The Journal of Animal & Plant Sciences, 25(4): 2015, Page: 1114-1120
ISSN: 1018-7081

STUDY OF GENETIC STABILITY IN *IN VITRO* CONSERVED *PODOPHYLLUM HEXANDRUM* USING RAPD MARKERS

A. Tariq, S. Naz, K. Shahzadi, S. Ilyas and S. Javed

Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan.
Corresponding Author: e.mail:drsnaz31@ hotmail.com

ABSTRACT

In the present study, micropropagation of *Podophyllum hexandrum*, an endangered species of Pakistan was carried out on basal media containing different combinations of auxins and cytokinins as an attempt for its conservation. The results showed that the media containing BAP and NAA enhanced shoot induction from rhizome segments. Maximum number of shoots was observed in media having higher concentrations of BAP and NAA i.e. 11.0 mg L^{-1} and 5.3mg L^{-1} respectively. On an average 84% test tubes were observed showing initiation of shoots within 24 ± 0.83^{a} days, media containing 3.0 mg L^{-1}IBA was found to be the best for rooting. RAPD analysis using 10-mer primers was also carried out to check genetic stability of the *in vitro* conserved plants in comparison to the mother plants kept in the green house as the control. The results showed no genetic alterations when compared in term of number of bands and their respective sizes thus confirming that the plants conserved *in vitro* did not go through any genetic changes.

Keywords: *Podophyllum hexandrum*; *in vitro* conservation; Genetic stability; molecular markers; RAPDs.

INTRODUCTION

The rich herbal heritage of Pakistan covers a major portion of the northern areas with a diverse climatic regime and almost 600 plants are reported to be used as sources of important medicines in these areas (Shinwari, 2010). In the rural areas, the Hakims or the traditional practitioners use these plants as domestic therapy system for a number of diseases (Usmanghani *et al*., 1984).

Podophyllum hexandrum (syn. *P. emodi*) commonly referred to as Kakora or Bankakri in Pakistan and internationally as Indian May apple or Himalayan May apple is a perennial plant. It occurs at 2000 – 4500 m in Tibet, Afghanistan, China India, Bhutan and Himalayan areas of Pakistan (Evans, 2002). It grows low to the ground and bears a few stiff branches at the tips of which, lobed and glossy green leaves are borne which droop down. During flowering season, they produce pink flowers and later bulbous fruits which are bright red/orange in color. Vegetative propagation is carried out by seed or through the rhizome after dividing it. The plant is tolerant of cold but not of drought (Nadeem *et al*., 2000).

A resin, known as Indian Podophyllum Resin is produced by the rhizome which is processed to yield a neurotoxin known as podophyllotoxin, or podophyllin. It prevents tumor growth, therefore used to synthesize teniposides (*Giri et al*., 2000) and etoposides (Canel *et al*., 2000). Testicular and lung cancer respectively are treated using these medicines (Stahelin and Warburg, 1991). Podophyllin is also significant as an alterative, purgative, emetic and when given in conjunction with belladonna and Hyoscyamus it acts as a bitter tonic (Allevi *et al*., 1993).

The populations of this species have decreased in number in the wild to such an extent that the plant now faces extinction. The root cause of this threat is increase in the rate of its collection due to an increase in the demand for rhizomes of *Podophyllum* by the pharmaceutical industries (Silva *et al*, 1998). Hence due to the threats it faces, it falls under the category A (likely to become extinct) of the endangered species (Ullah and Rashid, 2013). Serious efforts are needed for the conservation of this endangered plant with immense medicinal value.

Rare and endangered medicinal plants can be conserved and rapidly propagated using *in vitro* culture method (Rahman *et al*., 2009; Thomas and Shankar, 2009; Nalawade *et al*., 2003). These protocols are of great importance since they produce uniform planting material (Shahzad and Sahai, 2013).

PCR based DNA fingerprinting techniques such as ISSR (inter simple sequence repeats) , AFLP (amplified fragment length polymorphism) and RAPD (random amplified polymorphic DNA), are all very useful and cost effective approaches for assessment of genetic integrity as well as genetic variation plants (Williams *et al*., 1990; Zietkiewicz *et al*., 1994;Vos *et al*., 1995). Of the several types of markers used to check genetic stability of plants propagated *in vitro*, RAPD (Random amplified polymorphic DNA) is the cheapest and simplest as well as reliable marker system (Williams *et al*., 1990; Rout and Das, 2002; Martins *et al*., 2004).

