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Improvement of Anticancer Drug Camptothecin Production by Gamma Irradiation on Callus Cultures of *Nothapodytes Foetida*

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Abstract

Gamma irradiation can change plant biology and enhanced the secondary metabolites is now rarely investigated. Therefore, our study was aimed to explore the effects of low doses of gamma irradiation on improvement of camptothecin production by callus cultures of *Nothapodytes foetida*. Callus cultures were established on Murashige and Skoog's medium fortified with α-Naphthaleneacetic acid (10.74 µM) and N^6-benzyladenine (2.22 µM). After seven sub-cultures on same medium, callus cultures were irradiated at low doses ranging from 5 to 30 Gy. The gamma irradiation significantly boosted the production levels and increased the total of camptothecin and 9-methoxy-camptotheicin yields by 20-fold at 20 Gy and by only ~2-fold and ~9-fold at 10 and 15 Gy, respectively. At 20 Gy dose level of gamma irradiation influenced cell growth by ~2-fold, and also product synthesis and achieved 0.098% dry weight camptothecin and 0.0043 % dry weight 9-methoxy camptothecin that is ~20-fold more than that of non-irradiated callus cultures. It was revealed that low doses of gamma irradiation correlated with growth and product synthesis. The dose of 20 Gy was considered satisfactory for callus attenuation since irradiated cells above 25 Gy inhibited growth and productivity.. The present study is the first report of the gamma irradiation on the enhancement of alkaloid camptothecin production of *N. foetida* callus cultures.

Key words: *gamma irradiation, Nothapodytes foetida, callus cultures, camptothecin, 9-methoxy-camptothecin.*

Introduction

Nothapodytes foetida (Slemure) Wight is endanger plant species in the Western Ghats of India and contain two major alkaloids camptothecin and 9-methoxy camptothecin [1] (Fig. 1). Biological screening from the stem of *N. foetida* isolated alkaloid camptothecin and 9-methoxycamptothecin have significant anti-cancer activity[2]. Also, camptothecin is a potent antineoplastic compound with selectively targeting topoisomerase I-DNA inhibitor alkaloid by trapping the catalytic intermediate of the TopI-DNA reaction, the cleavage complex[3-5]. The discovery of hydrophilization of the camptothecin molecules that the primary cellular target for camptothecin is DNA topoisomerase-I had created renewed interest in the compound and led to the successful identification and development of the antitumor agents topotecan and irinotecan, which have been clinically approved for treatment of ovarian and colon cancers, respectively[6-7]. New camptothecin analogs exhibited antitumor activities superior to CPT-11 in human cancer xenograft models[8]. In addition, Li et al.[9] demonstrated that camptothecin, a traditional anti-tumor drug shown to possess anti-HIV-1 activity. The numbers of approaches are being used to improve the antitumor efficacy of camptothecin. In the past, many researches have been extensively worked to improve the biological profile of the potent anticancer compound camptothecin.

For several years, gamma irradiation has been attentive as new and speedy process to improve the growth and secondary metabolites in plant cell cultures. Gamma rays belong to ionizing radiation and interact to atoms or molecules to produce free radicals in cells and found to enhance plant growth and development by inducing physiological, biochemical,

cytological, genetic, and morphogenetic changes in cells and tissues[10-12]. Jan et al.,[13] reported that gamma dose applied to seedling of *Psoralea corylifolia* activated oxidative stress and control lipid peroxidation and proline accumulation. In addition, it was demonstrated that increased dose gamma irradiated seedling of *Vicia faba* and *Arabidopsis* plants induce high levels of reactive oxygen species generation[14-15]. These radicals can damage or modify important components of plant cells and have been reported to affect differently the morphology, anatomy, biochemistry, physiology and secondary products of plants depending on the irradiation level. Gamma irradiation has been successfully used in agriculture, medicine and biology in terms of biological effects induced by a counter intuitive switch over from low dose stimulation to high dose inhibition[16]. *In vitro* mutagenesis is a combination of *in vitro* cell culture and mutation induction, which provide the opportunity to increase the variability of an economically important cultivar used on plants in developing varieties that are agriculturally and have high productivity potential[17].

