

## INDUCTION OF CALLUS FROM DIFFERENT EXPLANTS OF *Bacopa monnieri* AND EFFECT OF ADJUVANT ON THE GROWTH RATE OF THE CALLI

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### ABSTRACT

Present work was carried out to observe the impact of growth regulators the auxin and cytokinin to observe their combined impact on callus induction and these calli were subcultured in the presence of adjuvant, here coconut milk to observe its impact on the growth rate of the calli. During the work it was noted that all the explants such as young leaf, mature leaf, and the internodes responded to different concentrations of 2,4-D and Kinetin, but highest percentage of response for swelling and callus induction was noted in the mature leaf explants when it was inoculated in MS + 1.5 mg/l 2,4-D + 0.4 mg/l Kinetin that was 94.68 and 90.34 respectively. This was followed by the internodal explants in the same medium and the percentage of response was 88.72 for swelling and 78.50 for callus induction. Here minimum response was in young leaf explants for swelling 78.25 and for callusing 73.46 respectively. Similar trends of response were noted in case of all the explants cultured in the medium supplemented with 1.0 mg/l 2,4-D + 3 mg/l Kinetin. Callus raised from mature leaf in MS + 1.5 mg/l 2,4-D + 0.4 mg/l Kinetin, was subcultured in the same medium with coconut milk as adjuvant, in the ratio of 0.5%, 1.0% and 1.5% which was 5ml/l, 10ml/l and 15ml/l. After three weeks the growth rate & biomass were calculated. It was observed that MS + 1.5 mg/l 2,4-D + 0.4 mg/l Kinetin and 1.5% coconut milk promoted better growth as was noted from the diameter and fresh weight of the calli in subculture. Here, the growth response, growth rate and weight of the calli were lowest in the medium containing 0.5% coconut milk.

**KEYWORDS:** *Bacopa monnieri*, Adjuvant, Coconut Milk, Sub Culture, Swelling, Explants, Diameter, Fresh Weight

**ABBREVIATIONS:** 2,4-D = 2, 4- Dichlorophenoxy Acetic Acid; KN = Kinetin; CM = Coconut Milk

*Bacopa monnieri* (L.) Pennel, commonly called as 'Brahmi' in local language, belong to the family *Serophulariaceae*. At Muzaffarpur plants are found from late June to October in wild population. They are generally found in moist shady places and due to their vigorous branching they form a mat like structure on the ground. They may grow in submerged conditions and the among the green leaves white flowers may attract anybody towards it. In addition to normal tap roots, several adventitious roots may be found at each node which is in touch of soil.

*Bacopa monnieri* (L.) Pennel, was placed second in priority list of the most important medicinal plants evaluated on the basis of their medicinal importance, commercial value and potential for further research and development (Shrivastva *et al.*, 1999). Tripathi *et al.*; (1996) reported that it acts as an antioxidant that stimulates brain circulation and also enhances the serotonin level in disorders, reducing stress. Mehta, (2017) reported that it is also used to treat asthma, insanity, epilepsy, hoarseness, enlargement of spleen, against snake bite, to cure rheumatism, leprosy, eczema and other skin diseases. It has been used as brain tonic in Ayurvedic system of medicine to improve memory, concentration, and learning as well as to cure mental illness (Srivastva *et al.*, 1999). Presence of

most important Saponins such as Bacoside A, B, C, and D in *Bacopa monnieri* has been reported by Majumdar *et al.*, (2011), Ahire *et al.*, (2013). Here Bacoside A is being used for the production of memory enhancing capsule. Ali *et al.*, (2001), Shukla *et al.*, (2007) reported that *Bacopa monnieri* is being used in phytoremediation of Heavy metal such as Cadmium, Mercury & Chromium.

