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EFFICIENT CALLUS FORMATION AND ANTHOCYANIN PRODUCTION FROM THREATENED MANGROVE SPECIES *XYLOCARPUS GRANATUM* KOENIG

Abdul Kader*, Sankar Narayan Sinha & Parthadeb Ghosh

Department of Botany, Faculty of Science, University of Kalyani, Nadia, West Bengal, India

**kaderfarakka@gmail.com*

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Present study was employed to conserve *Xylocarpus granatum* using *in vitro* technique. Nowadays tissue culture is also applied for production of various metabolites in several plant species. However, there is no report on the production of anthocyanin from *in vitro* study of this species. This investigation was carried out all over the year considering the leaf, internodal and nodal segments as explants for callus induction. These explants were cultured in various media like MS, LS, WPM and X with different combination of plant growth regulators like 2, 4-D, NAA and BA. Considering mangrove, the effect of NaCl at different concentrations like 0, 20, 40, 60, 80 and 100 mM on callus initiation, growth and anthocyanin production were also examined. Highest rate of callus and anthocyanin production was achieved with leaf explants collected during monsoon season. The MS medium found to be superior to other medium for callusing with 2.5 mg/L 2, 4-D and 1 mg/L BA in combination and 20mM NaCl addition. Anthocyanin production was exhibited after thirty days of inoculation in same media and plant growth regulator combinations. The addition of NaCl inhibited the anthocyanin production. The total anthocyanin content was 4.5 CV/g fresh weight of callus. During callus culture some globular embryo was also formed which was confirmed by histological studies. The present research gives an opportunity to examine the salt and salinity mechanism, and genetic transformation experiment at cellular level of this species. This protocol found to be very suitable for production of anthocyanin from callus of this species.

Keywords: Anthocyanin estimation, callus growth, explant browning, NaCl tolerance, seasonal influence

INTRODUCTION

Anthocyanins are water soluble flavonoid compounds synthesized via phenylpropanoid pathway (Yao et al. 2023). It is normally red, pink, violet and blue (Oren-Shamir 2009, Karahan et al. 2024). The medicinal and economical values of this compound are widely accepted. It is used as dye, antioxidant, anti-inflammatory and antitumor compound (Blando et al. 2005). So, it has great demand for pharmaceutical and dye industries. Nowadays, plant tissue culture is applied to produce anthocyanins in a number of plant species (Blando et al. 2005, Yao et al. 2023). This method is cheap, safe, advantageous and better alternatives over various chemical methods (Miyanaga et al. 2000). Recently, cyanidin 3, 5-di-O-glucoside (cyanin) an anthocyanin compound was isolated from callus of a mangrove species namely *Sonneratia ovata* which showed allelopathic activity against protoplast of lettuce (Sasamoto et al. 2018). The mangrove woody species are known to be tuff for all biotechnological investigations including the tissue culture studies such as micropropagation, callus culture and its cell suspension culture (Al-Bahrany & Al-Khayri 2003, Fukomoto et al. 2005, Kawana & Sasamoto 2008). Moreover, it is reported that the effectiveness of plant regeneration from callus of mangrove plants is extremely difficult and there is no report of regeneration from callus (Haron & Taha 2007, Hayashi et al. 2009).

Salinity is a vital ecological problem across the world for sustainable development of agriculture (Kader et al. 2022). To survive under saline condition, plants have developed several adaptive mechanisms to enhance tolerance against salt stress. Stress tolerance is a complex observable fact at the entire plant level as it appears on various levels of tissue organization whereas easy to study at the cellular level (Kumari & Parida

2018). In this circumstance, *in vitro* techniques are more functional than *ex vivo* method as it known all the regulatory routes for growth and development of the plant in a trouble-free and controlled environment (Yepes et al. 2018). So, to understand the saline and water logging tolerance, then the callus or cell culture of mangroves is an idle subject because mangrove species favour saline water for their growth, development and survival. It also tolerates water logging condition in many multiple times than other territorial plants. For these adaptive capabilities crop scientists have showed interest to insert these characters in food crops by several biotechnological applications (Kumar et al. 2018). Recently, salt-resistant rice varieties were developed by somatic hybridization technique using a callus of mangrove species (Kumar et al. 2018). However, there are limited reports are available on *in vitro* investigation of mangrove species as for explant death in culture medium (Al-Bahrany & Al-Khayri 2003, Hasegawa et al. 2011).