Previous studies by many researchers have shown that relatively low doses of gamma radiation on large number of plants accelerated cell proliferation, seed germination, cell growth, enzyme activity, nutrition, molecular and biological changes, stress resistance, crop yields and forest plants[17-25]. Recent evidence suggested that use of growth regulators, biotic and abiotic elicitors, feeding of precursors, UV, ultrasound to control the production of secondary metabolites in cell cultures such as Ajmalicine[26], phytoestrogens[27] , Precursor feeding[28], Growth regulators[29], UV[30], and ultra sound[31].

Conversely, it is recognized that low doses of gamma radiation promotes secondary metabolites in plant cell cultures and increased the production levels. Very limited numbers of studies have been investigated with the use of gamma irradiation on plant cell cultures to augment the cell growth and product synthesis[32-35]. Therefroe, the current study was conducted in order to investigate the effects of low doses of gamma irradiation on callus cultures of *N. foetida* for the production of anticancer drug camptothecin.

Materials and Methods

Plant material

Seeds of *Nothapodytes foetida* were obtained from Mahabaleshwar, Maharashtra State, India. Seeds were washed thoroughly with tap water. Thereafter, seeds were surface disinfected with 75% ethyl alcohol for 3 min followed by immersing in aqueous solution of 0.1% (w/v) mercuric chloride for 3 min and subsequently 5-6 washed with sterilized distilled water. Surface disinfected seeds were transferred aseptically on Murashige and Skoog's (MS) medium[36] for germination.

Tissue culture and conditions

Three-week-old germinated seedlings were employed for induction of callus cultures of *N. foetida*. The basal medium was fortified with α-Naphthaleneacetic acid (NAA) (10.74 µM) and N^6-benzyladenine (BA) (2.22 µM) and 3.0% sucrose. The pH of the medium was adjusted to 5.8 by 0.1 N NaOH or HCl before adding 0.8% agar. Culture medium was autoclaved at 121 ^{0}C at 103.42 kPa for 20 min. Cultures were maintained at 25±1 ^{0}C for 16 hr light provided by cool white fluorescent tubes (Phillips, Holland) to provide 40 µmol $m^{-2}s^{-1}$ light intensity.

Gamma Irradiation

N. Foetida callus cultures, three-week-old in solid medium, were placed in the irradiation chamber of gamma cell and irradiated at doses ranging from 5, 10, 15, 20, 25 and 30 Gy, at room temperature. The irradiation was carried out in a uniform source of $_{60}$Co gamma rays at Bhabha Atomic Research Centre, Mumbai, India. Callus cultures without irradiation were served as the control. After each dose the cultures were maintained at 25±1 ^{0}C for 16hr light provided by cool white fluorescent tubes (Phillips, Holland) to provide 40 µmol $m^{-2}s^{-1}$ light intensity and subsequently harvested on day 20 for growth and camptothecin contents.

Selection of friable callus cultures after gamma irradiation

After irradiation at low doses, callus cultures were maintained in culture room at an appropriate temperature and light intensity. Next day, three grams callus was transferred to 20 ml MS liquid medium supplemented with NAA (10.74 µM) and BA (2.22 µM) with 3% sucrose to giving an initial biomass to liquid ratio of 0.15 g/ml fresh weight. The suspension cultures were kept on a gyratory shaker at 120 RPM at 25±1^{0}C. After 24hr, suspension cultures (3 ml) were spread on Petri dishes containing 20 ml of the same medium. The plates were incubated at 25±1^{0}C under controlled photoperiod conditions for three weeks. Friable and fast growing callus were picked up and transferred to fresh medium. Callus cultures were established and after subsequent five subcultures taken up for further investigation.

Measurement of Growth Parameters
After harvest, callus cultures were gently pressed on filter papers (Whatman No. 1) to remove excess water for fresh weight (FW) determination. Cultures were dried to constant weight in an oven at 55^0C for 16hr to get the dry weight (DW). All experiments were replicated three times.

