RAPD Analysis of UV-B-Induced Variations
in Somaclones of *Vernonia cinerea*

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ABSTRACT

Plantlets were regenerated through callus-mediated organogenesis after UV–B irradiation (280–320 nm, 0.863–5.28 kJ m^{-2}) in *Vernonia cinerea* and were analyzed by Random Amplified Polymorphic DNA with 10 random decamers to assess UV–B induced polymorphism. A total of 45 different somaclones were selected based on difference in morphology compared to the parent plant. Out of the 10 primers chosen for initial screening of polymorphism in these 45 somaclones and one parent plant (total 46 genotypes), 8 primers gave amplified polymorphic products. A total of 1347 scorable bands were generated. The value of PIC (polymorphic information content) for each primer ranged from 0.238 to 0.361 with an average of 0.285. Pair-wise genetic similarity coefficients generated from all possible genotypes ranged from 0.3929 to 0.9667. All the calliclones exhibited up to a mean of 77.5% variation from the parent genotype except somaclone 14 that showed 97% similarity. As indicated by the genetic similarity indices, UV-B irradiation is a good way for generation of high frequency morphological and genetic variants during callus mediated shoot regeneration in *V. cinerea* and can be used for selection towards desirable traits, such as salt or drought stress.

INTRODUCTION

Vernonia cinerea L. (family Asteraceae) commonly called as Little Ironweed or Sahdevi is a herbaceous medicinal plant from tropical Asia (Sala 1995). The plant exhibits anti–cancer, anti-helminthic (Dhar *et al*. 1968); anti–diuretic (Adeboye *et al*. 1997); anti-inflammatory (Mazumdar *et al*. 2003); analgesic, antipyretic (Iwalewa *et al*. 2003) and antibacterial activities (Gupta *et al*. 2003); smoking cessation activity (Wongwiwatthananukit *et al*. 2009), toxic activity (Yoga *et al*. 2010). A large number of potent secondary metabolites including sesquiterpene lactones, various triterpenoids and cytotoxic vernolide A-B have been reported from roots and leaves of *V. cinerea* (Misra *et al*. 1984a, 1984b, 1984c, 1993; Jakupovic *et al*. 1986; Kuo *et al*. 2003; Lin 2005; Zhu *et al*. 2009). Besides in our previous studies we have reported that callus and cell suspension cultures of *V. cinerea* can be a potent source of alkaloid production (Maheshwari *et al*. 2007).

Genetic variations are required for improvement of plants towards various adaptive characters. This requires efficient procedures for inducing variations, selection of variants with improved genotypes and techniques for rapid micropropagation of these elite genotypes. Somaclonal variation describes epigenetic and genetic changes in plants that become apparent either during or after *in vitro* culture of plant cells, callus or organs. These are known to yield considerable level of genetic changes in regenerants (Larkin and Scowcroft 1981; Skirvin *et al*. 1993) and have proved valuable resource for genetic improvement of many important plants (Kukreja *et al*. 1991; Dhawan *et al*. 2003). Each cubic cm of callus is believed to be equivalent to a thousand shoot buds (Nabors 1982). Callus cultures undergo genetic changes like polyploidy, aneuploidy, mutations, gene amplification, chromosome breakage, deletions, inversions, translocations etc. that lead to generation of variants. Hence, during tissue culture stages, few changes that might be of interest may be heritable and result from changes in the plastid or nuclear genome. Some of these useful heritable variants can be selected and utilized for development of stable elite genotypes (Karp 1991).

The amount of variations generated *in vitro* are known to vary with the age of clone and use of selection pressures applied to cultures for stress conditions such as salt level, herbicides, microorganisms, specific metabolites and this can be further enhanced through the use of mutagens (Khanuja *et al*. 1998). However it is not possible to predict the type of somaclones valuability generated. These variations appear randomly in cultures and suitable screening procedures are required to identify genotypes with desired characters. The progress made in DNA marker technology has been tremendous and exciting. RAPD has great potential for generating a large number of markers representing a random sample of genome and it has efficiently been used to give reliable and reproducible results for estimating genetic variations (Chen *et al*. 1998; Rasheed *et al*. 2005; Mohamed 2007; Sheidai *et al*. 2008). Although it has some limitations concerning reproducibility and uncertain homology of co migrating fragments in gel electrophoresis (Rieseberg 1996), most of these are minimized by carefully adjusting the reaction and detection conditions (Olmos *et al*. 2002).

