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COMPARATIVE STUDIES ON THE IMPROVEMENT OF ANTHRAQUINONES USING VARIOUS PRECURSORS IN DIFFERENT*IN VITRO* SYSTEMS OF *RUBIA CORDIFOLIA*

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ABSTRACT
It is now well understood that the *in vitro*plant materials are functioning as prospective factories of future secondary metabolites.*Rubia cordifolia* is a plant noted for its red dyeing pigment AQ, from time immemorial, and the dye is chiefly located in its roots. The constant uprooting of the plant for AQs threatens its existence in natural stands. So, an attempt has been done to enhance the production of AQ in the*in vitro* conditions using various precursors viz., α ketoglutaric acid (AK), phenylalanine (PA), and piroxicam.In callus cultures,the addition 100 µM phenylalanine as a precursor resulted in 25-fold higher alizarin production over roots sampled from the naturally growing plant. In suspension culture, precursor feeding by 100 µM AK resulted from 27-fold (58.97 mg g-1 dw) productivity of alizarin and 26-fold productivity (18.68 mg g-1 dw) of purpurin over roots sampled from natural stands. Compared to cultures without AK feeding (control) 6 alizarin and 9-fold purpurin productivity was achieved in cell suspension culture. Thus, the present study is an initial step towards enhanced AQ production in *R. cordifolia* to meet the market demand for this particular dye and further research is needed to achieve 100-fold or more enhancements in AQ production.

Keywords: Anthraquinone, Alizarin, Purpurin, Callus culture, Suspension Culture, Rubia cordifolia

INTRODUCTION
Since time immemorial, man used his various resources for his own well-being. Even before settlement, his aesthetic sense forced him tomake various colours out of plant sources and he identified the curative properties of plant parts as well. Anthraquinones (AQs) is one such plant pigment, whose curative and coloring properties were identified much earlier in civilizations and its remnants can be found even in the clothes wound to Egyptian mummies. AQs have been identified from various plant sources and producedas a secondarymetabolite, and this dye molecule has gained its reign in the textile industry, cosmetics, food, etc. It is now widely exploited n traditional as well as modern systems of medicine.

An ever-increased commercial requirement for various secondary metabolites is witnessed by the world today. Anthraquinones (AQs) are one among them, with outstanding curative properties, and are well used in the treatment of various modern diseases. This molecule has always been a fascinating molecule for chemists to synthesize and access diversely substituted derivatives as therapeutic agents. AQs have been identified from various plant sources and this dye molecule has gained its reign in the textile industry, cosmetics, food, etc.

Rubia cordifolia is a plant well known for the red dye chiefly located in its roots. As theplant grows older, the dye content also increases. The constant uprooting of the plant for AQs threatens its existence in natural stands.It is now well understood that the *in vitro* plant materials are functioning as prospective factories of future secondary metabolites. So, in the present study,an attempt has been done tocompare the enhancedproduction of AQ in *R. cordifolia* using the precursor feeding method in two different culture systems.

MATERIALS AND METHODS
Callus initiation and callogenic responses: The nodalsegments were trimmed for desired size (1.5 cm2) and were inoculated in agar-gelled MS medium supplemented with different NAA (1.0, 2.5, 5.0, and 10.0 µM) concentrations. After the culture period (30 days) the ethanolic extracts of the

dried calli were prepared and were used to quantify alizarin and purpurin [1,2,3] content. The statistical significance of the metabolite content was then duly determined. The calli raised from nodal explants were subcultured on agar-gelled MS medium supplemented with 5 µM NAA six times and every 30 days interval growth stability and AQ production were tested..The growth index reflects the growth characteristic of the callus. The optimum growth rate was analyzed, and the data were set as a base for further investigation.

Initiation of cell suspension culture

Fine friable calli, characterized by stable growth and consistent production of AQ were used as the stock culture for the initiation of cell suspension cultures. Calli mass of about 300 to 500 mg fresh mass was transferred to 100 ml Erlenmeyer flask containing 30 ml MS liquid medium containing 3 % sucrose, and 5 µM NAA. The cultures were subjected to constant agitation on a rotary shaker at 130 rpm, the loosely adhered cells get dispersed and some of the callus mass remained as large clumps or unbroken masses were removed by cell dissociation sieve (mesh diameter 100 mm and opening size 140 mm, Sigma, St. Louis, US).

