

## EFFECT OF TOPOPHYSIS AND CARBON SOURCE ON ANTI-ALZHEIMER MEDICINAL PLANT (*BACOPA MONNIERRI*) IN VITRO GROWTH

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### ABSTRACT

*Bacopa monnieri* is used in traditional Ayurvedic medicine in India for centuries to control asthma, epilepsy and mental disorder. Various studies have shown that *Bacopa* is useful in controlling Alzheimer's disease, anxiety, and age-related cognitive decline. It is also used to improve mental function and memory, treat respiratory ailments, and heal stomach ulcers. The present study aimed to in vitro investigate the explants position and carbohydrate sources effect on the growth and development of *Bacopa monnieri* which were cultured in the MS nutrient medium supplemented with different concentrations (20, 30, 40 g\L) of the combination between the carbohydrates (sucrose, fructose, glucose) with the solid and the liquid media type and using stem of *Bacopa* plant containing of one node, two nodes and three nodes then cultured of two positions, horizontally and vertically method in the solid media. The incubation conditions were 25±2°C at 16 h/day photoperiod (2000-2500 lux). Glucose, 20 g\L, gave the best shoot number (148.75), shoot length (5.87), Growth value (4.25) and multiplication rate (15.00). Results showed that the best shoot number (17.00), Leaf number (76.00) and Node number (38.00) resulted from two nodes horizontally position. The best Shoot length (4.12) occurred due to one node horizontally position. The best root number (23.00) resulted from three node horizontally method. In vitro plantlets produced from rooting stage were transferred in plastic pots (8 cm diameter) containing peat, perlite and sand for acclimatization. The rooted plants were successfully acclimatized in different potting media and grew naturally in the greenhouse. The best planting medium for plant growth was peat moss +perlite mixture (2: 3).

**Keywords:** *Bacopa monnieri* Alzheimer's disease, Egyptian

### INTRODUCTION

*Bacopa monnieri* L. (Family Scrophulariaceae) also known as water hyssop, and "Brahmi," has been used in the ancient Indian medicine for thousands of years (Debnath *et al.*, 2006). In the folk medicine, it was used as a nootropic to improve memory function and to relieve anxiety or epilepsy. *Bacopa monnieri* has also been used as a cardiogenic, and to improve respiratory function in bronchial constriction cases. *Bacopa's* antioxidant contents may offer protection against free radical damage in cardiovascular disease and certain types of cancer (Mukherjee and Dey, 1966). Plant tissue culture techniques now play an important role in the clonal propagation and quantitative improvement of the medicinally important plant (Rao *et al.*, 1996). Vegetative propagation of the medicinally important plant is slow and

further hampered by specific habitat requirements and poor performance of propagules. Additionally, fieldcultivation is time consuming, labor intensive, season dependent and leaves the material vulnerable to natural calamities. Therefore, it is essential to develop alternative methods to ensure the selected plant resources availability. The present study aimed at studying the carbon source and topophysis (Topophysis, the effect on growth and differentiation of position of axillary buds along the shoot) effect on the in vitro growth and development of *B. monnieri*.

### MATERIALS AND METHODS

#### Plant material and sterilization of explants

This research was conducted in Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City during the period from 2013 to 2015. *Bacopa*

*monnieri* (L.) Pennell seeds were kindly donated from United State National Plant Germplasm System. Seeds were surface sterilized by cleaning thoroughly under running tap water for 15 min. followed by immersing in 1.5% (v/v) sodium hypochlorite for 15 min. They were washed thrice with sterile double distilled water and kept in laminar. Explants were cultured in a hormone free MS medium (**Murashige and Skoog, 1962**) containing 30g/L sucrose and 7.0 g/L agar, pH was adjusted to 5.7. The explants were grown in a 16 hrs light photoperiod under an irradiance of 3,000 lux, and an average temperature of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ .

