➔DOI : 10.15740/HAS/AJBS/9.2/178-187
e ISSN-0976-8343 | ■ Visit us : *www.researchjournal.co.in*

Asian Journal of Bio Science, Volume **9** | Issue 2 | October, 2014 | 178-187
**Received :** 26.02.2014; **Revised :** 13.08.2014; **Accepted :** 26.08.2014

**R**ESEARCH **P**APER

# Helping hand of biotechnology in conservation of medicinally important plant (*Asparagus racemosus*)

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Increase in human population, urbanization, cutting trees and use of plants for pharmaceutical purpose leads to destruction of medicinal plant. Not only destruction but some of the species become threaten for their existence. One of them is *Asparagus racemosus* whose roots and its extracts widely used for the preparation of medicines by the pharmaceutical companies. That's why, conservation efforts have been required which can be achieved with the help of various plant biotechnological techniques such as suspension culture. Not only for the conservation, it provides an alternative way of producing large amount of active compound directly in to the laboratory. For the establishment of suspension culture techniques a prime requirement is to have a standardize protocol for callus culture. This paper represents optimization of protocol for callus culture and suspension culture from *Asparagus racemosus*. It has been proved that the plant hormones play vital role for callus induction. In this work MS Medium with different combination of auxin (NAA, 2, 4 D) and cytokinines (BAP, Zeatin, Kinetin) have been tried for callus induction. The best friable callus induction, highest callus induction rate and excellent callus growth were obtained in MS medium supplemented with 1.0mg/L ZEATIN + 0.1mg/L BAP. Same medium was then utilized for the establishment of suspension culture. In cell suspension culture of *A. racemosus*, the maximum growth (7.7 g/l) were observed in suspension culture initiated from leaf explant and the highest saponin production (0.580 mg/g) were obtained from the biomass of suspension culture initiated from root explant at 18 days after culture.

**Key words :** Callus culture, Suspension culture, Secondary metabolites

**How to cite this paper :** Sherathiy, H.M., Kothiya, R.V., Malviya, B.J. and Sorathiya, P.V. (2014). Helping hand of biotechnology in conservation of medicinally important plant (*Asparagus racemosus*). *Asian J. Bio. Sci.*, **9** (2) : 178-187.

## INTRODUCTION

Medicinal plants are the most important source of life saving drugs since time immemorial for the majority of the diseases of the World's population. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modelled on plant substances. Even today, the World Health Organization estimates that up to 80 per cent of people still rely mainly on traditional remedies such as herbs for their medicines. Most of the pharmaceutical industry is highly dependent on wild populations for the supply of raw materials for extraction of medicinally important compounds (Chattopadhyay *et al.*, 2002). The genetic diversity of medicinal plants in the world is getting endangered at an alarming rate because of ruinous harvesting practices and over-harvesting for production of medicines and also due to extensive destruction of the plant-rich habitat which results into forest degradation, agricultural encroachment and urbanization. Hence, there is a strong need for proactive understanding in the conservation, cultivation, and sustainable usage of important medicinal plant species for future use.

*Asparagus racemosus* is known to possess active constituents such as galactose, arabinose, steroidal glycosides and saponin which have potential application in preparation of various medicines (Sinha and Biswas, 2011). Increase use of medicinal plant for pharmaceutical preparation leads to destruction at very high rate as an outcome of this result in to a threatened condition of their existence. *Asparagus racemosus* is one of the threaten species of medicinal plants. It is estimated that in India more than 500 tons of asparagus roots are needed every year for various pharmaceuticals purpose.

Saponin and its derivatives are secondary metabolites associated with many health benefits that include its

phytoestrogenic properties. *Asparagus racemosus* naturally shows the presence of high amount of saponin especially in their root. We intend to establish the *in vitro* production system for saponin to provide an alternative way and to overcome the burden on natural resources to fulfill the demands. Callus and cell suspension cultures of *Asparagus racemosus* were established to investigate the production of saponin *in vitro*.

The objective of the present studies was to test different growth regulator formulations to establish callus and cell suspension cultures and to develop long term maintenance on large scale. The evidence showed that there was no work done on establishment of such cultures. And it is the first time establishment of cell suspension cultures on a large scale through tissue culture techniques in *Asparagus recemosus*.

## RESEARCH METHODOLOGY

The present study was carried out at during the work plant shoot and root both were used as an explant for the initiation of callus culture :

**Collection of plant material :**

Plants of *Asparagus racemosus* were collected from Junagadh Agricultural University, Junagadh, Gujarat and maintained at the campus of M. and N. Virani Science College, Rajkot, Gujarat. Leaf and root were collected as explants from this grown plant whenever required during the work. Plant shoot and root both were used as an explant for the initiation of callus culture.

**Callus culture**

*Surface sterilization of explants :*

The explants first washed with tap water, followed by treatment with 2 per cent liquid detergent tween-20 for 15 minutes. After washing with tap water, the explants were further surface sterilized with 5 per cent sodium hypochlorite and 70 per cent ethanol under the laminar air flow. Then the explants were treated with 0.1per cent $HgCl_2$ for 2 to 3 minutes followed by 3-4 wash of sterile distilled water to remove the traces of $HgCl_2$ solution.

