PHILIPP AGRIC SCIENTIST
Vol. 101 No. 3, 251-260
September 2018

ISSN 0031-7454

Sucrose Enhanced Stigmasterol Production in Callus Cultures of *Wedelia biflora* (L.) D.C

Siti Norayu Idris[1], Abdul Bakrudeen Ali Ahmed[2,*] and Rosna Mat Taha[1]

[1]Tissue Culture Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2]Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh City, Vietnam
*Corresponding author: bakrudeen.ali.ahmed@tdt.edu.vn (e-mail); +91854339668 (telephone); +84 837755055 (fax)

This research is a portion of the Master of Science thesis and was funded by the University of Malaya Postgraduate Research Grant (PPP099-2013B).

Callus culture method was used to extract stigmasterol in mass amount. The current study aims to enhance the production of stigmasterol by manipulation of sucrose concentrations in Murashige and Skoog (MS) media. A combination of 2 mg NAA L^{-1} (an auxin) and 2.5 mg 6-benzylaminopurine (BAP) L^{-1} (a cytokinin) in MS medium was the best concentration to stimulate callus formation from stem explants of *W. biflora* with the fastest response (10 d), the highest biomass of callus (55 mg dry weight) and the best callus characteristic (green and compact). Application of 4% (w/v) sucrose in MS medium enhanced callus formation along with callus biomass and stigmasterol production (88 mg g^{-1}) compared with the control (58.3 mg g^{-1}) and in vivo grown stem (53.4 mg g^{-1}). Production of stigmasterol from *W. biflora* with reduced production time and cost is now possible with the methods obtained from the current study.

Key Words: callus induction, MS medium, secondary metabolites, stigmasterol, sucrose

Abbreviations: 2,4-D – 2,4-dicholorophenoxyacetic acid, BAP – 6-benzylaminopurine, HPLC – high performance liquid chromatography, IBA – indole-3-butyric acid, MS – Murashige & Skoog, NAA – naphthalene acetic acid

INTRODUCTION

Wedelia biflora belongs to the Asteraceae family, a type of creeping herb that is widely used in traditional medicine. Exploitation of its wild populations might be the easiest method to gain the medicinal benefits from W. *biflora*. However, by this method, supplies will be reduced periodically if no proper care is taken to ensure the sustainability of the species. On the other hand, callus tissue culture could be one of the best methods to ensure a continuous supply of raw material for extraction of bioactive compounds from it (Luchessini and Mensuali 2010). In addition, production of bioactive compounds from its wild population is minimal compared with *in vitro* cultures because researchers can regulate the production of selected bioactive compounds through tissue cultures (Dal Toso and Melandri 2011). Recently, Castro et al. (2016) reported a higher content of total phenol and flavonoids in callus derived from the leaves of *Byrsonima verbascifolia* (L.) D.C compared with the wild-grown leaf. Production of bioactive compounds is becoming more feasible with the application of callus culture because a number of explants can be obtained from a single plant; and this would subsequently be able to sustain the wild population of the plant.

A number of useful compounds have been identified and isolated from *W. biflora* such as sesquiterpenoids, frideline, epifriedelanol, quercetin derivatives, grandifloric acid and steroid (Huang et al. 2013; Khare, 2007; Thu et al. 2013). Khan et al. (2015a) and Khan et al. (2015b) suggested that distribution of bioactive compounds may vary at different embryonic phases or growth phases. Khan et al. (2015b) reported that brassicasterols detected in the lag phase of *Silybum marianum* L. adventitious root cultures, high amount of cinnamic acid, di-hydro kaempferol, punicic acid and lignin pinoresinol abundantly found in the log phase and malonic acid, prostaglandin A1 and phenyl acetic acid

were at the highest level in the stationary phase. Meanwhile, plant growth regulators could also affect the production of bioactive compounds. For instance, thiadiazuron was reported to enhance the production of silymarin in leaf callus of *Silymarin marianum* (Khan et al. 2014) and also the production of phenolic compounds (gallic acid, myricetin, caffeic acid, catechin and apigenin) in stem and leaf callus of *Fagonia indica* (Burm. f.) (Khan et al. 2016). In this study, production of stigmasterol was regulated by the manipulation of sucrose concentration in callus culture medium. Recent research by Doma et al. (2012) has reported a successful increase in production of withanolides from *Whitania somnifera* (L.) through manipulation of sucrose concentration. Doma et al. (2012) observed the accumulation of withaferin A in hairy root culture of the species incubated in a medium fortified with 2%, 3% and 4% sucrose with the highest accumulation at 4% sucrose and no accumulation observed at 6% sucrose.