Several researchers discussed the utilization and degradation of mangroves globally (Alongi 2002, Ren et al. 2009). So, to avoid several troubles of their *in vivo* propagation, restoration through *in vitro* study is very much necessary (Komiyama et al. 1996, Hasegawa et al. 2011). *X.granatum* Koenig (Meliaceae) is an evergreen tree found in the Sundarbans and tropical mangrove forests of south-east Asia, Africa, Australia, Malaysia, etc (Shahid-Ud-Daula & Basher 2009). This mangrove species is economically important for timber, fuel, furniture and interior decoration (Rouf et al. 2007). This species has various medicinal properties also such as fruits and seeds are allowed to treat diarrhoea, oil from seeds is applied for healthiness of hair and the bark is used to treat cholera (Haron & Taha 2007). Due to these medicinal and economical values, this species became threatened worldwide (Gopal & Chauhan 2006, Haron & Taha 2007, Simlai & Roy 2013)

In these contexts, the present investigation was an attempted for *in vitro* conservation of this species through callus. As *X. granatum* is a true mangrove species the salt tolerant degree of callus of this species is also evaluated. Additionally, the callus of this species was examined for production of anthocyanin. To our knowledge this study is the first report for production of anthocyanin as well as any secondary metabolite production from callus culture of this mangrove species.

MATERIALS AND METHODS

Screening for suitable explant for callus formation and their preparation

Different plant materials such as young leaf, nodal and inter nodal segments were collected from Indian Statistical Institute, Kolkata, West Bengal, India all over the year for this investigation. The explants were excised from branches and placed in water to bring into the laboratory. The explants were firstly washed with 2% (v/v) teepol to remove dust for 10 min and rinsed thrice with distilled water. Thereafter they disinfected with 0.15% (w/v) $HgCl_2$ for 10 min for surface sterilization and again washed for three times with sterile distilled water to remove any traces of $HgCl_2$ under a laminar air flow. After inoculation under a laminar air flow, the cultures were kept in darkness for different periods of time (3, 5, 9, 13 and 15 days) continuously to reduce phenol excretion.

Selection of superior culture media for callus induction

This study screened four various types of media for callusing such as X medium (Rao et al. 1998), Murashige and Skoog (MS) medium (Murashige & Skoog 1962), Linsmaier and Skoog (LS) medium (Linsmaier & Skoog 1965) and Woody Plant medium (WPM, Lloyd & McCown 1980). In these media 3% (w/v) sucrose and 0.8% agar were added for carbon source and solidification of media respectively. The pH of the medium kept at 5.7 before sterilization with autoclaving. To select a better media equal concentration (0.75, 1.25, 1.75 and 2.25 mg/L) of cytokinin like 6-benzyladenine purine (BA) and an auxin like-naphthalene acetic acid (NAA) were examined. The best performing media among them was selected for further studies.

Examination of different plant growth regulators for callus initiation and growth

In this study two different types of auxins at several concentrations (0.5, 1, 1.5, 2, 2.5 mg/L) were examined like 2, 4-dichloro phenoxy acetic acid (2, 4-D) and NAA alone or in combination with one cytokinin like BA at the concentrations of 0.5, 1, 1.5 mg/L to select better condition and concentration(s) of plant growth regulators for callus induction and growth. To examine the

effect of different growth regulators a control treatment (Media without plant growth regulator) was also performed. The cultures were kept at 25 ± 1 °C temperature with a relative humidity of 65 ± 5%. After explant browning treatment (Keeping the cultures in dark condition), the cultures were also exposed to 16 h photoperiod light under 80 µmol/m^2/s intensity with white, fluorescent lamps.

Degree of NaCl tolerant

After selection of better medium and plant growth regulator combination, the selected medium with optimized plant growth regulators was evaluated the degree of salt tolerant at various concentrations (0, 20, 40, 60, 80 and 100 mM) of NaCl. The callus formation rate (CFR) and callus growth (CG) was evaluated after 1 month and 2 months of incubation respectively in all experiments. They were estimated using the following formulas

$$\text{CFR} = \frac{\textit{Number of exlants having callus in medium}}{\textit{Total number of explants planted for callus in the same medium}} \times 100$$

$$\text{CG} = W_1\text{-}W_0$$

Where, W_0 is the initial explant weight and W_1 is the final callus weight.

Anthocyanin identification, extraction and estimation

Initial identification of the induced pigments was performed visually. Furthermore, the pigmented calli were treated with vapours of ammonium hydroxide (alkaline pH) or hydrochloric acid (acid pH). This experiment considers the identification of anthocyanin pigments through colour changes which is influenced by pH variation. The estimation of anthocyanin (colour value index, CV) was done according to the method of Irshad et al. (2018). Briefly, 100 mg of anthocyanin callus were crushed, and incubated the fine powder in 5 ml of methanol containing 1% concentrated HCl at 4 °C for 24 h. After centrifugation at 10000 rpm for 5 min in a cold centrifuge, the aqueous phase was used to take absorbance at 520 nm. The total anthocyanin pigment content was presented as CV/g fresh weight of callus. The calculation of CV is done with following formula

CV = 0.1 × Absorbance at 520 nm × Dilution factor

Histological studies

For histological observations, the callus was fixed in FAA (formaldehyde: acetic acid: ethanol; 100:50:50) solution for 8 days. The fixed calli were then washed for 45 min, thrice with double distilled water. Thereafter, the fixed calli were dehydrated through a diluted ethanol series (30%, 50%, 70%, 80% and 90%) for 30 min at all stages. The samples were then embedded in paraffin wax (Melting point/temperature 58 °C) and sectioned vertically at 10 µM thickness with a rotary microtome. The sections were placed at slides and considered to be dried for at least 15 min before staining. The specimen sections were firstly stained with hematoxylin-eosin and after that counter stained with safranin solutions. At last, the sections were observed under phase contrast microscope.

