and green color leaves. No variation was found in seed shape and leaves color (Table 1).

Table 1. Four qualitative traits of 24 genotypes of *Rhynchosia minima*.

S.no	Pod shape	Leaves color	Seed color	Seed shape
RM 001	Oval	Green	dark grey	oval shape
RM 002	Curved	Green	dark grey	oval shape
RM 003	Curved	Green	Grey	oval shape
RM 004	Curved	Green	light grey	oval shape
RM 005	Curved	Green	Grey	oval shape
RM 006	Curved	Green	dark grey	oval shape
RM 007	Curved	Green	Grey	oval shape
RM 008	Oval	Green	light grey	oval shape
RM 009	Curved	Green	Grey	oval shape
RM 010	Oval	Green	Grey	oval shape
RM 011	Curved	Green	dark grey	oval shape
RM 012	Oval	Green	dark grey	oval shape
RM 013	Curved	Green	Grey	oval shape
RM 014	Curved	Green	dark grey	oval shape
RM 015	Curved	Green	dark grey	oval shape
RM 016	Curved	Green	dark grey	oval shape
RM 017	Curved	Green	dark grey	oval shape

RM 018	Curved	Green	Grey	oval shape
RM 019	Curved	Green	Grey	oval shape
RM 020	Oval	Green	Grey	oval shape
RM 021	Oval	Green	Grey	oval shape
RM 022	oval	Green	Grey	oval shape
RM 023	curved	Green	Grey	oval shape
RM 024	curved	Green	Grey	oval shape

Quantitative traits

Descriptive statistics analysis was done for quantitative traits of genotypes of *Rhynchosia minima* in Excel 2013. The minimum range for internode length was 1.70 while maximum range from 4.10 with mean value 2.56, sample variance 0.57 and standard deviation 0.75.Biomass per plant showed maximum range from 8.00 while minimum range from 2.00 with sample variance 3.35, standard deviation 1.83 and mean value 3.96. In case of petiole length maximum range from 2.00 while minimum range from 0.20 with mean value 0.94, standard deviation 0.47 and sample variance 0.20.Maximum range for plant height was 56.00 while minimum range from 11.00 with sample variance 169.74, standard deviation 13.03 and mean value 33.54.Leaf length showed minimum range from 2.00 while maximum range from 6.00 with mean value 3.28, sample variance 1.02 and 1.01 standard deviation. Leaf width showed maximum range from 3.20 while minimum range from 1.00 with sample variance 0.36, standard deviation 0.60 and mean value 2.35.

Table 2. Descriptive statistics for nine quantitative traits of *Rhyncosia minima*.

Traits	Mean	Standard Error	Standard Deviation	Sample Variance	Range		CV%
					Minimum	Maximum	
IN	2.56	0.15	0.75	0.57	1.70	4.10	29.48
B/P	3.96	0.37	1.83	3.35	2.00	8.00	46.21
PtL	0.94	0.09	0.47	0.22	0.20	2.00	49.62
PH	33.54	2.66	13.03	169.74	11.00	56.00	38.84
LL	3.28	0.21	1.01	1.02	2.00	6.00	30.80
LW	2.35	0.12	0.60	0.36	1.00	3.20	25.62
PL	3.43	0.24	1.19	1.41	1.50	6.00	34.68
S/P	7.09	2.55	12.24	149.72	2.00	63.00	172.66
P/P	15.54	1.45	7.10	50.35	8.00	40.00	45.65

In case of pod length range from 6.00 maximum and 1.50 minimum with standard deviation 1.19, mean value 3.43 and sample variance 1.41.

Maximum range for seed per pod was range from 63.00 while minimum range from 2.00 with means value 7.09, standard deviation 12.24 and sample variance 149.72. Pod per plant showed maximum range from 40.00 while minimum range from 8.00 with sample variance 50.35, standard deviation 7.10 and mean value 15.54.

Remarkable variation was observed in plant height from 11 to 56 cm, biomass per plant observed 2.00 to 8.00 g, leaf length from 2.00 to 6.00, leaf width 1 to 3.20, pod length 1.50 to 6.00, seed per pod 2 to 63, pod per plant 8 to 40, petiole length 0.20 to 2.00 and internode length 1.70 to 4.10. (Table 2).

