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# Characterization of Podophyllotoxin Yielding Cell Lines of *Podophyllum hexandrum*

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**Abstract —** Callus cultures of *Podophyllum hexandrum* were initiated and established from juvenile explants isolated from axenic seedlings and mature explants isolated from 1 year old plants on B5 basal medium (at half strength) and modified MS medium supplemented with growth regulators. For initiating cell lines, cell suspension cultures were established with selected callus lines in B5 basal medium supplemented with growth regulators and cell lines were established by cell plating technique. Eight cell lines (RC1, RC2, RC3, RC4, RC5, RC6, RC7 and ZE) maintained as callus stocks in the laboratory have been characterized and compared on the basis of growth rate, morphology, podophyllotoxin content, ploidy, karyotype analysis and RAPD analysis. Morphologically the cell lines were of six different types like globular and dark brown, friable with rough surface and light brown, compact and yellow, compact and creamish, compact and dark brown in colour and creamish and highly friable in nature. The cell lines had variable growth rates with line RC1 being the fastest growing line. The lines also differed in podophyllotoxin accumulating capacity; line RC4 accumulated the highest amount of podophyllotoxin in 4 years of analysis followed by line RC2. Chromosome analysis from 2 and 4 year old cultures revealed the lines displayed the presence of diploid, hypodiploid and hyperdiploid cells in varying frequencies. An assessment of karyomorphology of the cell lines (except cell line ZE) indicated that the chromosomes in general were long (16.0 to 5.33µm) with median to subterminal or terminal region primary constrictions. Cell lines RC3, RC6 and RC7 showed the presence of chromosomes with secondary constrictions. The observed polymorphic banding pattern of the cell lines obtained with eleven RAPD markers reflected the genomic variability present in the cell lines. Primers OPA 01, OPA 02, OPA 04, OPA 12 and OPX 01 generated unique bands in some of the cell lines viz. RC1, RC2 and RC6, indicating that cell lines of *P. hexandrum* can be characterized and identified on basis of RAPD markers.

**Key words:** cell lines, lignan, podophyllotoxin, *Podophyllum hexandrum,* RAPD.

## INTRODUCTION

*Podophyllum hexandrum* Royle (Indian Podophyllum; Berberidaceae) is a commercial source of the highly valued lignan podophyllotoxin, which is used as a precursor for the synthesis of important antitumour drugs like etoposide (VP-16-213) and teniposide (VM-26), effective in the treatment of lung cancer, testicular cancer, a variety of leukemias and other solid tumours (Imbert 1998). The worldwide demand for podophyllotoxin is steadily increasing. But the occurrence of the plant is limited due to its long juvenile phase and poor fruit setting ability (Choudhary *et al.* 1998). Owing to its immense clinical importance new routes for total synthesis of podophyllotoxin have been discovered (Bush and Jones 1995; Berkowitz *et al.* 2000). But these are low yielding processes and not economically feasible. Thus isolation from plant source continues to be the only viable option (Canel *et al.* 2000). Due to reckless harvest from the wild there has been a steady decline in its natural population and the species has been declared as 'critically endangered' as per IUCN criteria (Airi *et al.* 1997; Samant *et al.* 1998). Thus biotechnological production of podophyllotoxin using plant cell cultures of *P. hexandrum* appears to be an attractive alternative.

There are very few reports on successful tissue culture of *P. hexandrum* which is a recalcitrant species *in vitro*. There is a single report on somat-

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ic embryogenesis in tissue culture of *P. hexandrum* (ARUMUGAM and BHOJWANI 1990). NADEEM *et al.* (2000) reported *in vitro* propagation of this species via multiple shoot formation and subsequent rooting. Callus cultures of this species were first established by VAN UDEN *et al.* (1989). Since then several studies have been carried out for improvement of podophyllotoxin accumulation in callus and cell cultures of *P. hexandrum* with limited success (CHATTOPADHYAY *et al.* 2002a; 2003c).

Cell cultures are dynamic entities from which groups of cells having desirable characteristics can be selected. Selection of cell lines with suitable genetic, biochemical and physiological characteristics is a necessary prerequisite for sustainable production of economically important phytochemicals through plant cell cultures and subsequent scale up of cultures to the industrial level. Enhancement of secondary metabolite accumulation in plant cell cultures based on selection of cell lines with desirable characteristics is well documented (EILERT *et al.* 1987; SMITH *et al.* 1987; JHA and JHA 1995; JHA *et al.* 1998; MUKHERJEE *et al.* 2000a; GHOSH *et al.* 2002b; FÜRDEN *et al.* 2005). RAPD markers have been used to detect DNA polymorphism in callus tissues as well as to identify different callus tissues and cell lines in some plant species (MANGOLIN *et al.* 2002; LAURA *et al.* 2003). We have successfully initiated and maintained cell cultures of *P. hexandrum* for over 6 years in our laboratory (MAJUMDER 2008) which have been used in the present study. The podophyllotoxin yielding cell lines have been characterized and compared on the basis of growth rate, morphology, podophyllotoxin content, ploidy, karyotype analysis and RAPD analysis in the present study. Such a study is a prerequisite for identification of high podophyllotoxin containing cell lines in this medicinally important plant species.

## MATERIALS AND METHODS

*Initiation and establishment of cell lines of P. hexandrum* - Callus cultures of *P. hexandrum* were established from juvenile explants (isolated from axenic seedlings) and mature explants (isolated from 1 year old plants) on B5 basal medium (GAMBORG *et al.* 1968) at half strength (half strength of respective macronutrients, micronutrients, vitamins, myoinositol, iron–EDTA; ½ B5 basal medium) and modified MS medium (MURASHIGE and SKOOG 1962) supplemented with growth regulators (MAJUMDER 2008). Cell suspension cultures were established with selected callus lines in B5 basal medium supplemented with growth regulators. Establishment and selection of cell lines were done by cell plating technique as reported earlier for *Coleus forskohlii* (MUKHERJEE *et al.* 2000a). With an aim of obtaining friable and fast growing cell lines with high podophyllotoxin synthesizing capacity selected cell lines were transferred onto different types of media *viz.* various basal media, modified media, variation of salts, sucrose concentration, supplemented with different growth regulators. Cultures were maintained in dark at 24±1 ºC with relative humidity of 55-60% and subcultured at 3 weeks interval. Eight cell lines (*viz.* RC1, RC2, RC3, RC4, RC5, RC6, RC7 and ZE) have been maintained for over 6 years as callus stocks on six different types of maintenance media (MS basal, modified MS and ½ B5 basal media supplemented with growth regulators) (MAJUMDER 2008).

*Determination of growth* - To study growth of each cell line, samples were harvested at regular intervals, dried (48 hours, 45 ºC) and weighed. Growth index (GI) was expressed as the ratio of final and initial dry weights (DW) of the callus masses.

