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Full Length Research Paper

Effect of plant growth regulators on callus multiplication and *in vitro* plant regeneration in *Bacopa monnieri* L.

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Current investigation deals with the tissue culture studies on *Bacopa monnieri* which has very high morphogenic potential. Its leaf explants respond very readily to treatment with auxins and cytokinins. Indefinite number of plantlets were regenerated from leaf margin, without intervening callus on Murashige & Skoog (MS) medium supplemented with indole-3- acetic acid (IAA) and kinetin (KN). Green embryogenic callus with indefinite number of micro shoots were developed on naphthalene acetic acid (NAA) supplemented medium when 2,4-dichlorophenoxyacetic acid (2,4-D) grown stem-derived callus was sub-cultured. During present investigation, stem (internode, node) explants exhibited callus induction, whereas, leaf explant showed a tendency to regenerate shoot and/or roots. *In vitro* grown plantlets were transferred to polypots containing sterile cocopeat and then series of steps were carried out for acclimatization. Survival rate was 100% when acclimatized plantlets were transferred to soil. Micro-propagated plantlets were without any morphological abnormalities.

Keywords: *Bacopa monnieri,* callus, IBA, IAA, KN, 2,4-D, NAA, embryoids, acclimatization.

INTRODUCTION

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Many medicinal plant species with their bioactive molecules are disappearing at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation and indiscriminate collection of the plant materials by the plant based pharmaceutical companies. Farnsworth et al., (1985) have listed many medicinal plants in a Bulletin of World Health Organization. Blumenthal et al., 2006, have reported a steady growth in total sales of herbal supplements in the United States .

In a 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, *Bacopa monnieri* was placed second according to a sector study by the Export-Import Bank of India,1997. According to an estimate, the annual requirement of the plant was projected to be about 12,700 tonnes of dry material, valued at approximately Rs. 15 billion *(*Ahmad 1993.)

Bacopa monnieri (L.) Pennell is also referred to as *Bacopa monniera*, Indian Pennywort (L.) Pennell, *Herpestes monnieria* L., *Gratiol amonnieria* belonging to the family Scrophulariaceae. It is commonly known as Water Hyssop, brahmi, jalbrahmi and nir- brahmi. The plant is used in the manufacture of various Ayurvedic drugs. In Ayurveda the plant is mainly known as " Brahmi", after Brahma, the creator God of the Hindu pantheon. It was around the 6th century A. Dthat *Bacopamonnieri* was initially mentioned in texts such as the Charaka Samhita, Atharva Veda and Susrut Samhita as

