International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(3): 1139-1143

Received: 07-03-2018
Accepted: 09-04-2018

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Standardized the protocol for *In-vitro* regeneration of *Bacopa monnierri* (L.): An endangered medicinal plant

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Abstract
Bacopa monnnieri is an herbaceous plant belongs to *Scrophulariaceae* family. Commonly found in India, Nepal, Sri Lanka, China, Taiwan, and Vietnam, and is also found in Florida, Hawaii and other Southern states of the USA.An experiment were conducted for standardization of *in-vitro* propagation techniques of *Bacopa monnieri* (L.), a medicinally herbs of India. Quality planting material is major factor for any economically important medicinal plants and micropropagation technique may play important role. Healthy nodal and axillary bud of the herb were used as explant with basic MS medium for shoot initiation of explant BAP (0.5 to 3 mg/l) was used. While concentration of BAP (0.5 to 3 mg/l), IAA (0.5 to 2 mg/l), NAA (0.5 to 2 mg/l) and KIN (0.5 to 2 mg/l) was used for multiplication. MS ½ strength medium with IBA (1-2 mg/l) used for rooting of plantlets. Maximum mean number of initiated plantlets 3 with shoot length 8 cm were found in MS medium treated with 2 mg BAP, while maximum mean number of multiplied plantlets 15 with shoot length 9 cm were found in MS medium treated with 1 mg BAP + 0.5 mg IAA. Maximum mean number of roots 12 with mean length 3 cmwas observed on half strength medium with 2 mg IBA. The rooted plantlets were successfully hardened in 1:1:1 ratio of sand: soil: vermicompost and successfully establishment in soil.
Then fully grown plants were harden to soil by mixing soil and coco peat in ratio 2:1.

Keywords: *Bacopa monnieri*, nodal segment, axillary bud, micropropgation, medicinal plant, plant growth regulators

Introduction
Ayurvedic medicines have got enormous applications in cosmetic, agriculture, pharma, medical and Food industry. The herbal preparations have contributed more specifically in these medicines. *Bacopa monnieri* (L) is a sprawling succulent ayurvedic tropical herb of the family *scrophulariaceae* commonly known as 'Bramhi' found in fresh and brackish waters, wet and marshy lands throughout India, Nepal, Sri Lanka, China, Taiwan, and Vietnam(Rathore *et al*., 2013). And is also found in Florida, Hawaii and other Southern states of the USA where it can be grown in damp conditions by the pond or bog garden.
It has been used for centuries in legends and traditional system of medicine as a memory enhancer (Shankar and Singh, 2000; Vijay *et al*., 2016), anti-inflammatory (Williams *et al*., 2014), analgesic, antidiarrhoeal, cytoxic activity (Afjalus *et al*., 2012), antipyretic (Bammidi *et al*., 2011), sedative and anti-epileptic agent (Srivastava *et al*., 2009). *B. monnieri* extract is a potential cognitive enhancer and neuroprotectant against Alzheimer's disease (Uabundit *et al*., 2010). The plant has been reported to contain several phyto constituents mainly flavonoids (luteolin and apigenin), alkaloids like brahmine, herpestatine and saponins. Saponins such as bacosides A, B, C, and D which are the active triterpinoid principles and known as 'memory chemicals'. Bacoside A, is a mixture of Bacoside A3, Bacoside II, *Bacopa*saponin C and an isomer of *Bacopa* saponin C.
B. monnieri placed second in a priority list of most important Indian medicinal plants on the basis of its medicinal properties, commercial value and potential for further research and development (Vijay *et al*., 2016 and Mehta *et al*., 2017). *B. monnieri* for meeting the estimated present requirement of 0.1 million quintal/year of the herb (Asha *et al*., 2013). Natural propagation is through the seeds. But their numbers are not sufficient to be used for large scale plantation (Kumari *et al*., 2014). The International Union for Conservation of Natural and National Resources has a long time ago listed *Bacopa monnieri* as a threatened species (Shatuti Rathore and Narendra Singh, 2013). Micro propagation through tissue culture is the

only way for the production of uniform planting materials. Plant tissue culture technique is being used for the conservation of germplasm of important and threatened medicinal plants.