The stock culture raised through the previous experiment was harvested after 30 days and approximately 400 - 500 mg was transferred to MS liquid medium containing 2.5 µM NAA. The growth of cells (fw), was analyzed by sampling cell cultures every five days interval up to a period of 30 days) and were used to plot growth curve of the cell suspension culture and was used to determine the most appropriate day of introducing elicitors to the medium.

Effect of different elicitors and precursors/inhibitors on AQ production

A time course cell number study revealed that the increase in cell number ceases by 25th day of culture. Based on this observation, elicitors were added on 20th day and the AQ accumulation was quantified on 30th day. The precursors at varying concentrations (50, 100, and 150 µM). were fed to the medium on the 20th day of culture through filter sterilization and on the 30th day the calli/cells were harvested and used for further investigation

RESULT AND DISCUSSION

The biosynthetic pathway of anthraquinones revealed that the first aromatic intermediate (o-succinyl benzoate) is formed from isochorismate and ∝–ketoglutarate (AK) in presence of thiamine pyrophosphate [4] and links with shikimate-mevalonate pathway. Thus ∝-ketoglutarate was selected as one of the precursors.Phenylalanine (PA) is an aromatic amino acid involved as a precursor in a wide variety of secondary metabolites, which functions as a substrate for phenylalanine ammonia-lyase that converts L-phenylalanine into trans-cinnamic acid, the first step of the biosynthesis of phenols in many plant species. The addition of precursors of AQ in the culture medium is an important strategy in cell culture techniques to improve the production of specialized metabolites in medicinal plants. Fresh weight accumulation was influenced by the media composition in which the calli grow. Statistical analysis revealed a highly significant ($p < 0.001$) effect of precursor addition in the culture medium for the production of AQ. In the present study, AK accumulated maximum fresh weight in 50 µM (909.83 mg) followed by 50 µM PA (864.03 mg), while higher concentrations of both precursors failed to enhance FW (Table 1). Dry weight data recorded a maximum (93.33 mg) at 50 µM PA and the least at 100 µM AK (54.68 mg). The growth rate conformed with these results. The precursors when applied to the medium, they function as substrates for enzymes involved in the biosynthetic pathway. PA supported the accumulation of increasing intracellular alizarin (Table 1). The highest accumulation of alizarin (54.55 mg g-1 dw) was noticed in 100 µM PA, with about 3-fold increase over control and 25-fold higher than roots of naturally growing plants (2.19 mg g-1 dw). Purpurin accumulation was highest in AK at 150 µM concentration (16.84 mg g-1 dw), which was about 4 times greater than the control and 24-fold higher than the root sample of naturally growing plant.

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Table 1: Effect of different precursors on AQ production in callus cultures of *R. cordifolia* raised on agar-gelled MS medium containing 5 µM NAA and varying conc. of precursors (α keto glutaric acid and phenylalanine)

Precursors	Concentration (µM)	Fresh Weight (mg)	Dry Weight (mg)	Alizarin (mg g^{-1} dw)	Purpurin (mg g^{-1} dw)
α keto glutaric acid (AK)	50	909.83±1.65^{a}	75.20±.15^{c}	26.40±.35^{d}	3.51±.01^{e}
	100	772.17±2.29^{e}	54.68±.17^{g}	26.71±.16^{d}	4.77±.07^{e}
	150	854.77±1.5^{c}	81.48±.13^{b}	45.64±.66^{bc}	16.84±.4^{a}
Phenyl alanine (PA)	50	864.03±1.69^{b}	93.33±.19^{a}	43.11±.51^{c}	7.65±.49^{cd}
	100	782.17±1.68^{d}	69.17±.30^{d}	54.55±.37^{a}	8.58±.15^{c}
	150	600.30±2.6^{f}	67.73±.49^{e}	45.68±.49^{bc}	11.26±.1^{b}
Control	0.0	647.20±1.28^{g}	64.90±.45^{f}	17.60±.13^{f}	4.42±.22^{e}
F value df (n-1)= 6		5879***	2771***	1741***	319.7***

