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Optimization of wedelolactone accumulation in shoot cultures of *Eclipta alba*

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Influence of different environmental factors on accumulation of wedelolactone, a potential anti-hepatotoxic principle of *E. alba* in shoot cultures was investigated. A significant increase in the content of wedelolactone due to kinetin treatment, temperature change and photoperiod alteration in shoot cultures was observed. Incorporation of phenylalanine in the medium also increased content of wedelolactone significantly in a dose-dependent manner.

Keywords: Kinetin, Phenylalanine, Phytohormones, Wedelolactone

Plants produce secondary metabolites not only for their survival but also for maintaining an ecological balance. Accumulation of these compounds is generally affected by biotic/abiotic signals. It becomes a difficult task to assess the effect of various epigenetic factors as regulatory controls in formation and accumulation of secondary metabolites of any plant in the cultivation field. Attempts have, therefore, been made constantly, to utilize *in vitro* culture techniques to unfold the epigenetic factors that influence the synthesis of medicinally important constituents of medicinal plants as there exists scanty knowledge on how to enhance the content of phytochemicals by manipulation of environment[1].

The whole dried herb powder of *Eclipta alba (Asteraceae)* is used in liver disorders especially in jaundice in Indian traditional medicines. *E. alba* grows in tropical and subtropical countries up to an altitude of 2000 meters and is one of the major ingredients of herbal formulations used clinically in the treatment of hepatic disorders in India[2], which contain wedelolactone as a main anti-hepatotoxic compound[3]. Reports on determinations of wedelolactone in micropropagated shoots and its isolation using preparative TLC and estimation in callus cultures by UV spectroscopy is available in literature[4,5]. Another report is also available on indirect organogenesis from callus derived from leaf, stem and root and presence of wedelolactone in regenerated shoots only, while it is absent in roots[6]. *In vitro* micropropagation of *E. alba* by single step nodal cutting technique to maintain clonal fidelity has also been reported[7]. Till date, however, no report is available to pinpoint the empirical variants that may affect the *in vitro* accumulation of wedelolactone in shoot cultures. Therefore, the present study was planned to identify different empirical factors that are involved in wedelolactone production and also to estimate it by sensitive HPTLC method.

Plant material—Nodal segments of *E. alba* were collected from the plants growing in the campus of the institute at 08 : 30 am and used as initial explants. The explants were sterilized using 0.1% (w/v) mercuric chloride solution with a drop of Tween-80 for 2 to 3 min and then washed thoroughly with sterile distilled water.

Generation of control cultures—The shoot cultures were raised from sterilized nodal explants on MS (Murashige and Skoog) medium supplemented with BAP (4.4 μM) and kept under 16/8 hr photoperiod (3000 lux) at 25°±2°C with periodical subculturing, once in a month[4]. *In vitro* shoots were harvested after 60 days, dried at 40°C, powdered and then analyzed for wedelolactone content, which served as control.

Effect of different epigenetic factors on wedelolactone production—The sterilized nodal segments were placed in 50 ml solutions of different concentrations (2.5, 4.5 and 13 μM) of various hormones such as kinetin, indole-3-acetic acid and naphthylacetic acid for 2 hr and shoot cultures were raised as mentioned above.

For temperature alteration study, *in vitro* shoot cultures were grown for 45 days under similar conditions as control cultures, and then cultures were allowed to grow at 20°, 30° and 37°C for next 15 days.

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In another set of experiments, *in vitro* grown shoot cultures were raised as per the reported method[4] for 30 days and then transferred to fresh medium of same composition, which were allowed to grow under three different photoperiods (continuous dark, 12 hr dark/light and continuous light cycles; 3000 lux) for another 30 days.

Effect of *p*H on wedelolactone accumulation was also studied by growing shoot cultures in media at *p*H 5.5, 6.0 and 6.2 following culture conditions as mentioned for control culture.

Effect of different amino acids on wedelolactone accumulation was also studied. Shoot cultures were generated in three separate media supplemented with 50 mg/l of phenylalanine, tryptophan or glycine.

In order to find out the effect of carbon source on wedelolactone formation, shoot cultures were generated with addition of fructose and glucose separately in the medium.

In all the above, shoot cultures were raised and maintained as per the established method using 10 replicates for each concentration and then analyzed for wedelolactone content after 60 days.

Estimation of wedelolactone by HPTLC—Methanol solution of reference standard of wedelolactone (Courtesy M/s Natural remedies, Bangalore, India) was applied (25- 75 ng/spot) on a pre-coated silica gel G 60 plate (E. Merk), using CAMAG TLC automatic sample applicator. The plate was developed in toluene: methanol: formic acid (11: 6: 0.1) as mobile phase. The plate was then dried and scanned at 254 nm using CAMAG TLC scanner. A calibration curve was plotted using peak areas versus concentration of wedelolactone.

