Legume Res., **37** (4) : 345-352, 2014

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com

ASSESSMENT OF SOMACLONAL VARIATION AND STABILITY IN *IN VITRO* REGENERATED GRASSPEA PLANTS USING SDS-PAGE

Surendra Barpete*, N.C. Sharma and Shiv Kumar[1]

Department of Biochemistry and Genetics,
Barkatullah University, Bhopal–462 001, India

Received: 05-04-2012 Accepted: 17-06-2013

ABSTRACT

Tissue culture may be one of the possible sources of variation for crop improvement. To assess variation and stability in regenerated plants, shoots were regenerated from the callus derived from axillary explants of 11 grasspea genotypes, and their shoot protein profiles were compared with those of seed cultured plants. The highest response of callus induction (87%) was observed when 2.0 mg/l 2,4-dichlorophenoxyacetic acid(2,4-D) and 0.25 mg/l BAP were supplemented in Murashige and Skoog's (MS) medium. α-naphthaleneacetic acid(NAA) and 6-benzylaminopurine(BAP) showed variable redifferentiation response along with callus formation. The MS medium supplemented with 0.5 mg/l BAP showed better multiplication and elongation of shoots. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed a unique protein band of 43 kDa in both tissue and seed cultured plants of Pusa 24. Polypeptide banding pattern of regenerated plants from 11 grasspea genotypes did not deviate from the banding pattern of parental seed protein. Similarity coefficient values ranged from 0.37 to 0.85 with a mean of 0.43 among the 55 genotypic combinations. Comparison of protein bands between calli raised regenerated shoots and parental seeds revealed the absence of somaclonal variation in regenerated plants, suggesting that the regeneration protocol used in the present study can be used for genomics enabled improvement in grasspea without the risk of additional variation or instability.

Key words: Axillary shoots, Grasspea, *In-vitro* culture, Regeneration, SDS-PAGE, Somaclonal variation.

INTRODUCTION

Grasspea (*Lathyrus sativus* L.), a member of the family Leguminosae (Febaceae), is an important protein-rich legume crop widely grown under subsistence farming in Indian subcontinent and Sub-Saharan Africa. Presently, it is reported to be grown on 1.5 million ha area with global production of 1.2 million tonnes (Kumar *et al* 2011). Properties such as tolerance to biotic and abiotic stresses make it an attractive food and feed crop under climate change scenario. In spite of these properties, limited research efforts have been directed towards grasspea improvement using conventional and biotechnological tools (Girma and Korbu 2012). Many countries including India and China had discouraged its cultivation in the past due to a disease 'neurolathyrism' caused by the presence of β-N-oxalyl-L-2,3diamino-propionic acid (ODAP)in grasspeaseeds (Rao1978;Hanbury *et al* 2000; Kumar *et al* 2011). In the recent past, the crop has received attention of researchers and policy makers for its improvement and as a source of genes for tolerance to drought and salinity (Kumar *et al*2011;Girma and Korbu 2012). Although the ODAP content in newly developed cultivars has been reduced through conventional plant breeding methods, its stability over environmentsstill remains a major challenge for grasspeabreeders.

Biotechnological tools offer great scope not only for the development of grasspea varieties that are either devoid of or have low levels of ODAP content but also as a source of economically important genes such as tolerance to drought, salinity and waterlogging for genomics enabled improvement

*Corresponding author's e-mail: surendrabarpete@gmail.com
[1]International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria

of agriculturally important crops (Girma and Korbu2012). Development of an *in vitro* regeneration protocol is considered an important step for genetic manipulation through transformation. Regeneration of many plant species including grasspeahas been reported from the callus derived from a variety of explants in various growth media Santha and Mehta 2001;Zambre *et al* 2002;Barik *et al* 2004, 2005; Barpete *et al* 2008, 2009;Kendir *et al* 2009). Barik*et al* (2005) and Barpete *et al* (2008) reported a high frequency adventitious plant regeneration protocol using epicotyl segment in grasspea.Using the regeneration protocol of Zambre*et al* (2002), Girma (2010)reported genetic transformation of two grasspea varieties of Ethiopian origin. There are reports of variation and instability among the regenerated grasspea plants using various explants and culture media (Van-Dorreste*et al* 1998, Santha and Mehta 2001), and the same has been used as a source of somaclonal variation for development of low ODAP varieties in grasspea (Kumar *et al* 2011). However, transformation studies require a regeneration protocol which is variation-free and stable.