Compared to other techniques employed for genetic improvement (recombinant DNA technology), the technique of somaclonal variation is much simpler and results in much genetic variability. Somaclonal variants may be distinguished by biochemical, physiological and genetic characteristics as well as by their morphological traits. However, their significance in improvement of plant varieties depends upon establishing a genetic basis for variation. Hence appropriate methods for creation of genetic variations must aim at the maximum number of desired mutations within a given population. In our previous *in vitro* studies on *V. cinerea* we have established a viable protocol for direct and indirect shoot regeneration methods of the species (Mahesh-

Original Research Paper

wari and Kumar 2006). The present study aim to highlight the mutagenic effects of UV-B radiations on the percent frequency of regeneration of variants generated through callus mediated propagation in *V. cinerea* and its detection using RAPD-PCR. UV radiation has often been used to study various physiologically relevant responses to DNA damages and is divided into UV-C (below 280 nm), UV-B (280-320 nm) and UV-A (320-390 nm). Besides, phenotypic characteristics of the *in vitro* obtained somaclonal variants of *V. cinerea* are also described. This variation can be used as a valuable tool for selection of callus for desirable traits in the future studies.

MATERIALS AND METHODS

Materials

All chemicals, reagents and plant growth regulators were purchased from Sigma-Aldrich, St. Louis, MI, USA and Merck Chemicals, India.

Callus regeneration

Leaf explants of *V. cinerea* collected from the plants grown in the greenhouse of School of Biotechnology, Devi Ahilya University, Indore were sequentially washed with 1% teepol (Sigma-Aldrich) detergent for 2 min and thereafter, under running tap water for 2 h. After that, the explants were treated with 5% (v/v) sodium hypochlorite (Merck Chemicals) for 15 min followed by 1% savlon (chemical name-chlorhexidine gluconate, purchased from Johnson and Johnson Ltd., Mumbai, India) for one min. The explants were surface sterilized by 0.1% aqueous mercuric chloride (Merck Chemicals) for 90 sec in a laminar airflow cabinet. The sterilized explants were thoroughly washed five times with sterile distilled water and inoculated aseptically on MS basal medium (Murashige and Skoog 1962) (Sigma-Aldrich) supplemented with 0.1–10 mg l^{-1} BA (N^6 benzyladenine) (Sigma-Aldrich) alone or in combination with 0.1–2.5 mg l^{-1} NAA (α-naphthalene acetic acid) (Sigma-Aldrich) for callus induction. The pH of the medium was adjusted to 5.80 ± 0.05 with 0.1 N NaOH or 0.1 N HCl before autoclaving at a pressure of 1.06 kg cm^{-2} for 20 min. All experiments were carried out in triplicates with 15 cultures per treatment. Sub-culturing was carried out every four weeks. All cultures were incubated at 25 ± 2°C with a 16-h photoperiod under cool white fluorescent tube lights (25 μmol m^{-2} s^{-1}) at 60–70% relative humidity.

UV-B treatment

Three week old calli regenerated in MS basal medium supplemented with 5 mg l^{-1} BA and 1 mg l^{-1} NAA were cut into pieces and 1 g was sub-cultured in the same medium contained in petri plates. Two sets of five plates each were exposed daily from the day of sub-culture up to a period of 7 days to UV–B light (280–320 nm, 0.863–5.28 kJ m^{-2}) for 15 min and 30 min respectively. UV-B exposure causes erythema and darkening of the skin. Prolonged exposures cause premature skin aging and increase the risk of skin cancer. Hence precaution must be taken while giving UV-B treatment to the cultures by avoiding direct contact of skin and other exposed parts of body to UV-B light. The number of shoot buds induced in the calli subjected to different durations of UV-B irradiation was counted each day. After the irradiation period of 7 days, the calli were transferred to 35 ml of the same medium contained in 100 ml capacity Erlenmeyer flasks and incubated for a period of 3 weeks for further growth. Number of shoots regenerated was counted every week and were observed for any morphological variations.

