OPTIMIZATION OF BIOMASS YIELD AND ASIATICOSIDE ACCUMULATION IN THE CALLUS CULTURES FROM THE LEAVES OF *CENTELLA ASIATICA* (L). URBAN

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ABSTRACT

Due to increasing evidences of potential medicinal and clinical applications of Asiaticoside, an active phytoconstituent of *Centella asiatica* leaves, it is an essential to produce this bioactive compound with optimal yield which can be achieved through invitro callus cultures from the young leaves. Callus growth, biomass yield and in vitro asiaticoside accumulation in callus cultures from young leaf explants of *Centella asiatica* was studied by the application of different plant growth regulators in the culture media. Callus induction was observed from young leaf explants on MS medium with different concentrations of 2,4-dichlorophenoxy aceticacid (2,4-D) and α-naphthalene aceticacid (NAA) supplied singly and in combination with different concentrations of kinetin, 6-benzylaminopurine. Optimum callus (84%) was developed on murashig-skoog (MS) medium with α-naphthalene aceticacid (2.0 mg/l) and kinetin (1.0 mg/l) which induced whitish green and compact callus and was found best for the optimal biomass yield (Fresh weight 1.58 g/20mL; Dry weight 110 mg /g FW) as determined by growth curve analysis and gave highest asiaticoside content(0.94 mg/gm dry weight) which was quantified by High-
Performance Liquid Chromatography (HPLC) on C18 reverse phase column using mobile phase water:acetonitrile (20:80) with the flow rate of 1 mL/min and read at 220 nm with PDA detector. Based on the callus growth curve, we also reported the suitable growth time point for callus harvesting in terms of biomass accumulation and the highest biomass yield was observed on 35th day after inoculation.

**Keywords:** Centella asiatica, asiaticoside, callus cultures, growth curve, biomass yield, High-performance liquid chromatography.

**INTRODUCTION**

*Centella asiatica* (L) an important medicinal plant belongs to the Umbelliferae family, widely distributed in India, South East Asia and other regions. The extracts of this plant having various medicinal properties due to the presence of various active compounds which include triterpenoid saponins, steroidal saponins, flavonoids, glycoalkaloids etc. Among triterpenoid saponins asiaticoside and madecassoside are of significant pharmaceutical interest\(^1\). Asiaticoside, a triterpenoid glycoside structurally consists a pentacyclic triterpene as sapogenin which is attached to three sugar molecules by glycosidic bond. It is widely used in wound healing due to stimulation of glycosaminoglycan and collagen synthesis\(^2\). It is also acts as a neuroprotectant used in the treatment of Alzheimer’s disease due to protect cells against beta amyloid-induced cell death\(^3\). It is having antiproliferative activity against tumor cells\(^4\). This glycoside has been reported to have activity against herpes simplex virus 1 and 2\(^5\) as well as mycobacterium tuberculosis and mycobacterium leprae\(^6\) and hence used in the treatment of leprosy and tuberculosis\(^7\). Asiaticoside also provides protection against chemical-induced inflammation and hepatotoxicity\(^8,9\). In vitro techniques are very useful in ensuring sustainable, optimized sources of plant-derived natural products. The ability of plants to produce certain bioactive substances is greatly influenced by the physical and chemical environments in which they grow. In the past few decades, secondary metabolite production from plant tissue culture has been identified as a tremendous resource for new drug development and clinical research in the fields of pharmacology and medicine. This is the systematic approach to study the effect of different concentrations and combinations of phytoregulators on the callus induction, growth rate of callus, fresh weight and dry weight of callus as well as asiaticoside content of the callus derived from young leaf explants.
MATERIALS AND METHODS

Chemicals
All the Plant growth regulators were purchased from Sigma Aldrich chemicals, Hyderabad, India. The medium components were purchased from Hi-Media chemicals, Hyderabad, India. The solvents were of HPLC grade and purchased from Merck, Hyderabad, India.

Plant material collection
Centella asiatica plants were collected from Tirumala hills, and identified with taxonomist, Department of Botany and maintained in the garden of the Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

Explant sterilization
The young leaves were excised and thoroughly washed under running tap water for 3-5 min, then leaves were disinfected with 1% teepol solution for 3 min and washed with distilled water. Under aseptic conditions explants were treated with 10% sodium hypochlorite solution for 60-90 sec followed by double distilled water. Surface sterilization was done by using 0.1% HgCl₂ for 30-45 sec followed by a final rinse (2-3 times) with double distilled water.