Bacopa monnieri L. needs also immediate conservation. Micro propagation technique provides new possibilities for *in vitro* propagation and multiplication of plants and also recognised as an efficient tool for rapid clonal propagation (Kumari *et al*., 2014). This technique have been utilised by (Jain and Vijaya Kumar, 2010). Keeping this idea mind the present work was done in the Plant Biotechnology Laboratory of University Department of Botany, B.R.Ambedkar Bihar University Muzaffarpur.

Materials and Methods

Collection of Explants

Plants of Bramhi were collected from Medicinal Garden of M. P. K. V. Rahuri University.

Sterilization of Explant

Nodal and axillary bud were cut and washed in running tap water to remove superficial dust particles and mud adhering to its surface. Explants were washed with liquid detergent Tween 20 (5/10 drops/ 100 ml) in a vial by gentle agitating conditions. The explants were thoroughly rinsed with distilled water for several times. Again these explant were dipped in to the 1% fungicide (Bavistin) for 20 min and then washed with distilled water. For surface sterilization, explants were transferred to sterile empty flasks under aseptic conditions and given a treatment of (0.5%) $Hgcl_2$ for 5 min then washed with sterile distilled water for 3/4 times. Finally explants were dipped in 70% ethanol for 30 sec subsequently they were washed in steriledistilled water. The procedure was carried out in the inoculation chamber under laminar air flow hood.

Preparation of MS Medium

Culture media was prepared as per described method of Murashige and skoog (1962) and different growth regulator was added as per requirement. For the initiation of explant BAP (0.5- 3 mg/l) while concentration of IAA (0.5 mg/l), NAA (0.5 mg/l) and KIN (0.5 mg/l) was used for multiplication. MS ½ strength medium with IBA (1-2 mg/l) used for rooting of plantlets.

Aseptic Inoculation of Explants

Nodal segments and axillary bud were cut properly and implanted vertically and horizontally on surface disinfected explants were inoculated on full strength MS medium fortified with different concentration of growth regulators. The culture were incubated at a constant temperature of 26± 2ºC with 16±1 h photoperiod (3000 lux).

Result and Discussion

Results

Surface sterilization and induction of axillary shoots

Treatment of explants with 0.5% for 5 minutes and Bavistin (1%) for 20 minutes resulted 100% contamination free viable cultures. BAP (0.5 to 3 mg/l) was used. MS ½ strength medium with IBA (1-2 mg/l) used for rooting of plantlets. Maximum mean number of initiated plantlets 3 with shoot length 8 cm were found in MS medium treated with 2 mg BAP, while Maximum mean number of roots 12 with mean length 3 cm was observed on half strength medium with 2 mg IBA (Table- 1). The rooted plantlets were successfully hardened in 1:1:1 ratio of sand: soil: vermicompost and successfully establishment in soil. Then fully grown plants were hardened to soil by mixing soil and coco peat in ratio 2:1.

Shoot Multiplication

Shoot multiplication is depend on different concentration. Sometimes BAP increasing is best for shoot or just opposite. Activated auxiliary shoots from the nodal explants and transfer to fresh medium containing BAP (0.5 to 3 mg/l) was used. While concentration of BAP (0.5 to 3 mg/l), IAA (0.5 to 2 mg/l), NAA (0.5 to 2 mg/l) and KIN (0.5 to 2 mg/l) was used for multiplication. When we lookresults in the present study showed the essential of plant growth regulators for *in-vitro* multiplication, as the shoots cultured on basal medium did not multiply and become dead. BAP and IAA at a concentration of 1.0 mg/l and 0.5 just gave an average of multiplied plantlets. maximum mean number of multiplied plantlets 15 with shoot length 9 cm were found in MS medium treated with 1 mg BAP + 0.5 mg IAA (Table- 2). Increasing the concentration of BAP and IAA, NAA and KIN, a decrease in shoot multiplication rate was observed.