*Media preparation for callus induction :*

For the induction of callus, leaves and root explant were cultured aseptically on MS basal medium supplemented with various concentration and combination of auxins like NAA, 2, 4, D and cytokinins like BAP, KIN, and zeatin. (Table A to F).

*Inoculation :*

After surface sterilization, the explants (leaf and root) were cut in to an appropriate size with the help of fine sterile stainless steel scalpel and forceps, and aseptically inoculated into a culture tube (25×150 mm) (Borosil, India) containing semi-solid MS medium supplemented with various hormone concentrations (Table A to F). All operations during inoculation including surface sterilization of explants were

**Table A : various concentrations of kinetin and NAA used for callus induction**

| NAA \ KIN | 0.1mg/L | 0.5mg/L | 1.0mg/L |
|---|---|---|---|
| 0.1 mg/L | $M_1$ | $M_2$ | $M_3$ |
| 0.5 mg/L | $M_4$ | $M_5$ | $M_6$ |
| 1.0 mg/L | $M_7$ | $M_8$ | $M_9$ |

**Table B : various concentrations of BAP and NAA used for callus induction.**

| NAA \ BAP | 0.1mg/L | 0.5mg/L | 1.0mg/L |
|---|---|---|---|
| 0.1 mg/L | $M_{10}$ | $M_{11}$ | $M_{12}$ |
| 0.5 mg/L | $M_{13}$ | $M_{14}$ | $M_{15}$ |
| 1.0 mg/L | $M_{16}$ | $M_{17}$ | $M_{18}$ |

**Table C : various concentration of zeatin with NAA used for callus induction**

| NAA \ ZEA | 0.1mg/L | 0.5mg/L | 1.0mg/L |
|---|---|---|---|
| 0.1 mg/L | $M_{37}$ | $M_{38}$ | $M_{39}$ |
| 0.5 mg/L | $M_{40}$ | $M_{41}$ | $M_{42}$ |
| 1.0 mg/L | $M_{43}$ | $M_{44}$ | $M_{45}$ |

**Table D : various concentration of 2, 4-D and BAP used for callus induction**

| 2,4,D \ BAP | 0.1mg/L | 0.5mg/L | 1.0mg/L |
|---|---|---|---|
| 0.1mg/L | $M_{19}$ | $M_{20}$ | $M_{21}$ |
| 0.5 mg/L | $M_{22}$ | $M_{23}$ | $M_{24}$ |
| 1.0 mg/L | $M_{25}$ | $M_{26}$ | $M_{27}$ |

**Table E : Various concentration of 2, 4-D and zeatin used for callus induction**

| 2, 4-D \ ZEA | 0.1mg/L | 0.5mg/L | 1.0mg/L |
|---|---|---|---|
| 0.1 mg/L | $M_{28}$ | $M_{29}$ | $M_{30}$ |
| 0.5 mg/L | $M_{31}$ | $M_{32}$ | $M_{33}$ |
| 1.0 mg/L | $M_{34}$ | $M_{35}$ | $M_{36}$ |

**Table F : Various concentrations of BAP and zeatin used for callus induction**

| BAP \ ZEA | 0.5mg/L | 1.0mg/L | 2.0mg/L |
|---|---|---|---|
| 1.0 mg/L | $M_{69}$ | $M_{70}$ | $M_{71}$ |
| 2.0 mg/L | $M_{72}$ | $M_{73}$ | $M_{74}$ |
| 3.0 mg/L | $M_{75}$ | $M_{76}$ | $M_{77}$ |

carried out inside a Laminar Air Flow cabinet.

*Incubation and maintenance of cultures :*

Leaves and root cultures were incubated in racks inside a culture room under controlled conditions of light and temperature. The cultures were exposed to continuous illuminations (4500 lux), cool white fluorescent lights, (Phillips, India) in a photoperiodic cycle of 16 hour dark and 8 hour light at 25±2°C. After an appropriate time period cultures were subculture on the same medium.

**Suspension culture :**

*Explant used* :

Friable callus produced from the callus culture of *A. racemosus* using leaf and root as an explant were utilized for the establishment of suspension culture.

*Initiation of suspension culture :*

Cell suspension cultures of *Asparagus racemosus* were initiated from callus tissue developed from root and leaf explants. One gram of friable green callus was excised aseptically. Then callus was slightly mashed and carefully transferred to 250ml glass bottle containing sterile 50ml liquid MS medium, supplemented with growth regulators at the same concentrations used to induce callogenesis (1.0mg/L ZEATIN + 0.1mg/L BAP). The pH of medium was adjusted to 5.8 before autoclaving the medium at 120ºC for 20 minutes. The bottles were agitated at 120 rpm on gyratory shakers and incubated at 25ºC under continuous low light (Behbahani *et al.*, 2011). After 15 days of incubation the medium needed to be replaced by fresh medium.

*Measurement of cell growth in suspension culture :*

Known volume of suspension culture was taken in a graduated centrifuge tube and tubes were centrifuge at 5000 rpm for 5 min. The volume of the pellet was recorded and packed cell volume (PCV) was calculated using following equation.

$$\text{PCV} = \frac{\text{Weight of the pellet}}{\text{Total weight of suspension}} \times 100$$

**Saponin analysis :**

*Saponin extraction :*

For extraction of saponin from natural roots, roots were dried and powdered. Such powdered (250 mg) were extracted with methanol by heating at boiling temperature. After filtration and evaporation to dryness, the residue was dissolved in 5 ml methanol and analysed by HPLC for detection and quantitation. Likewise saponin was also extracted from biomass of suspension culture by the same method. While supernatant of suspension was directly used for the HPLC analysis after filtration.