Selection of morphological variants

The morphological variants generated amongst the callus regenerated somaclones that exhibited difference in leaf shape, size, color or arrangement compared to the parental genotype under influence of UV-B radiations were selected. The selected somaclones were rooted and subjected to hardening and subsequently transferred to pots in greenhouse.

DNA extraction

DNA was extracted from the leaves of both the parent plant and 45 selected somaclones using CTAB buffer as described by Khanuja *et al.* (1999) with some modifications. Three gm fresh leaf tissue was lyophilized in liquid nitrogen and ground to fine powder using a pestle and mortar. The powder was transferred to 15 ml of preheated 2% CTAB extraction buffer in sterile polypropylene tube. The homogenate was incubated at 65°C in a water bath for 45 min, extracted with equal volume of chloroform: iso amyl alcohol (24: 1) and centrifuged at 5000 × g for 15 min. DNA was precipitated from the aqueous phase by mixing with 1/10 volume of 5M NaCl and equal volume of isopropanol. After centrifugation at 5000 × g for 15 min, the DNA pellet obtained was washed with 70% alcohol, air dried and subsequently dissolved in 50 μl TE buffer. After RNase treatment the DNA pellet was resuspended in 50 μl sterile deionised water. DNA quantity was estimated spectrophotometrically (Shimadzu UV-Vis Spectrophotometer UV-3600) by measuring absorbance at 260 nm.

PCR amplification

RAPD was performed using 10 random decamers as described by Williams *et al*. (1990). Sequences of the primers used are given in **Table 1**. PCR amplification of the 45 selected somaclonal DNA along with the parent DNA was carried out in a 25 μl reaction volume in the presence of 1X *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 20 p mole random primer, 20 ng template DNA and 1 unit of Taq DNA polymerase. Amplification was performed in a thermal cycler (Amplitron, USA) programmed for 1 cycle of denaturation at 94ºC for 5 min; 45 cycles of denaturation at 94ºC for 1 min, annealing at 37ºC for 1 min and extension at 72ºC for 1 min; followed by a final extension step of 72ºC for 5 min. The amplification products were resolved by electrophoresis in 1.5% agarose gels prepared in 1X TBE (Tris borate ethylene diamine tetra acetate) buffer using 0.5 μg/ml ethidium bromide as internal dye. A 500 bp DNA ladder was used as molecular size marker. Electrophoresis was carried out in 1X TBE buffer at 5 V/cm for 4 h and visualized using a Gel documentation system (Syngene, UK).

Table 1 Sequence of random primers used for amplification of genomic DNA of the donor and the somaclones.

Primer	Sequence
RAPD primer 10	GTGGGTGCCA
RAPD primer 11	GAGCGTCGCT
RAPD primer 12	TGGTCCAGCC
RAPD primer 13	TGCCGAGCTG
RAPD primer 14	GGTCCCTGAC
RAPD primer 15	GGGTAACGCC
RAPD primer 16	TCTGTGCTGC
RAPD primer 17	CAAACGTCGG
RAPD primer 18	GGACCCAACC
RAPD primer 19	ACCTGAACGG