In-Vitro Rooting

After 30 days of growth, rooting growth is rarely increase day by day in best culture. The multiple shoot clumps produced on this medium were transferred to solidified ½ strength MS medium with different concentration of IBA(1-2mg/l) for rooting of plantlets. Maximum mean number of roots was observed on in medium containing IBA (2 mg/l) (Figure E). On this medium an average no of roots 12 with length 3 cm was observed on half strength medium with 2 mg IBA (Table-3)

Hardening

After 4 weeks complete rooting medium, they were transfer to pots Soil: Sand: Manure (1:1:1) and maintained in greenhouse. In greenhouse is carefully monitored for growth, development and water done as must require. Long and healthy plants transferred to the field condition open ground where 100% growth rate was measured.

Table 1: Effect of plant growth regulator on *in- vitro* shoot induction

Treatment (T)	MS+ PGR (mg/l) BAP	No of shoots per Explants	Length of shoot (cm)	Shooting percentage (%)
T1	0.5	1	0.5	10%
T2	1.0	4	2.0	50%
T3	1.5	6	2.5	75%
T4	2.0	8	3.0	85%
T5	2.5	3	1.3	40%
T6	3.0	2	1.0	25%

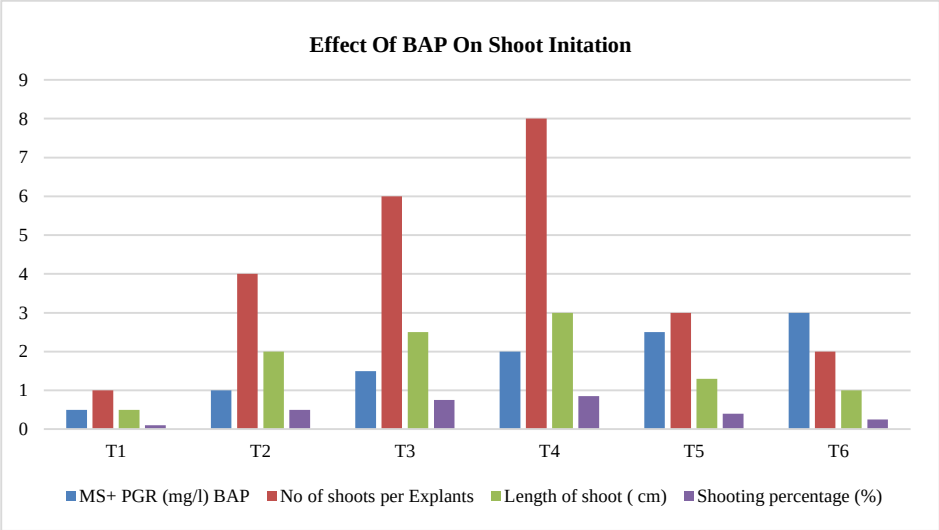

Fig 1: Effect of Growth Hormones (BAP) on shoot induction

Table 2: Effect of plant growth regulators on *in- vitro* shoot multiplication

Treatment (T)	MS+ PGR BAP+ IAA		No of shoots	Length of Shoots (cm)	Percentage
T1	1.0	0.5	15	9	95%
T2	1.5	1.0	9	5	70%
T3	2.0	1.5	4	3	50%
T4	2.5	2.0	4	3	50%
BAP+ NAA					
T5	1.0	0.5	8	5	70%
T6	0.5	1.0	6	5	50%
T7	2.0	1.5	4	3	40%
T8	2.5	2.0	3	2	20%
BAP+KIN					
T9	1.0	0.5	9	6	70%
T10	1.5	1	8	4	40%
T11	2.0	1.5	6	2	20%
T12	2.5	2.0	3	2	20%