Stigmasterol is a type of phytosterol along with β-sitosterol, ergosterol, campesterol and brassicasterol. Phytosterol or plant sterol is a naturally occurring phytoconstituent of plants. Stigmasterol is chemically similar to animal cholesterol and plays an important role in temperature tolerance in plants. Senthil-Kumar et al. (2013) found that a gene that catalyzed the conversion of β-sitosterol to stigmasterol (*Atcyp710A1*) in *Arabidopsis thaliana* (L.) was responsible for imparting low and high temperature tolerance in that species. Limited information is available on the stigmasterol content of *W. biflora*; however, stigmasterol was successfully found (in minimal amount) in *W. biflora* stem parts (Khare 2007) as well as *W. biflora* flower parts (Thu et al. 2013). Therefore, the current study aims to amplify the production of stigmasterol from *W. biflora* through tissue culture method to reduce time and cost of production.

MATERIALS AND METHODS

Plant Material

W. biflora plants were collected from the Botanical Garden of the University of Malaya, Kuala Lumpur, Malaysia where they had been planted. The young stems of *W. biflora* plants were used as the starting materials for callus induction. Prior to inoculation, the explant was washed in 5% (v/v) Teepol for 2 min followed by 20% (v/v) sodium hypochlorite for 2 min. Subsequently, the explant was surface sterilized with 0.1% (w/v) mercury chlorite for 1 min followed by 0.001% (w/v) carbendazim in 70% (v/v) ethanol for 1 min and thoroughly rinsed in sterile distilled water three times. The explant was air-dried on sterile tissue paper in a laminar air flow chamber and cut into 5–8 mm long pieces before inoculation onto fresh agar medium.

Callus Induction

For callus induction, MS media (Murashige and Skoog 1962) with 3% sucrose was supplemented with NAA (0.5–4 mg L^{-1}) or IBA (0.5–4 mg L^{-1}) or combinations of NAA and BAP (0.5–4 mg L^{-1}). MS media devoid of any hormones was used as control. All media were adjusted to pH 5.7 with 1 N NaOH and autoclaved at 121 °C for 20 min. An antifungal, 0.001% (w/v) carbendazim in 70% (v/v) ethanol, was added into the media and the media were dispensed into 60 mm specimen containers in the laminar air flow chamber under aseptic conditions. The sterile explant was inoculated horizontally for callus induction after the media was perfectly solidified. All cultures were incubated at 25 ± 2 °C under 16 h photoperiod. The callusing period, callusing frequency and color of callus were recorded from day 14 to day 42. The best hormone to obtain optimum callusing parameters was selected for the next experiment.

Manipulation of Callus Growth with Different Sucrose Concentrations

MS media supplemented with 2 mg NAA L^{-1} and 2.5 mg BAP L^{-1} was used in this experiment. Different sucrose concentrations (1%, 2%, 3%, 4% and 5%) were added to the MS media. MS media with 3% sucrose served as the control. Biomass of the callus (fresh and dry weights) were taken and recorded at day 14, 21, 28, 35 and 42. Callus from day 14, 21, 28, 35 and 42 were taken randomly, extracted with methanol and the accumulation of stigmasterol was estimated by using HPLC analysis.

Sample Extraction

The calli (2847 mg) were oven-dried until they were reduced to 360 mg (about 87% moisture loss) at 40 °C for 2 d. The samples were ground to produce fine homogenous powder using a mortar and pestle. The fine powder was soaked in 50 mL methanol at room temperature for 3 d, before it was centrifuged at 9000 rpm for 5 min. The supernatant was evaporated to dryness at room temperature for 3 d. The plant extracts were dissolved in methanol (50 mg mL^{-1}) and kept in glass vials at 4 °C until use.

HPLC Analysis

HPLC analysis was performed using JASCO HPLC System Manager equipped with UV-vis detector.

Table 1. Effects of application of NAA or IBA on callus formation from stem explants of *W. biflora*.