*Qualitative and quantitative analysis of podophyllotoxin content of the cell lines* - Podophyllotoxin was extracted from eight cell lines for 4 years following the method of HEYENGA *et al.* (1990) with modification and analysed by HPLC using standard samples of podophyllotoxin (Sigma). Pieces of calli from each cell line were dried (48 hours, 45 ºC), weighed and powdered in a mortar and pestle. Powdered samples (~0.5g DW) were heated with dehydrated ethanol on water bath for 3 hours after which the ethanol phase was evaporated to dryness. The residue was redissolved in 1.0 ml analytical grade methanol and filtered (Sartotius filters, 0.22µm) prior to analysis. The HPLC system consisted of a Shimadzu Liquid Chromatograph (LC-10AD), a Shimadzu UV-VIS detector (SPD-10A), a Supelco hypersil ODS column (150 x 0.46 mm) and a Supelco guard column (Pelliguard™) LC-18 kit with 2cm x 4.6mm cartridge. Isocratic elution was performed with acetonitrile/methanol/water (35:5:60, by volume) as the mobile phase, at a flow rate of 1ml $min^{-1}$. Podophyllotoxin was detected at 260 nm. Determination of podophyllotoxin was done by comparison with a calibration graph. The relationship was found to be linear over ten measurements at different concentrations. For analysis at least 500mg of the cultured material was used and the method was quantitative and reproducible. Identification of podophyllotoxin was done by retention time

and spiking with standard. Podophyllotoxin was identified by preparative HPLC and identified using $^1$H-NMR by comparing with literature values (JACKSON and DEWICK 1984a).

*Chromosome analysis* - Chromosome analysis of each cell line (from 2 and 4 year old cultures) was done following previously published methods (SIDDIQUE *et al*. 1990; ARUMUGAM and BHOJWANI 1994) with modification. To examine the cytological status of the cell lines, samples were pretreated in saturated solution of *para*-dichlorobenzene (10 ºC, 3½ hours), fixed in 1:3 acetic acid:ethanol (overnight), stained in 2% aceto-orcein and (N) HCl mixture (9:1) for one hour, squashed with 45% acetic acid and observed under a light microscope. The mitotic metaphase plates were photographed to prepare karyotypes from 1500X magnification, following the nomenclature of LEVAN *et al*. (1964). At least 75 metaphase plates were scored for each cell line.

*RAPD analysis* - DNA was isolated from 4 year old cell lines by cetyl trimethyl ammonium bromide (CTAB) based method developed originally by DOYLE and DOYLE (1987) with modification.

PCR was performed using 28 random decamer primers (Operon Kits A, Kits X; Operon Technologies Inc, Alameda, CA, USA). Reaction mixtures (25µl) contained 10X Taq DNA polymerase buffer [100 mM Tris (pH 9.0), 500 mM KCl, 0.1% gelatin], 2.5mM $MgCl_2$, 0.4mM dNTP, 25 picomole single random primer, 1.5U Taq DNA polymerase (Bangalore Genei, India) and 50ng template DNA. PCR amplification was performed in an automated thermal cycler (DNA Engine, MJ Research, PTC 200, Peltier Thermal Cycler). PCR conditions were: initial denaturation for 1 minute at 94 ºC, 45 cycles of denaturation for 1 minute at 94 ºC, annealing at 34 ºC for 1 minute and an extension of 72 ºC for 2 minutes followed by a final extension at 72 ºC for 7 minutes. Amplification products were resolved by electrophoresis in 1.2% (w/v) agarose gels in 0.5X TBE buffer, stained with ethidium bromide solution for visualization and documented on gel doc (Vilber Lourmat; DP-001.FDC). PCR amplifications were repeated at least thrice for RAPD profiling and only reproducible, well-marked amplified fragments were counted. The molecular sizes of the amplification products were estimated using a DNA ladder (GeneRuler™ 100 bp Plus, Fermentas).

*Statistical analysis* - Data were analysed by analysis of variance (ANOVA) to detect significant differences between means (SOKAL and ROHLF 1987). Means differing significantly were compared using the DUNCAN (1955) multiple range test (DMRT) at the 5% probability level. Variability around the mean was represented as ± the standard error. RAPD data analysis was done using Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) software package (version 2.1).

## RESULTS

*Morphology and growth rate of eight cell lines* - Morphologically the cell lines were of six different types. Cell lines RC2, RC3 and RC7 were globular, dark brown in colour; line RC4 was friable with rough surface and light brown in colour; line ZE was compact and yellow while line RC1 was compact and creamish in colour. Line RC5 was compact and dark brown in colour and RC6 was highly friable and creamish (Fig. 1).

The cell lines showed variable growth rates. Growth rate in each cell line was high following initiation; however, growth rate of all cell lines decreased attaining a steady growth rate after 2 years of culture. Comparison of the growth rates of the eight 2 year old cell lines over a period of 35 days revealed that cell line RC1 had the highest growth rate while cell line RC4 was the slowest growing line (Fig. 2). All cell lines have maintained their growth rate on maintenance medium for last 5 years.

*Podophyllotoxin content of eight cell lines* - Analysis of eight cell lines after 2 years of initiation showed that podophyllotoxin content varied considerably ($P \leq 0.05$) between cell lines (0.15% - 0.45% on DW basis) (Fig. 3). There was a decrease in content in all the lines till fourth year of initiation (0.03% - 0.2% on DW basis) after which the cell lines showed stability in podophyllotoxin content. Among the eight cell lines, line RC4 accumulated the highest amount of podophyllotoxin followed by line RC2.

*Cytological analysis of the cell lines* - Eight cell lines were analysed cytologically after 2 and 4 years (Table 1, Fig. 4). In addition to the normal diploid cells (2n=12) in varying frequencies hyperdiploid cells (cells with more than 12 chromosomes) were predominant in different cell lines like RC1, RC3, RC4, RC5, RC6, ZE. Although there was no change in the cytological status of lines RC1, RC5, RC6 and ZE which remained predominantly hyperdiploid even after 4 years, in lines RC3 and RC4 there was an increase in the frequency of hypodiploid cells (cells with fewer than 12 chromosomes). Interestingly, 2 year old cell lines RC2 and RC7 showed presence of hy-

