International Journal of Advance Research in Science and Engineering
Vol. No.6, Issue No. 06, June 2017
www.ijarse.com
IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

B. monnieri - MICROPROPAGATION USING VERMICOMPOST, ELUANT AND EXTRACTS OF VERMICOMPOST IN PLANT TISSUE CULTURE MEDIA

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ABSTRACT

Vermicompost produced by the activity of earthworms is rich in macro and micronutrients, vitamins and growth hormones. Vermicompost was evaluated with different methods of extractions like vermicompost only, eluant and vermicompost extract to use in the tissue culture media for micropropagation of B. monnieri. ANOVA, Student's t-test and Tukey's Honestly Significant Difference (HSD) Test were considered for analyses of significance of various parameters. The values for number of roots ranged from 4 in only vermicompost medium to 7 in vermicompost extract medium. This had F value of 92.67 and Pr > F of < 0.0001. The mean number of shoots formed in medium having vermicompost was 3.0 whereas in vermicompost extract it was 1.2. The level of significance has given the F value of 91.00 and Pr > F of <0.0001. Vermicompost extract has significantly supported higher nodes and leaves compared to eluant and only vermicompost. The significance level was Pr > F of 0.0011 and 0.0012 respectively. Significant increase in the weight of the plantlets (in milligrams) was observed in vermicompost extract as compared to other two treatments. The value for significance was at < 0.0001. Test for percentage response of survival of plantlets has shown that in all the three treatments there was 100 per cent survival of the explants. The results have shown that the response in vermicompost extract medium was significantly higher.

Keywords: B. monnieri, Eluant, Economical Plant Media, Vermicompost, Vermicompost Extract.

I. INTRODUCTION

Bacopa monnieri (L.), commonly known as "Brahmi" is a glabrous, sprawling succulent herb with ayurvedic medicinal importance in Indian subcontinent. The perennial creeper is a member of the family Scrophulariaceae and grows in damp and marshy places and on the banks of slow flowing rivers and lakes, ascending up to an altitude of 1,320 meters[1].The herb is known to improve memory cells and thus known as "memory booster" or "thinking person's herb". The rejuvenating herb with reputed drug value is used in folk and traditional system of medicine as a nerve, cardio and brain tonic to enhance concentration[2]. The principal active factors in *Bacopa* are

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ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

two steroidal saponins, bacosides A and B ("memory chemicals"), that help repair damaged neurons by enhancing proteins involved in the regeneration of neural-cell synapses[3]. Two new dammarane-type jujubogenin bisdesmosides, bacosaponins E and F of biological interest have also been isolated from this herb[4]. In India, brahmi is used to prepare important ayurvedic preparations like "Brahmighritam" and "Brahmirasayanam"[5]. On the basis of medicinal importance and potential for future research and development, *B. monnieri* is placed second in a priority species list of the most important medicinal plants[6]. This is one among 32 medicinal plants identified for cultivation and conservation by the NMPB[7], In recent years, the release of brahmi-based Memory Plus product-containing bacoside extracts increased the demand in the Indian market, which led to over-collection of the plant from the natural habitat. Multiple numbers of brahmi-based commercial ayurvedic preparations are already available in the market due to its therapeutic values[8]. *In vitro* regeneration and micropropagation of brahmi was reported by many researchers[9-15] and synthetic seed mediated plant conversion[16].

Vermicompost produced by the activity of earthworms is rich in macro and micronutrients, vitamins, growth hormones, enzymes such as proteases, amylases, lipase, cellulase and chitinase and immobilized microflora. The enzymes continue to disintegrate organic matter even after they have been ejected by the earthworms[17-21]. Vermiwash, a liquid nutrient obtained during vermicomposting has significant influence on plant growth and yield attributes[22-24]. Due to this, these vermiproducts have become key components of crop nourishment in organic and sustainable farming systems. Of the many species of earthworms evaluated for vermicomposting, *Eudrilus eugeniae (Kinberg.), Eisenia fetida (Savigny)* and *Perionyx excavatus (Perrier)* have been considered as the key species for organic matter recycling, throughout the world [25-30].