#### **Effect of Carbon Source on *in vitro* culture of *Bacopa monnieri***

Different concentrations (20, 30, 40 g/L) of the combination between the carbohydrates (sucrose, Fructose, Glucose) with the solid media type and the liquid media type were used in order to investigate their effect on *in vitro* growth of *Bacopa* shoots. The pH of the medium was adjusted to  $5.7 \pm 0.1$  prior to addition of 7.0 g/L agar (in solid medium). The medium of each treatment was distributed into culture jars (300 mL) where each one contained 40 mL. The explants were grown in a 16 hrs light photoperiod under an irradiance of 3,000 lux, and an average temperature of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . *In vitro* growth and development were estimated as Shoot number, shoot length, growth vigor and multiplication rate. Data were recorded for three subcultures and the experiment was repeated three times.

#### **Effect Of Topophysis ( Topophysis, the effect on growth and differentiation of position of axillary buds along the shoot) on *Bacopa monnieri* micropropagation.**

*In vitro* Stem of *Bacopa* plant containing of one node, two nodes, or three nodes were used then cultured of two position horizontally method and vertically method in the solid media. The pH of the medium was adjusted to  $5.7 \pm 0.1$  prior to addition of 8.0 g/l agar (in solid medium). The medium of each treatment was distributed into culture jars (300 mL)

where each one contained 40 ml. The explants were grown in a 16 hrs light photoperiod under an irradiance of 3,000 lux, and an average temperature of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . Shoot number, leaves number, shoot length, root number, node numbers were recorded after three subcultures and the experiment was repeated twice

#### **Acclimatization and transfer of plantlets to greenhouse**

Plantlets with well-developed roots were dislodged from the culture medium and the roots were washed gently under running tap water to remove the adhering medium. Plantlets were transferred to plastic pots (8 cm diameter) containing peat moss, sand, and perlite in different fourteen combinations as presented in Table (3). Plantlets were irrigated with distilled water every two days for two weeks followed by tap water for another two weeks. The plantlets were transferred to the greenhouse (day and night temperatures of the greenhouse were  $30.4^{\circ}\text{C}$  and  $18.5^{\circ}\text{C}$ , respectively). The average light level at the time of collection was 12000 Lux. An intermediate misting system was used with an initial higher frequency at 30 min intervals during daylight and at 60 min intervals overnight and reduced after 10 weeks to 60 min intervals during daylight, and 120 min intervals at night. Survival percentage, plant height, root number and length were recorded after eight weeks in acclimatization stage.

#### **Experimental design and data collection**

All experiments were conducted under a randomized block design including three replicates with 15 explants per treatment. Data were processed by analysis of variance (ANOVA) and comparisons between the mean values of treatments were made using least significant difference (L.S.D.) at the confidence level of  $P \leq 0.05$ . (**Snedecor and Cochran, 1989**).

## **RESULTS AND DISCUSSION**

#### **Effect of Carbon Source on *in vitro* culture of *Bacopa monnieri***

The present study was investigated the effect of various concentrations of carbon sources. Table (1) showed that using the different concentrations (20,30,40 g/L) of the combination between the carbohydrates (sucrose, Fructose, Glucose) with the solid media type and the liquid media type. 30 g/L Sucrose gave the best shoot number (82.75), Growth value (2.75), multiplication rate (8.25) and the longest shoot occurred due to 20 g/L sucrose (5.50) but, 20 g/L sucrose gave the lowest shoot number (54.25), Growth value (2.00), multiplication rate (5.75) and the least shoot length (5.37) occurred due to 40 g/L sucrose in the solid media type. Compare with the liquid media type 30 g/L sucrose gave the best shoot number (58.25), Growth value (2.25), multiplication rate (6.00) and the longest shoot (4.12) occurred due to 40 g/L sucrose but, 40 g/L sucrose gave the lowest shoot number (46.00), Growth value (2.00), multiplication rate (4.75) and the least shoot length (3.50) occurred due to 30 g/L sucrose. On the other hand, 30 g/L Fructose gave the best shoot number (66.25), shoot length (4.62), Growth value (2.50) and Multiplication rate (6.75) but, 40 g/L Fructose gave the lowest shoot number (42.75), shoot length (3.05), Growth value (1.75) and multiplication rate (4.25) in the solid media type compare with the liquid media type 30 g/L Fructose gave the best shoot number (49.00), shoot length (3.87), Growth value (2.00) and Multiplication rate (5.25) but, 40 g/L Fructose gave the lowest shoot number (23.50), shoot length (2.25), Growth value (1.00) and multiplication rate (2.50). While 20 g/L Glucose gave the best shoot number (148.75), shoot length (5.87), Growth value (4.25) and multiplication rate (15.00) but, 30 g/L Glucose gave the lowest shoot number (104.75), shoot length (4.67), Growth value (3.25) and multiplication rate (10.50) in the solid media type. Compare with the liquid media type 40 g/L Glucose gave the best shoot number (130.00), Growth value (4.00), multiplication rate (13.00) and the longest shoot (7.12) occurred due to 30 g/L Glucose but, 20 g/L Glucose gave the lowest shoot number (95.00), Growth value (3.00), multiplication rate (9.50) and the least shoot length (6.00) occurred due to 40 g/L Glucose. In general the solid media type gave the maximum results than the liquid media type on Bacopa plant.