Keeping in view the medicinal importance, increasing demand and threat of being extinct in near future the present work was aimed for *in vitro*

conservation of *P. hexandrum* through micropropagation. RAPD markers were then used for the comparison of genetic stability of the conserved plants and the controls.

MATERIALS AND METHODS

Collection of plant material: During the month of August, *Podophyllum hexandrum* plants were collected from eight different locations of Miandam Valley at different heights. Table 1 shows the locations as well as the accession numbers assigned to the plants collected from various altitudes. The plants were brought to the green house located in LCWU (Lahore College for Women University) Lahore, to be kept there as sources of explants for use in tissue culture. Some of the plants were used in DNA analysis.

Table 1. List of *Podophyllum* plants, their collection sites with respective altitudes and accession numbers assigned.

Sr. No	Accession #	Location	Altitude (ft)
1	PHmv 01	Miandam Valley	6000
2	PHmv 02	Miandam Valley	6500
3	PHsk 01	Swatookaley	8500
4	PHsk 02	Swatookaley	9500
5	PHsk 03	Swatookaley	11000
6	PHgk 01	Gujarookaley	8000
7	PHgk 02	Gujarookaley	8500
8	PHgk 03	Gujarookaley	10000

Tissue Culture:

Explant Sterilization: From the *Podophyllum* plants kept in the green house, rhizomes having active buds were obtained. The surface sterilization of rhizomes was done by washing them with household detergent and thorough rinsing with distilled water. After peeling, the rhizomes were dipped in sodium hypochlorite solution (20%) for 20 minutes after which the explants were washed with autoclaved distilled water at least thrice to remove any traces of bleach.

Culture medium and conditions: MS (Murashige and Skoog, 1962) medium was first prepared with addition of sucrose (3% w/v) sucrose and Phytagel (1.5 g/L), different amounts of auxins and cytokinins were then added to it. The pH of media was adjusted between 5.5 and 5.8 using 1 N HCL or 1 N KOH. After preparation, media were poured in glass test tubes, covered with polythene bags and autoclaved at 121°C for 20 min. A laminar air flow cabinet was first cleaned by wiping with 70% ethanol solution and then irradiating with UV light for 25 minutes before use and then inoculation was carried out inside it.

Inoculation of Rhizome segments: The rhizome segments were inoculated on basal media enriched with BAP, IAA, NAA, Kinetin and TDZ either individually or in combination, for shoot initiation. All the cultures were transferred to the culture room with 60 to 70% relative humidity at 25 ± 3°C with a light intensity of 2000 Lux under 16 hours light and 8 hours dark photoperiod.

Hardening Stage: For acclimatization of plantlets in green house, different types of mixtures i.e. autoclaved sand, compost and coco peat were used. There were thirty replicates for each treatment; the data regarding days to initiation, mean number of shoots and their lengths etc. was recorded regularly. COSTAT V.63 was then applied to carry out analysis of variance (ANOVA). The Duncan's multiple range test at 5% level was used to compare the mean values.

DNA isolation: The extraction of genomic DNA was done by following CTAB (hexadecyl trimethylammonium-bromide) method of Doyle and Doyle (1987) with some modifications as reported earlier by Naz *et al*. (2014).

DNA extraction of the leaves of plants generated through tissue culture was also extracted by following similar protocol, for a comparison of genetic profiles of the wild and the conserved plants.

RAPD Analysis: To optimize the RAPD markers for the plants (both control and experimental), ten oligonucleotide primers each having 10 bp were used for standardizing the PCR conditions. A DNA Thermocycler (Primus-96 Thermal cycler) was used for this purpose. A single PCR reaction was prepared by mixing 0.6 U of Taq DNA polymerase (Invitrogen Life technologies, USA) + $MgCl_2$ (1.5 mM) + dNTP (0.2 µM) + 1X PCR Buffer (10 mM TrisHcl pH 8; 50 mM KCl) + primer (0.5 µM) + DNA (20ng). The program of the thermocycler (Primus-96 Thermal cycler) consisted of an initial heating step (4 minutes at 94°C), followed by denaturation (30 seconds at 94 ºC), annealing (35 cycles at respective annealing temperatures for 1 minute); extension (1:30 minutes at 72°C) and finally an extension (7 minutes at 68°C) was carried out in the end. 2% (w/v) agarose gel stained with ethidiumbromide (0.5µg/ml) was used for the electrophoresis of PCR products which were then visualized under UV transilluminator (WEALTEC MD-20).