Tissue culture studies for micropropagation and callus regeneration have been done by Arumugam and Bhojwani (1990), Rani and Grover (1999), Martin (2004), Tsay Hsin-Sheng and Agarwal, (2005), Wood Ward and Bartel (2005), Sakakibara (2006), Bahadur *et al.*, (2007), Chaturvedi *et al.*, (2007), Monga *et al.*, (2008), Wang *et al.*, (2008), Siva *et al.*, (2009), Dash and Mandal (2010), Joshi *et al.*, (2010), Juan *et al.*, (2010), Showkat *et al.*, (2010), Ahmad *et al.*, (2011), Madhulkar *et al.*, (2011), Rao *et al.*, (2011), Rout *et al.*, (2011), Subashri and Kolipillai (2012), Jayakumar and Lingam (2013), Kalsaitkar *et al.*, (2014), Kaur *et al.*, (2013), Osman *et al.*, (2013), Sundriyal *et al.*, (2013), Kumari Ritika *et al.*, (2014), Subhashri and Pillai (2014), Thangavel *et al.*, (2014), Anita (2017), Aradkani *et al.*, (2017), Abiri *et al.*, (2017), Khattak *et al.*, (2017), Rajeev *et al.*, (2018) and Srivastva *et al.*, (2017). In the

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present work impact of plant growth regulators & adjuvant on the growth of callus have been studied.

## MATERIALS AND METHODS

The material used and methods followed to carry out the experiments have been described below.

### Location for the Plants

*Bacopa monnieri* (L.) was located in the campus of University Department of Botany, B.R. Ambedkar Bihar University, which was growing in wild habitat. The place was marked and unwanted weeds were removed from the surrounding of the plant which was present in a small area.

### Preparation of the Explants

Healthy branches from the plant of *Bacopa monnieri* were carefully excised with the help of pre-sterilized sharp blade. This was placed in a beaker containing distilled water and brought to the laboratory. Branches were taken out from the beaker and were placed in a wide mouth 2 L conical flask. The mouth was covered with the muslin cloth with the help of rubber bands. It was placed under running tap water. The materials were circulating within the flask but due to muslin cloth they were not coming out along with outgoing water current. This was done for 40 minutes so that the dust particles were washed away.

Above plant materials were taken out in a pre-sterilized Petri plate, placed in the aseptic condition of Laminar air flow cabinet. Here they were treated with 0.1%  $\text{HgCl}_2$  for two minutes in another conical flask. During this the flask was manually shaken vigorously. Materials were transferred in another conical flask containing sterilized distilled water. This was done thrice by changing the water. Shaking was done during this to remove even the trace of the adhering chemical.

The mature leaves, young leaves and internodal segments were cut with the help of pre-sterilized sharp blade. They were stored at low temperature wrapped in pre-sterilized and moistened cloth.

### Preparation of Culture Media

All the chemicals, plant growth regulators, used in these experiments were purchased from the suppliers of Hi-Media products. The basal medium was used as formulated by Murashige and Skoog (1962) desired amounts of

inorganic, micro and macro salts, the iron, and organic compounds were taken from the stock solutions, prepared earlier.

Desired concentrations of Auxin (2,4-D) and Cytokinin (Kinetin) here, 2,4-D, 0.5, 1.0 and 1.5 mg/l and Kinetin 0.2, 0.3 and 0.4 mg/l were added in the medium in three different sets. The agar 0.8% was used as gelling agent. The pH was adjusted to 5.8, before autoclaving, 30 ml above culture medium was dispensed in 250 ml culture flask each. They were plugged with cotton plugs and wrapped with Aluminum foil to avoid wetting of the plugs during autoclaving. Autoclaving was done at 15 lb pressure for 18-20 minutes. The temperature was expected to be  $121^{\circ}\text{C}$  at this pressure. All the culture flasks along with culture medium were allowed to cool at room temperature and then stored at low temperatures. They were used for inoculation after 3 days. Cultures showing any contamination were discarded after autoclaving.

### Inoculation of the Explants

Actually media were prepared before the explants were collected and sterilized for inoculation. All the tools to be used in inoculation such as forceps, scalpel, blade, scissor beakers and Petri plates were also autoclaved prior to their use. Inoculation was done in the aseptic conditions of Laminar air flow cabinet. 70% ethanol was used for sterilization of forceps and needles after each inoculation. Before removing of the plug from the culture flask it was heated above the spirit lamp and the leaf cut into small segment, the internodal segment were carefully placed on the surface of the culture and the mouth was properly plugged. Each experiment was repeated thrice, and each time there were 20 cultures. The inoculated culture flasks were incubated in the culture room where the temperature was controlled at  $26\pm 1^{\circ}\text{C}$ , relative humidity at 63-68% and the light at 3000 lux. Cultures were observed on an alternate day, flasks showing contamination were taken out and discarded after autoclaving.