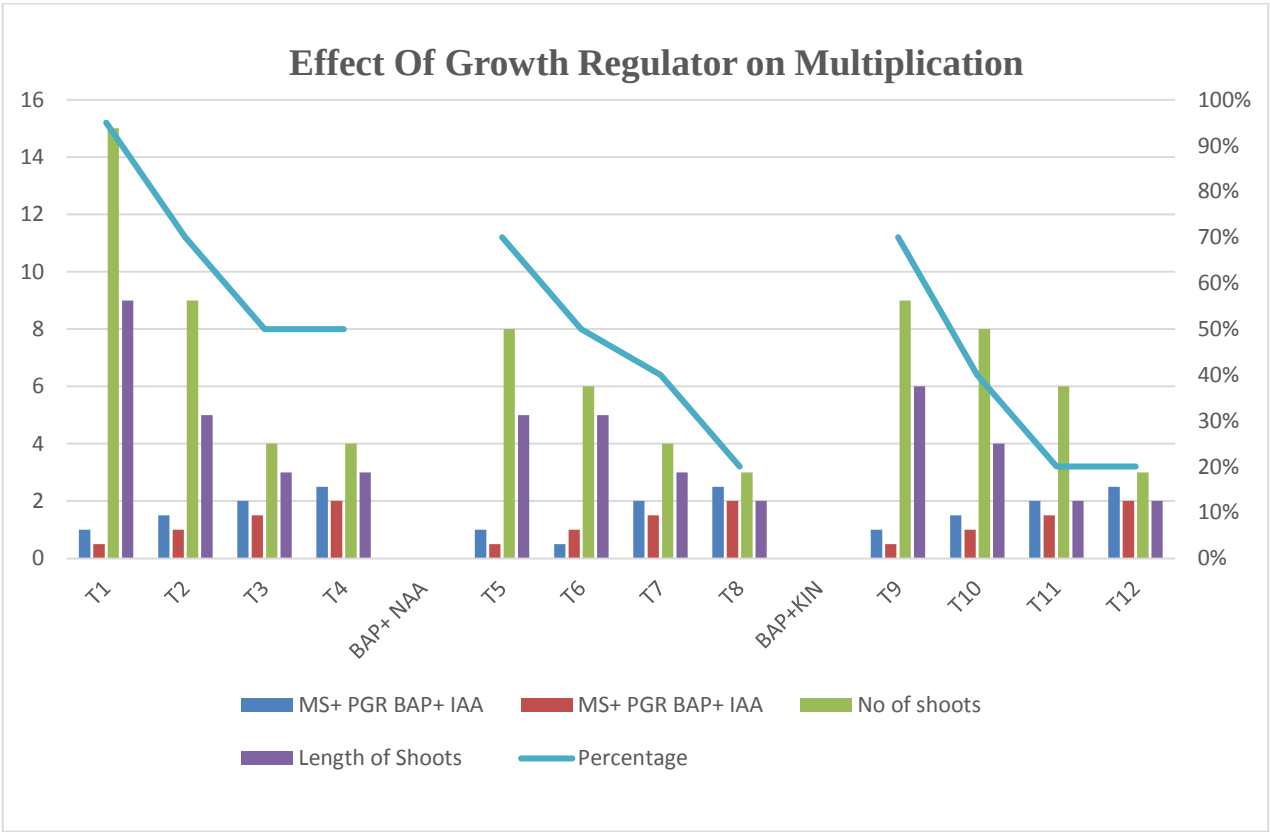

Fig 2: Effect of Growth Regulator on shoot multiplication

Table 3: Effect of IBA on rooting

Treatment (T)	½ Strngth MS + Hormone (mg/L) IAA	No. roots Per explant	Length of root(cm)	Rooting induction frequency (%)
T1	1.0	7	2	70%
T2	2.0	12	3	90%