The present investigation was planned to report a simple and rapid but novel method for *in vitro* multiplication of *B. monnieri* by using highly economical nutrient tissue culture media. All available medical literature[31] confirms that there has been no toxicity report associated with the use of *Bacopa* as a dietary supplement in children or adults[32]. Brahmi is especially suitable for students as it enhances the mind's ability to learn and to focus[33].

II. MATERIALS AND METHODS

2.1 Collection of Medicinal Plant Sample (Explants)

Explants from certified disease-free medicinal plants were obtained from Botanical Garden of University of Agricultural Sciences (UAS), Gandhi Krishi Vigyan Kendra, Bangalore. Plants were planted in potted soil at the institution.

2.2 Preparation of the MS Medium

Murashige and Skoog's [34] medium was used for the cultivation of *B. monnieri*, under *in vitro* conditions as reference standard medium.

The MS medium was purchased from the Sigma and final volume was made up with distilled water. The pH of the medium was adjusted to 6.0 using 1 N NaOH / HCl. About 20 ml of the medium was poured into sterile culture bottles. The culture bottles were autoclaved.

2.3 Use of aqueous extract of vermicompost as nutrient plant tissue culture medium

Three grams of vermicompost was mixed with 10 ml of distilled water, homogenized and the volume was made up to 10 ml with distilled water. Tubes were centrifuged at 3000 rpm for 10 minutes. The pH of the supernatant (extract) was made upto10 ml with distilled water and agar was added.

2.4 Modified method

Fresh vermicompost (30 per cent) was suspended in sterile distilled water and was placed on a stirrer for continuous agitation for 8 hours. After 24 hours, aqueous extract was collected containing humic and fulvic acids and was used to prepare the medium. This was supplemented with 9 grams / Litre of agar. Explants of *B. monnieri* were innoculated into this sterile medium under aseptic conditions.

2.4 Use of eluant as nutrient in plant tissue culture medium

Vermicompost (30 per cent) was suspended in known volume of distilled water and left undisturbed for 24 hours. Filtrate (eluant) was collected and used for media preparation.

2.5 Use of vermicompost as nutrient plant tissue culture medium

Vermicompost produced by the activity of epigeic earthworms on organic waste mix of plant litter, vegetable waste and cow dung slurry at the college campus was used for the present study. Vermicompost was sieved for collecting only the castings. Vermicompost (30 per cent) containing humic and fulvic acids were used to prepare the medium. Known volume of distilled water was added to vermicompost (30 percent).

All the above media samples were prepared and pH was maintained at 6.0 and was supplemented with 9 grams /Litre of agar and autoclaved. The medium was poured into washed and dried jars (approximately 50 ml) or test tubes (approximately 20 ml). They were then autoclaved at 121°C for 20 minutes at 15 psi pressure and transferred to the media storage room where they were kept under aseptic conditions till their further use.

2.6 Explant sterilization

B. monnieri chosen for *in vitro* micropropagation were washed under running tap water for 30 minutes in order to wash off the externally adhered soil, dust and other contaminants.

The nodal segments were cut into 1.5 cm to 2.0 cm length with single node and internode intact. These nodal and internodal cuttings were washed with 5 per cent (v/v) detergent solution (Teepol) for 10 minutes followed by a rinse with running tap water for several times.

In the laminar chamber the nodal segments of *B. monnieri* were further treated with 70 per cent alcohol for one minute followed by 0.1 per cent (w/v) Mercuric chloride treatment for 5 minutes. Aseptically explants were washed with sterile distilled water for three to four times and leaves were removed using sterile blade.

Sterile explants of *B. monnieri* were inoculated into the MS, vermicompost, vermicompost extract and eluant media jars. The experiments were carried out in culture rooms under 16 hours cycled cool white fluorescent 1ight of average 2500 lux (cool white fluorescent tube light 40 W) and at 25± 2 °C, with a photoperiod of 16 hours day light and 8 hours night breaks.