Statistical analysis

RAPD profiles were scored for presence (1) or absence (0) of bands for each primer-genotype combination. Bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous and were not scored. Data was entered in a binary matrix as discrete variables. Analysis of DNA profiles were done by means of determining similarity index (SI) for each primer using Jaccard's coefficient of similarity (Jaccard 1908) and a dendrogram was prepared using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA) algorithm. UPGMA method is valuable tool for the estimating the extent of genetic diversity and was routinely used in investigations involving genetic diversities (Asante and Offei 2003; Stedje and Ziraba 2003). The computer package NTSYS-pc (Rohlf 1989) was used for cluster analysis. The average SI for all pairwise comparisons (X_D) was calculated and used to estimate the probability of DNA fingerprints of two somaclones being identical by chance, employing the formulae $(X_D)^n$; where X_D = average SI and n = the number
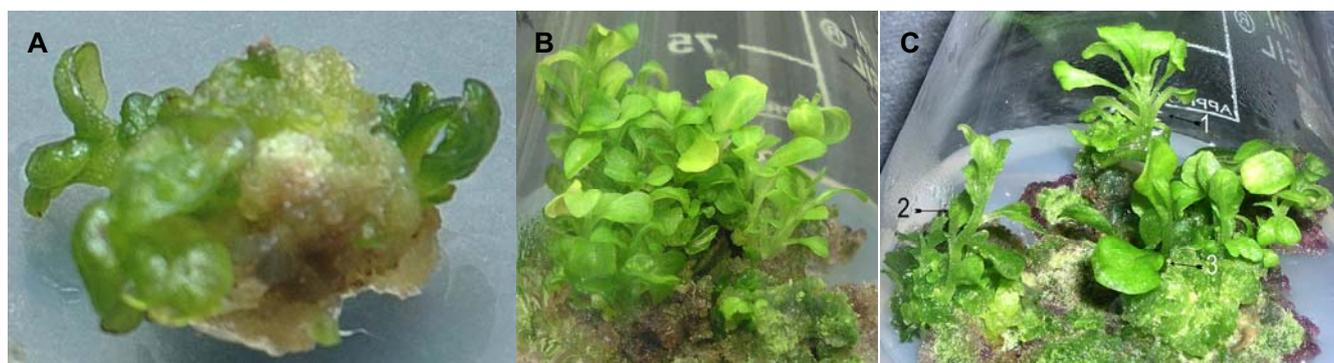
Fig. 1 Regeneration of somaclones through callus mediated organogenesis after UV-B irradiation. (**A**) Shoot bud induction in callus cultures after 7th day of UV-B treatment; (**B**) Elongated shoots after 4 weeks incubation; (**C**) Morphological variants amongst the selected somaclones, 1 – multileaved nodes, 2 – flattened stem, 3 – large sized leaves.

Table 2 Effect of UV-B treatment on number of shoots regenerated per gm of callus in *V. cinerea*.

Duration of UV-B treatment (min)	No. of shoot buds induced							No. of shoots regenerated		
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	2 Weeks	3 Weeks	4 Weeks
15	--	--	2.2 ± 0.45	3 ± 0	5 ± 0.70	7.4 ± 0.55	8.2 ± 0.45	10.4 ± 0.55	12.6 ± 0.89	15.6 ± 0.89
30	--	--	6.4 ± 0.55	8.2 ± 0.45	12 ± 0	13.4 ± 0.55	15.6 ± 0.89	18.2 ± 0.45	25.2 ± 0.45	30.2 ± 0.45

of marker loci as described by Ramakrishna *et al*. (1994). The polymorphic information content (PIC) was calculated by applying the formulae given by Powell *et al*. (1996) and Smith *et al*. (1997).

RESULTS AND DISCUSSION

Somaclonal variations have been reported in different plants and RAPD analysis has been widely used for analysis of culture derived regenerants (Welsh and McClelland 1990; Godwin *et al*. 1997; Linacero and Alves 2000; Ehsanpour *et al*. 2007; Shoja *et al*. 2010). A number of factors control the frequency of this variation, most important being the length of incubation period on callus induction medium and cultivar specificity (Soniya *et al*. 2001). Besides, callus regeneration mediated somaclonal variants may be the result of spontaneous mutations occurring during the tissue culture process, in particular during plant regeneration from callus; or induced by exposure of tissue to mutagens like γ-ray, UV-B, UV-C and X-ray, or chemical mutagens. Many different mutagenic agents have been used for inducing variations in tissue culture regenerated plants. Maliga (1981) reported a 4-fold increase in somaclonal variation by incorporation of mutagens in the culture medium. Gamma radiation has been used to induce variations in many plants including potato and thereafter the regenerated plants have been further characterized using RAPD markers (Wendt *et al*. 2001). In a study on potato by Ehsanpour *et al*. (2007) somaclonal variations were detected in callus exposed to UV-C radiation. In the present study UV-B radiations was hence used as source for inducing mutations during callus mediated plantlet regeneration in *V. cinerea*.