Callus induction and culture conditions
The sterilized leaves were dissected into small and equal parts (0.8 x 1.0cm) and inoculated on MS mediumsupplemented with different phytohormones such as 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA) and kinetin. The cultures were incubated at 21 ± 2 °C under photoperiod of 12-h light/12-h dark with light intensity 45-50 µ mol/m²/s. Sub-culturing was done with every 3 weeks interval for the friable and white friable colored calli on the same corresponding media.

Callus Growth measurement and Biomass yield
The frequency of the callus initiation was calculated based on the percentage of explants capable of being developed into the callus. The time taken (in days) for callus initiation was noted and the callus morphology was recorded. For growth measurement of calli, the proliferated callus was harvested and the fresh weight (FW) was recorded, the callus was dried in an oven at 40°C for 24 h and the dry weight (DW) was recorded using a monopan. Callus growth curve was recorded on fresh weight and dry weight basis at every 7day time interval from 14th day to 42nd day.
Extraction of Asiaticoside
For quantification of Asiaticoside callus cultures were oven dried at 40^\text{\textdegree}C for about 24 hrs and pulverized. Methanolic extracts of the callus were prepared by using soxhlet extraction method. The extracts were incubated at 4^\text{\textdegree}C for 24 hrs, then filtered and evaporated to dryness under vacuum. Then they were stored at 4^\text{\textdegree}C for HPLC analysis.

Chromatographic separation of asiaticoside
The chromatographic separation was carried out using Analytical Technologies Intelligent 3000 Series HPLC System equipped with PDA detector and the analysis was carried out with C18 column (5-µm size,250 × 4.6 mm in length) using mobile phase, water:acetonitrile (20:80) at an elution rate of 1.0 mL/min, and the column temperature was maintained at 25^\text{\textdegree}C. Asiaticoside content was detected by UV absorption at 220nm. The sample injection volume was 20 µL. Validation of quantitative method was done with samples consisting of three injections of 20 µL each.

Statistical analysis
All the experiments were designed randomly and the significance of differences among means was calculated using Duncan’s multiple-range test\textsuperscript{11} at the 5% probability (P≤0.05).

RESULTS
Callus induction
To determine the influence of phytoregulators on callus induction and biomass yield auxins, 2,4-D and NAA supplied singly as well as in combination with cytokinins kinetin and BA with different concentrations were used. After a week of inoculation, the explants started to swell before the callus developed. Callus was initiated in 14 days after inoculation on MS medium supplemented with 2.0 mg/L NAA in a combination of kinetin (1.0mg/L) and 2,4-D (2.0mg/L) alone also found to be good in callus induction and callus was initiated in 16 days. While long period (24 days) for callus initiation was observed on MS medium with 2.0 mg/L NAA alone. (Table 1). Maximum callusing (84%) was noticed on MS medium supplemented with 2.0 mg/L NAA and 1.0 mg/L kinetin followed by 2,4-D alone (82%). The other combinations viz 2.0 mg/L 2,4-D and 1.0 mg/L kinetin (74%) was found to be good where as 2.0 mg/L 2,4-D and 1.0 mg/L BA as well as 2.0 mg/L NAA and 1.0 mg/L BA was moderate or poor in inducing the callus. The auxin NAA (2.0mg/L) alone was very very poor in inducing callus.
Table: 1  Growth of callus derived from *Centella asiatica* leaves under different concentrations and combinations of phytoregulators [mg L\(^{-1}\)]. Growth was determined 14, 21, 28, 35 days after inoculation.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Percentage of callus induction</th>
<th>Time taken for callus initiation</th>
<th>Callus score</th>
<th>Callus morphology</th>
</tr>
</thead>
</table>
| MS + 2,4-D (2.0mg/l) | 82% | 16 days | 14= -
21=+
28=+++ 35=++++ | Whitish and compact
Whitish and compact
Whitish and friable |
| MS + NAA (2.0mg/l) | 53% | 24 days | 14= -
21= -
28=+
35=++ | _
Slightly Yellowish
Yellowish and loose |
| MS + 2,4-D (2.0mg/l) + kinetin (1.0mg/l) | 74% | 18 days | 14= -
21=+
28=++
35=+++ | Slightly Green
Whitish and compact
Whitish and friable |
| MS + 2,4-D (2.0mg/l) + BA (1.0mg/l) | 61% | 19 days | 14= -
21=+
28=+++ 35=++++ | Slightly Green
Whitish and friable
Whitish and loose |
| MS + NAA (2.0mg/l) + kinetin (1.0mg/l) | 84% | 14 days | 14= +
21=++
28=+++ 35=++++ | Slightly Green
Whitish Green
Whitish and compact
Whitish and compact |
| MS + NAA (2.0 mg/l) + BA (1.0mg/l) | 62% | 21 days | 14= -
21=+
28=++
35=+++ | _
Slightly yellowish
Yellowish and friable
Yellowish and loose |

