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Comparative Analysis of Genetic and Phytochemical Variations in *in vivo* and *in vitro* Cultures of Trachyspermum roxburghianum Using RAPD and GC-MS Techniques

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Abstract: ***The in vivo and in vitro plants of Trachyspermum roxburghianum subjected to GC-MS and RAPD studies revealed variations. The essential oil analysis revealed development of new components in in vitro plant. The RAPD studies showed the presence of new bands as well as absence of some bands in the in vitro plant on comparison with the in vivo plant. The present study clearly brings to light that somaclonal variation is associated with tissue culture of T. roxburghianum.***

Keywords: Apiaceae, RAPD, Coefficient of similitude, GCMS, Phytochemistry, genetic analysis

1. Introduction

Trachyspermum roxburghianum, medicinal plant of the family Apiaceae has remained obscure apart from its diverse medicinal uses. The plant is used in skin problems, chronic discharges. The oil from the seeds and herbage is used for flavouring purposes, treating cholera, stomach pain, dyspepsia and diarrhoea. The seeds are laxative, appetizer, anthelmintic, aphrodisiac, abortifacient, treat scabies, scorpion stings etc. The plant cures asthma in Yunani system of medicine. Random Amplified Polymorphic DNA studies (RAPD) in the *in vitro* and *in vivo* conditions are not reported so far. The Gas chromatography Mass Spectrometry analysis of the plant has been done by some workers, but the comparison of essential oil components of the *in vivo* and *in vitro* was again not attempted earlier. Hence the present study attempts to compare the variations developed by tissue culture at the genetic level and in the essential oil composition in contrast with the plant developing in the *in vivo* conditions.

2. Materials & Methods

The *in vivo* and *in vitro* (developed by tissue culture) plants were subjected to RAPD and GC-MS analysis. For RAPD studies, the DNA was extracted using Cetyl trimethyl ammonium bromide "CTAB" method of Doyle *et al. [*2] with minor modifications of Ausubel [1]. DNA was quantified and estimated spectrometrically.15 µl of isolated DNA is diluted in 3 ml of water and its optical density at 260nm wavelength was noted. The quantity of DNA is calculated using the formula:

Quantity of DNA (in micrograms) =A_{260} x quantity of DNA that corresponds to Optical Density one, where A_{260} is the optical density of the given sample at 260 nm wavelength.

AP-PCR combines PCR and primers of arbitrary sequences to amplify genomic DNA and produce a fingerprint. For the present reaction, 27 different oligonucleotide primers of OPA series and seven random primers of OPB, OPC and OPD series, Operon technologies Inc., Almeda, USA was used. The PCR was performed in 25 µl reaction mixture containing 3 µl of genomic DNA, 3.75 µl of the four DNTPs, 2 µl of each primer, 0.33 µl units of Taq polymerase, 2.5 µl of 10x reaction buffer (10mM 3-tris (hydroxymethyl) aminopropanesulphonic acid (pH8), 1.5 µl $MgCl_2$, 50mM KCl) and double distilled water (11.92 µl). The reaction mixtures were overlaid with 15 µl of mineral oil. Amplification was performed in an omnigene thermal cycler (MJ Research PTC 100) under programmed cycling conditions as follows: 1 cycle of 5 minutes at 94^0C, 33 cycles of 1 minutes at 94^0 C, 1 minute at 40^0 C and 1 minute at 72^0C, 1 cycle at 72^0 C for 15 minutes and at 15^0 C for 1 hour.10 µl of each reaction product was subjected to electrophoresis (99V for 2 hours) in 1.5% agarose gel in 1 x TBE (89 mM Trisborate, 10 mM EDTA pH8) and the DNA bands were stained by 6 µl of ethidium bromide. The gels were then photographed on an UV transilluminator.100 bp ladder (Genei, Bangalore), EcoR I + Hind III double digest of the λ phage DNA (New England, Biolab) were used as molecular weight standard.

For GC-MS studies, the fresh aerial plant parts of *T. roxburghianum* (DC.) Craib (both *in vivo* and *in vitro*) were collected at the time of flowering. Flaked and homogenized plant materials were hydro-distilled separately in a Clevenger apparatus for 4 hours at 100^0 C. The essential oil was quantified, dried over anhydrous sodium sulphate and stored in small amber coloured bottles at 4-6^0C. GC-MS of essential oil was carried out on a Shimadzu QP-5050 instrument at 700 V at 250^0C. GC Column: ULBON Hr-1, fused silica capillary 0.25 mm x 50 m with film thickness of 0.25 µ; carrier gas Helium; Flow rate 1.5 ml/min., Temperature programme: Initial temperature was 80^0 C for 1 minute and then heated at the rate of 5^0 C per minute to 250^0 C. Mass spectral identification was based on the associated computerized data. The data obtained from the qualitative

analysis on both *in vivo* and *in vitro* developed plants were subjected to numerical analysis to understand the chemical affinity of both by arriving at a numerical constant, the coefficient of similitude (CS), using the formula proposed by Sokall & Sneath [7].