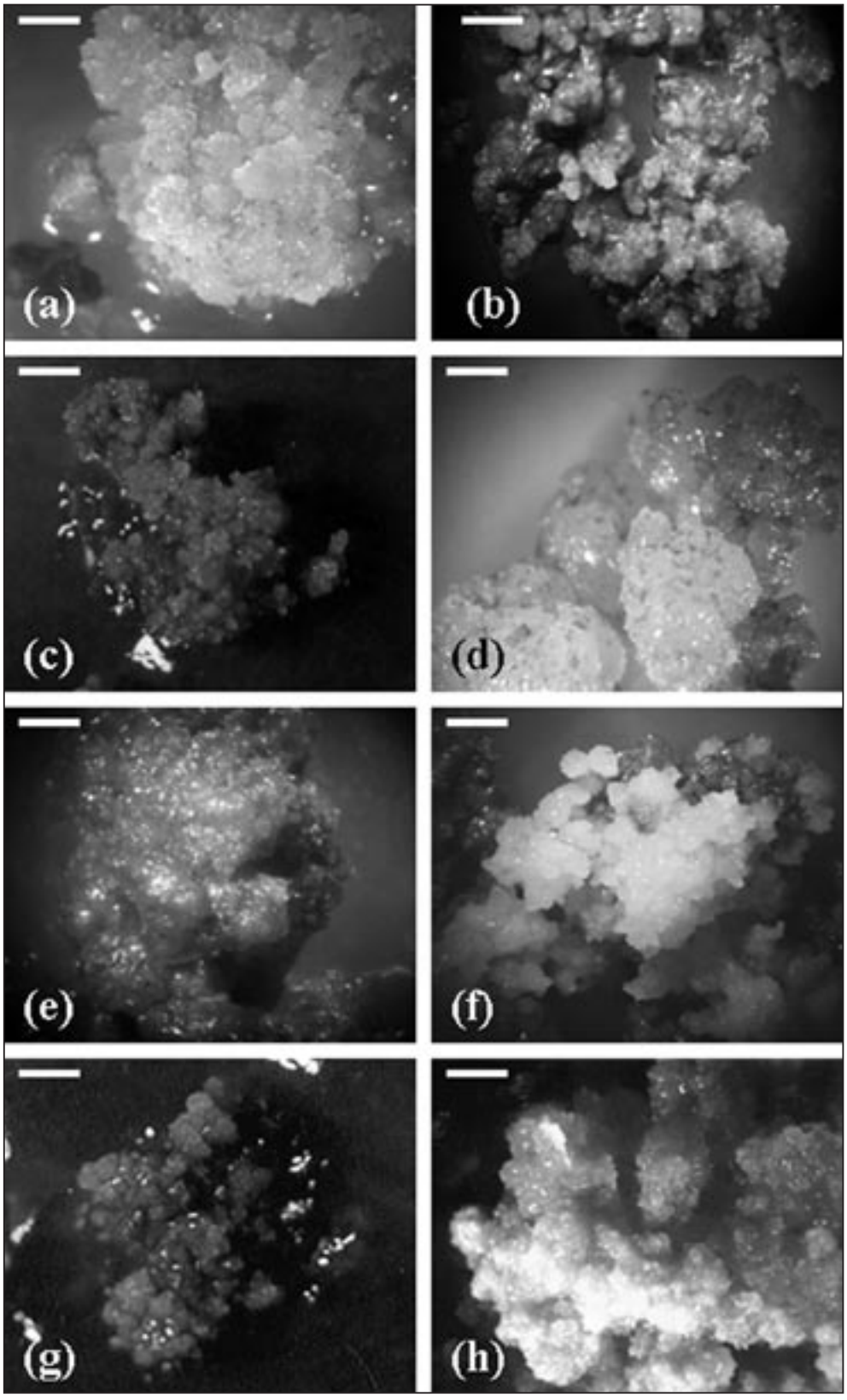


Fig. 1 — (a-h) - Six morphologically different types of cell lines. (a) Compact, creamish cell line RC1 (*Bar: 1.5mm*). (b,c) Globular, dark brown cell lines RC2, RC3 (*Bar: 1.5mm*). (d) Friable, light brown cell line RC4 (*Bar: 1.5mm*). (e) Compact, dark brown cell line RC5 (*Bar: 1.5mm*). (f) Highly friable, creamish cell line RC6 (*Bar: 1.5mm*). (g) Globular, dark brown cell line RC7 (*Bar: 1.5mm*). (h) Compact, yellow cell line ZE (*Bar: 1.5mm*).

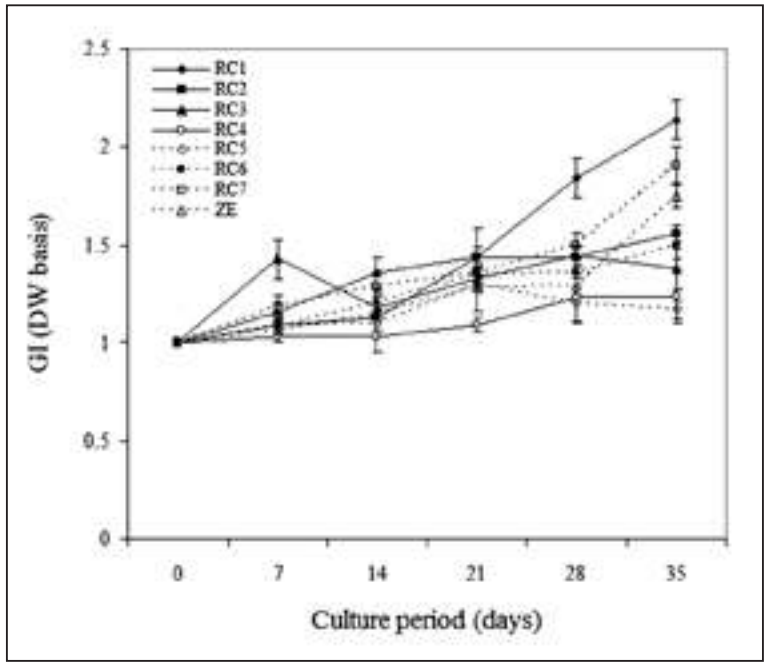


Fig. 2 — Comparative growth rates of eight 2 year old cell lines cultured in maintenance medium over a period of 35 days. All values are means ± standard error of three independent experiments (n=30).

