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Original Article

Genetic diversity of *Sauropus androgynus* L. shoot culture after UV-C treatment assessed using RAPD markers

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Abstract

In the present study, random amplification of polymorphic DNA is used to assess somaclonal variation in *Sauropus androgynus* L.. Shoot cultures treated with UV-C irradiation were investigated. The shoots of *S. androgynus* L. were cultured for six weeks on Murashige and Skoog (MS) medium containing 1 mg/l 6- benzyladenine (BA) for multiple shoot formation. Multiple shoots were treated with UV-C irradiation for 0, 5, or 10 min (0, 2.03 or 4.06 kJ m^{-2}) and wild type was included as control. The shoot cultures were further grown for three weeks. Then, DNA was extracted and used as template in RAPD analysis (n= 3). The results indicated that nine different decamer oligonucleotide primers generated 157 reproducible amplified products. The similarity coefficients calculated with Simqual subprogram of numerical taxonomy system in multivariate software ranged within 0.026-0.975. Cluster analysis divided four samples into two groups comprised of an "in" group and an "out" group. Maximum polymorphic bands were revealed by using UV-C irradiation for 5 min.

Keywords: genetic, *Sauropus androgynus* L., UV-C, RAPD

1. Introduction

Sauropus androgynus L. of the family Euphorbiaceae is used in traditional medicine to relieve fever, treat urinary problems, and increase breast milk production, and it has the highest nutrition, vitamin and positive antioxidant contents among vegetables (Benjapak *et al*., 2008; Singh *et al*., 2011). Tissue culture of *S. androgynus* L. is beneficial in solving the issue of slow growth due to pests. Growth regulators added in tissue culture formulas can stimulate the formation of antioxidants and induce genetic variation in the plant (Cetin, 2014; Ehsanpour *et al*., 2007; Fedoreyev *et al*., 2000; Pumchaosuan & Wongroung, 2008).

UV-C is radiation with a short wavelength (100-280 nm) and a comparatively high associated energy (Katerova *et al*., 2009). It is known that UV-C is acutely germicidal to microorganisms in water, on surfaces, or in air (Siddiqui *et al*., 2011). Moreover, UV-C radiation can induce DNA damage with specific types of mutation: base substitutions of cytosine (C) → thymine (T) at dipyrimidine sites (Ikehata & Ono, 2011), changed DNA patterns as a source of genetic variation of potato callus, and some changes at molecular level by the occurrence in some cases of new DNA bands or the disappearance of DNA bands (Cretescu & Petolescu, 2014; Ehsanpour *et al*., 2007).

Random amplification of polymorphic DNA (RAPD) is a PCR-based technique for identifying genetic variation. It involves the use of a single arbitrary primer in a PCR reaction, resulting in the amplification of many discrete DNA products. In addition, this process has been confirmed as a highly valuable technique (Cretescu & Petolescu, 2014; Ehsanpour *et al*., 2007). According to Kaeppler *et al*. (2000), somaclonal genetic variation results from micropropagated plant cultures. Genetic likeness and differences in plants propagated

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through tissue culture have been discovered through the productive application of RAPD (Carvalho *et al*., 2004; Martins *et al*., 2004; Modgil *et al*., 2005; Ramage *et al*., 2004). In this plant tissue culture process, the rate of somaclonal variation is enhanced by increasing number of subcultures in micropropagation protocols (Gaafar & Saker, 2006; Ehsanpour *et al*., 2007). Moreover, several studies (Biswas *et al*., 2009; Munir *et al*., 2011) have applied RAPD for assessing the genetic fidelity in strawberry and potato cultures.

This is an initial study of possible advantages in using UV-C irradiation on shoot cultures of *S. androgynus* L. with regard to genetic diversity assessed by using RAPD.