*HPLC analysis :*

20 µl of the extract was subjected to HPLC analysis on the Shimadzu LC-AT series, the mobile phase being acetonitrile:water, 35:65 at a flow rate of 1 ml/min (Bureau of Indian Standards BIS No. 14299-1995 and 14300-1995) with a reverse-phase analytical Chromega C-18 column (50 mm length × 4.6 mm diameter) with saponin retention time of 1.97 min. Saponin was detected at 215 nm by an SPD-10 AVP UV-VIS detector and quantified by comparing it with the peak of the external standard of saponin (95%, Sigma) and total saponin was calculated as the sum of intracellular and extracellular saponin accumulated.

## RESEARCH FINDINGS AND ANALYSIS

The finding of the study as well as relevant discussion have been summarized under following heads:

**Callus culture :**

Different concentration of NAA and KIN were tested for induction of callus in *Asparagus racemosus* in MS basal medium. Callus formation observed on leaf as well as root explants culture on MS medium supplemented with concentration 0.1mg/L KIN + 1.0mg/L NAA ($M_7$), 0.5mg/L KIN + 1.0mg/L NAA($M_8$) and 1.0mg/L KIN + 1.0mg/L NAA ($M_9$). Highest percentage of callusing (66%) and faster response was found with the $M_9$ hormonal combination in case of leaf explant and $M_7$ in case of root explant. The callus induction always priced by swelling of the explants. Callus produced by this concentration was white in colour, which later became brown (Table 1 and Fig. 1). Such callus was than subcultured on same medium for further growth and development.

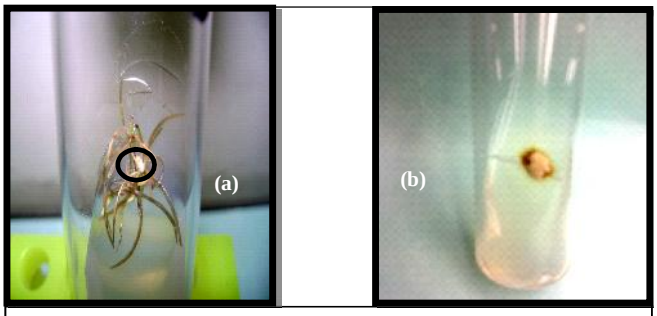



**Fig. 1 : (a) Callus induction from leaf explant on MS media supplemented with 1.0mg/L Kin and 1.0mg/LNAA (b) Callus induction from root explant on MS media supplemented with 0.1mg/L Kin and 1.0mg/LNAA**

In combination of BAP and NAA, the best callus induction rate (66%) was obtained in MS medium containing 0.5mg/L BAP + 0.1mg/L NAA ($M_{11}$) and 1.0mg/L BAP+0.1mg/L NAA($M_{12}$) with leaf explant. There was no difference in the percentage of explants producing callus (66%) among callus

induction observed. At the initial stage (7 to 10 days after incubation) of callus induction medium with BAP and NAA, the leaf explant swelled and friable callus formation was observed at three week (17 to 22 days) after incubation (Table 2 and Fig. 2). In case of root explant the callus induction was observed with $M_{13}$, $M_{14}$ and $M_{15}$ among which best in terms of rate of callus induction is $M_{13}$ (Table 2).

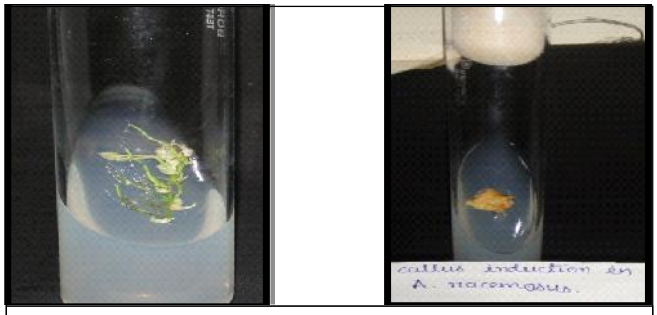


**Fig. 2 : (a) Callus induction from leaf explant on MS medium supplemented with 0.5mg/L BAP and 0.1mg/L NAA (b) callus induction from root explant on MS medium supplemented with 0.1mg/L BAP and 0.5mg/L NAA**

Different concentration of NAA and ZEATIN were tested for induction of callus in *Asparagus racemosus* in MS basal medium. Callus formation was observed on leaf as well as root explants on MS medium supplemented with $M_{43}$. While on $M_{44}$, $M_{45}$ callus formation was observed from root explant only and on $M_{37}$, $M_{38}$, $M_{39}$, $M_{40}$, $M_{41}$, $M_{42}$ only from leaf explant. Highest percentage of callusing (100%) was found with MS medium supplemented with $M_{42}$ in case of leaf explant and $M_{45}$ in case of root explant (Table 3 and Fig. 3). Callus generated from leaf and root explant shows the shoot formation as an outer growth of callus on long incubation (Fig. 3a).