Extraction of camptothecin
For determination of alkaloids, callus cultures were harvested and analysed for camptothecin and 9-methoxycamptothecin contents. Harvested biomass was dried in an oven at 55^0C for 16hrs subsequently powdered by Wily Mill (Model No. 4276, Thomas Scientific, USA). Powdered bio-material (100 mg) was extracted with 1000 µl of methanol followed by vortex for 3 minutes and sonication 10 min. After sonication, samples were centrifuged at 12000x*g* for 10 min. For analysis, clear supernatants were transferred to Eppendorf vials (1 ml) and applied directly on High Performance Liquid Chromatography (HPLC) to the camptothecin contents.

Quantification of alkaloids on HPLC
The HPLC analysis was performed on Jasco Liquid Chromatograph (PU-2080 Plus, Japan) equipped with auto sampler injector (AS-2055 plus Japan) with a 25 µL loop and a variable wavelength detector (Model No. UV-2075 plus, Japan). Data collection and integration were accomplished using Chrompass software. Separations were performed on Inertsil C18 (250 mm x 4.6 I.D, Sigma, USA) column. The camptothecin and 9-methoxy camptothecin were determined by using water:acetonitrile (60:40 v/v) as a mobile phase. The flow rate was 1 ml/min and the elution was monitored at 254 nm. The peak areas corresponding to camptothecin and 9-methoxycamptothecin were integrated by comparison with external standard calibration curves. The results of the five injections from the same samples at five concentrations (0.01µg–0.5µg) showed similar retention time. The analytical operation was completed in 10 min.

Chemicals
Camptothecin was obtained from Sigma Chemicals Co. USA, and 9-methoxy-camptothecin obtained from Yakult Central Institute for Microbiological Research, Japan. All other chemicals were of analytical grade and solvents of HPLC grade.

Statistical analysis
The influence of various treatments of gamma irradiation on growth and camptothecin content was analysed by one-way analysis of variance (ANOVA). Values are mean of three replicates from two experiments. The data were analyzed statistically by analysis of variance (ANOVA) and the difference between means of the samples was analysed by the least significant difference (LSD) at a probability level of 0.05.

Results and Discussion

Selection of friable Callus Cultures
In the present study, irradiated callus cultures were transferred onto MS medium supplemented with NAA (10.74 µM) and BA (2.22 µM) for three weeks. In addition, irradiated callus were also transferred on MS medium devoid of growth regulators. After three weeks of incubation, friable and oyster-whitish coloured callus induced, while no callus growth was obtained on MS medium devoid growth regulators. No shoots proliferations were occurred during same time period of incubation.

Growth curve of irradiated and non-irradiated callus cultures
Growth study was performed with gamma irradiated (20 Gy), which produced a high level of camptothecin and 9-methoxycamptothecin and non-irradiated callus cultures. Growth study was initiated with ~1 gm of callus cultures transferred in test tubes containing 20 ml MS solid medium supplemented with NAA (10.74 µM) and BA (2.22 µM) as an inoculum to achieve initial biomass ratio 50 gm/lit fresh weight. Every fifth day, three test tubes were harvested for determination of growth. In the present study, results revealed that non-irradiated cultures showed 5-fold increase in biomass on day 20 and thereafter started decline phase. When callus cultures were irradiated at 20 Gy, a lag phase for10 days followed by an exponential phase and reached stationary phase on day 20. Maximum cell growth was obtained by irradiated callus cultures that produced 8-fold biomass in three weeks. The present results revealed that low gamma irradiation doses improved cell growth as compared to that of non-irradiated callus. A representative curve was presented in Fig. 2.

Gamma irradiation effects on cells growth
The growth of irradiated callus cultures was examined by their fresh weight and dry weight on day 30.

The effect of low doses of gamma irradiation on this parameter was somewhat variable. A representative bar graph was presented in Fig. 3. The growth rate of competent cells falls off with increasing doses of radiation. A reduction of cell growth was achieved at 25 Gy to 30 Gy. The present results clearly revealed that there was a significant decrease in the cell growth of *N. foetida* with the increased in irradiation dose. It was concluded that high irradiation doses detrimental to the cell growth. However, callus growth was better at low doses of radiation up to 20 Gy and achieved 2.13 g/fresh weight and 0.497 mg/dry weight on day 30.