2. Materials and Methods

2.1 Plant material and in vitro culture

Shoots of *S. androgynus* L. were used as plant material. The shoots were cultured on MS medium supplemented with 1 mg /l BA for multiple shoots induction. All cultures were maintained in a culture room at 25 ± 3 ºC and 16 h photoperiod for 6 weeks, and shoot cultures of *S. androgynus* L. wild type were collected from the Science and Technology Center, Uttaradit Rajabhat University.

2.2 Exposure to UV-C irradiation

To investigate the effects of UV-C irradiation, a UV -C lamp was used. The UV-C illumination device consisted of an unfiltered germicidal UV lamp (El series UV-C lamp, UVP model UVS-28, Holland) located 29 cm above the radiation vessel. The different UV-C illumination doses (0, 2.03 and 4.06 kJm^{-2}) were obtained by altering the duration of exposure (5 or 10 min treatment groups) at a fixed distance. A non-illuminated sample was considered the control group. After illumination, the shoots were cultured in a room at 25 ± 3°C with a 16 h photoperiod for 3 weeks.

2.3 DNA extraction

Genomic DNA was extracted from four samples of *S. androgynus* L. shoot cultures treated with UV-C irradiation (0, 5, or 10 min) and the wild type, using cetyl trimethyl ammonium bromide (CTAB) method as described by Ehsanpour *et al*. (2007). DNA was quantified by spectrophotometric analysis, and DNA purity of 1.7 was calculated from OD260/OD 280 ratio. The DNA samples were checked by electrophoresis on 1% agarose gel in 0.5x Tris acetate ethylene diamine tetra acetic acid (TAE) buffer.

2.4 RAPD markers

RAPD amplifications were carried out by using nine declaimer RAPD primers from OPE, OPK and OPT series (Eurofin MWG, Germany) (Table 1). Polymerase chain reaction (PCR) mixture of 25 µl consisted of 25 ng/µl genomic DNA template (40 ng/µl determined by spectrophotometry), 25 pmol primer, 12.5 µl 2x PCR master mixture, and 10.5 µl of PCR water (TOYOBO, Japan).

Polymerase chain reaction conditions for the amplification had initial denaturation at 94ºC for 2 min; followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 45ºC for 30 seconds and extension at 68ºC for 1 min; with a final extension for 10 min at 68 ºC in a gradient MyGeneTM series Peltier Themal cycler (LongGene, HonhZhou).

Table 1. Primers used in the RAPD analysis

Sr. No.	Name	Sequence
1	OPE-02	GGTGCGGGAA
2	OPE-06	AAGACCCCTC
3	OPE-08	TCACCACGGT
4	OPE-09	CTTCACCCGA
5	OPE-10	CACCAGGTGA
6	OPE-12	TTATCGCCCC
7	OPE-17	CTACTGCCGT
8	OPE-18	GGACTGCAGA
9	OPE-19	ACGGCGTATG
10	OPK-03	CCAGCTTAGG
11	OPK-07	AGCGAGCAAG
12	OPK-08	GAACACTGGG
13	OPK-09	CCCTACCGAC
14	OPK-15	CTCCTGCCAA
15	OPK-16	GAGCGTCGAA
16	OPK-17	CCCAGCTGTG
17	OPK-19	CACAGGCGGA
18	OPK-20	GTGTCGCGAG
19	OPT-01	GGGCCACTCA
20	OPT-08	AACGGCGACA
21	OPT-12	GGGTGTGTAG
22	OPT-14	AATGCCGCAG
23	OPT-17	CCAACGTCGT
24	OPT-19	GTCCGTATGG
25	OPT-20	GACCAATGCC

Polymerase chain reaction generated amplimers were analyzed on 1.5% agarose gel in 0.5x TAE buffer. The gel was stained with ethidium bromide (0.6 mg/ml) solution and gel documentation was conducted by using Dolphin Doc Plus Gel Imagex System (Wealtec).

