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CLONAL FIDELITY STUDIES ON REGENERANTS OF *PSOPHOCARPUS TETRAGONOLOBUS* (L.) DC. USING RAPD MARKERS

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ABSTRACT
The molecular marker based methods are highly preferred over other methods to assess the clonal fidelity and genetic polymorphism. In this study, Random amplified polymorphic DNA (RAPD) markers were used to amplify DNA from *invitro* propagated plantlets of*Psophocarpus tetragonolobus* (L.) DC (Winged Bean) for evaluating the genetic stability. Seventeen arbitrary decamer primerswere used to find out the RAPD patterns existing in parent plant and *in vitro*regenerated plantlets. These 17 primers (C61-C77) yielded 170 products of which 44 were polymorphic (that is, 25.88% polymorphism).The dendrogram based on the Unweighted Pair Group Methodwith Arithmetic average (UPGMA) cluster analysis and Nei's similarity index depicted that the parent plant and the *in vitro* cultured plants have high degree of similarity.

INTRODUCTION

Eventhough*in vitro* propagation is one of the most suitable techniques for obtaining a large number of genetically homogenous plants, sometimes itcan lead to genetic variability known as somaclonal variation. The stress on the cells due to the activity of plant growth regulators during culturing of cells causes this variability. Somaclonal variation is beneficial forestablishment of variability in the base population especially in plants where natural variability is very low and it plays a significant role in crop improvement. *Psophocarpus tetragonolobus* (L.) DC or Winged Bean is a potential back yard crop, which is a highly protenaceous legume that can be used to overcome the problem of malnutrition. Still, the plant is an underutilized crop due to the high fiber content and pungent taste of the ripe seeds. Since the plant is cleistogamous, the variability in the crop is very low in nature. The present study was undertaken with an objective to assess if variability is spawned in *in vitro* regenerated plantlets of *Psophocarpus*.

In conventional screening practices there are many limitations like time factor, the changes might be heterozygous and recessive, and the changes occurred might not be phenotypic. Karyological analysis of regenerated plants even though reveal changes in ploidy levels and structural rearrangements, it does not reveal alterations in individual genes. Isozyme analysis also has limitations of availability of suitable markers. A precise determination of changes in a particular gene sequence resulting from tissue culture can be obtained by using DNA markers. DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann, 1983). The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics. The presence of various types of molecular markers, and differences in their principles, methodologies, and applications require careful consideration in choosing one or more of such methods. Restriction Fragment Length Polymorphism (RFLP) has the limitation that the analysis is limited only to those gene sequences used as a probe. Using the polymerase chain reaction (PCR) in conjunction with short primers of arbitrary sequence (Williams *et al.*, 1990), Randomly Amplified Polymorphic DNA (RAPD) markers are shown to be sensitive for detecting variations among individuals between and within species (Carlson *et al.*, 1991; Roy *et al.*, 1992). By using RAPD, we can determine the levels of variation in plant material at all stages of culture and growth, from single protoplasts to regenerated plants (Brown *et al.*, 1993, Al-Zahim*et al.*, 1999). RAPD analysis offers the markers of choice, since they have the advantage of being technically simple and cost effective as compared to other procedures.

Based on these facts, the present investigation was conducted to assess the clonal fidelity of the tissue culture regenerated plantlets using RAPD markers.

MATERIALS AND METHODS

Plants obtained through the various methods of tissue culture were subjected to RAPD analysis to find out the purity or genetic fidelity of the regenerated plants. Leaf materialsused for clonal stability studies of *Psophocarpus tetragonolobus* (L.) DC. were collected from tissue culture regenerants and the donor parent plant. Total genomic DNA from the young leaves of the plants was isolated by following Murray and Thompson's (1980) method using CTAB with appropriate modifications. 1.2 % PVP was added to the extraction buffer to remove phenolic contaminants and double chloroform extraction at 10000 rpm helped to remove polysaccharides. After ethanolic precipitation, DNA was resuspended in 100 ml of 1X TE buffer.Quantitative estimation and purity analysis of DNA was donespectrophotometrically.

