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In vitro production of camptothecin (an anticancer drug) through albino plants of *Ophiorrhiza rugosa* var. *decumbens*

Camptothecin (CPT) and its derivatives, topotecan and irinotecan, are known for their inhibitory activity on topoisomerase I in eukaryotic cells. Inhibition of replication, transcription and packing of double-stranded DNA containing adenoviruses, papova viruses, herpes viruses and parvoviruses by CPT has been established[1]. The efficacy of CPT derivatives against colon cancer[2], AIDS[3], uterine, cervical and ovarian cancer[4], and falciparum malaria[5] has also been reported. Originally CPT was isolated from the plant, *Camptotheca accuminata* and later it was found in *Nothopodytes foetdia*[6]. The roots of *Ophiorrhiza mungos* and *O. pumila* have been reported to produce CPT and 10-methoxy-camptothecin[7,8]. *Ophiorrhiza rugosa* var. *decumbens* is also found to produce CPT. The present study deals with *in vitro* propagation of spontaneously originated albino plant of *O. rugosa* var. *decumbens*, and the analysis of CPT yield in albino plants with respect to benzyl adenine (BA) concentrations in comparison with the green (normal) plants *in vitro*.

Production of secondary metabolites by suspension cultures, induction of untransformed and transformed roots, elicitation, immobilization and induction of variants with high productivity of the compounds of interest were much emphasized[9]. The potential of synthesis of secondary metabolites by albino plants has not been assessed so far. Albinos are reported to originate either spontaneously during *in vitro* culture[10] or through mutagenesis, short exposure to spectinomycin[11], proline and high temperatures[12]. Albinos are useful to study the gene regulation, especially related to chloroplast differentiation, palisade development, and as a standard marker in plant cytoplasmic genetics and also in studies of gene expression. The albinos also open possibilities to dissect out the key enzymes of metabolic pathways concerning a product of specific interest.

Table 1. Induction of shoots from leaf explants, albino and green plants on MS medium with different BA concentrations (sucrose 3%)

	Green leaf explants		Albino leaf explants	
BA (mg/l)	Number of shoots ± SD	Mean fresh weight (g) ± SD	Number of shoots	Mean fresh weight (g)
2	148.4 ± 5.89	8.8 ± 0.96	164.8 ± 4.17	2.6 ± 0.13
3	170.4 ± 9.65	12.8 ± 0.25	164.2 ± 10.27	2.9 ± 0.51
4	179.2 ± 4.96	16.8 ± 1.05	171.8 ± 5.19	4.8 ± 0.25
5	148.6 ± 3.6	8.2 ± 0.85	158.2 ± 4.58	2.2 ± 0.22
6	138.8 ± 5.44	6.6 ± 0.76	152.2 ± 3.97	1.7 ± 0.14

The number of shoots represents mean of twenty replicates. Incubation period is 28 days.

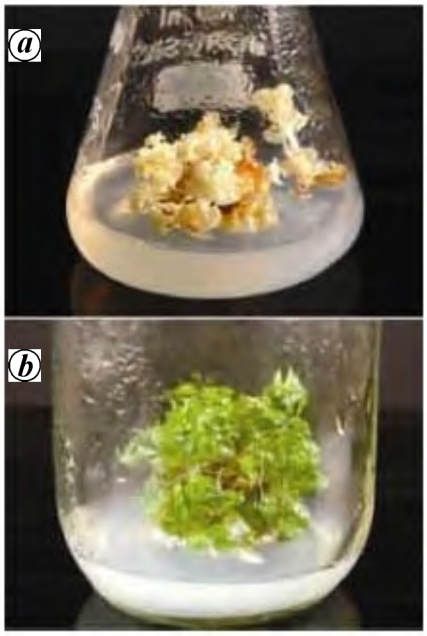

Figure 2. Albino multiple shoots (*a*) and normal multiple shoots (*b*) grown on MS medium with 4 mg/l BA.

Leaf explants excised from the albino plant originated spontaneously during *in vitro* propagation of *O. rugosa* var. *decumbens* on Murashige and Skoog (MS) medium with 4 mg/l BA were used for multiplication of albino plants. MS medium with different concentrations of BA (2–6 mg/l) was used for *in vitro* culture. The culture of leaf explants of green plants on the respective medium served as control. The influence of sucrose on CPT yield was studied by adding different concentrations of sucrose (3, 4 and 5%) to MS medium with 4 mg/l BA. In all other cases, the medium was supplemented with 3% sucrose. All cultures were incubated at 25 ± 2°C with 12 h photoperiod. Subcultures were performed at an interval of 28 days.

Albino as well as green plants grown on corresponding media were lyophilized and extracted separately with chloroform and the extracts were dried under vacuum. The residues obtained were redissolved separately in known volume of acetonitrile : water (24 : 1). CPT was analysed using an HPLC system (Shimadzu, Japan) consisting of two solvent-delivery systems LC 10AT VP, a UV detector SPD-10A VP and run with CLASS VP software. Separation was performed with a reversed-phase column (Purospher star column RP-18, end capped, 5 μm, 250 × 4.60 mm, Merck, Germany) at 25°C with a flow rate of 1 ml/min using an isocratic elution of acetonitrile : water (60 : 40) solvent system. Absorbance was monitored at 256 nm. The retention time of CPT was 5.8 min (Figure 1 *a* and *b*).