SDS-PAG Eanalysis using seed protein has been deployed successfully forassessment of genetic variation and phylogenetic relationships in several crops including grasspea Przybylska *et al* 2000;Sammour *et al* 2007; Zaher and Mustafa 2007; Celebi *et al* 2009; Hameed *et al* 2009; Emre*et al* 2010). This analysis has also been used for the assessment of variation and stability of regenerated plants inwheat (Kiarostami and Ebrahimzadeh 2001) and sugar beets (Bekheet *et al* 2007).Thepresent study reports a regeneration protocol in grasspea, and SDS-PAGE analysis for assessment of genetic variation between tissue- and seed cultured plants of 11 genotypes.

MATERIALS AND METHODS

Seeds of eleven genotypes of grasspea *viz.*, Pusa 24, Ratan, Pratik, IC120455, IC120500, IC120505,IC120507,IC120519, IC345392, IC345401, and IC345403 were obtained from National Bureau of Plant Genetic Resources,Regional station (Akola), India and Indira Gandhi Agricultural University (Raipur), India.The experiment was conducted at Department of Genetics, Barkatullah University (Bhopal), India. For *in-vitro* studies,seeds were grown on the full strength MS medium (Murashige and Skoog's 1962) gelled with 0.8% (m/v) agar (Hi-Media). The pH of the medium was adjusted to 5.8 prior to autoclaving. Cultures were kept in a growth chamber at 25°C under dark for two days and subsequently under fluorescent light (35 µmol m^{-2} s^{-1})following a 16/8 h light/dark cycle. After *in-vitro* seed germination, axillary shoots were excised and used as a source of explants for callus induction. The explant was inoculated on MS medium supplemented with three concentrations (1.0, 1.5 and 2.0 mg/l) either of 2,4-dichlorophenoxyacetic acid (2,4-D)or α-naphthaleneacetic acid(NAA) in combination with 0.25 mg/l6-benzylaminopurine(BAP)for callus induction. After 21 days of inoculation, observations were recorded on callus induction and redifferentiation response.In order to accelerate shoot multiplication and elongation from the callus, five concentrations (0.10, 0.25, 0.50, 1.00 and 2.00 mg/l) of BAP were supplemented to the medium and observations were recorded on shoot multiplication response, and average number and length of shoots.For root induction, only one genotype Pusa 24 was used with six concentrations (0, 0.25, 0.50, 0.75, 1.00 and 2.00 mg/l) of Indole-3-butyric acid (IBA) and 100 mg activated charcoal (AC) in the half-strength MS medium and root induction percentage, and number and length of roots per explant were recorded. All observations were recorded on five explants each replicated six times for each of the 11 genotypes after 21 days of inoculation.

Total protein was extracted from the healthy seeds and regenerated shoots of 11 genotypes following the procedure outlined by Sammour *et al* (2007). For quantitative determination of protein, 0.02 g dried flour was dissolved in 1000 µl extraction buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% sucrose and 0.002% bromophenol blue with 5% β-merceptoethnol) for 24 hat–4^0C. The extracts were then centrifuged for 10 minutes at 10,000 rpm prior to determination of protein concentration. For qualitative analysis of protein, 20 µl of the extracted protein whose final concentration was adjusted to 2 mg cm^3protein was boiled in a water bath for 5-10 min and loaded on Sodium dodecyl sulphate polyacrylamide gel electrophoresis(SDS-PAGE)

containing 12.5% resolving gel and 4% stacking gel (Lammeli 1970) using bromophenol blue as tracking dye. Electrophoresis was performed in the Medox-Bio electrophoresis unit at 25 mA until the tracking dye reached the bottom of the gel. The gels were destained in solvent composed of 7.5% glacial acetic acid and 5% methanol. The gels were visualized in white fluorescent light. Phosphorylase B (97.4 kDa), bovine serum albumin (66.0 kDa), ova albumin (43.9 kDa), carbonic anhydrase (29.0 kDa) and lactoglobulin (18.4 kDa) were used as marker proteins and subunit molecular weight of the protein bands of the samples was determined by correlating position of the marker proteins.The genetic diversity among the genotypes was estimated following the Jaccard's similarity matrix (Jaccard 1908) using binary data on protein banding patterns in NTSYS-PC 2.1 (Rohlf 1998). The similarity coefficients were used to construct a dendrogram depicting genetic relationship usingthe unweighted pair group mean average (UPGMA) method (Sneath and Sokal 1973). Simple correlation coefficients among protein bands, 100-seed weight and total seed protein werecalculated using the standard statistical method.