In a similar study, Ilczuk *et al.*, (2013) showed that tested several different sugars as carbon source on the efficiency of shoot proliferation and *in vitro* rooting of common ninebark (*Physocarpus opulifolius* (L.) Maxim.). Fructose, glucose, maltose and sucrose were tested at concentration ranging from 0–50 g dm<sup>-3</sup>. The highest number of shoots was produced on the fructose-containing medium. The concentration of 30 g dm<sup>-3</sup> appeared to be optimal; the rate of proliferation at 30 and 40 g dm<sup>-3</sup> were in fact similar, but the former produced a more favorable shoot length. The number of adventitious roots produced per shoot increased with increasing fructose concentration up to 30 g dm<sup>-3</sup>. Fructose can be therefore recommended as the best C-source for the *in vitro* shoot proliferation. On the other hand, Fayek *et al.* (2007) stated that the effect of carbon source (sucrose, fructose and glucose) was considered with proliferation of jojoba clones. Sucrose proved to be the best carbon source for shoot number while, glucose then fructose gave higher increase in shoot length and leaf number.

Carbohydrates play an important role in *in vitro* cultures as an energy and carbon source, as well as an osmotic agent. In addition, carbohydrate-modulated gene expression in plants is known (Koch, 1996). Plant gene responses to changing carbohydrate status can vary markedly. Some genes are induced, some are repressed, and others minimally affected. As in microorganisms, sugar-sensitive plant genes are part of an ancient system of cellular adjustment to critical nutrient availability. However, there is no evidence that this role of carbohydrate is important in normal growth and organized development in cell cultures.

The selection of sucrose as the most suitable energy source for cultures follows many comparisons between possible alternatives. Some of the first work of this kind on the carbohydrate nutrition of plant tissue was done by Gautheret (1945) using normal carrot tissue. Sucrose was found to be the best source of carbon followed by glucose, maltose

and raffinose; fructose was less effective and mannose and lactose were the least suitable. Sucrose has almost invariably been found to be the best carbohydrate; glucose is generally found to support growth equally well, and in a few plants, as in this study, it may result in better *in vitro* growth than sucrose, or promote organogenesis where sucrose will not; but being more expensive than sucrose, glucose will only be preferred for micropropagation where it produces clearly advantageous results. Multiplication of *Alnus crispa*, *A. cordata* and *A. rubra* shoot cultures was best on glucose, while that of *A. glutinosa* was best on sucrose (Tremblay and Lalonde, 1984; Tremblay et al., 1984; Barghchi, 1988). Direct shoot formation from *Capsicum annum* leaf discs in a 16 h day required the presence of glucose (Phillips and Hubstenberger, 1985). Glucose is required for the culture of isolated roots of wheat (Ferguson, 1967) and some other monocotyledons (Bhojwani and Razdan, 1983).

