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Influence of Light and Temperature on Secondary Metabolite Accumulation in Callus Cultures of *Helicteres Isora* L

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Abstract: *The present study reports the effect of light and temperature on secondary metabolite accumulation in callus cultures of an important medicinal plant Helicteres isora L. Callus cultures were exposed to varying light (complete darkness, light of different spectral quality viz.white, red, blue, yellow and temperature (30°C, 13°C and 24°C) regimes during their growth cycle. Influence of these conditions on calli growth, total phenol content (TPC), total flavonoid content (TFC) and betulinic acid (BA) accumulation was examined. Among the tested light regimes, although there was no significant difference in calli growth but the biochemical parameters varied. Highest TPC and TFC was recorded in calli exposed to blue light whereas the BA accumulation was maximum under white light conditions on 28 days of incubation. Exposure of calli to elevated temperature (29°C) favoured both calli growth as well as TPC, TFC but inhibited BA accumulation. On the contrary, incubation of calli to low temperature (13°C) throughout their growth cycle increased BA content by over 200% , at the same time caused decline in culture growth, TPC, TFC in comparison to the control calli maintained at 24°C .However, when the cultures were maintained at 24°C for first 15 days of growth cycle and then exposed to low temperature, BA accumulation was increased. It is suggested that variation in light and temperature conditions alters the internal environment of cell making them stressed and this contributes to their ability to accumulate secondary metabolites. The findings of present study proposes a strategy to enhance BA production in callus cultures of H.isora L, which makes it a potential source for extraction of BA.*

KeyWords*: Betulinic acid, Callus cultures, Growth index, HPLC, Physical environment*

I. Introduction

Helicteres isora L., belongs to the family Sterculiaceae and occurs in dry forests throughout central and western India, and some parts of south-east Asia. This plant commonly named as Indian Screw Tree has been used traditionally as a herbal drug for treatment of many ailments throughout south-east Asia since ancient times, in Ayurvedic, Unani, Siddha as well as folk and tribal medicines (Kumar and Singh, 2014). Almost all the parts of plant are used in Ayurveda.

Pharmacological studies of *H. isora* have revealed many medicinal properties viz. antidiabetic , hypolipidaemic , hepatoprotective, antioxidant and anticancer properties (Jain et al., 2014). *H. isora* contains a wide range of phytochemical viz. phenols, flavonoids, alkaloids, glycosides, saponins, betulinic acid (Loganayaki et al., 2013, Pagi et al., 2007). Betulinic acid is a pentacyclic triterpenoid and is highly valued for its anticancer activity against HIV and a variety of tumour cell lines (Cichewicz and Kouzi, 2004).

Our previous study (unpublished) showed the presence of low concentration of betulinic acid in callus cultures of *H. isora*. Elicitation by various abiotic and biotic factors is a widely used method to enhance secondary metabolite production in plant cell cultures (Namdeo, 2007). Therefore, the present work was undertaken to stimulate secondary metabolite synthesis in callus cultures of *H. isora* by varying light and temperature regimes.

II. Materials and Methods

2.1 Seed Germination and Callus Induction

The pods of *H. isora* L. were collected from plants growing in Kolhapur, Maharashtra, India. The seeds were removed from dry pods and soaked overnight in tap water. They were then treated with concentrated sulphuric acid for 10 min followed by washing with distilled water. For surface sterilization, seeds were treated with 0.1% mercuric chloride for 6 min, washed with sterile distilled water and inoculated on water agar (0.8% agar) for germination. Hypocotyl explants from the seedlings were inoculated on MS medium containing 3% sucrose, 0.8% agar, BAP(0.5mg/l) and 2,4-D(0.5mg/l) for callus induction. The pH of the media was adjusted to 5.8 prior to autoclaving at 121ºC for 20 minutes. Cultures were incubated at 24±1 ºC under 16 hours photoperiod. The resultant callus was subcultured on the same fresh medium for multiplication.

For each experiment, 1.5 g of callus was transferred onto the above described medium. For light modification experiments the cultures were maintained in complete darkness or under continuous light (40 µmol m^{-2} s^{-1}, 35 W white fluorescent tubes), applied directly or through colour filters (182 primary red, 010 yellow, 119 dark blue, Lee Filters, Andover, UK) and incubated at 24±1ºC. For temperature modification experiments, cultures were incubated at 30ºC, 24ºC and 13ºC. Each experiment was conducted in three replicates. The calli were harvested after 28 days and assessed for growth index, TPC, TFC and betulinic acid content.

2.2 Measurement of Relative Growth Rate (RGR)

Growth indices of callus were expressed as relative growth rate and calculated according to the formula: RGR = [(G1 - G0)/G0] X 100 % where, G1 is the callus fresh weight(g) at the end of a growth period and G0 is the fresh weight of the inoculum.