a medharasayana- class herb usually taken to sharpen intellect and to attenuate mental deficits. The herb was believed to have been used by ancient Vedic scholars to memorize lengthy hymns and scriptures.
In the last two decades, *B.monnieri* has been studied extensively (Gupta et al., 2004; Rajani et al., 2004; Mohapatra and Rath, 2005; Ganzerra et al., 2004) for its chemical constituents, and its activity has been established in several *in vivo* and *in vitro* models; randomized clinical trials have also been carried out. It is used in traditional medicine for various nervous disorders (Chopra et al.1956; *The Ayurvedic Pharmacopoeia*,1999). Traditionally it was used as brain tonic to enhance memory development, learning and concentration (Mukherjee and Dey, 1966; Russo and Borrelli, 2005). Research on anxiety, epilepsy, bronchitis and asthma, irritable bowel syndrome and gastric ulcers also supports the Ayurvedic uses of Bacopa (Singh and Dhawan,1997; Roodenrys et al., 2002; Prakash Om et al., 2008; Srinivasa et al., 2004). The beneficial effects of *Bacopa monnieri* on the intelligence and mental performance were further investigated at Banaras Hindu University in a trial carried out on 20 school children over a period of three months (Sharma et al., 1987).
Compounds responsible for the pharmacological effects of Bacopa include alkaloids, saponins and sterols. The main constituents responsible for Bacopa's cognitive effects are some saponins which include Bacosides A and Bacosides B (Chatterjee et al., 1963, 1965; Chakravarty et al. 2003; 2008;Zhou et al., 2007). These are a complex mixture of dammarane type of triterpenoidal saponins (Chatterjee et al., 1963; Jain and Kulshreshta, 1993; Rastogi et al., 2012). The bacosides aid in the repair of damaged neurons by enhancing kinase activity and ultimately nerve impulse transmission (Singh and Dhawan, 1997). Loss of cholinergic neuronal activity in the hippocampus is the primary feature of Alzheimer's disease. Bacosides appear to have antioxidant activity in the hippocampus frontal cortex and striatum (Bhattacharya et al., 2000). Bacopa also gives anticancer effect possibly due to inhibition of DNA replication in cancer cell lines (Elangovan et al.,1995)
A recent *in vitro* study has also demonstrated Bacopa extracts's specific anti-microbial activity against *Helicobacter pylori*, a bacteria associated with chronic gastric ulcers Goel et al., 2003.
Therapeutic doses of Bacopa are not associated with any side effects and has been used safely in Ayurvedic medicine for several hundred of years (Martis et al., 1992).
The natural habitat of Bacopa is deteriorating day by day and the plant itself has become endangered due to many reasons. *In vitro* regeneration holds tremendous potential for the production of high quality plant based medicine. Therefore, there is the need to encourage *in vitro* plant propagation which is considered one of the important strategies for *ex- situ* biodiversity conservation. The best commercial application of tissue culture techniques has been in the production of true to type plants at a very rapid rate compared to conventional methods (Ammirato, 1983) and tissue cultured plants are reported to grow faster and mature earlier than their seed propagated progenies (Vasil and Vasil,1980). Multiplication of plants as a result of tissue culture can occur through enhanced formation of axillary shoots and production of adventitious shoots either directly from the explant or through the intermediate stage of callus followed by rooting of individual shoots and also by somatic cell embryogenesis (Murashige, 1974; Redenbaugh et al., 1991b).
Moreover, plant tissue culture technique is an integral part of genetic transformation process (Vasil et al., 1992; Nisha et al., 2003), production of secondary metabolites, important bioactive (Zenk et al., 1975, 1977 ; Tal et al. , 1982) compounds and production of somaclonal variants by callus culture (Evans et al., 1984 b). Most significant application of callus tissue is accumulation of secondary metabolites which can be manipulated by biotransformation and number of other methods. Previous studies on plant tissue culture of *Bacopa monniera* have been carried out by Shrivastava and Rajani, 1999; Mohapatra and Rath , 2005; Nisha et al., 2003; Tiwari et al., 1998, 2001, 200 ; Majumdar et al., 2012 and they have reported its high morphogenic potential in tissue culture system, but reports on callus initiation, embryogenic callus formation and its profuse growth are less mentioned. The present investigation has been carried out for developing a micropropagation protocol after accessing the response of different explants in order to find out a suitable media with/ without supplementation of phytohormones which would facilitate rapid multiplication. The current investigation also deals with the production and regeneration of embryogenic callus.

MATERIALS AND METHODS

The present investigation has been carried out in the Department of Botany, Ranchi Women's College, Ranchi. The materials and methods used for the experiment are described in detail in the following sections;

Explant

Fresh plantlets were collected from the garden of Dr. S. K. Aggarwalla, MBBS doctor and a Herbal Practitioner from Amrita Family Health Centre

Lalpur, Ranchi. Taxonomic identification was carried out in the laboratory of Prof. Kunul Kandir, University Department of Botany, Ranchi University, Ranchi. Leaf, internode, nodes (stem) were taken as explants. Sometimes nodes with axillary buds were also taken.

Preparation of explants

Stem segments with leaves of approximately 25-30 cm. with 10-12 nodes obtained from garden grown plants were carefully excised using sterilized surgical blade or scalpel and brought to the laboratory as quickly as possible . Without cutting the plantlets into small pieces, stem segments were washed thoroughly in tap water so as to remove all dust and fungicide, if any, adhering to them. The plant material was treated with cetavelon (1:10) for 5 minutes. This was followed by washing with running tap water by continuous shaking to remove the traces of cetavelon. Last wash was done with distilled water.

