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Research Article

In-vitro culture and Anthocyanin Synthesis from *Tectonagrandis* L.f.

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Abstract

Anthocyanins are polyphenolic derivatives that are proven as food additive and as natural dye. It displays many biological potentialities such as antioxidant, anti-proliferative, anti-microbial and anti-inflammatory. *In vitro* culture of anthocyanin synthesis emerges as an interesting industrial proposal, since this can eliminate seasonality, geographical, annual variations in plants and also impact in yields due to pests and pathogens. In this juncture, the present study aims to *in-vitro* culture and anthocyanin synthesis from nodal and leaf explants of the woody plant *Tectonagrandis* L.f. Generally, clonal multiplication in woody plants was usually established via somatic embryogenesis, organogenesis or micropropagation. Initially, callogenesis was carried using leaves or nodal region as explants. Effective surface sterilization was done with mercuric chloride followed by the combination of sodium hypochlorite + Tween 20 + fungicide. Remarkable compact and friable callus proliferation was noticed with Naphthalene acetic acid (0.2 mg/L) and Benzylamino purine (1 mg/L) within 21 days from leaf explants of teak. Medium supplemented with 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) showedprofuse callus induction in both light and dark explants after 28 days, which was 73.3%. 2,4-dichlorophenoxyacetic acid + Kinetinyielded the highest amount (15.23 mg g^{-1} callus) of anthocyanins. Subsequent cultures of the calli on the above medium yielded stable production of anthocyanins. Callus subculture with low growth rate yielded better colour value of anthocyanin. Both callus growth and anthocyanin synthesis were inversely correlated. Future studies are designed to purify and fractionate different anthocyanin and to enumerate its biological potentialities.

Keywords: Anthocyanin; Callus; Growth hormones; Nodal explants; Teak leaves

Abbreviations

BAP	:	Benzylamino purine
CW	:	Coconut water
2,4-D	:	2, 4-dichlorophenoxyacetic acid
KIN	:	Kinetin
MS	:	Murashige and Skoog Medium
NAA	:	Naphthalene acetic Acid
WPM	:	Woody plant medium

Introduction

In-vitro plant regeneration technique has become popular and effective method to overcome the traditional methods of plant propagation. Tissue cultured plantlets show more ideal features than plants developed from seeds. The uniform growth rate and remarkable physiological potentiality of culture raised plantlets suggest the induction of useful characters present in the plant. Further, *in vitro* approaches have been applied for *in-situ* conservation of the germplasm. Ajay and Sudhakar [1] developed a simple, high frequency and reproducible protocol for the induction of adventitious shoot buds from *Jatrophacurcus* cultured on MS media supplemented with thidiazuron, BAP and IBA.

Anthocyanins belong to flavonoid group synthesized through a well-definedshikimic acid biosynthetic pathway. Biotic and abiotic elicitors induce the synthesis of flavonoids in *in vitro* culture systems. Anthocyanins by *in vitro* plant cultures technology have been proposed as an efficient source for its production. Production of anthocyanin in plant cell and tissue cultures has been reported for many plants [2,3]. Further, it is reliable and predictable to produce anthocyanin under controlled conditions. In addition, it can be applied at industrial levels and provides continuous supply of uniform quantity with optimal amount without seasonal constraints. Maharik et al. [4] selected an anthocyanin-accumulating cell line from callus derived from seedling and accumulated the