Hormone Concentration (mg L^{-1})	Intensity of Callus	Percentage of Callus Formation (%)	Days of Callus Formation	Nature of Callus	Callus Dry Weight (mg)
MS basal	--	0	--	--	11±2.5
NAA					
0.5	+	78.1 ± 2.1	18	GCC	39 ± 1.5
1	+	73.3 ± 1.7	18	GCC	38 ± 2.5
1.5	+	77 ± 2.5	18	GCC	33 ± 1.6
2	++	85.1 ± 3.3	18	GFC	48 ± 2.8
2.5	++	81.5 ± 2.8	18	GFC	43 ± 1.8
3	++	79.1 ± 2.5	18	GFC	30 ± 3.1
3.5	++	76.1 ± 2.1	18	GFC	36 ± 1.9
4	++	82.3 ± 2.3	18	GFC	46 ± 1.5
IBA					
0.5	+	57.8 ± 2.3	21	GFC	20 ± 1.6
1	+	63.2 ± 1.2	21	GFC	18 ± 1.5
1.5	++	65.5 ± 1.8	21	GFC	9 ± 1.9
2	++	49.6 ± 2.6	18	GFC	18 ± 2.5
2.5	++	58.8 ± 1.4	18	GFC	10 ± 3.1
3	++	55.1 ± 3.1	18	GFC	9 ± 1.7
3.5	++	62 ± 2.5	18	CFC	15 ± 1.9
4	++	56.5 ± 2.1	18	CFC	7 ± 2.8

GCC – Green compact callus; GFC – Green friable callus; IBA – Indole-3-butyric acid; NAA – α-naphthalene acetic acid
+ Meager (0.5–1 cm); ++ Moderate (1–1.5 cm); +++ Intense (>1.5 cm).
Values are mean ± SE of 10 replicates per treatment and repeated thrice.

Chromatographic separation was carried out using a C18 reversed phase column under isocratic conditions. The mobile phase was a 70:30 mixture of acetonitrile and methanol at a flow rate of 1 mL min^{-1}. The operative wavelength was set at 205 nm and the injection volume was 20 µL. Each sample was run for 16 min. The amount of stigmasterol in samples (in mg g^{-1}) was calculated using a standard curve of stigmasterol standard with known concentrations.

Statistical Analysis

All experiments were repeated three times with 10 replicates each. The effect of different treatments was quantified as mean ± SE and the data with different SE within a column were significantly different.

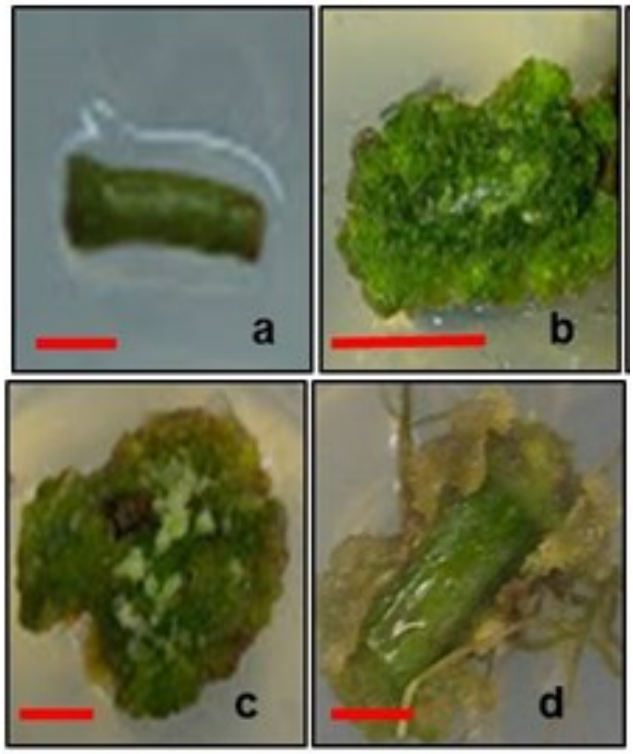

Fig. 1. a) Stem callus inoculated in MS media without any hormones; b) Green and compact stem callus inoculated in MS medium fortified with NAA; c) Green and compact stem callus inoculated in MS medium supplemented with combination of NAA and BAP; and d) Colorless stem callus with formation of roots inoculated in MS medium supplemented with IBA. Bars represent 0.5 cm. (BAP – 6-benzylaminopurine, IBA – indole-3-butyric acid, MS – Murashige & Skoog, NAA –

RESULTS

Callus Induction with NAA (Auxin) and BAP (Cytokinin)

Callus induction from stem explants of *W. biflora* has been successfully achieved. Callus initiation was observed from the cut end surface of stem explants as early as 12–14 d of incubation in all media tested, except for MS media without hormones (no callus observed, Fig. 1a). In general, application of NAA in MS media resulted in the highest frequency and fastest response for callus induction. The highest frequency of callus formation was observed in MS media supplemented with 2 mg L^{-1} NAA (85.1%) and produced green and compact callus (Table 1).