RESULTS AND DISCUSSION

In the present study, *in vitro* regeneration protocol of grasspea through axillary explant was standardized, andSDS-PAGE was used as a tool for the assessment of somaclonal variation among the regenerated plants by comparing their protein profileswith those of parent lines.

Shoot induction and multiplication using axillary explants: Axillary explants obtained from *in-vitro* grown seeds of11 grasspea genotypes showed,on an average, 43.34 to 86.66% callus induction and 46.15 to 64.70% redifferentiation response with different concentrations of auxin and cytokinin(Table 1). Supplementation of2,4-D in the medium gave better callus induction as compared to NAA across the genotypes but inhibited shoot regeneration. Callus induction from axillary explants was highest (86.66%) when supplemented with 2.0 mg/ l2,4-D and 0.25 mg/lBAP. This establishes the fact that 2,4-D induces dedifferentiation (Reinert and Bajaj 1977). This also depends on the endogenous level of plant growth regulators in the explant (Kumar *et al*1983).However, callus induction (56.67%) coupled with redifferentiation response (64.70%) was highest when supplemented with 1.5 mg NAA and 0.25 mg BAP. Medium containing 2,4-D induced a mixture of brownish and yellowish callus with friable texture. This loose callus showed proliferation but remained non-organogenic. In the MS medium supplemented with NAA and BAP, axillary explants produced aberrant leafy structures with green nodular callus and became organogenic with small shoot-like structures (Fig. 1). In successive cultural passages on the MS medium supplemented with BAP, many of these structures elongated into shoots with morphology resembling a normal plant (Fig. 2A). This suggests that for redifferentiation, NAA with BAP is more effective than 2,4-D with BAP (Bekheet *et al* 2007) as 2,4-D is reported to inhibit organogenesis (Reinert and Bajaj 1977).Regeneration of plant species including grasspeahas been reported from callus tissues derived from a variety of explants with varying

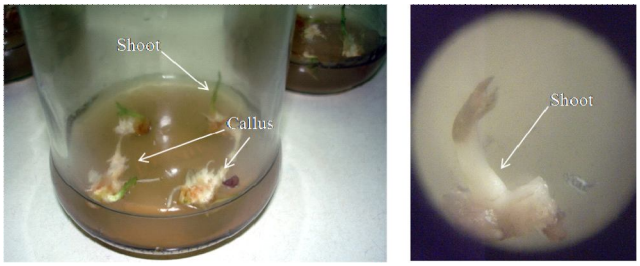

FIG. 1: *In vitro* shoot initiation from callus raised from axillary shoot explant in grasspea genotype IC345392.

TABLE 1: Callus induction and redifferentiationin grasspea using axillary explantsin MS medium supplemented with different concentrations(mg/l) of growth regulators.

Growth Media	Callusinduction (%)	Callus formation*	Redifferentiation response (%)
MS + 1.0 mg 2,4-D	66.66	+ +	0
MS + 1.5 mg 2,4-D	73.33	+ + +	0
MS + 2.0 mg 2,4-D + 0.25 mg BAP	86.66	+ + +	0
MS + 1.0 mg NAA + 0.25 mg BAP	46.66	+ +	57.14
MS + 1.5 mg NAA + 0.25 mg BAP	56.67	+ +	64.70
MS + 2.0 mg NAA + 0.25 mg BAP	43.34	+	46.15

