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Original Article

Effect of Some Phytohormones on Podophyllotoxin Production in Cell and Plantlets Cultures of *Linum album*

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Abstract

The natural lignan podophyllotoxin, a dimerized product of two phenylpropanoid moieties which occurs in a few plant species, is a pharmacologically important compound for its anticancer activities. Optimization of the *in vitro* cultures might lead to a renewable source of lignans for medicinal uses. In order to maximize efficiency of podophyllotoxin accumulation via *in vitro* cultures, the effect of different concentrations of α-Naphthalene acetic acid (NAA) (0, 0.4 and 1 mg/l), Kinetin (Kin) (0, 0.2 and 0.4 mg/l) and combinations of NAA and Kin [(0.4 and 0.2), (0.4 and 0.4), (1 and 0.2) and (1 and 0.4) mg/l] on growth and accumulation of podophyllotoxin in callus, cell culture and *in vitro* propagated plantlets of *Linum album* was evaluated. The highest growth and accumulation of podophyllotoxin in callus culture was obtained when it grown under dark condition in comparison to photoperiod. The biomass of cell and callus do not changed under various hormone concentrations. Content of podophyllotoxin was enhanced in cell and callus cultures supplemented with NAA 1 mg/l + Kin 0.4 mg/l. The experiments demonstrated Kin 0.2 or 0.4 mg/l stimulated multiplication and production of podophyllotoxin in shoot culture.

Key words: Podophyllotoxin, *Linum album*, α-Naphthalene acetic acid, Kinetin

Introduction

Podophyllotoxin (PTOX) is an important antitumor and antiviral agent isolated from *Podophyllum* species. PTOX itself has only a limited therapeutic use due to its high toxicity, but semi synthetic derivatives of this compound are medicinally applied against cancer [1]. The administration of PTOX derived drugs causes complex physiological reactions beyond the inhibition of DNA topoisomerase and tubulin polymerization [2]. Chemical synthesis of PTOX is not economic and their synthesis is highly expensive on a commercial scale; therefore PTOX is extracted from *Podophyllum* roots and rhizomes [3]. Besides the *Podophyllum* genus, PTOX is also found in other plant genera such as *Linum, Juniperus, Callitris* [2-4]. Previous studies confirmed the potential of cell culture of the endemic Iranian flax (*Linum album* Kotschy ex Boiss.), to produce this compound [5-7]. This system represents a potential renewable source for biosynthesis of PTOX that cannot be produced by microbial cells or chemical syntheses. The expression of many secondary metabolite pathways is easily altered by external factors such as nutrient levels, stress factors, light and growth regulators [8]. Medium optimization is an efficient strategy to increase secondary metabolites in scale up using cell suspension and bioreactor cultures. Phytohormones in medium culture play a crucial role in a variety of plant growth and developmental processes [9]. Numerous studies as ginsenoside in *Panax quinquefolius* L. [10] or berberine in the callus cultures of *Coscinium fenestratum* (Gaertn.) Colebr. [11] have been

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conducted to improve biomass and obtain a high yield of these secondary metabolites by applying phytohormones in medium culture. To our knowledge, there has not been any report so far on the evaluation of cell, tissue and organ culture of *L. album* with comparing PTOX production in response to exogenous plant growth regulators. For this reason, the main target of this study was to investigate the effects of exogenous application of different concentrations of phytohormones in cell, callus and micro-propagated cultures of *L. album* on the PTOX content and biomass production.

Material and Methods

Plant Material

The seeds of *L. album* Kotschy ex Boiss. were collected in July 2008 from Sohanak, Tehran. The plant material was identified by herbarium of Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran.