CS = Number of similar components x 100
Total number of components

3. Results

DNA was isolated from the parent plant and the *in vitro* plants. The two tissue cultured plants were designated as TC1 and TC2.3 µl of the template DNA was used for the preparation of reaction mixture and analysed using 27 primers of arbitrary sequences. Of the 27 primers used, ten successfully amplified the extracted DNA with consistent reproducible bands. The number of bands resolved per primer ranged from 2-12. The size of amplification products differed and ranged from approx.100 bp to 900 bp in the 1000bp ladder and approx.300 bp to 3000 bp in the 4000bp ladder. The RAPD finger print of the variant (TC1) differed from the parent plant with seven primers (OPA 02, OPA 06, OPA 09, OPA 11, OPC 03, OPC 11 & OPD 01). A few bands were found to be missing in the variant when these primers were used. Additional bands in the variant were also detected by this marker screening. Precisely, four bands for the primer OPA 02 (Fig. I) as well as OPA 11 (Fig. III) and three bands for OPA 09 (Fig. II) were absent in the variant (TC1) in comparison with the parent. TC1 also showed one additional band for OPA 09, five additional bands for OPA 11, OPA 06 (Fig. IV), than the parent. With OPC 03, one additional band was detected in the variant (TC1), which was absent in the parent as well as TC2. Using OPC 11, one additional band was observed in the TC1 and TC2, which again was not detected in the parent. The parent showed one additional band for OPC 03, two for OPC11 (Fig. V), and two for OPD 01 when compared to the variant TC1 and TC2. The reproducibility of these genomic DNA bands was consistent in successive repetitions. Since the amplification of TC1 was showing more difference than that of TC2, the former was considered as variant and used for GC-MS analysis.

The essential oils of *in vitro* and *in vivo* plants were analysed both quantitatively and qualitatively. The oil yield in the*in vitro* plant developed (1.2%) was slightly greater than the normal plant (0.9%). The essential oils were analysed by the GC-MS and the components detected were monoterpenes, sesquiterpenes, furan derivative, aryl alkyl aldehyde, alkanol, long chain acid with 16 carbon atoms, bicycloalkene, monoterpene alcohol and an epoxy compound. The GC-MS pattern of the *in vitro* essential oil (Fig. VII) was distinctly different when compared to that of parent plant (Fig. VI). The major component of *in vitro* plant was isothujol (16.42%) whereas that of *in vivo* plant was epiglobulol (19.55%). The *in vivo* plant is characterised by the presence of β-myrcene, limonene, $_{\gamma}$-terpinene, α-cubebene, α-caryophyllene, eudesma-4 [14] 11-diene (10.94%), aromadendrene, δ-cadinene, epiglobulol (19.55%), caryophyllene oxide, aromadendrene oxide, isobenzofuranone, β-methyl benzene propanol (15.74%), tetradecanol and n-hexadecanoic acid which were found to be absent in the *in vitro* plant. The essential oil of the *in vitro* plant possess ocimene, α-farnasene, 1, R, 3Z-9S-4, 11, 11-trimethyl 8-methylene bicyclo [7.2.0] undec-3-ene, isothujol (16.42%), β-farnasene, α-β-epoxycumene (14.72%), α-bergamotene which were not detected in the *in vivo* plant. The common components were thujene, δ-3-carene, β-terpineol, germacrene-D, cedrene, β-caryophyllene, α-neoclovene, α-bisabolene oxide and patchoulane and one unidentified compound. The total number of chemical components detected by GC-MS in both *in vivo* and *in vitro* grown plants were 32. However, the number of similar components were 10. Coefficient of similitude between *in vivo*and *in vitro* plants was found to be 31.2724.