RESULTS AND DISCUSSION

Micropropagation from Rhizome segments: Results of the present work showed that media having both BAP and NAA at higher concentrations exhibited maximum shoot regeneration (Table 2). The most effective media for micropropagation was the one having 5.3 mg $L^{-1}$of NAA and 11 mg L^{-1} of BAP, in this medium 84% shoot

initiation was recorded in maximum test tubes(21 ± 0.83[a]), days taken for bud break were also the lowest i.e. 24 ± 0.83[a] as compared to other media. However when BAP and NAA were used in combination with other growth hormones like IAA or TDZ the results showed that there was a gradual decrease in all parameters as it can be seen in Table 2. When BAP and IAA were used the percentage frequency of shoot initiation (16, 19, 35, 54, 20, 20) as well as days to bud break (34±0.83[a], 31±0.70[a], 24±0.83[a],33±0.83[a], 32±0.89[a], 37±0.70[a], 31±0.70[a], 27±0.70[a], 25±0.89[a]) were lower as compared to the other media. Similar results were obtained in media with BAP + TDZ and NAA + TDZ, with frequency of shoot initiation being lower (18% and 24%) and days taken for shoot initiation (45) being greater. However, TDZ when used alone in mg L^{-1} also gave better results in terms of frequency of shoot initiation i.e. 68% as compared to other media, days taken for shoot initiation were also lesser i.e. 17±0.1[a].

Shoots started appearing within a month of inoculation, but they were transferred to the rooting media after almost a month to allow elongation of shoots to a significant length. 1/2 strength basal (MS) media with varying concentrations of IBA from 0.5 to 3.0 mgL^{-1} was used as rooting media. Maximum rooting was observed in medium with 3.0 mgL^{-1} of IBA. Thus indicating that IBA alone was quite effective for induction of roots in the micro shoots at a comparatively greater concentration.

The two main problems that naturally hinder rapid multiplication of *P. hexandrum* are slow growth (Chattopadhyay *et al*., 1995; Kushwaha *et al*., 2008) and seed dormancy (Kharkwal *et al.*, 2008) can be eradicated by applying micropropagation.

Micropropagation has many advantages over the orthodox techniques of vegetative propagation including increase in proliferation and infection free plants (Perez *et al*., 2002), as well as provision of the natural products at any time of the year irrespective of the season (Sidhu, 2010). It is also reported that since micropropagation occurs through explants containing organized meristem, the threat of genetic instability is generally low (Shenoy and Vasil, 1992).

Shoot initiation using rhizome explants of *Podophyllum* was attempted using various combinations of growth regulators and the most effective media was observed to be MS medium supplemented with BAP and NAA at high concentration i.e. 2.68 µM and 11.1 µM (Chakraborty *et al*., 2010), as inferred from the current study. *Podophyllum peltatum* was propagated *in vitro* by using rhizome tips as explants that developed into plantlets. In this case as well, high concentrations of BAP (4.4 µM) and NAA (2.5 µM) were reported to be effective when used together (Moraes-Cerdeira *et al*., 1998).

Table 2. Formation of shoots from rhizome explants of *P. hexandrum* on MS medium having different concentrations of IAA, BAP, NAA and TDZ.

Concentrations of plant growth regulators					Days to shoot initiation±S.E	No. of TT showing shooting±S.E	Frequency of shoot initiation (%)
IAA+ BAP mg L^{-1}	BAP+NAA mg L^{-1}	BAP+TDZ mg L^{-1}	NAA+TDZ mg L^{-1}	TDZ mg L^{-1}			
2.5+1					34±0.83[a]	4±0.70[a]	16
3.0+1					31± 0.70[a]	6±0.83[a]	19
3.5+1					33±0.83[a]	9±0.89[a]	35
4.0+1					32±0.89[a]	15±0.83[a]	54
4.5+1					37±0.70[a]	5±0.70[a]	20
3+5.0					31±0.70[a]	8±0.83[a]	20
	5+5.1				27±0.70[a]	12±0.89[a]	35
	7+5.2				25±0.89[a]	15±0.7[a]	40
	11+5.3				24±0. 83[a]	21±0.83[a]	84
1+0.1					43±0.83[a]	5±0.54[a]	20
		1.5+0.1			45± 0.86[a]	3±0.53[a]	18
		2+0.1			47± 0.70[a]	2±0.56[a]	15
		2.5+1			50± 0.86[a]	2±0.50[a]	14
3+0.01					45± 0.1[a]	6±0.54[a]	24
			2+0.01		47± 0.1[a]	4±0.53[a]	22
			1+0.01		45± 0.5[a]	3±0.50[a]	21
			1+0.03		41± 0.53[a]	2±0.53[a]	20
0.1					50± 0.54[a]	17±0.1[a]	68
		0.09			55±0.53[a]	14±0.50[a]	63
		0.06			59±0.70[a]	11±0.53[a]	51
		0.04			62±0.83[a]	9±0.56[a]	45

