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ORIGINAL PAPER

Effects of donor plants and plant growth regulators on naphthoquinone production in root cultures of *Impatiens balsamina*

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Abstract The effects of type of explant (leaves and roots), donor plants, and plant growth regulators on naphthoquinone (NQ) production of *Impatiens balsamina* L. root cultures were evaluated. The root cultures were initiated in liquid Gamborg's B5 medium supplemented with 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA), 0.1 mg l^{-1} kinetin (Kn) and 1.0 mg l^{-1} 6-benzyladenine (BA). The present investigation indicated that the root cultures established from the leaf explants produced higher total NQ content [1.01 ± 0.046 mg/g dry weight (DW)] than those established from the root explants (0.62 ± 0.023 mg/g DW). The leaf explants of four *I. balsamina* strains including white flower plant (IbW), pink flower plant (IbP), violet flower plant (IbV) and red flower plant (IbR) were used to establish the root cultures. Based on HPLC analysis, IbP strain produced the highest total NQ content (3.39 ± 0.072 mg/g DW), while IbR strain produced the lowest one (1.45 ± 0.055 mg/g DW). The root cultures established from the IbP explant were capable of producing higher content of total NQs (2.76 ± 0.093 mg/g DW) than those established from the other strains. The results suggest that the tissue cultures initiated from the high-yielding donor plants should be capable of producing higher content of secondary compounds than those initiated from low-yielding donor plants. In addition, plant growth regulator manipulation exhibited that a combination of 0.1 mg l^{-1} NAA, 1.0 mg l^{-1} Kn and 2.0 mg l^{-1} BA is capable of increasing NQ production (2.97 ± 0.072 mg/g DW) in *I. balsamina* root cultures.

Keywords High-yielding · Medium manipulation · Lawsone · Lawsone methyl ether · Bilawsone · Naphthoquinone

Abbreviations

HPLC	High Performance Liquid Chromatography
BA	6-Benzyladenine
B5	Gamborg's B5 medium
DW	Dry weight
Kn	Kinetin
NAA	α-Naphthalene acetic acid
LME	Lawsone methyl ether
NQ(s)	Naphthoquinone(s)

Introduction

Impatiens balsamina L. (Balsaminaceae) has long been used in Thailand as a traditional folk medicine. The leaves are usually used for the treatment of thorn or glass-puncture wounds, abscesses, ingrown nails and chronic ulcers caused by allergic reaction of detergents (Farnsworth and Bunyapraphatsara 1992). Naphthoquinones (NQs); lawsone and lawsone methyl ether (LME) (Fig. 1) are the major active compounds that possess strong antimicrobial (Tripathi et al. 1978; Kang and Moon 1992; Yang et al. 2001), anti-anaphylaxis (Ishiguro et al. 1994; Ueda et al. 2003), anti-allergic (Oku and Ishiguro 2002), and anti-inflammatory (Reanmongkol et al. 2003) activities. Bilawsone (Fig. 1) has been reported as a non-natural

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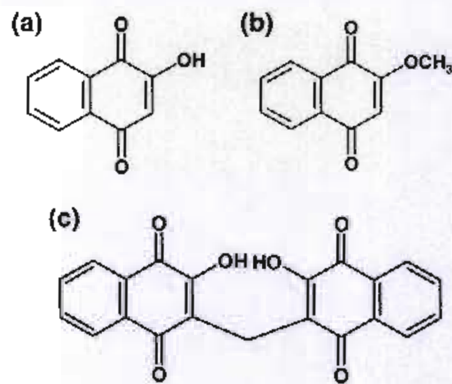

Fig. 1 Chemical structures of NQs: a lawsone b LME and c bilawsone

occurring NQ found in *I. balsamina* root cultures (Panichayupakaranant et al. 1995). This compound has been reported as an antipruritic agent (Oku et al. 2002).

I. balsamina root cultures have been established in order to study their ability to biosynthesize secondary metabolites. The root cultures have been found to produce three NQs; lawsone, LME and bilawsone as well as three coumarins; scopoletin, isofraxidin and di-isofraxidin (Panichayupakaranant et al. 1995, 1998). Plant strain selection and medium manipulation are the strategies to improve secondary metabolite production in plant cell cultures. Statistically high-producing plants give rise to high-producing cell lines (Dörnenburg and Knorr 1995; Bourgaud et al. 2001). Our previous report successfully established NQ producing *I. balsamina* cell suspension cultures by using NQ high-yielding donor plants as the initiated explant (Panichayupakaranant 2001). In this paper, we describe the selection of the explant as well as the NQ high-yielding donor plants for initiation of the NQ high-producing *I. balsamina* root cultures. Plant growth regulator manipulation in order to increase NQ production in the root cultures is also reported herein.