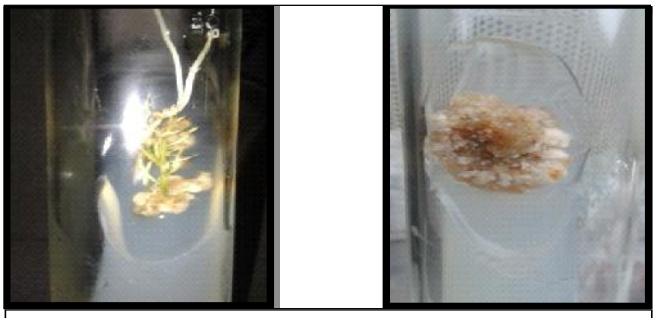


**Fig. 3 : (a) Callus induction from leaf explant on MS medium supplemented with 1.0mg/L Zeatin and 0.5mg/L NAA (b) callus induction from root explant on MS medium supplemented with 1.0mg/L Zeatin and 1.0mg/L NAA**

MS medium supplemented with different levels of 2, 4, - D and BAP were tried to induce callus, callus formation from leaf explants observed on almost each combination except $M_{20}$ and $M_{26}$. Best and fastest callus initiation was shown on MS medium supplemented with 1.0mg/L BAP +

**Table 1 : Effect of NAA and Kinetin for callus induction**

| Sr. No. | Concentration of growth hormones | Explant | Initiation of callus | % of callus | Outer growth |
|---|---|---|---|---|---|
| $M_1$ | 0.1mg/L KIN + 0.1mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_2$ | 0.5mg/L KIN + 0.1mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_3$ | 1.0mg/L KIN + 0.1mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_4$ | 0.1mg/L KIN + 0.5mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_5$ | 0.5mg/L KIN + 0.5mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_6$ | 1.0mg/L KIN + 0.5mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_7$ | 0.1mg/L KIN + 1.0mg/L NAA | Leaf | 24 days | 33% | NO |
| | | Root | 25 days | 66% | NO |
| $M_8$ | 0.5mg/L KIN + 1.0mg/L NAA | Leaf | 22 days | 66% | NO |
| | | Root | 30 days | 33% | NO |
| $M_9$ | 1.0mg/L KIN + 1.0mg/L NAA | Leaf | 16 days | 66% | NO |
| | | Root | 25 days | 33% | NO |

NO = Not Observed

**Table 2 : Effect of BAP and NAA for callus induction**

| Sr. No. | Concentration of growth hormone | Explant | Initiation of callus | % of callus | Outer growth |
|---|---|---|---|---|---|
| $M_{10}$ | 0.1mg/L BAP + 0.1mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_{11}$ | 0.5mg/L BAP + 0.1mg/L NAA | Leaf | 17 days | 66% | NO |
| | | Root | NO | - | - |
| $M_{12}$ | 1.0mg/L BAP + 0.1mg/L NAA | Leaf | 22 days | 66% | NO |
| | | Root | NO | - | - |
| $M_{13}$ | 0.1mg/L BAP + 0.5mg/L NAA | Leaf | NO | - | - |
| | | Root | 30 days | 66% | NO |
| $M_{14}$ | 0.5mg/L BAP + 0.5mg/L NAA | Leaf | NO | - | - |
| | | Root | 33 days | 33% | NO |
| $M_{15}$ | 1.0mg/L BAP + 0.5mg/L NAA | Leaf | NO | - | - |
| | | Root | 32 days | 33% | NO |
| $M_{16}$ | 0.1mg/L BAP + 1.0mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | - | - |
| $M_{17}$ | 0.5mg/L BAP + 1.0mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | - | - |
| $M_{18}$ | 1.0mg/L BAP + 1.0mg/L NAA | Leaf | NO | - | - |
| | | Root | NO | - | - |

NO= Not Observed

**Table 3 : Effect of Zeatin and NAA for callus induction**

| Sr. No. | Concentration of growth hormones | Explant | Initiation of callus | Per cent of callus | Outer growth |
|---|---|---|---|---|---|
| $M_{37}$ | 0.1mg/L ZEATIN + 0.1mg/L NAA | Leaf | 39 days | 66% | Shoot formation |
| | | Root | NO | - | - |
| $M_{38}$ | 0.5mg/L ZEATIN + 0.1mg/L NAA | Leaf | 41 days | 66% | Shoot formation |
| | | Root | NO | - | - |
| $M_{39}$ | 1.0mg/L ZEATIN + 0.1mg/L NAA | Leaf | 39 days | 66% | Shoot formation |
| | | Root | NO | - | - |
| $M_{40}$ | 0.1mg/L ZEATIN + 0.5mg/L NAA | Leaf | 39 days | 33% | Shoot formation |
| | | Root | NO | - | - |
| $M_{41}$ | 0.5mg/L ZEATIN + 0.5mg/L NAA | Leaf | 39 days | 66% | Shoot formation |
| | | Root | NO | - | - |
| $M_{42}$ | 1.0mg/L ZEATIN + 0.5mg/L NAA | Leaf | 39 days | 100% | Shoot formation |
| | | Root | NO | - | - |
| $M_{43}$ | 0.1mg/L ZEATIN + 1.0mg/L NAA | Leaf | 39 days | 33% | Shoot formation |
| | | Root | 47 days | 33% | NO |
| $M_{44}$ | 0.5mg/L ZEATIN + 1.0mg/L NAA | Leaf | NO | - | - |
| | | Root | 45 days | 66% | NO |
| $M_{45}$ | 1.0mg/L ZEATIN + 1.0mg/L NAA | Leaf | NO | - | - |
| | | Root | 42 days | 100% | NO |