It is known that sucrose has dual roles as carbon source and osmotic agent in plant cells/ organs cultures. In plants, sugars can be used as signaling and have regulatory functions like hormones, and sucrose transport and hydrolysis play key regulatory roles in sugar signal generation. Generally, more consumption of sucrose would benefit root growth and metabolite biosynthesis. However, the osmolality of culture medium under high sucrose concentration (3.0–4.0%) could cause the loss of cell viability by the dehydration and promote the diffusion of phytochemicals from root tissues into the liquid medium, thus leading to the low content at high sucrose concentration. (Koch 2004; Rolland et al. 2002).

#### **Effect Of Topophysis on *Bacopa monnieri* micropropagation**

The current study was investigated the effect of explant position and number of nodes per explants on *in vitro* growth. Table (2) showed that regarding using stem of *Bacopa* plant containing of one Node, two Node and three Node then cultured of two position

Horizontally method and Vertically method in the solid media. Data in table showed that the best shoot number (17.00), leaf number (76.00) and node number (38.00) resulted from two nodes horizontally position. The best Shoot length (4.12) occurred due to one node horizontally position. The best of Root number (23.00) resulted from three node horizontally method. But, the lowest of Shoot number (8.25), Leaf number (45.00), Root number (13.50) and Node number (22.50) occurred due to one node stem horizontally position. The least length of shoot of *Bacopa* plant occurred due to three node stem horizontally position. On the other hand, compare with the vertically position Shoot number, Leaf number, Shoot length and Node number are increasing by ascending when the best shoot number (8.75), Shoot length (5.00), Leaf number (52.25) and Node number (26.25) resulted from three node stem of *Bacopa monnieri* but, the best root number occurred due to two node stem of *Bacopa* plant. One node stem vertically method of *Bacopa* gave the lowest Shoot number (6.00), Leaf number (33.50), Shoot length (3.20), Root number (10.50) and Node number (16.62).

In a similar study, Shirdelet al ., (2011) compared the effect of nodal position *in vitro* growth of Dog Rose it can be propagated in three nodal positions (Lower, middle and terminal) on the stem on MS media the results showed that maximum bud break percentage and highest shoot length were observed in lower nodal position. Minimum bud break percentage and shoot length were observed in middle nodal position. Growth or regeneration from explants can be influenced by the way that the explant is placed into or upon the medium. The effects noted may sometimes be due entirely to polarity; on other occasions they can be attributable to the effect of positioning on the availability of nutrients and growth regulators to those parts of the explant which are competent. Strictly positional effects on regeneration (e.g. where similar buds from the top and bottom of a plant behave differently *in vitro*) are examples

of topophysis (an effect of position upon the observed characteristics of a plant). In experiments with trees, positional effects are sometimes further divided into cyclophysis (differential effects exhibited by parts of the same age, but different position) and periphysis (distinctive characteristics shown by parts of the same age and position which have been subjected to different physical exposure) (Oleson, 1978; Pierik, 1987).

The organogenesis obtained on root or stem sections is also liable to differ according to whether the explants are normally orientated or inverted on the medium. Shoots regenerated from the upper (proximal) surface when dandelion root sections were placed distal side (i.e. the side that would normally be nearest to the root tip) downwards on the medium. Reversed, with the proximal side in contact with the agar, they produced a mound of callus from the upper (distal) surface plants with respect to morphological and growth characteristics. All the micropropagated plants were free from external defects. The high response may be due to the ability of the mixture to provide enough moisture and aeration to the plants, thereby resulting in good root growth. The mixture possesses cation exchange properties, thus, it can hold and make available ammonium, potassium, calcium and magnesium to the growing plants. Perlite, when combined with peat moss promotes faster root growth and provides quick anchorage to young roots (Hartmann *et al.*, 2007). Perlite is also an important commodity in the potting mixture when mixed with peat moss. The addition of perlite to peat moss increases the amount of air (oxygen) held in the peat moss, as well as the amount of water retained by the peat moss. This obviously improves the growing conditions for plants (Donahue and Miller, 1990).