Materials and methods

Plant materials

Impatiens balsamina was grown in the Botanical Garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The plants were divided into four groups according to their flower color: including white flower plant (IbW), pink flower plant (IbP), violet flower plant (IbV) and red flower plant (IbR). The voucher specimens (IbW specimen no. SKP 021 09 02.01 01; IbP specimen no. SKP 021 09 02.02 01; IbV specimen no. SKP 021 09 02.03 01; IbR specimen no. SKP 021 09 02.04 01) were deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Seed germination

Seeds of *I. balsamina* were separately collected from four *I. balsamina* donor plants and sterilized by rinsing with 70% (v/v) ethanol for 5 s, immersion in 20% (v/v) Clorox® solution for 15 min and rinsing three times in sterile distilled water. Seeds were germinated on hormone-free B5 solid medium with 20 g l^{-1} sucrose, at 25 ± 2°C in with 16 h light/8 h dark.

Root cultures

I. balsamina root cultures were initiated from the leaves or roots of the plantlets in solid B5 medium supplemented with 0.1 mg l^{-1} NAA, 0.1 mg l^{-1} Kn, 1.0 mg l^{-1} BA, 20 g l^{-1} sucrose and 8 g l^{-1} agar. The root cultures were transferred into 250-ml Erlenmeyer flasks containing 50 ml of liquid B5 medium supplemented with the same hormonal composition. The cultures were incubated on a rotary shaker (80 rpm), at 25 ± 2°C in with 16 h light/8 h dark. Maintenance of the cultures was carried out by periodic subculture at 3 weeks intervals.

Extraction of NQs

The leaves of *I. balsamina* plantlets (1-month-old) were separately collected according to the plant strains and dried at 50°C. The cultured roots were harvested after 1 month of subculture and dried at 50°C. The dried leaf or root powders (0.5 g) were extracted with 50% chloroform in methanol (20 ml) under reflux conditions for 1 h and then filtered. The filtrates were evaporated to dryness in vacuo. The residues were reconstituted and adjusted to 10 ml with methanol and subjected to HPLC analysis.

HPLC analysis of NQs

HPLC analysis was carried out using the method as previously reported (Sakunphueak and Panichayupakaranant 2008). Briefly, the HPLC apparatus consisted of an Agilent 1100 series equipped with photodiode-array detector and autosampler. Data analysis was performed using Agilent 3D ChemStation software (Agilent, USA). Separation was achieved at 25°C on a Supelco Discovery® C18 column (5 μm, 4.6 × 150 mm). The mobile phase consisted of 2% aqueous acetic acid–methanol (gradient from 25% methanol to 55% methanol in 50 min) and was pumped at a flow rate of 1 ml min^{-1}. The injection volume was 20 μl. The quantitative wavelength was set at 280 nm. The calibration curves of lawsone, LME and bilawsone were established

from the authentic samples (3.25–56 μg ml^{-1}). Lawsone, LME and bilawsone exhibited linearity over the evaluated ranges with the linear equation of $Y = 91.838X - 116.8$ ($r^2 = 0.9999$), $Y = 94.567X - 92.173$ ($r^2 = 0.9998$) and $Y = 65.847X - 32.73$ ($r^2 = 1$), respectively. Each calibration point was carried out in triplicate.

Plant growth regulator manipulation

The root cultures initiated from the IbP strain were transferred into liquid B5 media supplemented with 0.1 mg l^{-1} NAA, 0.1 mg l^{-1} Kn, with a variation of BA concentration (0.1, 0.5, 1.0 and 2.0 mg l^{-1}). After three successive subcultures, the one-month old root cultures were harvested and subjected to HPLC analysis of NQs using the method as described above. The combination of plant growth regulators that gave the highest NQ producing root cultures was used for subsequent manipulations of Kn (0.1, 0.5, 1.0 and 2.0 mg l^{-1}) and NAA concentrations (0.1, 0.5, 1.0 and 2.0 mg l^{-1}) in the same manner of BA.