After 10 – 14 days of culture on rooting media, the rooted plantlets were transplanted to pots or trays for hardening prior to their final transfer to soil. After this the plants were carefully planted in the polybags containing vermicompost and soil mixtures in 1:1 ratio.

2.7 Experimental Data Analysis

ANOVA, Student's t-test and Tukey's Studentized Range or Tukey's Honestly Significant Difference (HSD) Test were considered for analyses of significance of various parameters and were carried out to compare the mean number of nodes, leaves, roots and shoots formed in the vermicompost extract, eluant and vermicompost media with respect to *B. monnieri.* The 'P' values less than 0.05 were considered as indicative of significance. The analysis was performed using SAS statistical software.

III. RESULTS AND DISCUSSION

Adventitious shoot buds were induced from nodal and stem explants of *B.monnieri* on MS basal medium supplemented with 3.0 mg/L BAP alone which showed highest rate of shoot regeneration with this treatment (Fig 1A, 1B). Low concentration of NAA combined with high concentrations of BAP resulted in shoot initiation, whereas root induction required low concentration of BAP and high amount of IAA (Table 1). It was found that the response of the explants to BAP or IAA individually or in combination with MS media was more or less the same and the final weight of the plantlets ranges 131.4 milligram to 154.0 milligram. The results have shown that the growth response in vermicompost medium was significantly higher than those from MS medium (Table 2).

Vermicompost was evaluated with different methods of extractions like use of vermicompost only, eluant and extract of vermicompost to use in the tissue culture media for micropropagation of *B. monnieri* (Fig 1D, 1E, 1F). Vermicompost extract has supported significantly higher growth of roots, shoots, nodes and leaves over the eluant and vermicompost media. The values for number of roots ranged from 4 in only vermicompost medium to 7 in vermicompost extract medium. This had F value of 92.67 and Pr > F of < 0.0001 (Table 3). Use of vermicompost as such in medium was significantly higher in the formation of shoots (Table 4). This indicates that some biomolecules that are in vermicompost to help in shoot initiation has not been extracted into vermicompost extract medium. This mean number of shoots formed in medium having vermicompost was 3.0 whereas in vermicompost extract it was 1.2. The level of significance has given the F value of 91.00 and Pr > F of <0.0001. In case of the development of nodes and leaves from the nodal explants of *B.monneiri*, vermicompost extract has significantly supported higher growth compared to eluant and only vermicompost (Table 5). The significance level was Pr > F of 0.0011 and 0.0012 respectively. Plantlets have shown significant variation among the treatments. Significant increase in the weight of the plantlets (in milligrams) was observed in vermicompost extract as compared to other two treatments (Table 6). The value for significance was at < 0.0001. Tukey's Studentized Range (HSD) Test for percentage response of survival of plantlets has shown that in all the three treatments there was 100 per cent survival of the explants (Table 7).

Some studies speculated that the growth responses of plants from vermicompost appeared more like hormone-induced activity associated with the high levels of nutrients, humic acids and humates in vermicompost[35-36].

Some studies have also reported that vermicompost contained growth promoting 'auxins', 'cytokinins' and flowering hormone gibberellins[37-39]. Growth regulators were analysed in worm castings[40]. It contained gibberellins (GA3) 2.75 microgram/gram, cytokinins (IBA) 1.05 microgram/gram and auxins (IAA) 3.80 microgram/gram. Bano *et al.* (1987) studied the nutrient status of the vermicompost (Vee Comp E 83 UAS). It was found to be rich in all mineral nutrients. It contained N (0.75 per cent), P (0.37 per cent), K (0.4 per cent),

Mg (0.38 per cent), Zn (0.16 per cent), Cu (0.02 per cent), Fe (1.38 per cent) and organic carbon (4.0 to 5.04 per cent). Vermicompost is rich in vitamins, enzymes, antibiotics and growth hormones, which provides balanced nutrients to the plant making them resistant against pests[41].

Growth promoting activity of vermicompost was tested using a plant bioassay method. The plumule length of maize (*Zea mays*) seedling was measured 48 hours after soaking in vermicompost extract and in normal water. The marked difference in plumule length of maize seedlings indicated that plant growth promoting hormones are present in vermicompost[42-43].

