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RESEARCH ARTICLE

ANTHRAQUINONE PRODUCTION IN CELL CULTURES OF *MORINDA CITRIFOLIA* L. THROUGH COTREATMENT WITH ELICITORS AND PRECURSORS

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SUMMARY

Morinda citrifolia (Rubiaceae) commonly known as noni, is chiefly valued as source of neutraceuticals. In the present work, experiments were conducted to initiate a cell suspension culture from adventitious roots. It is followed by standardization of suitable media for the production of fine cell suspension and to evaluate the effect of elicitors and precursors for the production of anthraquinones (AQ) in cell suspension cultures of *M. citrifolia.* Suspension cultures were raised by incubating the culture on a gyratory shaker (Orbital shaking incubator, RIS 24-BL, REMI, India) at 100 rpm at 25 ± 2° C 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. Friable callus maintained in MS medium supplemented with 2.5 µM NAA derived from adventitious root explants was used as the stock culture for the initiation of cell suspension cultures. The AQ production in different concentrations of NAA (0.5, 2.5, 5 µM) were analysed to determine the optimum concentration of NAA for productive fine cell suspension. The effect of elicitors and precursors for enhancing anthraquinone production were analysed. Five biotic elicitors (yeast extract, pectin, xylan, chitosan, alginic acid) and three precursors (phenylalanine, α-ketoglutaric acid, shikimic acid) on anthraquinone accumulation in cell cultures were evaluated. Among the elicitors analysed, alginic acid showed highest intracellular AQ production (46.9 mg/g dw). Among various precursors tested, addition of α-ketoglutaric acid showed highest intracellular AQ production (53 mg/g dw). The addition of α-ketoglutaric acid showed 4.8-fold enhancement in AQ production as compared to control cultures.The present work demonstrates that biotic elicitors and precursors are potent factors for AQ production without much loss in biomass in *M. citrifolia* cell suspension culture.

Keywords: *Morinda citrifolia*, anthraquinone, cell suspension culture, elicitors, precursors.

INTRODUCTION

Natural product, cosmetic and pharmaceutical industries are now giving more emphasis to plant cell culture based production of secondary metabolites. It can be effective to harness more inexpensively than extracting either the whole plant growing under natural conditions or chemical synthesis of the product. The advantage of plant cell culture technology is that it can ultimately provide a continuous,

reliable source of natural products. The cell cultures typically accumulate large amounts of secondary metabolites only under specific conditions (Mulabagal & Tsay 2004). In order to obtain high yields that is desirable for commercial exploitation, efforts have now focused on isolating the biosynthetic activities of cultured cells, achieved by optimizing culture conditions, selecting high yielding strains, employing precursor feeding, transformation methods and immobilization techniques (Dicosmo & Misawa 1995). *Morinda citrifolia* (Rubiaceae) commonly known as noni, is chiefly valued as source of neutraceuticals. It contains several medicinally active compounds, e.g. anthraquinones, terpenoids, alkaloids, sitosterol, carotene, flavones and glycosides that exhibit various therapeutic effects such as antibacterial, antiviral and anticancer activities (Wang et al. 2002, Treetip et al. 2008, Sreeranjini & Siril 2011). The root of the plant is a valuable source of natural dye anthraquinone (AQ) (Komaraiah et al. 2005). Since the roots contain maximum level of AQ, large scale extraction of dye from roots can cause depletion of natural stands. Alternatively, various plant cell culture techniques can be utilized for the large scale production of AQ through in vitro techniques, without imposing pressure on naturally growing *M. citrifolia*. In the present work, experiments were conducted to initiate a cell suspension culture from adventitious roots. It is followed by standardization of suitable media for the production of fine cell suspension and to evaluate various factors affecting production of AQ in cell suspension cultures of *M. citrifolia.*

MATERIAL AND METHODS

Initiation of cell suspension culture

Friable callus maintained in MS medium supplemented with 2.5 µM NAA derived from adventitious root explants was used as the stock culture for the initiation of cell suspension cultures. Friable piece of callus (~300−500 mg fw) was transferred to 100 ml Erlenmeyer flask containing 30 ml MS liquid medium. When loosely adhered cells in the callus mass get dispersed in to the medium, removal of unbroken callus material and large clumps were done through filtration using cell dissociation sieve (mesh diameter 100 mm and opening size 140 mm, Sigma, St. Louis, US). The suspension raised in liquid medium was repeatedly subcultured for six times to fresh medium in every two wks interval. Approximately 100−200 mg fw of cell suspension were inoculated to fresh medium on every 15 d interval. Suspension cultures were raised by incubating the culture on a gyratory shaker (Orbital shaking incubator, RIS 24-BL, REMI, India) at 100 rpm at 25 ± 2° C 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.