Note: *Callus induction response + = low, + + = moderate, + + + = high

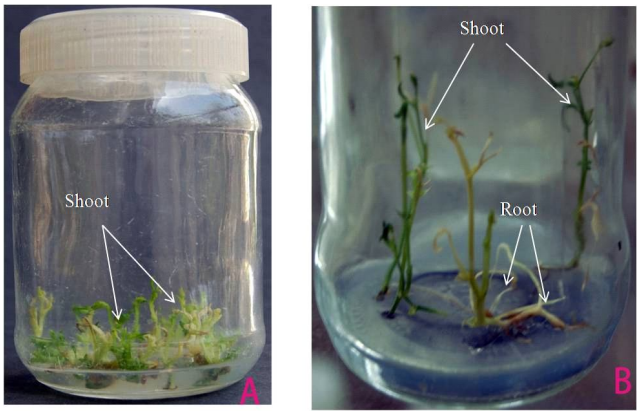

FIG. 2: (A) Multiplication of shoots raised from callus obtained from axillary shoot explant, and (B) *In vitro* root induction in regenerated shoot of grasspea genotype Pusa 24.

degree of regeneration in earlier studies (Zambre *et al* 2002; Kendir*et al*2009).

Shoot multiplication response while subculturing redifferentiated shoots ranged from 27% with 2 mg/l BAP to 68.6% with 0.5 mg/l BAP (Table 2). After 21 days of subculturing, the average number of shoots from a single regenerated shoot ranged from 3.1± 0.74 to 7.4± 0.78. The average length of such shoots varied from 1.20± 0.05 to 3.80± 0.06 cm. The highest shoot multiplication response coupled with greater number of large shoots was observed with 0.50 mg/lBAP using callus raised shoots from axillary explants. High response for shoot multiplication was associated with greater number and length of shoots in all replications. Callus obtained from an equal number of initial shoot explants produced six to seven-fold elongated shoots.Earlier studies in grasspea also showed high frequency of shoot proliferation with BAP using various explants including calli raised shoots (Malik *et al*1993), cotyledonary node (Barik *et al* 2004), epicotyl (Barik *et al* 2005) and immature zygotic embryo (Kendir *et al* 2009).In other studies, multiple shoot induction response was observed with the MS medium containing BAP in combination with 10^{-8} mg/l picloramusing stem explant in grasspea (Sinha *et al* 1982). These shoots were used for further studies on root induction and protein profile analysis.

Root induction: For root induction studies, only one genotype Pusa 24 was used. The shoots of Pusa 24 obtained from the callus subcultured on the half strength MS medium supplemented with 100 mg/l AC and different concentrations of IBA showed 33.3 to 80% root induction (Table 3). MS medium supplemented with 100 mg/lACwithout IBA showedminimum (33.3%) root induction response.Some of regenerated plants initiate roots without special treatments while other requires a medium supplemented with different growth regulators essentially of an auxin nature. Different plant species may vary in auxinrequirement for adventitious root formation. The presence of AC in the medium is known to improve root induction response due to adsorption of inhibitors that are produced during the process of morphogenesis (Reinert and Bajaj 1977).The induction of root,

TABLE 2: Shoot multiplication and elongation of callus raised shoot from axillary explants in grasspea after 21 days of subculture in MS Medium supplemented with different concentrations of BAP.

Concentration of BAP (mg/l) in MS medium	Shoot multiplication response (%)	Average no. of shoots* (Mean ± S.E.)	Average length(cm)of the multiplied shoots (Mean ± S.E.)
0.10	49.60	5.10 ± 0.65	2.10 ± 0.05
0.25	54.30	5.30 ± 0.63	2.60 ± 0.06
0.50	68.60	7.40 ± 0.78	3.80 ± 0.06
1.00	34.60	4.20 ± 0.82	1.70 ± 0.08
2.00	27.00	3.10 ± 0.74	1.20 ± 0.05

Note: * Based on six replicates of five explants each.

TABLE 3: Root induction response in shoots obtained from callus derived axillary explant of grasspeagenotypePusa 24 subculturedin half MS medium supplemented with 100 mg/l AC and different concentrations of IBA after 21 days of inoculation.

IBA concentration(mg/l)	Root induction(%)	Number of roots*Mean± SE	Root length*Mean± SE
0	33.3	2.78 ± 0.65	1.8 ± 0.04
0.25	60.0	4.53 ± 0.53	4.1 ± 0.05
0.50	66.7	5.73 ± 0.67	4.6 ± 0.05
0.75	80.0	6.80 ± 0.58	5.3 ± 0.06
1.00	53.3	3.47 ± 0.43	3.4 ± 0.07
2.00	0	0	0

Note: * Based on six replicates of five explants each.