pigment only under continuous illumination. Similarly, in vitro production of anthocyanin is regulated by hormonal combinations like auxin and cytokinin [5]. MS medium fortified with different concentrations of abscisic acid resulted in a synergistic increase in anthocyanin production in terms of pigment content [6]. Cell cultures of *Hibisbussabdariffa* have also been used for studies on the production of primary or secondary metabolites [7]. Mikhajo et al. [8] selected an anthocyanin-accumulating cell line from callus derived from seedling of *Solanumtuberosum*. Maryam et al. [9] reviewed the role of tissue culture techniques in the conservation of rare and endangered species. Zuraida et al. [10] efficiently developed a protocol for *in vitro* culture in *Piper crocatum* i.e., MS media containing BAP + 2,4-D supplemented with charcoal was the most effective in shoot initiation, proliferation and showed less in browning. Rahman et al. [11] showed effective callus induction with multiple shoots in *Plectranthus amboinicus* cultured in Murashige and Skoog medium supplemented with different concentrations of BAP, NAA and KIN. Bakhtiar et al. [12] developed in vitro callus induction and micropropagation of *Thymus persicus*, an endangered medicinal plant. Mahipal et al. [13] successfully carried *in vitro* regeneration of shoots and ex vitro rooting of an important medicinal plant *Passiflora foetida* L. through nodal segment cultures.Sahu et al. [14] micropropagated *Vitexnegundo*: a multipurpose dynamic medicinal plant via callus induction. Saryam et al. [15] evaluated in-vitro micropropagation of *Hemidesmus indicus* using different culture media and hormonal combinations. In Vitro direct shoot regeneration of plantlet in *Oenotherabiennes* using kinetin and NAA hormonal combinations was carried by Sattar et al. [16]. All these research output substantiate the role of *in vitro* culture as an efficient method for the micropropagation and elicitation of phytochemicals from plants.

Tectonagrandis L.f (Lamiaceae) an economically important, deciduous tree predominantly distributed in tropical and sub-tropical regions of Southeast Asia. Teak is a slow growing tree with poor germination rate that make difficult to propagate naturally and does not meet the demand of supply at present. Most of the protocols were developed to multiply shoots by means of shoot tip and axillary bud cultures [17]. Common problem encountered from field-sourced materials includes exudation of phenolic substances into culture medium leads to the risk of contaminations. Also, repeated cycles of sub-culturing increases the risk of verification phenomena among the in vitro plantlets which ends in genetic abnormalities and reduced regeneration capacity [18].The young leaf of teak is reddish in colour due to rich anthocyanin content. Greeshma et al. [19] analyzed the bioactive compounds by GC-MS from the young leaves of *Tectonagrandis* L.f. for the first time.Hence, the present investigations propose to develop a reproducible and fast protocol for large scale production of callus from teak which may be induced for increased production of commercially important anthocyanin.

Materials and Methods

The fresh, young leaf and node from the tip of the healthy teak (*Tectonagrandis* L.F.) stump stocks from the natural habitat were collected. The leaves and nodal segments were washed for 10 min in ethanol solution at 20% v/v, followed by washing in sterile distilled water to disinfect the surface of the collected material. Subsequently, the material was standardized using different sterilization solutions like mercuric chloride (0.05% to 0.2%), sodium hypochlorite (NaOCl-5% to 10%) and calcium hypochlorite (at 2%-5%w/v). 2 drops of Tween 20(as additive) and methyl 2-benzimidazolecarbamate (0.05%) fungicide as additional disinfectant solution were used in combination with above sterilants to remove the fungal contaminants. Duration of treatment varied from 2 to 20 min.

The sterilized explants were then aseptically placed on the surface of Murashige and Skoog (MS) medium or Woody plant medium (WPM) supplemented with different concentrations of phytoregulators in 3% (w/v) of sucrose concentration [20,21]. The pH of the medium was adjusted to 5.7-5.8 with 0.1 N NaOH or HC1 prior to adding 0.8% agar and autoclaved at 121^0C for 20 min. MS medium supplemented with 2,4-D, naphthalene acetic Acid (NAA), Kinetin and 6-benzylaminopurine (BAP) alone or in combinations were used for callus induction. Coconut water (CW) was also supplemented in the MS medium to analyze its role in callus initiation and growth. The plain WPM and basal MS media without any growth hormone were treated as control for leaf and nodal explants. WPM was not used only in case of nodal explants. All the cultures were raised in wide mouth conical flasks or test tubes and maintained in warm white fluorescent light of 20-40 µE m^{-2}/S^1 intensity for 16 h photoperiod at 25 ± 2^0C temperature. Callus induction percentage was calculated for each hormonal treatment with the following formula:

