Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Asian Journal of Plant Science and Research, 2013, 3(3):131-138

ISSN : 2249-7412
CODEN (USA): AJPSKY

Production of anthraquinones from adventitious root derived callus and suspension cultures of *Morinda citrifolia* L. in response to auxins, cytokinins and sucrose levels

Sreeranjini S. and E. A. Siril*

Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, Kerala, India

---

ABSTRACT

Various parameters for the in vitro production of anthraquinones through callus and cell suspension cultures of Morinda citrifolia were standardized using adventitious roots as the explant source. .Nodal explant derived cultures were established in MS medium containing BAP (18 µM). The shoots were harvested from fast proliferating six-week-old M. citrifolia cultures were rooted adventitiously in IBA (24 µM) containing MS medium. Four-week-old adventitious roots were excised and cultured on MS medium containing NAA (2.5 µM) produced maximum callus (486.5mg fw / 10 ml solid medium) and anthraquinone (20.26 mg/g dw) by 45th day of culture. Callus subjected to suspension cultures in MS medium supplemented with 2.5 µM NAA in combination with 0.5 µM BAP lead to sustained production of anthraquinones (16.99 mg/g dw). Among the different carbon sources studied, 5% sucrose resulted in maximum anthraquinone (16.17 mg/g dw) production in cell cultures. Anthraquinone production based on adventitious root derived callus and cell suspension cultures are proved to be a promising approach to produce this pharmaceutically important natural pigment.

Key words: Anthraquinones, adventitious roots, callus culture, cell suspension culture.

INTRODUCTION

Anthraquinones are important class secondary metabolites traditionally been used as medicine [1] due to antitumor [2], immunomodulatory [3] and antioxidative activities [4]. This class of natural dyes and its derivatives are extremely resistant to light induced fading. Major source of anthraquinones are members of family Rubiaceae [5]. The roots of *Morinda citrifolia* a member of family Rubiaceae is known to contain substantial amount of anthraquinones.

Morinda citrifolia, commonly known as noni, is native to Southeast Asia (Indonesia) and Australia and is distributed throughout the tropics [6]. Noni have a broad range of therapeutic effects including antibacterial, antiviral, antifungal, antitumor and immune enhancing effects. The major phytochemical principle that is responsible for therapeutic properties is the anthraquinones. Previous reports suggests presence of several anthraquinone constituents like nordamnacanthal, morindone, rubiadin, rubiadin-1-methyl ether, morinaphthalenone, 5, 15-dimethyl morindol, morindicone and morinthone in *M. citrifolia* [7,8,9]. Demand for the *M. citrifolia* derived anthraquinones in modern pharmacopeia as well as traditional system of medicines in the East are getting increased in the recent past. In this scenario relaying on natural stands of the source plant for the metabolites will evoke issues such as seasonal variations, increased labour and consequent high production cost. Moreover, it causes depletion of *M. citrofolia* resources in its natural habitats. Alternatively, *in vitro* cell culture based system can be explored. The strategy has proved in many cases, economically viable and environmentally sustainable. Since roots of *M.citrifolia* have been reported to be a rich source of anthraquinones, in the present study an attempt was made to use *in vitro*-raised roots as initial plant material for large-scale production of anthraquinones.

MATERIALS AND METHODS

Initiation of adventitious roots

Nodal segments derived from 3-year-old tree growing in swamps near Aakulam tourist village (latitude 8º31.505'N; longitude 76º53.942'E; altitude 8m above sea level) Thiruvananthapuram District Kerala State, India was used for *in vitro* shoot proliferation on MS [10] medium containing 3% sucrose, 18µM BAP solidified with 0.7% agar (Hi Media, Mumbai). The nodal segments from young branches were excised and washed thoroughly under running tap water for 30 minutes, subsequently immersed in a suspension of carbondazim fungicide (0.2% w/v; Bavistin; Mfg. BASF, Mumbai, India) for one hour in a gyratory shaker (100 rpm) followed by several rinses in distilled water. These explants were successively immersed in 70% ethanol for 30 seconds and 0.1% (w/v) mercuric chloride for 6 minutes followed by several rinses in sterile distilled water. Nascent shoots thus obtained were segmented and nodal parts were subcultured every 6 weeks, facilitated stable rate of multiplication (i.e. @ 7.2-5.5 no. of shoots/culture). Individual shoots (<3cm) with four or more expanded leaves from the third subculture onwards were harvested and transferred to agar free MS medium supplemented with 3% sucrose and auxins (IAA, IBA or NAA) in varying concentrations (12, 24 or 36 µM) for adventitious root induction. MS medium without auxin served as the control. The microshoots were supported with filter paper bridge in the liquid medium. Four weeks after planting, frequency of root induction, root length (cm) and number of roots were recorded.