Time-courses of growth and NQ production

The root cultures (1.0 g fresh weight) were transferred to B5 liquid medium supplemented with 0.1 mg l^{-1} NAA 1.0 mg l^{-1} Kn and 2.0 mg l^{-1} BA. The roots were harvested every 3 days for the period of 30 days. The dry weights of the roots were recorded after drying at 50°C for 24 h. The dried roots were extracted and subjected to quantitative analysis of NQs using the methods as described above. These data were plotted to produce growth and NQ production curves.

Statistical analysis

All experiments were repeated three times. Data were subjected to one-way analysis of variance, and means were compared using Tukey's test for multiple comparisons using the statistical package SPSS version 15.0. Data obtained from the explant selection experiment were compared by independent-sample *T*-test using the same statistical package.

Result and discussion

Effect of explant on NQ production

It has been demonstrated that the leaves of *I. balsamina* produce higher NQs content than the roots (Panichayupakaranant and De-eknamkul 1992b). In this study, we investigated the effect of explants that possesses different potential of NQ production on NQ production in *I. balsamina* root cultures. Our finding indicated that the root cultures that initiated from different explant exhibited different NQ production. The leaf explant could provide the root cultures that produced significantly higher NQ content than those initiated from the root explant (Table 1). This result suggests that a starting plant material that possesses higher potential of secondary metabolite production may provide a higher secondary metabolite producing plant tissue culture.

Effect of donor plants

A variation of NQ content in *I. balsamina* leaves was assessed in four strains of *I. balsamina*. The plant strains were categorized by their flower colors. HPLC analysis of NQs in the leaves of *I. balsamina* plantlets revealed that all 4 strains produced lawsone, LME and bilawsone. In addition, lawsone and LME are found as the main NQs in the leaves of *I. balsamina* (Fig. 2). A variation of NQ production among the plant strains was observed. The content of NQs in IbP was significantly higher than those of the other strains (Table 2). Among these, IbR produced the least content of NQs. All four strains of plantlets were used to establish the root cultures of *I. balsamina* in order to investigate the effect of the donor plants on NQ production of the root cultures.

After several subcultures, the root cultures of *I. balsamina* were examined for their potential of NQ production using HPLC. The HPLC-chromatograms of the cultured root extracts were different from those of the intact plants (Fig. 2). The cultured root extracts contained lawsone and bilawsone as the major NQs, while the intact leaf extracts contained lawsone and LME as the major NQs. Although the root cultures produced higher amount of lawsone and

Table 1 NQ contents in *I. balsamina* root culture established from the leaf and root explants of IbR

Explants	NQ content (mg/g DW) [mean ± SE] ($n = 6$)			
	Lawsone	LME	Bilawsone	Total NQ
Leaves	0.52 ± 0.032*	0.14 ± 0.001*	0.35 ± 0.007*	1.01 ± 0.046*
Roots	0.29 ± 0.011	0.11 ± 0.002	0.22 ± 0.003	0.62 ± 0.023

* Significantly different ($P < 0.05$) when compared to means within the same column