Data collection and statistical analysis

Each experiment was conducted with 12 replicates and repeated three times. Data were analysed by one way analysis of variance (ANOVA) and the difference between means were scored using Duncan's Multiple Range Test $P \leq 0.05$ (Duncan 1955) on the statistical package of SPSS (Version 10).

RESULTS AND DISCUSSIONS

Selection of explants for callus initiation

Among the different explants screened for callusing, leaves were found to be excellent for this purpose (Figure 1a). However, few amounts of callus were formed at nodal and inter nodal segments (Figure 1b).

For tissue culture studies, selection of explants is a vital process for any plant species, especially for woody plant species (Karunaratne et al. 2014, Kader et al. 2022). Leaves are exhibited to be excellent for callus formation due to several factors, including their accessibility, strong meristematic potential, and ability to response on media very quickly and efficiently. Leaf cells, during juvenile or early developmental stages, possess high meristematic capability which promotes cellular de-differentiation to form

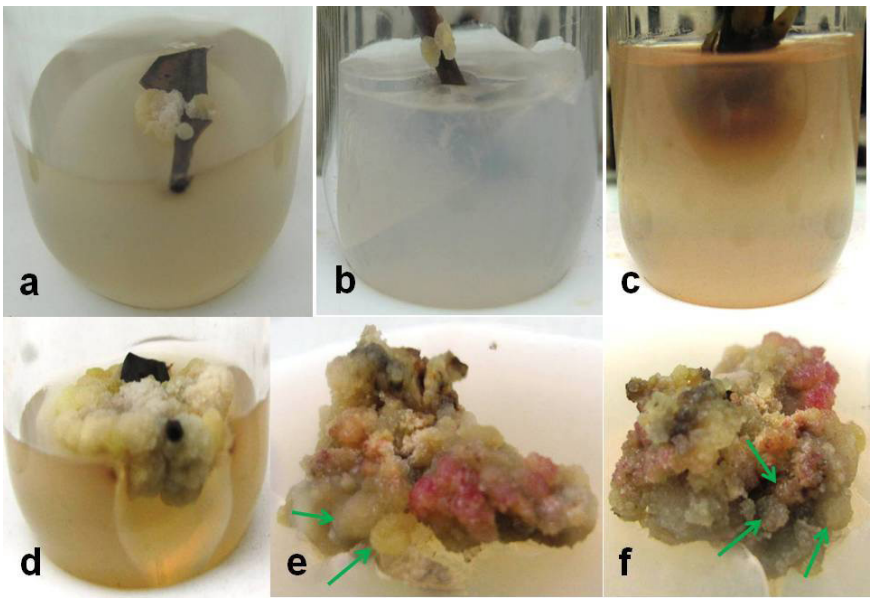

Figure 1 Callus initiation on different explants, media discoloration and anthocyanin formation of *Xylocarpus granatum*: (a) White callus formation after 15 days of inoculation using leaf explants on MS media supplemented with 1.25 mg/L both for BA and NAA; (b) Callus initiation at nodal explant after 15 days of inoculation using 2.25 mg/L both for NAA and BA in combination; (c) Media discoloration caused by secretion of phenolic compounds after 8 days of inoculation; (d) Callus growth obtained on MS medium supplemented with 2.5 mg/L 2, 4 - D and 1 mg/L BA in combination without NaCl; (e) Anthocyanin production on MS medium supplemented with 2.5 mg/L 2, 4 - D and 1 mg/L BA in combination without NaCl , green arrow showing some globular embryos; (f) Development of globular embryo indicated by green arrow.

callus. However, the efficiency depends on the plant species, the age of the leaf explant, and the type of culture media performed (Karunaratne et al. 2014). Previously, Haron and Taha (2007) documented that leaves were found to best for callus induction of *X. granatum*.

