Journal of Horticultural Science & Ornamental Plants 11 (2): 66-83, 2019
ISSN 2079-2158

DOI: 10.5829/idosi.jhsop.2019.66.83

Micropropagation, Conservation, Phytochemicals and Genetic Characterizations of *Bacopa monnieri* L. In Egypt

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Abstract: *Bacopa monnieri* is a rare and promising pharmaceutical uses plant. So, the aim of this investigation is to establish an efficient procedure for micropropagation and plant preservation *via* synthetic seeds, estimate the genetic characterization as well as, quantitative phytochemical determination. Several factors affect micropropagation, like BA and IBA concentrations, MS strength, explant types, explant orientation on the medium and acclimatization system, were examined. In the multiplication, the highest shoot proliferation and growth vigor were obtained when big cluster used as an explant and culture medium was 3/4 MS + 0.5 mg/l BA. In the rooting, the highest roots number was achieved from 2.0 mg/l IBA in the presence of AC. Roots number, root length and growth vigor significantly affected by different types of explant, the big cluster was superior in the most measured parameters. The success of the acclimatization of *B. monnieri* was enhanced by the examined acclimatization systems, the system which included culture jars with 150 ml water possessed the highest acclimatized number followed by culture mills and pots. The colour of the flowers was white in pots and field, while it was light purple in the water system. The short-term preservation *via* synthetic seeds established through examined the effect of $CaCl_2.2H_2O$ concentrations and the ions exchanged period. The round shape, good texture and the highest germinated and vital synseeds (97.00%) resulted from 100 mM of $CaCl_2.2H_2O$ when the ions exchanged period was 15 or 20 min. The high concentration of $CaCl_2.2H_2O$ and the long period of ions exchanged minimized the deterioration of germinated synthetic seeds for 150 days. Genetic characterizations of the regenerated plants after the conservation were determined through eleven RAPD and thirteen ISSR primers. The total number of amplified fragments produced by using RAPD primers was 44 AF, while ISSR produced 33 AF. The quantitative analysis *via* HPLC revealed that Chlorogenic acid and Gallic acid were the main phenolic components (329.49 µg/g and 310.88 µg /g, respectively.). While, Rutin with a concentration of 517.58 µg/g was the main of flavonoid compounds. The *B. monnieri* extract (BME) diminished the antioxidant defense system of lung carcinoma (A549) cells after treated with 100 µg/ml BME for 24 hours. At the end of treatment, treated cell *via* BME decreased glutathione (GSH) with 32% in compared with control due to GSH peroxidase. Also, there was a decrease in the level of SOD-1. The blockage may be an indicator of reduced intracellular oxidative stress supplied by BME and therefore, the expression of SOD-1 was decreased concordantly. The percentage of MDA of treated cells is higher than controls. This indicates that the lipid peroxidation occurs due to increased ROS because of BME.

Key words: *Bacopa monnieri* · Micropropagation · Acclimatization system · Preservation · Synthetic seeds · RAPD · ISSR · HPLC · GSH · SOD-1 · ROS · lung carcinoma

INTRODUCTION

Bacopa monnieri is an annual creeping herbal plant, which belongs to Family: Scrophulariaceae, commonly known as Brahmi in India. It is growing in Nepal, Srilanka, China, Taiwan, Florida, Southern States of the USA and in wet and marshy regions of the Eastern Mediterranean coastal and North Sinai, Egypt [1, 2]. It is extensively used

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in folk medicine as nerve relaxing and memory enhancing, anti-inflammatory, anti-epileptic agent, anticancer and antioxidant activities, analgesic and antipyretic [3-6]. In addition, *Bacopa monniera* contains numerous alkaloids like nicotine, brahmine and herpestine as well as bacosides A, B, C and D, which are triterpenoid principles famous as "Memory chemicals" [7-9]. Also, *B. monnieri* used in phytoremediation programs to remove heavy metals [10]. In nature, the plant needs much water requirement for its proper growth and development. [11]. *B. monnieri* is not propagated through seeds because of their short viability (two months) and seedling death frequently. Vegetative propagation using stem cuttings is slow and produces a poor performance of propagules [11, 12]. Micropropagation is an efficient method for rapid clonal propagation, a constant supply of plant material, sustainable conservation of rare plants and a new combination of high technology like gene-transformed ones [13-17].