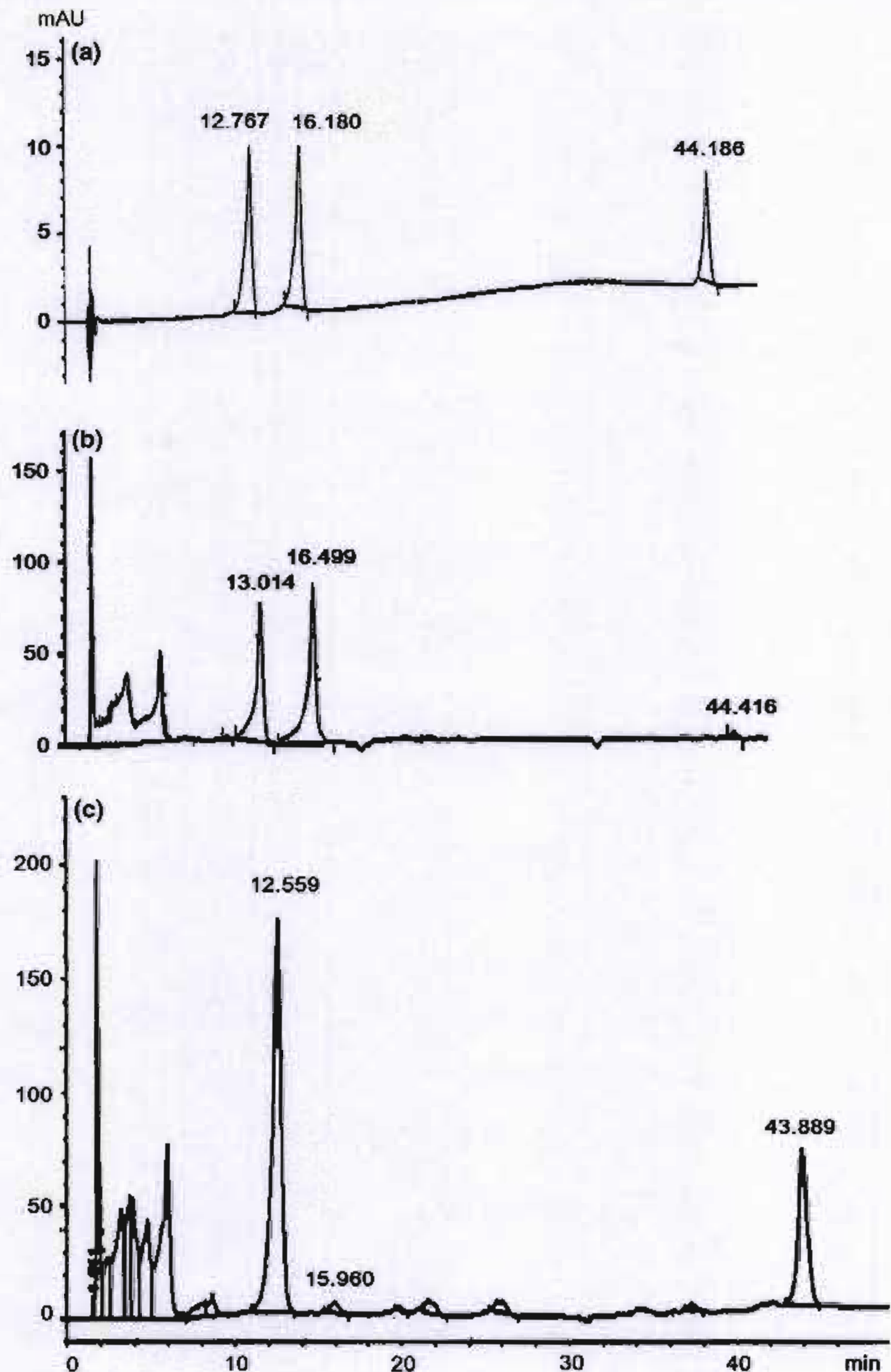

Fig. 2 HPLC Chromatograms of **a** authentics: lawsone (1), LME (2), and bilawsone (3); **b** intact leaf extract; and **c** root culture extract

bilawsone, it produced lower amount of LME than the leaves of the intact plant (Tables 2, 3). This may be due to a lack of *o*-methyltransferase enzyme, a key enzyme in the final step of LME biosynthesis (Panichayupakaranant and De-eknamkul 1992a). The obtained NQ high-producing root cultures can be used as a material of choice for biosynthetic studies of NQs in *I. balsamina*. They can be used for detection of *o*-succinylbenzoyl-CoA ligase activity which converts *o*-succinylbenzoic acid (OSB) to be its activated form, OSB-Co-A ester in the biogenesis of

Table 2 NQ contents in the leaves of various *I. balsamina* strains

Plant strains	NQ content (mg/g DW) [mean ± SE] ($n = 16$)			
	Lawsone*	LME*	Bilawsone*	Total NQ*
IbR	0.50 ± 0.027d	0.89 ± 0.057c	0.06 ± 0.003b	1.45 ± 0.055c
IbV	1.01 ± 0.027b	1.27 ± 0.071b	0.06 ± 0.002b	2.34 ± 0.061b
IbP	1.50 ± 0.064a	1.81 ± 0.079a	0.09 ± 0.003a	3.39 ± 0.072a
IbW	0.84 ± 0.039c	1.37 ± 0.068b	0.07 ± 0.002b	2.28 ± 0.052b

Means followed by the same letter within a column are not significantly different according to Tukey's test

* Significantly different ($P < 0.05$) when compared to means within the same column

Table 3 NQ contents in root cultures of *I. balsamina* established from the various plant strains

Plant strains	NQ content (mg/g DW) [mean ± SE] ($n = 16$)			
	Lawsone*	LME*	Bilawsone*	Total NQ*
IBR	0.75 ± 0.027d	0.11 ± 0.004b	0.10 ± 0.005b	0.96 ± 0.031d
IBV	2.04 ± 0.067b	0.14 ± 0.003a	0.14 ± 0.016a	2.32 ± 0.071b
IBP	2.49 ± 0.081a	0.12 ± 0.003b	0.16 ± 0.005a	2.76 ± 0.093a
IBW	1.70 ± 0.019c	0.09 ± 0.007c	0.16 ± 0.007a	1.95 ± 0.024c