Callus induction (%) = Total Number of explants with callus induction /Total No. of explants inoculated for each treatment X 100

The callus growth rate was recorded per day basis of callus induction and graded by comparison infour categories as excellent (++++), good (+++), average (++) and poor (+). The callus morphology such as color, appearance, texture, pigmentation etc. was noted per hormone treatments.

Nodal segments (0.5-1 cm length) were cultured on MS medium supplemented with BAP with different concentrations (0, 0.5, 1, 1.5, 2, 2.5 mg/L) combined with three concentrations of NAA (0, 0.2 and 0.5 mg/L) for shoot initiation.

Selection of anthocyanin-producing callus line

The anthocyanin-producing callus line was isolated by selectively sub-culturing a variant red pigment containing cell clus-

ter that appeared spontaneously in one of the stock culture of the wild callus line. The cell aggregate cloning method was repeatedly applied for six generations until uniform pigmented callus tissue was obtained. Anthocyanin-producing cell line was sub-cultured every month into the fresh medium for six consecutive passages before subjecting to growth and production studies.

Callus growth measures

For all growth measurement experiments, after each sub-culturing, the adhered agar was removed from callus tissues and then the fresh weight of calli were calculated. The growth index (GI) W/Wo, where Wo and W denote aggregates fresh weight before and after the cultivation, respectively. Calli were then placed in an oven at 60^0C until a constant weight which represents the dry weight (DW).

Extraction and quantification of total anthocyanin

Anthocyanin content of freeze-dried calli was determined using the pH-differential method of Abeda et al. [7].

Colour value determination

The colour value of anthocyanin was estimated according to the method described by Chaudhary and Mukhopadhyay [22].

Statistical analysis

Results were analyzed using Sigma Plot statistical software (version 12), and were expressed as mean ± SD. The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Statistical P value < 0.05 was considered to be significant.

Results and discussion

The major hurdle accounted with teak culture was related with its high endogenous fungal contaminations. Effective surface sterilization was carried by initial treatment with ethanol followed by 0.1% $HgCl_2$ solution for 5 min and washed repeatedly with sterilized water. These treated explants were further immersed in 3% sodium hypochlorite solution + 2 drops of Tween 20 + 0.05% fungicide for 20 minand then washed thrice with sterile deionized water. This protocol yielded 95% contamination free cultures with 90% survival cultures. Longer duration of sterilization treatments resulted browning and explant death. The protocol is simpler compared to the sterilization protocol of Ramesh et al. [17] i.e., teak shoot explants were treated with 1% Bavastin, 0.1% Streptomycin, 70% ethanol, 0.05% mercuric chloride, 5% sodium hypochlorite + Tween-80 and finallyrinsed in sterile distilled water. In the present study, the sterilization protocol was adopted to that of Mendoza de Gyves et al. [23] for micropropagating and *in vitro* rooting of teak. However, the concentration of sterilants were less than that of the previous study.