17 decamer primers (C 61 – C 77) from IDT,Coralville**,** USAwere used for DNA amplification. RAPD assay was carried out in a final volume of 25ml reaction mixture containing 2.5 ml 10X amplification buffer (10mM Tris HCl, pH - 9.0, 1.5 mM $MgCl_2$, 50mM KCl and 0.01% gelatin), 200mM each of dATP, dTTP, dGTP,dCTP, 1.0U Taq DNA polymerase (Finnzymes, Helsinki, Finland.), 15 pMoles of 10mer primer and 50ng of genomic DNA. Amplification was performed in Thermal Cycler PTC 100 (MJ Research, USA). The PCR programme consisted of an initial denaturation for 2 min. at 94^{0}C followed by annealing and extension of primers for 2 min. at 36^{0}C and 2 min. at 72^{0}C respectively. This was followed by 38 cycles of 1 min. denaturation at 94^{0}C followed by annealing and extension of primers for 1 min. at 36^{0}C and 1 min. at 72^{0}C respectively. For ensuring the completion of reaction, the mixture was kept at 72^{0}C for 6 minutes.

After amplification samples were loaded and electrophoresed on 1.2% agarose gels. As ethidium bromide had been incorporated in the gel, DNA bands were visualized and documented using an Alpha Chemi Imager Gel Documentation System.Amplified products that were reproducible and consistent in performance were scored for data analysis.

POPGENE (Version 1.32), a computer program, was used to estimate standard genetic variability measures from data obtained through RAPD assay. The presence and absence of amplicons in the gels are scored as 1&0 respectively for the RAPD data analysis. Nei's (1972) original measures of genetic identity and genetic distance were calculated from this data. Cluster analysis using UPGMA method was applied to estimate genetic distance values from Nei's genetic similarity values to generate a phenogram based on those values.

RESULTS AND DISCUSSION

The plants regenerated through various methods of tissue culture were analyzed for their purity with the parent plant. 17 primers (C61-C77) were employed for the RAPD analysis and they yielded 170 products of which 44 were polymorphic (25.88% polymorphism; Table: 1, 2; Fig. 1-5). Mean value of the products per primer was 10, whereas, the mean value of polymorphism per primer was 2.58. The primer C71 produced the maximum number of products of 14 and C77 produced the minimum number of 5 products. C69 and C 71 showed the maximum and minimum polymorphism of 61.54% and 7.14% respectively.

Table: 1 Primers and the number of bands produced in RAPD analysis.

No.	Primer	Sequence 5'→3'	No. of bands produced	No. of polymorphic bands
1	C 61	TTC GAG CCA G	7	2
2	C 62	GTG AGG CGT C	8	2
3	C 63	GGG GGT CTT T	7	2
4	C 64	CCG CAT CTA C	10	2
5	C 65	GAT GAC CGC C	13	2
6	C 66	GAA CGG ACT C	7	1
7	C 67	GTC CCG ACG A	10	4
8	C 68	TGG ACC GGT G	13	2
9	C 69	CTC ACC GTC C	13	8
10	C 70	TGT CTG GGT G	10	1
11	C 71	AAA GCT GCG G	14	1
12	C 72	TGT CAT CCC C	11	3
13	C 73	AAG CCT CGT C	13	3
14	C 74	TGC GTG CTT G	9	1
15	C 75	GAC GGA TCA G	11	4
16	C 76	CAC ACT CCA G	9	3
17	C 77	TTC CCC CCA G	5	3
Total No. of bands			170	44

Table: 2 Data of RAPD analysis

Sl No	Parameters	Result
1	Number of assay units	17
2	Number of products	170
3	Average Number of products /assay	10
4	Average number of polymorphic products/assay	2.58
5	Percentage polymorphism	25.8

Analysis of the RAPD data using Nei's (1972) original measures of genetic identity and genetic distance showed that the regenerants and the parent plant showed a similarity value ranging from 0.99 to 0.84, with a mean value of 0.91 (Table:3). There were 17 polymorphic loci and the percentage of polymorphic loci was 100%. From the dendrogram and the similarity matrix, it is clear that the parent plant and the regenerants show a high degree of similarity.

