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ASSESMENT OF THE VARIANTS PRODUCED THROUGH TISSUE CULTURE TECHNIQUES IN *WITHANIA SOMNIFERA* (L.)Dunal BY RAPD PCR METHOD

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ABSTRACT

Withania somnifera (L.)Dunal is regenerated from callus cultures on MS medium supplemented with BAP and 2,4-D.The random amplified polymorphic DNA (RAPD) assay was performed to assess the genetic diversity among *Withania somnifera* (L.)Dunal regenerant and its mother plant. The 12 decamer primers were used in this study. Out of 158 bands obtained 29 were polymorphic. The combination of RAPD data and the simple matching coefficient grouped the species in clusters. The data derived from RAPD marker helped in a comprehensive understanding of the genetic relationship between *Withania somnifera* (L.)Dunal variants.

KEYWORDS: *Withania somnifera*, RAPD, Regenerants, *soma*clonal variation.

INTRODUCTION

Withania somnifera(L.)Dunal is commonly known as ashwagandha and belongs to family Solanaceae. All the parts of the plants have shown remarkable significance in the field of pharmacology as it contains withanolides A and D. Ashwagandha withanolides are anti-inflammatory and enhance the body's defence against infections and tumour (Jaffer et al. 1988, Devi and Sharada, 1992). Its roots are prescribed for hiccups, female disorders, cough,

rheumatism, dropsy, low energy and arthritis (Trivedi et al. 2015). The roots are also used as sedative for senile debility and for the prevention and inhibition of Alzheimer's disease (Kirtikar and Basu, 1975).

Propagation of ashwagandha is done mainly by seed but seed viability is limited to one year.As the viability of seeds is less and because of viral attacks on seedlings, there is overwhelming demand for an alternative to traditional propagation method of cultivations. Tissue culture techniques can play an important role in conservation, clonal propagation and qualitative improvement of this medicinally important plant (Sivanesan 2007).

Tissue culture is meant for the production of true to type plants. Plants grown in an *in vitro* system are exposed to a controlled environment. Since the chemical substance are in the synthetic media are not mutagenic, however due to sort of environmental stress some physiological changes may take place in plantlets raised through tissue culture. Identification of variants through tissue culture is for useful aspects, for example a variant of foliages and flowers in ornamental plants, disease resistant cultivars in crop improvement and phytochemical variation in medicinal plants has been considered as useful variations (Devika and Nareshchandra, 2012).

RAPD (Random Amplified Polymorphic DNA) analysis has been demonstrated to be sensitive in detecting variation among individuals (Sheidai *et al.*, 2010). RAPD markers have been applied to many plant species to evaluate the clonal fidelity and genetic stability of the micropropagated plants. Somaclonal variation may be detected by using molecular marker such as RAPD and AFLP and by cytological studied (Sahijram *et al.* 2003).

MATERIALS AND METHODS

Callus derived from leaf explants inoculated on MS medium fortified with BAP (2.5mg/l) and 2, 4-D (1.0mg/l) showed the initiation of shoot buds in a week of inoculation. After inoculation cultures were transferred into dark chamber of the growth room. On the initiation of shoot buds, these cultures were shifted to the racks to the same growth room provided with light. Plants were allowed to grow up to 10-12cm in the culture vessels (Figure 1). These plantlets were made agar free by washing their delicate roots in sterile water mixed with fungicides and were transferred to the plastic cups. Each cup covered by multiholed plastic bag and was sent to green house for acclimatization. Leaves of four month's old tissue culture (Figure 2) and its mother plants (Figure 3) were used for DNA extraction.

Figure 1: Different stages of tissue culture.

Figure 2: Regenerated plant

Figure 3: Mother plant

DNA extraction

Genomic DNA of both genotypes (mother plant and regeneranted plant) were isolated from new young leaves by following CTAB method. Freshly harvested young and tender leaf samples (1gm) were ground in liquid N_2 using mortar and pestle. Approximately 350 mg of the grounded leaf samples were quickly transferred in to 1.5ml of microcentrifuge tube and equal volume (W/V) (350µl) of hot (65°C) 2X CTAB buffer was added in to the