Means followed by the same letter within a column are not significantly different according to Tukey's test

* Significantly different ($P < 0.05$) when compared to means within the same column

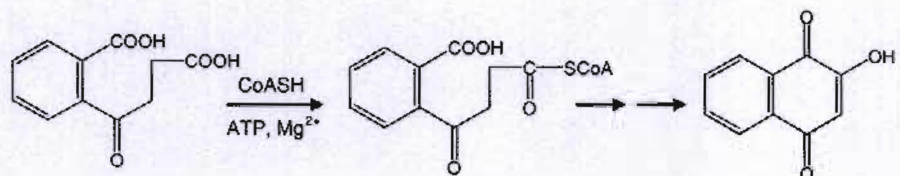

Fig. 3 Biogenesis of lawsone from OSB via it activated form, OSB-CoA ester

lawsone (Fig. 3). However, the strategies to increase LME production in the root cultures should be further investigated.

Regarding the effect of the donor plants, the root cultures established from NQ high-yielding strain (IbP) significantly produced higher total NQ content than those established from NQ low-yielding strains (Table 3). In addition, the root culture established from IbR produced the lowest amount of total NQs. These results support our hypothesis that the root cultures initiated from the NQ high-yielding donor plants are capable of producing higher amount of NQs than those initiated from the NQ low-yielding strains.

Effect of plant growth regulators

Our previous report has demonstrated the production of lawsone, LME and bilawone in *I. balsamina* root cultures that cultured in B5 medium supplemented with 0.1 mg l^{-1} NAA, 0.1 mg l^{-1} Kn and 1.0 mg l^{-1} BA (Panichayupakaranant et al. 1995). However, the content of NQs produced by the root cultures as well as a medium manipulation to improve NQ production in the root cultures have not been investigated yet. In this study, we investigated the effect of the plant growth regulator concentration on the NQ production in *I. balsamina* root cultures. Variation of BA concentrations in the culture medium (supplemented with 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} Kn) demonstrated that the NQ production in the root cultures was gradually increased by the increasing of BA concentration (Table 4). The optimum BA concentration that produced the highest NQ content in the root cultures was 2.0 mg l^{-1}. Thus, the further determination of Kn concentration was performed by fixing of BA and NAA at 2.0 and 0.1 mg l^{-1}, respectively. Variation of Kn concentrations indicated that NQ production was reached to the highest content when Kn concentration was 1.0 mg l^{-1} (Table 4). An increasing of Kn concentration up to 2.0 mg l^{-1} resulted in a decreasing of

Table 4 Effect of plant growth regulator on NQ contents in *I. balsamina* root cultures

Plant growth regulator (mg l^{-1})			NQ content (mg/g DW) [mean ± SE]			
NAA	Kn	BA	Lawsone	LME	Bilawsone	Total NQ
0.1	0.1	0.1	0.22 ± 0.006*d	0.11 ± 0.004*b	0.17 ± 0.008*c	0.49 ± 0.010*d
0.1	0.1	0.5	0.35 ± 0.006*c	0.09 ± 0.008*b	0.19 ± 0.016*c	0.63 ± 0.031*c
0.1	0.1	1.0	0.55 ± 0.012*b	0.15 ± 0.006*a	0.33 ± 0.022*b	1.03 ± 0.037*b
0.1	0.1	2.0	1.60 ± 0.027*a	0.15 ± 0.004*a	0.50 ± 0.024*a	2.25 ± 0.029*a
0.1	0.1	2.0	1.72 ± 0.081#b	0.15 ± 0.005#b	0.47 ± 0.012#c	2.34 ± 0.093#b
0.1	0.5	2.0	1.82 ± 0.032#b	0.17 ± 0.011#a	0.41 ± 0.020#c	2.40 ± 0.077#b
0.1	1.0	2.0	2.14 ± 0.070#a	0.17 ± 0.005#a	0.62 ± 0.009#a	2.93 ± 0.101#a
0.1	2.0	2.0	1.88 ± 0.056#b	0.16 ± 0.003#b	0.57 ± 0.003#b	2.61 ± 0.088#b
0.1	1.0	2.0	2.12 ± 0.081†a	0.18 ± 0.015†a	0.67 ± 0.042†a	2.97 ± 0.072†a
0.5	1.0	2.0	1.33 ± 0.026†b	0.11 ± 0.009†b	0.35 ± 0.020†b	1.79 ± 0.063†b
1.0	1.0	2.0	0.94 ± 0.070†c	n.d.	0.32 ± 0.040†b	1.26 ± 0.126†c
2.0	1.0	2.0	0.19 ± 0.038†d	n.d.	0.17 ± 0.003†c	0.36 ± 0.044†d