number of roots and root length increased with the increase of IBA concentration from 0.25mg to 0.75 mg/lin the medium.The highest root induction (80%) coupled with maximum number of roots per shoot (6.80± 0.58) and root length (5.3± 0.06 cm) was observed with the half strength MS medium supplemented with 100 mg AC and 0.75 mg IBA (Fig. 2). The number of roots per shoot ranged from 2.78± 0.65 without growth regulator (IBA)to 6.80± 0.58 with IBA (0.75 mg/l). Similarly, the mean root length per shoot ranged from 1.8± 0.04 cm without IBA to 5.3± 0.06 cm with IBA (0.75 mg/l). Furthermore, higher concentration of IBA (1 mg/l) resulted in reduction of root induction response as well as in root number and length. Root induction was completely inhibited with 2 mg/l IBA in the medium.Root induction was low when the medium had no growth regulator. Roy *et al* (1991) also induced rooting in the regenerated shoots ingrasspeausing IBA supplemented MS medium. Barik *et al* (2004, 2005) found it necessary to supplement IAA, IBA or IPA in the half strength MSfor root induction in the regenerating shoots. Sujatha and Kumar (2007)reported root induction in shoots of *Carthamus tinctorius*and *C. arborescens* using axillary explants on half strength MS medium supplemented with IBA along with phloroglucinol. The frequency of regenerated plantlets successfully transferred in potsunder greenhouse condition was 53.14%.

SDS-PAGE analysis of regenerated plants of 11 grasspea genotypes: SDS-PAGE analysis of seed protein and callus raised plantlets of 11 genotypes showed 11 to 19 protein bands (Figs. 3 and 4). Perusal of protein profiles of regenerants and their parent seeds showed similar banding pattern in all the genotypes. This suggeststhat the regeneration protocol used in the present study does not cause genetic variation in the regenerated shoots. Therefore, the regeneration protocol suggested in the present study can be used effectively in the genomics-enabled grasspea improvement using recombinant DNA technologies. Similar results were reported in wheat (Kiarostami and Ebrahimzadeh 2001; Bapat *et al* 1992) and sugarbeet (Bekheet *et al* 2007) where no significant variation was observed between tissue- and seed cultured plants. Protein bands were minimum (11) in Ratan and maximum (19) in Pusa 24 and IC345392 (Table 4). Pusa24 had

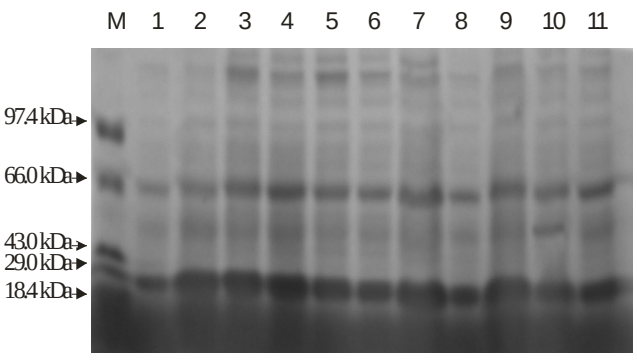

FIG.3: Electrophoretogram of seed protein of 11 grasspea cultivars: M (Protein marker), 1 (IC345403), 2 (IC120455), 3 (IC120505), 4 (IC345392), 5 (IC345401), 6 (IC120519), 7 (IC120507), 8 (IC120500), 9 (Pusa24), 10 (Pratik), 11 (Ratan).

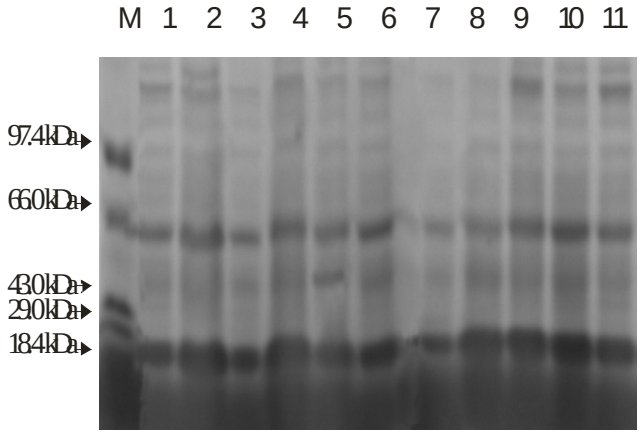

FIG.4: Electrophoretogram of callus raised shoots protein of 11 grasspeaL. genotypes: M (Protein marker), 1 (IC345401), 2 (IC120519), 3 (IC120507), 4 (IC120500), 5 (Pusa24), 6 (Pratik), 7 (Ratan), 8 (IC345403), 9 (IC120455), 10 (IC120505), 11 (IC345392).