2.3 Preparation of Crude Callus Extracts

The fresh callus was dried in oven at 70°C and then ground in mortar pestle. The fine powder so obtained was extracted with ethanol in 1:10 ratio by keeping overnight on a mechanical shaker. The mixture was centrifuged at 8000 rpm for 10 min, supernatant was collected and concentrated using rotavapour. The obtained solutions were filtered through 0.2µm Millipore syringe filter for HPLC. The extracts thus prepared were stored at -20°C until further use.

2.4 Total Phenolic Content (TPC)

The TPC was determined by a Spectrophotometric method using 1N Folin-Ciocalteu (FC) reagent and 7% sodium carbonate (Jain et al., 2014). The absorbance was recorded at 760 nm. The phenolic content of each sample had been determined from the standard curve of Gallic Acid. The TPC is expressed as milligrams of Gallic Acid Equivalent per gram of Sample (mg GAE/g).

2.5 Total Flavonoid Content (TFC)

The total flavonoid content was estimated by spectrophotometric method, which detects the amount of coloured complex formed between the flavonoids and aluminium ions. The sample was reacted with 2% aluminium chloride and the absorbance was recorded at 368 nm (Pai et al., 2013). The flavonoid content of each sample had been determined from the standard curve of Rutin. The total flavonoid content was thus expressed as milligrams of Rutin Equivalent per gram of sample (mg RE/g).

2.6 Detection and Quantification of Betulinic acid using RP-HPLC

Detection and quantification of betulinic acid was done using reverse-phase HPLC (High Performance Liquid Chromatography). Waters HPLC (Model 2487) instrument with a UV spectrophotometer detector was used. The column was a 15 cm hypersil C18 reverse phase column with 5µ particle packing. The separation was carried out in isocratic mode using acetonitrile and water (85:15) as the mobile phase with the flow rate of 1ml/min and the detection was done at 280 nm.

The standard of BA was prepared in ethanol (1 mg/ml) and it was diluted to 50 ppm. About 20µl of standard was injected. Ethanolic extracts of callus were injected and BA was detected based on its retention time as identified by standard chromatogram. The concentration of BA was estimated from the peak area measurements and the output was given in the units of ppm. The results were converted from ppm to µg/g. All the solvents and chemicals used were of HPLC grade.

III. Data Analysis

The experiment was arranged in a completely randomized design with three replications for each treatment. Data were subjected to analysis of variance (ANOVA) using SPSS (ver. 14, SPSS Inc., Irvine, Calif.) and the treatment means were separated with Duncan's Multiple Range Test(DMRT) at $P<0.05$.

IV. Results and Discussion

Influence of light on secondary metabolism in cell cultures is well known (Ramakrishna and Ravishankar, 2011). The present investigation demonstrated that the light regime did not significantly influence the growth of *Helicteres isora* callus. Under all the light conditions, the calli showed fast growth with RGR values exceeding 200% after 28 days experiment (Table 1). On the other hand, light conditions significantly influenced the TPC, TFC and BA content. The presence of light stimulated the accumulation of examined

compounds. The highest amount of TPC, TFC were found in calli maintained under blue light. Similar to our results beneficial effect of blue light on the accumulation of plant phenolics has also been reported in *in vitro* cultures of *Sausureamedusa* (Guo et al 2007), *C. subternata* (Kokotkiewicz etal.,2014) and *Ruta graveolens* (Szopa et al., 2012).

In the present study, light significantly influenced the accumulation of betulinic acid in callus. Maximum accumulation was recorded in cultures illuminated with white light (Table 1) whereas it was inhibited under dark conditions. Further, accumulation of betulinic acid could be correlated with high growth rate (both achieved after 28 days) in cultures maintained under white light. Higher BA accumulation could be due to white light induced-upregulation of genes involved in BA biosynthesis pathway as explained by Liu et al. (2011) for terpenoid accumulation in *Catharanthus roseus* seedlings.

Table 1: Effect of light regimes on growth and biochemical parameters of callus cultures of *H.isora* L. maintained at control (24°C) temperatur3

Light conditions	Relative growth rate (%)	Total phenol Content (mg GAE g^{-1})	Total Flavonoid Content(mg RE g^{-1}))	Betulinic Acid content (µg g^{-1})
Darkness	327.4[a]	7.58[d]	8.94[c]	Not detected
White light	400[a]	21.43[b]	117.68[a]	6.46[a]
Red	346.8[a]	12.0[c]	98.00[b]	5.03[b]
yellow	309.7[a]	13.76[c]	93.76[b]	5.13[b]
Dark blue	297.5[a]	24.07[a]	120.45[a]	5.33[b]

Means followed by different letters differ significantly at 5% level of probability, DMRT.

High temperature (30 ºC) favoured both calli growth as well as TPC and TFC contents in *H.isora* in the present study. Around two-fold increase in calli Gi and three-fold increase in TPC, TFC was recorded at 30 ºC as compared to control temperature (24° C). Stimulatory effect of high temperature (32°C) on biomass yield and isoflavone content has also been reported in *Puereria candolli* callus(Thanonkeo and Panichayakul, 2006). In contrast to this, Kokotkiewicz et al., 2014) reported inhibition of callus growth in *C. subternata* at 32ºC. BA content was reduced to 39 % at high temperature as compared to control (24 ºC).

