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# ANALYSIS OF THE VARIANTS PRODUCED THROUGH TISSUE CULTURE TECHNIQUES IN *WITHANIA SOMNIFERA* (L) DUNAL. BY DNA FINGER PRINTING EMPLOYING RAPD METHOD

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**ABSTRACT**
*Withania somnifera* (L) Dunal.var.JA20 (Jawahar Asgund 20), JA134 (Jawahar Asgund134), GLV(Gujarat Local Variety) and wild plants were regenerated from callus cultures on MS medium supplemented with BAP and 2,4-D. DNA finger printing by PCR method with randomly amplified primers was carried out for mother plants and regenerated plants. Of 10 different primers from OPBE series, 3 primers namely OPBE 09-GGAAGCGTCC, OPBE 15-CTTTGCGCAC and OPBE 18-GGGAAAAGCC produced polymorphisms. Minor variation was observed between mother plants and regenerants of *W.somnifera* (L) Dunal var.JA20, JA134 and GLV. The regenerated and mother plants of JA20 variety showed minor variation in the first band and third band. Regenerated and mother plants of JA134 variety showed variation between first bands. GLV depicts variation between 3rd, 4th and 5th bands and similarity was observed between 1st and 2nd bands. There was no variation between regenerants and mother plants of wild plants selected for the present investigation.
**Keywords:** *Withania somnifera,* somaclonal variation, RAPD. Regenerants, Mother plants

## INTRODUCTION

Ashwagandha [*Withania somnifera* (L) Dunal.] an important medicinal herb has got immense value in Ayurveda, Siddha and Unani mode of medications. It is traditionally used to treat the symptoms and conditions like, chronic fatigue, bone weakness and tension, loose teeth, thirst, impotency, premature ageing, debility, constipation, senility, rheumatism, nervous exhaustion, memory loss, neurodegenerative disorders and spermatorrhoea[1,2]. Plant is propagated usually by seeds. As the viability of seeds is less and because of viral attacks of seedlings, there is overwhelming demand for an alternative to traditional propagation method of cultivations. Industries adopt tissue culture methods. 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. RAPD has been used widely and is proved an efficient tool for assessing genetic stability in the tissue culture process[3-7]. Our study is an attempt to screen the genetic variability of regeneratated plants of Ashwagandha var.JA20, JA134, Gujarat Local Variety and wild plants and its mother plants by DNA fingerprinting with RAPD method by using 10 decamers of OPBE series. These types of studies will help to analyze the variants for useful traits interns of their phytochemical contents, drought tolerance and disease resistance.

## MATERIALS AND METHODS

Callus derived from leaf explants inoculated on MS medium fortified with BAP (2.5mg/l) and 2, 4-D (1.0 mg/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. 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 and its source plants were used for DNA extraction.

**DNA extraction**

DNA extraction was carried out using modified SDS DNA extraction protocol.2g of fresh leaf tissue frozen quickly in liquid nitrogen and finely powdered with pestle and mortar. The tissue was not allowed to thaw until the extraction buffer was added. 0.25g of PVP was added while grinding and mixed with it. The fine tissue powder was transferred to the centrifuge tube containing preheated 10ml extraction buffer and 40µl mercaptoethanol and incubated at 60° C for 1h with intermittent shaking at 15 minutes intervals. After incubation, equal volumes of chloroform: isoamyl alcohol mixture (24:1v/v) was added and homogenized by gentle inversion. DNA pellet was spooled in to a sterile 1.5ml microcentrifuge tube (Eppendorf). The pellet was washed with 70% ethanol 3 times and dried at room temperature. The pellet was dissolved in TE buffer (pH 8.0) and stored in refrigerator.

**Purification of DNA**

2.5µl of RNase was added to the DNA sample and kept at 37°C for removing RNA from DNA mixture.500 µl chloroform: Isoamyl alcohol (24:1) was added to the same centrifuge tube and mixed well for 1 minute and then

centrifuged at 14500 rpm for 15 minutes at 4°C. Supernatant was transferred to a clean eppendorf tube, 700µl of absolute alcohol and 20µl of 3M potassium acetate was added and kept at 20°C for 1 h. It was centrifuged at 14500 rpm for 20 minutes. Pellet was transferred to a sterile eppendorf tube and washed with 70% alcohol for 3 times. Pellet was dried at room temperature and then dissolved in TE buffer pH7.8 and stored in refrigerator for subsequent use. Quantification of DNA was carried out using Spectronic GENESYS-5 Spectrometer.