Callus initiation

Four-week-old adventitious roots from the microshoots were excised and approximately 10-15 mg of roots per tube were cultured on MS medium supplemented with 30g/l sucrose, 7g/l agar and varying concentrations (0.5, 2.5, 5, 7.5 or 10 µM) of different plant growth regulators *viz.,* 2, 4-D, NAA or IBA (Sigma Chemical Co., Bangalore, India). To develop callus cultures from the roots, 25 x 150mm culture tubes wtih polypropalene cap was used. The pH of the medium was adjusted to 5.8 before autoclaving at a pressure of 1.08 kg cm^2 for 20 minutes. The cultures were maintained in a growth chamber at 25±2ºC under 16 h photoperiod at 50µmol$m^{-2}s^{-1}$ irradiance provided by cool white fluorescent tubes (Philips, India) and 60-65% relative humidity. Callus initiated on MS medium supplemented with varying levels of auxins were transferred to corresponding fresh medium on every 15th day. Total biomass of the callus (fresh weight), growth index and total anthraquinones were determined on every 15th day, up to a period of 90 days. The growth index was calculated by GI = Wf-Wo /Wo where Wf is the final fresh weight and Wo is the initial fresh weight.

Suspension culture

The initial screening of callus raised in various auxin types and concentrations showed varying levels of anthraquinone. Accordingly, the callus which mounted a highest anthraquinone level was used to initiate cell suspension culture. Suspension cultures were started by transferring friable piece of callus (300-500mg fresh weight) in 100 ml Erlenmeyer flasks containing 30 ml MS medium. When the cells from the callus get dispersed in to the medium, removal of unbroken callus material and large clumps were done through filtration using cell dissociation sieve with mesh diameter 100 mm and opening size 140 mm (Sigma, St. Louis, US). The hormone type and concentration, level of sucrose and pH of the medium was kept the same as in the optimized level for callus multiplication and maintenance medium. Cultures for raising suspension were incubated on a gyratory shaker at 100 rpm at 25±2ºC under 16 h photoperiod at 50 µmol$m^{-2}s^{-1}$ irradiance provided by cool white fluorescent tubes (Philips, India) and 60-65% relative humidity. The suspension raised in liquid medium was repeatedly subcultured for six times by transferring approximately 100-200 mg fresh weight of cells to fresh medium in every two week interval. The repeated cultures facilitated formation of fine cell suspensions. At this stage, freely suspended cells were collected and approximately 100-200 mg fresh weight of cells / 30 ml MS medium in 100 ml conical flask was used for further experiments. In the first experiment, the suspension (approx. 100-200mg/fw) raised in 2.5 µM NAA medium were transferred to MS medium supplemented with 2.5 µM NAA along with IBA, BAP or kinetin (0.5 or 2.5 µM) and sucrose (3%). The anthraquinone productivity of these cultures was quantified on 15th and 30th days of culture. The cells suspended in 2.5 µM NAA alone were kept as the control in this experiment.

In the second experiment, the effect of three carbon sources *viz.,* sucrose, fructose and galactose (3% and 5%) was tested for the production of anthraquinones. The stock culture (apprx. 100-200 mg/fw) was transferred to liquid medium containing varying types and concentrations of carbohydrates. Three flasks per treatment were harvested at 15th and 30th day and fresh weight and total anthraquinones of each sample were analysed.

Quantitative analysis of anthraquinones

Analysis of anthraquinone was done according to Hagendoorn [11]. Dried callus and filtered dry cell suspension (0.05 to 0.2g) was extracted twice with 80% ethanol for 45 minutes in a water bath incubator shaker at 80^{o}C (KEMI Co. Ltd., Mumbai, India). The ethanolic extracts were collected by centrifuging the tubes at 1500 rpm for 10

minutes and the supernatant was collected. More than 99% of the anthraquinones were extracted from the cells by this method. The absorption was determined at 434 nm on UV-visible spectrophotometer (UV-1700, Schimadzu, Japan) and anthraquinones was estimated using the alizarin (Fluka Analytical, St. Louis, US) as pure standard.

Statistical analysis

All experiments were conducted using a completely randomized block design (CRBD) method. For *in vitro* shoot multiplication, rooting and subsequent callus initiation, each treatment composed of three replications and each replication block was represented by ten culture tubes. For cell suspension cultures, three conical flasks were considered as single replication. The significance of treatments was determined by performing Analysis of variance (ANOVA) [12]. The mean separation was done according to Duncan's Multiple Range test ($p<0.05$).

RESULTS

Effect of auxins on adventitious root induction

Microshoots of *M. citrifolia* cultured on auxin added media showed significant ($P<0.001$) response in terms of rooting percentage, root number and length of roots. Among the three auxins tested, IBA showed significantly ($p<0.05$) high root induction showed 100% rooting on MS medium supplemented with 24 or 36 µM IBA. Among the two concentrations, 24 µM IBA resulted in a prolific adventitious rooting (23.16 ± 1.79 and root growth (3.13 ± 0.20 cm) within four weeks of culture (Fig 1a). Addition of NAA (36 µM) in rooting medium showed relatively lower response compared to IBA (Table 1). Among varying concentrations of IAA, 36 µM added medium produced 13.33 ± 1.25 roots/ shoots and were elongated to 2.06 ±0.34 cm. Maintaining rooted shoots for 7-8 weeks in auxin containing medium caused turning yellow coloured roots to brown and become fragile. However, *in vitro* developed adventitious roots can be used for callus production and later initiation of cell suspension from developed callus. Among the three auxins tested, adventitious roots raised on IBA (24 µM) supplemented medium was selected for callus induction.