Incubation of calli at low temperature showed significant increase in calycosin and its glucoside content in *A. membraceous* seedlings (Pan et al.,2007) and bioflavonoid in *C.ternata* (Kokotkiewicz et al., 2014). In contrast, in the present study exposure of callus to 13 ºC lowered RGR, TPC and TFC and increased betulinic acid content (Table 2). Reduced calli growth indicated sensitivity of calli to temperature changes. Thus, despite the higher production of BA, the above experimental approach has no practical significance. In order to enhance BA accumulation without compromising calli growth, the calli were subjected to low temperature only after 15 days of the incubation at normal temperature (24ºC). This approach proved better as it helped to achieve relatively high growth rate as well as BA accumulation(ca 300%).

Table 2**:** Effect of temperature regimes on growth and biochemical parameters of callus cultures of *H.isora* L. under white light conditions

Temperature (°C)	Relative growth rate (%)	Total Phenol Content (mg GAE g^{-1})	Total Flavonoid Content (mg RE g^{-1})	Betulinic Acid content (µg g^{-1})
30	758.4[a]	52.58[a]	330.94[a]	3.96[c]
24	400[b]	21.43[a]	117.68[a]	6.46[a]
13	86.8[c]	6.20[b]	54.20[c]	21.0[a]
24ºC for first 15 days followed by exposure to 13°C	387.8[b]	16.6[a]	97.54[b]	18.4[b]

Means followed by different letters differ significantly at 5% level of probability, DMRT.

In conclusion, light and temperature strongly influenced the TPC, TFC and betulinic acid content in *H. isora*. The best results in terms of calli growth and BA accumulation were obtained by incubating the cultures at 13ºC during the second half of growth cycle.

References

[1] Cichewicz R. H.; Kouzi S. A. Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. Med. Res. Rev. 24: 90– 114; 2004
[2] Guo B, Liu Y-G, Yan Q, Liu C-Z (2007) Spectral composition of irradiation regulates the cell growth and flavonoid biosynthesis in callus cultures of Saussurea medusa Maxim. Plant Growth Regul 52:259–263.
[3] Jain,A. , Sinha,P. and Desai,N.(2014). Estimation of flavonoid, phenol content and antioxidant potential of Indian screw tree (*Helicteres isora* L.). Int. J. of Pharma. Sci. and Drug Res. 5(4):1320-30.
[4] Kokotkiewicz, A., Bucinski, A. , Luczkiewicz, M. (2014). Light and temperature conditions affect bioflavonoid accumulation in callus cultures of *Cyclopia subternata* ogel(honeybush).Plant Cell Tiss Organ Cult (2014) 118:589.
[5] Kumar, N. and Singh,A.(2014). Plant profile, phytochemistry and pharmacology of Avartani (*Helicteres isora* Linn.): A review,"Asian Pacific J. Trop. Biomed. 4(1):S22-S26.
[6] Loganayaki,N., Siddhuraju,P. and Manian, S.(2013).Antioxidant activity and free radical scavenging capacity of phenolic extracts from *Helicteres isora* L. and *Ceiba pentandra* L,"J. Food Sci. *and* Tech. 50(4):687-695.
[7] Namdeo, A.G. (2007).Plant Cell Elicitation for Production of Secondary Metabolites: A Review. Pharmacognosy Reviews. 1(1):69-79.
[8] Pagi,B. Lahiri SK., Yadav, GK., Mamta. B. (2010). Development and Validation of HPTLC Method for Determination of Betulinic Acid in *Helicteres isora* root Extract Kashmir. Int. J. of Chem.Tech. Res. 2(2): 851-855.
[9] Pan H, Fang C, Zhou T, Wang Q, Chen J (2007) Accumulation of calycosin and its 7-O-b-D-glucoside and related gene expression in seedlings of *Astragalus membranaceus* Bge. var. mongholicus (Bge.) Hsiao induced by low temperature stress. Plant Cell Rep 26:1111–1120.
[10] Ramakrishna A, Ravishankar GA (2011) Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal Behav 6:1720–1731
[11] Szopa A, Ekiert H, Szewczyk A, Fugas E (2012) Production of bioactive phenolic acids and furanocoumarins in *in vitro* cultures of *Ruta graveolens* L. and *Ruta graveolens* ssp. Divaricata (Tenore) Gams. under different light conditions. Plant Cell Tiss Organ Cult 110:329–336.
[12] Thanonkeo S, Panichajakul S (2006) Production of isoflavones,daidzein and genistein in callus cultures of *Pueraria candollei* Wall. ex Benth. var. mirifica. Songklanakarin J Sci Technol28:45–53.