Means within a column followed by the same letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. *** F value highly significant ($p \leq 0.001$)

Piroxicam is a potent anti-inflammatory chemical used in cancer cell research. PX showed a significant ($p < 0.001$) effect on parameters such as fresh weight, dry weight, and intracellular and extracellular AQ accumulation. The highest fresh weight (817.97 mg) and dry weight (107.9 mg) were recorded in 150 µM PX.. Among different piroxicam treatments, alizarin accumulation was the highest level at 100 µM (Table 2) and is 19-fold higher than roots of naturally growing roots. Purpurin accumulation also showed significant ($p < 0.05$) enhancement (18-fold) in 100 µM piroxicam treated cultures (Table 20) over naturally growing plants. These reports strongly support that PX is an effective promoter of anthraquinones synthesis in *R. cordifolia* cultures.

According to Zaprometov [5], precursor feeding is effective only if they are capable ofpiercing the cell and if their formation in the cultured cell is limited.In the present investigation, precursor addition has a positive influence on the accumulation of AQ in *R. cordifolia* callus cultures. Previous reports suggest that AK can enhance the accumulation of anthraquinone in *Morinda citrifolia* [6] and *Oldenlandia umbellata* [7]. According to Shin and Chi [8] α-ketoglutarate (100 mg L-1) and shikimic acid (500 mg L-1) enhanced the bioaccumulation of pigments in *R. cordifolia*. However, in *R. akane* [9] there was no significant increase in AQ by the addition of these precursors in the medium. *In vitro* production of radio-labeled taxol revealed PA as the best precursor for taxol production [10] and colchicines in callus and roots of *Gloriosa superba* [11].

Table 2: *In vitro* production of AQ through callus developed on agar-gelled MS medium supplemented with 5 µM NAA and varying conc. of piroxicam (PX)

Piroxicam (µM)	Fresh Weight (mg)	Dry Weight (mg)	Alizarin (mg g^{-1} dw)	Purpurin (mg g^{-1} dw)
50	580.47±3.32^{d}	68.21±.38^{c}	40.23±.68^{b}	10.44±.12^{b}

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100	785.8±1.35[b]	98.13±.24[b]	49.83±.32[a]	12.33±.25[a]
150	817.97±1.16[a]	107.9±.15[a]	42.13±.35[b]	10.87±.46[b]
Control	647.20±1.28[c]	64.90±.45[c]	17.60±.13[c]	4.42±.22[c]
F value df (n-1)= 2	3750.6***	5709.4***	2182.4***	282.4***

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 highly significant ($p < 0.001$)

Cell suspension raised in MS medium supplemented with varying concentrations of NAA showed a significant ($p < 0.001$) effect on biomass, alizarin, and purpurin content. Biomass accumulated per 30 ml media showed the highest value in 2.5 µM NAA added medium (Table 3). The addition of NAA at 5 µM concentration resulted in a reduction in growth. While examining the production of both alizarin and purpurin it was noticed that yield of metabolites is much lower than in callus cultures. A maximum amount of intra-cellular alizarin (10.81 mg g-1 dw) and purpurin (1.12 mg g-1 dw) was recorded in 2.5 µM NAA added medium. On the basis of fresh biomass, AQ content NAA concentration 2.5 µM was selected for further experiments.

Table 3. Effect of varying conc. of NAA on growth and AQ production in cell suspension cultures of *R. cordifolia*

NAA Conc. (µM)	Biomass (fw mg/30 ml media)	Alizarin content (mg g^{-1} dw)	Purpurin content (mg g^{-1} dw)
1.0	585.52d	8.55b	0.86b
2.5	768.33a	10.81a	1.12a.
5.0	728.83b	8.23b	1.04a
10.0	649.13c	4.57c	0.84b
15.0	431.17e	3.40c	0.65c
F value df (n-1) =4	97.35***	776.27***	143.38***