Surface Sterilization

Surface sterilization of explant was done under perfect aseptic condition in a Laminar air flow cabinet. Explants were treated with 0. 1% HgCl2 for few minutes followed by rinsing thrice with sterile distilled water. After surface sterilization the stem segments with leaves were cut into nodal segments of 1.0-1.5 cm. After undergoing the said processes, leaves, nodes and internodes were ready for inoculation.

Culture Media

The basal culture medium used in the present study was Murashige and Skoog culture medium (Murashige and Skoog, 1962). The chemicals (inorganic and organic salts, growth regulators agar etc.) used for preparing various media were of analytical grade i.e., from Loba, Merck and Sigma. Desired concentration of growth regulators were added in hot and homogenized semi solid medium. After adjusting the pH (5.8), the medium was poured into culture tubes/conical flask which were thoroughly washed, rinsed and oven-dried. Sterilization was done for 15-20 min. at a pressure of 1.1 kg/cm^2 (121˚ c). After sterilization culture vials were kept in an air - conditioned culture room.

Inoculation

Autoclaved inoculation tools like forceps, scalpel, blade, scissors and petriplates were used for transferring the explants onto the medium.

Culture Condition

Each experiment with a minimum of 10 cultures was repeated 3 times and maintained in the culture cabinets in diffuse fluorescent light at temperature 25+_2 *C and at 50%- 60% humidity. Time to time sub culture onto fresh media with same composition was done after 50 days interval for further multiplication. The cultures were observed every day for shoot, root and callus formation.

Planting out and Acclimatization

After proper root development *in vitro* grown plantlets were taken out in such a way that no damage was caused to the root system. Roots were washed gently under running tap water to remove the adhering medium and were transferred to potting mixture contained in small plastic pots.

Preparation of Potting Mixture

Initially small plastic pots with a hole for facilitating drainage, were filled with autoclaved cocopeat mixture. Plantlets were nourished with MS nutrient solution (with macro and micro nutrient at half strength) having pH 5.8 at weekly intervals .

Control of Temperature and Humidity

It was achieved by covering the transplanted plantlets with transparent plastic covers and the pots were kept under shade. Spraying of cold water at an interval of four to five hours using a hand sprayer with fine mist nozzle was done in order to maintain high humidity (90-100%) and mild temperature (25+_2) inside the plastic cover.

Removal of Polythene Bag or Cover

After 15-20 days when the plants showed initial signs of establishment in the pot, the polythene covers were removed for one to two hours daily and gradually the time was increased on daily basis. After 20-25 days of initial planting, covers were totally removed. Spraying of water was done at regular intervals. After 40-45 days of initial planting, the individual plantlets were transferred to larger plastic pots containing garden soil, farmyard manure and cocopeat mixture in equal proportion.

Transfer to Soil

The plantlets were ready for transfer in the garden soil after another forty five days. There was 100% transplantation success in the polypots. Similarly there

Table 1. Callus induction and proliferation from different explants of Bacopa monnieri L. after 35 days. Mean ±S.D. n=30.

S.No.	Media	Stem explants(internode)			leaf explants			Node explant		
		No. of explant	No. of explant responded	% of explants responded	No. of explant	No. of explant responded	% of explants responded	No. of explant	No. of explant Responded	% of explants responded
1	MS+0.05mg/L 2,4-D	30	5.13±1.13	16.33	30	8.53±1.22	30%	30	19.93±1.33	66.6%
2	MS+0.5mg/L 2,4-D	30	14.13±1.01	50%	30	3.3±2.13	10%	30	13.23±1.10	42.6%
3	MS+1.5mg/L 2,4-D	30	29.63±1.32	100%	30	14.7±0.99	50%	30	28.56±1.81	87.5%
4	MS+2.5mg/L 2,4-D	30	0	0%	30	0	0%	30	0	0%
5	MS+5.0mg/L 2,4-D	30	0	0%	30	0	0%	30	8.1±1.15	26%

was 100% transplantation success in the soil. The regenerated plantlets were morphologically similar to their mother plants (Fig. 11)