Gamma irradiation effects on camptothecin and 9-methoxy camptothecin production

The callus cultures of *N. foetida* exhibited variable responses to gamma irradiation doses. Camptothecin and 9-methoxy camptothecin content of *N. foetida* callus cultures showed a volumetric increment with increased gamma irradiation doses (Fig. 4 & 5). The results of the present study revealed that the gradual increase the camptothecin content correspond to the incremental increase of low doses of gamma irradiation. The content of alkaloids was optimum when callus cultures irradiated with 20 Gy and produced the highest amount of camptothecin (0.98% dry wt) and 9-methoxy camptothecin (0.0038% dry wt). However, decrement of content was observed when callus cultures were exposed to high gamma strength. It was also found that there was no significant difference in alkaloid content among the callus cultures irradiated with 5 Gy and non-irradiated callus cultures. Results achieved from the present study suggested that ionizing radiation augment the product synthesis and significant increase in camptothecin content by callus cultures of *N. foetida*.

Gamma irradiation intervenes secondary metabolites is an alternative and attractive method to influence the production of high value bioactive compounds. In the past extensive studies have been carried out to increase the yield of content of irradiated plants and cell cultures[25,37,38]. According to the results obtained in the present study, it was observed that steady increment of camptothecin production with gradual increase of gamma doses. Nevertheless, the high gamma strength adversely affects the growth and production levels. This result revealed that there was a direct correlation between gamma doses and product synthesis to improve the camptothecin content in irradiated callus cultures. Our results are in agreement with El-Beltagi et al. [34] who demonstrated that higher gamma doses positively enhanced the secondary metabolites of flavonoids in *Rosmarinus officinalis* callus culture. Similarly, the callus and shoots of *Cucumis melo* growth decreased with the increasing dosage of gamma irradiation[38].

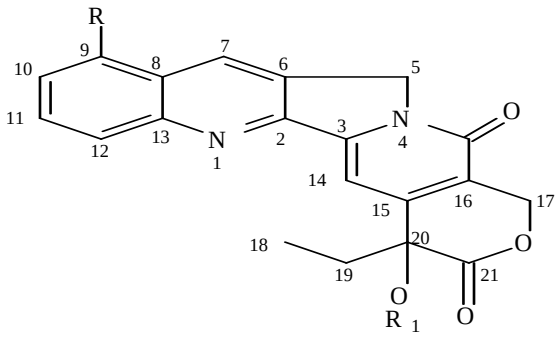
An irregular distribution of the content of bioactive compounds in irradiated and non-irradiated cell cultures reported inconsistency in different plants and cell cultures. In accordance with the present results showed that irregular distribution of camptothecin content in an irradiated and non-irradiated callus culture of N. foetida. It was found that total camptothecin and 9-metoxy camptothecin yields by 20-fold more than that of non-irradiated callus cultures. Our results are in agreement with Chung et al.,[33], found that similar type of irregular distribution of shikonin content in irradiated and non-irradiated cell suspension cultures of Lithospermum erythrorhizon and the gamma irradiation significantly stimulated the shikonin biosynthesis of the cells and increased the total shikonin yields by 400% at 16 Gy. Similarly, in accordance with the results obtained by El-Betagi et al.[34] reported that higher doses of gamma irradiation of 20 Gy positively enhanced secondary products accumulation of total phenols and total flavonoids in Rosmarinus officinalis callus culture. These results could indicate that low doses of gamma irradiation have some vital role to promote the yield of secondary metabolites. As mentioned by Ling et al.,[39], irregular distribution of rosmarinic acid content in irradiated at 40 and 50 Gy and non-irradiated callus culture of Orthosiphon stamineus. Irradiated callus cultures of N. foetida showed an increase of camptothecin and 9-methoxy camptothecin. The camptothecin content of irradiated callus cultures with 20 Gy was increment by ~20 fold over that of non-irradiated callus cultures. Results of the present study of increase of camptothecin content in callus due to exposure to ionization radiation is an agreement with findings of Jaisi et al.,[40] who reported that increase in ~4 fold higher of plumbagin by root cultures of Plumbago indica when exposed to gamma ray irradiation at a dose level of 20 Gy.