Dendrogram (Table: 4) shows the presence of a single cluster with two sub clusters and a single out layer. The results show that the plants derived through shoot tip culture, axillary bud proliferation and cotyledon culture show less variation than the callus regenerated plants. The first subcluster consists of the parent plant and the plants regenerated from shoot tips, axillary buds and cotyledons and the second subcluster consists of callus regenerants. The outer layer is also a callus regenerant. Eventhough the callus derived out layer remained as a separate entity, there was no significant variation from the rest of the plants showing a similarity index of 0.85 to 0.91 with an average of 0.88. This does not significantly vary from the total similarity index, which varies from 0.99 to 0.84 with an average of 0.91.

PCR based methods like RAPD are increasingly being used in the analysis of genetic diversity (Nair *et al.,* 2002) and for testing the purity (Gupta and Roy, 2002) of crop plants, because of the relative ease with which PCR assays can be done. One of the advantages of this method is that there is no need for any prior knowledge about the genome. In India, molecular markers are being used mainly for characterization

Table: 3 Nei's Original Measures of Genetic Identity and Genetic distance.

pop ID	1	2	3	4	5	6	7	8	9	10
1	****	0.9864	0.9557	0.9677	0.8683	0.9624	0.9168	0.9264	0.8944	0.8671
2	0.0137	****	0.9687	0.9808	0.8907	0.9653	0.9316	0.9293	0.9165	0.8931
3	0.0453	0.0318	****	0.9654	0.8401	0.9381	0.8796	0.9140	0.8654	0.9009
4	0.0328	0.0194	0.0352	****	0.8757	0.9715	0.9088	0.9399	0.8940	0.8900
5	0.1413	0.1158	0.1742	0.1327	****	0.8815	0.9506	0.9370	0.9188	0.8899
6	0.0383	0.0353	0.0639	0.0289	0.1262	****	0.9301	0.9620	0.9150	0.8528
7	0.0869	0.0708	0.1282	0.0956	0.0506	0.0725	****	0.9439	0.9697	0.8930
8	0.0764	0.0733	0.0900	0.0620	0.0650	0.0387	0.0577	****	0.9286	0.9080
9	0.1117	0.0872	0.1446	0.1120	0.0847	0.0888	0.0308	0.0741	****	0.9029
10	0.1427	0.1130	0.1043	0.1165	0.1167	0.1592	0.1131	0.0966	0.1022	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