Table 3. Correlation among the nine quantitative traits of *Rhyncosia minima*.

	IN	B/P	PtL	PH	LL	LW	PL	S/P	P/P
IN	1								
B/P	0.008	1							
PtL	-0.102	-0.065	1						
PH	0.019	-0.342	0.009	1					
LL	0.312	0.011	-0.042	0.198	1				
LW	0.037	0.006	-0.317	-0.095	0.080	1			
PL	0.376	0.386	-0.011	0.169	0.237	-0.348	1		
S/P	0.153	0.133	0.282	-0.191	0.064	0.091	-0.236	1	
P/P	-0.313	-0.125	0.100	0.230	-0.089	-0.378	-0.104	-0.093	1

Correlation analysis of traits

For nine agro-morphological traits of *Rhynchosia minima* correlation was measured by using SPSS software. Total 36 correlation coefficient values was observed in which 20 was positively correlated while 16 was negative correlated.

Table 4. Total genetic diversity present in 10 bands of *Rhyncosia minima*.

Bands	F	P%	A%	TGD%
B-1	11	45.8	54.2	0.54%
B-2	11	45.8	54.2	0.54%
B-3	13	54.2	45.8	0.46%
B-4	17	70.8	29.2	0.29%
B-5	14	58.3	41.7	0.42%
B-6	15	62.5	37.5	0.38%
B-7	18	75	25.0	0.25%
B-8	16	66.7	33.3	0.33%
B-9	14	58.3	41.7	0.42%
B-10	15	62.5	37.5	0.38%

In correlation petiole length was negatively correlated with internode length and biomass per plant whereas leaf length show positive correlation with internode length, biomass per plant and plant height. Leaf width was positively correlated with leaf length, biomass per plant and internode length. Pod length showed negative correlation with leaf width and petiole length. Pod per plant showed negative correlation with internode length, biomass per plant, leaf length, leaf width, pod length and seed per pod positively correlated with internode length, biomass per plant, petiole length, leaf length and leaf width (Table 3).

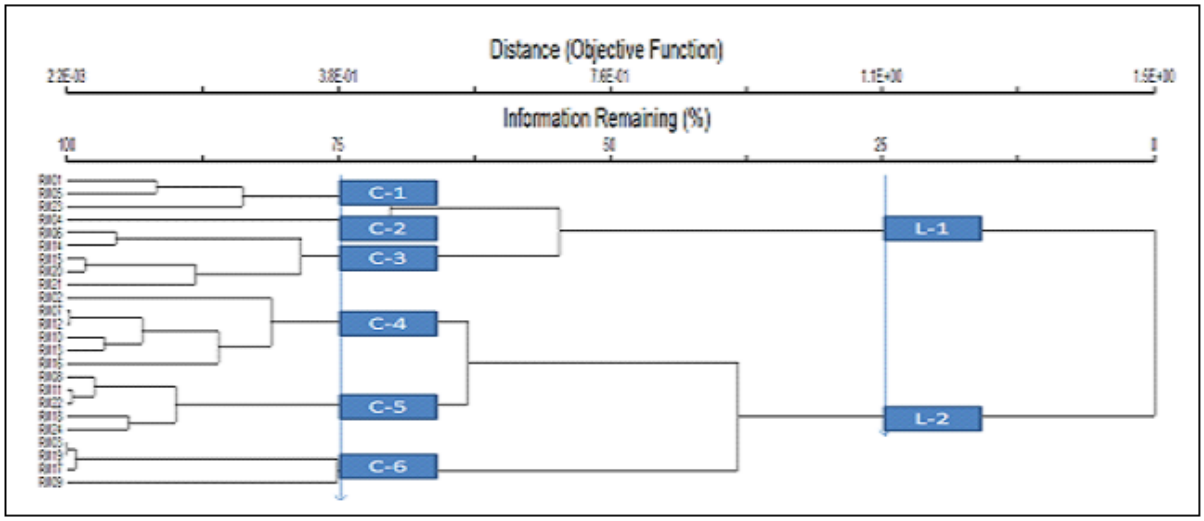

Fig. 1. One way cluster analysis for quantitative traits.

Cluster analysis of morphological data

The morphological data of 24 genotypes of *Rhynchosia minima* was evaluated through PC-ORD software for the building of phylogenetic tree.