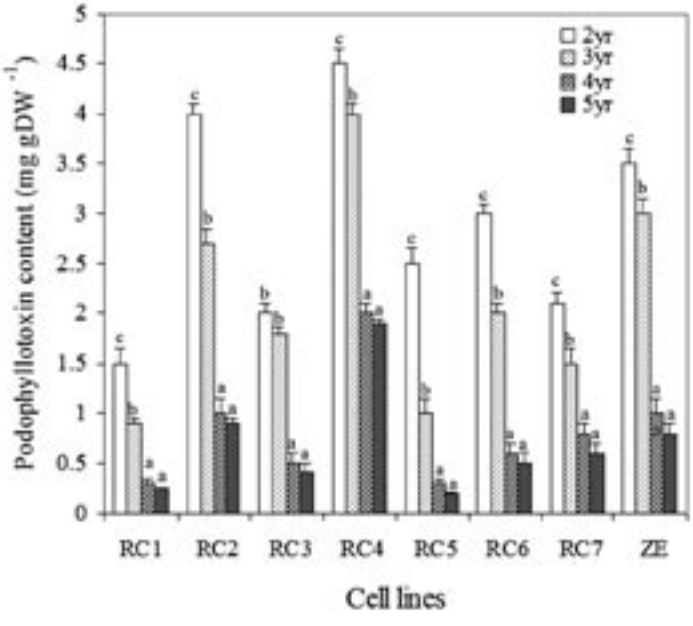


Fig. 3 — Podophyllotoxin content of eight cell lines. All values are means ± standard error of three independent experiments (n=30). Bars with different letters are significantly different from each other at $P \leq 0.05$ according to ANOVA and DMRT for each cell line.

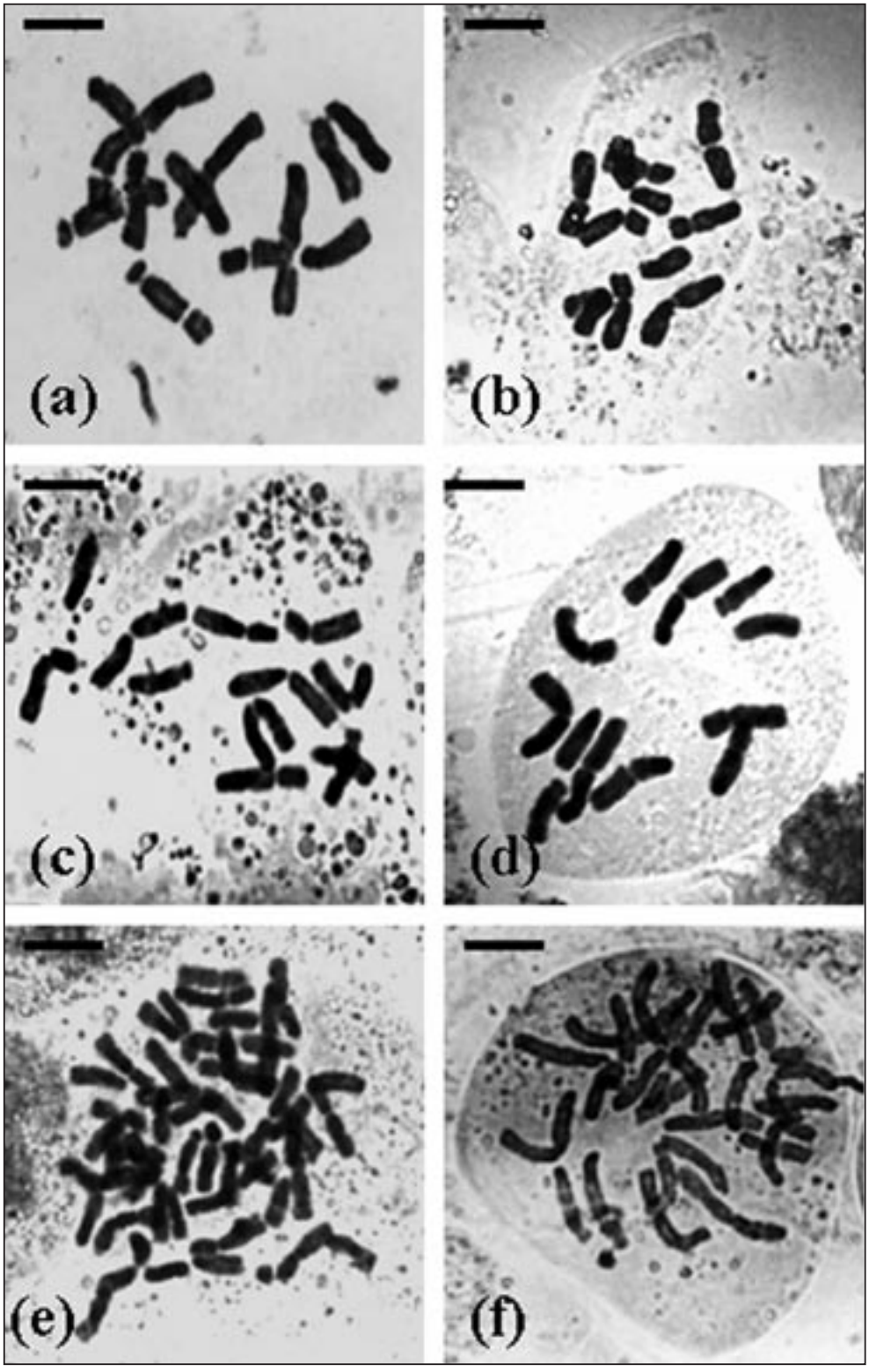


Fig. 4 — (a-f) - Metaphase plates from 2 and 4 year old cell lines. (a) Hypodiploid plate showing 9 chromosomes from 2 year old cell line RC4 (*Bar: 5µm*). (b) Hypodiploid plate showing 10 chromosomes from 4 year old cell line RC4 (*Bar: 5µm*). (c-d) Diploid plates showing 12 chromosomes from 2 year old cell lines (c) RC3 (d) RC4 (*Bar: 5µm*). (e-f) Hyperdiploid plates from 2 year old cell lines (e) RC2 (f) RC4 ((*Bar: 5µm*).

TABLE 1 — Cytological status of eight cell lines (2 and 4 year old cultures)[a].

| Cell lines | Age (years) | Frequency (%) of different ploidy levels | | |
|---|---|---|---|---|
| | | Hypodiploid (< 2n) | Diploid (2n) | Hyperdiploid (> 2n) |
| RC1 | 2 | 3 | 2 | 95 |
| | 4 | 5 | 3 | 92 |
| RC2 | 2 | 65 | 12 | 23 |
| | 4 | 30 | 17 | 53 |
| RC3 | 2 | 10 | 10 | 80 |
| | 4 | 57 | 30 | 13 |
| RC4 | 2 | 29 | 10 | 61 |
| | 4 | 47 | 17 | 36 |
| RC5 | 2 | 37 | 25 | 38 |
| | 4 | 33 | 27 | 40 |
| RC6 | 2 | 35 | 3 | 62 |
| | 4 | 32 | 4 | 64 |
| RC7 | 2 | 53 | 13 | 34 |
| | 4 | 26 | 8 | 66 |
| ZE | 2 | 3 | 3 | 94 |
| | 4 | 2 | 1 | 97 |

[a] *P. hexandrum*: diploid with 2n=12
Data based on an average of 75 metaphase plates per cell line.

podiploid cells in high frequency, which were rapidly eliminated in 4 year old cultures with simultaneous increase in the frequency of hyperdiploid cells. Chromosome number counts of hypodiploid cells revealed that cells with 9 and 10 chromosomes were of most frequent occurrence in the different cell lines (40-55%). Chromosome numbers in other hypodiploid cells ranged from 5 to 11. With regard to hyperdiploids, in a few cases the exact chromosome number could not be resolved. To avoid any error in presentation, all such cells with high chromosome numbers have been classed under hyperdiploids.