[Means that have same letter in a column were not significantly different using Duncan's Multiple Range test at $P < 0.05$ %. Each treatment had 30 test tubes; S.E: Standard error]

Micropropagation can therefore be used as a tool for carrying out *in vitro* conservation of endangered medicinal plants within a limited time. As in case of *Psoralea corylifolia,* an endangered medicinal plant of family *Fabaceae* which has been propagated *in vitro* using apical meristems as explants. Highest shoot regeneration of 95% was observed in MS medium containing 12 µM BAP with 10.0 µM NAA and 15.0 µM Kn (Pandey *et al*., 2013).

A micropropagation protocol for *Saussurea involucrata* Kar. et Kir., an endangered Chinese medicinal plant, has also been developed. Shoot organogenesis occurred from *S. involucrata* leaf explants inoculated on medium with 10 µM BAP and 2.5 µM NAA (Guo *et al*., 2007).

Assessment of Genetic stability by RAPD analysis: RAPD profiles of the plants developed from *in vitro* cultures were compared to those of the control plants (collected from field) in order to confirm genetic stability. The DNA banding pattern for all the selected RAPD markers is reflected in Table 3, the results were based only on clear and reproducible bands. Ten RAPD primers were screened with the DNA of the donor plants and only eight generated bands that were discrete and reproducible; these primers were then used for the genetic analysis of the conserved plants. Table 3 shows results of RAPD analysis in terms of primer sequences, total number of light and dark bands and their size range while the amplification patterns of different primers are reflected in Fig. 2. a-c. The Eight RAPD primers resulted in a total of 297 scorable band classes; there sizes range between 600 bp to 1900 bp. An average of 37 bands per RAPD primer was obtained varying from 30 bands (P3 & P10) to 50 bands (P4) as shown in Table 3. All the primers yielded monomorphic bands for both *in vitro* grown plants and their counterparts in the greenhouse (Fig. 2).

The genetic stability of the regenerated plants was determined by analyzing the number of bands and their reproducibility in the amplified banding patterns.

The amplified banding pattern of the plants micropropagated form rhizome segments were identical to those of control plants for eight out of ten primers used and this technique can be used for conserving the germplasm of endangered *Podophyllum hexandrum* (Table 3; Fig. 2. a-c).

RAPDs, SSRs, ISSRs and AFLPs are all PCR based molecular markers which are considered to be reliable for the analysis of variations in the DNA sequences of plants (Rahman and Rajora, 2001).

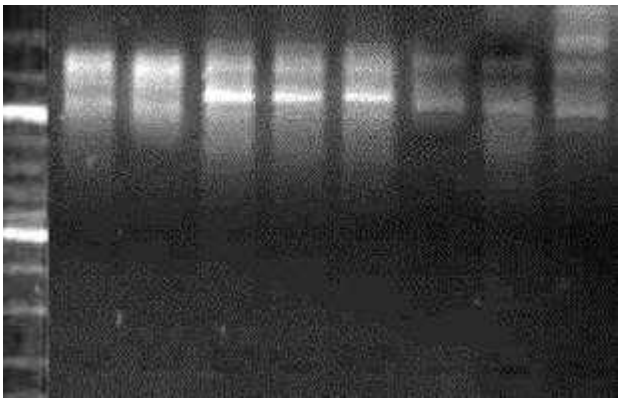
Genetic uniformity a prerequisite of *in vitro* conservation is confirmed through molecular analysis (Alizadeh and Singh, 2009). Lack of genetic disparity in micropropagated plants by analyzing RAPD profiles and comparing the number of bands and their reproducibility to the control plants has been reported in many studies (Rout and Das, 2002; Martins *et al*., 2004; Venkatachalam *et al*., 2007). RAPD profile of ginger conserved *in vitro* was studied using ten primers and no polymorphism was detected among the conserved plants thus indicating genetic stability (Geetha, 2002). Similarly genetic uniformity was also reported in micropropagated *Mantisia spathulata* using RAPD markers (Bhowmik *et al*,. 2009). Tissue cultured *Alpinia galanga* plantlets when subjected to assessment of genetic stability using RAPD markers produced banding profiles that were monomorphic and similar to those of the mother plant (Parida *et al*., 2011).