Analysis of growth characteristics of cell suspension in NAA medium

To determine the optimum concentration of NAA for productive fine cell suspension, approximately 100−200 mg fw of cells raised in 2.5 µM NAA containing medium were transferred to MS medium with different concentrations of NAA (0.5, 2.5 or 5 µM). Cells inoculated in growth regulator free MS medium served as control. AQ production was analysed by harvesting cell cultures on every fifth d up to a period of 40 d.

Quantitative analysis of AQs

Analysis of AQs was done according to Hagendoorn et al. (1994). Dried cells (100−200 mg) were extracted twice with 80% ethanol for 45 min in a boiling water bath at 80° C (KEMI water bath incubator shaker). The ethanolic extracts were collected by centrifuging the tubes at 1500 rpm for 10 min. The absorption was determined at 434 nm on UV visible spectrophotometer (Shimadzu; Model No.UV-1700) and were estimated using the standard graph of alizarin (Fluka Analytical, US).

Effect of elicitors and precursors

Five biotic elicitors, yeast extract, pectin, xylan (SRL, Mumbai) chitosan and alginic acid (Sigma- Aldrich, St. Louis, US) and three precursors, phenylalanine, α-ketoglutaric acid (SRL, Mumbai) and shikimic acid (Sigma- Aldrich, St. Louis, US) were filter sterilized and fed to the suspension culture on 20th d of culture. AQ accumulation was evaluated on 35th d of incubation.

Statistical analysis of the data

All the experiments were conducted using a completely randomized block design (CRBD) method. Each treatment composed of three replications and each replication block was represented by three conical flasks per treatment. One way ANOVA was performed to determine significance of treatments and also to determine interaction of factors (Snedecor & Cochran 1962). The mean separation was done according to Duncan's multiple Range Test ($P < 0.05$).

OBSERVATIONS

Initiation of suspension culture

Production of friable callus was necessary for the initiation of cell suspension culture. Callus which developed in 2.5 µM NAA supplemented with MS medium, upon subculture resulted in multiplication of friable callus containing AQ. This friable callus was used as the initiation material for the production of fine cell suspension. Suspension cultures were started by transferring friable piece of callus (300−500mg fresh weight) in 100 ml Erlenmeyer flasks containing 30 ml MS medium. The repeated cultures facilitated formation of fine cell suspensions and maintained as stock cell suspension culture.

AQ accumulation in 2.5 µM NAA medium

AQ accumulation also showed significant differences among various NAA concentrations tested. Control set raised in MS hormone free medium showed least accumulation of AQs as compared to NAA treated cultures and this indicates that auxin (NAA) treatment can enhance AQ accumulation in cell cultures of *M. citrifolia.* From 15th d onwards significant difference ($P < 0.05$) in AQ accumulation was noticed in each treatments. In 0.5 µM NAA supplemented medium, maximum AQ accumulation (7.36 mg/g dw) was noticed on 30th d followed by a slight decrease in AQ accumulation from 35th d onwards. Addition of 2.5 µM NAA in the medium resulted maximum production of AQ (11.09 mg/g dw) on 25th d of incubation. NAA treatments at 5 µM concentration produced maximum AQ (5.5 mg/g dw) on 25th d of incubation. Even though biomass increase was noticed to be high in 5 µM NAA supplemented medium compared to cultures in 0.5 µM NAA, AQ accumulation was found to be high in 0.5 µM NAA treatments. Cultures raised in 5 µM NAA supplemented medium showed significant reduced level of AQ accumulation compared to other NAA treatments. Among the three concentrations of NAA tested, 2.5 µM, resulted in highest AQ accumulation on 35th d of culture (Table 1).

Effect of elicitors and precursors

Among the various elicitors tested, alginic acid resulted highest AQ accumulation (46.9 mg/g dw). Addition of alginic acid resulted in 4.2- fold increase in intracellular AQ production compared with the control cultures. The three precursors analysed here showed enhanced AQ production compared to control cultures. Addition of α-ketoglutaric acid produced 53 mg/g dw AQ on 35th d. The addition of α-ketoglutaric acid showed 4.8-fold enhancements in AQ production compared to control cultures (Fig. 1).