Seed Germination

The seeds were thoroughly washed in running tap water for 5 to 8 min and subsequently were treated with 0.1% sodium hypochlorite for 10 minutes after rinsed with sterile distilled water 3 times, sterilization followed by hydrogen peroxide 3/3% for 10 minutes, seeds were washed six times with autoclaved distilled water to remove the traces of sterilizing agents and in the final step seeds treated with70% alcohol for 1 min and rinse with distilled water. Sterilized seeds were treated with GA3 (500mg/l) for an hour and cultured on free-hormone Murashige and Skoog (MS) [12] medium supplemented with sucrose (3%) and placed in the growth room (25± 1ºC and photoperiod of 16 h light and 8 h dark) for germination.

Induction of Callus

For callus induction, four weeks old *in vitro* grown plantlets of *L. album* were harvested and its 1cm explants were cultured on MS solid medium containing NAA (0, 0.4, 1 mg/l); Kin (0, 0.2, 0.4 mg/l) and their combinations [(0.4 and 0.2), (0.4 and 0.4), (1 and 0.2) and (1 and 0.4) mg/l], then they were placed under dark condition or photoperiod of 16 h light and 8 h dark. After four weeks, induced callus were harvested and weighed for fresh weight. All the media were adjusted to pH 5.8 and solidified with 0.6% agar before being autoclaved for 20 minutes under 20 psi. at 121 °C.

Establishment of Cell Suspension Culture

Suspension culture was initiated by transfer of friable callus in to liquid medium (30 ml in 100 ml Erlenmeyer flask) containing NAA (0.4) or (1) mg/l and NAA + Kin [(0.4 and 0.2), (0.4 and 0.4), (1 and 0.2) and (1 and 0.4) mg/l] then was incubated on rotary shaker (125 rpm, 25 °C) under dark condition. Cells (2 g) were subcultured in the same medium every 10 days. Growth was evaluated by measuring the fresh weight (FW) was measured after filtration at regular intervals 4, 8 and 12 days. The cells were collected for extraction and HPLC analyses.

Plantlet Culture

Four weeks old *in vitro* grown plantlets of *L. album* were harvested and its 1cm explants were cultured on MS solid medium containing NAA (0, 0.4, 1 mg/l); Kin (0, 0.2, 0.4 mg/l) and their combinations [(0.4 and 0.2), (0.4 and 0.4), (1 and 0.2) and (1 and 0.4) mg/l], then they were placed under photoperiod of (16 h light and 8 h dark). After 30 days, shoot growth and PTOX accumulation were investigated.

Extraction of Podophyllotoxin

Powdered cell, callus and shoots (2 g) were extracted by sonication in methanol (80% v/v) during 1 h. Dichloromethane (4.0 ml) and water (4.0 ml) was added to obtain a partition of compounds between two layers. The dichloromethane fractions were then collected, dried and dissolved in 1.0 ml of HPLC grade methanol and then injected into the HPLC [4].

HPLC Analysis

The presence of PTOX in the samples was verified by comparison of the RT and UV spectral peaks of the sample with those of an authentic sample. UV spectra were measured on-line using a Thermo Quest. The HPLC system was equipped with a spectra system KO 6000 LP photodiode array detector. A Grom-Sil 120 ODS-3 ST, 5 µm C18 (250 mm × 4.6 mm) column. A guard column Grom-Sil 120 ODS-3 ST, 5 µm C18 (50 mm × 4.6 mm) was used to safeguard the analytical column. The detector was set at 290 nm. In the present work all injection volume was 20 µl. All the calculations for quantitative analysis were performed with linear regression external standardization by measurement of peak areas.

Results and Discussion

Callus Culture

Callus Induction and Growth

Linum album explants were cultured in MS supplemented with different concentrations of NAA, Kin and NAA+ Kin for 30 days in dark or photoperiod. Our results showed significantly greater callus formation when the cultures were in darkness (Fig. 1). Salehi et al. [13] reported in root explant greater callus production in darkness. Summart et al. [14] reported callus grown under dark condition had higher cell mass than that under light (16 h light and 8 h dark).