The addition of vermicompost, including that produced from agrowastes, medicinal, and aromatic plants[44] to soil can increase the population of beneficial microbes[45]. The earthworm castings contain higher percentage (nearly two fold) of both macro and micronutrients than the garden compost.

A two-stage culture procedure has been developed[46] for highly efficient shoot regeneration from leaf and internode explants of *B. monnieri*. Adventitious shoot buds were obtained on the shoot induction medium containing Murashige and Skoog's (MS) basal salt supplemented with 1.5 mg/L thidiazuron and 0.5 mg/L naphthalene acetic acid (NAA). Further subcultured on multiplication medium containing 0.5 mg/L BAP produced more shoots (13.5) and longer shoots (7.8 cm) with more nodes (6). Best response of root induction with more number of roots (16.5) and longer roots (8.7 cm) was observed in half strength MS basal medium supplemented with 1.0 mg/L IBA (indole-3-butyric acid) and 0.5 mg/L phloroglucinol. *In vitro* obtained plants were transferred to the field after hardening with a 100 per cent survival rate [46].

Bud initiation was seen immediately after two days of inoculation on vermicompost extract, humin and vermicompost media by showing a small newly sprouted bud, which proliferates into shoot buds with leaves during 21-25 days which were placed in the culture room under the standard conditions of temperature (25 ± 2°C). Shoot bud initiation was observed visually on the ninth day of incubation in all replicates in the MS media having different concentrations of BAP and KIN[47]. The response of the explant to the new test media is much easier than that is observed in the regular MS media used in tissue culture studies.

IV. CONCLUSION

The growth responses were most probably due to hormone-like activity of humic acids from the vermicompost. The study has indicated that by standardizing the technique, it is possible to develop the plants through micropropagation in an economical way. Cost analysis carried out during this study confirms that vermicompost is more economical (Rs. 10.227/- per litre) compared to conventional MS medium (Rs. 66.576/- per litre) used in plant tissue culture[48]. It can reach farmers as affordable plantlets, to develop in agricultural fields for mass production.

V. ACKNOWLEDGEMENTS

The authors thank Prof Rajanna (Botanical garden, Gandhi Krishi Vigyan Kendra, Bengalure) for supplying the plant samples to conduct research work. Authors thank Mount Carmel College, Bengaluru for providing lab and other chemical supplements for successful completion of the research work.

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ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

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IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

ABBREVIATIONS

Abb1: MS—Murashige and Skoog

Abb2: KIN—kinetin

Abb3: 2,4-D-2,4—Dichlorophenoxy acetic acid

Abb4: GAEs—gallic acid equivalent

Abb5: QE—Quercetin equivalents

Abb6: BA—butyric acid

Abb7: NAA—Naphthalene acetic acid

Abb8: IAA—Indole acetic acid.

Abb9: IBA- Indole-3-butyric acid

Abb10: BAP- 6-Benzyl amino purine

Table 1: Growth parameters of *B. monnieri* analysed after third week of incubation on MS Basal medium containing growth regulators

Growth Parameters	MEDIA		
	MS+IAA+BAP $\bar{X}$±SE	VC extract only	t-statistical value
Number of roots	3.8±0.2	6.8±0.2	0.00008**
Number of shoots	1.2±0.2	1.2±0.2	1.0000
Number of nodes	1.6±0.24	6.4±0.6	0.0007**
Number of leaves	3.2±0.48	12.8±1.2	0.0007**
Weight of plantlets	15.4±1.51	437±6.6	0.00007**
Percentage response	78.6±0.97	99.6±0.2	0.0000009**

Note: MS - Murashige and Skoog medium
IAA - Indole3-acetic acid
BAP - 6-Benzyl amino purine

Table 2: Student's t-test to compare the means of growth parameters of *B.monnieri* micropropagated on MS medium with hormones and vermicompost extract medium without chemical supplements

VC=Vermicompost
Note: P≤0.05: * Significance at 5per cent level
**** Significance at 1per cent level**

Table 3: Tukey's Studentized Range (HSD) Test for development of roots from nodal explants *B.monnieri* grown on vermicompost extract, eluant and vermicompost media.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Types of Media	2	18.53	9.26	92.67	<.0001
	Mean	**N**	**Tukey Grouping**		
VC extract	7.0000	5	A		
			A		
Eluant	5.0000	5	B		
			A		
VC only (30 per cent)	4.4000	5	C		

Note: VC=Vermicompost.

