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Different wavelengths light to induce physiological changes callus for the biosynthesis of gymnemic acid in *Gymnema sylvest*

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ABSTRACT: A callus culture of *Gymnema sylvest* was cultivated under different light conditions (blue, white, red and green light). Green-white coloured gymnemic acid containing cell groups were formed on the surface of the callus culture under blue light respectively. The phytohormone kinetin enhanced the gymnemic acid formation in blue light, but was unable to induce gymnemic acid in darkness. The phytohormone 2,4-D induced the formation gymnemic acid under all light conditions studied. Analysis of the callus growth phase under all treatments revealed that gymnemic acid accumulation was maximum (12.22 mg/g d.w) in the callus during stationary phase of 45 days, grown in MS medium with 2,4-D (1.5 mg/l) and KN 0.5 mg/l under white fluorescent light. Blue light enhanced the gymnemic acid accumulation up to 4.4 fold of that found under white fluorescent light and 2.8 fold of that found in intact leaves. Present findings concluded that blue light can be used as tool for enhancing pentacyclic tri-terpenoids in batch culture of *G. sylvest*.

INTRODUCTION

Gymnema sylvest (Retz) R.Br. Ex Roemer & Schultes is a woody climber belonging to the family Asclepiadaceae. It is distributed in Deccan peninsula parts of northern, western India, tropical Africa, Vietnam, Malaysia, Sri Lanka and it is available in Japan, Germany and USA as health food. It is a traditional medicine in India for diabetes mellitus, and used as a stomachic and as a diuretic (1). In Japan, there are *Gymnema* Teas and *Gymnema* chewing gum made from *G. sylvest* leaves which are promoted as a natural method for controlling obesity, insulin secretion, diabetes and inhibiting the taste of sweetness (2). It helps to maintain blood glucose homeostasis through increased serum insulin levels provided by repair or regeneration of the endocrine pancreas (3). Several products are marketed under brand names such as Body Slatto Tea[®], *Gymnema*[®], *Gymnema* Tea[®], *Gymnema* Diet[®], Sugar Off[®], Glucoset[™], Cindrome X[™], and PiliSoft[™]. *Gymnema sylvest* was propagated *in vitro* using axillary node and inter nodal explants; among that axillary node explants showed higher multiple shoots (57.2 shoots/ explant) than inter nodal explants (4). Furthermore, Veerashree et al. (2012) reported that yeast extract, pectin, and chitin elicitors enhanced the gymnemic acid via cell suspension culture in *G. sylvest* (5). Current advances in plant biotechnology make cell and organ culture a promising approach and have been successfully applied to the production of valuable secondary metabolites, including pharmaceuticals, pigments, and other chemicals (6). Light is an important factor affecting growth, organogenesis and the formation of plant products including both primary and secondary metabolites. In addition, light is also involved in regulating the secretion mechanism of secondary metabolites (7). Light irradiation was established in which MS medium supplemented with plant growth regulators was used for callus growth, and modifying the shaking speed, pH and medium play an important role in the production of gymnemic acid (8). A series of experiments were carried out to investigate the influence of different wavelength lights on callus

growth and gymnemic acid accumulation by varying the irradiation period and intensity under light supplied by illumination and radiation energy in the course of cultivation. The present report describes the establishment of callus from leaf explant of *G. sylvest* and the enhancement of gymnemic acid accumulation using various wavelengths of light (blue, red and green light) as an inducing stress factor.

MATERIALS AND METHODS

Plant materials and *In vitro* culture conditions

Gymnema sylvest plants collected from Pachamalai hills, Tiruchirappalli, India were maintained in the botanical garden of Department of Plant Science, Bharathidasan University, Tiruchirappalli. The young leaf explants collected from *G. sylvest* were sterilized with alcohol 70 percent (v/v) for 30 s, 3 percent sodium hypochlorite (v/v) for 2 min and 0.1 percent mercuric chloride (w/v) for 3 min, followed by a three times rinse with sterile distilled water. Sterilized leaf explants were cultured on MS medium (9) supplemented with various concentrations and combinations of growth regulators, i.e. 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KN) in 100 ml culture tubes and were maintained at 25°C under control white fluorescent light (380-780 nm, Philips Company, India; 16 h photoperiod) for callus induction and gymnemic acid production.