The phylogenetic tree divided the genotypes into two linkages (L-1 and L-2). Linkage-1 comprises of 3 clusters (cluster-1, cluster-2 and cluster-3) which contain total 9 genotypes of *Rhynchosia minima* i.e. RM001, RM005, RM023, RM004, RM006, RM014, RM015, RM020, and RM021. Linkage-2 contain 3clusters (cluster-4, cluster-5 and cluster-6) which consist of total 15 genotypes i.e. RM002, RM007, RM012, RM010,RM013, RM016, RM008, RM011, RM022, RM018, RM024, RM003, RM019, RM017, RM009 (Fig 1 and 2).

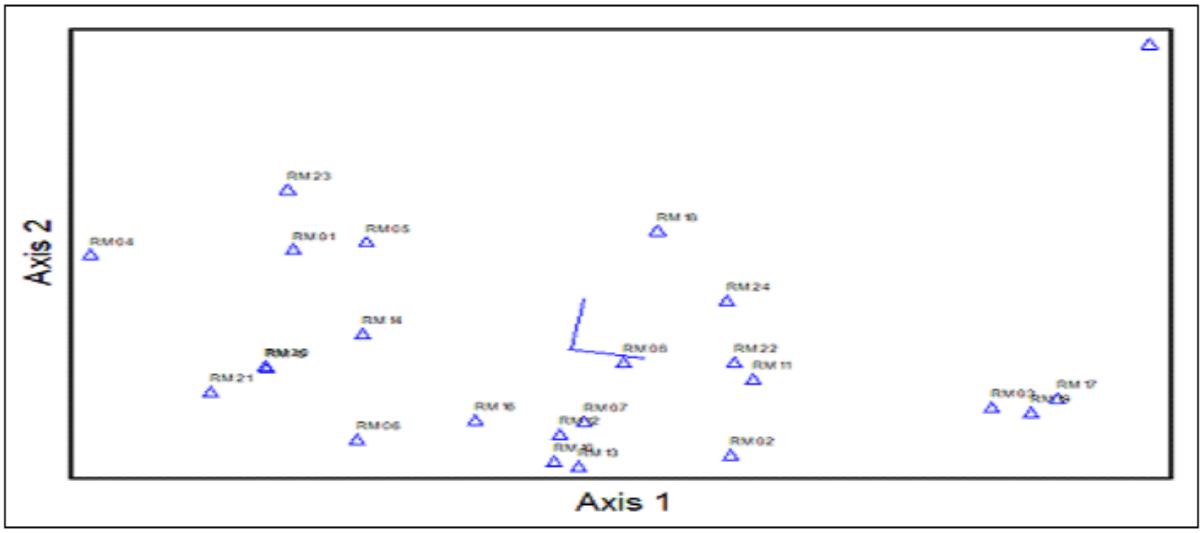

Fig. 2. PCA for morphological quantitative data.