OBSERVATION AND RESULTS

The results of various experiments carried out to standardize the optimum culture conditions for production of maximum number of plantlets of *Bacopa monnieri* L. through tissue culture method are described as follows:

Explant choice and sterilization

Results of the trials on screening various explants of *Bacopa monnieri* for initiating plantlet release and callus production are presented in Table 1 and 2. Preliminary results indicated that the nodal segments with leaf of 1 cm. or more in length was most suitable for initiating shoot buds. Single oblong leaf was also suitable for shoot bud proliferation as well as for plantlet regeneration. Trials to induce callusing in various explants were quite effective .

Surface Sterilization

Since the field grown material is known to be heavily infected with microbes and spores, special care was taken for surface sterilization of the explants. 0.1% Mercuric chloride ($Hgcl_2$) for few minutes (2 minutes-5 minutes) was found to be the most effective sterilizing agent. Increasing the treatment time led to death of explant. Ethyl alcohol and chlorine water were not effective sterilizants in case of *Bacopa monnieri* .

Effect of Auxins

2,4-D: In almost all the concentration of 2, 4-Dichlorophenoxy acetic acid [2,4-D (0.05-5 mg/l)], callus was induced from the entire surface of the leaf, node and internode segments though growth of callus was inhibited after some time in the same medium. Stem segment (internode) was the most effective explant in 2,4-D(1.5 mg/L) supplemented medium for compact callus formation (Table- 1). When these 2,4-D grown calli were transferred to secondary medium supplemented with NAA, plantlet regeneration was observed. So growth of compact callus was never followed by morphogenic response in the same media. In few cultures presence of green embryogenic callus could also be seen. (Fig. -1)
NAA: Leaf, node and internode segments cultured on MS medium supplemented with different concentration (0.05-5.00 mg/L) of NAA (Naphthalene acetic acid) exhibited callus formation as well as plantlet formation. Just after inoculation hypertrophy of explants was observed. Friable Callus started to grow from the leaf margin. After callusing microshoots were observed with differentiation of roots also. Direct plantlet regeneration with callus formation was observed in medium supplemented with 1.5 mg/L NAA showing maximum height (10.3±1.41cm) of plantlet with maximum number (4.10±1.15) of roots (Table-2). It was observed that with the further increase in concentration of NAA number of shoot root formation was decreased. 1.5mg/l NAA was found to be the most suitable growth regulator for direct plant regeneration through callus formation. When 2 ,4-D grown callus subculture was performed on NAA supplemented medium, plantlets were observed (Fig 2,3,4). 2.5 mg/LNAA was the most suitable concentration for regeneration of plantlets with thick roots, from callus explants (Fig 4).

Effect of Combination of Growth Regulators

IAA + KN: When leaf, node and internode explants were cultured on the medium supplemented with

Table 2. Effect of different growth regulators on *in vitro* plantlet regeneration of *Bacopa monnieri* L. Mean± S.D. n=30.