TABLE 1: Effect of NAA on anthraquinone production in cell suspension cultures of *M. citrifolia.*

NAA (μM)	Total anthraquinone (mg/g dw) ± S.E. (Days of culture)							
	5	10	15	20	25	30	35	40
Control	2.3 # 0.3^{a}	2.56 ± 0.3^{a}	2.1 ± 0.25^{b}	2.1 ± 0.30^{b}	1.91 ±0.25^{c}	1.79 ± 0.28^{d}	1.61 ± 0.19^{d}	1.33 ± 0.05^{d}
0.5	2.5 ± 0.2^{a}	2.73 ± 0.2^{a}	4.36 ± 0.64^{a}	4.96 ± 1.0^{a}	6.46 ± 0.37^{b}	7.36 ± 0.84^{b}	6.46 ± 0.21^{b}	5.9 ± 0.20^{b}
2.5	2.3 ± 0.4^{a}	2.3 ± 0.43^{a}	3.4 ± 0.41^{b}	5.4 ± 1.21^{a}	10.4 ± 0.46^{a}	10.86 ± 0.63^{a}	11.09 ± 0.45^{a}	10.04 ± 0.17^{a}
5	1.7 ± 0.15^{a}	1.76 ± 0.1^{a}	2.3 ± 0.08^{b}	2.9 ± 0.08^{ab}	5.5 ± 0.37^{b}	4.43 ± 0.69^{c}	3.28 ± 0.13^{c}	3.27 ± 0.23^{c}
Df(n-1)= 3	1.34^{NS}	1.85^{NS}	6.72*	5.87*	87.41**	36.28***	224.3***	27.3***

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

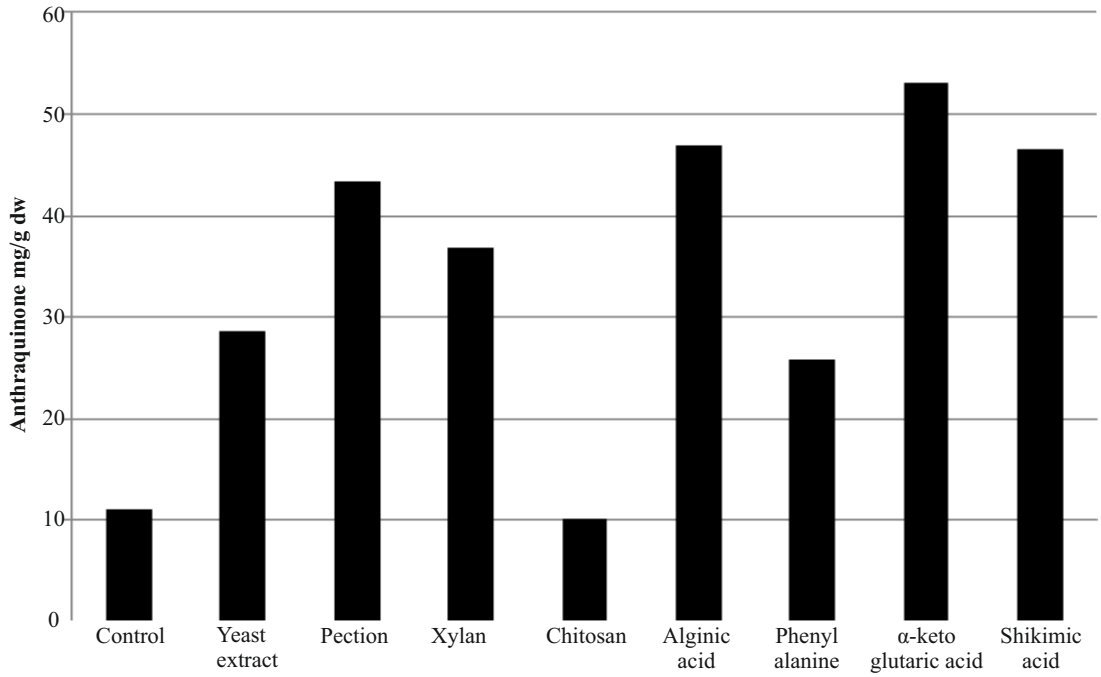

Fig.1: Effect of treatments on accumulation of anthraquinones in *M.citrifolia* cell cultures.

DISCUSSION

Plant cell culture technology has enhanced the usefulness of plants as renewable resources of valuable chemicals. There has been considerable interest in plant cell cultures as a potential alternative to traditional agriculture for the industrial production of secondary metabolites (Dicosmo & Misawa 1995). Plant growth regulators are effective triggers of secondary metabolism at in vivo conditions (Bohm 1980). Since the production of secondary metabolites in plant cell cultures is a function of both cell multiplication and division, growth regulators have a major role in determining the potential productivity of a given culture (Kurz & Constabel 1979). In the present study, NAA in the medium enhanced AQ accumulation and resulted in the production of fine cell suspensions.