**Polymerase Chain Reaction:** Polymerase chain reaction was conducted in Eppendorf PCR Thermal Cycler. 10 decamers of OPBE series (Operon Technologies Inc., Almeda, CA, USA) used for PCR amplification. They are OPBE01-CACTCCTGGT, OPBE02-GCCTGTAGTG, OPBE06-CAGCGGGTCA, OPBE08-GGAAGCGTCC, OPBE09-CCCGCTTTCC, OPBE14– CTTTGCGCAC, OPBE15- TTCGGCGATA, OPBE17GGGAAAAGCC, OPBE18 -CCAAGCCGTC and OPBE19 AGGCCAACAG. Amplification reactions (15 µl final volume) contained 5 ng of genomic DNA, 13 µl of PCR Master mix (Sterile water 10.3 µl, 0.75 µl10-mer primer, PCR buffer1.5 µl, 0.3µl dNTPs and 0.15 µl *Taq* polymerase) added to each eppendorff tube containing 2 µl of genomic DNA. Amplification was performed in a programmable thermocycler (Model- PTC100: MJ research Inc., Waltham MA, USA) with 35 cycles of 95ºC for 45 sec, 37ºC for 1 min, and 72ºC for 2 min with an initial denaturation at 94ºC for 5 min and a final extension at 72ºC for 15 min.

## RESULTS AND DISCUSSION

Polymerase chain reaction (PCR) amplification of discrete loci with single, random sequence,oligonucleotide primers have become popular because of its simplicity and ease of use in modestly equipped laboratory[8,9]. Figure 1 shows the mother plants and Figure 2 shows in vitro generated plants taken for the present study. The RAPD amplification reaction is performed with genomic DNA of regenerated and mother plants of *Withania somnifera* (L)Dunal. With an arbitrary oligonucleotide primer, it results in the amplification of several discrete DNA products. Randomly Amplified Polymorphic DNA (RAPD) based detection of genetic polymorphism was carried out for mother plants as well as plants regenerated from callus of *W.somnifera* (L) Dunal. var.JA20, JA134, Gujarat local variety and wild plants. RAPD based detection of polymorphism[8,9] has found successful application in describing somaclonal variability in regenerated individuals of several plant species[10,11]. In the present study callus was formed from the leaf explants on MS medium supplemented with 2, 4-D used to regenerate the plantlets. Callus was incised aseptically and inoculated on MS medium supplemented with BAP (2.5mg/l and 2, 4-D (1.0mg/l) produced plantlets. These plantlets were examined through RAPD analyses. DNA extracted from fresh leaves of *in vitro* regenerated plantlets and mother plants of *Withania somnifera* (L) Dunal all the varieties and wild plants. Band patterns of *in vitro* raised plantlets and mother plants of Ashwagandha were compared. Plus and minus signs were used for the presence or absence of bands in agarose gel. 10 different primers were tested, of which three primers of OPBE series showed amplification. RAPD was performed on the basis of standard protocol[8]. PCR mastermix was taken in PCR vials and overlaid with a layer of mineral oil (Sigma chemical. Co.). PCR amplification was performed in a automated eppendorf gradient PCR thermal Cycler and programmed for 40 cycles. OPBE09 (CCC GCTT TCC), OPBE15 (TTC GG CG ATA) and OPBE 18 (CC AA GCCGTC) showed polymorphism in mother plants and regenerated plants, of molecular weight 2915,3059 and 2973. Initial five bands were considered for the distinction between mother plants and regenerates. In scoring of bands, intense bands considered as Plus (+) and very faint bands were considered as Minus (-). The first band produced by OPBE09, OPBE15 and OPBE 18 in regenerates, but the third band was absent. In the mother plants first, 2nd and 3rd bands were observed between regenerates and mother plants. Primer OPBE 09, OPBE15 and OPBE18 produced five bands. Var.JA134 first, second, third, fourth and fifth bands were intense. Hence one character (trait) may be recessive in mother plants, which may be dominant in regenerates (Figure 3 and Table 1). First band in *in vitro* regenerated plantlets and mother plants showed minor variation.

Polymorphic bands produced by OPBE 09, OPBE15 and OPBE18 primers in var GLV showed first band as faint in both the mother plants and regenerates. Second, third, fourth and fifth bands were intense in mother plants (Figure 3 and Table 1). The polymorphic bands of regenerants and mother plants showed variation in first, third, fourth and fifth bands. Intense bands observed in regenerants may be due to the appearance of new character (trait). So no allelic variation was produced between regenerants and mother plants of Gujarat local variety. Polymorphic bands were produced by OPBE O9, OPBE15 and OPBE18 primers in *W.somnifera* (L) Dunal. Wild plant showed similar band pattern between regenerants and mother plants. First band was missing in both the mother plants and the regenerants. Second band was intense in both the mother plant and regenerants. Third, fourth and fifth bands were also missing in mother plants and regenerants (Figure 3 and Table 1). Minor variations were noticed between mother plants and regenerants of *W.somnifera* (L) Dunal var.JA20, JA134 and Gujarat local variety.