	LEAF EXPLANT			
MEDIA	**No. of plantlet/explant**	**Height of plantlet(cm)**	**No. of roots/plantlet**	**Length of root/plantlet(cm)**
NAA(mg/L)				
0.05	2.10 ± 1.06	5.60 ± 1.03	1.41 ± 1.03	1.65 ± 0.70
0.5	1.03 ± 1.91	6.80 ± 1.76	2.13 ± 0.97	2.20 ± 0.84
1.5	4.20 ± 0.76	10.3 ± 1.41	4.10 ± 1.15	2.80 ± 0.47
2.0	2.96 ± 1.09	9.20 ± 2.04	3.20 ± 1.29	2.13 ± 0.68
2.5	1.45 ± 0.86	7.10 ± 1.58	2.10 ± 1.72	1.50 ± 1.21
IBA+KN(mg/L)				
0.5+0.5	2.46 ± 1.00	3.96 ± 1.37	1.20 ± 0.66	1.73 ± 0.86
1.0+2.5	2.73 ± 1.17	5.66 ± 1.76	3.36 ± 1.60	2.16 ± 0.94
2.5+2.5	4.66 ± 1.49	10.63± 1.34	4.66 ± 0.84	2.73 ± 1.04
2.5+1.0	4.13 ± 1.35	8.71± 1.28	3.73 ± 1.04	2.10 ± 0.75
5.0+1.0	2.10 ± 1.02	2.20 ± 0.96	2.33 ± 0.95	1.36 ± 0.92
IAA+KN(mg/L)				
0.5+0.5	2.30 ± 0.71	4.20 ± 1.10	1.03 ± 0.99	1.26 ± 1.11
1.0+2.5	2.71 ± 1.05	6.10 ± 2.36	3.06 ± 1.33	2.56 ± 1.56
2.5+2.5	4.10 ± 1.10	8.50 ± 1.67	4.83 ± 0.91	2.83 ± 1.36
2.5+1.0	3.81 ± 1.51	7.54 ± 0.98	3.36 ± 1.24	2.50 ± 1.27
5.0+1.0	2.70 ± 1.25	2.43 ± 1.19	2.66 ± 1.15	1.36 ± 1.29

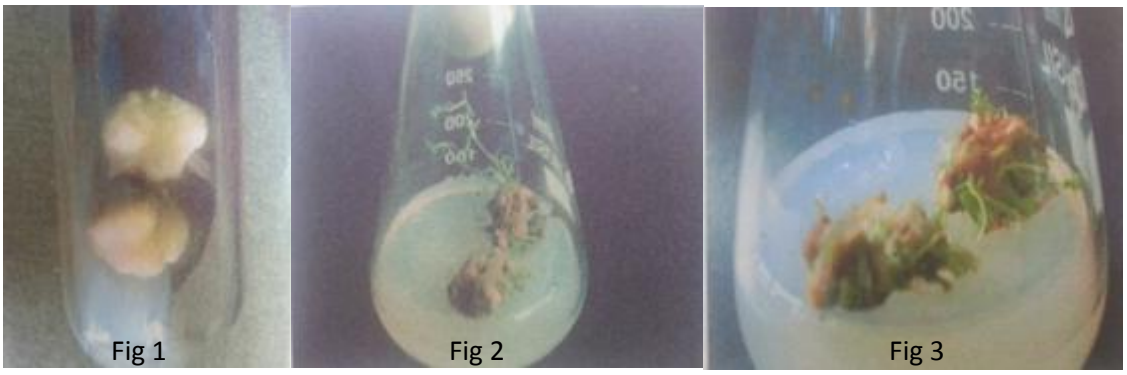

Figure 1. 1 month old internode (stem) explant culture on MS+ 2,4-D (1.5 mg/L) showing callus growth, **Figure 2,3.** 1 ½ month old 2,4-D grown stem-derived callus culture on MS+ NAA (1.5 mg/L) showing plantlets.

combination of Indole-3-acetic acid [IAA(1 mg/L)] and 6-furfurylaminopurine i.e. Kinetin [KN (2.5 mg/L)], direct plantlet regeneration was observed from leaf explants without callus formation with maximum 8.50±1.67cm plantlet height (Table 2). Leaf margin exhibited initiation of indefinite number of microshoots (shoot buds). These microshoots were developed into mature plantlets within a month without any subculture. During present investigation no secondary medium was required for the maturation of microshoots and primary medium supplemented with IAA and KN was sufficient for initiation as well as for maturation of shoot buds without intervening callus (Fig 8,9). In this combination branching of shoots was not observed.

IBA+KN: When leaf, node and internode explants were cultured on the medium supplemented with combination of IBA (2.5 mg/L) and KN (2.5 mg/L), direct plant regeneration was observed from the leaf explants with maximum plantlet height of 10.63±1.34 cm (Fig 6, 7). Initially hypertrophy and marginal curling of leaf explant was observed which was followed by simultaneous growth of embryogenic callus and shoot bud formation (Fig 5) . Within two weeks few shoot buds attained full length plantlets with normal stem branching and indefinite number of roots. Leaf margin exhibited maximum number of shoot bud induction in this combination of growth regulators but all shoot buds were not able to attain full length. This might be due to non-availability of sufficient nutrients because of over population of shoot bud.