*Structural variations in chromosomes* - An assessment of the karyomorphology of the cell lines also revealed structural chromosomal variations among the cell lines. Studies on chromosome complement (from actively dividing root tip cells obtained from germinating seeds) by SIDDIQUE *et al*. (1990) revealed that the 12 chromosomes could be grouped into six duplets on the basis of overall length, centromeric position and details of NOR (nucleolar organizing region). The haploid set comprised of one metacentric, three sub-metacentric and two acrocentric chromosomes, with the metacentric chromosomes being the longest and the acrocentric ones being the smallest in the complement; four chromosomes had secondary constrictions. The normal karyotype of *P. hexandrum* (from root tip squashes of germinated zygotic embryos) as reported by ARUMUGAM and BHOJWANI (1994), also comprised of six homologous pairs with two median, six sub-median and four sub-telocentric chromosomes including two pairs with secondary constrictions. Since the present study involved analysis of chromosomes from callus cultures only, an accurate description of karyotype of all hyperdiploid or hypodiploid cells was not possible. Karyotype analysis of diploid cells of each cell line except ZE has been presented in Table 2. Chromosomes in general were long (16.0 to 5.33µm) as reported earlier by SIDDIQUE *et al*. (1990) with median to sub-terminal or terminal region primary constrictions (Fig. 5). In addition to median, sub-median (in all lines analysed) and sub-terminal chromosomes (in lines RC1, RC2, RC4, RC5 and RC6) as reported earlier, the cell lines analysed also showed the presence of chromosomes with constrictions in the median region (lines RC1, RC2, RC3, RC4 and RC6) and terminal region (lines RC1, RC2, RC4, RC6 and RC7). Thus cell lines RC1, RC2, RC4 and RC6 were similar in having median, submedian, subterminal chromosomes alongwith chromosomes with constrictions in the median and terminal regions. In contrast to earlier reports, cell line RC3 lacked any sub-terminal chromosome or chromosome with terminal region constriction. In conformity with previous results by SIDDIQUE


Fig. 5 — (a-g) - Karyotypes of different cell lines (a) RC1 (b) RC2 (c) RC3 (d) RC4 (e) RC5 (f) RC6 (g) RC7. Chromosomes with secondary constrictions are underlined.

*et al.* (1990), in all cell lines excluding line RC6, median chromosomes were the longest in the complement wherese in line RC6 chromosomes with constrictions in the median region were the longest. Also, in cell lines RC1, RC2, RC4, RC5 and RC6 sub-terminal chromosomes were the smallest in the complement as reported earlier (SIDDIQUE *et al.* 1990). Cell lines RC1, RC2 and RC5 had two unpaired chromosomes each. In line RC1, one unpaired chromosome was extremely unusual with two constrictions, both sub-median, at opposite ends of a very small

TABLE 2 — Karyotype of diploid metaphase plates (2n=12) from 2 year old cell lines.

| Cell line | Karyotype |
|---|---|
| *P. hexandrum* root tip [a] | 1M*+1M+1sm*+5sm+2st+2st* |
| RC1 | 4M+1m+4sm+1st+2t |
| RC2 | 2M+2m+4sm+3st+1t |
| RC3 | 2M+2m+6sm+2sm* |
| RC4 | 2M+2m+4sm+2st+2t |
| RC5 | 4M+5sm+3st |
| RC6 | 2M+2m*+4sm+2st+2t |
| RC7 | 4M+4sm+2sm*+2t |

[a] SIDDIQUE *et al.* (1990)
M, median chromosome; m, median region chromosome; sm, sub-median region chromosome; st, sub-terminal region chromosome; t, terminal region chromosome (according to LEVAN *et al.* 1964). Chromosomes with secondary constrictions are marked with an asterisk.

middle arm. Such an unusual chromosome has not been reported earlier in *P. hexandrum*. The other unpaired chromosome was sub-terminal. In line RC2, one unpaired chromosome was sub-terminal and the other one with constriction in the terminal region, whereas in line RC5, one unpaired chromosome was sub-median and the other one sub-terminal in nature. Among all the cell lines studied, lines RC3, RC6 and RC7 showed the presence of a pair of chromosomes each with secondary constrictions. In lines RC3 and RC7, the secondary constrictions were in the long arms of sub-median chromosomes, while in line RC6, the short arms of chromosomes with median region primary constrictions had the secondary constrictions (Fig. 5). Previous reports by SIDDIQUE *et al.* (1990) indicated that out of four chromosomes possessing secondary constrictions, two were meta-/sub-metacentrics with secondary constrictions in the short arms whereas the remaining two were acrocentrics with secondary constrictions in the long arms.

*RAPD analysis* - Isolation of genomic DNA from callus tissues was difficult. After several tedious trials with different DNA extraction protocols (DELLAPORTA *et al.* 1983; BOUSQUET *et al.* 1990; MANGOLIN *et al.* 2002) DNA was finally extracted from fresh callus tissues (4 years old) by cetyl trimethyl ammonium bromide (CTAB) based method. The yield of DNA in the eight cell lines ranged from 800-2900µg $ml^{-1}$ for 1g callus tissue with $A_{260}/A_{280}$ ratio varying from 1.79-1.92, indicating that the DNA samples were relatively pure.

Initially 28 primers were screened for amplification using 8 DNA samples. Out of 28 primers 17 primers failed to produce amplification

products or gave weak fragments. The remaining 11 primers produced clear intense bands. These were chosen for variability analysis based on the quantity of their amplified fragments and their polymorphism. The amplified products using 11 primers namely OPA 01, OPA 02, OPA 03, OPA 04, OPA 09, OPA 10, OPA 12, OPA 16, OPX 01, OPX 02 and OPX 04 were separated in agarose gels (Fig. 6). The data generated using these primers were analysed for similarity or variability studies. 11 primers gave rise to 445 products with an average of 40.5 products out of which 52 bands were scored for polymorphic analysis in total with an average of 7.1 bands per primer, the minimum being 4 bands for the primers OPA 03 and OPA 09 and the maximum being 12 bands for the primer OPX 02 (Table 3). An average level of 64.7% polymorphism (25% for OPA 09 to 90% for OPX 01) was observed. The approximate size of the largest fragment was 2.5kb (OPA 02 and OPX 04) whereas the smallest recognizable fragment was 0.3kb in size (OPA 04).