IN VITRO CALLUS GROWTH CURVES

Callus cultures were optimized and evaluated quantitatively for their appearance nature, biomass and gymnemic acid content at the end of their respective growth cycle. MS media treating with various combinations of auxins, cytokinins, and in combinations, fresh and dry weight of the calli was determined at 15, 25, 35, 45 and 55 days.

At regular intervals for all treatments, each callus was harvested by careful separation from the media using metal spatulas, and fresh and dry weight was promptly recorded. The optimum concentration (2,4-D 1.5 mg/l + KN 0.5 mg/l) was selected, based on the callus biomass (dry and fresh weight) and callus nature (green compact). Above mentioned optimum concentration was used in further studies. The optimum concentration (2,4-D 1.5 mg/l + KN 0.5 mg/l) initiated calli was maintained under light stress conditions viz. blue light (455-495 nm), green light (495-575 nm), red light (625-780 nm) and white light (380-780 nm) for 55 days to measure the effect on gymnemic acid accumulation in callus culture. The wavelengths light intensities irradiated directly and indirectly passes on the surface of cultivation vessels (culture tube) was measured using a Digital Light Meter (0-50,000 lux, Cole Parmer Company, India) and rate of expression given in wavelength. For determination of fresh weights, the calli were gently pressed onto filter paper to remove excess water and subsequently weighed. They were then dried in an oven at 60°C for 24 h, upon which the dry weight was promptly recorded.

PHYTOCHEMICAL ANALYSIS

Extraction, sample preparation, and chromatographic analyses by HPTLC and HPLC were performed as follows. Briefly, *in vivo* leaf and *in vitro* callus (500 mg d.w) were extracted 5 times with methanol. The combined methanol extract was centrifuged at 5000 × g for 10 min at room temperature, then the supernatant was carefully pipetted into fresh Eppendorf tubes without disturbing the inter-phase residues and centrifuged again. The resulting green-coloured methanol supernatant (4 ml) was evaporated, dried and the residue obtained (ca. 6 mg) was redissolved in MeOH (5.0 ml), of which 20 µL were subjected to HPTLC and HPLC analysis, respectively, using authentic gymnemic acid as a standard. HPTLC (high performance TLC; Camag, Switzerland) was equipped with a sample applicator Linomat IV for quantification of gymnemic acid. HPTLC plates are characterized by smaller particles ($\leq 10\mu\text{m}$), thinner layers ($\leq 150\mu\text{m}$) and smaller plates ($\leq 10\mu\text{m}$ developing distance). In addition, the particle size distribution of the sorbent is narrower than for conventional TLC layers. EMD Silica Gel 60 F254 fluorescent TLC plate and developed in a TLC chamber using the appropriate mobile phase. 10 samples were applied on each HPTLC (10 cm) at a start line 8 mm from the bottom of TLC plate, including eight lanes (each lane occupy 3 mm) of *in vitro* callus and one lane of *in vivo* leaves with standard gymnemic acid (1 mg/ml of methanol) (20 µL) one land. During experiment, each lane distance was 6 mm exactly using for successful separation. 10 samples were applied on each plate at a start line 8 mm from the bottom, including nine lanes of *in vitro* callus and *in vivo* leaves with standard gymnemic acid (20 µL). The mobile phase (isopropyl alcohol: chloroform: methanol: acetic acid 5: 3: 1: 0.5; v/v/v/v) was allowed to run up to 80 mm, and detection was by UV light at a wavelength of 200 nm using a TLC scanner III, integration and quantification was performed using CAT 4.0 software. Methanol extracts of *in vivo* leaves and *in vitro* callus culture extracts were analysed by HPLC. This system consisted of a two 510 Pumps, a 7725 Rheodyne auto injector, a DUG-12 A degasser, SCL-10 Avp system controller and a Spectromonitor 486 wavelength UV/VIS detector. Calibration curves were established using purified gymnemic acid (obtained from Prof. Yoshikawa, Kyoto Pharmaceutical University, Japan) as a standard. Gymnemic acid determination was performed according to a method described by Imoto et al. (1991) (10). HPLC analysis of intact leaves and callus sample (20 mL) was carried out with the following conditions: Water HPLC system (Shimadzu model, Kyoto, Japan), C₁₈ (ODS) reverse phase column (150 mm X 4.6 mm i.d., 5 µM particle size),

mobile phase - water / methanol (35:65, v/v), 0.1% (v/v) acetic acid, flow rate: 1 ml/min, detection was by UV at a wave length of 230 nm.