Table 3. List of primers with their sequences, total number of bands and their size range.

Primer No.	Sequence	Total bands	No. of dark bands	No. of light bands	Size range (bp)
P1	CATCGCCGCA	---	---	---	---
P2	TTCCGCCACC	---	---	----	---
P3	CAGGCCCTTC	30	12	18	800-1800
P4	TGCCGACCTG	50	16	14	700-1100
P5	CAGCACCCAC	48	19	06	800-1700
P6	GTGTGCCCCA	27	14	13	700-1000
P7	GTGACGTAGG	41	16	25	600-1500
P8	CAATCGCCGT	29	20	09	500-1000
P9	CCACAGCAGT	42	18	24	700-1300
P10	AGCGCCTTGT	30	23	07	900-1900

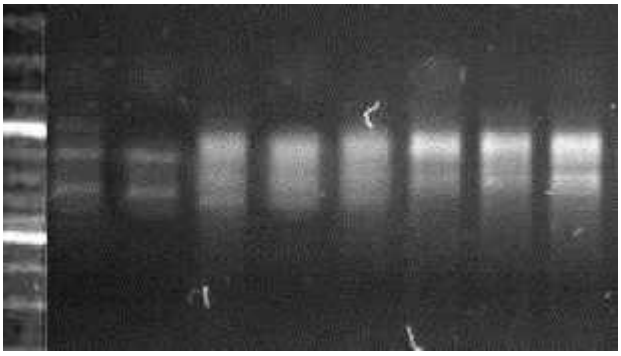
It was therefore concluded from the results of the present study that the plants multiplying on a medium optimized for *in vitro* growth do not necessarily undergo any genetic change. This was clearly reflected in the monomorphic patterns of DNA fragments generated using RAPD markers, which are well documented for determining genetic integrity in many plant species (Devarumath *et al*., 2002; Rout, 2002; Rehman, 2002). RAPD markers are considered to be a better option because they amplify different regions of the genome and

the simultaneous analyses of such regions gives a better interpretation of the genetic stability of the *in vitro* grown plants (Palombi and Damiano, 2002; Martins *et al*., 2004).



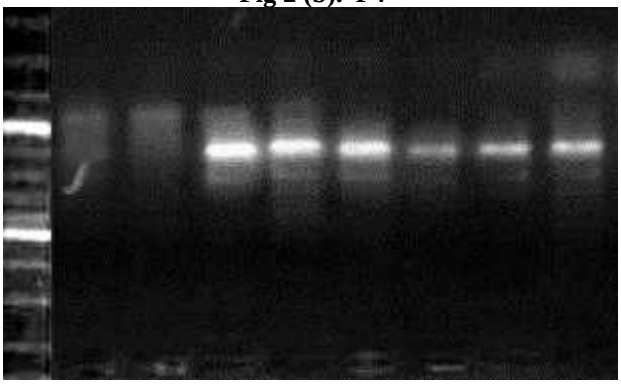
L V1 V2 V3 V4 V5 V6 V7 V8

Fig 2 (a). P3



L V1 V2 V3 V4 V5 V6 V7V8

Fig 2 (b). P4



L V1 V2 V3 V4 V5 V6 V7 V8

Fig 2 (c). P5

Fig 2: DNA banding pattern of wild and tissue cultured plants after polymerization with three primers (P3,P4, P5), a (donor plants), b &c (Tissue cultured plants). L: Ladder, V1: PHmv01, V2: PHmv02, V3: PHsk01, V4:PHsk02, V5: PHsk03, V6: PHgk01, V7: PHgk02, V8: PHgk03.

REFERENCES

Alizadeh, M. and K. S. Singh (2009). Molecular assessment of clonal fidelity in micropropagated grape (*Vitis* spp.) rootstock genotypes using RAPD and ISSR markers. Iran. J. Biotechnol.7:37 - 44.

Allevi, P., M. Anastarin, P. Claffrede, E. Begati, and P. Macdonald (1993). Sterioselective glucosidation of *Podophyllum* lignans - A new simple synthesis of etoposide. J. Org. Chem. (58): 4175 - 4178.