Primer OPX 01 produced three bands of lengths 1000bp, 950bp and 900bp in the lines RC2, RC3, RC4, RC5, RC6, RC7 and ZE; but these three bands were totally absent in line RC1, which instead showed the presence of a unique band of 850bp. Primer OPX 02 generated two bands (1500bp and 1000bp) in the seven lines RC2, RC3, RC4, RC5, RC6, RC7 and ZE which could not be detected in line RC1 which has been a consistently low yielding line in 2-5 years of analysis. Primer OPA 04 was able to generate two amplification products (1350bp and 300bp) which were present in lines RC2 and RC1 respectively. Primer OPA 02 generated one amplification product (2500bp) that was unique to line RC2. Primer OPA 01 produced 2 fragments (2000bp and 1550bp) which were unique to line RC2 whereas primer OPA 12 produced one unique product (900bp) in line RC6.

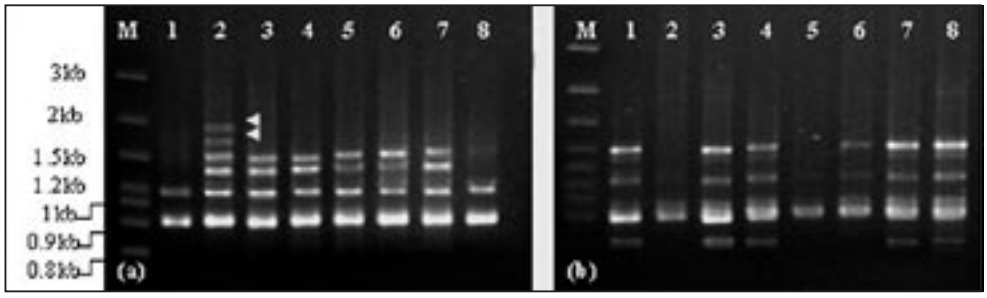


Fig. 6 — (a-b) - RAPD profiles of 8 different cell lines generated by primers (a) OPA 01 (b) OPA 16. Lanes 1-8: cell lines RC1-ZE. Lane M: molecular marker (GeneRuler™ 100 bp Plus, Fermentas). Positions of unique bands are indicated by *arrows*.

TABLE 3 — List of primers producing polymorphic bands in the cell lines.

| Primer | Sequence 5´→3´ | No. of bands produced | No. of polymorphic bands |
|---|---|---|---|
| OPA 01 | CAGGCCCTTC | 6 | 4 |
| OPA 02 | TGCCGAGCTG | 7 | 4 |
| OPA 03 | AGTCAGCCAC | 4 | 3 |
| OPA 04 | AATCGGGCTG | 8 | 7 |
| OPA 09 | GGGTAACGCC | 4 | 1 |
| OPA 10 | GTGATCGCAG | 5 | 3 |
| OPA 12 | TCGGCGATAG | 6 | 3 |
| OPA 16 | AGCCAGCGAA | 6 | 5 |
| OPX 01 | CTGGGCACGA | 10 | 9 |
| OPX 02 | TTCCGCCACC | 12 | 8 |
| OPX 04 | CCGCTACCGA | 10 | 5 |

To assess genetic similarity or distance in eight cell lines tested, similarity matrix was calculated based on Jaccard's Similarity Coefficient. A dendogram (Fig. 7) was generated following cluster analysis with pairwise distance between the cell lines under study using NTSYSpc software with UPGMA algorithm. The genetic distance ranged from 0.6 to 1.85. Among the lines, line RC3 was closely related to line RC7 (least genetic distance of 0.6) and maximum distance (1.85) between line RC1 and RC3, RC7 revealed that they have least genetic similarity. Three distinct clusters could be identified. Lines RC3 and RC7 were clustered with RC5, RC4, ZE and RC6 forming the first cluster. Line RC2 was distantly related with this cluster with a distance coefficient of 1.48 and line RC1 formed the third cluster with a distance coefficient of 1.85 with the rest.

As the purpose was to characterize cell lines on the basis of limited number of primers used in the present study, the cell lines can be identified on the basis of unique bands produced by primers as follows (Table 4).

## DISCUSSION

*Correlation between morphology, growth and podophyllotoxin content of the cell lines* - A great deal of heterogeneity was noted in the cell lines of *P. hexandrum* in terms of morphology, growth rate and podophyllotoxin content. Selection and maintenance of fast growing cell lines capable of accumulating high levels of podophyllotoxin would not only solve the limitation in the supply of this potent antineoplastic lignan presently needed for clinical use, but will also help conserve the large number of *P. hexandrum* plants that need to be harvested in order to isolate the compound. Also, the availability of fast growing cell lines is a key factor in scale up operations. In the present study, the eight cell lines differed substantially in their biosynthetic capacities. Similar variation in secondary metabolite synthesizing capacity has been noted among cell lines of *Catharanthus roseus, Taxus canadensis*, *T. cuspidata, T. wallichiana*, *Coleus forskohlii* (HALL and YEOMAN 1987; JHA and JHA 1995; KETCHUM and GIBSON 1996; MUKHERJEE *et al*. 2000a; GHOSH *et al*. 2002b). In our study, cell lines which showed high productivity following their initiation were characterized by gradual de-

TABLE 4 — Primers producing unique bands in the cell lines

| Cell line | Primer number | Size of unique band (bp) |
|---|---|---|
| RC1 | OPA 04 | 300 |
| RC1 | OPX 01 | 850 |
| RC2 | OPA 01 | 2000, 1550 |
| RC2 | OPA 02 | 2500 |
| RC2 | OPA 04 | 1350 |
| RC6 | OPA 12 | 900 |