RESULT AND DISCUSSION

A balanced combination of plant growth regulator plays an important role in regulating cellular and subcellular differentiation (11). Callus cultures were initiated from leaves of *G. sylvestre* and grown on MS solid medium supplemented with various concentrations and combinations of auxins and cytokinins. Successful biomass was obtained in MS medium supplemented with 2,4-D (1.5 mg/l) and KN (0.5 mg/l) of heterogeneous callus (hard cells) showed green compact callus (144 mg/l dry weight) and can't be separated easily by metal spatula. Whereas NAA (1.0 mg/l) and KN (1.5 mg/l) were produced green compact with white friable callus (139 mg/l dry weight) and showed homogenous callus (free cells) can be separated easily (Figure 1).

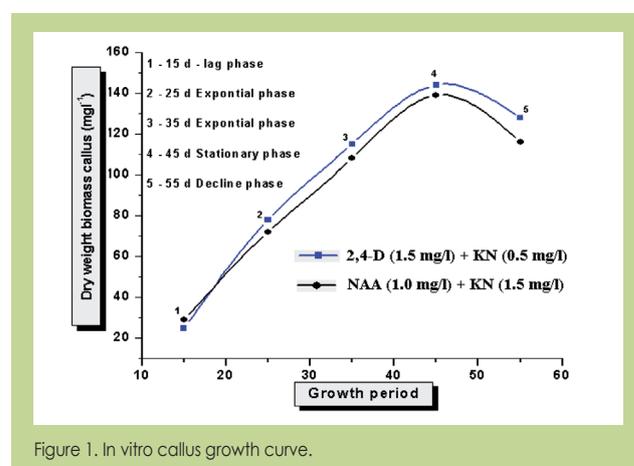


Figure 1. In vitro callus growth curve.

Among the various concentrations of auxins and cytokinins tried in callus induction, the optimum concentrations of 2,4-D (1.5 mg/l) with KN (0.5 mg/l) induced green compact natured callus with a maximum biomass at 45 days in terms of fresh and dry weight. *G. sylvestre* callus growth curve was sigmoid type and four growth phases can be distinguished in different days (15, 25, 35, 45 and 55 days). In the log phase (0-15 days), *in vitro* callus was slowed at the initial state; the callus biomass was drastically reduced over the other phase. In the lag phase (15-25 days; 91 mg/l dry weight), callus initiation and proliferation were observed by profound cell division. At 25-35 days (exponential phase), biomass (105 mg/l dry weight) with green compact nature was significantly increased. The maximum level of callus biomass (144 mg/l dry weight) in the stationary phase (35-45 days) of the callus growth suggests the cellular membrane stabilization. At the decline phase (45-55 days) the callus

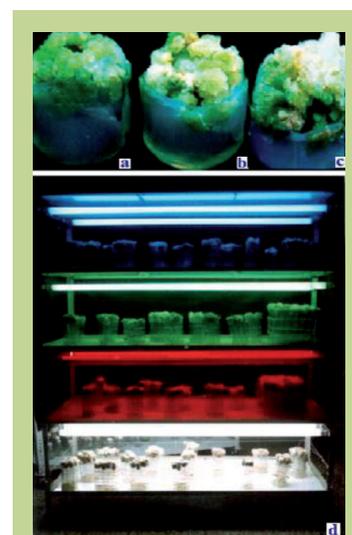


Figure 2. Callus growth on different wavelengths light stresses. a. Blue light (1.8x); b. Red light (1.8x); c. Green light (1.6x); d. Wavelengths light experiment setup.

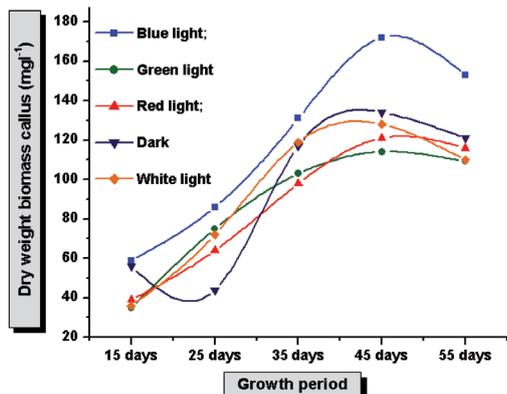


Figure 3. Wavelengths light callus biomass growth curve.