Prevention of browning problem of explants in medium

After inoculation all the cultures were placed in dark continually for 15 days and yielded suitable result for this species. Tissue blackening or browning (Figure 1c) is one of the vital constrained associated with tissue culture studies of woody plants as well as mangrove species (Kawana & Sasamoto 2008, Arumugam & Panneerselvam 2012). The cause of browning due to oxidation of phenolic compounds secreted from the excised ends of various explants by the activities of different enzymes like peroxidises and polyphenol oxidases. Thereafter, the oxidized compounds bind to several proteins. This way it inhibits various enzyme activities of explants and leading to toxic death. Moreover, browning leads to reduce explants regeneration capacity by interfering with cell division of explants (Singh 2018, Al-Mayahi et al. 2018). Several strategies have been proposed to reduce/omit this problem such as addition of antioxidants or absorbent like activated charcoal, polyvinol peroxidise (PVP), ascorbic acid, potassium citrate etc or chelating agent like ethylene diamine tetra acetic acid (EDTA) in medium. Furthermore, low temperature, repeatedly change of culture medium is also suggested to decrease the tissue blackening according to some literatures (Altan et al. 2010, Singh 2018, Al-Mayahi et al. 2018). However, in this study placement of culture in dark yielded the reasonable results as the occurrence of media discoloration varies among cultivars, species and the physiological condition of the plant/tissue. Similar type of finding was also shown by various authors with the study of mangrove species (Haron & Taha 2007, Sasamoto et al. 2018, Sasamoto et al. 2020). From this observation it may reveal that dark treatment is the superior way to reduce browning problem for mangrove species.

Screening for suitable culture media

The callus was initiated within 6-11 days after inoculation using equal combinations of auxin (NAA) and cytokinin (BA) in all the media (Figure 2). However, X medium required 13-20 days for callusing. The Figure 2 reveals that MS medium found to be superior among the other medium tested for callusing. From this result, the MS media was selected for all the rest experiments.

Literature studies indicated that for *in vitro* investigation of mangrove species then MS media is highly preferable (Al-Bahrany & Al-Khayri 2003, Haron & Taha 2007, Kawana & Sasamoto 2008, Arumugam & Panneerselvam 2012). MS medium is found to be superior for callusing because its high concentration of macronutrients, particularly nitrates and ammonium, provides a robust and non-limiting nutrient environment for the rapid and undifferentiated cell division characteristic of callus formation. In contrast, WPM and LS media are formulated for different purposes and contain lower salt concentrations that may not be optimal for many species. Although Rao et al. (1998) developed a fresh medium named 'X' for cultivation of a mangrove species *Excoecaria agallocha*. However, for induction of calli of *X. granatum* this media is not found to be fruitful. This media resulted delay callus response and reduced callus induction rate. From this observation it may assume that X media is not suitable for cultivation of *X. granatum*.

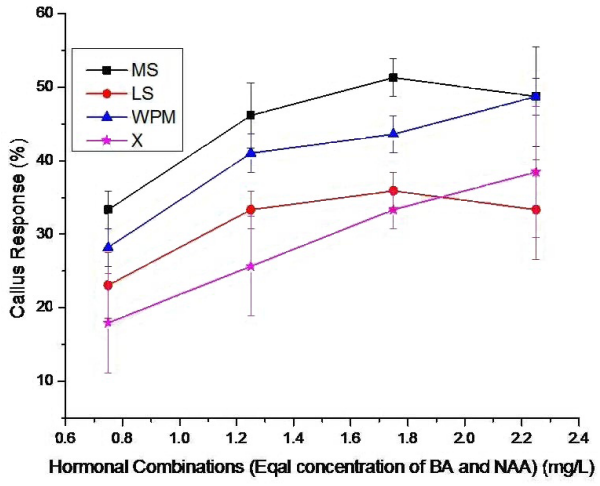

Figure 2 Media selection for better callus initiation of *X. granatum* using four different medium formulated with 0.75, 1.25, 1.75 and 2.25 mg/L both for NAA and BA.

Examination of different plant growth regulators for callus initiation and growth

The callus initiation rates at different plant growth regulator combinations on MS media are tabulated on Table 1. In this Table the texture of callus is also shown. This table shows that there is no callus formation on MS media without plant growth regulator. The NAA found to be superior to 2, 4-D for callus initiation at lower concentration. The addition of BA both in two auxins had boosted the callus formation rate. The table exhibits that the best callus formation rate (91.76%) resulted on MS medium formulated with 2.5 mg/L 2, 4-D and 1 mg/L BA in association (Figure 1d). The production of anthocyanin also found to be better at these plant growth regulator combinations also. The NAA treated callus was white and hard in nature and lack of anthocyanin based on this study.

Previously Haron and Taha (2007) reported that MS medium supplemented with 10 mg/l 2, 4-D and 10 mg/l NAA yielded 87.5% of callus after 14 days in culture using leaf as explants. In this view, present investigation found to be more advanced for both callus induction rate and fast callus response with low concentration of plant growth regulators in same medium and explant used.