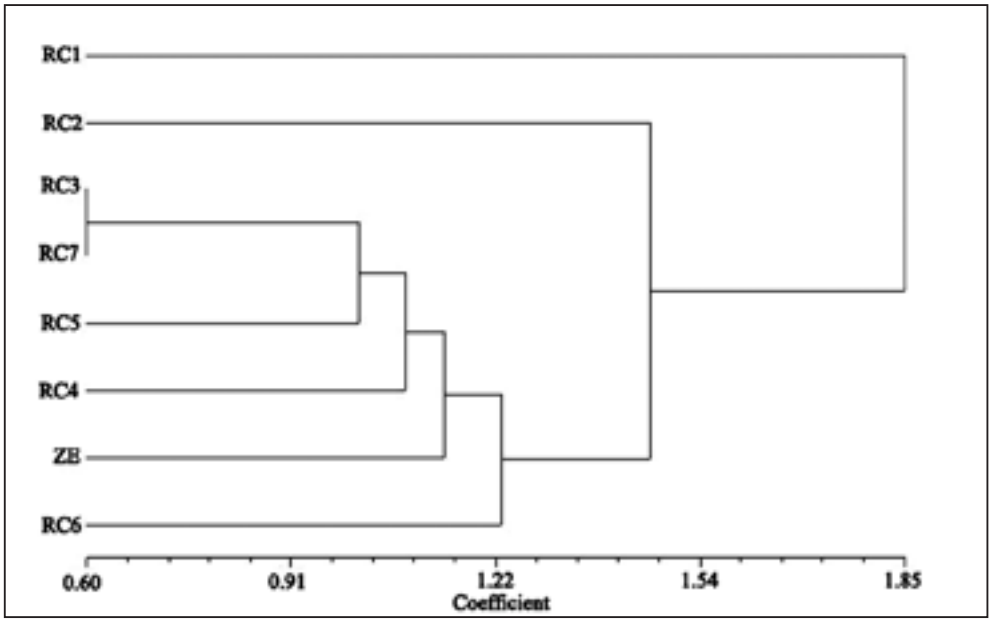


Fig. 7 — Dendogram showing relationship among eight different cell lines of *P. hexandrum* generated by cluster analysis of RAPD profiles obtained with 11 decamer primers.

crease in productivity till fourth year of initiation. The instability of secondary metabolite accumulation is a ubiquitous problem in plant cell cultures and is a widely documented phenomenon (DEUSNEUMANN and ZENK 1984; MORRIS 1986; HOLDEN *et al.* 1988; PIEHL *et al.* 1988; SCHRIPSEMA and VERPOORTE 1992; WHITMER *et al.* 2003; QU *et al.* 2006). The loss in productivity of plant cell cultures may be due to the fact that prolonged subculture selects rapidly growing cells which are metabolically less active (WHITMER *et al.*, 2003). However, in the present study, stability in podophyllotoxin accumulation in the cell lines was noted after 4 years of initiation, although the level of accumulation was much lower than initial cultures. Thus the age of the cell lines was a crucial factor in determining podophyllotoxin productivity.

An inverse correlation between growth rate and secondary metabolite accumulation in plant cell cultures has been noted in many cases (LINDSEY and YEOMAN 1983; FETT-NETO *et al.* 1993; 1994; LUO *et al.* 1999). In conformity with these studies, in the present study, cell line RC4 which accumulated the highest level of podophyllotoxin, had a slow growth rate. On the other hand, fast growing cell line RC1 accumulated low levels of podophyllotoxin. Thus optimal culture conditions need to be determined empirically for each cell line to obtain high yielding fast growing cell lines for podophyllotoxin production.

Variation in secondary metabolite accumulation in morphologically distinct cell cultures has been frequently reported (WATTS *et al.* 1984; PIEHL *et al.* 1988; GALNEDER and ZENK 1990; WICKREMESINHE and ARTECA 1993; GHOSH *et al.* 2002b). Previous reports indicate that dark brown coloured cultures of *P. hexandrum* accumulated high amounts of podophyllotoxin; a complete loss in podophyllotoxin accumulation was noted when the cultures turned yellow/green (VAN UDEN *et al.* 1989). In the present study, light brown, friable cell line RC4 accumulated the highest amount of podophyllotoxin for 4 years and can be distinctly differentiated from dark brown coloured cell lines like RC2, RC3, RC5, RC7 or yellow and creamish cell lines like RC1, RC6 and ZE. Additionally, lines RC4 and RC6 were friable in nature and most suitable for scale up studies in batch/continuous cultures (RAZDAN 2003; FLORYANOWICZ-CZEKALSKA *et al.* 2006).

*Cytological status of the cell lines* - Cytological analysis of eight cell lines revealed a wide variation of chromosome numbers as expected. The diploid number of *P. hexandrum* is 2n=12 (KUROSAWA 1971; SIDDIQUE *et al.* 1990; ARUMUGAM and BHOJWANI 1994). The present study revealed that abnormalities in chromosome behaviour leading to the formation of hypo- and hyperdiploid cells were present in the different cell lines. Variation in the frequencies of diploid, hypo- and hyperdiploid cells was noted among and within the cell lines according to the age of the cultures. ARUMUGAM and BHOJWANI (1994) reported stability in chromosome number in 18 month old callus cultures of *P. hexandrum* with all cells showing the diploid chromosome number, while both numerical and structural variations were observed by them in 3 year old calli. Callus tissues have a unique potential of generating variation *in vitro* (KUMAR and MATHUR 2004). Chromosome instability is of most frequent occurrence in callus cultures. Numerical chromosome variation during callus culture is evidently attained by some kind of endoreduplication and anaphase nondisjunction (MUKHOPADHYAY and SHARMA 1990). Genome multiplication is affected by certain spindle anomalies giving rise to cells with abnormal chromosome numbers, the frequency of which increases with time as a result of multiplication of divisional errors (LAVANIA and SRIVASTAVA 1988). Mixoploid nature of the source explants and culture conditions have also been suggested as some of the other causes leading to chromosomal variation *in vitro* (PHILLIPS *et al.* 1994). In the present study lines RC1, RC5, RC6, ZE were predominantly hyperdiploid as expected in long term cell cultures (JHA 1989). Changes in the ploidy levels of lines RC3 and RC4 from hyperdiploid to hypodiploid could be as a result of a mechanism leading to chromosome reduction or as a result of hypodiploid cells becoming predominant due to different growth rates (JHA 1989). Conversely, 2 year old cell lines RC2 and RC7 which were dominated by hypodiploid cells became hyperdiploid in 4 year old cultures. This shift in frequency might be due to a setback of proliferation of hypodiploid cells in the dividing cell population.

In addition to numerical variations, the present study also revealed structural chromosomal alterations in the cell lines. The karyomorphology of *P. hexandrum* from actively dividing root tip cells was described by SIDDIQUE *et al.* (1990) and ARUMUGAM and BHOJWANI (1994) to be composed of six homologous pairs with two median, six sub-median and four sub-telocentric chromosomes. The chromosome complement of the cell lines analysed in the present study however had chromosomes with constrictions in the median region (for example in cell lines RC1, RC2, RC3, RC4 and RC6) and terminal region (for example

in cell lines RC1, RC2, RC4, RC6 and RC7) together with median, submedian and sub-terminal chromosomes as reported earlier. Structural chromosomal changes might be brought about by segmental interchanges, thereby changing the positions of the centromeres (ARUMUGAM and BHOJWANI 1994). Chromosomal instability in plant cell cultures has been recorded for several other species (OGIHARA 1982; JHA and SEN 1987; JHA 1989; MUKHOPADHYAY and SHARMA 1990; ZIAUDDIN and KASHA 1990; KUMAR and MATHUR 2004).