Nutrient medium composition and salt strength affected the micropropagation of *B. monnieri*. Various explants were used in micropropagation, *i.e.*, shoot tip, nodal and leaf. Nodal explants of *Bacopa monnieri* cultured on MS were superior on those which cultured on B5 media in shoot multiplication and plantlet regeneration [18]. In addition, the number of shoots, the number of roots and callus formation differed according to different combinations of growth hormones [19]. For shoot regeneration, BA was used and found to be superior over other implemented cytokinins in some earlier reports on *B. monnieri* [20-25]. Shoots directly proliferated on MS fortified with 2.0 mg/l BA [26]. Increasing the concentration of cytokinin from 0.5 to 2 mg/l, showed a gradual increase in the number of shoots [12, 21]. The best shoot induction from nodal explant resulted from MS supplemented with 3.0 mg/l BA. Subculture the induced shoots on MS fortified with 1.0 mg/l GA_3 maximized the average shoot length (6.4 cm) [27]. Nodal explants showed better responses than the shoot tip explants and enhanced the number of multiple shoots on 1.5 mg/l BA [28]. While Asha *et al.* [24] reported that basal MS medium produced higher shoot length and elongated internodes. On the contrary, the higher BA concentration (2.5 and 3.0mg/l) inhibited the number of shoots [29]. Another report estimated that low concentrations of BA (0.2 mg/l) induced shoots multiplication [20]. The most suitable cytokinin combination for shoots multiplication was BA and Kn at 0.5 mg/l. Nodal explants more enhanced shoots number than shoot tips in the same medium composition (18.0 and 15.0, respectively) [23, 30]. While the maximum number of shoots (32.3 ± 0.41 shoots/internode) was obtained on MS basal medium supplemented with 3.0 mg/l TDZ within 30-32 days [31]. MS medium fortified with 1 mg/l BA and 0.5 mg/l IAA gave the maximum multiplied number of plantlets (10.00 ± 2.58) which was 6.1 ± 1.91 cm in length [5].

For root induction of the regenerated shoots, there was a need for auxin in the culture medium. The developed shoots produced healthy rooting at the half and the full strength of MS basal medium. [20, 29]. On MS supplemented with 0.5 mg/1 NAA and 1 mg/1 IBA, the roots were induced. Surviving percent of hardening plantlets raised to 96% of the rooted shoots. The produced plants did not show any morphological change or variation compared with the mother plant [32]. For root induction, 90% rooting plantlets was obtained from ¼ strength of liquid MS medium + 1.0 mg/l IBA + 2% sucrose [21]. In another report, the survival rate of plants which well established in the greenhouse was 100%. No morphological variations were observed and produced plants resembled the mother plants in habitat [24]. Half strength of MS medium fortified with 100 mg/l activated charcoal gave the maximum number of roots of *Bacopa monnieri* [31, 5].

In another investigation, root stimulation and acclimatization stage are done in one step to save time and cost of micropropagation which leads to eliminating of an *in vitro* rooting step [33, 34, 27]. Also, IBA showed positive effects on root induction and parameters of *Bacopa monnieri*, the best rooting achieved from MS + 2.0 mg/l IBA. During the acclimatization stage, the maximum survival percent was 90% [23, 30].