Anthocyanin identification and estimation

Thirty days after inoculation (After second subculture) reddish anthocyanin pigments were accumulated on callus surface (Figure 1e, 1f). Callus was maintained with subcultures at 15 days of intervals in a fresh medium supplemented with 2.5 mg/L 2, 4-D and 1 mg/L BA. The highest anthocyanin production was noticed after third subculture period. In exposure to acidic pH the anthocyanin contain callus were turned into reddish orange and in alkaline pH it became green in nature. The absorbance spectra (UV spectra) of extracted anthocyanin pigments are presented on Figure 3. The estimation of total anthocyanin content was 4.5 CV/g fresh weight of callus.

Table 1 Rate of callus initiation of *X. granatum* in MS medium at different concentrations of plant growth regulators (mg/L).

2, 4-D (mg/L)	BA (mg/L)	NAA (mg/L)	% of callus response	Texture of callus
0	0	0	00.00±0.00^{k}	-
0.5	0	-	43.07±4.43^{j}	Hard, compact, white
1	0	-	45.63±2.56^{ij}	Hard, compact, white
1.5	0	-	53.32±2.56^{ghij}	Hard, compact, white
2	0	-	63.58±2.56^{cdefgh}	Hard, compact, white
2.5	0	-	58.45±4.44^{efghij}	Hard, compact, white
0.5	0.5	-	55.89±2.56^{fghij}	Hard, compact, white
0.5	1	-	61.02±2.56^{defghi}	Hard, compact, white
0.5	1.5	-	55.89±2.56^{fghij}	Hard, compact, white
1	0.5	-	63.58±2.56^{cdefgh}	Hard, compact, white
1	1	-	66.15±4.43^{bcdefgh}	Hard, compact, white
1	1.5	-	66.15±4.43^{bcdefgh}	Hard, compact, white
1.5	0.5	-	68.71±2.56^{bcdefg}	Hard, Compact, reddish
1.5	1	-	78.96±2.56^{abc}	Hard, compact, reddish
1.5	1.5	-	66.15±4.43^{bcdefgh}	Hard, compact, white
2	0.5	-	71.27±2.56^{bcdef}	Hard, compact, reddish
2	1	-	78.97±6.78^{abc}	Hard, compact, reddish
2	1.5	-	73.84±8.88^{bcde}	Hard, compact, reddish
2.5	0.5	-	73.84±8.88^{bcde}	Hard, compact, reddish
2.5	1	-	91.79±6.78^{a}	Hard, compact, reddish
-	0	0.5	50.76±4.44^{hij}	Hard, Compact, White
-	0	1	53.32±2.56^{ghij}	Hard, Compact, White
-	0	1.5	55.89±5.13^{efghi}	Hard, compact, white
-	0	2	61.02±2.56d^{efghi}	Hard, compact, white
-	0	2.5	58.45±4.44^{efghij}	Hard, compact, white
-	0.5	0.5	63.58±2.56^{cdefgh}	Hard, compact, white
-	1	0.5	68.71±6.78^{bcdefg}	Hard, compact, white
-	1.5	0.5	58.45±4.44^{efghij}	Hard, compact, white
-	0.5	1	76.40±6.78^{bcd}	Hard, compact, white
-	1	1	73.84±4.43^{bcde}	Hard, compact, white
-	1.5	1	63.58±6.78^{cdefgh}	Hard, compact, white
-	0.5	1.5	58.45±4.44^{efghij}	Hard, compact, white
-	1	1.5	63.58±2.56^{cdefgh}	Hard, compact, white
-	1.5	1.5	63.58±2.56^{cdefgh}	Hard, compact, white
-	0.5	2	76.40±2.56^{bcd}	Hard, compact, white
-	1	2	81.53±4.44^{ab}	Hard, compact, white
-	1.5	2	66.15±4.43^{bcdefgh}	Hard, compact, white
-	0.5	2.5	61.02±6.78^{defghi}	Hard, compact, white
-	1	2.5	61.02±2.56^{defghi}	Hard, compact, white

Here NAA: α - Naphthalene Acetic Acid, 2, 4-D: 2, 4-Dichlorophenoxyacetic acid, BA: 6-benzyladenine purine. The values in the second last column are Mean ± SE followed by the letters within the column indicating the level of significance at P≤0.05 by Duncan's Multiple Range Test

Sasamoto et al. (2018) produced anthocyanin from red callus of a mangrove species *Sonneratia ovata*. They also found that the anthocyanin was initiated after 1.5 months of culture which may correlate with this study. In the present study anthocyanin produced in only a fraction of the cells in the callus. The reasons may be hereditary gene expression levels related to fluctuate among the individual cells. Moreover, physiological state of each cell may experience a different microenvironment in the medium (Miyanaga et al. 2000; Mathur et al. 2010). The CV index is used to estimate anthocyanin in callus as the extracts contained several anthocyanins which can fluctuate under different cultural conditions (Irshad et al. 2018). The present investigation reports first time an appropriate and dependable approach for anthocyanin production in callus cultures of *X. granatum* using leaf as explant till now to the best of our information.