NO = Not Observed

0.5mg/L 2, 4-D ($M_{24}$). In case of root explant callus formation was observed on $M_{19}$, $M_{20}$ and $M_{21}$ among which $M_{21}$ proven to be best as it given fastest response and highest callus induction percentage (100%).The callus initiated was initially white but turned to brown and shows the bud formation after long time incubation (Table 4 and Fig. 4).

**Fig. 4 : (a) Callus induction from leaf explant on MS media supplemented with 1.0mg/L BAP and 0.5mg/L 2,4,D (b) callus induction from root explant on MS media supplemented with 1.0mg/L BAP and 0.1mg/L 2,4,D**

Different concentration of 2, 4, D and ZEATIN were tested for induction of callus, Callus formation from leaf explant were observed on MS medium supplemented with 0.1mg/L ZEATIN + 1.0mg/L 2,4,D ($M_{31}$), 1.0mg/L ZEATIN + 0.5mg/L 2,4,D ($M_{33}$) and0.1mg/L ZEATIN + 1.0mg/L 2,4,D ($M_{34}$) after 30 days of incubation. Callus produced by this concentration was white in colour, which later became brown (Table 5 and Fig. 5). While no callus induction was observed on any of the treatment of this combination from root explant during the incubation time.

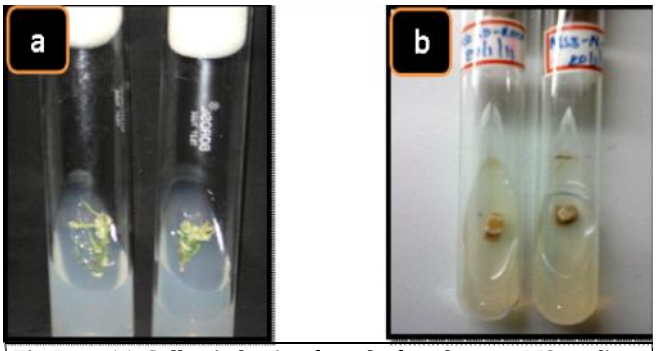



**Fig. 5 : (a) Callus induction from leaf explant on MS medium supplemented with 0.1mg/L Zeatin and 0.5mg/L 2,4-D (b) No callus induction from root explant on MS medium supplemented with 0.1mg/L Zeatin and 0.5mg/L 2,4-D**

Different concentration of ZEATIN and BAP were tested for induction of callus in *Asparagus racemosus* in MS basal medium. Callus formation observed within 3-4 weeks on leaf as well as root explants cultured on MS medium supplemented with $M_{69}$, $M_{71}$, $M_{72}$, While $M_{73}$ and $M_{74}$ induced

**Table 4 : Effect of BAP and 2, 4-D for callus induction**

| Sr. no. | Concentration of growth hormones | Explant | Initiation of callus | Per cent of callus | Outer growth |
|---|---|---|---|---|---|
| $M_{19}$ | 0.1mg/L BAP + 0.1mg/L 2,4,D | Leaf | 48 days | 66% | Shoot bud formation |
| | | Root | 55 days | 66% | Shoot bud formation |
| $M_{20}$ | 0.5mg/L BAP + 0.1mg/L 2,4,D | Leaf | NO | - | - |
| | | Root | 55 days | 100% | Shoot bud formation |
| $M_{21}$ | 1.0mg/L BAP + 0.1mg/L 2,4,D | Leaf | 50 days | 100% | Shoot bud formation |
| | | Root | 52 days | 100% | Shoot bud formation |
| $M_{22}$ | 0.1mg/L BAP + 0.5mg/L 2,4,D | Leaf | 45 days | 100% | Shoot bud formation |
| | | Root | NO | - | - |
| $M_{23}$ | 0.5mg/L BAP + 0.5mg/L 2,4,D | Leaf | NO | - | - |
| | | Root | NO | - | - |
| $M_{24}$ | 1.0mg/L BAP + 0.5mg/L 2,4,D | Leaf | 9 days | 100% | Shoot bud formation |
| | | Root | NO | | - |
| $M_{25}$ | 0.1mg/L BAP + 1.0mg/L 2,4,D | Leaf | 50 days | 100% | Shoot bud formation |
| | | Root | NO | | |
| $M_{26}$ | 0.5mg/L BAP + 1.0mg/L 2,4,D | Leaf | NO | - | - |
| | | Root | NO | - | - |
| $M_{27}$ | 1.0mg/L BAP + 1.0mg/L 2,4,D | Leaf | 50 days | 66% | - |
| | | Root | NO | - | - |

NO= Not Observed

the callus only from leaf explant (Table 5 and Fig. 6). Best and fastest callus initiation was shown on MS medium supplemented with $M_{72}$. The callus induction always priced by swelling of the explants. Callus produced by this concentration was friable and white in colour.