(Booth and Satchuthananthavale, 1974). Adventitious roots formed from *Acinidia deliciosa* callus formed shoots at their proximal end if they were detached from the callus and inserted vertically with the root tip in the agar medium, but produced shoots along much of their length from the proximal end if placed horizontally (Revilla and Power, 1988). When the basal ends of white spruce hypocotyls sections were placed in the medium, almost all the explants produced scale-like organs which could be subcultured to give buds and shoots, but only roots were formed on 50% of the sections if they were cultured with their apical ends downwards (Campbell and Durzan, 1978). Discs cut from the inflorescence stalk of gladiolus developed callus and root primordia from their basal ends when that end was placed on the medium, but if explants were cultured in an inverted position, regeneration was more rapid. Roots were again formed at the proximal end (now uppermost), and one or two buds developed at the distal end (in the medium). Subsequent leaf growth turned the explants over. Subcultured correctly orientated on the same medium, explants eventually developed clusters of cormlets and one or two shoots (Ziv *et al.*, 1970). It was similarly advantageous to invert scape sections of asparagus (Takatori *et al.*, 1968) and *Narcissus* (Seabrook *et al.*, 1976) to obtain any morphogenetic response. It has been suggested that inversion may overcome the polar transport of natural auxin, but this was not the case with tulip. Stalk explants elongated only when they were inverted on a medium containing IAA, and growth was due to basipetal (polar) auxin movement (Gabryszewska and Saniewski, 1983).

#### Acclimatization and growth of plantlets

The rooted plants were successfully acclimatized in different potting media and grew naturally in the greenhouse. Referring to Table 3, maximum root length and plant height were obtained on plants grown in peat moss + perlite mixture (2: 3). There was no detectable variation among the acclimatized

**Table (1): Effect of Carbon Source on *in vitro* growth of *Bacopa monnierra***

Carbon source	conc	Shoot number			Shoot Length			Growth vigor			Multiplication rate		
		Solid	Liquid	Mean	Solid	Liquid	Mean	Solid	Liquid	Mean	Solid	Liquid	Mean
<b>Sucrose</b>	20	54.25 FGH	57.00 FG	55.62 CD	5.500 BC	3.750 FG	4.62 BC	2.000 GH	2.250 FGH	2.12 CDE	5.750 FG	5.750 FG	5.75 CD
	30	82.75 DE	58.25 FG	70.50 C	4.625 DE	3.500 FG	4.06 CD	2.750 DEF	2.250 FGH	2.50 C	8.250 DE	6.000 EFG	7.12 C
	40	59.25 FG	46.00 FGHI	52.62 D	5.375 BCD	4.125 EF	4.75 B	2.000 GH	2.000 GH	2.00 DE	6.250 EFG	4.750 FGHI	5.50 CD
<b>Fructose</b>	20	65.00 EFG	31.75 HI	48.37 DE	3.375 FG	3.750 FG	3.56 D	2.250 FGH	1.250 IJ	1.75 EF	6.750 EF	3.250 HI	5.00 DE
	30	66.25 EF	49.00 FGH	57.62 CD	4.625 DE	3.875 EFG	4.25 BC	2.500 EFG	2.000 GH	2.25 CD	6.750 EF	5.250 FGH	6.00 CD
	40	42.75 GHI	23.50 I	33.12 E	3.050 GH	2.250 H	2.65 E	1.750 HI	1.000 J	1.37 F	4.250 GHI	2.500 I	3.37 E
<b>Glucose</b>	20	148.8 A	95.00 CD	121.87 A	5.875 B	6.125 B	6.00 A	4.250 A	3.000 CDE	3.62 AB	15.00 A	9.500 CD	12.25 A
	30	104.8 CD	102.5 CD	103.62 B	4.675 CDE	7.125 A	5.90 A	3.250 CD	3.250 CD	3.25 B	10.50 CD	10.00 CD	10.25 B
	40	111.3 BC	130.0 AB	120.62 A	5.700 B	6.000 B	5.85 A	3.500 BC	4.000 AB	3.75 A	11.25 BC	13.00 AB	12.12 A
<b>Mean</b>		81.66 A	65.88 B		4.75 A	4.50 A		2.69 A	2.33 B		8.30 A	6.66 B	
		L.S.D 5% A=16.37 B=7.716 AB=23.15			L.S.D 5% A=0.600 B=0.2832 AB=0.8495			L.S.D 5% A=0.4884 B=0.2302 AB=0.6907			L.S.D 5% A=1.691 B=0.7973 AB=2.392		