Synthetic seeds are a polymer contained embryos or explants (shoot tip or axillary bud). Synthetic seeds formation (texture, hardness and germination ability) varied according to the concentrations of sodium alginate (Na-alginate), calcium chloride ($CaCl_2.2H_2O$), duration of ions exchanged or the components which Na-alginate dissolved in [17]. Synthetic seeds can be utilized in multi biotechnology purpose such as clonal propagation with high plant uniformity, saving elite and rare plants through germplasm conservation, delivery and transportation of plantlets, easy storage with avoiding of human errors, save money and human efforts, economized the requirements of space, *etc.* [15, 5, 35]. Many medicinal climbers are broadly micropropagated and conserved *via* synthetic seeds [36-38]. The synthetic seeds formation depends on the formation of Ca-alginate polymer, which could be regulated through the concentrations of either Na-alginate or calcium chloride as well as the duration period which allowed ions exchanged to occur. Alginate

matrix composition affected the formation and the germination of synthetic seeds, 3 or 4% Na-alginate produced round synseeds with good texture and easy to be handled with 90.0% germination ability [39, 15, 31, 17]. Another research conducted that round synthetic seeds were obtained at 2.5% Na-alginate and 100 mM calcium chloride. Synthetic seeds composition affected regeneration, synseeds contained MS and sucrose was significantly superior compared with Na-alginate dissolved in water. The encapsulated shoot tips of *B. monnieri* were preserved for six months at low temperature (4°C) without growth regulators. Monthly decline of the regeneration ability of synthetic seeds was observed during storage duration [2]

Medicinal plants have applied in many nutritional, pharmaceutical, medicinal and health promoting fields; this ethnopharmacological usage confidently warrants their compatibility and biosafety for human [40]. Many traditional and modern food applications were reported for *Bacopa monnieri* powders and extracts, which could indicate the potential biosafety of Brahmi for human uses [41]. Plants ordinarily protect themselves from invaders and microorganisms via the production of secondary metabolites, which generally represent miscellaneous arrays derived from alkaloid, phenylpropanoid, isoprenoid and fatty acid/polyketide pathways [42]. Therefore, these are the main reasons for screening plants as potential sources for antioxidant agents.

Glutathione peroxidase catalyzing the removal of hydrogen peroxidase by catalyzing the reaction of it's with glutathione (GSH) as the following reaction

$H_2O_2 + 2\ GSH\ 2\ H_2O_2 + GSSG$

GSH peroxidase catalyzes the reduction of other hydroperoxides and thus it has a broader protective spectrum [43]. It is well-known the antioxidant enzyme SOD catalyzes the dismutation of O_2 into O_2 and H_2O_2 [44].

The aim of this investigation is to establish an efficient procedure for micropropagation and plant preservation *via* synthetic seeds, estimate the genetic characterization as well as, phytochemical quantitative determination..

MATERIALS AND METHODS

Source of Plant Materials: The plant seeds were introduced from U.S. National Plant Germplasm System (Accession: NSL 454600 *Bacopa monnieri* (L.) Pennell. The tissue culture and molecular investigations were implemented in Plant Biotechnology Department Laboratories.

Plant Materials: Shoot tips, nodes and clusters of the *in vitro* germinated seeds were used as explants in the investigations.

Culture Medium Composition and Sterilization: Murashige and Skoog medium (MS) [45] was used in all implemented investigations. MS medium was fortified with 30 g/l sucrose and 6 g/l agar. The pH value of the media was adjusted to 5.8 and distributed into culture jars (350 ml); 50 ml for each jar, prior to sterilization for 20 min at 121°C and pressure 1.2 Kg/cm^2.

Incubation Conditions: For all investigations, cultures were incubated at 26±2°C and light intensity 2000 lux and photoperiod was adjusted as 16/8 light and dark.

Multiplication of *Bacopa monnieri*: Effect of MS strength and BA concentrations on the multiplication and growth parameters of *Bacopa monnieri* during multiplication stage: Five shoot tips were cultured on different strengths of MS medium combined with various concentrations of benzyl aminopurine (BA) (0.0, 0.5, 1.0 and 1.5 mg/l). Each treatment included five jars as replicates and each jar contained six explants. After four weeks, shoots number, shoot length and growth vigor were recorded.

Effect of the Types of Explant on the Multiplication and Growth Parameters of *Bacopa monnieri* During Multiplication Stage: Effect of various explants; shoot tips, single nodes, double nodes, small clusters (about 3 compact cut shoots) and big clusters (about six compact cut shoots) on multiplication and growth parameters; *i.e*., shoot length (cm) and growth vigor were examined. Each treatment included five jars as replicates and each jar contained six explants; six explants of shoot tips, single nodes or double nodes, two explants of the small cluster or one explant of the big clusters.