Effect of NaCl on callus initiation, growth and anthocyanin production

The effects of NaCl concentration on the callus initiation and growth were compared after second subculture i.e., 30 days later after inoculation (Figure 4). From this study it was found that *X. granatum* exhibited best callus response and growth at 20 mM NaCl on MS medium supplemented with 2.5 mg/L 2, 4 - D and 1 mg/L BA in combination (Figure 4). It was also displayed that NaCl inhibited the production of anthocyanin.

Several reports are available which showed promotion of callus initiation and growth using different concentrations of NaCl on study of *in vitro* investigation of mangrove species. Sasamoto et al. (2020) reported that 100 mM of NaCl in medium enhanced the callus growth of *Avicennia alba*. The results of this study regarding NaCl were also presented by Haron and Taha (2007) with the same species. They displayed that addition of 0.1 g/L NaCl in medium reduced the callus formation rate of *X. granatum* as compared to devoid of NaCl in MS medium. However, present study opposed this observation and revealed that addition of limited quantity of NaCl in optimized growth regulator MS medium would increase the callus initiation rate and growth of callus. Usually, anthocyanins are secreted to reduce different types of stress. Considering the true mangrove species of this study it may assume that anthocyanins are produced due to lack of NaCl in the medium which favours their growth in natural habituate environment. After adding NaCl in medium the anthocyanins disappeared. From this observation it may seem that the induction of anthocyanin, probably due to a nutritional stress condition of this species.

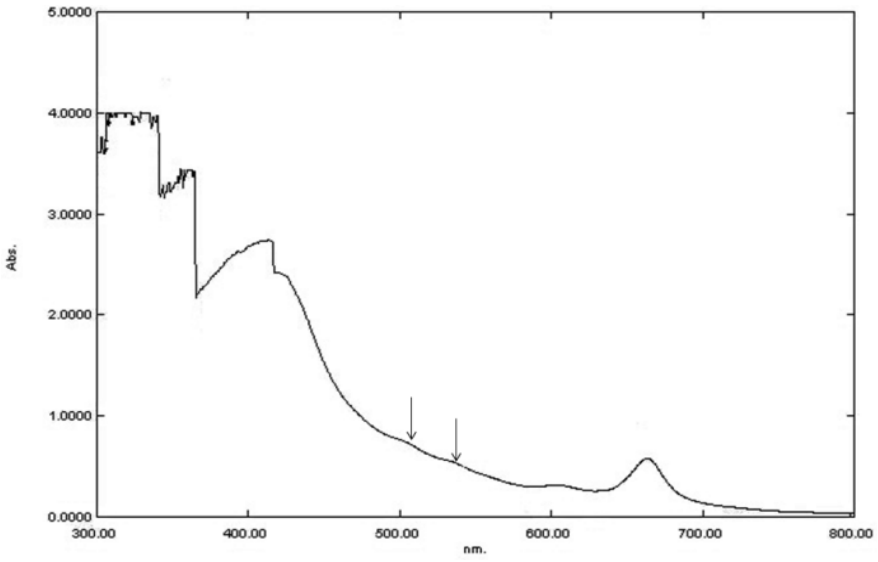

Figure 3 The absorbance spectra of extracted anthocyanin. Black arrow indicates peak at nearly 520 nm and 540 nm.