4. Discussion

Of the 27 primers used in the present study, ten primers showed successful amplification. Failure of primers or probes tested in the investigation to detect variation may indicate that either the gene or altered gene responsible for the abnormality has no homology with the primers or probes/ the abnormality in the clones may be caused by an epigenetic phenomenon i. e., a change in the expression of the gene (due to hypo or hypermethylation) according to Philips *et al.* [5], but not the content of the gene. Polymorphism in the amplified bands were observed in the present study. Polymorphism in the amplification products represent changes in the sequence of primer binding site or change which alter the size or prevent the successful amplification of a target DNA. DNA amplification products, which represent one allele per locus could result from changes in either the sequence of the primer binding site or changes which alter the size and prevent the successful amplification of target DNA according to Rout *et al. [*6]. Presence of RAPD markers at a specific locus in both genotypes indicate a high level of homology at that site. The sequence difference between two genotypes is expressed as the absence of the marker and thus as band differences in the RAPD fingerprint as explained by Williams *et al.* [8]. According to Patnaik *et al. [*4] culture stress may induce variations in tissue cultured plants which are sometimes associated with useful agronomic characters such as oil yield, oil content etc. The lesser value of coefficient of similitude (31.2724) obtained on comparing of the essential oils of parent and the variant, shows the dissimilar nature of essential oil composition of these oils. The dissimilarity may be probably due to variation in the biosynthetic pathways of the essential oils, which are genetically controlled. In both samples, the major component was different. This indicates that the genetic changes due to culture stress of hormones used, affected the biosynthetic pathway of major components. the absence of some constituents may be due to some hindrance in the biosynthesis of these components. The appearance of certain new compounds in the essential oil of variant may be due to the triggering of certain diverged biosynthetic pathways. The marked difference in the essential oil composition may be due to the fact that biosynthesis of volatile aromatic chemicals is genetically controlled as explained by Heffendehl& Murray [3].

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Illustrations Captions:

Fig. I RAPD fingerprint of OPA 02 primer
Fig. II RAPD fingerprint of OPA 09 primer
Fig. III RAPD fingerprint of OPA 11 primer
Fig. IV RAPD fingerprint of OPA 06, OPB15, OPB 18 and OPC 03 primers (from left to right)
Fig. V RAPD fingerprint of OPC 10, OPC 11, OPD 07 and OPD 01 primers (from Left to right)
Fig. VI Gas chromatogram of *in vivo* plant
Fig. VII Gas Chromatogram of *in vitro* plant