biomass was drastically reduced (119 mg/l dry weight) as compared to other phases. Light irradiation has remarkable effect on plant cell and tissue growth and secondary metabolite biosynthesis. The action of light on higher plants occurs mainly two aspects. First, light provides the energy source required by plant through photosynthesis. Second, light is a signal received by photoreceptor to regulate the growth and differentiation and metabolism (12). In the present study, light irradiation (blue, red, green and white light) affected callus morphogenesis of *G. sylvestre* leaf (Figure 2). Under different wavelengths light, the MS medium supplemented with optimum concentrations (2,4-D (1.5 mg/l) + KN (0.5 mg/l) showed higher biomass at stationary phase of 45 days (Figure 3). Cryptochrome is a technique and the wave length (450 nm) was closed to the blue light, and this treatment more Pr (blue form that absorbs red light) transformed into Pfr (blue-green form that absorbs far-red light) for phenylethanoid glycoside

production (13). Red light was drastically reduced the callus growth and the biomass showed in sigmoid order 31 mg/l, 59 mg/l, 85 mg/l, 122 mg/l and 102 mg/l of dry weight could be registered at 15, 25, 35, 45 and 55 days. Hence, green light treated to the MS medium supplemented with optimum concentrations induced 116 mg/l of dry weight at 45 days of culture. In addition, the callus cell size was higher in blue light than red light followed by green light. Green and red light induced callus was green friable and showed lesser biomass (dry weight) than blue light. In specific blue light (cryptochrome) stimulates chlorophyll, carotenoid synthesis, activation of gene expression and increase phenyl alanine ammonium lyase activity (14). White light induced green compact callus and increased biomass than green and red light. Dark treatment yielded white friable callus and biomass drastically reduced than blue, red, green and white light treatment. In HPTLC, the samples analysed time was shorter (6 min), when compared to TLC (40 min). Gymnemic acid (Figure 4) successfully separated in the following mobile phase of isopropyl alcohol: chloroform: methanol: acetic acid (5:3:1:0.5; v/v/v/v). *In vivo* leaf and *in vitro* callus extracts sample curve was linear; the correlation coefficients had good linearity between concentration and area, it could be helpful to calculate the gymnemic acid content in the respectable sample.

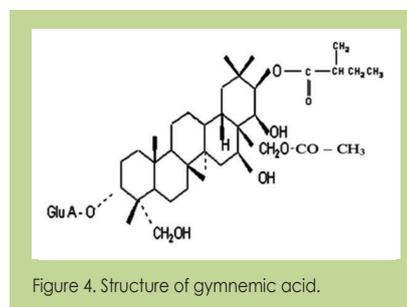


Figure 4. Structure of gymnemic acid.

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In our study, NAA (1.0 mg/l) and 2,4-D (1.5 mg/l) are combined with KN (0.5 mg/l) increased the gymnemic acid content, when compared to auxins alone determined by Rf value (ratio-to-front) of HPTLC. Gymnemic acid was significantly increased in the MS medium supplemented with auxins and cytokinins where concentrations derived from leaf explants of *G. sylvestre* were determined in HPTLC (15). For HPLC analysis, *in vivo* leaf and *in vitro* callus methanol extracts (20 µl) were uploaded in HPLC system to quantify gymnemic acid under retention time. UV spectrophotometer peak area data were compared with standard gymnemic acid. The gifted gymnemic acid (standard) had shown single peak (Figure 5) in the following mobile phase 0.1 percent acetic acid; water/methanol (v/v) (35:65 HPLC grade) and the stability and impurity were characterized at initial, middle and end of HPLC experiment.

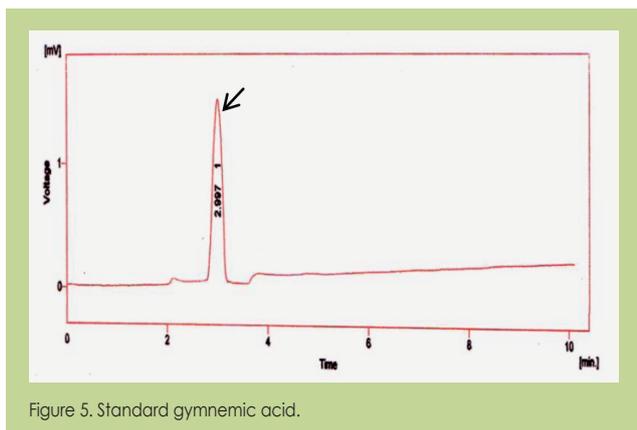


Figure 5. Standard gymnemic acid.