The callus could be induced using MS media from both young fresh leaves nearer to terminal buds as well as nodal explants (Figure 1A and 1B).Various kinds of phytohormone and their combinations induced callus with differentrates. Optimal callus induction was noticed with leaf when compared to nodal explants irrespective of media or hormones employed. This may be due to the fact that leaves are more immature and therefore highly meristematic. Maximum callus biomass was obtained in MS medium supplemented with BAP (1 mg/L) + NAA (0.2 mg/L). In the present analysis, fast initiation of callus was recorded on 21st day using MS medium with leaf explants. The hormonal treatments (MS medium with 2 mg /L 2,4-D + 0.5 mg/L kinetin) and MS with NAA (3 mg/L) + BAP (0.5 mg/L) could induce optimalcallus with an average period of 28th and 30th days, respectively (Figure 2A,B; Table1). Mean while, using woody plant medium (WPM), callus induction was delayed i.e., with an average period of 42 days after incubation for all replicates. Medium callus multiplication rate (++) was observed with (WPM + 2.5 mg/L NAA + 0.5 mg/L BAP). The maximum callus induction percentage (60.2%) in WPM was recorded in case of MS medium with 2,4-D (2 mg/L) + kinetin (0.5 mg/L) (Table 1) followed by others with an average of 41% (Table 1). The r values were 0.49 and 0.43, respectively at 1% significant level. The 30% of induction percentage was recorded (r-value= 0.29 at 1% significant level) MS medium supplemented with coconut water (20 ml/L). Coconut water is a proven natural growth regulators influencing in-vitro growth of plant tissues [24]. However, in the present study coconut water did not showed remarkable induction of callus but induced callus multiplication. In case of nodal explants, callus induction took place on 35th day with an average of 28 days in the MS nutrient medium (Table 1).The best induction percentage (72%) was recorded in MS (Table 1). Generally, the treatments with hormonal combinations MS + 2,4-D (high) + kinetin (low) maybe considered as optimum for callus induction and growth for woody species. Further, poor callus induction was recorded in nodal explants with 2, 4-D alone (3 mg/L) or the combinations of NAA (1 mg/L) + BAP (0.5 mg/L) i.e., 23% only (r value = 0.31 at 1% significant level). After 21stday of inoculation on the culture media, significant callus induction was noticed with BAP (1 mg/L) + NAA (0.2 mg/L) and 2,4-D (2 mg/L) + kinetin (0.5 mg/L) for leaf and nodal explants 92.5% and 75% respectively. The minimum callus induction percentage was recorded to be 40.5% with other combinations for leaf and nodal explants. Fully grown callus could be maintained in both induction and basal MS medium for longer periods in terms of healthy greenish and friable callus for easy multiplication (Figure 3A). On the contrary, in the MS and WPM media with 2,4-D, NAA or kinetin no significant callus initiation was noticed with leaf and nodal explants. Similarly, callus initiation was minimal in the basal MS and WPM media without hormonal combinations.

Figure 1A: In vitro culture of nodal region from teak.

Figure 1B: In vitro callus initiation from leaves of teak.

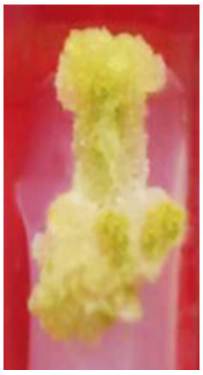

Figure 2 A and B: In vitro callus initiation from basal region ofnode from teak.

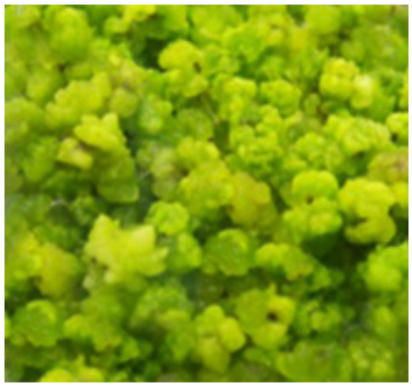

Figure 3A: Green compact callus of teak.