The day after which callus was initiated, percentage of explants responding for it, colour of the calli etc. were noted. Explants showing swelling was also noted and percentage of response was calculated. The data obtained for different treatments have been placed in the tables 1-3.

Impact of coconut milk on biomass production of callus in subculture was also studied. Fresh coconut milk was extracted from green fruits. This was deproteinated after boiling. It was filtered through the muslin cloth and filtrate was stored at low temperature. Subculture of calli was done in the same medium in which better growth was obtained. Here, the coconut milk was added at 0.5%, 1.0% and 1.5%. The period of incubation was fixed that was 3 weeks. The colour of the callus, its diameter, weight of calli and percentage of response was calculated.

The data obtained were placed in table 4. Callus with coconut milk was used as control. 200 mg fresh callus was inoculated in culture medium without coconut milk was taken as control.

## RESULTS AND DISCUSSION

Data collected on the basis of experiments and observations have been presented in tables 1-4 and graph 1 & 2. From the table it may be noted that among the explants, mature leaf was found more responsive with respect to callus induction. When inoculated in MS + 0.5

mg/l 2,4-D + 0.2 mg/l Kinetin the percentage of response was 68.25, followed by the internodal explants that was 62.72%. Lowest percentage of response was observed in young leaf explants which was 52.38% only. This pattern of response for callusing was also found in case of cultures in MS + 1.0 mg/l 2,4-D + 0.3 mg/l Kinetin. Here the percentage of response in the adult leaf explants was 73.65% followed by internodal explants 68.54% and young leaf explants 64.32%. Maximum percentage of response for callus induction among the mature leaf explants was also observed in MS + 1.5 mg/l 2,4-D + 0.4 mg/l Kinetin which was 90.34% followed by internodal explants 78.50% and young leaves 72.46% respectively. It may be noted from the table that most suitable concentrations of plants growth regulators for the induction of callus in three different explants was MS + 1.5 mg/l 2,4-D + 0.4 mg/l Kinetin, followed by MS + 1.0 mg/l 2,4-D + 0.3 mg/l Kinetin. Lowest percentage of response with respect to callus induction was noted in case of MS + 0.5 mg/l 2,4-D + 0.2 mg/l Kinetin. Time taken for initiation of callus was the maximum 14 days for young leaf used as explants, in the medium where lowest percentage of response was noted.

**Tables 1: Medium – MS + 0.5 mg/l 2,4-D + 0.2 mg/l Kinetin**

Explants	% Swelling	% of Callus induction	Time taken	Colour of the callus
Young leaf	66.54	52.38	14 days	Cream white
Matured leaf	74.38	68.25	12 days	Greenish
Internode	71.65	62.72	12 days	Yellow green

**Tables 2: Medium – MS + 1.0 mg/l 2,4-D + 0.3 mg/l Kinetin**

Explants	% Swelling	% of Callus induction	Time taken	Colour of the callus
Young leaf	68.75	64.32	13 days	Dull white
Matured leaf	79.45	73.65	11 days	Green white
Internode	72.80	68.54	11 days	Yellow white