microcentrifuge tube and mixed thoroughly by vigorous shaking for 2 min. Approximately 700µl of ice cold chloroform: isoamyl alcohol (24:1) was added into the microcentrifuge tube, mixed well by inversion and centrifuged at 12,000 rpm for 5 min. The top aqueous phase was collected using cut tips into a new microcentrifuge tube and 1/5th volume of 5% CTAB solution was added in the microcentrifuge tube and mixed well by gentle inversion. Further, equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed well by inversion and centrifuged at 12,000 rpm for 5 min. The top aqueous phase was collected using cut tips into a new microcentrifuge tube and equal volume of CTAB precipitation buffer was added, mixed and incubated on ice for 5 min. are incubation, microcentrifuge tube was centrifuged at 12,000 rpm for 5 min and supernatant was discarded. Fifty microliters of high salt was added into the microcentrifuge to dissolve the pellet. DNA was precipitated by addition of ice cold ethanol (2.5 volumes of the supernant) and mix gently by inversion. Microcentrifuge tubes were centrifuged at 12,000 rpm for 15 min and supernatant was discarded. DNA pellet was washed with 70% ethanol, air dried and dissolved in 30 µl of TE buffer ($T_{10}E_1$).

Data Analysis

RAPD analysis of both genotypes was conducted by using 12 decamer arbitrary primers obtained from Operon Technologies, California. RAPD amplifi cation was performed in 25 µl volume containing 1X Taq DNA Polymerase buffer, 200 µM dNTPs mixture, 0.5 µM primer, 25ng of template DNA and 1U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a thermal cycler. The reagents were mixed thoroughly and then placed on a Thermalcycler for cyclic amplification and the conditions for amplification was programmed as follows: The thermal profile set comprised a denaturation step of 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 30 s at 34°C, an extension at 72°C for 2 min and a final extension at 72°C for 10 min. Amplification products were then subjected to electrophoresis in 1.2% agarose gel using 1 X TBE and detected by ethidium bromide staining, viewed under UV light and photographed with Gel documentation system. The RAPD markers observed agarose gel electrophoresis were converted into a matrix of binary data, where the presence of the band was scored as 1 and the absence of band was scored as 0. Using NTSYS so ware, a dissimilarity matrix was calculated utilising Jaccard's (1908) coefficient. The matrix was converted to a dissimilarity matrix corresponding to the complement (dissimilarity=1−similarity). Cluster analysis based on the dissimilarity matrix, was performed using un-weighted pair group method arithmetic averages (UPGMA) of the

NTSYS–PC version 2.2 (Rohlf, 2005).

RESULTS AND DISCUSSION

RAPD analysis was performed to discriminate the different variants by using 12 random primers. The amplification profiles of different primers and level of polymorphism observed in different variants were as described in Table 1**.** The primer OPA05 amplified 10 fragment of size ranging from 100 bp to 6.00 kbp. Out of total 10 bands, none of band were observed polymorphic. The primer OPA06 amplified 17 fragments with 23% polymorphism. The primer OPA07 produced 2 polymorphic bands out of total 17 bands obtained in these genotypes studied. The primer OPA10 yielded 14 band but none of polymorphic bands. The primer OPA11 showed 4 polymorphic bands with 33% polymorphism. The primer OPA14 generated 4 polymorphic with 28% polymorphism. The primers OPA17 and OPA19 polymorphism was found to be 37% and 50%, respectively. The primer OPB01 generated total 12 bands of size ranging from 250 bp to 8.00 kbp in all the genotypes studied. The primer OPB06 generated 13 bands (500 bp to 8.00 kbp) in all the genotypes studied. Out of the 13 bands generated by OPB06, 2 bands were found to be polymorphic with 15.38% polymorphism . The primer OPC05 generated 5 bands of size ranging from 250 bp to 4.00 kbp. Out of total 5 bands, 1 bands were found to be polymorphic with 20% polymorphism. The percentage polymorphism was found to be zero in the primer OPC12 while generated total 16 bands of size ranging from 250 bp to 8.00 kbp in all the genotypes studied (Figure 4-5).

Table 1: Sequences and analysis of RAPD markers used for characterization of different variants.

Sl. No.	Primer	Primer Sequence(5'-3)	Total Number Of Band	Number Of Polymorphic Band	Polymorphic Percentage (%)
1.	OPA05	AGGGGTCTTG	10	0	00
2.	OPA06	GGTCCCTGAC	17	4	23
3.	OPA07	GAAACGGGTG	17	2	11
4.	OPA10	GTGATCGCAG	14	0	00
5.	OPA11	CAATCGCCGT	12	4	33
6.	OPA14	TCTGTGCTGG	14	4	28
7.	OPA17	GACCGCTTGT	16	6	37
8.	OPA19	CAAACGTCGG	12	6	50
9.	OPB01	GTTTCGCTCC	12	0	00
10.	OPB06	TGCTCTGCCC	13	2	15
11.	OPC05	GATGACCGCC	05	1	20
12.	OPC12	TGTCATCCCC	16	0	00