Leaf	Concentration (mg/L)	Callus induction (%)	Callus growth grade
Hormonal combinations			
MS medium	0	1.8	-
MS + 2,4-D	0.5	4	+
MS + 2,4-D	1	13	+
MS + 2,4-D	2	43.3	+
MS + 2,4-D	3	25	+
MS + 2,4-D	4	29	+
MS + 2,4-D	5	31	+
BAP + NAA	0.5 + 0.2	57	+ +
BAP + NAA	1 + 0.2	92.5	+ + + +
BAP + NAA	0.5 + 1	50	+ +
BAP + NAA	0.5 + 2	60	+ + +
2,4-D + KIN	2 + 0.5	73	+ + + +
2,4-D + KIN	1 + 1	60	+ + +
2,4-D + KIN	1 + 2	52	+ +
2,4-D + KIN	2 + 1	48	+
2,4-D + KIN	3 + 1	41	+
2,4-D + KIN	2 + 2	5	-
2,4-D + KIN	2 + 3	32	+
2,4-D + KIN	3 + 4	29	+
2,4-D + KIN	4 + 5	18	+
2,4-D + KIN	5 + 6	13.2	+
MS + CW(ml/l)	5	23	+
MS + CW(ml/l)	10	25	+
MS + CW(ml/l)	15	28	+
MS + CW(ml/l)	20	30	+

Table 1: Hormonal combinations and percentage of callus induction in teak.

Excised nodal segment inoculated on basal MS medium failed to produce multiple shoots. Where in excised node inoculated on MS medium supplemented with either BAP (0.5 -2.5 mg/L) produced less multiple shoots, but in the presence of both BAP (2.0 mg/L) and NAA (0.5 mg/L) the multiple shoot regeneration efficiency was higher (80%) (Table 2). MS medium supplemented with BAP alone showed minimum induction of multiple shoots. The highest percent of shoot induction (80%) was obtained in MS medium supplemented with BAP (2.0 mg/L) and NAA (0.5 mg/L) and 15.4 shoots per culture were obtained (Table 2). Multiple shoots produced in *Thymus persicus* [12], *Vitexnegundo* [14], *Passiflorafoetida* [13], were less than the present results. Mean while, *Hemidesmusindicus* [15] and *Oenotherabiennes* [16] produced remarkable multiple shooting (%) than that of teak.

Leaf	Concentration (mg/L)	Callus induction (%)	Callus growth grade
Hormonal combinations			
MS medium	0	0	0
BAP + NAA	0 + 0	0	0

BAP + NAA	0.5 + 0	9.5	1
BAP + NAA	0.5 + 0.2	22.7	2.7
BAP + NAA	1 + 0.2	32	3.5
BAP + NAA	1.5 + 0.2	36	4
BAP + NAA	2 + 0.2	44	5.5
BAP + NAA	2.5 + 0.2	40	4.4
BAP + NAA	0.5 + 0.5	50	6
BAP + NAA	1 + 0.5	64	9
BAP + NAA	1.5 + 0.5	74	11
BAP + NAA	2 + 0.5	80	15.4
BAP + NAA	2.5 + 0.5	40	4.3

Table 2: Nodal culturing in MS media supplemented with different combinations of BAP and NAA in teak for multiple shoot initiation.

Robinson and Maheswari [25] successfully produced *in vitro* multiple shoot induction from nodal explants of *Capsicum annum* kandhari variety with 2,4-D+kinetin combination. *In vitro* adventitious shoot regeneration, cryopreservation, protoplast culture, shoot tip and nodal culture, and somatic embryogenesis propagation technologies in many economically important species were reviewed by Pijut et al. [26]. Narasimhan et al. [27] attempted *in vitro* culture of teak, jack, mulberry in MS medium containing glycine and gibberellic acid. Similarly, Omar and Novak, [28]; Rao and Vaidyanath, [29] and Litz et al. [30] were successfully established calliin date palm, sesamum and gymnosperm specieswith the combinations of 2,4-D and Kinetin. Kumariet al. [31] initiatedMS medium supplemented with 2,4-D and Kinetin in rubber plant. The present study also reports callus initiation and multiplication with leaf and nodal teak explants with BAP+NAA; 2,4-D + kinetin in MS media. Similarly, enhanced rate of callusing in *Aquilariamalaccensis* has been reported by Saikia et al. [32] using 2,4-D and kinetin combinations.