The frequency of shoots developed from leaf explants cultured on MS medium with different concentrations of BA was similar in albino and green plants (Table 1; Figure 2 *a* and *b*). In both cases, MS medium supplemented with 4 mg/l BA developed the highest number of shoots (Table 1). Shoot buds from the explants were initiated within 10–15 days. After 25 days, the leaves turned reddish. Above the optimal BA level (4 mg/l), there was a decrease in the number of shoots (Table 1). Though the albino plants were grown under light, no development of chlorophyll pigment was noticed during the study.

CPT yield showed significant difference in albinos and normal plants grown on medium with similar BA concentrations. Production of CPT increased with respect to BA concentration. The highest CPT level was recorded from normal and albino plants when the medium was supplemented with 6 mg/l BA and CPT yield was 0.311 and 1.04 mg/g DW respectively, for these plants. In the case of optimal shoot multiplication medium, both in normal and albino plants, though there was increase in biomass (16.8 ± 1.05 and 4.8 ± 0.25 g respectively), CPT yield was less (0.037 and 0.558 mg/g DW respectively). Sucrose concentrations has significant influence on the production of CPT. Albino plants in MS medium with 4 mg/l BA and 3% sucrose produced higher amount of CPT (0.558 mg/g DW) compared to 4% (0.207 mg/g DW) and 5% (0.198 mg/g DW) sucrose.

Albinism is common in higher plants. In addition to the natural origin, development of albinos has been reported to occur by mutagenesis, high temperatures[12] or short exposure to spectinomycin[11]. Albinism is considered as a standard marker in plant cytoplasmic genetics[13]. Plastid-encoded chlorophyll deficiency is shown to result from point mutations[14] or deletions[15]. Plastid ribosome deficiency due to nuclear mutations is also documented as a cause of albinism[13].

In the present study, albinos that originated spontaneously during *in vitro* culture are stable even after prolonged growth under illumination. Although several reports pertaining to albinos were documented, the higher production of secondary metabolites through albinos in comparison to green plants has not been accomplished so far. In the present study, CPT yield in multiple shoots of albinos was higher compared to green shoots on DW basis. Supplementation of 4 mg/l BA was optimal for multiple shoot production in both albinos and green plants. Increasing BA concentrations showed elevated production of CPT. The multiple shoots developed from albinos and green shoots on MS medium containing 6 mg/l BA produced the highest amount of CPT. As in the present study, an enhancement in the yield of secondary metabolites with corresponding decrease of shoot induction in the medium with higher levels of phytohormones than the optimal medium for shoot induction, has been reported with normal plants of other species[16]. In-

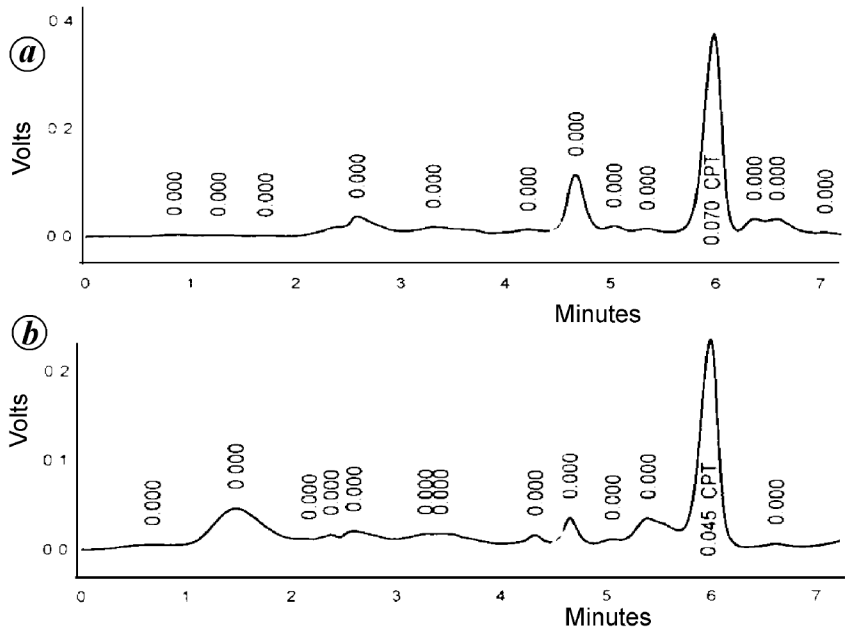

Figure 1. Comparative HPLC chromatogram of extracts of normal and albino multiple shoots of *O. rugosa*. ***a***, Chromatogram of extract of normal shoots. About 1.124 g DW was used for extraction. The extract was redissolved in 5 ml acetonitrile : water solution. Then 20 µl of this sample was injected into the column. ***b***, Chromatogram of albino shoots. About 0.216 g DW was used for extraction. The extract was redissolved in 5 ml acetonitrile : water solution. Then 20 µl of this sample was injected into the column.

creased production of secondary metabolites on media favouring reduced growth is considered to be a stress response. Medium supplemented with 3% sucrose was beneficial for CPT production (0.558 mg/g DW) through albinos. Positive and negative influence of sucrose in secondary metabolite production has already been proved[17].

Albino plants seem to be a good source of CPT. Exploration of albinos in comparison to green plants using modern biochemical and molecular tools will provide insight into the link between secondary metabolism and plastid genome. Besides, albinism enables to unravel the genes required for chloroplast differentiation and palisade development. Propagation of albinos provides an elegant method of sustainable production of CPT *in vitro*.

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