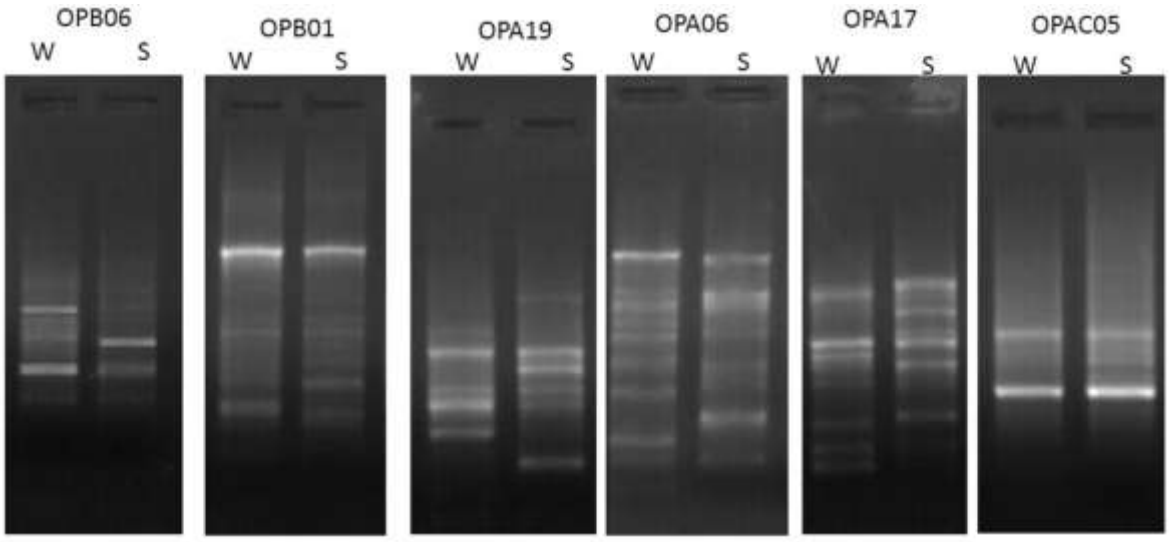

Figure 4: RAPD bands of mother and regenerants plant.

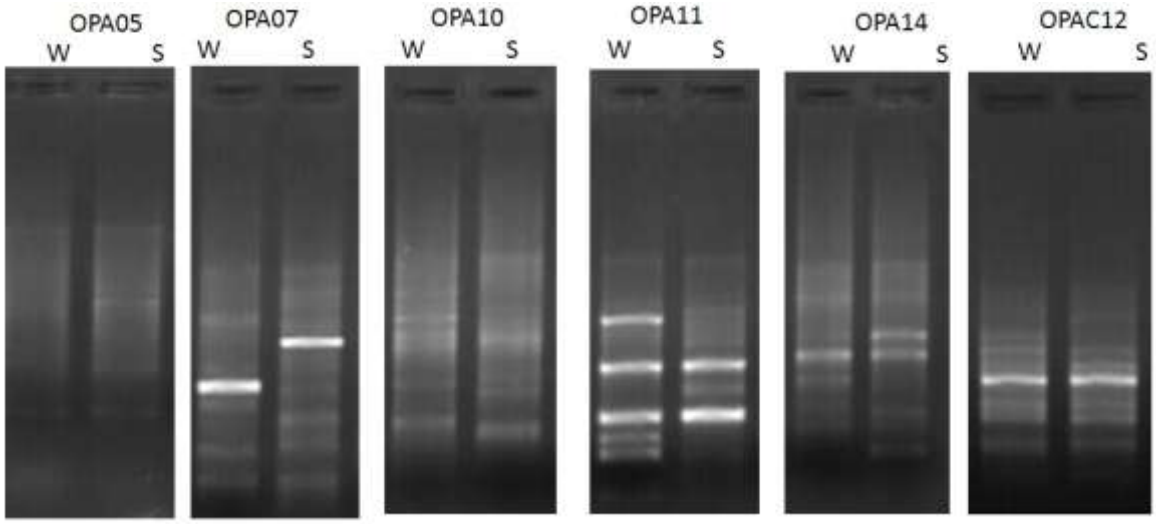

Figure 5: RAPD bands of mother and regenerants plant.

W=Control

S=Tissue culture plant

CONCLUSION

These types of studies will help to analyze the variants for useful traits in terms of their phytochemical contents, drought tolerance and disease resistance.In the present study it was observed that variants can be generated in tissue culture plants. They are often shown in regenerated plants but character was not inherited in future generations. *W.somnifera* (L.)Dunal var regenerated plants DNA variation by primers suggest that the variants that generated in *in vitro* raised plantlets to be analyzed in future generations/ progeny for useful traits.

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