On the basis of maximum biomass and number of shoot regenerated, callus line obtained in MS basal medium supplemented with 5 mg l⁻¹ BA and 1 mg l⁻¹ NAA was selected for mutagenic studies. Since induction of shoot buds in callus started in the 4th week of culture, 3 week old callus cultures were employed for UV-B treatment. Amongst the two different doses of UV-B treatment given to callus cultures (15 and 30 min), exposure time of 30 min was more effective. Shoot buds started to appear on the 3rd day of treatment (**Fig. 1A**) and a mean of 30 shoots were regenerated per gm of callus (**Fig. 1B**) after 3 weeks incubation period in the calli exposed to UV-B light for 30 min for a period of 7 days (**Table 2**).

Of the 10 RAPD primers used, 8 generated amplified polymorphic products. Amplification of the 46 genotypes with these 8 RAPD primers produced distinct polymorphic banding patterns in all the genotypes examined and yielded

a total of 1347 scorable bands. Out of these 46 were monomorphic while the rest 1301 were polymorphic exhibiting 96.56% polymorphism (**Table 3**). The number of scorable bands generated by a single primer per genotype ranged from 1 to 5. The size of the amplified products ranged from 75–1500 bp. The PIC value for each primer ranged from 0.238 to 0.361 with an average of 0.285 (**Table 3**). Maximum number of amplified fragments was observed with RAPD primers 13, 14 and 16 whereas least number with RAPD primers 11 and 15. Portions of gels showing typical amplification products produced by RAPD primer 12 are given in **Fig. 2**.

Analysis of the coefficient of genetic similarity among the different somaclones indicated that all the regenerated plants exhibited a varied degree of genetic difference from the parent plant as well as among themselves. Since, even single base change at the primer annealing site is manifested as appearance or disappearance of RAPD bands, it could be suggested that UV-B radiations have induced varied amount of genetic changes in the different regenerated plants. Some of the molecular changes appeared identical in regenerated plants. The reason for such similarity is that these plants are derived from the same callus and the variations observed in the RAPD pattern may be due to different causes including loss/ gain of a primer annealing, due to point mutations or by the insertion or deletion of sequence or transposable elements (Peschke *et al*. 1991). The difference in intensity of different clones might be due to the accumulation of two or more slightly different bands due to low resolving power of agarose, although RAPD technique can potentially detect single base pair mutations or deletions at the level of primer target and also insertions or deletions within the amplified fragments (Williams *et al*. 1990).

Pair-wise genetic similarity coefficients generated from all possible genotypes ranged from 0.3929 to 0.9667. Lowest genetic similarity coefficient was observed among somaclones 33 and 30; 44 and 8; 44 and 15; 44 and 18; 44 and 30 indicative of maximum variations between the pairs. The highest genetic similarity coefficient 0.9667 was observed between somaclone 39 and 16 indicative of maximum genetic similarity between these clones (97%). Therefore somaclones 39 and 16 are represented as most similar pairs amongst all pair wise combinations between *V. cinerea* parent plant and regenerated somaclones. According to similarity matrix somaclone 1 and 43 were most genetically diverse clones (59% genetic similarity) and represent a true image of the extent of variations induced by UV-B radiations and culture medium during callus mediated shoot re-

Table 3 Somaclonal polymorphism in calliclones regenerated from callus irradiated with UV-B light through RAPD profiling in *V. cinerea*.

Primer	Total no. of bands	% polymorphism	Base pair size of fragment(s)	Individual PIC	Average PIC
RAPD1	138	100	700	0.489	0.361
			600	0.455	
			500	0.139	
RAPD2	92	50	550	0	0.238
			400	0.476	
RAPD3	184	100	1200	0.364	0.312
			700	0.158	
			400	0.363	
			300	0.364	
RAPD4	230	100	600	0.350	0.280
			400	0.288	
			250	0.258	
			150	0.158	
			75	0.350	
RAPD5	230	100	1500	0.226	0.293
			800	0.226	
			700	0.287	
			600	0.440	
			300	0.288	
RAPD6	92	100	600	0.097	0.251
			500	0.405	
RAPD7	230	100	1000	0.385	0.294
			700	0.364	
			500	0.123	
			400	0.364	
			250	0.340	
RAPD8	184	100	1200	0.194	0.249
			800	0.385	
			500	0.258	
			200	0.158	

Fig. 2 Portions of gels showing typical amplification products produced by RAPD primer 12. M- Marker lane; 1-2 – Wild type DNA lane; S1-S45 – Somaclonal DNA lanes.

generation.