Enhancement of secondary metabolites by elicitation is one of the few strategies recently adapted by commercial cell culture ventures (Savitha et al. 2006). The effect of elicitors on cell growth is dependent on elicitors used, concentrations or conditions, plant species and the stage at which the elicitors are applied (Cai et al. 2012). Among the five biotic elicitors analysed in the study, alginic acid recorded the maximum AQ production in suspension cultures. Enrichment of medium with precursors has been reported to trigger enhancement of metabolite production (Veeresham & Kokate 1997). Any compound, whether endogenous or exogenous, that can be converted by an organism or living system into the investigated product or secondary metabolite or useful compounds is known as precursor (Veeresham 2004). Supplementation of precursors in the medium has been reported to have an enhanced effect on specific metabolite production in cell cultures. AQs are anthracene derivatives that have two keto groups, usually in positions 9 and 10. The A and B ring of AQs are originated from the carbon skeleton of shikimic and α-ketoglutaric acid via isochorismate/o-succinylbenzoate pathway. The C ring is originated from 2-c methyl-d-erythritol 4-phosphate (MEP) pathway (Quevedo et al. 2010). In the present study, α-ketoglutaric acid and shikimic acid enhanced AQ production. The present work demonstrates that biotic elicitors and precursors are potent factors for AQ production without much loss in biomass in *M. citrifolia* cell suspension culture. Production of anthraquinones from cell suspension cultures under in vitro conditions is a promising approach which relives pressures on natural stands of the source plant.

REFERENCES

BOHM H 1980 The formation of secondary metabolites in plant tissue and cell cultures *Intl Rev Cytol Suppl* **11B** 183−208

CAI Z, KASTELL A, MEWIS I, KNORR D & SMETANSKA I 2012 Polysaccharide elicitors enhance anthocyanin and phenolics acid accumulation in cell suspension cultures of *Vitis vinifera Plant Cell Tiss Org Cult* **108** 401−409

DICOSMO F & MISAWA M 1995 Plant cell and tissue culture: Alternatives for metabolite production *Biotech Advances* **13** 425−453

HAGENDOORN M J M, LINUS H W & SEGERS G J 1994 Accumulation of anthraquinones in *Morinda citrifolia* cell suspensions- A model system for the study of the interaction between secondary and primary metabolism *Plant Cell Tiss Org Cult* **38** 227−234

KOMARAIAH P, KAVIKISHORE P B, CARLSSON M, MAGNUSSON K E & MANDENIUS C F 2005 Enhancement of anthraquinone accumulation in *Morinda citrifolia* suspension cultures *Plant Sci* **168** 1337−1344

KURZ W E W & CONSTABEL F 1979 Plant cell cultures a potential source of pharmaceuticals *Adv Appl Microbiol* **25** 209−240

MULABAGAL V & TSAY H S 2004 Plant Cell Cultures - An alternative and efficient source for the production of biologically important secondary metabolites *Int Appl Sci Engg 2* **1** 29−48

QUEVEDO C, PERASSOLO M, ALECHINE E, CORACH D, GIULIETTI A M, TALOU J R 2010 Increasing anthraquinone production by overexpression of 1-deoxy-D-xylulose-5-phosphate synthase in transgenic cell suspension cultures of *Morinda citrifolia Biotech Lett* **32** 997−1003

SAVITHA B C, TIMMARAJU R, BHAGYALAKSAMI N, RAVISHANKAR G A 2006 Different biotic and abiotic elicitors influence betalain production in hairy root cultures of *Beta vulgaris* in shake flask and bioreactor *Process Biochem* **41** 50−60

SNEDECOR G W & COCHRAN W G 1962 *Statistical methods* 4th ed Iowa State University Press Ames Iowa

SREERANJINI S & SIRIL E A 2011 Evaluation of anti-genotoxicity of the leaf extracts of *Morinda citrifolia* Linn *Plant Soil Env* **57** 222−227

TREETIP R, SUMON T & PRANEE N 2008 *In vitro* genotoxic and antigenotoxic studies of Thai Noni fruit juice by chromosomal aberration and sister chromatid exchange assays in human lymphocytes *Songklanakarin J Sci Tech* **30** 583−589

VEERESHAM C 2004 *Medicinal Plant Biotechnology* CSB Publishers New Delhi

VEERESHAM C & KOKATE C 1997 Taxol A novel anticancer agents from cell cultures of *Taxus* sp *Indian Drugs* **34** 354−359

WANG M Y, BRETT J W, JENSEN C J, NOWICKI D, SU C, PAUL A K & ANDERSON G 2002 *Morinda citrifolia* (Noni): a literature review and recent advances in Noni research *Acta Pharmacol Sinica* **23** 1127−1141