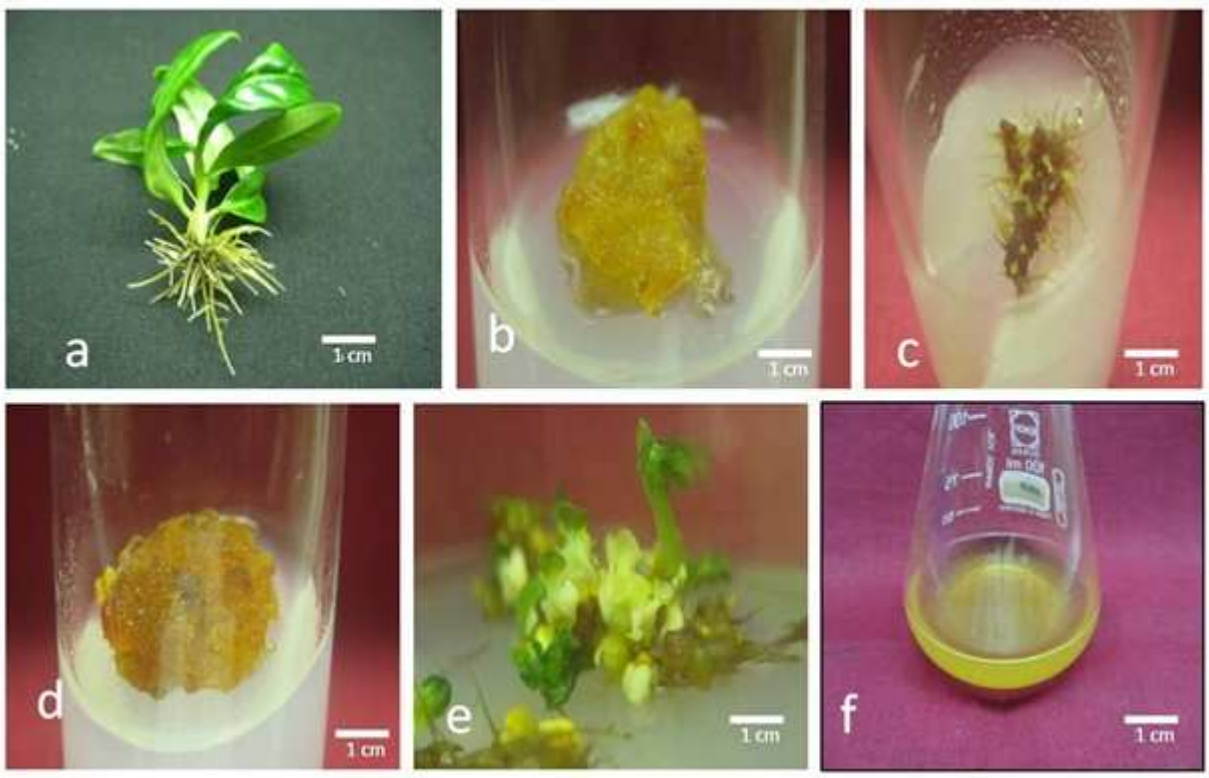


Figure 1 a- Adventitious rooting in 24µM IBA treated microshoots (scale bar = 1.2 cm), b- Callus development in 2,4-D supplemented medium (scale bar =1.78 cm), c- Callus development along with rooting in IBA supplemented medium (scale bar=1.78 cm), d- Callus development in NAA supplemented medium (scale bar =1.78 cm), e- Shoot induction from roots inoculated in MS basal medium (scale bar = 0.6 cm), f- Cell suspension raised in 2.5 µM NAA along with 0.5 µM BAP (scale bar=3.8 cm).