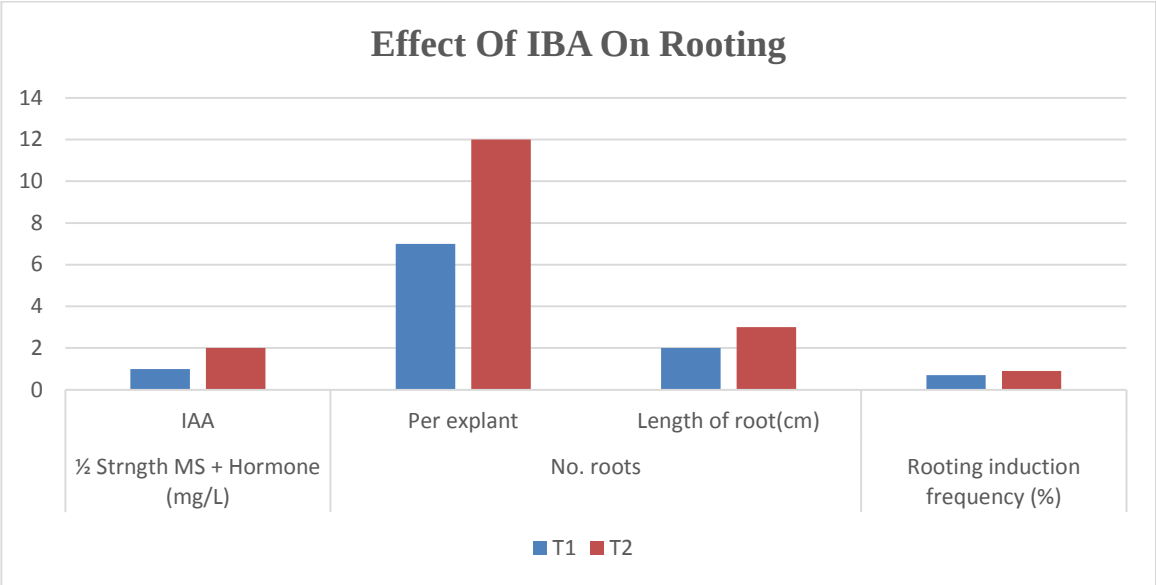

Fig 3: Effect of Growth Hormone (IBA) On Rooting

Discussion

Bacopa monnieri is a medicinal plant used in Ayurvedic medicine for thousands of years to treatment for mental illness, asthma, anxiety, and age- related, antioxidant, stress, cough and cold etc. Mercuric chloride is highly antimicrobial against for both fungi and bacteria at low concentration (0.1%) it is perhaps the most effective disinfectivee agent for soil- borne fungi (Vijay *et al*., 2016). In this study of years to treatment for mental illness, asthma, anxiety, and age- related, antioxidant, stress, cough and cold etc. Mercuric chloride is highly antimicrobial against for both fungi and bacteria at low concentration (0.1%) it is perhaps the most effective disinfectivee agent for soil- borne fungi (Vijay *et al*., 2016). In this study, 100% contamination free viable cultures were obtained by treatment of explants with 0.5% $Hgcl_2$ for 5 minutes. From the present studies MS media proved to be the best culture medium for the establishment of shoot culture in *B. monnieri* plant. This work was undertaken in order to learn the tissue culture technique for medicinally important plant *Bacopa* which is the mostly used for memory enhancing purposes. Final observation after 3-4 weeks showed that MS media supplemented with 2 mg/l of BAP proved to be most capability in shoot induction. On this medium an average 8 shoots with mean shoot length 3 cm were obtained. Numerous reports of BAP as bud inducer at concentrations ranging from 1.0- 5.0 mg/l have already published (Sharma *et al*., 2010; Kaur *et al*., 2013; Vijay *et al*., 2016; Shrivastava *et al*., 1999). These present results are supported by the findings of other workers who have also observed and experimentally found the positive influence of MS medium for optimum shoot and root multiplication in different *Bacopa* species. Activated shoots from nodal explants and transfer to fresh medium containing 1.0 mg/l BAP and 0.5 IAA to establish a stock of shoots used for *in- vitro* multiplication. This observation supported by previous studies on *B. monneiri* (Vijay *et al*., 2016; Vijaykumar *et al*., 2010; Kumari*et al*., 2010; Jain *et al*., 2013; Asha *et al*., 2013; Kaur *et al*., 2013).½ strength MS medium with IBA used for root induction. Maximum rooting recorded in media containing 2 mg/l IBA. Tissue culture raised plants are need acclimatization before field transfer. For this purpose *in- vitro* regenerated plantlets were shifted to pots and kept in greenhouse for a month. The protocol resulted in development of healthy plants without any need of intermediary hardening treatment. After 4 weeks completed rooting medium, they were transfer to pots soil: sand: manure (1:1:1) and maintained in greenhouse. So, this technology is effective as it produces thousands of plants in a short span of time.