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 JA20, JA134, GLV and wild plants DNA by 3 primers suggest that the variants that generated in *in vitro* raised plantlets to be analyzed in future generations/ progeny for useful traits.

**Table 1: Scoring of RAPD band in mother plants and regenerants in *W. somnifera* (L)Dunal. var . JA20 and JA134**

| **Primer** | **JA20 R** | | | | | **JA20 M** | | | | | **JA134 R** | | | | | **JA134 M** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| OPBE 09 | − | + | − | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + |
| OPBE 15 | − | + | − | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + |
| OPBE 18 | − | + | − | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + |
| Primer | **GLV 20 R** | | | | | **GLV 20 M** | | | | | **Wild R** | | | | | **Wild M** | | | | |
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| OPBE 09 | − | + | + | + | + | − | + | − | − | − | − | + | − | − | − | − | + | − | − | − |
| OPBE 15 | − | + | + | + | + | − | + | − | − | − | − | + | − | − | − | − | + | − | − | − |
| OPBE 18 | − | + | + | + | + | − | + | − | − | − | − | + | − | − | − | − | + | − | − | − |

JA 20 Mother plant

JA134 Mother plant

GLV Mother plant

Wild Mother plant

**Figure 1: Mother plants of *Withania somnifera* (L.) Dunal var. JA20, JA134, GLV and wild plants**

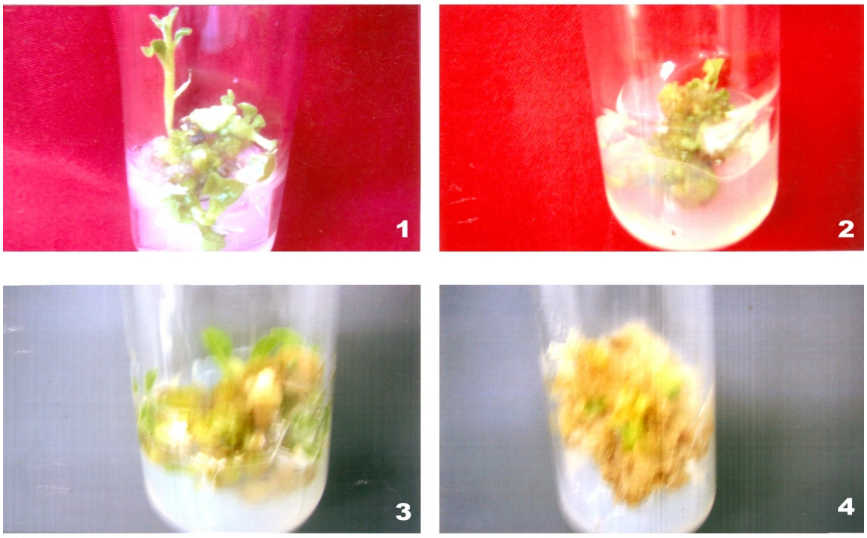


**Figure 2: Initiation of Shoot buds in Callus.**

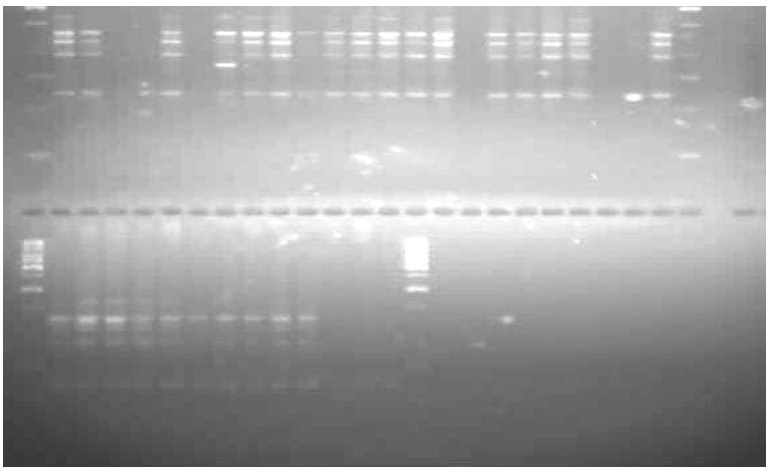

**Figure 3: RAPD bands of Mother and regenerants of JA20, -JA134,-GLV and Wild plant.**

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