**Tables 3: Medium – MS + 1.5 mg/l 2,4-D + 0.4 mg/l Kinetin**

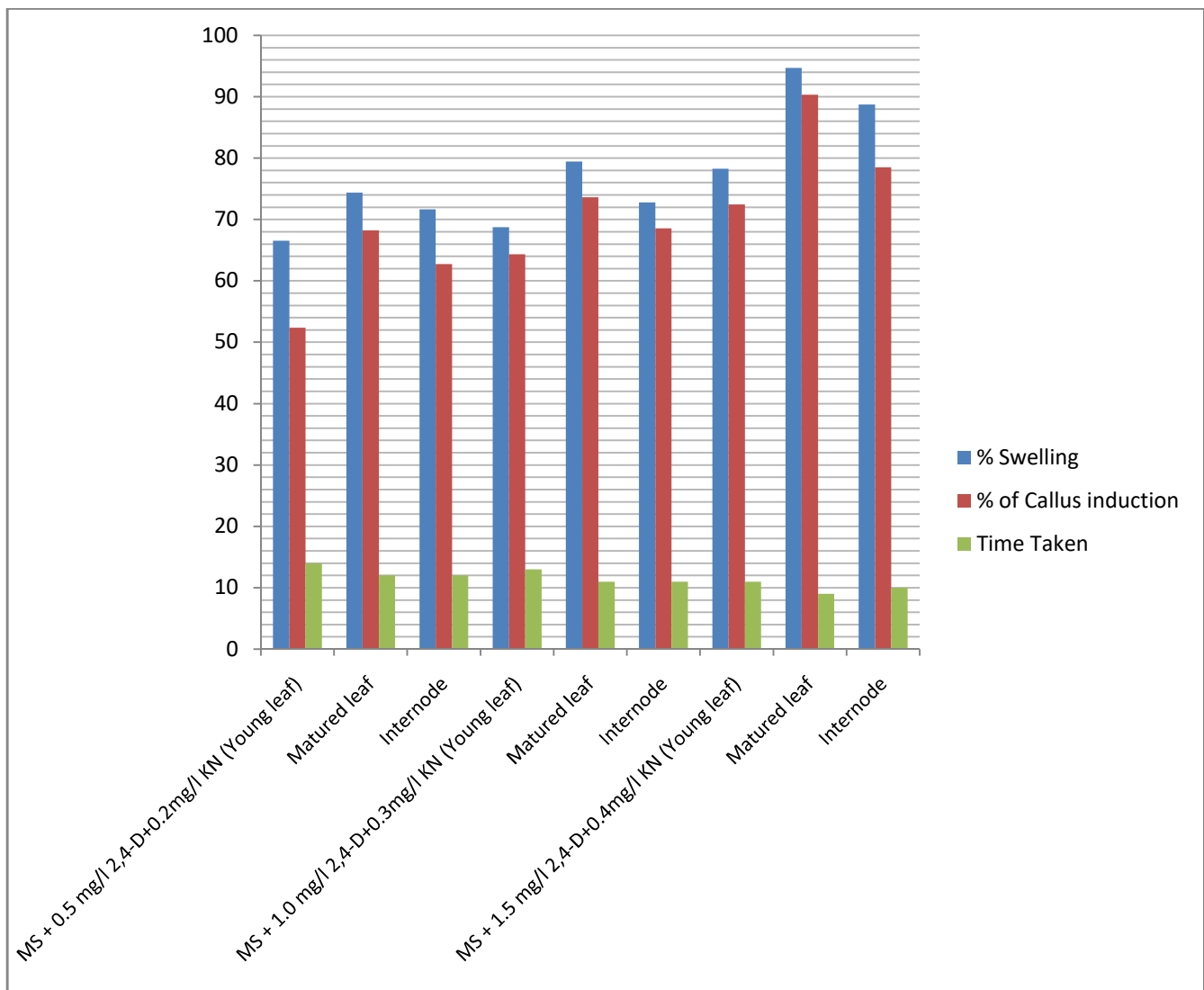
Explants	% Swelling	% of Callus induction	Time taken	Colour of the callus
Young leaf	78.25	72.46	11 days	white
Matured leaf	94.68	90.34	9 days	Green white
Internode	88.72	78.50	10 days	Yellow white