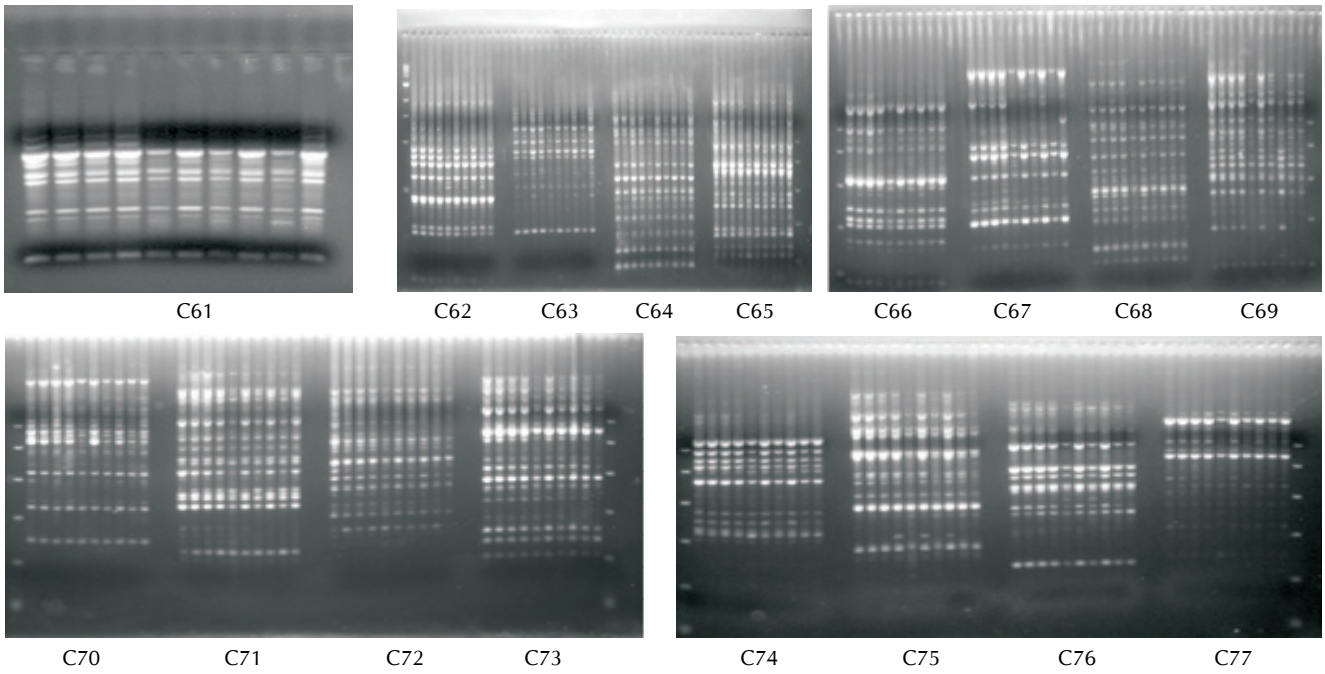

Figure: 1 RAPD profile of various regenerants along with control using 17 different primers C61 – C77. 1 – Parent plant; 2 – Shoot tip regenerant;3- Axillary bud regenerant;4 – Right cotyledon regenerant; 5 – Left cotyledon regenerant;6- 10 – Callus regenerants.

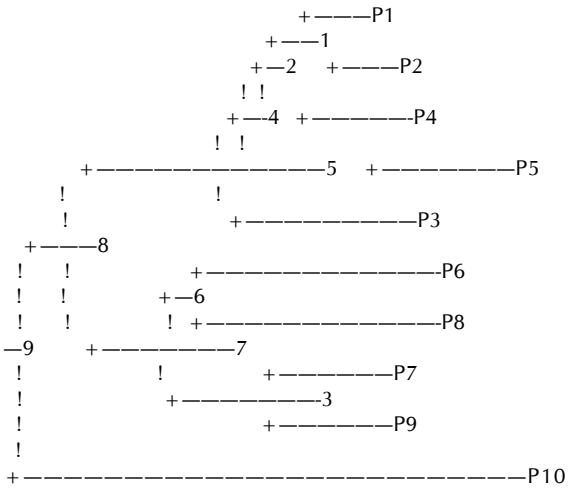

Figure 2: Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA—Modified from NEIGHBOR procedure of PHYLIP Version 3.5.

of the available germplasm through DNA fingerprinting and estimation of genetic diversity, testing genetic purity, selection of parents for hybridization studies and for molecular assisted breeding (Nair *et al.,* 1996; Bhat*et al.,* 1999; Negi*et al.,* 2000; Nair *et al.,* 2002; Sharma *et al.,* 2002).

Cluster analysis is a standard method for analyzing the relatedness of individuals and hence grouping them, from measured data (Melchinger*et al.,* 1992). This has an advantage over other grouping methods like Principle Component Analysis (PCA) in that, the number of related groups in the material under study does not have to be known in order to carry out the analysis. The main assumption made is that, two individuals or cultivars, which group together at a particular level, share a common ancestor more recently than those which only join at a high level (Wilkie*et al.*, 1993). In the present dendrogram, the regenerants were grouped into a single main cluster along with the control, suggesting a close relatedness of the regenerants with the parent plant.

The close relatedness of the shoot tip and cotyledon derived regenerants to the parent plant can be explained by the fact

that most of the shoot tips contained homogeneous cells capable of forming adventitious buds and regenerate plants with stable genotypes (Salmia, 1975; Papes*et al.,* 1983). The inertness of the well differentiated apical or bud meristems to a limited or no variations under tissue culture conditions are very well documented in many studies (Shenoy and Vasil, 1992; Isabel *et al.,* 1993; Rani and Raina, 1998; Jayanthi and Mandal, 2001; Anand, 2003). However, the identification of variability in micropropagated plants derived from the same donor mother plant as in *Populus deltoids* (Rani *et al.*, 1995) and *Piper longum*(Paranie*t al.*, 1997), using RAPD provides evidence for the existence of variants.

From the present study, it could be concluded that this species exhibits a high level of clonal stability and other methods must be employed for inducing variation for crop improvement programmes. Artificial methods for creating variation is necessary in this plant because the plant is cliestogamous i.e. the fertilization occurs even before the flower opens, and thus only self-pollination is taking place.

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