2.5 Data analysis

The DNA profiling of RAPD was scored as present (1) or absent (0). The RAPD based estimates of genetic distance, the number of polymorphism bands, percentage of polymorphism bands, and phylogenetic tree analysis by UPGMA method, were computed using POPGENE version 1.32 (Yeh & Yang, 1999)

3. Results and Discussion

In a prior study Petchang (2014) reported that shoot culture of *S. androgynus* L. treated with UV-C irradiation can have elevated antioxidant activities, total phenolics and total flavonoids contents. However, it was found that UV-C irradiation for 10 min damaged the cells and was harmful to the plants.

In other reports Munir *et al*. (2011), Bordallo *et al*. (2004), Ehsanpour *et al*. (2007) and Cretescu & Petolescu (2014) analyzed somaclonal genetic variation by RAPD in potato callus. The authors found that somaclonal variations were induced by different growth regulators and UV-C irradiation. UV-C radiation used on tissue culture induced some changes

at molecular level, seen by the occurrence in some cases of new DNA bands, or by the disappearance of DNA bands in other cases. The reports suggest the usefulness of RAPD fingerprints in assessing plant DNA diversity with high accuracy.

In our results, RAPD primers were applied to evaluate somaclonal genetic variability in four different samples of shoot cultures of *S. androgynus* L., obtained through *in vitro* propagation exposed to UV-C irradiation (0, 5, or 10 min) and wild type. A difference in the intensity and resolution of banding patterns at different annealing temperatures was observed. Nine of the 25 primers (Table 1) produced sharp DNA bands at 45ºC annealing temperature. In total 157 reproducible bands were generated with OPE, OPK, and OPT primers, ranging from 500 bp to 2500 bp in size. The number of bands and percentage of polymorphism generated are summarized in Table 2. 16 polymorphic bands were produced by seven decamer primers (OPE18, OPE19, OPK15, OPK16, OPK17, OPK 19 and OPT20), while two primers (OPT1 and OPT8) gave no polymorphic bands. It was observed that the overall percentage of polymorphism is 10% (Table 3). There was a difference in intensity of polymorphic bands generated by different primers. Differences in band intensity occur because each primer hybridizes to different extents with target DNA, and undefined target DNA may exist in multiple copies per genome. Similar results have already been reported by Skroch and Nienhuis (1995), showing that RAPD bands amplified by one primer vary in intensity from those amplified by another primer.

Figure 1 is a representative RAPD profile analysis, indicating high percentage of the polymorphic bands generated with OPE18, OPK17 and OPT20 primers. RAPD patterns obtained with the primers OPE18, OPK 17 and OPK 20 were used in analyzing the genetic distance of the four groups with SIMQUAL subprogram of NTSYSpc 2.20e software. The distances between the populations of *S. androgynus* L. are illustrated with a dendrogram (Figure 2). Cluster analysis divided the four samples into two groups, an "in" group and an "out" group. The in group comprised *S. androgynus* L. treated with UV-C irradiation for 0 or 10 min and the wild type. The out group consisted of *S. androgynus* L. treated with UV-C irradiation for 5 min, which caused the most genetic difference. The genetic distance correlation coefficients are in the range 0.026-0.975 ($p < 0.01$) (Table 3), which is considered to include relatively high genetic distances.

In a prior study RAPD analysis was conducted on the genetic resources of *Plantago* spp. to assess its genetic variability (Singh, Lal, & Shasany, 2009). It was reported that variation in growth regulator concentrations and their proportion in culture media, culturing time, nutrients (Modgil *et al*., 2005), plant species, explants used, culturing conditions, and UV-C irradiation (Ehsanpour *et al*., 2007; Shuangxia, 2008) can all be factors inducing somaclonal genetic variation in micropropagated plants. Analysis of the somaclonal variations by RAPD has been reported for some other plants as well.

The experiments were repeated three times. Further, 2x Quick Taq HS DyeMix (TOYOBO) was used for amplification reactions instead of PCR mixture made by adding individual components separately, to avoid errors that can affect reproducibility.