**Tables 4: Medium – MS + 1.5 mg/l 2,4-D + 0.4 mg/l Kinetin**

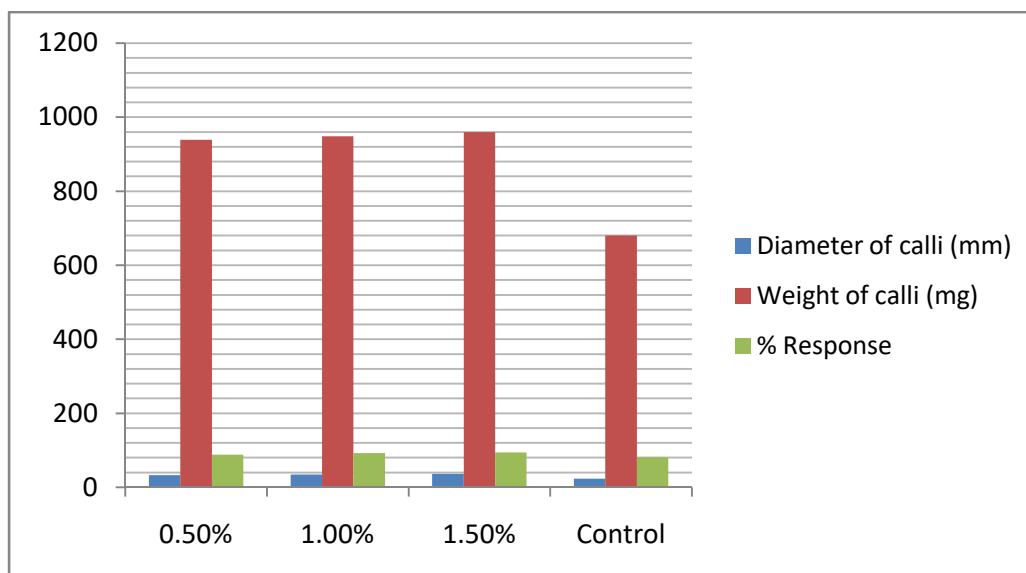
Coconut Milk	Period of incubation 3 weeks (Colour)	Diameter of calli (mm)	Weight of the calli (mg)	% Response
+ 0.5%	Greenish	32.45	938.74	88.25
+ 1.0%	Dark Green	34.80	948.65	92.54
+ 1.5%	Dark Green	36.38	959.72	94.30
Control	Green White	23.72	680.54	81.40

Initial weight of callus inoculated was 200 mg. Data are mean of three replicates, each containing 20 cultures.

**Graph 1: Showing % Swelling, % of Callus induction & time taken with different explants at different concentrations of 2, 4-D and KN supplemented with MS Medium for callus induction**



**Graph 2: Showing diameter of calli, weight of calli and % response in culture supplemented with different concentration of Coconut milk.**



Similarly, minimum periods taken for callus initiation was taken by mature leaf explants that was 9 days in the medium where maximum response was found. This was followed by the internodal explants that were 10 days. Young leaf explants took 11 days for initiation in the medium.

Experiments were also done to evaluate different concentrations of coconut milk on growth and fresh weight of the callus. Data were presented in the table 4. From the table it was clear that MS + 1.5 mg/l 2,4-D + 0.4 mg/l Kinetin + 1.5 Coconut milk, induced maximum growth where the diameter was 36.38 mm, the fresh weight was 959.72 mg and the response was 93.30% after three weeks of inoculation of similar amount of callus in the control as well as in 1.0% and 0.5% coconut milk.

This was followed by MS + 1.5 mg/l + 0.4 mg/l Kinetin, where the diameter was 34.80 and in 0.5% Coconut milk it was only 32.45. The fresh weight was 948.65 mg and 938.74 mg respectively. The percentage of response was 92.54 and 88.25%. From the table it was observed that in the same medium without coconut milk the growth was 23.72 mm, the weight was 680.54 mg and the response was 81.40%.

## DISCUSSION

In the present work, it was found that mature leaf explants revealed highest frequency of callus induction than rest of the two explants that were young leaves and the internodes. Among different concentrations of 2, 4-D at 1.5 mg/l and 0.4 mg/l Kinetin gave better results than that of the other concentrations. Woodward and Bartel, 2005; Sakakibara, 2006; reported that auxins and cytokinins in the culture medium regulate various aspects of plant cell dedifferentiation and differentiation. Generally, auxins are used for callus induction and proliferation and both auxins and cytokinins are required for redifferentiation of callus into organized cell (Wang *et al.*, 2008).

Callus induction has been reported by different workers in different medicinal plants. Sharath *et al.*, (2007) reported that 2,4-D at different concentrations induced callus in leaf explants of *Bacopa monnieri*. They found that both the lower 0.25 mg/l and the higher 2.0 mg/l concentrations had lower effect on callogenesis. Das and Mandal (2010) observed enhanced rate of callus induction in *Stevia rebaudia* Bert in the MS medium supplemented with 2.0 mg/l 2,4-D + 0.5 mg/l BAP.