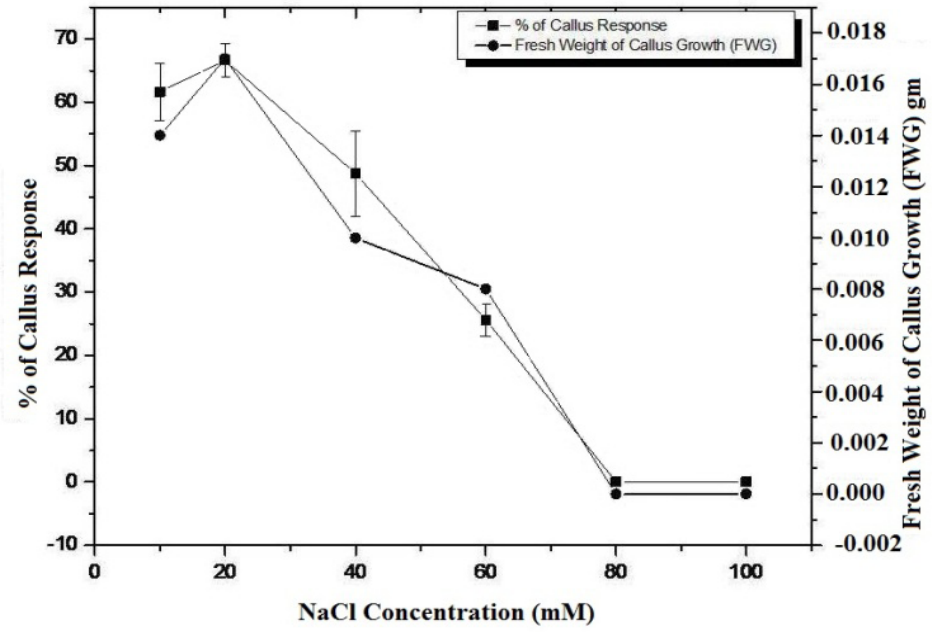

Figure 4 Effect of salt concentration on callus initiation and growth on MS medium supplemented with 2.5 mg/L 2, 4 - D and 1 mg/L BA in combination of *X. granatum*.

Seasonal effect on callus formation

This study was carried out all over the year of different seasons viz., during monsoon (July-September), after monsoon (November-February) and before monsoon (March-June) to check the seasonal effect for callus initiation and anthocyanin accumulation on MS medium supplemented with 2.5 mg/L 2, 4 - D and 1 mg/L BA in combination using leaf explant. The Figure 5 shows that, for *in vitro* investigation particularly for callus formation and anthocyanin induction of this species rainy season was most excellent time. Figure 5 also exhibited that after monsoon season yielded lowest result for callusing based on this study.

Literature studies found that seasonal collection of explants had heavy impacts on *in vitro* investigation (Fei et al. 2000, Kartsonas & Papafotiou 2007). The health of explants varied in different seasons as it causes change in temperatures, humidity, rainfall, photoperiod etc. It is varied among the species, genus and cultivars also. Several reports showed that collection of explants during summer/rainy season exhibited the finest results for *in vitro* investigation as for meristematic activity of tissues in explants (Rutkowska-Krause et al. 2003, Chitra & Gudipalli 2005, Kartsonas & Papafotiou 2007). A lot of tree species which are examined during rainy season (Active growth time) induces marvellous growth in *in vitro* conditions as physiological condition of tissues of tree species are varied due to alteration of season (El-Morsey & Millet 1996).

Histological study

The histological view of produced callus is shown on Figure 6. The Figure 6 shows that in same callus both embryogenic and non-embryonic cells are situated (Figure 1e, 1f). The Figure 6 displays that the embryogenic calli had stained, small and compact isodiametric cells (Indicated by green arrow) whereas non-embryonic calli exhibited large and highly vacuolated cells (Indicated by sky blue arrow). Furthermore, embryonic calli exhibited dense cytoplasm with little vacuoles where nuclei undergoing constant cell division and formed the mitotic cells zone (MCZ). The existence of several starch granules indicated high metabolic activity of cells in callus (Black arrow).