Means followed by the same letter within a column are not significantly different according to Tukey's test

n.d. not detect

*, #, † significantly different ($P < 0.05$) when compared to means within the same column

NQ production. Thus, the further determination of NAA concentration was performed by fixing of BA and Kn concentrations at 2.0 and 1.0 mg l^{-1}, respectively. In contrast to cytokinin, increasing of NAA concentration caused decrease in both NQ production and growth. In addition, at high concentration of NAA, the root cultures were dedifferentiated to be callus. This dedifferentiation of the root cultures may cause a functional impairment of NQ biosynthesis pathway. Our finding on the effect of high concentration of NAA that suppressed NQ production seems to agree with the previous reports on the inhibitory effect of auxin on NQ production of *Echium lycopsis* callus cultures (Fukui et al. 1983) and *Drosophyllum lusitanicum* cell suspension cultures (Nahálka et al. 1996) as well as anthraquinone production of *Morinda citrifolia* cell cultures (Stalman et al. 2003). These finding thus suggest B5 supplemented with 0.1 mg l^{-1} NAA, 1.0 mg l^{-1} Kn and 2.0 mg l^{-1} BA as an appropriate medium for NQ production of *I. balsamina* root cultures.

Time-courses of growth and NQ production

The growth cycle of *I. balsamina* root cultures during a period of 30 days demonstrated that there was a very short lag phase of growth followed by a rapid growth of exponential phase (9 days) and linear phase (15 days). This resulted in a continuous increase of biomass throughout the period of 24 days. Thereafter, the dried biomass weights became constant and then gradually decreased, indicating that the root cultures had reached the stationary and decline phases, respectively (Fig. 4). The root cultures attained

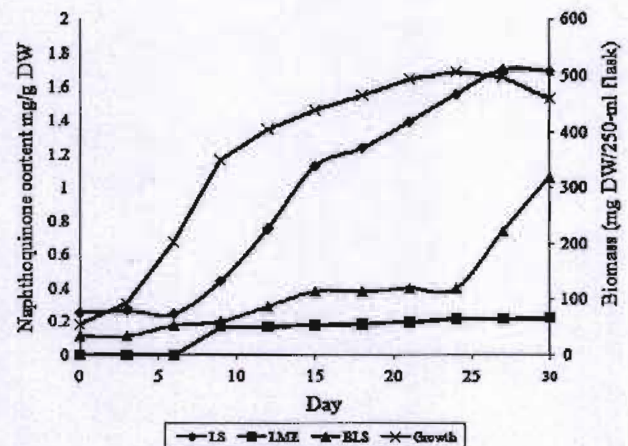

Fig. 4 Time-course of growth and NQ production of *I. balsamina* root cultures

their highest dry biomass weight of 507.8 mg/250-ml flask at day 24, equivalent to about nine times of the inoculated dry biomass.

The formation of all NQs in the root cultures seemed to begin in the late exponential phase or early linear phase (day 9) of the growth. Lawsone was actively biosynthesized throughout the linear and stationary phases. The highest level of lawsone was observed at day 27 and then began to decline at the end of the growth cycle. In addition, bilawsone was initially accumulated in small amount throughout the linear phase. However, it was actively biosynthesized when the root cultures reached the stationery and decline phases. This may be due to a need of high

lawsone accumulation to trigger the biosynthesis of bilawsone. In contrast, LME was initially biosynthesized in the late exponential phase with a constant rate of production and a small amount of accumulation throughout the growth cycle. These results suggested that the biosynthesis of lawsone is operational in the early stage of growth and throughout the growth cycle. Subsequently, the biosynthesis of bilawsone is functional after the biosynthesis of lawsone is at the maximum. In contrast, the biosynthesis of LME is not working in the root cultures even if they produce high amount of lawsone, a precursor of LME biosynthesis. This could be due to a limit of *o*-methyltransferase enzyme expression in the root cultures of *I. balsamina*.

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