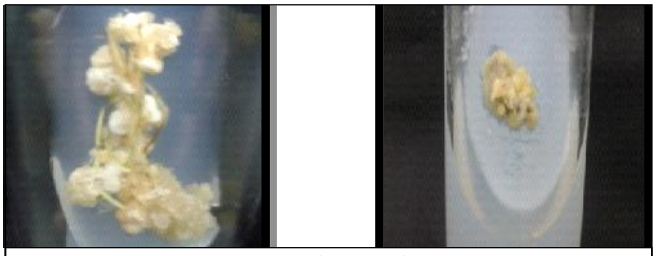


**Fig. 6 : (a) Callus induction from leaf explant on MS medium supplemented with 1.0mg/L Zeatin and 0.1mg/L BAP (b) callus induction from root explant on MS medium supplemented with 1.0mg/L Zeatin and 0.1mg/L BAP**

**Suspension culture :**

Suspension culture was carried out by using callus produced from leaf and root explants of *A. racemosus* and assessment of growth in culture was done by measuring the packed cell volume as shown in Table 7. The cell suspension

**Table 5 : Effect of Zeatin and 2, 4-D for callus induction**

| Sr. No. | Concentration of growth hormones | Explant | Initiation of callus | Per cent of callus | Outer growth |
|---|---|---|---|---|---|
| $M_{28}$ | 0.1mg/L ZEATIN + 0.1mg/L 2,4,D | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_{29}$ | 0.5mg/L ZEATIN + 0.1mg/L 2,4,D | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_{30}$ | 1.0mg/L ZEATIN + 0.1mg/L 2,4,D | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_{31}$ | 0.1mg/L ZEATIN + 0.5mg/L 2,4,D | Leaf | 30 days | 33% | NO |
| | | Root | NO | | |
| $M_{32}$ | 0.5mg/L ZEATIN + 0.5mg/L 2,4,D | Leaf | 33 days | 33% | NO |
| | | Root | NO | | |
| $M_{33}$ | 1.0mg/L ZEATIN + 0.5mg/L 2,4,D | Leaf | 30 days | 33% | NO |
| | | Root | NO | | |
| $M_{34}$ | 0.1mg/L ZEATIN + 1.0mg/L 2,4,D | Leaf | 30 days | 33% | NO |
| | | Root | NO | | |
| $M_{35}$ | 0.5mg/L ZEATIN + 1.0mg/L 2,4,D | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_{36}$ | 1.0mg/L ZEATIN + 1.0mg/L 2,4,D | Leaf | NO | - | - |
| | | Root | NO | | |

NO=Not Observed

**Table 6 : Effect of Zeatin and BAP for callus induction**

| Sr. No. | Concentration of growth hormones | Explant | Initiation of callus | % of callus | Outer growth |
|---|---|---|---|---|---|
| $M_{69}$ | 0.1mg/L ZEATIN + 0.1mg/L BAP | Leaf | 30 days | 33% | NO |
| | | Root | 35 days | 66% | NO |
| $M_{71}$ | 0.5mg/L ZEATIN + 0.1mg/L BAP | Leaf | 28 days | 66% | NO |
| | | Root | 32 days | 100% | NO |
| $M_{72}$ | 1.0mg/L ZEATIN + 0.1mg/L BAP | Leaf | 22 days | 100% | NO |
| | | Root | 30 days | 100% | NO |
| $M_{73}$ | 0.1mg/L ZEATIN + 0.5mg/L BAP | Leaf | 25 days | 100% | NO |
| | | Root | NO | | |
| $M_{74}$ | 0.5mg/L ZEATIN + 0.5mg/L BAP | Leaf | 25 days | 80% | NO |
| | | Root | NO | | |
| $M_{75}$ | 1.0mg/L ZEATIN + 0.5mg/L BAP | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_{76}$ | 0.1mg/L ZEATIN + 1.0mg/L BAP | Leaf | NO | - | - |
| | | Root | NO | | |
| $M_{77}$ | 0.5mg/L ZEATIN + 1.0mg/L BAP | Leaf | NO | - | - |
| | | Root | NO | | |

NO= Not Observed

**Table 7 : PCV of suspension culture**

| Sr. No. | Days | Packed cell volume (PCV) from leaf | Packed cell volume (PCV) from root |
|---|---|---|---|
| 1. | 0 | 0.99 | 0.1 |
| 2. | 3 | 1.25 | 1.22 |
| 3. | 6 | 1.40 | 1.35 |
| 4. | 9 | 1.70 | 1.60 |
| 5. | 12 | 2.60 | 2.40 |
| 6. | 15 | 4.0 | 3.45 |
| 7. | 18 | 4.20 | 3.8 |
| 8. | 21 | 4.25 | 3.9 |

cultures depict a typical sigmoid curve with an initial lag phase, followed by an exponential phase (extending up to 4 weeks), terminating in a stationary phase characterized by no cell growth but increased secondary metabolite accumulation (extending up to 4 weeks) Fig. 7.