In addition, irradiated callus cultures showed a steady decrement of camptothecin and 9-methoxy camptothecin content with the increment of irradiation strength at 25 Gy and 30 Gy. The camptothecin and 9-methoxy camptothecin content for irradiated callus with 30 Gy was decreased by ~4 fold over the non-irradiated callus cultures. Similarly, Bajaj[41], high irradiation dosage of gamma irradiation caused inhibition of tissue culture growth along with the failure of RNA and subsequently the

failure of protein synthesis. The total soluble protein, carbohydrate, soluble free amino acids and total DNA content and band intensities both decreased with increasing doses of gamma irradiation is anticipated to be the main effect of gamma irradiation on the viability of Oryza sativa seed[42]. Also, Jan et al.,[13] found that the mean percentage germination of Psoralea corylifolia seeds and early germination with low doses that seeds irradiated at higher doses. Reduction in seed germination with gamma radiation might be due to an increase in the production of active radicals responsible for seed lethality. The effect of gamma irradiation on the growth of Capparis spinosa shoots in vitro was also studied. A 10 Gy dose of gamma irradiation stimulated growth of shoots up to 200% and increased shoot rooting percentage from 75 to 100% [17]. On the contrary, Al-Safadi and Simon[32] reported that gamma irradiation stimulated Daucus carota shoot formation at low doses, and inhibited shoot formation at high doses. Our present results are in agreement with the earlier reports that high gamma irradiation dosage caused inhibition of callus growth and production levels.

Conclusion

In the present study, we have focused on the improvement of topoisomerase I-DNA inhibitor alkaloid camptothecin production of *N. foetida* using low doses of gamma irradiation is reported for the first time. Based on our finding, the high gamma strength found sensitive to callus cultures while, low doses of gamma irradiation stimulated product synthesis and obtained significant increment in camptothecin and 9-methoxy camptothecin content in callus cultures. Our results provide some evidence to establish high yield cell lines of callus cultures for initiation of suspension cultures to scale up in bioreactor.



C P T : R & R 1 =H

9 − M e C P T : R = O M e , R 1 = H

Fig. 1: Chemical Structure Camptothecin and 9-methoxycamptothecin

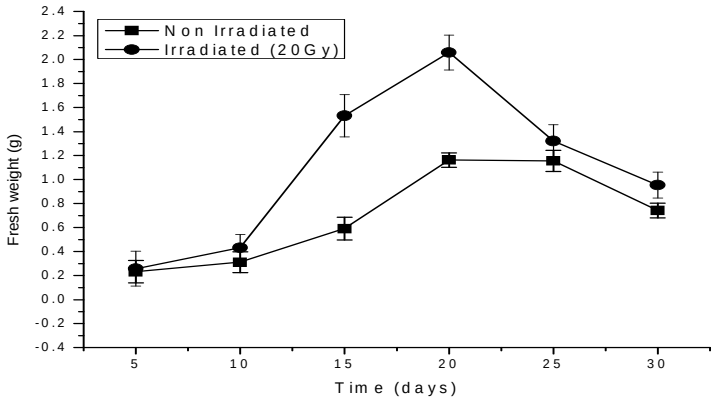


Fig. 2. Growth curve of irradiated and Non-irradiated callus cultures of *N. foetida*

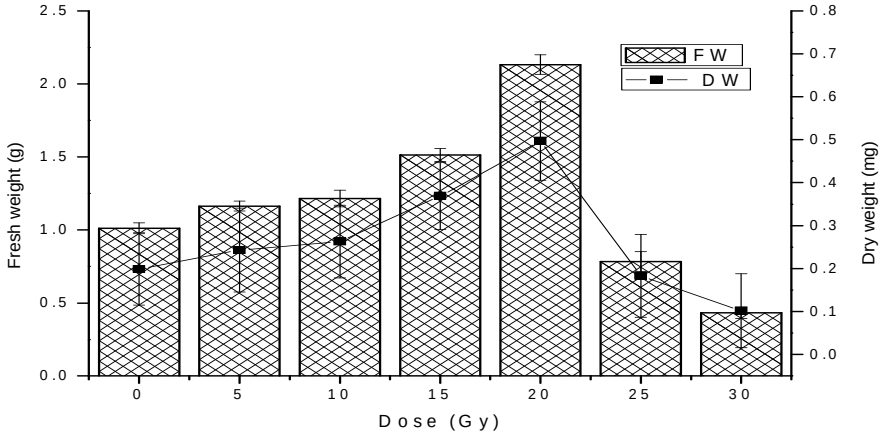


Fig. 3. Callus cultures of *Nothapodytes foetida* were irradiated with gamma radiation (5 to 30 Gy) and cells growth was observed by fresh weight and dry weight. The observation was obtained from triplicate determinations. These results were representative from three independent replicates (± SE).