Bhowmik, S. S., S. Kumaria, S. R. Rao, and P. Tandon (2009). High frequency plantlet regeneration from rhizomatous buds in *Mantisia spathulata Schult*. and *Mantisia wengeri Fischer* and analysis of genetic uniformity using RAPD markers. Indian J. Exper. Biol. 47:140-146,

Canel, C., R. M. Moraes, F. E. Dayan, and D. Ferreira (2000). Molecules of interest 'Podophyllotoxin'. Phytochem. 54:15–20.

Chakraborty, A., D. Bhattacharya, S. Ghanta, and S. Chattopadhyay (2010). An efficient protocol for *in vitro* regeneration of *Podophyllum hexandrum*, a critically endangered medicinal plant. Indian J. Biotechnol. 9: 217-220

Chattopadhyay, S., S. K. Datta, and S. B. Mahato (1995). Rapid micropropagation for *Mucapuriens f. puriens L.Plant.* Cell. Rep. 15: 271-273.

Devarumath, R. M., S. Nandy, V. Rani, S. Marimuthu, N. Muraleedharan, and S. N. Raina (2002). RAPD, ISSR and AFLP fingerprints are useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite Tea clones representing *Camellia sinensis* (China type) and *C. assamica* spp *assamica* (Assam India type). Plant. Cell. Rep. 21: 166-168.

Doyle, J. J. and J. L. Doyle (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull.19:11-15.

Evans, W. C. (2002). Trease and Evans Pharmacognosy.13th ed. BailliereTindall, London. 654 – 656.

Geetha, S. P. (2002). *In vitro* technology for genetic conservation of some genera of Zingiberaceae. Ph.D Thesis. Calicut University, Calicut, Kerala, India.

Giri, A. and M. L. Narasu (2000). Production of Podophyllotoxin from *Podophyllum hexandrum*: A potential natural product for clinically useful anticancer drugs. Cytotechnol.34:17–26

Guo, B., M. Gao, and C. Z. Liu (2007). *In vitro* propagation of an endangered medicinal plant *Saussurea involucrata* Kar. et Kir.Plant.Cell. Rep.26(3):261-5.

Kharkwal, A. C., R. Kushwaha, O. Prakash, R. Ogra, and K. Bhattacharya (2008). An efficient method of propagation of *Podophyllum hexandrum*: An endangered medicinal plant of the western Himalayas under ex situ conditions. J. Nat. Med. 62: 211-216.

Kushwaha, R., S. Pandey, S. Chanda, A. Bhattacharya, and P. S. Ahuja (2008). Temperature dependent growth and emergence of functional leaves: An adaptive mechanism in the seedlings of the Western Himalayan *plant Podophyllum hexandrum*. J Plant Res. 121: 299-309.

Martins, M., D. Sarmento, and M. M. Oliveira (2004). Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers. Plant. Cell. Rep. 23: 492 - 496.

Moraes-Cerdeira, R. M., C. L. Burandt, J. K. Bastos, N. P. D. Nanayakkara, and J. D. McChesney (1998). *In vitro* propagation of *Podophyllum peltatum*. Planta. Medica. 64: 42–45.

Murashige, T. and F. Skooge (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15:473-479

Nadeem, M., L. M. S. Palni, A. N. Purohit, H. Pandey, and S. K. Nandi (2000). Propagation and conservation of *Podophyllum hexandrum*Royle: an important medicinal herb. Biol. Cons. 92:121-129.

Nalawade, S. M., A. P. Sagare, C. Y. Lee , C. L. Kao, and H. S. Tsay (2003). Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. Bot. Bull. Acad. Sinica. 44:79-98.

Naz, S., K. Shahzadi, S. Rashid, F. Saleem, A. Zafarullah, and S. Ahmad (2014). Molecular characterization and phylogenetic relationship of different Citrus varieties of Pakistan. J. Anim Plant. Sci. 24(1): 315-320.

Palombi, M. A. and C. Damiano (2002). Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev). Plant. Cell. Reports. 20 (11): 1061-1066.

Pandey, P., R. Mehta, and R. Upadhyay (2013). *In vitro* propagation of an endangered medicinal plant *Psoralea corylifolia Linn.* Asian. J. Pharm. Clin. Res. 6(3): 115-118.