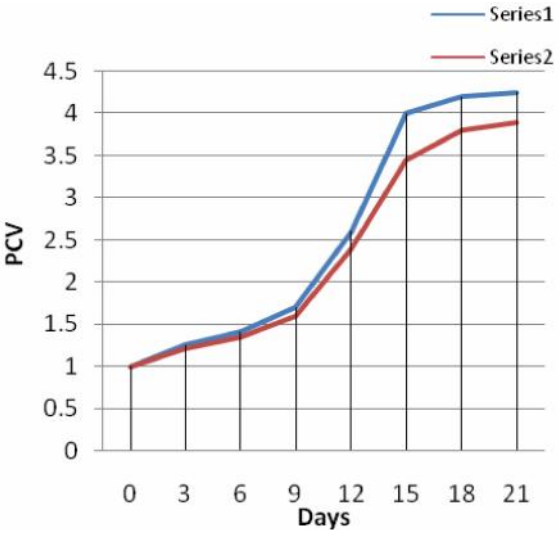
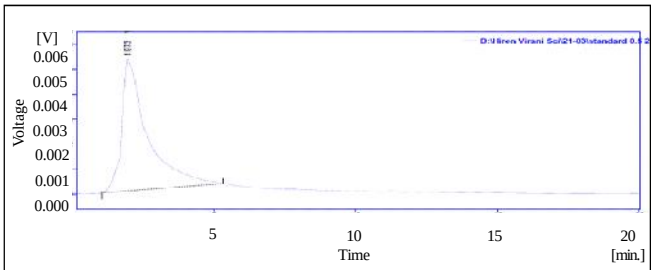



**Fig. 7 : Curve of suspension culture with packed cell volume versus time**

## Saponin analysis :

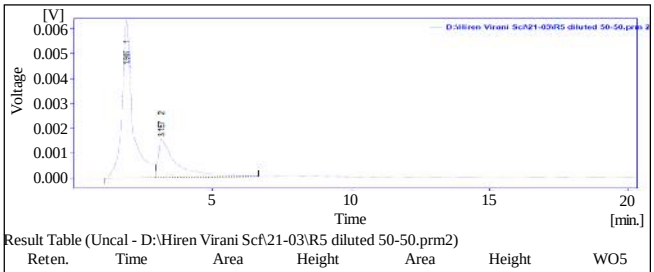
The identification of saponin was done by comparing its retention time and UV spectrum with the standard saponin. The standard of saponin was purchased from Sigma. The saponin concentration was estimated by interpolation of the peak area with a calibration curve constructed for standard saponin. Saponin content of the analyzed samples was expressed as mg $g^{-1}$ of dry weight as shown in Table 8. The presented results are the means of three replicates. Data of the HPLC analysis are shown in image 8 to 10. In suspension culture established from leaf callus of *A.resemosus*, the content of saponin within the culture medium (extracellular) were 0.170± 0.35 mg $ml^{-1}$ culture supernatant and within the cell biomass were 0.545± 0.14 mg $g^{-1}$ culture supernatant and in suspension culture established from root callus of *A.resemosus*, the content of saponin within the culture medium (extracellular) were 0.175± 0.11mg $ml^{-1}$ culture supernatant and within the cell biomass were 0.580± 0.20mg $g^{-1}$ culture supernatant. Saponin content [1.22± 0.150 mg $g^{-1}$ dry wt.] of saponin was observed within natural root extract by HPLC.




Result Table (Uncal - D:\Hiren Virani Scf\21-03\standard 0.5 2)

| Reten. | Time [min] | Area [mV.s] | Height [mV] | Area [%] | Height [%] | WO5 [min] |
|---|---|---|---|---|---|---|
| 1 | 1.973 | 672.962 | 10.580 | 100.0 | 100.0 | 0.74 |
| Total | | 672.962 | 10.580 | 100.0 | 100.0 | |

**Fig. 8 : Chromatogram for standard saponin**



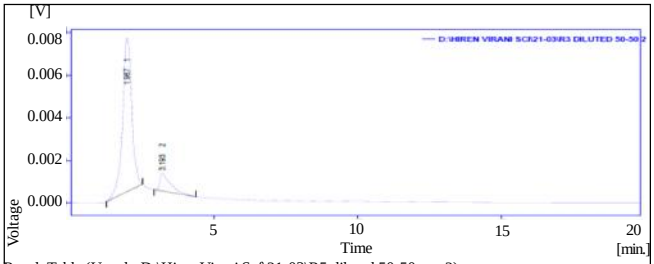

Result Table (Uncal - D:\Hiren Virani Scf\21-03\R5 diluted 50-50.prm2)

| Reten. | Time [min] | Area [mV.s] | Height [mV] | Area [%] | Height [%] | WO5 [min] |
|---|---|---|---|---|---|---|
| 1 | 1.907 | 3432.121 | 125.869 | 71.3 | 80.4 | 0.30 |
| 2 | 3.157 | 1382.046 | 30.780 | 28.7 | 19.6 | 0.49 |
| Total | | 4814.167 | 156.649 | 100.0 | 100.0 | |

**Fig. 9 : Chromatogram for natural root extract**

**Table 8 : Comparative study for of naturally produced saponin with saponin produced from suspension culture**

| Sr. No. | Source | | Concentration of saponin through HPLC analysis (mg/g or mg/ml) |
|---|---|---|---|
| 1. | Natural root | | 1.22± 0.150 |
| 2. | Suspension culture from leaf callus | Culture medium | 0.170± 0.35 |
| | | Biomass extract | 0.545± 0.14 |
| 3. | Suspension culture from root callus | Culture medium | 0.175± 0.11 |
| | | Biomass extract | 0.580± 0.20 |




