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Light-induced biochemical variations in secondary metabolite production and antioxidant activity in callus cultures of *Stevia rebaudiana* (Bert)

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ABSTRACT

Stevia rebaudiana (*S. rebaudiana*) is a very important species with worldwide medicinal and commercial uses. Light is one of the major elicitors that fluctuate morphogenic potential and biochemical responses. In the present study, we investigated the effect of various spectral lights on biomass accumulation and secondary metabolite production in callus cultures of *S. rebaudiana*. Leaf explants were placed on Murashige and Skoog (MS) medium and exposed to various spectral lights. 6-Benzyle adenine (BA) and 2, 4-dichlorophenoxy acetic acid (2, 4-D; 2.0 mg l^{-1}) were used for callus induction. The control light (16/8 h) produced optimum callogenic response (92.73%) than other colored lights. Compared to other colored lights, control grown cultures displayed maximum biomass accumulation (5.78 g l^{-1}) during a prolonged log phase at the 18th day of growth kinetics. Cultures grown under blue light enhanced total phenolic content (TPC; 102.32 μg/g DW), total flavonoid content (TFC; 22.07 μg/g DW) and total antioxidant capacity (TAC; 11.63 μg/g DW). On the contrary, green and red lights improved reducing power assay (RPA; 0.71 Fe(II) g^{-1} DW) and DPPH-radical scavenging activity (DRSA; 80%). Herein, we concluded that the utilization of colored lights is a promising strategy for enhanced production of antioxidant secondary metabolites in callus cultures of *S. rebaudiana*.

1. Introduction

Stevia rebaudiana is one of the medicinal and emerging commercial herbs in the Asteraceae family that is cultivated worldwide [1]. The medicinal importance of *S. rebaudiana* can be judged from "yerba mate" (herbal remedy for heartburn) that is used by the Guarani tribes of Paraguay and Brazil since a long time ago [2]. During plant development, the leaves accumulate steviosides which add 300 times more sweetness than normal sugar [3,4]. Among different compounds in the leaves of *S. rebaudiana*, stevioside is one of the sweetest compounds present in larger quantities and nowadays commonly used in various commercial products [5]. As compared to glucose, there are no receptors for stevioside content that is why it is considered as zero caloric. Therefore, steviosides are very useful for diabetic patients and also helpful in weight reduction.

Various in vitro cultures have been exploited for stevioside production [6,5,7]. Callus culture is a more effective substitute compared to whole micropropagation for accumulation of secondary metabolites. However, the biosynthetic pathways of these secondary metabolites are markedly influenced by various elicitors. The addition/exposure of these elicitors to culture media may modulate the production of secondary metabolites. Mostly, the abiotic and biotic stresses alter the accumulation of bioactive compounds in higher quantities as compared to naturally growing plants [8].

Among various elicitors, light quality/quantity greatly influence plant architecture development, morphogenetic responses and synthesis of valuable bioactive compounds [9]. Optimum light intensity and selective wavelength enhanced the production of diosgenin in callus cultures of *Dioscorea deltoidea* [10]. However, the physiological and morphological responses of plants towards light quality are greatly varied depending upon the plant species [11]. Tariq et al. [9] have reported a significant variation in both morphogenic and biochemical attributes in callus cultures of *Artemisia absinthium* subjected to various monochromatic lights. It is a well known fact that light plays a key role in primary and secondary metabolism and various plant developmental processes [12–14]. Many reports have suggested that light sources directly stimulated the production of important secondary metabolites including anthocyanins, artemisinin, caffeic acid derivatives and flavonoids [12,15–17]. The inhibitory effects of light on nicotine and shikonin production were also reported [18]. Besides its synergistic/antagonistic effects on secondary metabolites, light also plays a key role in regulating the secretion mechanism of secondary metabolites [11].

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Medicinal plants are small natural factories that synthesize and release various bioactive compounds under specific conditions. These plant based compounds are gaining more attention as potential drugs, nutraceuticals, pharmaceuticals and food additives [19]. Plants under stress conditions either release antioxidative enzymes or phenolics and flavonoids as a defense system [20]. Plant polyphenols represent the principal group of natural antioxidants among various classes of secondary metabolites that are considered to be more valuable as compared to carotenoids and vitamins [21]. Plant based flavonoids are well reputed for their antioxidant properties due to their redox capacity. It has been implicit that a flavonoid rich diet inversely affects lipid peroxidation, cell aging and cancer [22]. These phenolics and flavonoids play a key role in scavenging toxic reactive oxygen species (ROS) [23]. These compounds have numerous uses in pharmacological activities like antioxidant, anti-carcinogenic, to cure cardiovascular diseases and promote the immune system by ingesting it directly or indirectly from various medicinal plants [24]. In vitro cultures are among the best options for production of antioxidant compounds [25]. Antioxidants are compounds having the ability to block or minimize the oxidation process through inhibition of the initiation of oxidizing chain reactions [26].

To the best of our knowledge, there are no previous reports regarding the effect light illumination of specific wavelength on callus induction, biomass accumulation and production of antioxidant secondary metabolites in *S. rebaudiana*. Therefore, the main objective of the present study was to evaluate the proliferation of callus cultures under various sources of illumination having different wavelengths, suitability of a specific spectrum for a particular biochemical response and correlation between total phenolics, flavonoids and antioxidant activities.