Callus induction was observed at all treatments containing NAA and NAA+ Kin, whereas callus fresh weight was not affected by change in applied concentrations after 30 days (Fig. 1). No callus induction was observed in free-hormone MS medium and Kin was failed to stimulate callus formation when applied alone. Many researchers observed auxins (IBA, NAA and 2,4-D) as the best growth regulator for callus induction [15]. Auxins stimulate RNA metabolism and induce the transcription of messenger RNA which code the proteins that are required for the chaotic cell proliferation and ultimately the callus formation [16]. Nahid et al. [17] reported that 2,4-D, NAA and IBA induce cell division and enlargement at optimum concentrations which is associated with increase in the activities of autolytic and synthetic enzymes by effecting cell wall plasticity and by synthesizing new cell wall materials. In the media supplemented with NAA (0.4 or 1 mg/l) root-like emerged from the callus. Other researchers have been showed the effect of phytohormones on callus induction and plant cell growth. Ahmad et al. [18] demonstrated in callus culture of *Podophyllum hexandrum* that MS medium supplemented with NAA 1.5 and 6-Benzylaminopurine (BAP) 0.5 µM were effective for both initiation and sustained growth of callus tissue. Ionkova et al. [19] reported that the medium with 0.4 mg/l or 2.0 mg/l NAA with 0.1 mg/l Kin is appropriate for callus formation in explants of *L. linearifolium*. Caretto et al. [20] showed in *Artemisia annua* MS basal medium supplemented with 2 mg/l 2,4-D and 0.15 mg/l 6-BAP as the optimal medium composition for obtaining rapidly growing green and friable callus.

Podophyllotoxin Production in Callus

Our results showed that callus culture accumulated only trace amounts of PTOX when it was grown in the photoperiod whereas dark had a significant effect on increasing PTOX (Fig. 2). Hobss and Yeoman [21] reported transfer of *Nicotiana glauca* callus from the dark into the light caused a decrease in alkaloid accumulation, while moving cultures from the light into the dark resulted in an increase in alkaloid content. Chattopadhyay et al. [22] also observed that in suspension cultures of *Podophyllum hexandrum* the biosynthesis of podophyllotoxin was favored when plant cells were cultivated in the dark.

Podophyllotoxin Production in Plantlets

In shoot cultures, PTOX production was significantly higher in MS basal medium supplemented with only Kin (0.2 mg/l or 0.4 mg/l) respectively 1.26 and 1.1 mg/g or with NAA (0.4 mg/l + Kin 0.2 mg/l) 1.1 mg/g PTOX (Fig. 4). The product yield of PTOX in medium culture supplemented with similar ratio of Kin and NAA was comparable to MS free hormone. Kovacevic and Grubisic [35] showed in *Frangula alnus* highest metabolite production (1731 mg/100 g) of total anthraquinone was in the shoots grown on the MS medium with addition of NAA 0.1 mg /l and thidiazuron (TDZ) 0.1 mg /l. Gadzovska et al. [36] reported, in multiple shoots of *Hypericum* cultivated on medium with cytokinin N6-benzyladenine (0.5 mg/l BA), phenolic and flavonoid contents were significantly high on day 4 and 14 respectively (40-60 mg/g) and (9-13.6 mol/g) compared to control shoots. We observed PTOX content in plantlet appeared to 18.5 times higher than that in callus culture. This result was in consistent with the findings of Vasilev and Ionkova [37] in *Linum strictum* that the higher yields of 6-MPTOX was observed in shoot cultures. This is probably due to the differentiation of the shoot compared to undifferentiated callus culture.

Cell Suspension Culture

In the previous experiments revealed that in the medium supplemented with 0.2 mg/l or 0.4 mg/l Kin and in the free hormone medium, callus formation did not occurs, also in photoperiod condition accumulation of PTOX was very poor. Therefore these treatments and photoperiod were eliminated in cell suspension cultures. Cell cultures were analyzed to determine growth and their ability to produce PTOX at regular intervals; during 12 days. Growth of the cells was not affected by applied hormones (Fig. 5). Our results revealed PTOX production was significantly different between 4 day and 8 days of culture condition (Fig. 6). Analysis of cell culture showed PTOX accumulation in the presence of individual NAA as well as NAA + Kin.