Changes in ploidy levels are often accompanied by conspicuous changes in the accumulation of secondary metabolites (LAVANIA 2005). DEUS-NEUMANN and ZENK (1984) cited examples of different plant species, cell cultures of which showed variation in production of their respective secondary metabolites and suggested that genetic variation was one of the main factors contributing to this instability. In the present study, variation in podophyllotoxin content among the different cell lines was noted which may be attributed to the differences in ploidy levels or karyomorphology among the cell lines. Reports indicate variation in secondary metabolite accumulation according to ploidy for *in vitro* cultures of *Catharanthus roseus*, *Hypericum perforatum*, *Datura stramonium*, *Artemisia annua* (DEUS-NEUMANN and ZENK 1984; ELLÁROVÁ *et al*. 1997; BERKOVA *et al*. 2003; JESUS-GONZALEZ and WEATHERS 2003). It is noteworthy that cell lines RC1 and ZE were cytologically similar in terms of ploidy level but differed in podophyllotoxin accumulation from 1st to 5th year of analysis. This difference could be due to explant source from which they were initiated or medium composition on which they were maintained. Cell line RC4 showed increase in diploid and hypodiploid cells with age and decrease in hyperdiploid cells. Such characteristic is similar to line RC3. However the two lines significantly differed in their capacity to accumulate podophyllotoxin.

*RAPD analysis* - Isolation of genomic DNA from the cell lines of *P. hexandrum* proved to be difficult. Major problems encountered were degraded or denatured DNA or poor DNA yields unsuitable for PCR amplification. In general, medicinal and aromatic plants contain high amounts of polysaccharides, polyphenols and other secondary metabolites which interfere with DNA isolation procedures and downstream reactions like DNA restriction, amplification and cloning (KHANUJA *et al*. 1999; PADMALATHA and PRASAD 2006). In the present study, the CTAB method followed resulted in isolating high amounts of good quality DNA free of contaminants and coloured pigments from the eight different cell lines of *P. hexandrum*, amenable to PCR amplification. CTAB based methods have been employed for isolating DNA from other plants containing high amounts of polyphenols and polysaccharides like *Pinus radiata*, *Emblica officinalis*, *Terminalia belerica*, *T. chebula*, *Taxus baccata*, *T. canadensis*, *T. cuspidata* and *T. wallichiana* (STANGE *et al*. 1998; SAIKIA *et al*. 2000; COLLINS *et al*. 2003; WARUDE *et al*. 2003; HANANIA *et al*. 2004).

It has been established that *in vitro* culture can result in severe effects on the genetic constitution of plants (LARKIN and SCOWKROFT 1981). High degree of somaclonal variation is one of the major problems of *in vitro* plant cultivation (BORDALLO *et al*. 2004). Tissue culture condition imparts a physiological stress which may be due to explant source, genotype, alterations in plant growth regulator concentrations, duration of *in vitro* culture, variations in diurnal rhythm, nutrition etc. (EVANS and SHARP 1988; BORDALLO *et al*. 2004; MODGIL *et al*. 2005) leading to epigenetic and genetic variation. The variations may be due to changes in structure and/or number of chromosomes, point mutations, chromatin loss, DNA amplification, activation of transposable elements etc. (RAO *et al*. 1992). Several strategies can be used to assess the genetic integrity of *in vitro* cultures, but most of them have certain limitations. Karyological analysis cannot reveal alterations in specific genes or small chromosomal rearrangements. Although isozyme markers provide a convenient method for the detection of genetic changes, they are subject to ontogenic variation, are limited in number and only DNA regions coding for soluble proteins can be sampled. Restriction fragment length polymorphism (RFLP) markers are useful for sampling various regions of the genome and are potentially unlimited in number. However, use of RFLP markers is time consuming, expensive and requires large amounts of plant tissue (ISABEL *et al*. 1993). On the other hand, random amplified polymorphic DNA (RAPD) analysis is a reliable and cost efficient PCR-based methodology requiring small quantities of material; the specific DNA fingerprints obtained are independent of ontogenic expression and most of the genome can be sampled with a potentially unlimited number of markers (ISABEL *et al*. 1993). RAPD markers have been used to genetically profile different plant species like almond, hybrid potatoes, *Digitalis obscura*, hybrid strawberries, *Artemisia annua*, *Echinacea purpurea* (BAIRD *et al*. 1992; BARTOLOZZI *et al*. 1998; GAVIDIA *et al*. 1996; DEGANI *et al*. 1998; SANGWAN *et al*. 1999; BAUM *et al*. 2001) etc. and

to detect polymorphism among tissue culture regenerated plants (HASHMI *et al.* 1997; SONIYA *et al.* 2001; BENNICI *et al.* 2004; MARTINS *et al.* 2004) and callus tissues (EVANS and SHARP 1988; STOCKINGER 1996; NKONGOLO *et al.* 1998; MANGOLIN *et al.* 2002). Also, the unique polymorphic profiles of DNA markers serve as distinguishing tags for species, genotypes, varieties, undifferentiated callus tissues or somaclones (MARTIN *et al.* 1997; MANGOLIN *et al.* 2002). In addition, like any molecular marker, RAPD profiles are independent of age, physiological status, tissues or environmental influences (VIRK *et al.* 1996). RAPD markers have been used to differentiate *P. hexandrum* populations from Chamba and Kullu into distinct region specific clusters (SHARMA *et al.* 2000) and in discriminating seven *P. peltatum* accessions collected from three different sites in Lafayette County, Mississippi (LATA *et al.* 2002). In the present study, besides being used to detect DNA polymorphism in the eight different cell lines of *P. hexandrum*, RAPD markers were also used to generate useful DNA fingerprints to facilitate cell line identification. Molecular identification of different cell lines is an important parameter for marking lines which are highly productive. The observed polymorphic banding patterns obtained with 11 decamer primers reflect the genomic variability present in the cell lines. In a similar study, RAPD markers were used to detect DNA polymorphism in callus tissues of *Cereus peruvianus* as well as to identify callus tissues maintained in different hormone combinations (MANGOLIN *et al.* 2002). LAURA *et al.* (2003) reported screening of genetic variation in callus cultures of *Cyclamen persicum* and selection of the most stable embryogenic cell lines using RAPD analysis. Selection of the most suitable cultivar for synthetic seed production through RAPD analysis of calli of commercial potato cultivars has also been reported (EVANS and SHARP 1988). In the present study, some of the primers (viz. OPA 01, OPA 02, OPA 04, OPA 12 and OPX 01) generated unique bands in the cell lines RC1, RC2 and RC6; but high podophyllotoxin producing cell line like RC4 could not be marked by any of the effective primers. A broader screening of markers may help in establishing a relationship between high producing lines and unique profiles, thereby marking cell lines as elite genotypes, which can be selected and maintained for podophyllotoxin production in higher amounts through further optimization.

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