Rout *et al.*, (2011) induced callus in leaf explants of *Bacopa monnieri* in MS + 2.0 mg/l BAP alone and MS + 2.0 mg/l BAP + 1.0 mg/l NAA. Similar result was obtained with MS + 1.5 mg/l Kinetin+ NAA. All these findings are

in agreement with the findings of present work as also a combination of 2,4-D + Kinetin gave better result with respect to callus induction in leaf explants when cultured in MS medium along with the above plant growth regulators.

Osman *et al.*, (2013) used MS + 1.0 mg/l 2,4-D + 0.3 mg/l BAP and found 100% callus induction in the leaf explants of *Lycium barbatum* L., an important herb used in traditional Chinese medicine. Kalsaitkar *et al.*, (2014) induced callus in leaf explants of *Bacopa monnieri* in MS + 2,4-D + Kinetin, at different concentration. Panathulu *et al.*, (2014) found maximum induction of callus in leaf explants of *Centella asiatica* in MS + 2.0 mg/l 2,4-D + 0.5 mg/l BAP.

Priya Dharishini *et al.*, (2014) found that even 0.4 mg/l 2,4-D when supplemented in MS medium could induce callus in leaf explants of *Bacopa monnieri*. Thangavel *et al.*, (2014) reported that when leaf explants of *Decalepis hamiltonii* was cultured in MS + 10.0 µM 2,4-D, there was maximum callus induction. Mehta (2017) reported that MS + 1.5 mg/l 2,4-D, gave better result with respect to callus induction in leaf explants of *Bacopa monnieri* L. In the present work also leaf explants when cultured in MS + 1.5 mg/l 2,4-D + 0.4 mg/l Kinetin gave better response with respect to callus induction as here the highest percentage of explants induce calli and the time taken for callus induction was also the minimum. Therefore, findings of the present work corroborate with the findings of the above workers.

For higher biomass production of callus, different adjuvant has been used by different workers. Siva *et al.*, (2009) used different percentage of coconut milk in medium containing plant growth regulators that induce callus in *Oldenlandia umbellata* leaf explants. They concluded that biomass of calli increased with higher growth rate than that of the calli cultured in control. The weight of calli varied according to the concentration of coconut milk supplemented in the medium. Findings of the present work are with agreement of the aforesaid findings as here also the biomass varied along with the concentration of the coconut milk, added in the medium.

Das and Mandal (2010) reported that if amino acids are supplemented in the medium, used for sub culture of calli, the rate of proliferation of the calli used for sub culture. They noted that among the amino acids used

glutamine supplemented at the rate of 50 mg/l gave best result.

Here the percentage of response was 88.09 and the weight increased considerably than that of the control. These workers also used casein hydrolysate as an adjuvant to observe its effect on biomass production of calli, cultured in the suspension culture.

Thangavel *et al.*, (2014) subcultured the calli raised from leaf disc of *Bacopa monnieri* L. in MS medium supplemented with 6 µM NAA + 2.0 µM BAP + 10% coconut milk + 2% Sucrose + 1.2% Agar + 0.05% Polyvinylpyrrolidone and found that 0.5 g of callus after 20 days of inoculation became 2.9 g. In the present work also in MS + 1.5 mg/l 2,4-D + 0.4 mg/l KN + 1.5% coconut milk, 200 mg callus became 959.72 mg. Therefore, present finding corroborates with the findings of above workers.

## CONCLUSION

Experiments for induction of calli in different explants of *Bacopa monnieri* were performed. It was found that among the explants, mature leaves were much responsive than the young leaves and the internodal explants. Likewise, MS + 1.5 mg/l 2,4-D + 0.4 mg/l KN was most favorable culture conditions for callus induction. The calli were subcultured for biomass production. Here MS + 1.5 mg/l 2,4-D + 0.4 mg/l KN + 1.5% Coconut milk favoured much faster growth. Enhanced biomass may be utilized for the extraction of the important secondary metabolite, Bacoside A, which is being used in the production of memory enhancing capsules.

## ACKNOWLEDGEMENT

Author is grateful to the Head, Department of Botany, B. R. A. Bihar University, Muzaffarpur for their kind support in the form of infrastructure facilities of the Department to conduct the study.

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