The Effect of the Explant Orientations on Culture Medium on the Multiplication and Growth Parameters of *Bacopa monnieri* During Multiplication Stage: Effect of two orientations *i.e*., vertical and horizontal orientations, were examined.

In all investigations implemented during multiplication stage, shoots number, shoot length (cm) and growth vigor which were determined according to the method of Pottino [46], were recorded after four weeks.

Root Induction of *Bacopa monnieri*:

Determination the Effects of IBA Concentrations and Activated Charcoal on the Root Induction and Parameters of *Bacopa monnieri*: The regenerated shoot tips were transplanted into rooting medium; 3/4 MS supplemented with different concentrations of indole-3-butyric acid (IBA) (0.0, 0.5, 1.0 or 2.0 mg/l) combined with presence or absence of 1 g/l activated charcoal.

Determination the Effects of the Explant Types on the Root Induction and Parameters of *Bacopa monnieri*: The effect of explant types (shoot tips, single nodes, double nodes, small clusters and big clusters) on rooting formation were examined, different explant types were transplanted on 3/4 MS medium supplemented with the recommended dose of IBA which recommended in the previous investigation.

All rooting investigations included 10 jars for each treatment as replicates and each jar contained either 12 explants in the case of shoot tips, single nodes or double nodes or four explants in the case of small clusters or two explants in the case of big clusters. After four weeks, roots number/explant, root length (cm) and growth vigor were recorded.

Acclimatization Stage

Effect of Several Acclimatization Systems and Different Explant Types on the Success of Acclimatization and Mass Production: Plantlets resulted from different explant types, were collected from culture jars and washed using running tap water to remove any residual of rooting medium and soaked in antifungal solution (0.2% Rizolex for 5 min). To determine the suitable acclimatization system for the hardening of *Bacopa monnieri*, plantlets were transplanted into different acclimatization systems as following:

- Pots filled with garden culture medium (PGM): Garden culture medium; consisted of sand, peatmoss and perlite (1:1:1 v/v), were put in 10cm pots, after transplanting, the pots were capped with transparent polyethylene bags; after transplanting the plantlets resulted from the various explant types to provide suitable relative humidity around plantlets, then, bags were gradually removed.
- Cultural mills filled with garden culture medium (MGM); consisted of sand, peatmoss and perlite (1:1:1 v/v). Culture mill contained 96 wells. After transplanting, plantlets were watered and covered with polyethylene bag which gradually removed.
- Culture jars contained water: Jars contained 150 ml water and cup of fome as a supporting material which prevented platelets to sink were used. Plantlets were translocated in jars contained water and foam cup after removing the rooting medium and well washing of plantlets under running tap water. Culture jars which contained plantlets did not covere.

Ten explants of each type were transplanted in each system as replicate. After the acclimatization stage, survival number and percent of each treatment was calculated and shoot length (cm) were measured (cm). The acclimatized plantlets were transplanted into the field; soil medium under drip irrigation system.

Conservation *via* Synthetic Seeds

The Effect of Calcium Chloride Concentrations and the Duration of Ions Exchange on Synseeds Formation and the Conservation Period: Synseeds formation implemented through dissolve 4% (W/V) of Na-alginate in MS medium; which contained 30g/lsucrose+0.5mg/l BA and preparation of different concentrations (50, 75 and 100 mM) of $CaCl_2.2H_2O$. Sterilization of the solutions in the autoclave prior to be used. Also, preparation of *Bacopa monnieri in vitro*-nodes explants should be done. Prepared *in vitro* nodes were soaked in Na-alginate solution. Then, drops of Na-alginate solution contained nodes were dropped into different concentrations of $CaCl_2.2H_2O$ to allow the occurrence of ions exchange [15]. The effect of the ions exchange durations (10, 15 or 20 min) on the polymerization process were examined. Then synseeds were collected and washed in sterilized distilled water to remove any excess of $CaCl_2.2H_2O$ and partially air dried on sterilized tissue for two minutes. Synthetic seeds were planted in Petri dishes contained basal MS medium (10 synseeds/dish and three replicate for each treatment) and incubated in 26±2°C, 2000 lux, 16/8 light/dark photoperiod. Synthetic seeds texture and shape were observed, also, survival number and percentage of syneeds/Petri dish, germination and vital synseeds percent were recorded after 60 and 150 days.