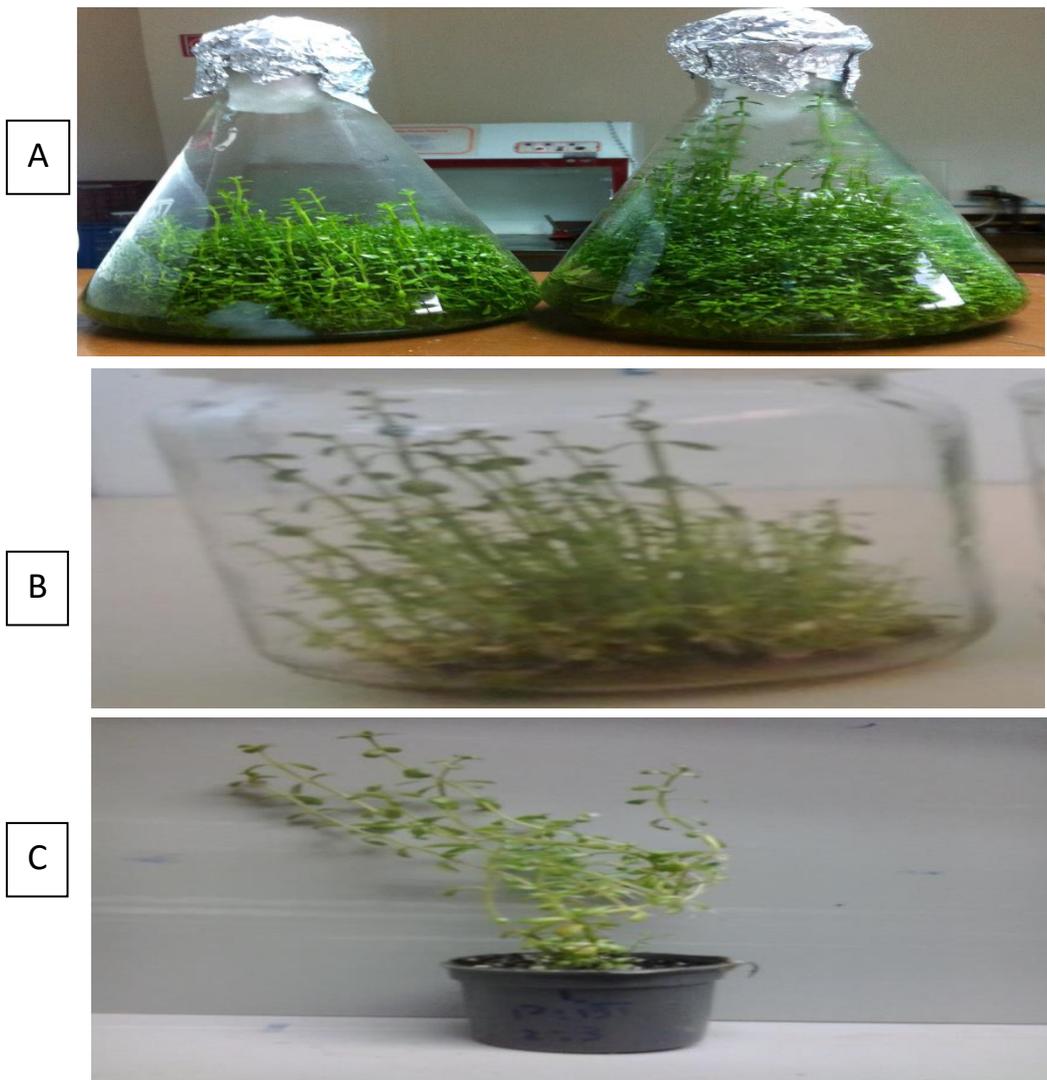
Growth vigor was estimated according to **Pottino (1981)** as the following: 1= no growth 2= below moderate growth 3= moderate growth 4= good growth