2. Materials and Methods

2.1. Leaf Explant Collection and Sterilization

Fresh leaves were excised from 50-day old nursery grown plants of *S. rebaudiana* at Ground and Garden Nursery, Department of Horticulture, The University of Agriculture Peshawar. Leaves were gently dipped in autoclaved distilled water to retain viability. These leaf explants were surface decontaminated according to the protocol of Aman et al. [6]. Healthy leaves were exposed to 70% ethanol (1 min) and 0.2% mercuric chloride (2 min) to remove surface contaminants. Subsequently, the leaves were rinsed three times with sterile distilled water to minimize the content of mercuric chloride and ethanol. These surface sterilized explants were dried on sterilized filter paper to remove surplus water.

2.2. Establishment of Callus Cultures Under Different Colored Lights

To study the effect of various spectral lights on callogenic frequency, approximately 3–4 mm^2 leaf pieces were inoculated on Murashige and Skoog (MS; Phytotechnology Lab, USA) [27] media augmented with BA (2.0 mg l^{-1}) and 2, 4-D (2.0 mg l^{-1}) (from the experiment of Aman et al. [6]). MS-media without plant growth regulators (PGRs) was used as the control (MS0). The MS-media was supplemented with 3% sucrose (Merck), solidified with 8 g l^{-1} agar (Agar Technical LP0013, Oxoid, Hampshire, England), the pH was adjusted to 5.8 by using a pH meter (Eutech Instruments pH 510, Singapore) and finally all the media were autoclaved (Systec VX 100, Germany) at 121 °C for 25 min. The different monochromatic colored lights used were: green lights (40 W Litex; 480–670 nm), yellow lights (36 W, Philips Ltd.; 530–780 nm), blue lights (220 V; 50 Hz, Keliang Ltd.; 380–560 nm) and red lights (25 W, BINXIANG; 610–715 nm), while white fluorescent tube lights (20 W, Toshiba FL20T9D/19; 380–780 nm) with a 16/8 h photoperiod and light intensity ranges from ~40–50 μmol m^{-2} s^{-1} were used as the control. These cultures were maintained in a growth room at a temperature of 25 ± 1 °C. Each treatment was divided into three independent experiments. Each experiment was designed on Completely Randomized Design (CRD). After 30 days of callus establishment, the averages were randomly recorded using each replication as % callus induction.

2.3. Callus Growth Kinetics and Biomass Accumulation

During growth kinetics, data was collected from 30 day old culture with 3 days interval. A growth curve was established for the rapidly growing callus biomass in response to each colored light. After 30 days of culture establishment, fresh callus was harvested from solid media for determination of fresh weight (FW). After FW determination, the callus was dried at 50 °C in an oven (Thermo Scientific; Germany) and subsequently the dried weight (DW; Sartorius digital balance; Germany) was determined (Fig. 1). Fresh and dry weights of the calli were expressed in gram/liter (g l^{-1}).

3. Analytical Methods

The dried calli obtained from various colored lights were powdered by using a mortar and pestle for extract preparation. The total phenolic content (TPC) was determined according to the protocol of Ahmad et al. [23]. During phenolic determination, 0.03 ml of extract and 0.1 ml of Folin–Ciocalteu reagent were slowly mixed with 2.55 ml sterile water. Before incubation in the dark for 30 min, the mixture was centrifuged at 10,000 rpm for 15 min. The supernatant was collected and passed through 45 μm membrane filter paper in a UV visible spectrophotometer cuvette (Shimadzu-1650; Japan). Gallic acid (Sigma; 1.0–10 mg ml^{-1}) was used for standard curve establishment. The absorbance of each callus extract and gallic acid was monitored at 760 nm. The results were expressed as GAE mg/g DW of callus. The total flavonoid content (TFC) was determined according to the protocol of Ahmad et al. [23]. For flavonoid determination, 0.25 ml extract, 0.075 ml $AlCl_3$ (5% *w/v*) and 0.5 ml NaOH were slowly mixed with 1.25 ml sterile water. The mixture was centrifuged at 10,000 rpm for 15 min and then kept in the dark for 30 min. The absorbance of each sample was measured at 510 nm with a UV–visible spectrophotometer. Rutin (Sigma; 1.0–10 mg ml^{-1}) was used for establishment of the standard calibration curve. The total flavonoid content was expressed as RE mg/g DW of callus.

DPPH-radical scavenging activity (DRSA) in different callus cultures exposed to colored lights was determined according to the protocol of Ahmad et al. [26]. Briefly, 5 mg of each callus extract was independently dissolved in 20 ml HPLC grade methanol. The DPPH solution was

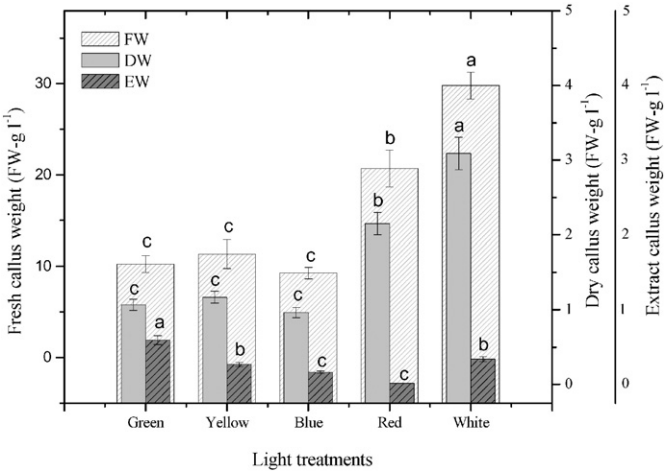

Fig. 1. Fresh weight, dry weight and extractive values of callus cultures exposed to different spectral lights. Data was collected from three replications. Mean values (±SE) with common alphabets are significantly different at $P < 0.05$.