Statical Analysis: Complete randomized design implemented in the investigations which including one or two factorials. Data were analyzed by using MSTAT software ver. 2.2. Differences among observed data were compared using the least significant difference (LSD) at 5% level [47].

Genetic Characterization of Conserved Plants: The conserved plants were genetically characterized after planted in feild using both random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) PCR based techniques.

DNA Extraction: Meristematic leaves; about 200 mg, were grounded to a fine powder using liquid nitrogen. Mini Kit of i-genomic Plant (protocol A), from iNtRON Biotechnology Co. was implemented to DNA extraction. The concentration of isolated DNA was 50 ng/µl (as described by protocol). The insurance of the quality of the isolated DNA proved through electrophoresis separation of 10 µl of isolated DNA in 1% agarose gel.

RAPD and ISSR Techniques: Eleven RAPD primers and thirteen ISSR primers (Table, 7) were got from Bio Basic Inc. used in 25 µl PCR reaction. The reaction contained 100 ng of isolated DNA, 12.5 µl master mix (i- TaqTM, iNtRON Biotechnology), RAPD or ISSR primer (2 µl) and 4 µl PCR buffer with 1.5 mM of $MgCl_2$. The final volume was 25 µl. PCR program conditions were implemented as described by Hamza *et al.* [48].

Electrophoresis of the DNA: The electrophoresis of PCR products was carried out in 1.5% agarose gel for both techniques. The agarose gels including DNA were stained using ethidium bromide as described by Sambrook and Russel [49]. To determine the DNA fragments molecular weight, the DNA ladder (1-Kb plus blue DNA Ladder, GeneOne.Co.) was used. UV transilluminator was used to photo-record the agarose gel.

Analysis of DNA Electrophoresis: The amplified fragments were recorded as present (1) or absent (0) fragments for both RAPD and ISSR primers. Data were analyzed according to Rohlf [50].

The Phytochemicals Composition of Conserved Plants *Bacopa monnieri* Extract (BME) Preparation: *Bacopa monnieri* was dried with hot air at 45°C for 24 h, then dried materials were powdered using an electrical grinder and the powder was sieved to get ~60 mesh particle size. Three grams BM were immersed in 25 ml of 70% ethanol and agitated at 230 xg for six h, using a rotary shaker. BME was filtered in a Buchner funnel through filter papers, Whatman No. 41, to eliminate the plant particles, which were re-extracted with 25 ml of the solvent and filtered and the total extracts were combined and subjected to flash evaporation at reduced pressure at 40°C to discard about 90% of solvent till constant weight was attained. The final dry extract was further dried under vacuum in a desiccator, weighed and powdered. BME powder was then suspended in distilled water, by vigorous agitation at 45°C, to reach a final concentration of 10% (w/v). Finally, the extract solution was sterilized using a syringe filter (0.22 m pore size, sterilized) and kept at 4 °C, in sterile dark bottles a described by Tayel *et al*. [51].

Quantitative Determination of BME Phytochemicals: The phytochemical analysis of BME was conducted in the food Chemistry Lab., Food Technology Research Institute, National Research Center, Giza, Egypt. The quantification of phenolic contents in BME was carried out according to the method illustrated by Spigno *et al*. [52] whereas flavonoid contents were determined according to Mattila *et al.* [53]. HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using Eclipse Plus C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase is of water (A) and 0.02% tri-floro-acetic acid in acetonitrile (B) with a flow rate of 1 ml/min. The mobile phase was programmed successively in a linear gradient as following: 0 min (80% A); 0-5 min (80% A); 5-8 min (40% A); 8-12 min (50% A); 12-14 min (80% A) and 14-16 min (80% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 µl for each of the samples. The column temperature was maintained at 35°C.