Even though the application of NAA alone did produce callus in stem explants of *W. biflora*, the intensity of the callus produced was very low (less than 1.5 cm in size; Fig. 1b). Therefore, the intensity of callus production was improved by addition of BAP (cytokinin) in MS media (Fig. 1c). This study verified that combination of 2 mg L^{-1} NAA with various concentrations (0.5–4 mg L^{-1}) of BAP resulted in faster callus induction (in 10¬–12 d) and produced green and compact callus. Among all the BAP concentrations used (from 0.5–4 mg L^{-1}), only 2.5 mg L^{-1} BAP gave the best results in terms of callus nature and biomass (55 mg dry weight) when combined with 2 mg L^{-1} NAA (96.2% callusing, Table 2). From this experiment, we concluded that stem explants of *W. biflora* were best inoculated in MS medium supplemented with a combination of 2 mg L^{-1} NAA and 2.5 mg L^{-1} BAP. This hormone combination will be used in this entire study to produce callus from stem explants of *W. biflora*.

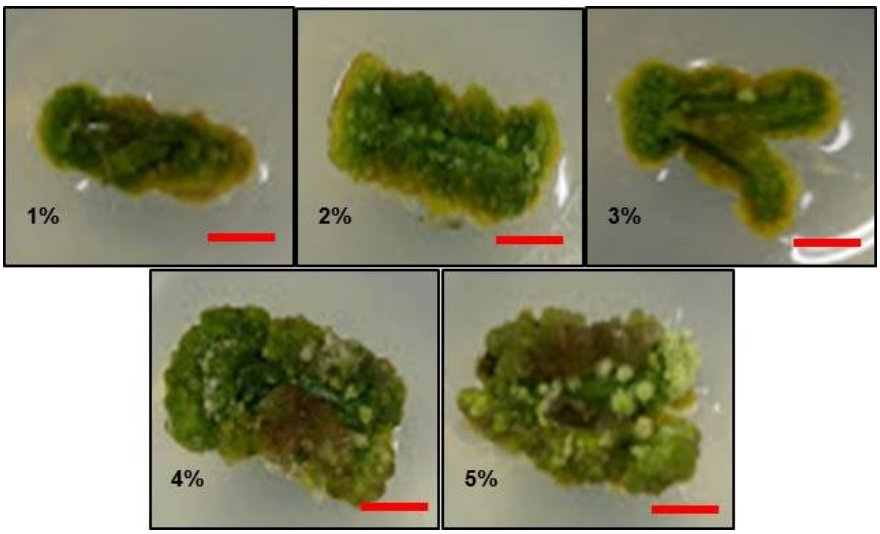

Fig. 2. Stem callus inoculated in MS medium supplemented with combination of 2 mg NAA L^{-1} and 2.5 mg BAP L^{-1}, fortified with different sucrose concentrations from 1% to 5% (w/v). Application of 3% (w/v) sucrose concentration served as control treatment. Bars represent 0.5 cm. (BAP – 6-benzylaminopurine, MS – Murashige & Skoog, NAA – naphthalene acetic acid).

Effects of Sucrose Concentrations and Incubation Temperature on Callus Growth

Sucrose is a source of carbon (energy) needed by the explants to grow optimally in a particular growth medium. Scarcity of carbon source will cause the explants to grow slower or even not at all, while too much carbon source will become toxic to the explants. This experiment used MS medium fortified with 1%, 2%, 3% (control), 4% and 5% sucrose concentration. In this experiment, addition of extra sucrose was expected to produce more callus than application of

Table 2. Improvement of callus formation by combination of NAA with various concentrations of BAP.