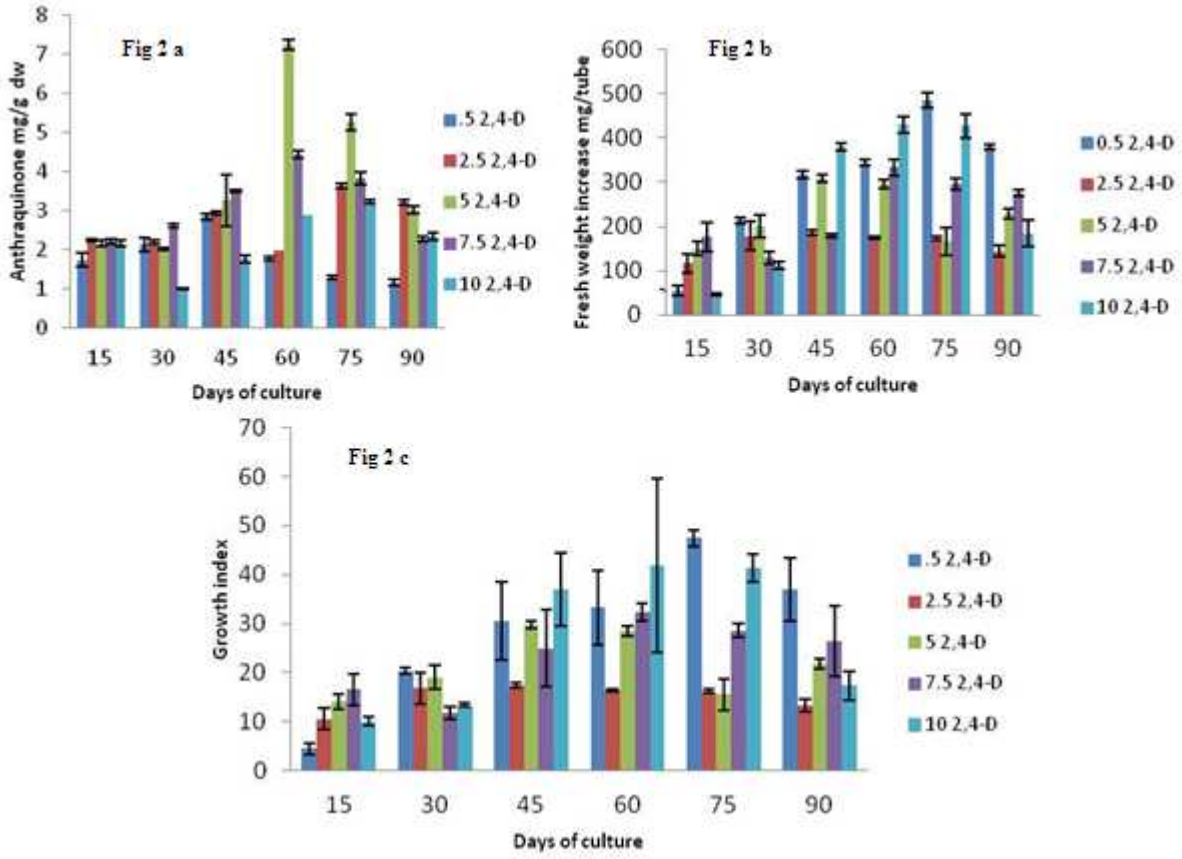
Table 1. Effect of auxins on *in vitro* rooting of *M. citrifolia* microshoots in liquid MS medium after 4 weeks of culture

Auxin Type	Concentration (µM)	Percentage of rooting (%)	Mean number of roots/shoot	Mean root length (cm)
Control	0.0	38.88±11.11^{c}	4.50±1.36^{f}	1.11±0.27^{c}
IBA	12	94.43±5.56^{a}	15.66±1.74^{bc}	1.43±0.14^{bc}
	24	100.00±0^{a}	23.16±1.70^{a}	3.13±0.20^{a}
	36	100.00±0^{a}	8.50±1.17^{def}	1.43±0.11^{bc}
NAA	12	88.86±11.13^{ab}	6.33±0.91^{f}	1.18±0.14^{c}
	24	94.43±5.56^{a}	12.16±2.27^{bcd}	1.46±0.15^{bc}
	36	83.30±0^{ab}	13.33±0.61^{b}	1.16±0.10^{c}
IAA	12	66.66±0^{b}	6.50±1.17^{ef}	1.58±0.29^{bc}
	24	88.86±11.13^{ab}	10.83±1.62^{cde}	2.10±0.28^{b}
	36	83.30±9.64^{ab}	13.33±1.25^{bc}	2.06±0.34^{b}
Treatment Df (n-1)	9	6.57***	14.22***	7.66***
Auxin type (T) Df (n-1)	2	5.76*	17.37**	8.64**
Auxin con (C) Df (n-1)	2	2.07^{NS}	12.65**	5.59*
TXC Df(n-1)	8	0.92^{NS}	12.73**	4.13*

*Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. *F value significant at p□0.05 level, **significant at p□0.01 level , *** significant at p<0.001 level*