**Table (2) Effect Of Topophys on *in vitro* growth of *Bacopa monnieri* during multiplication stage**

	<b>Shoot N.</b>			<b>Leave N.</b>			<b>Shoot L.</b>			<b>Root N.</b>			<b>Node N.</b>		
	<b>H.</b>	<b>V.</b>	<b>Mean</b>	<b>H.</b>	<b>V.</b>	<b>Mean</b>	<b>H.</b>	<b>V.</b>	<b>Mean</b>	<b>H.</b>	<b>V.</b>	<b>Mean</b>	<b>H.</b>	<b>V.</b>	<b>Mean</b>
<b>1Node</b>	8.250 BC	6.000 C	7.125 B	45.00 C	33.50 C	39.25 B	4.125 B	3.200 C	3.662 A	13.50 B	10.50 B	12.00 B	22.50 C	16.75 C	19.63 B
<b>2Node</b>	17.00 A	7.750 C	12.38 A	76.00 A	43.50 C	59.75 A	3.875 BC	4.000 BC	3.938 A	20.25 A	11.75 B	16.00 AB	38.00 A	21.50 C	29.75 A
<b>3Node</b>	14.25 AB	8.750 BC	11.50 AB	70.00 AB	52.25 BC	61.13 A	3.375 BC	5.000 A	4.188 A	23.00 A	11.50 B	17.25 A	35.00 AB	26.25 BC	30.63 A
<b>Mean</b>	13.17 A	7.500 B		63.67 A	43.08 B		3.792 A	4.067 A		18.92 A	11.25 B		31.83 A	21.50 B	
<b>L.S.D 5%</b> <b>A=4.43</b> <b>B=3.61</b> <b>AB=6.26</b>			<b>L.S.D 5%</b> <b>A=14.33</b> <b>B=11.70</b> <b>AB=20.26</b>			<b>L.S.D 5%</b> <b>A=0.6058</b> <b>B=0.4946</b> <b>AB=0.8567</b>			<b>L.S.D 5%</b> <b>A=4.53</b> <b>B=3.69</b> <b>AB=6.40</b>			<b>L.S.D 5%</b> <b>A=7.13</b> <b>B=5.82</b> <b>AB=10.09</b>			

**Table ( 3 ). Effect of planting media on survival percentage and plant growth and development of *Bacopa monnierra* produced through tissue culture techniques during acclimatization stage after 8 weeks**

Planting media			Survival %	Plant height cm	Root No	Root length cm
Perlite	Peat moss	sand				
1	1	0	90%	9.800 D	14.20 DE	3.500 C
1	2	0	100%	12.40 BCD	21.00 ABC	6.400 A
1	3	0	100%	14.30 ABC	23.40 A	5.600 AB
2	1	0	100%	13.70 ABC	15.40 DE	5.800 AB
2	3	0	100%	17.20 A	21.80 AB	4.300 BC
3	1	0	100%	17.20 A	19.00 ABCD	5.500 AB
3	2	0	90%	14.40 ABC	22.40 AB	5.000 ABC
0	1	1	100%	12.80 BCD	17.40 BCD	3.700 C
0	2	1	100%	17.40 A	17.80 BCD	3.700 C
0	3	1	60%	14.60 ABC	15.00 DE	3.600 C
0	1	2	100%	16.00 AB	15.40 DE	4.400 BC
0	3	2	100%	15.40 ABC	16.40 CDE	5.500 AB
0	1	3	80%	11.80 CD	11.60 E	3.500 C
0	2	3	80%	15.00 ABC	14.60 DE	3.600 C
<b>L.S.D 5%</b>				<b>3.71</b>	<b>5.10</b>	<b>1.59</b>



**Fig. 1. Mass propagation of *Bacopa monnieri*.**

- a- In vitro growth of two node shoot on horizontal position on MS free medium (30 days old).
- b- In vitro growth on MS medium containing 20 g/L glucose (30 days old).
- c- Hardened plants in plastic cups (8 weeks old).

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