Callus induction of *Careya arborea* Roxb. – An Important medicinal plant

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ABSTRACT

The purpose of this study was to standardize a medium protocol for induction and maintenance of callus from seeds of *Careya arborea* Roxb. Fourteen types of media (Murashige and Skoog, 1962) were prepared according to various concentrations of growth regulators (2,4-D-0.5-2.0 mgL⁻¹; BAP-0.2-0.5mg L⁻¹, Kn-0.1-0.4 mgL⁻¹ & NAA-0.5-2.0 mgL⁻¹) to standardize a medium protocol for callus induction from *Careya arborea* Roxb. seeds. The explants (seeds) were surface sterilized, cultured and incubated at 24±2°C, under 16 hrs photoperiod, 8 hrs dark, 2000 Lux of light intensity and 60% of relative humidity. Explants that were cultured on MS 1962 medium supplemented with 2.0 mgL⁻¹ of 2,4-D combination with 0.5 mg L⁻¹ BAP, 0.2 mg L⁻¹ Kn and 1.0 mg L⁻¹ NAA have high frequency callus induction (95%) compared to those that were cultured on thirteen types of media. In this amend 2.0 mg L⁻¹ of 2,4-D combination with 0.5 mg L⁻¹ BAP, 0.2 mg L⁻¹ Kn and 1.0 mg L⁻¹ NAA was suitable concentrations in MS medium for development of callus from the seeds of *Careya arborea* Roxb at high frequency level hence, the medium protocol was standardized. Further, the same medium was used for maintenance of callus in the laboratory (in vitro) conditions.

Key words: *Careya arborea* Roxb, Plant Tissue Culture, Callus, BAP and Growth regulators.

INTRODUCTION

*Careya arborea* Roxb. is commonly known as ‘Wild Guava’ (Kumbhi) belongs to the family Lecythidaceae and a medium sized deciduous tree. *Careya arborea* Roxb. has a wide range of medicinal values, an oldest medicinal herb and found in many places of the world [1]. India is well known worldwide for its Ayurvedic treatment and *Careya arborea* Roxb. is widely available in India [2, 3]. It is throughout India growing natural in planes: Sub-Himalayan tract, from Jammu eastwards to West Bengal, Madhya Pradesh and Tamil Nadu. In Karnataka it is distributed in Belgaum, Bellary, Chikmagalur, Chitradurga, Hassan, Mysore, North Kanara, Shimoga and South Kanara districts. The *Careya* genera have three species they are (1) *Careya sphaerica* Roxb. (2) *Careya herbacea* Rox. (3) *Careya arborea* Roxb. *Careya arborea* Roxb. is reported to be useful in tumours, cough, bronchitis, toothache, wounds, colic, intestinal worms, dysentery, leucoderma, epilepsy, ulcers and smallpox. Medicinal herbs have therapeutic property due to the presence of secondary metabolites (chemical substances) in one or more parts (leaves, bark, root and seed) of these plants. *Careya arborea* Roxb. plant has been extensively investigated, and chemical constituents from the barks, leaves and seeds have been reported to include triterpenoids, flavonoids, cumarin, saponins and tannins [4]. Stem bark of *Careya arborea* Roxb. is traditionally used in the treatment of tumors, bronchitis, skin disease, epileptic fits, astringents, antidote to snake-venom, abscesses, boil and ulcer [1]. Fruits are used as decoction to promote digestion. Leaves and flowers are used in the form of past to cure several skin diseases [5]. It is also used
as remedy for diarrhea, dysentery with bloody stools and ear pain. Leaf paste and pulp used as poultice rapidly heals ulcers and root is used for the treatment of tuberculosis and skeletal fractures.

The plant has been considered ethonobotanically important due to its use in traditional and health care system for curing severe diseases like skin diseases, bronchitis, tuberculosis, diarrhea, dysentery, ulcer etc. Most prominent medicinal properties are the presence of triterpenoids [6, 7], flavonoids [8], cumarin [9], saponins [10] and tannins has great demand in pharmaceutical industries. Considering the high economical and pharmacological importance of secondary metabolites, industries are deeply interested in utilizing plant tissue culture technology for large scale production of these substances. Plant tissue culture is an important frontier area in plant biotechnology and to support the production of an enormous array of phytochemical compounds in the laboratory conditions [11, 12]. The use of tissue culture for large scale production of plants, improvement of crops, conservation of valuable germplasm and production of secondary metabolites has been well documented. However, many plants are producing pharmaceutically or pharmacologically valuable secondary metabolites, which are extremely expensive to obtain by extraction from the plant. The in vitro derived phytochemical compound has many selective advantages over the normal and synthetic source of production.