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

Effect of precursors and piroxicam

The effect of precursors like ∝-ketoglutarate and phenylalanine on AQ production in suspension culture revealed a significant ($p < 0.001$) effect. In presence of 100 µM AK, alizarin and purpurin were produced significantly ($p < 0.05$) higher quantities than the control. Alizarin production (58.97 mg g^{-1} dw) in 100 µM α keto glutaric acid supplemented medium was about 27 fold higher than roots sampled from naturally growing plants and 6 fold greater than control, while purpurin showed 9 fold increase over control and was 26 fold higher than roots sampled from its source plant (Table 4). The effect of piroxicam on suspension culture showed increased fresh weight accumulation (809.3 mg) and dry matter (67.8 mg). There is a relationship between biomass and secondary product formation. Intracellular alizarin (30.39 mg g^{-1} dw), and purpurin (8.97 mg g^{-1} dw) were accumulated in MS medium fortified with 100 µM PX and 2.5 µM NAA. PX-mediated improved production may be due to the combined action of PX and NAA. Thus the present study confirms that both AK and PX are superior PA to produce AQ constituents in the cell suspension cultures. In *R. cordifolia*, inhibitors like cantharidin [12], lovastatin, piroxicam [13], etc. induced AQ production in different

culture systems. In the present investigation, PX was found to have an inducer role in the synthesis of AQ in the culture system.

Table 4: Effect of different precursors and piroxicam (100 µM) on AQ production in suspension cultures of *R. cordifolia* raised in MS medium containing 2.5 µM NAA

Precursors used	Fresh weight (mg)	Dry weight (mg)	Growth rate	Alizarin (mg g^{-1} dw)	Purpurin (mg g^{-1} dw)
AK	682.50±2.05^{b}	72.12±.14^{a}	0.37±00^{b}	58.97±.18^{a}	18.68±.30^{a}
PA	725.0±5.88^{a}	44.67±.49^{c}	0.45±.01^{a}	11.95±.07^{b}	4.26±.04^{b}
PX	809.3±2.3^{a}	67.8±.33^{b}	0.62±.00^{a}	30.39±.44^{a}	8.97±.10^{a}
Control	549.07±1.22^{c}	48.23±.17^{b}	0.10±.00^{c}	9.67±.33^{c}	2.06±.04^{c}
F value df (n-1) = 4	627.111***	2343.902***	627.11***	15783.2***	2598.3***

Means within column a followed by the same letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. ***highly significant ($p < 0.001$) F value

SUMMARY AND CONCLUSION

For callus initiation, leaf explants were selected with 73.3% callogenic response in MS medium containing 2.5 µM NAA where 1.29 g fresh weight was obtained within 60 days of the culture period. In order to obtain friable calli, the response of different explants was compared and found thatnodal explants cultured on MS medium supplemented with 2.5 µM NAA produced (78.3%) reddish-orange friable calli. Nodal segment-derived callus cultured in MS medium with 2.5 µM NAA produced 17.76 mg g^{-1} dw alizarin and 3.8 mg g^{-1} dw purpurin. Callus developed in this were continuously subcultured every 30 days interval, over six times in the same medium and both alizarin and purpurin production was consistent and stable in subculture IV onwards. AQ accumulation in the callus was further improved by the addition of precursors. The addition of phenylalanine (100 µM) resulted in increased production of alizarin (54.55 mg g^{-1} dw) which is 3 fold increase over control and 25-fold higher than roots of naturally growing plants (2.19 mg g^{-1} dw). Even though piroxicam is a metabolic inhibitor, its addition enhanced intracellular accumulation of AQ at 100 µM in 49.83 and 12.33 mg g^{-1} dw for alizarin and purpurin respectively.

Cell suspension culture of *R. cordifolia* was raised from a fast-growing, nodal segment-derived callus in MS medium supplemented with 5 µM NAA. Cell growth pattern showed significant enhancement of alizarin and purpurin after the 20th day of subculture, hence the addition of precursors was maintained on the same day itself. Addition of a precursor, 100 µM AK significantly enhanced the production of both AQ constituents alizarin (58.97 mg g^{-1} dw) and purpurin (18.68 mg g^{-1} dw). Through cell suspension cultures which are fed with 100 µM α keto glutaric acid (AK), 6 folds increase in alizarin production and 9 folds increase in purpurin production over control was achieved. The enhanced production level of alizarin through AK feeding is 27-fold higher than roots sampled from the naturally growing plant, while purpurin productivity is 26-fold higher than roots sampled from its source plant.

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