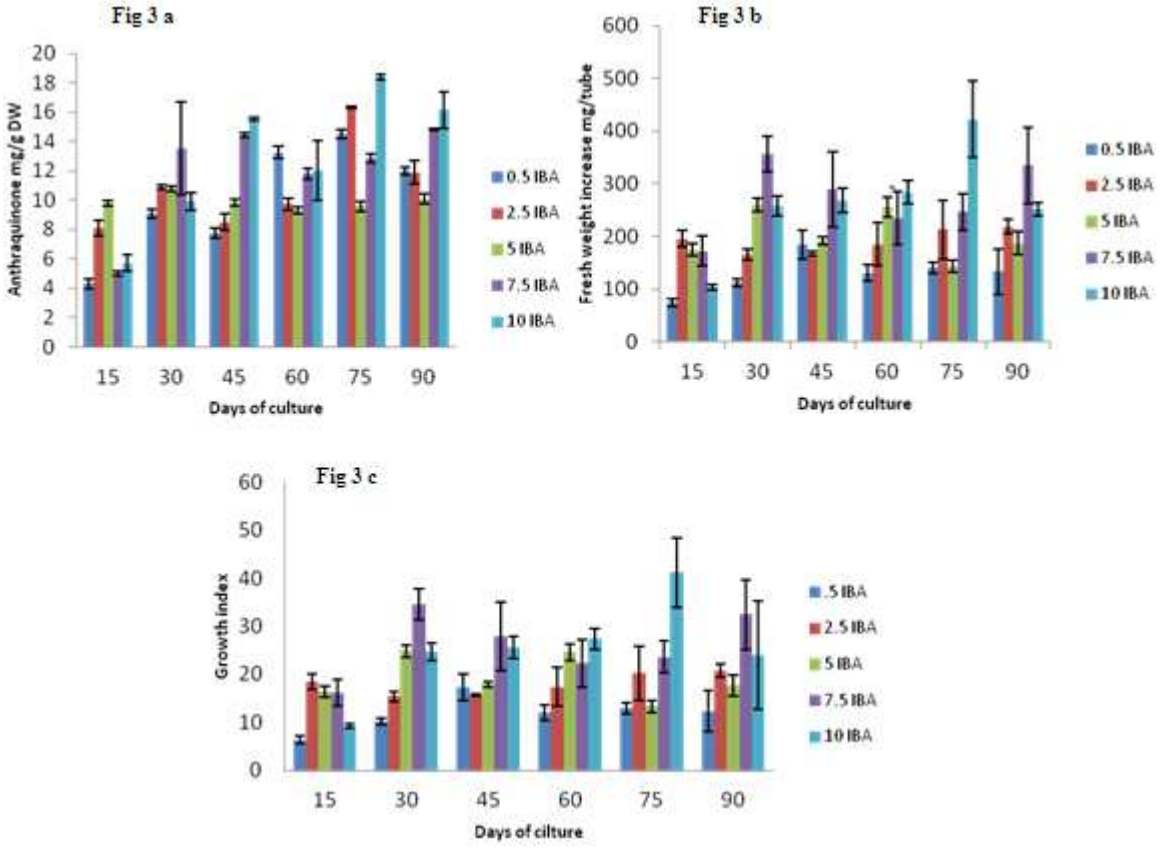
Effect of 2, 4-D

Callus initiation was noticed when adventitious roots were inoculated on MS medium containing 2, 4-D, NAA or IBA at varying concentrations (0.5, 2.5, 5, 7.5 or 10 µM). Auxin type and concentration had significant (P<0.001) effect on callogenic response as well as AQ production. A significantly (P<0.001) high AQ production (7.23 mg/g dw) was noticed in 5 µM 2, 4-D on 60th day of culture (Fig. 2,a). Reduction in AQ accumulation was noticed on 90th day in all the treatments. Plant cell biomass (485 mg fw/10 ml solid medium) and growth index (47.4 ± 1.65) was significantly (P<0.05) high due to the addition of 0.5 µM 2, 4-D in MS medium as recorded on 75th day of culture (Fig 2,b and c). The callus developed in MS medium containing 0.5 µM 2, 4-D, showed significantly (p<0.05) different biomass and growth index. Among the five different concentrations of 2, 4-D, 0.5 µM resulted in the production of least amount of AQ (1.15 ±0.027mg/g dw) on 90th day of culture. Irrespective of various 2, 4-D concentrations (2.5, 5, 7.5 and 10 µM), production of anthraquinones at different day intervals showed significant variation . Yellow coloured callus (Fig 1 b) was a peculiar feature of callus induced from the adventitious roots of *M. citrifolia* in 2, 4-D supplemented medium. Roots inoculated in hormone free MS medium showed development of shoots (Fig 1 e)



Effect of IBA

Brown coloured callus along with yellow roots was a characteristic feature of callus developed due to IBA supplementation in the medium (Fig 1 c). All the concentrations showed an increase in biomass and growth index from the 15th day and decreased biomass and growth index towards the end of 90th day. AQ accumulation was found to be increasing up to 75th day in all the cultures and showed significant ($P<0.05$) variation among different concentrations. Among the five different concentrations of IBA tested, 10 µM resulted in the production of 18.4 mg/g dw of AQ in callus cultures (Fig 3,a). Biomass (422.4 mg fw) and growth index (41.2) was found to be highest in 10 µM IBA supplemented medium on 75th day of culture (Fig 3, b and c).



Effect of NAA

Different concentrations of NAA tested, showed significant ($P<0.05$) variations in biomass and growth index at each intervals up to 90th day. Friable callus with yellow orange colouration was noticeable feature of NAA added medium (Fig 1 d). Highest quantity of AQ (20.25 mg/g dw) was recorded in 45-day- old callus developed in 2.5 µM NAA (Fig 4,a). In this medium biomass (486.5 mg fw) and growth index (47.6) was high compared to other NAA concentrations (Fig 4.b and c). Increasing concentrations of NAA caused a significant ($P<0.05$) decline in the anthraquinone synthesis. Callus developed in 2.5 µM NAA supplemented MS medium was used to initiate cell suspension culture.

