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Light-induced fluctuations in biomass accumulation, secondary metabolites production and antioxidant activity in cell suspension cultures of *Artemisia absinthium* L.

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ABSTRACT

Light is an important factor influencing plant morphogenesis and biochemical pathways, including biosynthesis of primary and secondary metabolites. In the present study, we investigated the differential effect of light on biomass accumulation and secondary metabolites production in cell suspension cultures of *Artemisia absinthium* L. A prolonged log phase of 21 days was followed by light-grown cultures. Light-grown cultures displayed 3.9-fold maximum increase (8.88 g/l) in dry biomass on day 30 of culture which was comparable to 3.7-fold maximum increase (9.2 g/l) on day 27 in dark-grown cultures. Compared to dark grown-cultures, enhanced levels of total phenolic content (5.32 mg/g DW), total phenolic production (42.96 mg/l) and total secondary metabolites (6.79 mg/g) were found in light-grown suspension cultures during the log phase of growth. Further, a positive correlation among maximum levels of antioxidant activity (63.8%), total phenolic production (42.96 mg/l) and total secondary metabolites (6.79 mg/g DW) was displayed by light-grown suspension cultures.

1. Introduction

Artemisia absinthium L. (wormwood) is a well known medicinal plant, mentioned in almost all books of herbal medicine in the Western world [1]. It has been considered as a "general remedy" for all diseases and was referred to as "A herb of Mars" for its over-arching medical powers [2]. The plant has traditionally been used as anti-helmintic, choleretic, antiseptic, balsamic, depurative, digestive, diuretic, emmenagogue and in treating leukaemia and sclerosis [3]. Phytochemical studies have shown the presence of terpenes, antioxidant phenolics, flavonoids, and other biologically active compounds [4]. The dry leaves and stem contain, among others, 0.25–1.32% essential oil, absinthin, anabsin, artemisinin, anabsinthin, artabsin, and matricin [5].

Plant secondary products are of immense use as potential drugs, nutraceuticals, and food additives [6]. Among different classes of secondary metabolites, plant polyphenols constitute the largest group of natural antioxidants [7]. These compounds posses biological properties like antioxidant, anti-aging, anti-carcinogen, protection from cardiovascular, immune/autoimmune diseases and brain dysfunctions viz. Parkinson's, Alzheimer's, Huntington's diseases, etc. [8,9]. Phenolics may have a direct contribution in the antioxidant activity [10]. The antioxidant potential in various medicinal plants has been shown mainly due to phenolic compounds [11–16]. Likewise, different species of the genus *Artemisia* including *A. absinthium* L. have shown phenolics associated antioxidant activities [4,17–19]. Furthermore, the importance of flavonoids as antioxidants and their role in antimalarial and anticancer activities of *Artemisia annua* has been reviewed [20].

Cell suspension cultures offer a simple system to study growth and production kinetics that can help to evaluate and implement optimal conditions for the production of a number of high value medicinal compounds in good quantities [21]. Light plays an important role in almost all plant developmental processes and provides the fundamental building blocks for growth, development, primary and secondary metabolism [22–24]. Secondary metabolites production can be efficiently stimulated by optimizing *in vitro* conditions including light sources [25]. The stimulatory effects of light on accumulation of secondary metabolites, including flavonoids [26], anthocyanins [27], artemisinin [28] and caffeic acid derivatives [22] have been shown. On the other hand, the inhibitory effects of light on the accumulation of secondary metabolites such as nicotine and shikonin [29] were also reported. In addition to its stimulatory and inhibitory effects on secondary metabolites, light is also involved in regulating the secretion mechanism of secondary metabolites [30].

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The exploitation of *in vitro* cultures for secondary metabolites enhancement is a promising approach to overcome various limitations posed by wild plants. The present study was aimed to investigate the effect of light on total phenolic and total flavonoid accumulation and to find out a correlation of these secondary metabolites with antioxidant activities in cell suspension cultures of *A. absinthium* L. under continuous light conditions.

2. Materials and methods

2.1. Cell suspension cultures establishment

Cell suspension cultures were established as has been described in our previous study [31]. Briefly, leaf explants from 28-day old seed derived plantlets were cultured on Murashige and Skoog (MS) [32] medium supplemented with TDZ 1.0 mg/l and NAA 1.0 mg/l to obtain friable calli. To prepare inoculum culture, 35-day old yellowish friable calli were cultured in liquid MS media with the same composition of plant growth regulators. The cultures were placed in gyratory shaker (25 °C, 120 rpm) in dark for the development of stock cell suspension cultures. Fine cell suspension cultures were collected after a period of 14 days. Subsequent experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml MS media with 30 g/l sucrose, 1.0 mg/l TDZ and 1.0 mg/l NAA in combination and 1.5 g fresh cell suspension was inoculated in each flask. The same protocol was repeated for the establishment of inoculum and cell suspension cultures in continuous light. Observations and data recording of the growth kinetics were performed with an interval of 3 days for 39 days period. Triplicate flasks were used in all experiments.