Result Table (Uncal - D:\Hiren Virani Scf\21-03\R5 diluted 50-50.prm2)

| Reten. | Time | Area | Height | Area | Height | WO5 |
|---|---|---|---|---|---|---|
| | [min] | [mV.s] | [mV] | [%] | [%] | [min] |
| 1 | 1.967 | 3226.370 | 144.474 | 55.4 | 59.5 | 0.32 |
| 2 | 3.193 | 425.235 | 16.442 | 11.6 | 10.2 | 0.34 |
| Total | | 3651.609 | 160.915 | 100.0 | 100.0 | |

**Fig. 10 : Chromatogram of suspension culture cell biomass extract**

**Callus culture :**

NAA was tried with Kin, BAP and Zeatin for callus induction showed which more effective response when tried with zeatin. It gave faster response when tried with KIN and BAP but the percentage of callusing were poor in both the combination. While when tried with zeatin it gave high percentage of callusing but taken more time for induction of callus, also it showed formation of shoot from callus when incubated for long period of time. No callus was formed during the whole culture period at the lowest concentration (0.1, 0.5mg/l) (Table A) of NAA when tried with Kin in both root and leaf explant. NAA when tried with BAP, it's more effective in lower concentration (0.1 mg/l) for callus induction from leaf induction from root explant. While in case of root explant and at 0.5mg/l concentration for callus explant, NAA was only effective in higher concentration (1.0 mg/l) when tried with zeatin. It is observed that induction of callus required more time from root as compared to leaf in each combination.

2, 4-D were tried with BAP and zeatin for the induction of callus from leaf as well as root, it was more effective with BAP when percentage of callusing were compared. It also shows shoot bud formation with BAP when grown on medium for long period of time without sub culturing. And when tried with zeatin, no callus induction was observed from root explant at any concentration and at the lower concentration (0.1mg/l) of 2, 4-D from leaf explant. And with leaf explant percentage of callusing were poor but time required for callus induction was less as compared to previous combination. Surprisingly one of the combinations with BAP ($M_{24}$) has given very fast response for callus induction from leaf irrespective of other combination.

When BAP were tried with zeatin surprisingly this combination has proven to be the best for the callus induction from both root as well as leaf explant. Growth rate of callus was fastest and percentage of callusing highest at lower concentration of BAP of this combination then the other combination tried. This might be due to endogenous presence of auxin as BAP and Zeatin being a cytokinin is proven to be very good callus inducer. Callus produced by this concentration was white in color and friable as compared to other which was further utilized for the establishment of suspension culture.

**Suspension culture :**

Suspension cultures initiated from leaf explant were grown more faster and accumulated a greater biomass in media containing 1.0mg/L ZEATIN + 0.1mg/L BAP over a period of 30 days as compare to those initiated from root explant Table 7. For maintenance, suspension culture is necessary to subculture them because the cultures tended to form cell clusters of a few cells to aggregates. Cells grown in suspension culture were entered in exponential phase after a lag phase of a week (8 days) with a 3 fold increase in PCV. After the log phase cells were remained in stationary phase till the observation period. The colour of suspensionculture was also turned into yellowish. Both the suspension culture was checked for the presence of secondary metabolite, (saponin) by HPLC (*i.e.* the qualitative determination), which shows the positive result.

**Saponin analysis :**

HPLC analysis had shown that though the growth rate of suspension culture initiated from leaf induced callus was high, but the amount of saponin was more in case of suspension culture initiated from root induced callus (Table 8). The above results prove that *in vitro* cultures can be used as an alternative source for saponin. However, further work need to be done on establishment of suspension cultures, secretion of saponin into liquid media and evaluation of bioactivities to validate the protocol for scale up studies.

**Conclusion :**

The production of secondary metabolites from the cell suspension culture system is one of the most promising methods in the medicinal industry. Not only do the cells from the callus or suspension grow faster, but the products produced can be extracted more easily than from the cells of the intact plants, and the quantities of the product produced in some plants species are much higher than those of the field-grown plants. A cell suspension culture could be a more suitable alternative for the pharmaceutical industry for obtaining the important bioactive compounds. Tissue and cell suspension culture techniques have made industrial production of some natural products possible. Use of cell suspension cultures as metabolic factories, instead of whole plants, offers an alternative way for the production of saponin without the destruction of natural plants. To reach the aim of obtaining yields in concentrations high enough for commercial exploitation, there will be a need of further work regarding optimizing the cultural conditions, selection of high-producing strains, precursor feeding, transformation methods, and immobilization techniques. Also the availability of optimize

protocol for basic techniques like callus culture also help full to develop other tissue culture techniques such as plant regeneration, hairy root culture and micropropagation, which serves as an important tool to full fill the various scientific need for the betterment of environment and human in future.

**Acknowledgement :**

The author thanks to Dr. Shivani Patel, head of the Department of Biotechnology, Shree Manibhai and NavalbenVirani Science College, Rajkot, India for providing guidance and laboratory.

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