Preparation and quantification of wedelolactone in test sample—*In vitro* shoots were harvested after 60 days and dried at 40°C, dry powder (100 mg) was extracted with methanol (10 ml) and 10 µl of methanol extract was applied (band width: 5mm, distance between the bands: 5 mm) on a pre-coated silica gel G60 HPTLC plate (E- Merk). The plate was developed using 10 ml of toluene: acetone: formic acid (11: 6: 0.1) as mobile phase, dried and then scanned at 254 nm using CAMAG TLC scanner and peak areas were recorded. Wedelolactone was quantified in test samples from calibration curve.

The determinations were made on 60 days old *in vitro* shoots, because tissues of young plants often contain relatively high levels of secondary metabolites[8]. All the results have been presented in Table 1. Production of secondary metabolites is greatly influenced by environmental, nutritional, and hormonal factors as anthraquinones synthesis was altered significantly in cell cultures[9].

When nodal explants were treated with different concentrations of kinetin, indole-3-acetic acid and naphthylacetic acid for 2 hr, a significant increase in wedelolactone production was observed. It was also noted that the increase in wedelolactone content was directly proportional to the concentration of kinetin and shoots grown were apparently found more elongated

Table 1 — Percentage of wedelolactone production in the shoot cultures in MS medium containing BAP 4.4 µ*M*

[Values are mean ± SD of 3 readings]

Treatment	% Wedelolactone produced
Hormone (µ*M*)	
Kn	
2.5	0.080 ± 0.009
4.5	0.092 ± 0.007*
13.0	0.112 ± 0.008**
IAA	
2.5	0.080 ± 0.004
4.5	0.081 ± 0.006
13.0	0.080 ± 0.001
NAA	
2.5	0.080 ± 0.007
4.5	0.080 ± 0.008
13.0	0.080 ± 0.006
Amino acids Conc. (50 mg/l)	
Phenylalanine	0.116 ± 0.009***
Tryptophan	0.081 ± 0.007
Glycine	0.080 ± 0.003
Temperature	
20° ± 2°C	0.080 ± 0.008
30° ± 2°C	0.091 ± 0.001*
37° ± 2°C	0.110 ± 0.008**
Carbohydrate (3%)	
Sucrose	0.083 ± 0.003
Fructose	0.071 ± 0.003
Glucose	0.062 ± 0.004
Photoperiod	
Dark	0.080 ± 0.005
12 dark / 12 light	0.093 ± 0.006*
Light	0.080 ± 0.009
***p*H**	
5.5	0.077 ± 0.007
6.0	0.074 ± 0.002
6.2	0.082 ± 0.005
Control	0.081 ± 0.008

Significant at *$P < 0.01$; **< 0.002; ***< 0.001

and slender, while the leaves were dark green may be due to more chlorophyll accumulation. The result obtained corroborates the report that zeatin has enhanced the alkaloid production in *Catharanthus roseus* suspension cultures[10] and a linear correlation between solasodine productivity and chlorophyll content occurs in shoot cultures of *Solanum* species[11].

A few of the aromatic amino acids such as L-phenylalanine, tryptophan, and tyrosine generally serve as precursors in the production of alkaloids, flavonoids etc. In the present study, incorporation of these amino acids in the culture medium was tried because wedelolactone is a flavonoid and perhaps, one of these may act as precursor in its production. Significant enhancement in wedelolactone content was found when phenylalanine was added to the culture, whereas no increase was observed with tryptophan and glycine. These results suggested that phenylalanine might be involved in the biosynthesis of wedelolactone.

Alteration in temperature is reported to induce modification of physical and chemical properties of lipids, which influence the activities of the enzymes involved in the biosynthesis of secondary metabolites[12]. In one of the earlier studies on *C. roseus* liquid medium culture, the production of indole alkaloid increases at 16°C, but at 27°C it decreases[13]. In the present investigation, increase in temperature resulted in significant increases in wedelolactone at two temperature (30°±2°C and 37°±2°C).

Light intensity, spectral quality and length of daily exposure produce considerable impact on secondary metabolite production[14]. It is reported that light exerts an influence on flavonoid pathway[15]. In the present study, when cultures were exposed for three different, constant dark, constant light and 12 hr dark/light photoperiods, a significant increase in wedelolactone production was observed at 12 hr dark/light photoperiod and the shoots generated had less accumulation of chlorophyll as compared to control.

Sugars have important signaling functions on secondary metabolite production as the carbon source and their relative concentrations affected the secondary metabolite synthesis[16]. In order to observe the effect of other carbon sources, glucose and fructose were also used in the culture medium at 3% (w/v) concentration. The medium supplemented with 3% (w/v) sucrose was found to produce maximum amount of wedelolactone as compared to other tested sugars. The probable reason may be the osmotic strength generated by sucrose favored wedelolactone production.

The present study reveals that variation of empirical factors significantly alters the accumulation of wedelolactone in *E.alba* shoot cultures and provide scope for detailed study to identify other regulatory parameters that may be involved in the wedelolactone production.

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