TABLE 4: 100-seed weight, number of protein bands in the electrophoretogram and total seed protein content in 11 grasspeagenotypes.

Cultivars	100-seed weight (g)	No. of protein bands	Seed protein content(%)
IC120455	6.22	15	24.6
IC120500	7.05	17	26.1
IC120505	5.52	17	22.4
IC120507	6.51	15	24.3
IC120519	5.85	18	25.1
IC345392	6.26	19	26.5
IC345401	5.39	18	27.0
IC345403	5.08	13	23.4
Pusa 24	7.48	19	27.8
Pratik	6.96	18	27.2
Ratan	6.59	11	28.2
Mean ± SE	6.26± 0.22		25.7± 1.87

acharacteristic band of 43 kDa indicating uniqueness of the genotype.Correlation studies showed that number of protein bands did not have significant association with seed protein content and 100-seed weight. However,a significantly positive correlation between100-seed weight and seed protein content (r= 0.62*) was noticed among the genotypes.

The estimated similarity coefficient values ranged from 0.37 to 0.85 with a mean of 0.43 among 55 genotypiccombinations (Table 5).Two combinations, namely Pusa24/IC20507 and IC120500/IC120519 showed maximum similarity (0.85) whereas four combinations, IC120519/ IC345401, IC120507/IC345401, IC120519/ IC120505 and IC120507/IC120505 showed minimum similarity (0.37) coefficient values. Ratan showed 80% similarity with Pratik and 83% with Pusa 24. However, Pratik showed 80% similarity with IC345403 and 57%with IC120519 and IC120507.Two genotypes, IC345401 and IC120505, showed low similarity index values with most of the remaining genotypes.The UPGMA dendrograms obtained from the similarity matrices of regenerants and parental genotypes showed similar clustering pattern, and therefore, dendrogram based on seed protein is only presented in Fig. 5.The dendrogram showed two main clusters each with three subclusters of genotypes.Cluster I had four genotypes (IC120455, IC345403, IC120505 and IC345401) with mean similarity index of 64%whereas, cluster II had seven genotypes (Pusa 24, IC120507, IC120500, IC120519, Pratik, Ratan and IC345392) with mean similarity index of 70%.Pusa 24 and IC120507 were grouped together with 85% similarity index in subclusterIIA; IC120500 with

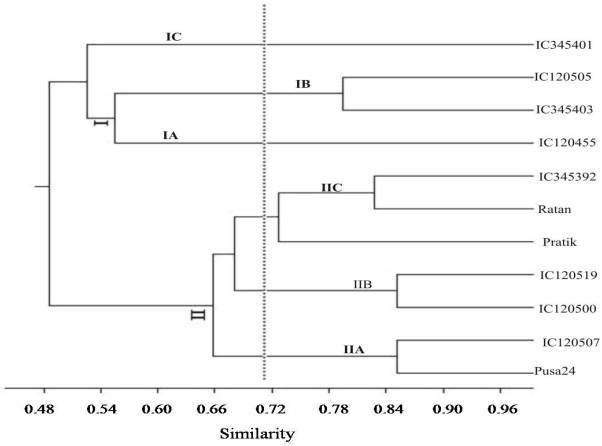

FIG. 5: Dendrogram showing genetic relationship among 11 grasspeagenotypes based on total seed protein.

IC120519 with 85% similarity indexin sub-cluster IIB; and Pratik, Ratan and IC345392 in sub-cluster IIC. Genotypes in Cluster I formed three subclusters, two with a single genotype and one with two genotypes (IC345403and IC120505). In the present study,Jaccard's similarity coefficient valuesand clustering pattern in dendrograms were similar for both tissue and seed raised plants but indicated fair degree of genetic variation among the genotypes irrespective of the protein isolated from regenerated plants or seed raised plants.Pedigree of different genotypes grouped in cluster II revealed that Pusa 24, Prateek and Ratan have common parent, which might be one of the reasons for their grouping in the same cluster. Thus, the research findings suggest that the regeneration protocol used in the present study can be employed for genetic transformation studies in grasspea as it does not generate somaclonal variation.