The pH of all media was adjusted to 5.8 (Eutech Instruments pH 510, Singapore) before autoclaving (121 °C, 20 min, Systec VX 100, Germany). To establish suspension cultures in light, cultures were placed in continuous light with intensity of 40 μmol m^{-2} s^{-1} and temperature in the shaker was maintained at 25 ± 1 °C. Murashige and Skoog basal medium (MS0) was used as control.

2.2. Analytical methods

To investigate dry biomass accumulation (DBM), each cell suspension culture obtained with an interval of 3 days in response to continuous light and dark was oven dried (60 °C, 24 h) over a 39 days period.

Dried cell suspension culture samples were extracted as has been described previously [31]. Briefly, each finely ground dried sample (100 mg) was mixed with 80% (v/v) methanol (10 ml). The mixtures were sonicated (10 min; Toshiba, Japan) 3 times with a resting period of 30 min in between and centrifuged (8000 rpm, 10 min). The supernatants were collected and either immediately used for analysis or stored at 4 °C.

Total phenolic content was determined using Folin–Ciocalteu reagent according to the protocol of Velioglu et al. [33]. Absorbance was measured at 725 nm using UV/VIS-DAD spectrophotometer (Halo DR-20, UV–VIS spectrophotometer, Dynamica Ltd., Victoria, Australia). The calibration curve (0–50 μg/ml, R^2 = 0.968) was plotted using gallic acid as standard and the TPC was expressed as gallic acid equivalents (GAE)/g of dry weight.

Total flavonoid content was determined using aluminum chloride colorimetric method as described by Chang et al. [34]. Absorbance of the reaction mixtures was measured at 415 nm using UV/VIS-DAD spectrophotometer. The calibration curve (0–40 μg/ml, R^2 = 0.998) was plotted using quercetin as standard. The TFC was expressed as quercetin equivalents (QE)/g of dry weight.

For antioxidant activity determination, the DPPH free radical scavenging assay (RSA) as described by Abbasi et al. [35] was used. Absorbance of the mixtures was recorded at 517 nm by spectrophotometer. For background correction, a methanolic solution of DPPH that had decayed with no resultant purple color (2 mg of butylated hydroxyanisole (BHA) dissolved in 4 ml of methanol with 0.5 ml of DPPH solution added) was used instead of pure methanol. The radical scavenging activity was calculated by the following formula and expressed as %age of DPPH discoloration:

$$\% \text{ scavenging DPPH free radical} = 100 \times (1\text{-AE/AD})$$

where AE is absorbance of the solution when an extract was added at a particular concentration and AD is the absorbance of the DPPH solution with nothing added.

2.3. Experimental design and data analysis

All experiments were conducted in a completely randomized design and were repeated twice. Each treatment was consisted of three replicates. Mean values of various treatments were subjected to analysis of variance (ANOVA) and significant difference was separated using Duncan's Multiple Range Test (DMRT). SPSS (Windows version 7.5.1, SPSS Inc., Chicago) was used to determine the significance at $P < 0.05$. Pearson correlation coefficients were determined using GraphPad Prism 5.01. Figures were generated using Origin 8.5.

3. Results and discussion

3.1. Effect of light on biomass accumulation

Cell suspension cultures of *A. absinthium* L. established under continuous light followed relatively longer lag and log phases of 9 and 21 days, respectively, over a 39 days culture period, compared to cultures established under continuous dark. Maximum dry biomass accumulation of 8.88 g/l was observed on day 30 which was comparative to dry biomass of 9.03 g/l on day 30 under continuous dark (Fig. 1).

Starting with the initial values of 2.27 g/l and 2.47 g/l on day 0, almost doubling in dry biomass with the values 4.7 g/l and 5.8 g/l was observed on day 15 under light and dark, respectively. However, log phase under continuous light displayed relatively lower values for dry biomass until the start of stationary phase. Stationary phases of both cultures showed significantly similar dry bio-

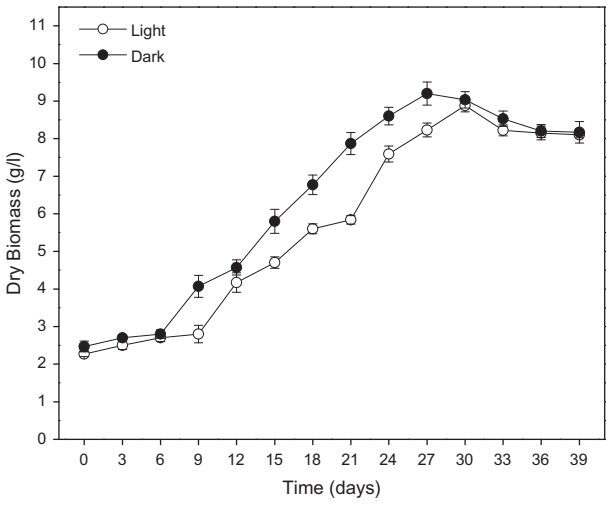

Fig. 1. Biomass accumulation of cell suspension cultures of *Artemisia absinthium* L. on MS medium supplemented with 1.0 mg/l TDZ + 1.0 mg/l NAA under continuous light and dark. Values are mean ± standard error of three replicates.