Hormone Concentration (mg L^{-1})	Intensity of Callus	Percentage of Callus Formation (%)	Days of Callus Formation	Nature of Callus	Callus Dry Weight (mg)
*2 NAA	++	85.1 ± 3.3	18	GFC	48 ± 2.8
2 NAA + BAP					
0.5	++	82.3 ± 2.5	12	GFC	49 ± 1.5
1	++	87.1 ± 3.2	12	GFC	36 ± 2.1
1.5	++	89.1 ± 2.1	12	GFC	39 ± 1.8
2	++	91.5 ± 2.4	12	GFC	20 ± 0.7
2.5	+++	96.2 ± 2.8	10	GFC	55 ± 1.9
3	+++	95.1 ± 3.5	10	GCC	45 ± 1.6
3.5	+++	92.4 ± 2.8	10	GCC	25 ± 1.2
4	+++	93.1 ± 2.1	10	GCC	25 ± 2.3

*The best hormone concentration selected from Table 1.
GCC – Green compact callus; GFC – Green friable callus; NAA – α-naphthalene acetic acid; BAP – Benzylaminopurine
+ Meager (0.5–1 cm); ++ Moderate (1–1.5 cm); +++ Intense (>1.5 cm).
Values are mean ± SE of 10 replicates per treatment and repeated thrice.

normal sucrose concentration (3%). For stem explants of *W. biflora*, the higher the concentration of sucrose, the more callus formation was stimulated with optimum production at 4% sucrose concentration (Table 3). The callus formed from stem explants inoculated with 4% sucrose was green in color, compacted, and more than 1.0 cm in size (Fig. 2). Application of 5% sucrose concentration also stimulated the production of intense callus from stem explants. However, a browning effect was observed after 21 d of culture, which suggested necrosis of the callus cell. Therefore, 4% sucrose concentration in MS media was the optimum condition for callus production from stem explants, followed by 5%, 3% (control), 2% and 1%.

Growth Curve and Biomass Analysis

The growth curve of callus biomass was of the sigmoid type which consists of a lag phase, an exponential phase, a stationary phase and a decline phase (Fig. 3). The lag phase occurred until the 21st day in incubation followed by the exponential phase from day 21 until day 35. The stationary phase was not obviously shown as the decline phase occurred simultaneously until day 42 and onwards. Therefore, the callus biomass peaks were at day 35. The production of stigmasterol was directly proportional to the growth of callus (Fig. 4). Small quantities of stigmasterol were detected during the lag phase (day 14) in both stem and leaf callus. Its production began to increase at the beginning of the exponential phase (day 21) and was optimum during the late exponential phase (day 28). The production of stigmasterol gradually decreased during the stationary phase (day 35) and remained low during the decline phase onwards. Based on the results obtained, calluses that were 28 d old, which was at the exponential phase, were chosen for the subsequent experiments because the cells at this age were healthy, rapidly dividing and produced higher stigmasterol content.

Accumulation of Stigmasterol (HPLC Analysis)

During analysis of stigmasterol content using the HPLC method, all the extracts showed the presence of stigmasterol but in different concentrations. The extracts were analyzed simultaneously with known concentration of standard stigmasterol to confirm the detection of the correct compound. The standard curve of known concentration of stigmasterol obeys the Beer Lambert's law in the concentration range from 100 mg L^{-1} to 600 mg

Table 3. Callus formation and stigmasterol content in stem callus manipulated with different sucrose concentrations.

Sucrose Concentration (%)	Color of Callus	Callus Dry Weight (mg)	Stigmasterol Content (mg g^{-1})
1	Green	37 ± 4.1	14.4 ± 6.6
2	Green	55 ± 3.8	31.7 ± 8.9
3 (Control)	Green	56 ± 2.6	58.3 ± 7.1
4	Green	196 ± 5.3	88 ± 9.8
5	Green	161 ± 4.9	44.3 ± 5.2
In vivo stem	-	-	53.4 ± 8.2

Values are mean ± SE for 10 replicates per treatment and repeated thrice.

L^{-1} at 205 nm. The concentration and absorbance of the standard showed a good linearity with correlation coefficient (r^2) of 0.9789 (Fig. 5).

Stigmasterol content was analyzed in each treatment of callus and presented in Table 3. The amount of stigmasterol increased when the callus of *W. biflora* was treated with different sucrose concentrations. The best sucrose concentration for stem callus was 4% which yielded 88 mg g^{-1} stigmasterol, followed by 3% (58.3 mg g^{-1} stigmasterol), *in vivo* grown stem (53.4 mg g^{-1} stigmasterol), 5% (44.3 mg g^{-1} stigmasterol), 2% (31.7 mg g^{-1} stigmasterol) and 1% (14.4 mg g^{-1} stigmasterol). Therefore, the production of stigmasterol in stem callus of *W. biflora* could only be improved by regulation of sucrose concentration on MS medium. This study verified that application of 4% sucrose concentration into MS media supplemented with 2 mg L^{-1} NAA and 2.5 mg L^{-1} BAP is the best method to increase the production of stigmasterol from stem callus of *W. biflora*.