The influence of light and darkin terms of callus morphology was also studied. Interestingly, the callus remained cream or whitish in dark while, on light (20-40 µE m^{-2}/ S^{1}) it turn into green (Figure 3A). Similarly, colour variation in terms of white, cream, brown greenand also textures likeloose and friablecalli were noticed when MS media supplemented with varieddoses of 2,4-D + Kinetin in dark/light regime (Figure 4A,B&C).Further, media supplemented with high NAA and low BAP produced yellow to cream coloured and compact callus. MS medium supplemented with coconut wateryielded blackish white and compact callus however, the callus obtained on WPM was pale yellow or brown globular and compact.

Figure 4 A,B,C: Cream compact callus of node from teak.

As the second phase, sub culturing of calliwas attempted with 2,4-D, KIN, BAP, NAA, coconut water either singly or in combinations. Interestingly, 2,4-D+KIN combination did not enhance callus growth but, irrespective of explants, the combination began to alter the colour from 30th day of inoculation from brownish to pink pigmentation (Figure 5A & B). The obtained results suggest that 2,4-D and KIN probably induce anthocyanin biosynthetic pathway [4,33-35]. Table 3 shows the production of anthocyanin in callus from the both explants used on MS with different combination of hormones. It is worth to note that the medium containing 1.0 mg/L 2,4-D and 2.0 mg/L KIN yield callus with remarkable anthocyanin contents irrespective of theexplants used (Figure 5C). However, leafexplants yielded 0.06 to 15.23 mg/g anthocyanin levels when compared to node (0.8-8.96 mg/g). The results tempt to state that BAP + NAA produced optimal callus growth whereas 2,4-D+KIN yielded remarkable anthocyanin content.

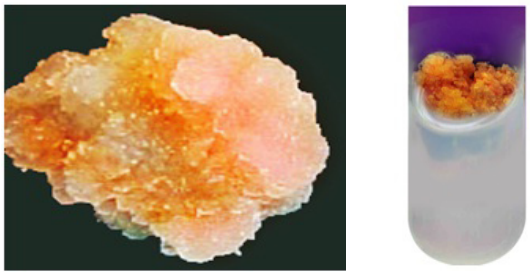

Figure 5A,5B: Coloured callus initiated from node.

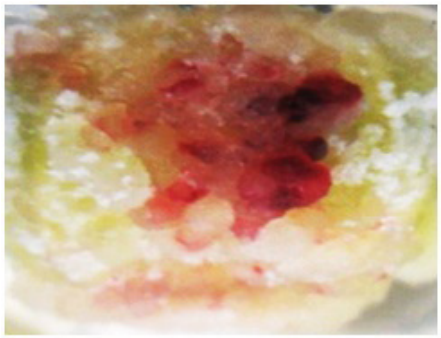

Figure 5C: Induction of pink colouration from leaf callus.

Leaf	Concentration (mg/L)	Anthocyanin content (mg/g callus)
Hormonal combinations		
MS medium	0	0
MS + 2,4-D	0.5	0.06
MS + 2,4-D	1	0.09
MS + 2,4-D	2	0.12
MS + 2,4-D	3	0.19
MS + 2,4-D	4	0.23
MS + 2,4-D	5	0.24
BAP + NAA	0.5 + 0.2	0.27
BAP + NAA	1 + 0.2	6.84
BAP + NAA	0.5 + 1	3.3
BAP + NAA	0.5 + 2	4.2
2,4-D + KIN	1 + 1	8.92
2,4-D + KIN	1 + 2	15.23
2,4-D + KIN	2 + 1	9.8
2,4-D + KIN	3 + 1	7.6
2,4-D + KIN	2 + 2	4.2
2,4-D + KIN	2 + 3	3
2,4-D + KIN	3 + 2	2.6
2,4-D + KIN	3 + 4	3.8
2,4-D + KIN	4 + 5	4.9
2,4-D + KIN	5 + 6	2.59
MS + CW(ml/l)	5	0.38
MS + CW(ml/l)	10	0.41
MS + CW(ml/l)	15	0.42
MS + CW(ml/l)	20	0.28

Table 3: Sub-culturing of calli in MS media supplemented with different hormonal combinations and respective anthocyanin content in teak.