Cell suspension culture

Cell suspensions raised in 2.5 µM NAA along with 0.5 or 2.5 µM BAP, KIN or IBA showed significantly ($P<0.001$) varying growth response (Table 2). Medium supplemented with 2.5 µM NAA , produced 10.86 mg/g dw AQ on 30th day of culture. Types and concentrations of growth hormones showed significant ($p<0.001$) interaction on anthraquinone accumulation. The cells suspended in 2.5 µM NAA along with 0.5 µM BAP produced maximum AQ (16.99mg/g dw) within 30 days culture period (Fig 1 f). The least amount of AQ accumulation (3.44 mg/g dw) was recorded in medium containing 2.5 µM NAA and 2.5 µM KIN. Increased AQ accumulation was noticed in all the treatments on 30th day than on 15th day of incubation.

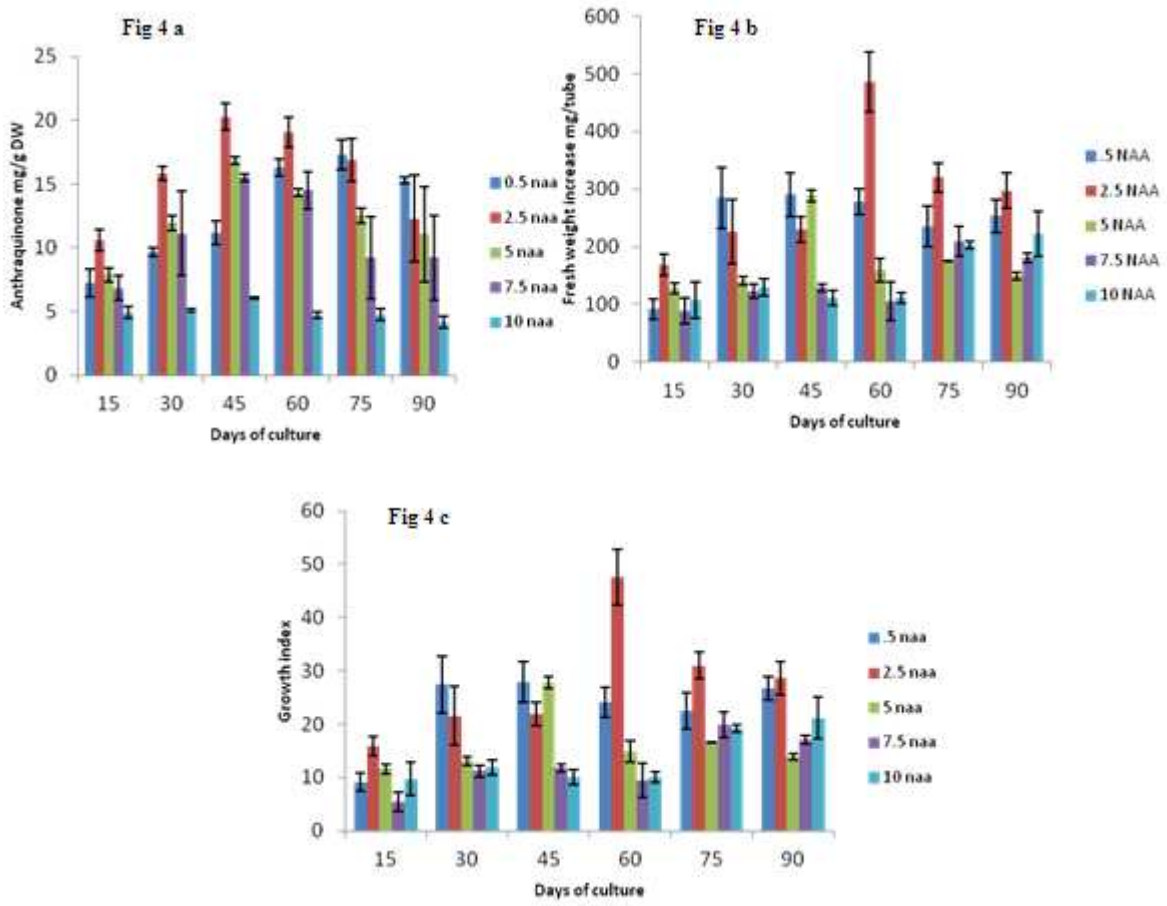


Table 2. Effect of NAA in combination with BAP, kinetin or IBA on biomass and anthraquinone production in suspension cultures on 15th and 30th day after incubation