To best of our knowledge, there is no information concerning the in vitro callus of Careya arborea Roxb. Therefore, an objective of the presented investigation was to standardize a medium protocol for induction (indirect method) and maintenance of callus from the seeds of Careya arborea Roxb. In addition, an important point is that in vitro callus of Careya arborea Roxb. is a key step to enhance the production of secondary metabolites, for genetic transformation and to study of somaclonal variations in laboratory conditions.

MATERIALS AND METHODS

Plant material
Careya arborea Roxb. seeds were used as source of explants throughout this experiment.

Surface sterilization
The seeds (explants) were thoroughly washed under running tap water for 5 minutes to remove the traces of dust. Then the seeds were treated with solutions of Indofil (fungicide) 2 g L⁻¹ and antibiotic K-cyclin (streptomycin + tetracycline) 200 mg L⁻¹ for 2 hours followed by rinsing twice with distilled water. Next, the seeds were treated with concentrated hydrochloric acid (1ml of conc. HCl plus 9 ml of distilled water) for 30–45 minutes to break the hard coat of the seeds followed by rinsing twice with distilled water. Now, the broken hard coat of the seeds was removed and, the seeds were then washed and cleaned carefully under running tap water for 30 minutes to remove all the phenolic compounds. Then, the explants of Careya arborea Roxb were transferred to laboratory for further sterilization process. In front of the laminar air flow chamber followed the following procedure for surface sterilization of seeds:

1. Place the explants in a beaker (sterilized) and wash the seeds with sterile distilled water for 1 minute.
2. Now, the seeds were allowed to soak (by stirring on magnetic mini-stirrer) in 70% (v/v) ethanol for 30-45 sec. followed by sterile distilled water twice.
3. Finally, the seeds were soaked in 0.12% HgCl₂ solution along with 1 drop of Tween 20 for 12 minutes followed by rinsing thrice with sterile distilled water.
4. After surface sterilization, keep the sterilized seeds in distilled water in petridish to prevent drying.
5. Now, disinfect the forceps and scalpels by flaming using the alcohol lamp for 10–15 sec.
6. Finally, the surface sterilized seeds (explants) were placed on the MS culture medium with a sterile scalpel.

Preparation of culture medium
Murashige and Skoog [13] was used as the basic culture media and fourteen types of media were prepared according to different concentrations of growth regulators (2,4-D, NAA, Kn & BAP (Sigma made)) (Table-1). 25 ml of distilled water was added in to each conical flask (250 ml capacity). Sucrose was added and stirred it to dissolve completely, and then the stock solutions of major, minor, iron source, vitamins and hormones were added one by one. The final volume of known quantity (100 ml) was obtained by adding sterilized double distilled water. Agar was added to the boiling media slowly and gradually with constant stirring to avoid formation of any clumps. All the media were adjusted the pH value between 5.75 - 5.80 by using of 1N HCl or 1N NaOH before autoclaving. Then the media were dispensed into culture vessels, plugged with polypropylene caps and autoclaved at 121°C.
temperature and 15 lbs pressure for 15 minutes. Sterilized media were allowed to cool, kept for contamination observations and used for inoculation.

**Inoculation and Culture conditions**

Surface sterilized seeds were inoculated onto culture vessels containing 40 ml MS media supplemented with different concentrations and combinations of plant growth regulators (Table-1) for callus induction at 25°±2°C. Each media formulation was inoculated by 15 seeds (2 or 3 seeds per culture vessel). All the culture vessels were placed under illumination, provided by white fluorescent tube light (2000 lux), and exposed to 16 hrs of photoperiod, 8 hrs of dark period and 60% of relative humidity. Callus induction rate on each media formulation was calculated and the data were recorded after every week, for six weeks.

**RESULTS AND DISCUSSION**

A highly efficient (standardized) protocol for the callus induction of *Careya arborea* Roxb was established. Two different concentrations of auxins like NAA & 2,4-D and cytokinins like Kinetin and BAP were used to standardize a medium protocol for the induction and maintenance of callus from the seeds of *Careya arborea* Roxb. (Table -1). Surface sterilized seeds (explants) were cultured on MS medium supplemented with different concentrations of auxins such as 2,4-D and NAA (auxins) in combination with 6-BAP and Kn (cytokinins). After six weeks, callus initiation from the seeds was observed in the fourteen types of media. No callus induction was observed in the two medium types (CaRM-1 & CaRM-14) and recorded (Fig-2 & Table-1). Low callus induction frequency (17% to 28%) was observed in the two medium types (CaRM-2 & CaRM-4) and recorded (fig-2 & Table-1). High callus (CaRM-12) induction frequency (95%) (Fig-1,2 & Table-1) was observed on the modified MS medium 1962 MS supplemented with 2.0 mg L\(^{-1}\) of 2,4-D combination with 0.5 mg L\(^{-1}\) BAP, 0.2 mg L\(^{-1}\) Kn and 1.0 mg L\(^{-1}\) NAA.