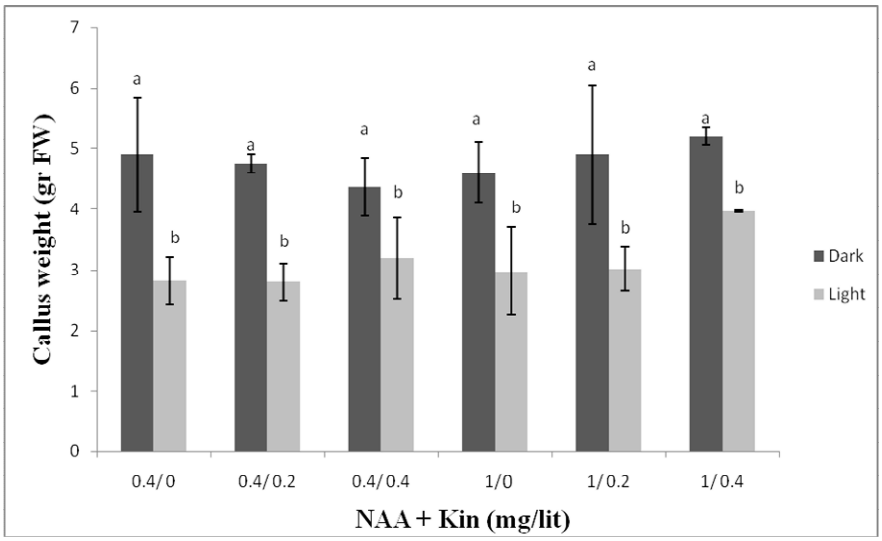

Fig. 1 Influence of dark and light on callus formation on *Linum album* explants exposed to different concentration of phytohormones after 30 days.

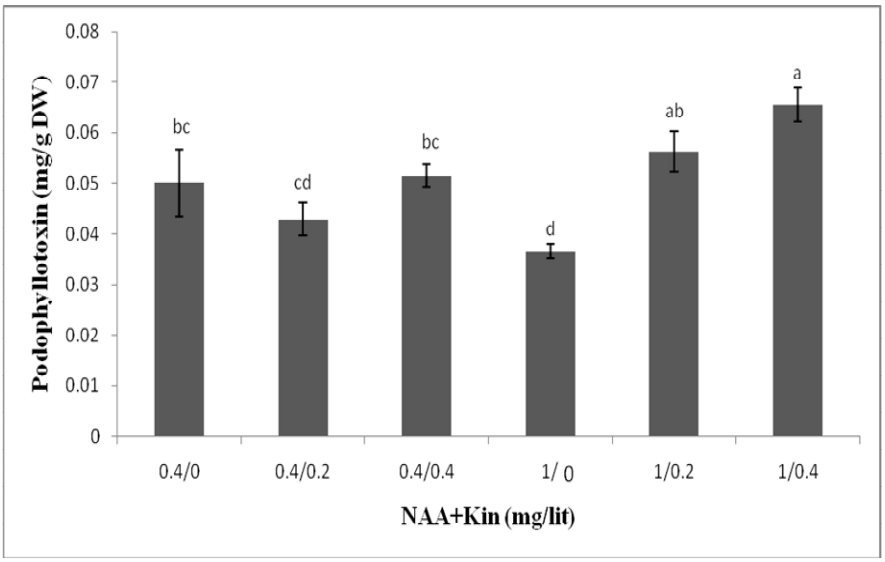

Fig. 2 Influence of various concentrations of NAA, Kin and NAA+Kin on accumulation of PTOX in callus cultures in dark after 30 days.