DISCUSSION

Auxins and cytokinins were used to induce the formation of callus from stem explants of *W. biflora* in this study. Callus was chosen instead of wild plant for production of stigmasterol because the conditions for callus growth are easier to control. Bathoju and Giri (2012) studied the production of stigmasterol in callus and *in vitro* regenerated plant of *Chlorophytum borivilianum* Santapau & R.R. Fern. They found a 5.4-fold increase in stigmasterol production in plants regenerated from somatic embryos compared with stigmasterol production in undifferentiated callus of the same species.

In this study, only stem explants were used because, according to Yaacob et al. (2013), stem explants were the best starting material to induce callus compared with other explants. The explants were exposed to the treatment of auxins alone or in combination with cytokinins. Application of auxins and cytokinins in combination (particularly combination of 2 mg NAA L^{-1} and 2.5 mg BAP L^{-1}) resulted in better callus production compared with application of auxins alone. This result concurs with most of the previous studies, especially studies on plants within the same family such as *Eclipta*

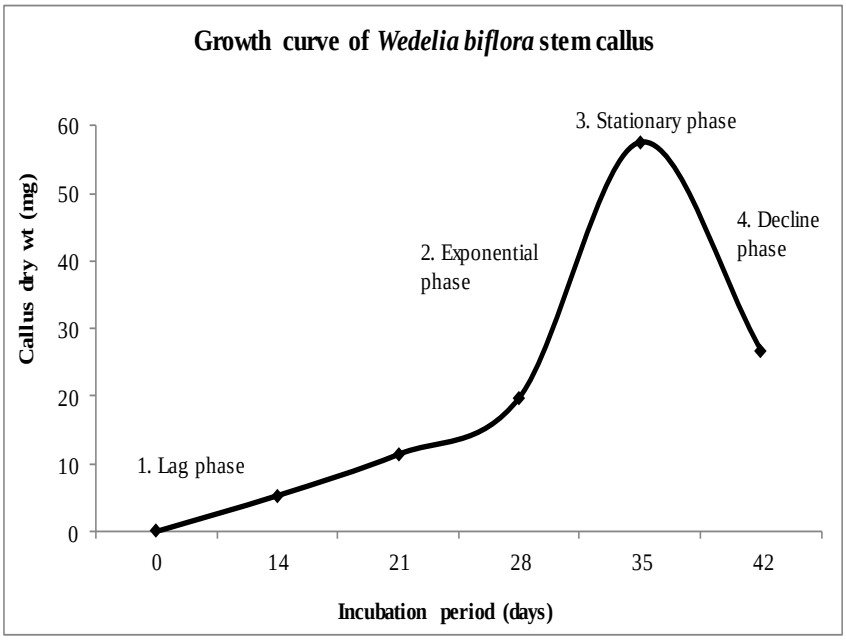

Fig. 3. Sigmoid-shaped growth curve of *W. biflora* stem callus with four distinct phases.

alba (L.) Hassk (Sharma et al. 2013) and *Leuzea carthamoides* (Willd.) Iljin (Zand et al. 2014). Sharma et al. (2013) reported that leaf callus of *E. alba* was best induced with a combination of 1 mg L^{-1} 2,4-dicholorophenoxyacetic acid (2,4-D) and 0.5 mg L^{-1} BAP while Zand et al. (2014) documented that a combination of 1 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BAP was the best to induce callus from leaf explants of *L. carthamoides*.

Application of MS media devoid of any hormone failed to induce callus in stem explants of this species. This result indicates that production of callus from stem explants of *W. biflora* is dependent on application of plant growth regulators in MS medium. Most of the species from this genus, such as *Wedelia calendulacea* (L.) Less. and *Wedelia chinensis* (Osbeck) Merr., were dependent on the application of plant growth regulators to induce callus formation. Leaf callus of *W. calendulacea* was initiated by addition of 2 mg L^{-1} 2,4-D in MS medium (Sharmin et al. 2014). On the other hand, Dhas et al. (2016) reported that application of 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP optimized callus production in *W. chinensis*.