During present investigation this was the most significant result of plantlet formation (Fig 6,7).

Figure 4. 1 ½ month old 2,4-D grown stem-derived callus culture on MS+ NAA (2.5 mg/L) showing plantlets
Figure 5. 1 ½ month old leaf culture on MS+ IBA (1.5 mg/L)+ KN (2.5 mg/L) showing green embryogenic callus with microshoots
Figure 6,7. 1 month (6) and 3 month (7) old single leaf with stem culture on MS+IBA (2.5 mg/L) + KN (2.5mg/L) showing plantlet regeneration.

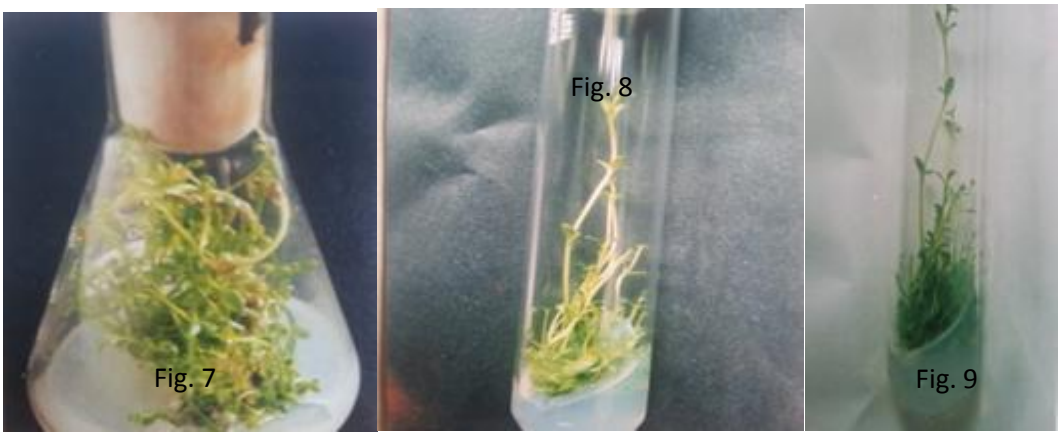

Figure 6,7. 1 month (6) and 3 month (7) old single leaf with stem culture on MS+IBA (2.5 mg/L) + KN (2.5mg/L) showing plantlet regeneration
Figure 8,9. 1 ½ month old leaf explant culture on MS+ IAA (1 mg/L) +KN (2.5 mg/L) showing plantlet regeneration without intervening callus

These plantlets were the best suited plantlets for planting out, acclimatization and for transferring to soil. There was 100% survival rate of these plantlets in the soil.

Standardization of Physical Conditions

Culture Medium: In the present investigation semi-solidified culture medium was suitable for micropropagation. An interesting observation was that cultures could be maintained up to six and a half months with subsequent subculturing. The aim was to get indefinite numbers of plantlets and these were obtained simply by using semi-solidified media. That is why liquid medium was not used in the present investigation.
Light and Temperature: Low intensity of light (500 lux) always induced callus formation whereas comparatively high intensity (1000 lux) caused morphogenesis.

Complete plantlets were observed by incubating the cultures in 16/8 hours light / dark period at 25±2˚C.

DISCUSSION

Plant tissue culture is a biotechnological tool for solving the problem of propagation of multipurpose and endangered medicinal plants in India (Yadav et al., 2012)
Explant type, its age, concentration and combination of growth regulators play an important role in standardization of protocol for micropropagation. Species or even the cultivar behave differently to the chemicals and its proportion. Different explants of the same species also respond differently to the composition of media, they are being subjected to