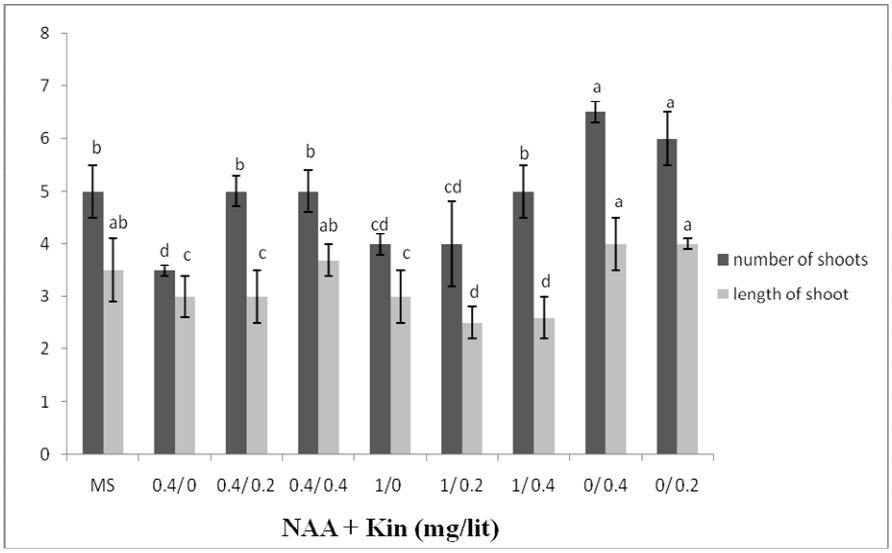

Fig. 3 Influence of various concentrations of NAA, Kin and NAA+Kin on growth and development shoot of *L.album* after 30 days.

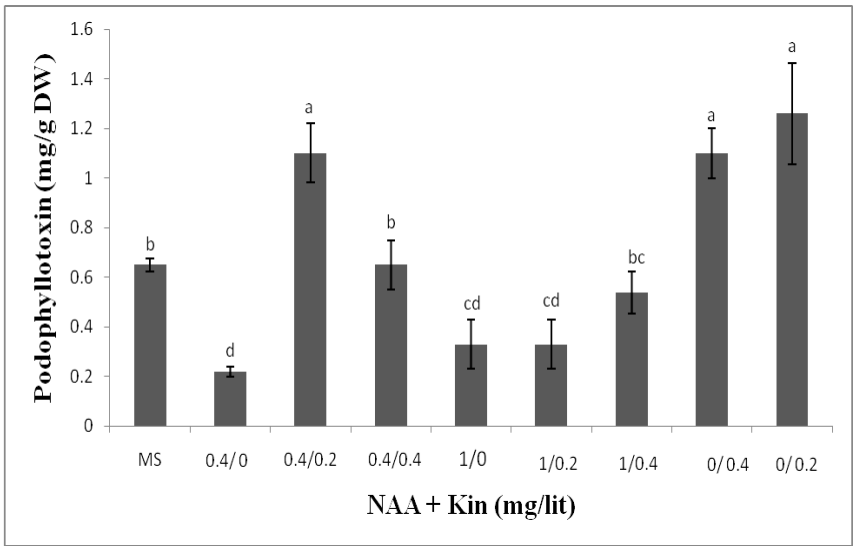

Fig. 4 Influence of various concentrations of NAA, Kin and NAA+Kin on accumulation of PTOX in shoot cultures after 30 days.