- = No callus; +=Low amount; +++=Good; +++=High; +++=Intense or very high

**Callus morphology**

Callus exhibited different morphological changes (Table 1) such as, slight green, greenish, whitish and compact, white and friable, whitish and loose, and yellow loose callus. For combinations such as 2.0 mg/L NAA and 1.0 mg/L kinetin as well as 2.0mg/L 2,4-D alone the morphological changes from 2\(^{nd}\) week to 6\(^{th}\) week were almost similar and white and compact callus was observed and shown in Fig. 1.a-d. Yellowish and loose callus was observed for other concentrations and combinations of plant growth regulators. Sub cultured
calli on MS medium with NAA and kinetin was white and compact in morphology and shown in Fig. 1e.

Fig :1. Callus derived from the leaves of *C.asiatica* on MS medium with NAA (2.0mg/L) and kinetin(1.0mg/L) (a) represents leaf explant inoculated (b) callus was initiated after 14 days (c) whitish Greenish callus after 21 days (d) White and Compact callus after 35 days of inoculation. (e) Whitish and compact callus after two weeks of sub culturing on MS medium containing NAA (2.0mg/L) and kinetin (1.0mg/L).

**Callus Biomass yield**

The growth of callus was determined based on the fresh and dry weights of the callus cultures. Successful biomass was obtained by the use of NAA (2.0 mg/L) with kinetin (1.0mg/L) (FW-1.58 g/20mL; DW-110 mg/gFW) followed by 2.0 mg/L of 2,4-D (FW-1.52 g/20mL; DW-105 mg/gFW) alone and 2,4-D (2.0 mg/L) with kinetin (1.0mg/L) (FW-1.36 g/20mL; DW-91 mg/gFW). However, the combinations such as 2,4-D (2.0mg/L) with BA(1.0mg/L) and NAA with BA as well asNAA alone were resulted poor in biomass yield (Table 2). During the measurement of callus biomass at various time intervals, the batch callus culture was continuously examined by taking a subculture at two weeks interval to prevent cell death and browning of media.
Table 2. Effect of different phytoregulators on Biomass accumulation and asiaticoside content after 5 weeks of inoculation. Data represents mean ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan’s multiple range test at P ≤ 0.05.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Callus fresh wt (g/20ml)</th>
<th>Callus dry weight (mg/g FW)</th>
<th>Asiaticoside content (mg/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS + 2,4-D (2.0mg/l)</td>
<td>1.52 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>105 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.94 ± 0.03&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>MS + NAA(2.0mg/l)</td>
<td>0.72 ± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>56 ± 0.06&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.78 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MS + 2,4-D(2.0mg/l) + kinetin (1.0mg/l)</td>
<td>1.36 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>91 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.90 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MS + 2,4-D(2.0mg/l) + BA (1.0mg/l)</td>
<td>0.84 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
<td>69 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.75 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MS + NAA(2.0mg/l) + kinetin(1.0mg/l)</td>
<td>1.58 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.96 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MS + NAA(2.0 mg/l) + BA (1.0mg/l)</td>
<td>0.89 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>67 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.81 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Asiaticoside production

Quantification of asiaticoside was done by comparing the peak areas of the test samples with that of standard. The maximum accumulation of biomass was recorded by using NAA with kinetin (Table 2). Asiaticoside production in the callus cultures also followed the similar trend and the highest asiaticoside yield from callus was obtained on the MS medium containing 2.0 mg/L NAA with 1.0 mg/L kinetin. The good accumulation of asiaticoside was also recorded by using 2.0mg/L 2,4-D alone and 2.0 mg/L 2,4-D with 1.0 mg/L BA. But combinations such as 2,4-D with BA and NAA with BA as well as NAA alone were not suitable for biomass yield and asiaticoside production (Table. 2).