TABLE 5: Genetic similarity indices among grasspeagenotypes based on protein electrophoresis data.

Genotype	IC345403	IC120455	IC120505	IC345392	IC345401	IC120519	IC120507	IC120500	Pusa24	Pratik	Ratan
IC345403	1										
IC120455	0.83	1									
IC120505	0.80	0.66	1								
IC345392	0.57	0.50	0.42	1							
IC345401	0.50	0.42	0.60	0.66	1						
IC120519	0.50	0.44	**0.37**	0.62	0.37	1					
IC120507	0.50	0.44	**0.37**	0.62	0.37	0.55	1				
IC120500	0.57	0.50	0.42	0.71	0.42	**0.85**	0.62	1			
Pusa 24	0.57	0.50	0.42	0.71	0.42	0.62	**0.85**	0.71	1		
Pratik	0.80	0.66	0.60	0.66	0.60	0.57	0.57	0.66	0.66	1	
Ratan	0.66	0.57	0.50	0.83	0.50	0.71	0.71	0.83	0.83	0.80	1

Figures in bold represent range (low and high values) of thegenetic similarity indices

REFERENCES

Bapat, S.A., Rawal, S.K., Mascarenhas, A.F. (1992).Isozyme profiles during ontogeny of somatic embryos in wheat (*Triticum aestivum*). *Plant Sci*. **82:**235-242.

Barik, D.P., Mohapatra, U., Chand, P.K.(2005).High frequency *in-vitro* regeneration of *Lathyrussativus* L. *Biol. Planta*. **49:**637-639.

Barik, D.P., Naik, S.K., Mohapatra, U., Chand, P.K. (2004). High frequency plant regeneration by *in-vitro* shoot proliferation in cotyledonary node explants of grasspea (*Lathyrussativus*L.). *In vitro Cell Dev. Biol. Plant* **40:**467-470.

Barpete, S., Sharma, N.C., Parmar, D., Dhingra, M. (2009).*In vitro* induction of multiple shoots and plant regeneration of Khesari Dal (*Lathyrussativus* L.) using cotyledonary node explant.*Indian Appl. Pure Biol.***24:**81-86.

Barpete, S., Sharma, N.C., Parmar, D., Dhingra, M. (2008).*In-Vitro* Regeneration of *Lathyrussativus* L.*Nat. Life Sci.***05:**235-238.

Bekheet, S.A., Taha, H.S., Matter, M.A. (2007).*In vitro* regeneration of sugar beet propagules and molecular analysis of the regenerants. *Arab J.Biotechnol.***10:**321-332.

Celebi, A., Acik, L., Aytac, Z. (2009). Biosystematics studies among *Ebenus*L. species based on morphological, RAPD-PCR and seed protein analyses in Turkey. *Pakistan J. Bot*.**41:**2477-2486.

Gharyal, P.K., Maheshwari, S.C. (1980). Plantlet formation from callus cultures of a legume, *Lathyrussativus* cv. L.S.D.-3.*Pflanzen Physiol.***100:**359-362.

Girma, D., Korbu, L.J. (2012). Genetic improvement of grass pea (*Lathyrussativus*) in Ethiopia: an unfulfilled promise. *Plant Breeding* 131:231-236. *doi:10.1111/j.1439- 523.2011.01935.x*.

Girma, D. (2010). Ethiopian Grass Pea (*Lathyrussativus* L.) Started the Genomics Era.Lambert Academic Publishing, Koeln, Germany.

Hameed, A., Shah, T.M., Atta, B.M., Iqbal, N., Haq, M.A., Ali, H. (2009).Comparative seed storage protein profiling of Kabuli chickpea genotypes. *Pakistan J. Bot.***41:**703-710.

Hanbury, C.D., White, C.L., Mullan, B.P., Siddique, K.H.M. (2000). A review of the potential of *Lathyrussativus* L and *L. Cicera* L seed for use as animal feed.*Anim. Feed Sci. Tech*.**87:**1-27.

Jaccard, P. (1908).Nouvellesrecherchessur la distribution florale.*Bull. Soc.Vaudoise Sci. Nat.***44:**223-270.