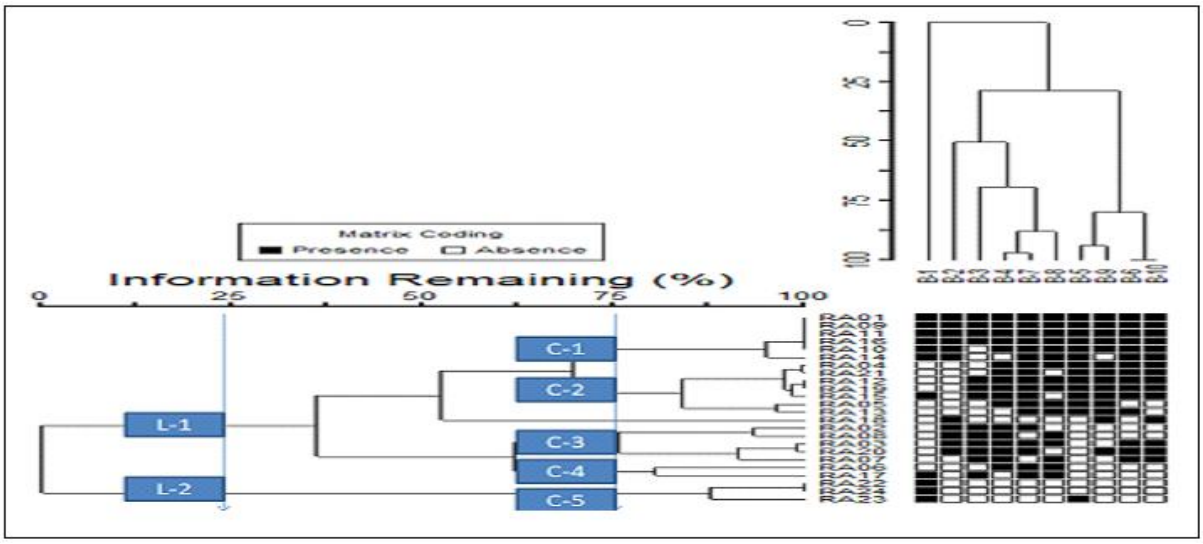

Fig. 3. Two-way Clusters Analysis based of seed storage proteins profile in genotype. Dendogram tee indicates genetic relationship of 24 genotype. Genotype based on bands.

Biochemical analysis

During the present work, a total of 24 genotypes of *Rhynchosia minima* were tasted through SDS-PAGE analysis in which 10 reproducible bands was observed. Similarly to the work of Noor *et al.*(2018) who worked on 100 genotypes of *Rhynchosia minima* and find out 8 reproducible bands. Dendogram tree was constructed for 24 landraces by mean of software PCORD and PCA, which divided the landraces into different clusters on the basis of similarity and differences. 24 genotypes divided into two linkages which were further divided into 5 clusters. The cluster one contain 6/24 genotypes, cluster 2 contain 8/24, cluster 3 contain 5/24, cluster 4 2/24 and cluster 5 has 3/24 genotypes respectively. Our result was supported by the work of (Noor M *et al*; 2018). Total 10 reproducible bands were observed in 24 genotypes of *Rhyncosia minima (fig.3.5)*. High level of variation was observed in B-1(0.54%) and B-2 (0.54%) followed by B-3 (0.46%), B-5 (0.42%) and B-9 (0.42%). similarly low level of variation was observed in B-7 (0.25%) followed by B-4 (0.29%), B-8 (0.33%), B-6 (0.38%) and B-10 (0.38%) (Table. 4).

On the basis of variation present in seed storage protein, the genotypes of *Rhynchosia minima* verified through ward method two way cluster analysis and principle component analysis.

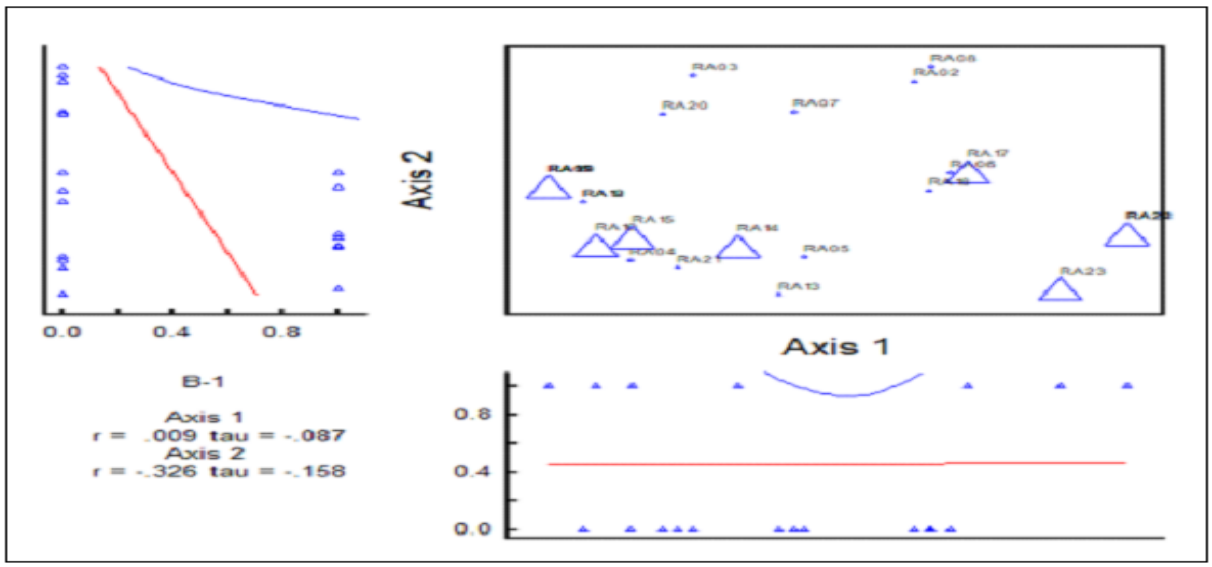

Fig. 4. PCA for seed storage protein of 24 genotype.