Callus Growth curve

This study aimed to determine the most suitable growth time point for callus harvesting with the highest biomass yield. Measured calli fresh weight and dry weight at various time intervals (14, 21, 28, 35 and 42<sup>nd</sup> day). During the lag phase, there is a little callus growth and biomass yield of C.asiatica until the day 18 of inoculation. The exponential growth phase started on the day 18 and continued up to 35 day, then the culture entered the stationary growth phase (Fig. 2). The highest biomass obtained on day 35, followed by day 28 and the lowest biomass obtained on day 15. It is noted that although fresh content is increased on day
but the dry weight is drastically reduced. These two observations clears the paradox for most suitable growth time point for callus harvesting i.e. at 35\textsuperscript{th} day after inoculation.

Fig: 2. Growth curve of callus on fresh weight and dry weight basis during different culture periods in the milligram proliferation medium (MS + 2.0mg/L NAA + 1.0mg/L kinetin). Data represents mean values±SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at \( P \leq 0.05 \) according to Duncan’s multiple range test (DMRT).

DISCUSSION

Traditional uses of natural compounds has received much attention because of their potential medicinal, clinical, pharmaceutical and neutraceutical properties and they are believed to be safe for human use as well as environmentally non toxic. \textit{Centella asiatica} is an important medicinal plant which constitutes pharmaceutically active compounds, but it derives its pharmaceutical value mainly due to the presence of asiaticoside and its derivates structurally comes under the category of pentacyclic triterpenoid glycosides present in the leaves mainly. Asiaticoside has antispasmodic, circulation stimulatory, strong diuretic and wound healing properties and is used in the treatment of leprosy, tuberculosis, asthma, bronchitis, ulcer, anxiety, and mental disorders\textsuperscript{12}. Considering its medicinal properties and due to increasing pharmaceutical demand of this herb, the requirement for a tissue culture technique as an alternative production system was crucial for biomass and asiaticoside production.

In this study we have reported the effect of auxins alone as well as combination of auxins with cytokinins on in vitro callus cultures from young leaf explants of \textit{C.asiatica} in terms of callus induction, callus morphology, biomass yield and asiaticoside content. The auxin 2,4-D was very good in inducing white and compact callus and took short duration (16 days) for
callus initiation and it produced 82% of callus response. 2,4-D treatment also resulted in good biomass production and asiaticoside content. But the auxin NAA was very poor in inducing callus which is yellowish and loose and took very long time (24 days) for callus initiation. It only produced 53% of callus response. Further investigation was carried out using combinations of auxins with cytokinins. Whitish green calli was observed in the medium supplemented with 2.0 mg/L 2,4-D with 1.0 mg/L kinetin and calllogenesis was noticed on 18th day, producing an 76% callus response and resulted in good biomass yield and asiaticoside content. The application of 2.0 mg/L of 2,4-D with 1.0mg/L BA was poor in inducing the and resulted in less biomass yield and asiaticoside content.

A further screening of suitable growth regulators for C.asiatica callus induction was conducted through culturing of young leaf explants on the MS medium supplemented with the combination of 2.0 mg/L NAA and different cytokinins, particularly kinetin and BA. Highly proliferating whitish green calli was observed in the medium supplemented with 2.0 mg/L 2,4-D with 1.0 mg/L kinetin which induced the callus with in 14 days producing 84% callus response and resulted in high biomass yield and asiaticoside content where as the application of 2.0 mg/L of NAA with 1.0mg/L BA was not suitable for callus induction in terms of callus initiation time, growth, biomass yield and asiaticoside content.

**CONCLUSIONS**

Tissue culture based methods are viewed as an alternative strategies for the production and optimization of bioactive compounds. The manipulation of nutritional requirements and supplementation of different concentrations of phytoregulators and elicitors known to have stimulating effect on production of bioactive compounds. Although there were few reports on C.asiatica roots13,14, there is little is known about callus induction and asiaticoside content in the leaf cultures under different nutritional and phytohormone concentrations. In the present study we could achieve the highest biomass yield and asiaticoside content in the callus cultures from young leaf explants on MS medium with NAA and kinetin. This approach may help for the production of asiaticoside on large scale for pharmaceutical applications and increasing marketing demands.

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REFERENCES