Meyer and Van-Staden [36] induced anthocyanin from callus cultures of *Oxalis linearis* using the same combination. It is interesting to state that there is an inverse correlation between callus growth and anthocyanin accumulation. Accumulation of anthocyanin in callus was seen during the final subculturing i.e., after 4 months only. The highest increase in the anthocyanin content in callus was associated with low growth rate. In effect, at the end of the growing period (subculture 3 to 6) the colour value (CV) varied from 2.9 to 13.81 (Table 4). Taha et al. [37] obtained similar anthocyanin content from calli cultures of ornamental plants. Maharik et al. [4] also noticed similar feature in *in vitro* culture of *Crataegussinaica.*

Subculturing days	Colour value (CW/g DW)
1	0.94
2	1.6
3	2.9
4	4.5
5	6.9
6	8.65
7	10.2
8	11.5
9	12.3
10	13.8

Table 4: Sub-culturing days and respective colour value.

Meanwhile, in *Ajugarepens* cell cultures showed a reverse trend i.e., replacement of 2,4-D with NAA induced anthocyanin with decreased calli mass [38]. Moreover, Mori et al. [39] reported that in strawberry BAP yielded optimal growth of callus but its growth rate did not showed any correlation with anthocyanin content. Sakamoto et al. [40] reported high 2,4-D with low KIN stimulated anthocyanin pigmentation without affecting cell growth in *Aralia cordata* cultures. So it can be interpreted that anthocyanin, the secondary metabolite synthesis may be variously altered in plants with different hormonal combinations [36,41,42]. Jadhav and Deodhar [43] successfully propagated aromatic woody *Mesuaferrea* using varied hormonal combinations. Shahinozzaman et al. [44] also established multiple shoots in *Acacia mangium*using node as explants. Mozahim et al. [45] designed a similar protocol for shoot regeneration from leaf petioles of neem trees. Ramanathan et al. [46] produced leaf culture from *Aegle Marmelos*. However, Senthilkumar [47] displayed different hormonal combinations with charcoal and sucrose in the MS medium for in vitro micropropagation of teak. Sudha and Ravishankar [48] showed elicitation of anthocyanin production in callus cultures of carrot with the involvement of calcium channel modulators. Mihai et al.

[49] designed a possible strategy for the enhancement of anthocyanin biosynthesis in grape callus using chemical elicitors.

Calli of teak after two months of subculture produced colouredcalli according to the hormonal combinations. In callus line derived from the medium containing 1.0 mg/L 2-4 D and 2.0 mg/L KIN, coloured cell lines frequently appeared after 90 daysof subculture. The accumulation of coloured pigment which is the anthocyanin has been reinforced between four and six subcultures. However during the sub-culturing, the anthocyanin-containing cell lines also produced colourless cell cluster on their surface. When these colourless parts were isolated, they retained their whitish colour during the following passage, but sometimes new pigmented areas were formed.

Conclusion

Calli initiation and growth were best with leaf explants on MS medium supplemented with 1.0 mg/LBAP + 0.2 mg/L NAA. But, the highest anthocyanin yield was obtained on MS medium added with 2.0 mg/L 2,4-D + 1.0 mg/L KIN. The result suggests that the calli growth and anthocyanin synthesis are inversely correlated. The present study clearly reveals an optimization protocol foranthocyanins production from callus culture. These compounds have numerous pharmacological properties and could be used as dietary supplements. Callus culture that allows the production of secondary metabolites opens the way for mass production of anthocyanins without destroying the *in vivo* that are their usual sources. Further studies are warranted to fractionate anthocyanin by GC-MS, LC-MS and to analyze their biological activities.

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