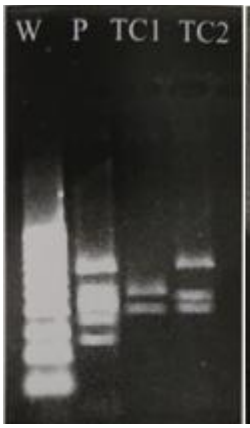

Figure I

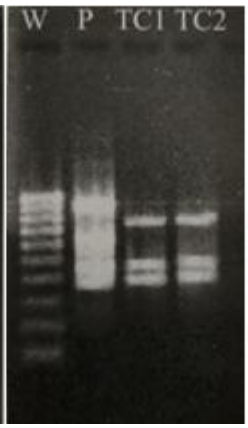

Figure II

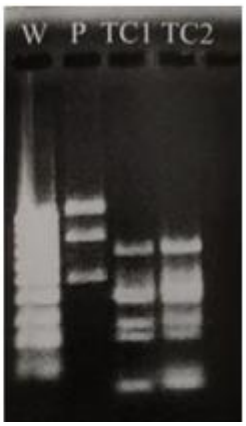

Figure III

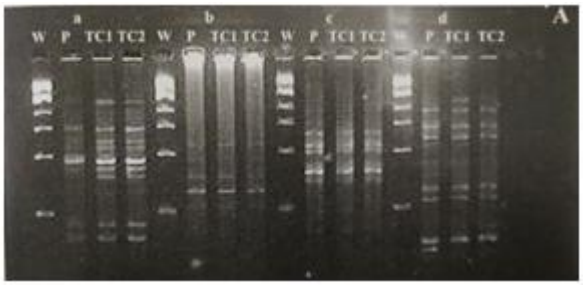

Figure IV

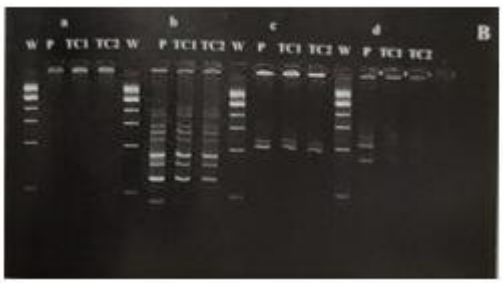

Figure V

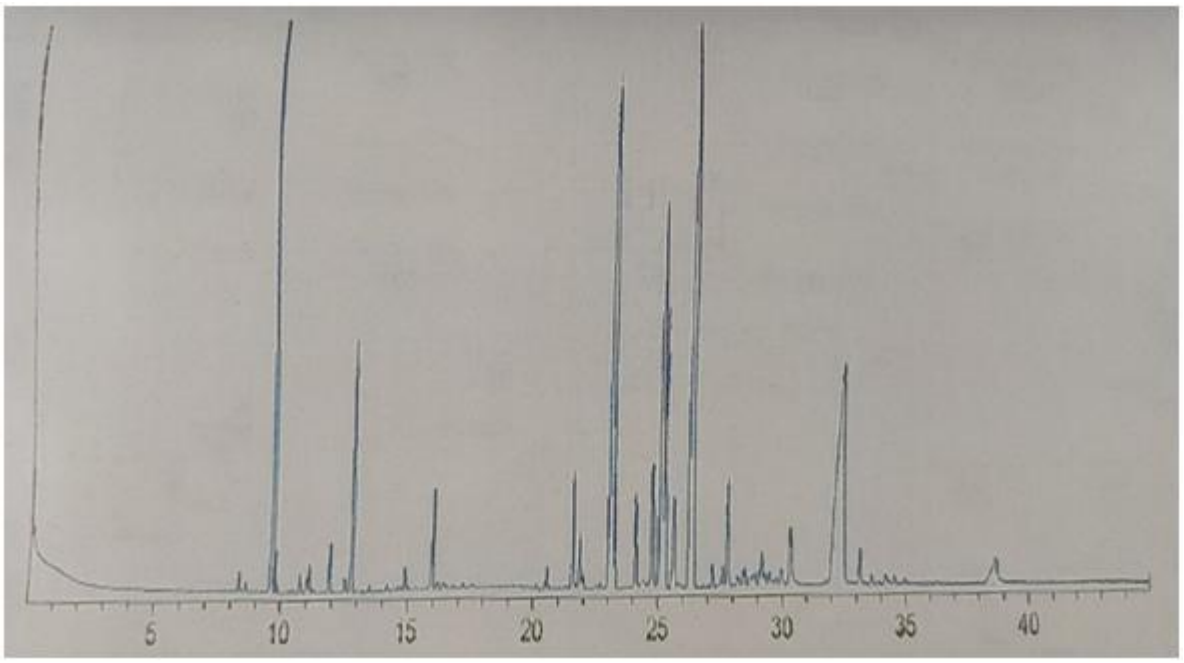

Figure VI

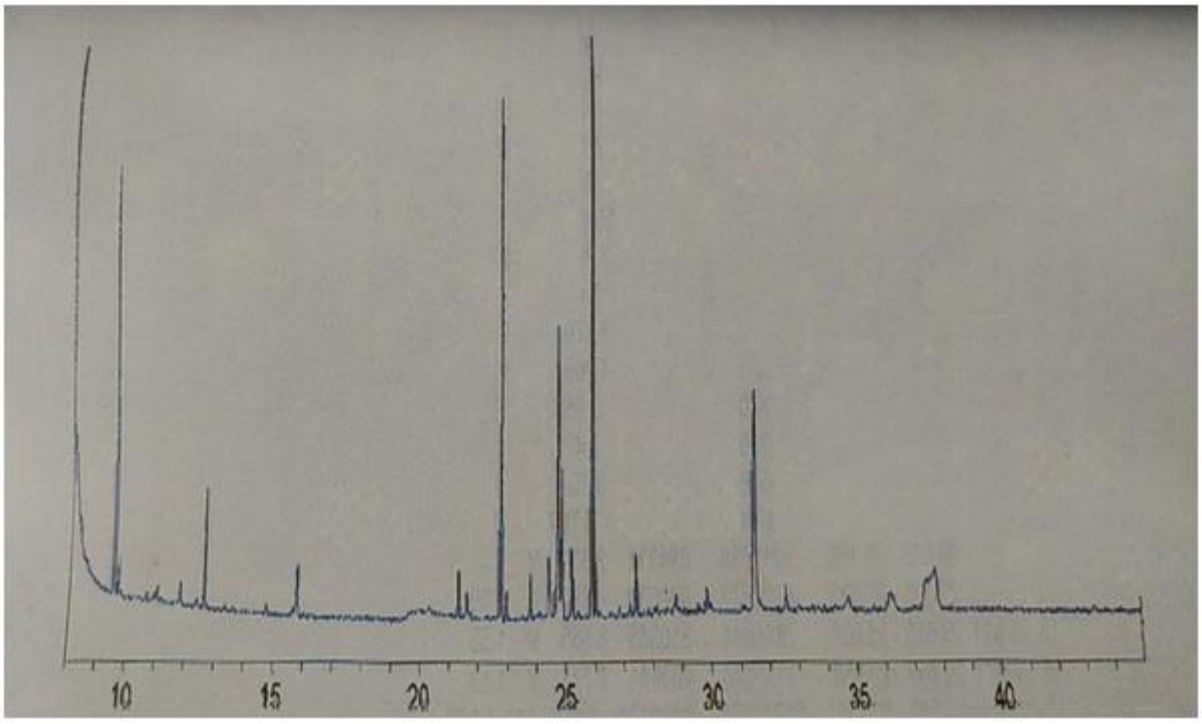

Figure VII