Amongst the callus regenerated somaclones after UV-B treatment, many morphological variations were observed compared to the parent plant (**Fig. 1C**). It is reported that negative effects of UV-B radiation results in deformed morphological parameters. Exposure to UV-B decreased plant height, leaf area and plant dry weight, increased auxiliary branching and leaf curling (Barnes *et al.* 1988; Dai *et al.* 1995; Greenberg *et al.* 1997; Furness *et al.* 1999). Dai *et al.* (1995) reported that after a few weeks of UV-B exposure, leaf area and plant dry weight of rice were significantly reduced. High levels of UV-B clearly decreased the relative growth rate and nitrogen productivity, as leaf area ratio, leaf area productivity and leaf nitrogen productivity were all decreased (Zuk-golaszewska *et al.* 2003). In our study the variations that appeared fairly stable upon hardening and field transfer were flattened stem, high leaf plus stem ratio, multi-leaved nodes, abnormal shape of leaves, light green color of leaves, long sized leaves, long plus abnormal shaped leaves, pigmented stem, smaller leaves and small pigmented leaves represented by somaclone number 1-10,

42 and 43. The remaining somaclones possessed less distinct characteristics. Tests for the utility and robustness of the molecular markers generated are based on molecular classification and morphology grouping. In the present study clustering based on RAPD method almost matched that based on morphological variations. The dendrogram (**Fig. 3**) represented 2 major clusters; cluster 1 constituted of 44 genotypes including the parent plant at 59% similarity. Cluster 2 comprised of somaclones 42 and 43 at the 67.5% similarity level. The major clusters were further divided into sub-clusters and sub sub-clusters (**Table 4**). Sub sub-cluster $1A_1$ consisted of somaclone 1 having flattened stem grouped along with 36 and 26 at 83% similarity indicating that these three somaclones are genetically similar. In the same way sub sub-cluster $1A_2$ contained somaclone 2 having abnormal shape of leaves that was grouped along with 6, 16, 39, 8, 17, 33, 18, 23, 24 at 81% similarity having either long sized leaves or long plus abnormal shaped leaves. The sub sub-cluster $1A_3$ contained somaclone 3 having multi-leaved nodes grouped along with 38, 22, 25, 10, 40, 9, 41, 12, 45, 19, 34 at 79.5% similarity all having
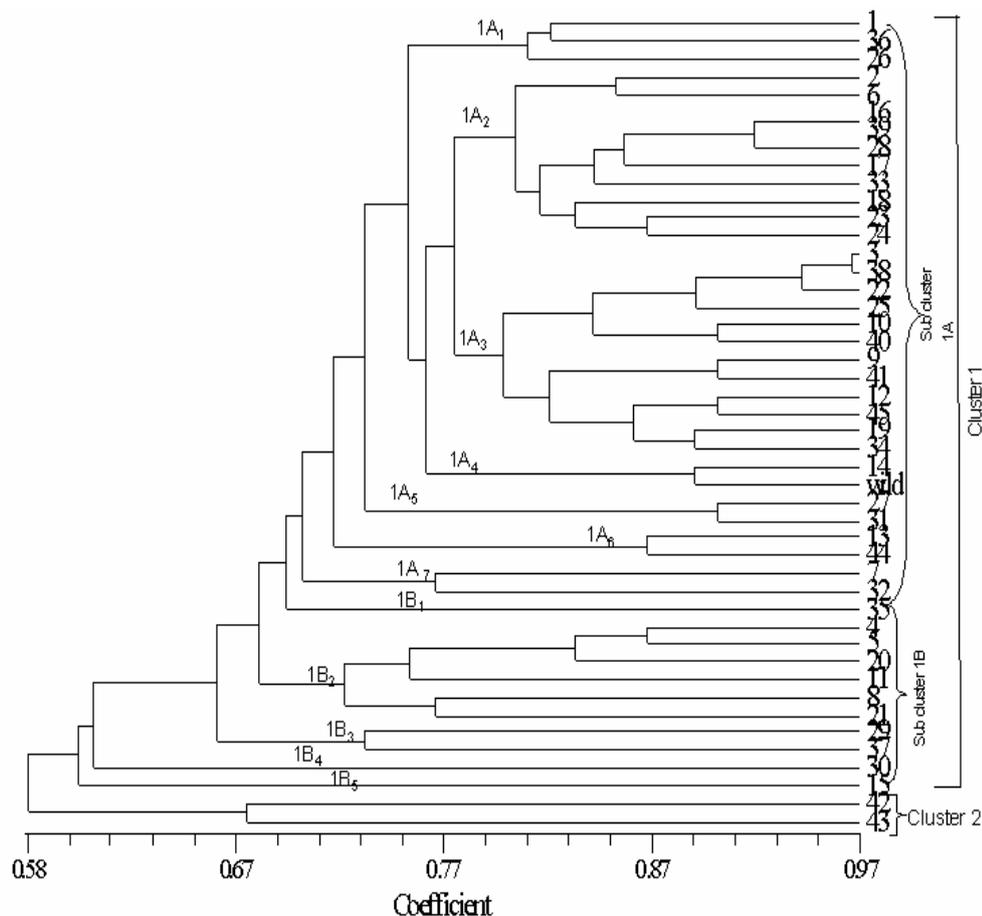
Fig. 3 Dendrogram depicting the clustering of the 45 somaclones and wild genotype based on UPGMA. The major clusters and sub-clusters are marked on the right hand side of the dendrogram whereas sub sub-clusters are depicted on left hand side. Numbers 1-45 designate the 45 different somaclones that were selected based on morphology differences and difference in RAPD profiles.