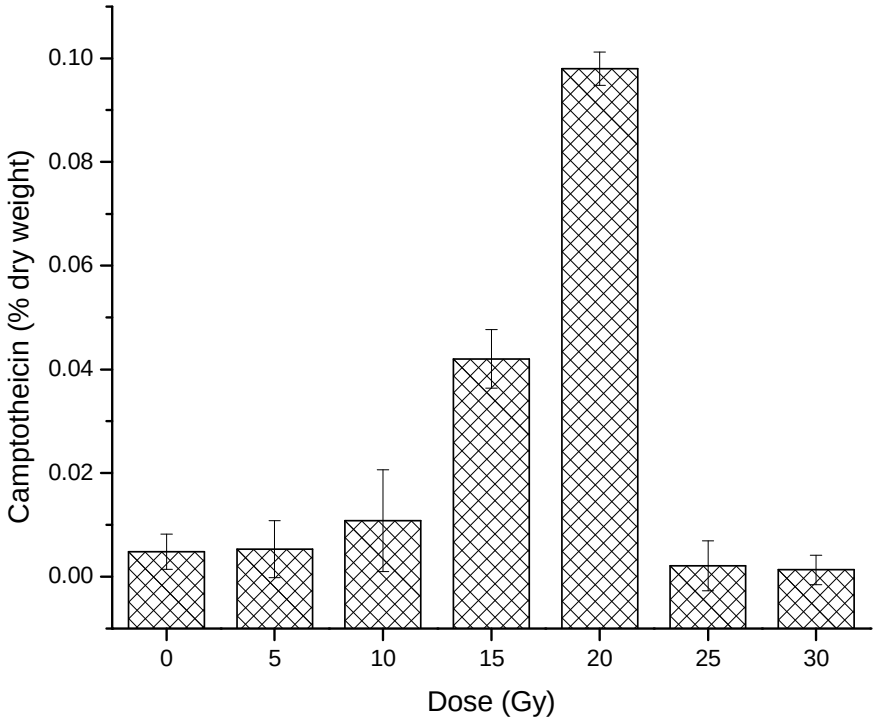


Figure 4: Effect of γ-rays treatment (low doses 5 to 30 Gy) on callus cultures of *Nothapodytes foetida*. High Performance Liquid Chromatography was performed to evaluate the content of camptothecin. The observation was obtained from triplicate determinations. These results were representative from three independent replicates (± SE).

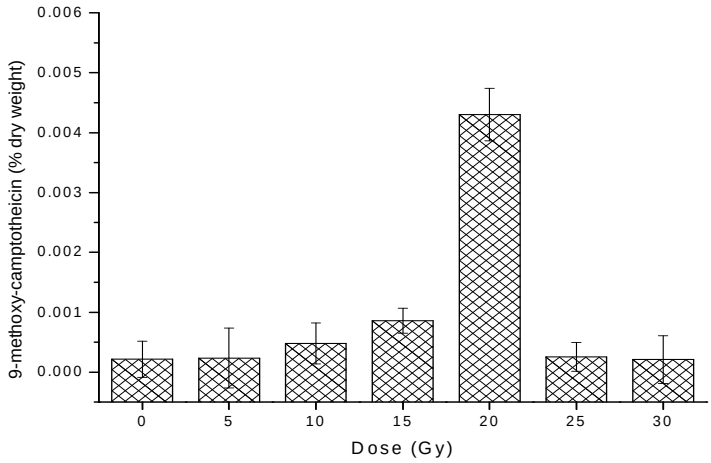


Figure 5: Effect of γ-rays treatment (low doses 5 to 30 Gy) on callus cultures of *Nothapodytes foetida*. High Performance Liquid Chromatography was performed to evaluate the content of 9-methoxy-camptothecin. The observation was obtained from triplicate determinations. These results were representative from three independent replicates (± SE).

"Cite this Article"

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