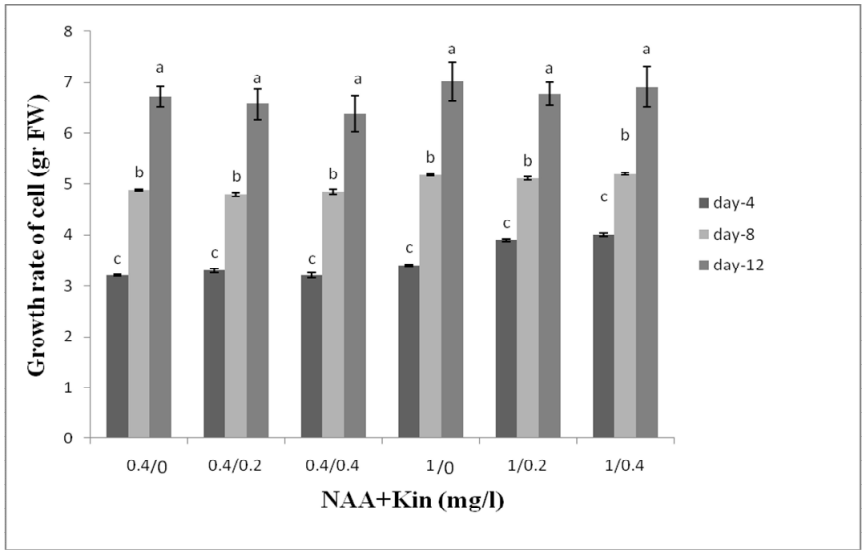

Fig. 5 Influence of various concentrations of NAA, Kin and NAA+Kin on biomass of *Linum album* cell culture after 4, 8 and 12 days.

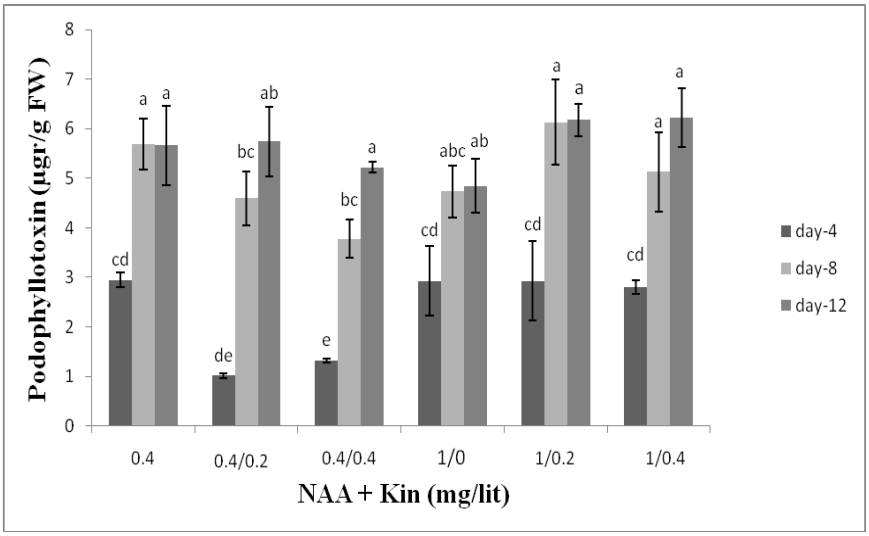

Fig. 6 Influence of various concentrations of NAA, Kin and NAA+Kin on accumulation of PTOX in cell culture after 4, 8 and 12 day.

In cell culture similar to callus culture, in MS supplemented only with NAA, root like was emerged. Smollny et al. [29] reported that 0.4 mg/l NAA was ideal concentration for cell culture of *L. album*. In *P. hexandrum* cells culture production of podophyllotoxin was influenced by IAA [5].

Conclusion

PTOX production in callus, cell and plantlet cultures of *L. album* was influenced by changing in concentration of plant growth regulators. Callus and cell cultures produce higher PTOX in darkness than photoperiod condition. The content of PTOX in plantlet culture was significantly higher than in callus culture. According to this study, *in vitro* cultures can be used as an alternative source for PTOX production. However, efforts need to maximize PTOX production in cultures of *L. album* by adopting different culture techniques such as immobilization, elicitation and precursor feeding.

Acknowledgement

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