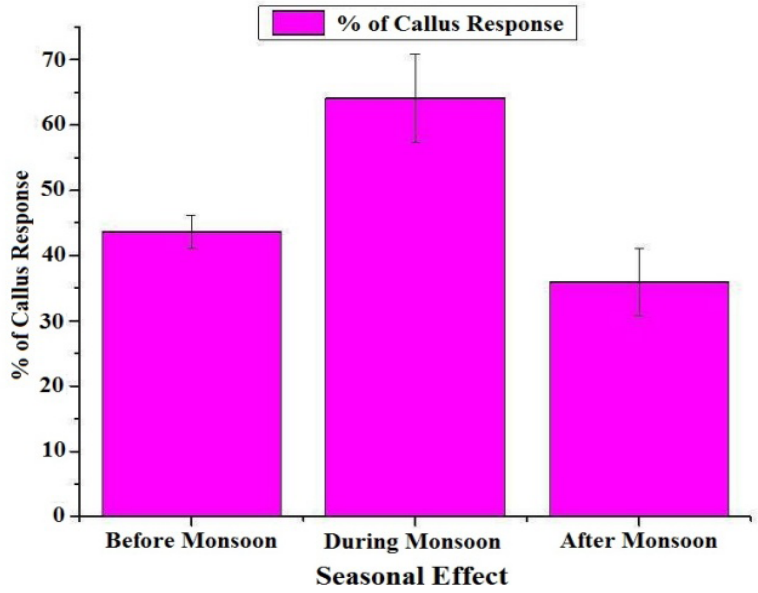

Figure 5 Seasonal effect on callus induction and growth of *X. granatum*

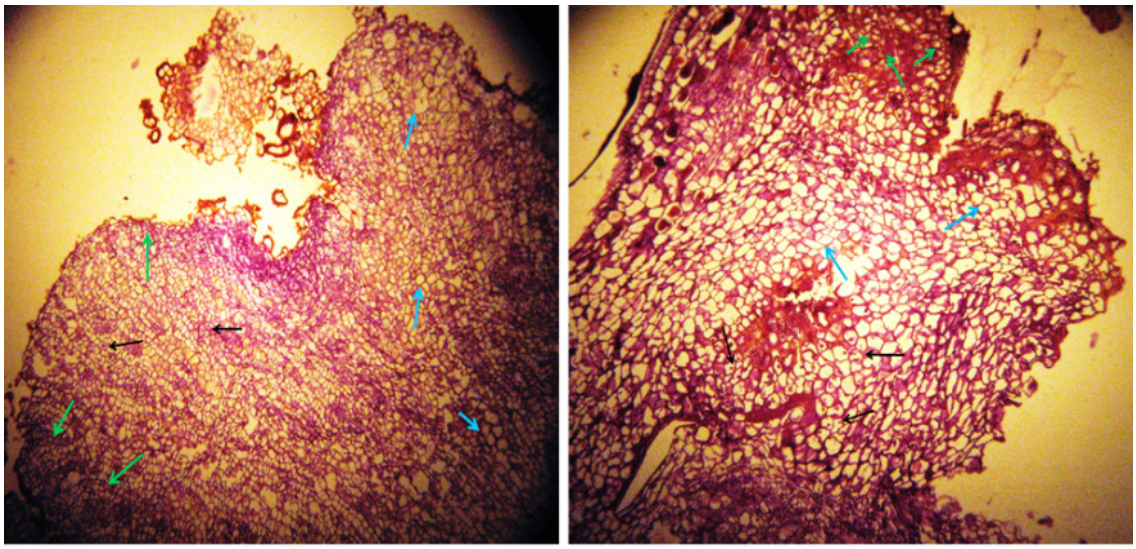

Figure 6 Histological images of *X. granatum* callus. Common view of 1 month old protuberance produced at the proximal part vertical section, green arrow indicates calli containing both small meristematic cells with highly-stained nucleus in mitotic cells zone (MCZ), blue arrow indicates non embryogenic cells contained large vacuole, black arrow indicating the starch granules.

Previous studies on anthocyanin production from callus of different plants is found to be related with depletion of medium components like nitrate, phosphate or addition of elicitors like various growth regulators, salicylic acid, methyl jasmonate etc (Meyer & Staden 1995, Karaaslan et al. 2013, Tarrahi & Rezanejad 2013). It is also suggested that the light and dark condition is also affect the anthocyanin production (Karaaslan et al. 2013, Ai et al. 2016). From this experiment it may seem that anthocyanin was developed due to light and dark conditions also because during this study cultures were kept at dark continuously for 15 days to remove browning of explants. It was

also observed that anthocyanins were produced in light, but not in the dark condition. This finding may correlate with the results of Miura et al. (1998). The nature and amount of growth regulators used in this study had vital role in anthocyanins production. The leaf as explant, MS medium with 2.5 mg/L 2, 4-D and 1 mg/L BA in combination, lack of NaCl and dark condition at starting incubation period of cultures were found to be suitable for the production of anthocyanin pigmented callus from *X. granatum*. Previous studies related to anthocyanin production from callus of different plants showed that biomass acceleration and anthocyanin accumulation involve various media conditions (Addition of elicitors or depletion of medium component) to provoke a change from the growth state to the metabolite induction state. This may prevent the protocol for efficiency of uses in commercial production. However, this study showed that anthocyanin accumulation was connected to callus growth on the same cultural medium. It requires no addition of extra compound which act as elicitors or depletion of medium component. So, this study is an appropriate and reliable method for in vitro anthocyanin production. For stress related physiological studies, mangrove callus and its suspension cultures are very much promising (Inoue et al. 2015). In this context, the present research shows an opportunity to examine the salt and salinity mechanism, and genetic transformation experiment at cellular level of this species. Current study is undertaken to conserve the *X. granatum* as callus is being widely accepted for afforestation programmes (Ahuja 1991). This investigation is an opening experiment for micropropagation of *X. granatum*. At present many efforts have been employed to establish micropropagation from this callus by transferring them into medium containing several concentrations of different plant growth regulators, coconut water (Natural source of cytokinin), casein etc.

CONCLUSIONS

This study described an attempt to conserve this threatened species through callus. This protocol found to be very suitable for production of anthocyanin from callus also. The efficient amount of callus and anthocyanin produced through leaf explant culturing on MS medium formulated with 2.5 mg/L 2, 4-D and 1 mg/L BAP in combination. This study revealed that the monsoon season exhibited better time for both callus induction and anthocyanin production. Addition of 20 mM NaCl reported to be advantageous for callusing. However, the addition of NaCl inhibited the anthocyanin production. The anthocyanin identification was performed through colour changes caused by pH variation and UV spectra. This protocol yielded 4.5 CV/g fresh weight of callus of anthocyanin. This technique displayed some globular embryo which was confirmed by histological slides.

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