The dendogram tree separated the genotypes into two linkages (L-1 and L-2). Linkage-1 comprise of 4 clusters (cluster-1, cluster-2, cluster-3 and cluster-4) whereas linkage-2 contain only one cluster i.e. cluster-5.Cluster-1 of linkage-1 consist of 6 genotypes which is RM001, RM09, RM011, RM16, RM10 and RM14. Cluster-2 contain 8 genotypes include RM04, RM12, RM21, RM19, RM15, RM05, RM13 AND RM18. Cluster-3 contains 5 genotypes i.e. RM02, RM08, RM03, RM20 and RM07. Cluster-4 contains only 2 genotypes i.e. RM06 and RM17.Cluster-5 of linkage-2 consist of 3 genotypes including RM22, RM24 and RM23 (Fig.3 and 4).

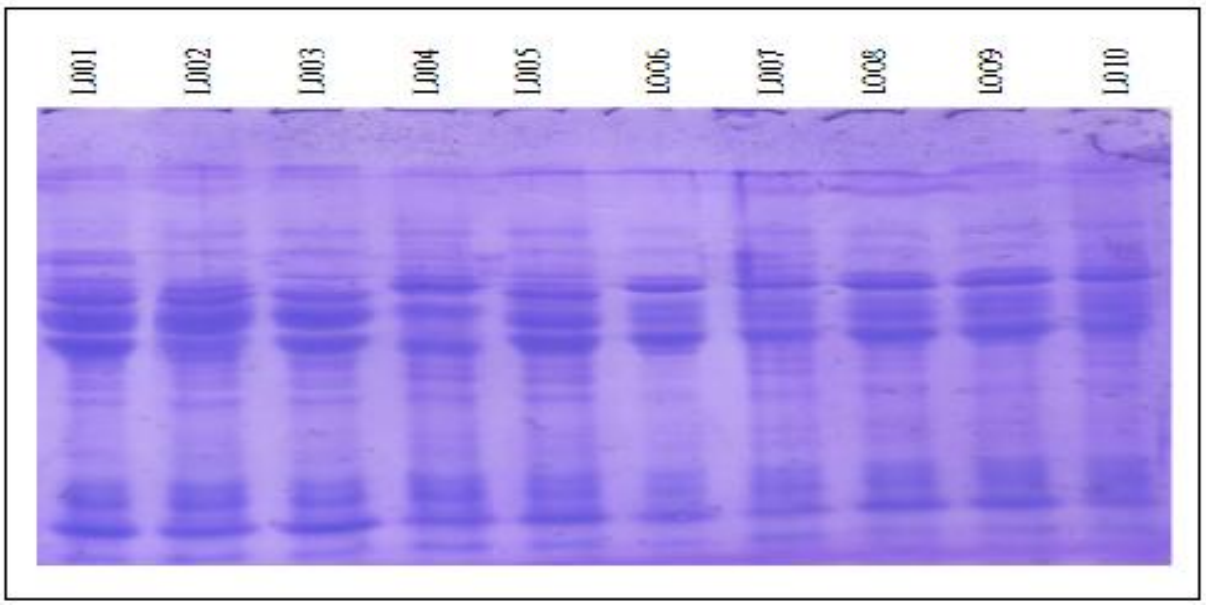

Fig. 5. The gel show the protein bands pattern and their existing, of 10 landraces out of 24 *Rhyncosia minima*.

Conclusion

A significant genetic diversity was found in qualitative characters on the basis of frequency distribution except seed shape and leaves color show no variation. On behalf of these traits descriptive statistics, correlation and cluster analysis was made the quantitative traits was observed with significant variation for all the traits. Their genetic diversity studied through the biochemical analysis by SDS-PAGE. There was significant diversity among the

genotypes. A total 10 bands were found, all the protein bands were polymorphic, there were no monomorphic bands found.

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