Treatment of NAA (particularly 2 mg L^{-1}) has indeed produced intense callus formation from stem explants of *W. biflora*. Rashmi and Trivedi (2014) reported that explants from *Nerium odorum* L. produced more intense callus formation with application of NAA than any other auxins. However, combination of 2 mg NAA L^{-1} with various concentrations of BAP (a type of cytokinin) accelerated and increased the production of callus even more from stem explants of *W. biflora*. Yang et al. (2008) found that more callus was produced from *Leonurus heterophylus* Sw. when NAA was combined with BAP, up to four times compared with NAA application alone. This result proved that regulation of auxins and cytokinins with a certain concentration further increased callus production. In our study, combination of a lower concentration of auxins (2 mg NAA L^{-1}) and a higher concentration of cytokinins (2.5 mg BAP L^{-1}) resulted in better callus formation from stem explants of *W. biflora*. Angulo-Bejarano and Paredes-Lopez (2011) in contrast reported that a similar ratio of auxins and cytokinins in growth medium will provide better callus response, especially in *Opuntiaficus-indica* (L.) Mill. The variety of results shows that the effects of auxins and cytokinins ratios vary in different plants.

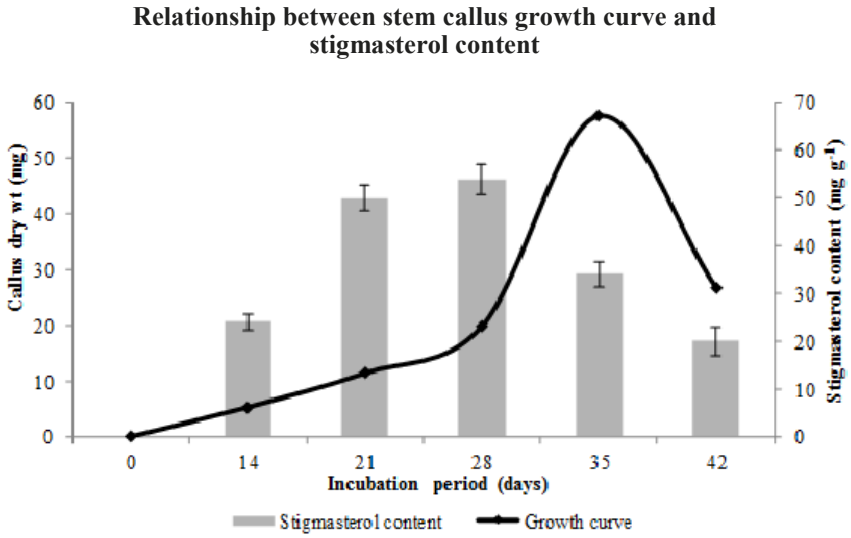

Fig. 4. Stigmasterol content in stem callus with phases in callus growth curve.

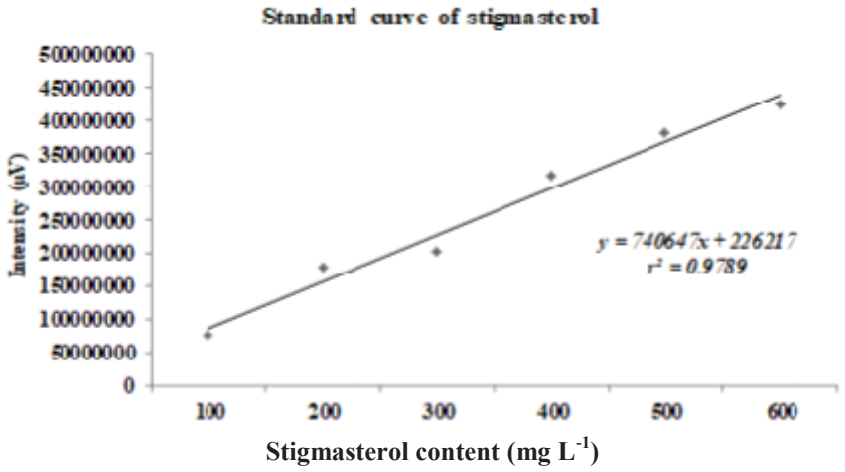

Fig. 5. Standard curve of standard stigmasterol used to estimate stigmasterol content in stem callus.