Hormones (µM)				15th day	15th day	30th day	30th day
NAA	BAP	KIN	IBA	Biomass (g FW/30 ml)	Anthraquinone (mg/g dw)	Biomass (g FW/30 ml)	Anthraquinone (mg/g dw)
2.5	0	0	0	3.16±0.14^{a}	2.4±0.04^{a}	10.11±0.63^{a}	10.86±0.63^{cd}
2.5	0.5			1.70±0.11^{cd}	0.88±0.14^{c}	4.89±0.16^{b}	16.99±1.50^{a}
2.5	2.5			1.38±0.03^{d}	1.95±0.02^{ab}	2.81±0.45^{cd}	8.74±0.32^{d}
2.5		0.5		1.84±0.04^{bc}	1.65±0.09^{b}	2.99±1.17^{cd}	13.3±0.25^{b}
2.5		2.5		1.68±0.08^{cd}	0.99±0.09^{c}	1.87±0.31^{d}	3.44±0.64^{f}
2.5			0.5	1.51±0.23^{cd}	1.57±0.07^{b}	4.27±0.13^{bc}	12.41±0.79^{bc}
2.5			2.5	2.09±0.05^{b}	1.82±0.09^{b}	3.23±0.20^{bcd}	6.3±0.25^{e}
Treatment Df(n-1)= 6				24.13***	8.62***	24.1***	36.63***
Type (T) Df(n-1)= 2				2.87^{NS}	8.06*	4.24^{NS}	18.79**
Con (C) Df(n-1)= 1				0.12^{NS}	7.83*	10.12*	165.71***
TXC Df(n-1)= 5				8.53*	40.22**	0.56^{NS}	41.89***

*Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. *F value significant at p□0.05 level, **significant atp□0.01 level , *** significant at $p<0.001$ level*

Effect of carbon sources

Significant ($p<0.001$) differences in anthraquinone accumulation was noticed in cells suspended medium containing different carbon sources at varying concentrations on 30th day of records. Among the different carbon sources studied, biomass and anthraquinones accumulation was found to be highest in MS medium enriched with 5% sucrose (Table 3). Fresh weight of cells on 15th (3.28 g/30 ml) and on 30th day (11.56 g/30ml) in 5% sucrose fortified medium was significantly ($P<0.05$) high. Anthraquinone accumulation was found to be 3.64 and 16.17 mg/g dw on 15th and 30th day respectively in 5% sucrose added medium. Accumulation of anthraquinone at early stage of incubation (15th day) in different carbon sources supplementation did not show any significant difference.

Table 3. Effect of carbon sources on biomass and anthraquinone production in suspension cultures on 15th and 30th day after incubation

Carbon source (%)			15th day	15th day	30th day	30th day
Sucrose	Fructose	Galactose	Biomass (g FW/30 ml)	Anthraquinone (mg/g dw)	Biomass (g FW/30 ml)	Anthraquinone (mg/g dw)
3			3.16±0.14^{ab}	2.4±0.41^{bc}	10.12±0.64^{ab}	10.86±0.63^{b}
5			3.28±0.08^{a}	3.64±0.32^{a}	11.56±0.37^{a}	16.17±1.88^{a}
	3		1.77±0.10^{d}	2.05±0.31^{c}	4.29±0.96^{c}	3.04±0.34^{c}
	5		2.83±0.09^{b}	2.90±0.15^{abc}	3±0.17^{c}	2.41±0.15^{c}
		3	2.83±0.15^{b}	2.97±0.09^{ab}	10.42±1.34^{a}	8.92±0.44^{b}
		5	2.46±0.01^{c}	2.57±0.09^{bc}	7.65±0.87^{b}	8.64±0.37^{b}
Treatment Df(n-1)= 5			25.12***	4.39*	18.12***	35.57***
Type (T) Df(n-1)= 2			36.12**	2.10^{NS}	41.09***	79.24***
Con (C) Df(n-1)= 1			8.9*	6.88*	1.66^{NS}	4.37^{NS}
TXC Df(n-1)= 5			22.2**	5.30^{NS}	3.33^{NS}	7.51*

*Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. **F value significant at p□0.05 level, **significant atp□0.01 level , *** significant at $p<0.001$ level*

DISCUSSION

Prolific rooting of microshoots planted in IBA supplemented medium was noticed in the present study. IBA has been widely used as a root induction hormone under *in vitro* and *in vivo* condition [13, 14, 15]. IBA is a synthetic and stable auxin can easily metabolized by plants. In a previous work on *M. citrofolia*, effectiveness of IBA to induce adventitious root from leaf explants was reported [16]. Induction of roots from microshoots using IBA has been reported in many rubiacean members like *Anthocephalus cadamba* [17], *Randia dumetorum* [18] and *Cephaelis ipecacuanba* [19]. Callus cultures raised from parent tissue can be maintained for an extended period by subculture and can therefore represent a convenient form of long-term maintenance of cell lines. Further, such cultures can be used for initiating suspensions [20]. Results in the present study indicate that anthraquinone synthesis is regulated in a different way by the type, concentration and incubation period in the three auxin treated callus cultures of *M. citrifolia*. Among the three auxins used for callus cultures, NAA and IBA resulted in the production of substantial amount of anthraquinone compared to 2, 4-D. Earlier reports also suggest suppressive effect of 2, 4-D to anthraquinones production, while NAA promoted the production [21,22]. Stalman [23] has demonstrated that *M. citrifolia* cells grow in an equal rate both 2, 4-D and NAA, but anthraquinone accumulation was found to be high in NAA supplemented medium. In the present work, also the NAA addition has resulted in stable production of AQ and could be subcultured indefinitely without any change in growth rate and anthraquinone level.