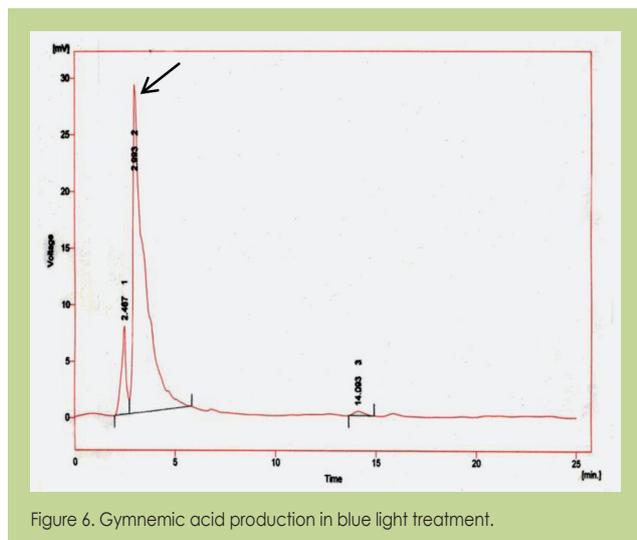


Figure 6. Gymnemic acid production in blue light treatment.

Our study described, the gymnemic acid content was significantly increased in *in vivo* leaf extracts (19.52 mg/g dry weight) than *in vitro* callus of MS medium supplemented with optimum concentrations (2,4-D (1.5 mg/l) + KN (0.5 mg/l); 12.22 mg/g dry weight). Maximum gymnemic acid content was observed in optimum concentration with blue light treatment (53.94 mg/g dry weight; Figure 6) followed by red light (8.90 mg/g dry weight; Figure 7) and green light (5.72 mg/g dry weight; Figure 8).

In this paper, we demonstrated that in *Gymnema sylvestre* the gymnemic acid accumulation was increased when the callus was treated with 2,4-D 1.5 mg/l + KN 0.5 mg/l and further enhanced when the callus was grown under blue light. Thus it appears that blue light may be used as a tool for enhancing secondary metabolite accumulation in callus culture. Although the precise mechanism as to how these factors affects gymnemic acid production remains to be determined, a

combination of these factors used for production of valuable compounds via *in vitro* abiotic stresses is a promising strategy. The above results will be useful in designing systems for the large-scale production of gymnemic acid.

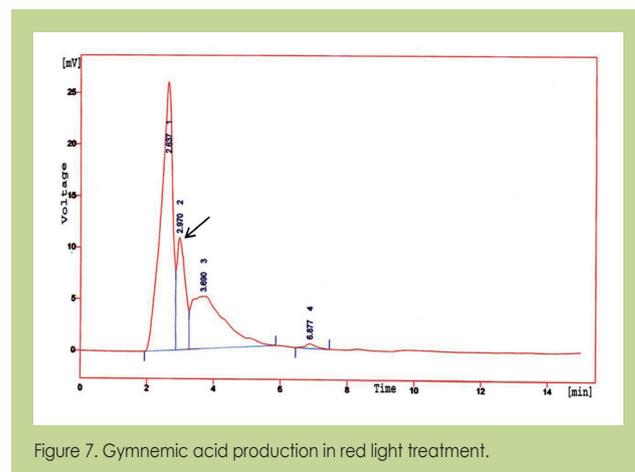


Figure 7. Gymnemic acid production in red light treatment.

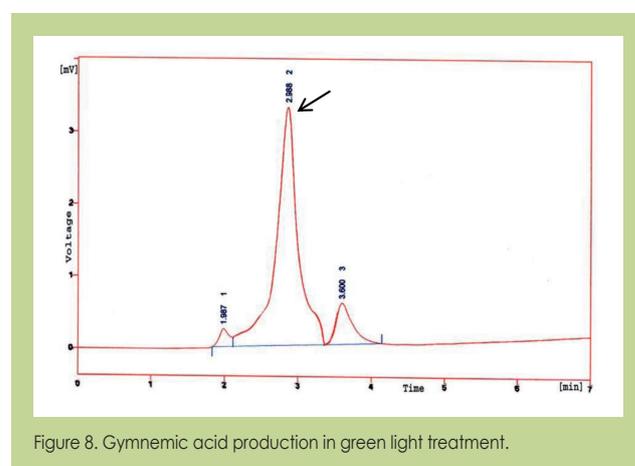


Figure 8. Gymnemic acid production in green light treatment.

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