Conclusion

Bacopa monnieri has always been a topic of interest of researchers. From tissue culture point of view several studies have been performed to propagate the plant *in- vitro*. The highlights of study are use of low concentration of plant growth regulators and minimization of time required for field transfer of tissue culture raised plantlets. Apart from this *in-vitro* propagation of *Bacopa monnieri*. The *Bacopa* research will give a new insight of research in medicinal components of plants through various advance techniques.

A B

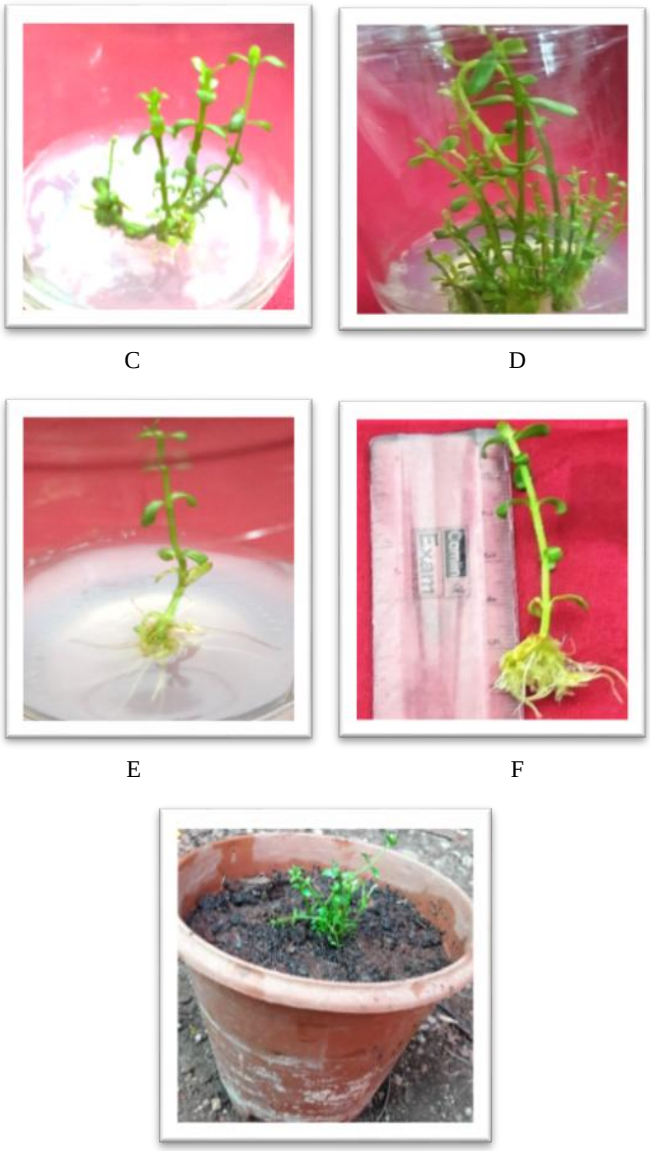

Fig 4: *In-vitro* Plant Regeneration of *Bacopa monnieri:*– **(A)** Explant inoculated on MS media, **(B)** Initiation of shoots on MS media supplemented with BAP, **(C)** Multiple shoot from on MS media supplemented with BAP+ IAA **(D)** Multiplication of plantlet **(E**) Rooting of plantlet on MS media with IBA **(F)** Measurement of shoot and root length **(G)** Hardening of regenerated plant in Soil: Sand: Manure

Acknowledgment
The authors are thankful to the K. K. Wagh College of Agricultural Biotechnology, Nashik (Affiliated toMPKV, Rahuri) for providing the excellent facility to carry out the research work as well as thankful to Prof. P. T. Yeole for their valuable guidance.

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