W. biflora and its related species are known to have medicinal properties due to the presence of medicinal secondary metabolites. A study carried out by Mishra et al. (2011) revealed that the ethanolic extract of whole *W. chinensis* plant contains glycosides, alkaloids and flavonoids while the petroleum ether extract

consists of steroids and the aqueous extract contains glycosides and saponins. The chemical components of *W. chinensis* aerial parts were isolated and identified as (3β)-oleanolic acid 3-(β-D-glucopyranosiduronic acid 6-methyl ester); and (3β)-3-hydroxy-30-noroleana-12,20(29)-dien-28 -oic acid 3-(β-D-glucopyranosiduronic acid) 6-methyl ester and structures elucidated using the spectral values (Li et al. 2012). Another study by Banu and Nagarajan (2013) revealed that the bioactive compounds present in *W. chinensis* (Osbeck) Merrill leaf extract are 2-tridecanone, n-(methyoxyphenylmethylene) carbamic acid ethyl ester and 9,12,15-octadecatrienoic acid methyl ester. In this study, stigmasterol was extracted from the whole plant of *W. biflora* as well as its callus. Methanolic extracts of related *Wedelia* species were reported to have potential use in medicinal field such as anti-leukaemia activity of *W. trilobata* methanolic extract (Venkatesh et al. 2016) and antibacterial activity of *W. chinensis* methanolic extract especially against *Bacillus cereus* (Darah et al. 2013).

Manipulation of abiotic stress conditions, for instance, manipulation of sucrose concentration, could also affect the production of callus. Baque et al. (2012) agreed with this finding when they found that manipulation of sucrose concentration in root suspension cultures of *Morinda citrifolia* L. could enhance the production of anthraquinone, phenolics and flavonoids. The manipulation of this external factor not only increased the callusing frequency, but was also able to regulate the production of secondary metabolites in the callus. The growth of the callus was measured by the fresh and dry weights; and a growth curve was plotted and analyzed to determine the most suitable growth time point for callus harvesting with the highest stigmasterol content. There was a limited number of reports found on production of stigmasterol in the callus of any plants. However, Flores-Sanchez et al. (2002) reported that overall sterol synthesis was higher during the exponential phase of *Uncaria tomentosa* (Willd.) D.C cell suspension cultures. Similarly, Ponis et al. (2006) reported that stigmasterol was produced during the exponential phase of microalgae growth, while most other secondary metabolites were produced during the stationary or decline phase (Miguel-Chavez et al. 2003). The reports from Flores-Sanchez et al. (2002) and Ponis et al. (2006) support the results obtained from the stem callus of *W. biflora*. The exponential phase is a phase where the cells proliferate rapidly and consume a lot of energy. In this period, secondary metabolites such as flavonoids and triterpenes are actively produced (Tan et al. 2010) to support the actively growing cells. Synthesis of sterols occurred actively during the exponential phase of cellular growth in order to provide enough sterols for the new membrane of the cells and to support cellular stability (Hartmann et al. 2000).

Sucrose is the most common carbon source used in tissue culture applications because of its easy availability and ease of absorption by plant cells. Normal concentration of sucrose used is 3% (w/v) as determined by Wilson et al. (1994) for optimum carbon supply to support plant cell growth during the in vitro incubation period. Application of 4% (w/v) sucrose concentration in MS media supplemented with 2 mg NAA L^{-1} and 2.5 mg BAP L^{-1} resulted in intense callus formation and highest stigmasterol accumulation (88 mg g^{-1}) compared with the application of normal sucrose concentration (58.3 mg g^{-1}) and *in vivo* grown stem (53.4 mg g^{-1}). Increase in secondary metabolites production with application of 4% (w/v) sucrose in growth media was also reported by Praveen and Murthy (2012) in *W. somnifera*. They recorded optimum production of whitanolide A in hairy root culture of W. somnifera at 4% sucrose concentration in all tested ranges of concentrations (1–8%).

CONCLUSION

Callus formation from stem explants of *W. biflora* has been efficiently established through the combination of 2 mg NAA L^{-1} and 2.5 mg BAP L^{-1} in MS medium. Callus from this hormone combination showed the fastest response, highest biomass and was green and compact in nature. Overall, the growth curve of the stem callus of *W. biflora* was sigmoid in shape with the peak (stationary phase) at 35 d old. Investigation of stigmasterol accumulation in stem callus showed the highest stigmasterol content during the exponential phase of the callus growth curve (28 d old). Application of 4% sucrose (w/v) successfully increased the production of stigmasterol in stem callus. Therefore, stem explants of *W. biflora* can be inoculated in MS medium supplemented with a combination of 2 mg NAA L^{-1} and 2.5 mg BAP L^{-1} with 4% (w/v) sucrose concentration in order to have bulk callus formation as well as increased amount of stigmasterol production. These novel findings are crucial for the extraction of this valuable secondary metabolite (stigmasterol) on a larger scale.

ACKNOWLEDGMENTS

The authors would like to thank the University of Malaya for providing financial support from PPP grant (PG099-2013B).

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