Table 4 Various clusters and the respective genotypes of somaclones of *V. cinerea*.

Major cluster	Sub cluster	Sub-sub cluster	Somaclones
Cluster 1	Sub cluster 1A	$1A_1$	1, 36, 26
		$1A_2$	2, 6, 2, 6, 16, 39, 8, 17, 33, 18, 23, 24
		$1A_3$	3, 38, 22, 25, 10, 40, 9, 41, 12, 45, 19, 34
		$1A_4$	14, wild
		$1A_5$	27, 31
		$1A_6$	13, 44
		$1A_7$	7, 32
	Sub cluster 1B	$1B_1$	35
		$1B_2$	4, 5, 20, 11, 8, 21
		$1B_3$	29, 37
		$1B_4$	30
		$1B_5$	15
Cluster 2	--	--	42, 43

different number of leaves per node. Somaclone 14 was grouped along with wild genotype indicating almost similar morphology and 89% genetic similarity in sub sub-cluster $1A_4$. Somaclone 7 having light green color of leaves was grouped with 32 in sub sub-cluster $1A_7$ exhibiting 77% genetic similarity. Sub sub-cluster $1B_2$ contained somaclone 4 having smaller leaves, 5 with small pigmented leaves grouped along with 20, 11, 8, 21 with 72% genetic similarity indicating that small leaf size character is common in all these to a large extent. Major cluster 2 contained somaclone 42 and 43 at a similarity value of 67.5% having high leaf plus stem ratio.

As indicated by the SI, up to 97% variation has been induced by the mutagenic effect of UV-B radiations during callus mediated regeneration. All the generated calliclones differentiated from the parent genotype and exhibited 58-97% variation. The study showed that UV-B irradiation serves as an excellent way for inducing a very high fre-

quency variation in calliclones and is useful in selection of variants with elite genotypes. RAPD markers are good to quantify the extent of genetic variations in a given population of somaclones as well as further identification of genotypes.

In our study, additional or missing DNA bands were detected due to UV-B radiation and possibly consequent pyrimidine dimer formation. In conclusion, UV-B radiations can increase somaclonal variation which might be useful for selection of callus for desirable traits, such as salt and drought tolerance or secondary metabolites. Besides, somaclonal variation in the callus cultures can be a promising source for creating new cultivars in plants and for isolating cell lines with high capacity of producing desired secondary compounds.

ACKNOWLEDGEMENTS

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