The type of carbon source and its concentration influenced the growth and anthraquinones production in *M. citrifolia* cell cultures. Among the three carbon sources studied, 5% sucrose showed better anthraquinone accumulation than fructose and galactose. Sucrose not only plays an important nutritive role but might have a regulatory role in the synthesis of secondary products in the cells. Smeekens [24] reported that sugars have important signalling functions throughout the plant life cycle. In an early work [25], it is reported that sucrose favoured anthraquinone production compared to glucose.

In conclusion, present study demonstrated that 24 μM IBA is the best auxin source for the induction of adventitious roots from microshoots of *M. citrifolia*. Further, by using *in vitro* derived adventitious roots, callus can be produced in MS medium containing auxin. Callus that is proven its AQ production capacity can be used for raising cell suspension. The *in vitro* biomass (i.e., callus and cell suspension) can be used as enhanced production system of anthraquiones.

Acknowledgement
The authors are thankful to the University of Kerala, Thiruvananthapuram, India for granting University JRF (No: Ac.E1/5825/09) and we thank Dr Ashalatha S Nair, Professor and Head, Department of Botany and Dean, Faculty of Science, University of Kerala for the facilities provided.

REFERENCES

[1] Han YS, Van Der Heijden R, Verpoorte R, *Plant Cell Tissue Organ Cult*, **2001**, 67,201-220.
[2] Montoya NSC, Comini LR, Sarmiento M, Becerra C, Albesa I, Arguello GA, Cabrera JL, *J Photochem Photobiol Biol*, **2002**, 78,77-83.
[3] Nordin H, Lajis J, Mooi LY, Abdullah A, Sukari MA, Ali AM, *Asia Pacific Mol Biol Biotechnol*, **2003**,11,3-7.
[4] Wang MY, Brett JW, Jensen CJ, Nowicki D, Su C, Paul AK, Anderson G, *Acta Pharmacol Sin*, **2002**, 23,1127-1141.

[5] Borotto J, Coll J, Rivas M, Blanco M, Concepcion O, Tandro YA, Hernandez M, Trujillo R, *Plant Cell Tiss Org Cult*, **2008**, 98,181-187.
[6] Nelson SC, *Species Profile for Pacific Island Agroforestry*, www.traditionaltree.org,**2006**.
[7] Farine J P, Legal L, Moreteau B, Le Quere JL, *Phytochemistry*, **1996**, 41,433-438.
[8] Kamiya K, Tanaka Y, Endang H, Umar M, Satake T, *Chem Pharm Bull*, **2005**, 53, 1597-1599.
[9] Siddiqui BS, Sattar FA, Begum S, Gulzar T, Ahmad F, *Arch Pharm Res*, **2007**, 30,793-798.
[10] Murashige T, Skoog F, *Physiol Plant*, **1962**,15, 473-497.
[11] Hangendoorn MJM, Jamar DCL, Van der plas LHW, *Plant Cell Culture Protocols*, Humana Press, New York Inc, pp 383-392.
[12] Snedecor GW, Cochran WG, *Statistical methods* (4th edn.) The Iowa State University Press, Iowa,**1962**.
[13] Mehta J, Ansari R, Syedy M, Khan S, Sharma S, Gupta N, Rathore R, Vaishnav K, *Asian J Plant Sci Res*, **2012**, 2 (5), 620-626.
[14] Mehta J, Naruka R, Sain M, Dwivedi A, Sharma D, Mirza J, *Asian J Plant Sci Res*, **2012**, 2 (4), 518-523.
[15] Abirami H, Suresh Kumar P, *Asian J Plant Sci Res*, **2013**, 3 (2), 99-106.
[16] Baque MA, Hahn E, Paek K, *Plant Biotechnol Rep*, **2010**, 4,109-116.
[17] Kavitha M, Kalaimagal I, Mercy S, Sangeetha N, Ganesh D, *Journal of Forest Science*, **2009**, 25, 111-118.
[18] Begum F, Mohammed DIK, Paul RN, Masfique M, Shyamole R, *Indian Journal of Experimental Biology*, **2003**, 41,1479-1481.
[19] Jha S, Jha TB, *Plant Cell Reports*, **1989**, 8,437-439.
[20]Brown JT, In: Pollard J W, Walker J.M (eds) *Methods in molecular biology*,Humana Press Inc., New Jersey , **1990**, 6,57-63.
[21] Zenk MH, Schulte U, E I-Shagi H, *Naturwissenschaften*, **1984**,71,266.
[22] Leistner E, In: YPS Bajaj (ed.) *Biotechnology in Agriculture and Forestry, Medicinal and Aromatic plants* Springer-Verlag, Berlin Heidelberg,**1995**, 33,296-307.
[23] Stalman M, Koskamp A, Ludered R, Vernooy JHJ, Wind JC, Wullems GJ, Croes A F, *J. Plant Physiol*, **2003**, 160, 607-614.
[24] Smeekens S, *Plant Mol.Biol*, **2000**,51,49-81.
[25] Zenk MH, E I-Shagi H, Schulte U, *Planta Med*. (Suppl), **1975**, 79-101.