4. Conclusions

UV-C irradiation of shoot culture of *S. androgynus* L. induced genetic diversity based on RAPD makers. RAPD analysis was show to be a reliable tool for the assessment of somaclonal variation in shoot culture of *S. androgynus*. L.

Table 2. Number of bands and percentage of polymorphism generated on using OPE, OPK, OPT primers for RAPD analysis.

S.No.	Primer	Total bands	Monomorphic bands	Polymorphic bands	Rare bands	Unique bands	Polymorphism (%)
1	OPE-18	11	8	3	0	0	27
2	OPE-19	22	20	2	0	0	9
3	OPK-15	17	16	1	0	0	6
4	OPK-16	22	19	3	0	0	14
5	OPK-17	13	10	3	0	0	30
6	OPK-19	9	8	1	0	0	11
7	OPT-01	22	28	0	0	0	0
8	OPT-08	16	16	0	0	0	0
9	OPT-20	19	16	3	0	0	16
	Total	157	141	16	0	0	10

Table 3. Genetic distance between populations estimated for cultured *S. androgynus* L. shoots treated with UV-C irradiation with untreated wild type included, analyzed with POPGEN version 1.32

Population	UV-C 0 min	UV-C 5 min	UV-C 10 min	Wild type
UV-C 0 min	****	0.952	0.975	0.975
UV-C 5 min	0.050	****	0.930	0.930
UV-C 10 min	0.026	0.076	****	0.950
Wild type	0.026	0.073	0.052	****

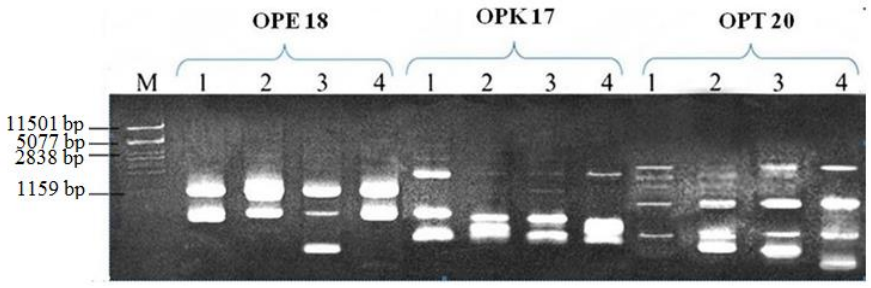

Figure 1. Representative RAPD profile of genomic DNA extracted from tissue culture of *S. androgynus* L. after four alternative UV-C irradiation treatments, observed with OPE18, OPK17 and OPT20 primers, M: marker λ/Pst 1, 1: UV-C irradiation for 0 min (Control group), 2: UV-C irradiation for 5 min, 3: UV-C irradiation for 10 min, 4: wild type

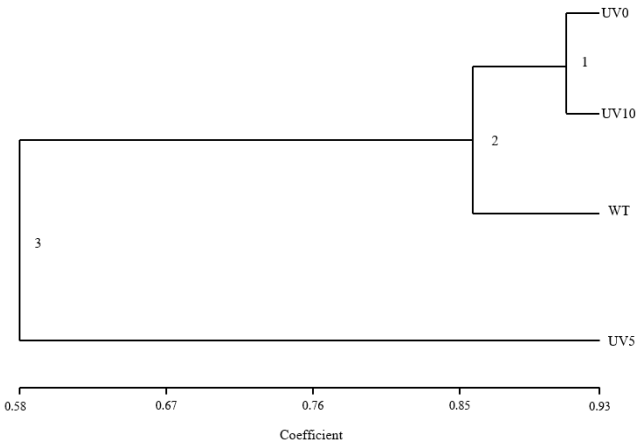

Figure 2. Dendrogram generated by the amplified products of four samples when using the 3 primers OPE18, OPK17 and OPT20, and analyzed with POPGEN version 1.32

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