**Table-1: Type of medium and effect of plant growth regulator combinations on callus induction.**

<table>
<thead>
<tr>
<th>Media Code</th>
<th>Plant Growth Regulators</th>
<th>Callus induction Intensity</th>
<th>Callus induction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAP (mg L(^{-1}))</td>
<td>Kn (mg L(^{-1}))</td>
<td>NAA (mg L(^{-1}))</td>
</tr>
<tr>
<td>CaRM-1</td>
<td>-</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>CaRM-2</td>
<td>-</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>CaRM-3</td>
<td>-</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>CaRM-4</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>CaRM-5</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>CaRM-6</td>
<td>-</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>CaRM-7</td>
<td>0.2</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>CaRM-8</td>
<td>0.3</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>CaRM-9</td>
<td>0.5</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>CaRM-10</td>
<td>0.3</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>CaRM-11</td>
<td>0.5</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>CaRM-12</td>
<td>0.5</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>CaRM-13</td>
<td>0.3</td>
<td>0.4</td>
<td>2.0</td>
</tr>
<tr>
<td>CaRM-14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**CalRM: Careya arborea Roxb Medium;**

*Callus induction intensity: No Callus (-); Low (+); Moderate (++); High (+++).*

The results showed that the effect of four types of growth regulators (2,4-D, BAP, Kn and NAA) treatment in MS media on callus formation from the seeds of *Careya arborea* Roxb. And also the results indicated that the callus growth from the seeds was significant effected by the type and concentration of growth regulator.

Most frequently 2,4-D (auxin) is used to initiate the callus growth, the addition of traces of 2,4-D in the medium, to initiate callus from tissues of several crops have been demonstrated by many scientists [14,15,16,17]. In this study, a medium protocol for induction and maintenance of callus from *Careya arborea* Roxb. was reported. It was found the modified MS medium 1962 MS supplemented with 2.0 mg L\(^{-1}\) of 2,4-D combination with 0.5 mg L\(^{-1}\) BAP, 0.2 mg L\(^{-1}\) Kn and 1.0 mg L\(^{-1}\) NAA (Figure-1&2) was superior to the MS containing 2.0 mg L\(^{-1}\) of 2, 4-D combination with 0.5 mg L\(^{-1}\) BAP and 2.0 mg L\(^{-1}\) NAA (Table-1) for high potential of Light yellow-greenish callus.
Figure-1: Callus induction from the seeds of *Careya arborea* Roxb. (A) Inoculated seed on Medium (B) Callus initiation from the seeds cultured in dark on MS supplemented with 2.0 mg L\(^{-1}\) of 2,4-D combination with 0.5 mg L\(^{-1}\) BAP, 0.2 mg L\(^{-1}\) Kn and 1.0 mg L\(^{-1}\) NAA after 8 days (C) Yellow-greenish callus from the seed on MS supplemented with 2.0 mg L\(^{-1}\) of 2,4-D combination with 0.5 mg L\(^{-1}\) BAP, 0.2 mg L\(^{-1}\) Kn and 1.0 mg L\(^{-1}\) NAA after 29 days.
The callus induction rate, in all the concentrations of NAA & Kn, it is suggested that the lower concentration of NAA and Kn were required to proliferate efficient callus from the seeds of *Careya arborea* Roxb. Lowest concentration of NAA is useful in order to avoid any somaclonal variation at the time of regeneration. The modified MS medium 1962 without any growth regulators (control) was not capable to induce callus from the seeds (Fig-2 & Table-1). Depending on the concentration and combination of growth regulators were used, a wide range of variation in frequency of callus induction was observed (Fig-2). Among the different combination of auxins and cytokinins tested, 2, 4-D in combination with BAP, NAA and Kn has been proved to be better in terms of inducing high frequency of callus induction (Fig-2).

The seeds of *Careya arborea* Roxb. were induced the best callus (95%) on modified MS basal medium 1962 was supplemented with 2.0 mg L\(^{-1}\) 2,4-D in combination with 0.2 mg L\(^{-1}\) kinetin, 0.5 mg L\(^{-1}\) 6-BAP and 1.0 mg L\(^{-1}\) NAA (Fig-1&2). And the callus was transferred to the same fresh modified MS medium supplemented with 2.0 mg L\(^{-1}\) 2,4-D, 0.2 mg L\(^{-1}\) kinetin, 0.5 mg L\(^{-1}\) 6-BAP and 1 mg L\(^{-1}\) NAA for maintenance of callus in laboratory conditions.

**CONCLUSION**

Callus culture system offers may advantages as model system for several biopharmaceutical investigations. Hence in the present investigation standardized a medium protocol has been devised for *in vitro* induction and maintenance of callus from the seeds of *Careya arborea* Roxb. This protocol will be useful for conducting genetic transformation, and inducing somaclonal variation in *Careya arborea* Roxb.

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**REFERENCES**