Table 4: Tukey's Studentized Range (HSD) Test for development of shoots from nodal explants *B.monnieri* grown on vermicompost extract, eluant and vermicompost media

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ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Types of Media	2	12.13	6.06	91.00	<.0001
	Mean	**N**	**Tukey Grouping**		
VC only (30 per cent)	3.0000	5	A		
VC extract	1.2000	5	B		
			B		
Eluant	1.0000	5	B		

Note: VC=Vermicompost.

Table 5: Tukey's Studentized Range (HSD) Test for development of nodes and leaves from nodal explants of *B.monnieri* grown on vermicompost extract, eluant and vermicompost media.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Nodes	2	18.53	9.26	12.64	0.0011
Leaves	2	72.40	36.20	12.34	0.0012
	Mean number of nodes	**Mean number of leaves**	**N**	**Tukey Grouping**	
VCextract	6.4000	12.800	5	A	
				A	
Eluant	5.8000	11.400	5	A	
VC only	3.8000	7.600	5	B	

Note: VC=Vermicompost.

IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

Table 6: Tukey's Studentized Range (HSD) Test for development weight of *B.monnieri* plantlets grown on vermicompost extract, eluant and vermicompost media.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Types of Media	2	134314.53	67157.26	39.29	<.0001
	Mean	**N**	**Tukey Grouping**		
VC extract	371.20	5	A		
Eluant	276.20	5	B		
VC only	140.60	5	C		

Note: VC=Vermicompost.

Table 7: Tukey's Studentized Range (HSD) Test for percentage survival of hardened plantlets of *B. monnieri* micropropagated on vermicompost extract, eluant and vermicompost media.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Types of Media	2	0	0	.	.
	Mean	**N**	**Tukey Grouping**		
Eluant	100.0	5	A		
			A		
VC only	100.0	5	A		
			A		
VC extract	100.0	5	A		

Note: VC=Vermicompost.

International Journal of Advance Research in Science and Engineering
Vol. No.6, Issue No. 06, June 2017
www.ijarse.com
IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

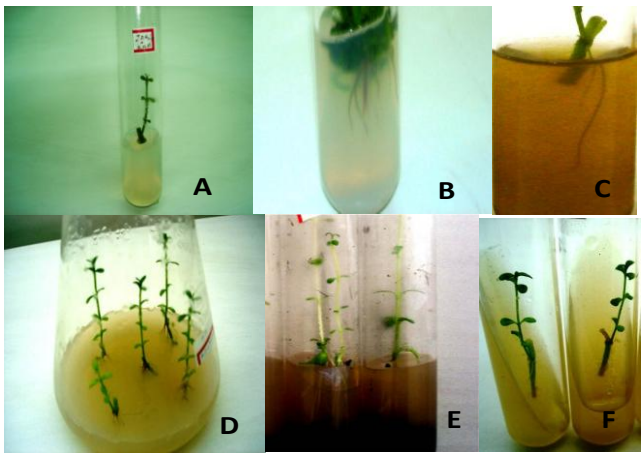

Fig 1 A: *B.monnieri* shoots developed on MS medium supplemented with 3mg/L BAP+0.5mg/I NAA
B: Rooting of *B.monnieri* on MS medium supplemented with 1mg/L BAP+3mg/L IAA
C: Rooting of *B.monnieri* on vermicompost extract medium.
D: *B.monnieri* shoots developed on vermicompost extract medium.
E: Micropropagation of *B.monnieri* on only vermicompost medium.
F: Micropropagation of *B.monnieri* on eluant.