Parida, R., S. Mohanty, and S. Nayak, (2011). Evaluation of genetic fidelity of in vitro propagated greater galangal (*Alpinia galangal L*.) using DNA based markers. Int. J. Pl. An. Env. Sci. (3):123-133.

Perez-Bermudez, P., H. U. Seitz, I. Gavidia (2002). A protocol for rapid micropropagation of endangered Isoplexis. *In vitro*. Cell. Dev. Biol. Plant. 38: 178-82.

Rahman, M. H. and O. P. Rajora (2001). Microsatellite DNA somoclonal variations in micropropagated trembling aspen (*Poplustr emuloides*). Plant. Cell. Rep. 20: 531-534.

Rahman, M. M., M. N. Amin, F. Ishiguri, S. Yokota, R. S. Sultana, Y. T. Kazuya, and I. N. Yoshizawa (2009). *In vitro* plantlet regeneration of ‘‘dwarf’’ Indian olive (Elaeocarpus robustus Roxb.): a fruit plant of Bangladesh. Plant. Biotechnol. Rep. 3:259–266.

Rehman, I. (2002). Ethnomedicinal Studies of Miandam Valley (Ph. D Thesis) Deptt of Botany, Government Jahanzeb Post Graduates College, Saidu Sharif Swat.

Rout, G. R and G. Das (2002). Assessment of genetic integrity of micropropagated plant of Plumbago*zeylanka*by RAPD markers. Biol.Plantarum.45:27-35

Rout, G. R. and G, Das (2002). An assessment of genetic integrity of micropropagated plants of *Plumbago zylenica* by RAPD markers. Biologia. Plantarum. 45(1):27-32

Shahzad, A. and A. Sahai (2013). Recent trends in biotechnology and therapeutic applications of medicinal plants. Springer Netherlands, 305-321.

Shenoy, V. B. and I. K. Vasil. (1992). Biochemical and molecular analysis of plants derived from embryo genic tissue cultures of napiergrass (Penisetum purpureum K. Schum.). Theor. Appl. Genet. 83:947-955.

Shinwari, Z. K. 2010. Medicinal plants research in Pakistan. J. Med. Pl. Res. 4(3): 161-176.

Sidhu, Y. (2010). *In vitro* micropropagation of medicinal plants by tissue culture. Plymouth. Student. Sci. 4: 432–449.

Silva, C. G., J. B. Power, K. C. Lowe, and M. R. Davey, (1998). Plant regeneration from root explants of the medicinal plant *Podophyllum hexandrum* cultured in liquid medium. 1998, Biological Sciences,Nottingham.

Stahelin, H. F. and A. Von Warburg (1991). The chemical and biological route from podophyllotoxin-glucoside etoposide. Cancer. Res. 51 (5)-15.

Thankappan, S. S. and V. M. Patell (2011). *In vitro* propagation studies and genetic fidelity assessment of endangered medicinal wild Yam-*Dioscorea prazeri*.Plant. Omics. J. 4(4):177-189.

Thomas, T. D. and S. Shankar (2009). Multiple shoots induction and callus regeneration in *Sarcostemma brevistigma* Wight & Arnott, a rare medicinal plant. Plant. Biotechnol. Rep. 3: 67-74.

Ullah, A. and A. Rashid (2013). Conservation status of threatened medicinal plants of Mankial Valley Hindukush Range, Pakistan.Int. J. Biodivers. Conserv. 6(1): 59-70.

Usmanghani, K.., M. Shameel, M. Sualeh, K. H. Khan, and Z.A. Mahmood (1984). Antibacterial and antifungal activities of marine algae from Karachi seashore of Pakistan. Fitotrap. 55:73-77.

Venkatachalam, L., R. V. Sreedhar, and N. Bhagyalakshmi (2007). Genetic analysis of micropropagatedand regenerated plantlets of banana as assessed by RAPD and ISSR markers. In Vitro Cell. Dev. Biol. 43:267-274.

Vos, P. R., M. Hogers, M, Bleeker, V. D. L. Reijans, M. Hornes, A. Fritjers, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau (1995). AFLP: a new concept for DNA fingerprinting. Nucleic Acids Res. 23: 4407–4414.

Williams, J. G. K.., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey (1990). DNA Polymorphisms amplified by arbitrary primers and useful as genetic markers. Nucl. Acids. Res. 18: 6531-6535.

Zietkiewicz. E., A. Rafalski, and D. Labuda (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics.20: 176-183.