Cell Line: Human lung epithelial cells (A549) were grown in Dulbecco modified Eagle medium (DMEM) fortified with 4 mM L-glutamine, 100 U/mL penicillin/streptomycin and 10% BSA. Cell line was obtained from (VACSERA, Giza, Egypt) and was maintained at 37°C in a 5% CO2 incubator as described by Khalil *et al*. [54].

Oxidative Stress and Antioxidants Biomarkers: A549 cells were exposed to BME for 24 hours. After the treatment, cells were washed and harvested in cold phosphate buffer saline at 4°C. The harvested cell pellets were lysed in cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1% Triton and 2.5 mM sodium pyrophosphate]. Following centrifugation (15, 000 x g for 10 minutes at 4°C) the supernatant (cell extract) was maintained on ice until assayed for oxidative-stress biomarkers. The extent of membrane LPO was determined by measuring the formation of malondialdehyde (MDA)

using the method of Ohkawa *et al*. [55]. MDA is one of the products of membrane LPO. A mixture of 0.1 mL cell extract and 1.9 mL of 0.1 M sodium phosphate buffer (pH 7.4) was incubated at 37°C for 1 hour. The incubation mixture, after precipitation with 5% TCA, was centrifuged (2300 × g for 15 minutes at room temperature). The supernatant was collected; 1.0 mL of 1 %(v/v) TBA was added to the supernatant and placed in the boiling water for 15 minutes. After cooling to room temperature, the absorbance of the mixture was at 532 nm and expressed in nmol/mg protein using a molar extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$. While, GSH level was quantified using Ellman's reagent described by Ellman [56], the assay mixture consisted of phosphate buffer, DTNB and cell extract. The reaction was monitored at 412 nm and the amount of GSH was expressed in terms of nmol/mg protein. SOD activity in cells was detected by commercial determination kit (Abcam). A549 were seeded in 6-well plate at a density of 1×106 cells/well and with BME for 24 h. Control group was treated with phosphate-buffered saline (PBS) in the medium. Cells were collected and dissolved in physiological solution. Then, cells were disrupted using ultrasound equipment and centrifuged at 6000 rpm for 10 min. The supernatants were used to determined enzyme activity.

RESULTS

Effect of MS Strength and BA Concentrations on the Multiplication and Growth Parameters of *Bacopa monnieri* During Multiplication Stage: Strength of MS medium significantly affected the number of shoots/shoot tip, 3/4 MS strength was superior followed by full and 1/2 MS strength (6.0, 5.5 and 5.0 shoot/shoot tip, respectively). Also, BA concentrations positively affected shoot multiplication, the highest shoots number was observed from 1.0mg/l BA followed by 0.5 mg/l BA (6.0 and 5.9 shoots/shoot tip, respectively) with no significant differences between them (Table, 1). The interaction between MS strength and BA concentrations revealed that the highest shoots number obtained from 3/4 MS strength + 0.5 mg/l BA (6.6 shoots/shoot tip), followed by 3/4 MS +1.0 mg/l BA and 1/2 MS + 1.0mg/l BA (6.2 and 6.0 shoots /shoot tip, respectively) with no significant difference among them. Shoot length enhanced by high MS strength insignificant way. While increasing BA concentrations negatively affected the shoot length of *B. monnieri*. The combination between MS strength and BA concentrations indicated that 3/4 MS strength + 0.5 mg/l BA and full MS strength + 0.5 mg/l BA significantly augmented shoot length (12.20 and 11.40 cm, respectively). Also, growth vigor of *B. monnieri* was positively related to MS strength. While the effects of BA concentrations were not significant. The interaction between the two factors proved that full and 3/4 MS in combination with all BA concentrations ascending growth vigor which ranged from 4.40 to 4.80.