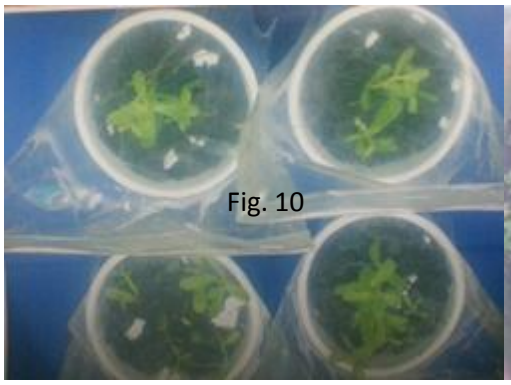

Fig 10. Micropropagated plantlets in plastic pots for hardening
Figure 11. Micropropagated plantlets in garden soil

(Schenk and Hildebrandt, 1972). During the present investigation different concentrations of 2,4-D responded to give pure calli from stem explants which were never differentiated on the same primary medium until calli were subcultured onto secondary medium. It was found that NAA supplemented secondary medium even without kinetin was sufficient for plantlet formation from callus explant, though plant growth was always intervened by callus in this condition. When leaf explant was cultured onto the medium containing IAA (1mg/l) and KN (2.5 mg/l) plantlets were developed from the leaf margin without intervening callus. Leaf margin exhibited green embryogenic callus formation when growth regulators were slightly changed in type and strength. If IBA was supplemented instead of IAA with KN, green globular shaped structures were observed on leaf margin. These globular shaped and heart shaped embryoids could be seen on the surface of leaf calli which developed into indefinite number of microshoots in 30 days. These microshoots grew into plantlets within another 15 days on the same medium. Previous work (Himisha Dixit and Ajay Thakur 2017) reveals shoot multiplication in BAP and IAA supplemented media, which were rooted when subcultured onto IBA incorporated secondary media. Establishing the requirement of one primary medium and another secondary medium for plantlet regeneration. Direct plantlet regeneration and callus formation was not observed by them. In the present study no secondary medium was required and combination of IBA (2.5 mg/l) and KN (2.5 mg/l) in the nutrient medium was sufficient for initiation, proliferation and maturation of microshoots. Another investigation (Mohapatra and Rath 2005) for shoot development was observed with BAP (6- benzylamino purine or 6- benzyladenine) which was followed by use of IBA for root formation from the base of shoots. The present study revealed shoot and root formation in the same primary medium with precocious development of shoots. Shrivastava and Rajani 1999 studied several shoot primordia emerging from the leaf explants surface, exhibited a potential for repeated harvesting of the shoots from the original leaf explants as the latter continued to expand and regenerate new shoots by repeated periodical subculturing onto BA supplemented fresh medium. However they did not study rooting of shoots and callusing response during their work. Behera S. et al. (2015) observed about 114.2 shoots/node with an average shoot length of 6.4 cm on GA3 supplemented MS medium when BA grown nodal explant derived shoot buds were subcultured. They further studied *in vitro* rooting by adding different concentrations (0.1-1.0 mg/L) of IBA in half strength of MS medium as well as *ex vitro* rooting either by planting the excised *in vitro* shoots (i) directly into the plastic pots containing garden soil or (ii) after treating the shoots with different concentration (1.0- 5.0 mg/L) of IBA, prepared in tap water for 1 hr. They further achieved *ex vitro* rooting mediated plant acclimatization of *Bacopa monnieri* in garden soil without any auxin treatment in shoots, which had taken only two weeks in contrast to seven weeks as reported by Caesar et al. 2010. Previous workers did not study the effects of 2,4-D and NAA on plantlet formation whereas in the present work NAA alone onto media exhibited plantlet formation with callus growth in the same primary medium.

CONCLUSION

During the present work an efficient protocol has been developed for plantlet regeneration of *Bacopa monnieri* by using single primary medium which saves time and tissue culture cost. The regenerated plants can be useful for constant supply of raw materials for secondary metabolite extraction. This will reduce

the pressure on natural population of this valuable medicinal plant species and thus be indirectly useful for conservation of this plant species. Callus tissue has its own significance as it can be exploited for the production of soma-clonal variants, synthetic seeds production as well as for the production of secondary metabolites and certain bioactive compound.

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