Kendir, H., Sahin, D.N., Khawar, K.M., Ozcan, S. (2009).*In-vitro* plant regeneration from Turkish Grasspea (*Lathyrussativus* L.) using immature zygotic embryo explant.Agricult. Environ.Biotechn. **23:**1177-1180.

Kiarostami, K.H., Ebrahimzadeh, H. (2001).Changes of proteins and oxidative enzymes in seeds *in vitro* regeneration plants of three Iranian cultivars of wheat (*Triticumaestivusm* L.).*Pakistan J. Bot.* **33:**257-266.

Kumar, S., Bejiga, G., Ahmed, S., Nakkoul, H., Sarkar, A. (2011).Genetic Improvement of grasspea for low neurotoxin (â-ODAP) content.*Food Chem.Toxicol.***49:**589-600.

Kumar, S., Reddy, T.P., Reddy, G.M.(1983). Plantlet regeneration from different callus culture of Pigeon pea (*Cajanuscajan*L.). *Plant Sci.Lett.***32:**271-278.

Lammeli, U.K. (1970).Cleavage of structural proteins during the assembly of the head of bacteriophage T4.*Nature* **227:**680–685.

Malik, K.A., Ali-Khan, S.T., Saxena, P.K. (1993).High frequency organogenesis from direct seed culture in *Lathyrus*. *Ann. Bot.***72:**629-637.

Murashige, T., Skoog, F. (1962).A revised medium for rapid growth and bioassays with tobacco tissue culture.*Plant Physiol.***15:**473-497.

Przybylska, J., Przybylsk, Z.Z., Kranjewski, P. (2000). Diversity of seed globulins in *Lathyrussativus* L. and some related species. *Genet.Resour.Crop Evol.***47:**239-246.

Rao, S.L.N. (1978).A sensitive and specific colorimetric method for the determination of alpha, beta-ODAP, the *Lathyrussativus* neurotoxin. *Ann.Biochem*.**86:**386-395.

Reinert, J., Bajaj, Y.P.S. (1977).Applied and Fundamental Aspects of Plant Cell, Tissue and Oragan Culture. Springer-Verlag Berlin, Heidelberg, New York.

Rohlf, F.J. (1998). NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System. Version 2.1 Exeter Publications, NY.

Roy, P.K., Singh, B., Mehta, S.L., Barat, G.K., Gupta, N., Kirti, P.B., Chopra, V.L. (1991). Plant regeneration from leaf disc of *Lathyrussativus*. *Indian Exp. Biol.* **29:**327-330.

Sammour, H.R., Mustafa, A.E.Z., Badr, S., Tahr, W. (2007). Genetic variability of some quality traits in *Lathyrus* Spp. Germplasm. *ActaAgricult. Slovenica* **90:**33-43.

Santha, I.M., Mehta, S.L. (2001). Development of low ODAP somaclones of *Lathyrussativus*. *Lathyrus Lathyrism Newslett*. **2:**42.

Sinha, R.R., Das, K., Sen, S.K. (1982). Plant regeneration from stem-derived callus of the seed legume *Lathyrussativus* L. *Plant Cell Tissue Organ Cult.* **2**:67-76.

Sneath, P.H.A., Sokal, R.R. (1973). Numerical taxonomy: The principles and practice of numerical classification. - Freeman and Company, San Francisco.

Sujatha, M., Kumar, D.V. (2007). *In vitro* bud regeneration of *Carthamustinctorius* and wild *Carthamus* species from leaf explants and axillary buds. *BiolPlanta*. **51:**782-786.

Van-Dorrestein, B., Baum, M., Abd-El-Moneim, A.M. (1998). Use of somaclonal variation in *Lathyrussativus* (grass pea) to select variants with low ODAP concentration. In: Proceedings of the 3rd European Conference on Grain Legumes, Valladolid, Spain, p. 364.

Zaher, E.A., Mustafa, M.A. (2007). Genetic variation among Egyptian cultivars of *Viciafeba* L. *Pakistan J BiolSci* **10:**4204-4210.

Zambre, M., Chowdharey, B., Kuo, Y.H., Montagu, M.V., Angenon, G., Lambein, F. (2002). Prolific regeneration of fertile plants from green nodular callus induced from meristematic tissue in *Lathyrussativus* L. (grasspea). *Plant Sci*. **163:**1107– 1112.