Effect of the Types of the Explant as Well as the Explant Orientations on the Growth Parameters of *Bacopa monnieri* During Multiplication Stage: Five types of explant; shoot tip, single node, double nodes, small cluster and big cluster, were examined. Explant types affected shoots proliferation; big cluster explant maximized shoot number (62.6 shoots/jar) followed by small cluster (47.0 shoots/jar), double nodes (22.8 shoots/jar), single node (11.4 shoots/jar) and shoot tip (5.3 shoots/jar). On the other hand, shoot length adversely affected, shoot tip, single node and double nodes enhanced shoot length (11.8, 12.8 and 12.4 cm, respectively) with no significant differences, while, small and big clusters inhibited the shoot length (9.4 and 8.8 cm, respectively). The reduction of shoot length which resulted from small and big clusters may be due to the high multiplication ability of these explants when compared with other explants. Growth vigor of produced shoots was improved by the most explants, except for big cluster which resulted in the lowest growth vigor (3.6) but still good vigor because of the suitable culture medium. Concerning, the explant orientation, both shoot number and shoot length were not significantly affected by explant position. While growth vigor was significantly maximized (4.9) when explants orientation was horizontal.

Determination the Effects of IBA Concentrations and Activated Charcoal on Rooting Parameters of *Bacopa monnieri*: Data in Table 3 and Figure 3 cleared that increasing IBA concentrations positively affected roots induction and growth parameters of *B. monnieri*. Also, adding activated charcoal to rooting medium enhanced rooting and growth parameters. Interaction between IBA concentrations and the presence of activated charcoal (AC) in the rooting medium revealed that, the highest roots number (7.76 roots/plantlet) obtained from 2.0 mg/l IBA in presence of AC, while the tallest root length (10.38 cm) was observed when IBA concentrations was 1.0 mg/l without AC. The highest plantlet (10.33 cm) resulted from 1.0 mg/l IBA in presence of AC.

Table 1: Effect of MS strength and BA concentrations on the multiplication and growth parameters of *Bacopa monnieri* during multiplication stage

	Shoot number /shoot tip				Shoot length (cm)				Growth vigor*			
	BA Concentrations (mg/l) (B)				BA Concentrations (mg/l) (B)				BA Concentrations (mg/l) (B)			
MS strength (A)	0.0	0.5	1.0	Mean (A)	0.0	0.5	1.0	Mean (A)	0.0	0.5	1.0	Mean (A)
Full	5.0	5.8	5.8	5.5	10.80	11.40	9.00	10.40	4.40	4.80	4.60	4.60
¾	5.2	6.6	6.2	6.0	10.60	12.20	9.40	10.73	4.80	4.60	4.60	4.70
½	5.0	5.2	6.0	5.0	9.60	8.20	7.80	8.53	3.80	3.40	3.00	3.40
Mean (B)	5.07	5.9	6.0	---	10.33	10.60	8.73	---	4.33	4.28	4.07	---
LSD at 5%	A: 0.38	B: 0.4	AxB: 0.69	---	A: 0.71	B: 0.71	AxB: 1.22	---	A: 0.38	B: NS	AxB: 0.66	---

• *Growth vigor was determined as described by Pottino [46], (5 = Excellent, 4 = very good, 3 = good, 2 = moderate and 1 = less than moderate)

Table 2: Effect of the types of explant as well as explant orientation on the multiplication and the growth parameters of *Bacopa monnieri* during multiplication stage

Treatments		Shoot number/jar	Shoot length (cm)	Growth vigor*
Explant types	Shoot tip	5.3	11.8	4.6a
	Single Node	11.4	12.8	4.8a
	Double Nodes	22.8	12.4	4.4ab
	Small cluster	47.0	9.4	4ab
	Big cluster	62.6	8.8	3.6b
LSD at 5%		1.8	1.43	0.8
Explant orientations	Vertical	24	12.96b	4.3b
	Horizontal	25	13.66a	4.9a
LSD at 5%		NS	NS	0.44

• *Growth vigor was determined as described by Pottino [46], (5=Excellent, 4=very good, 3=good, 2=moderate and 1=less than moderate)

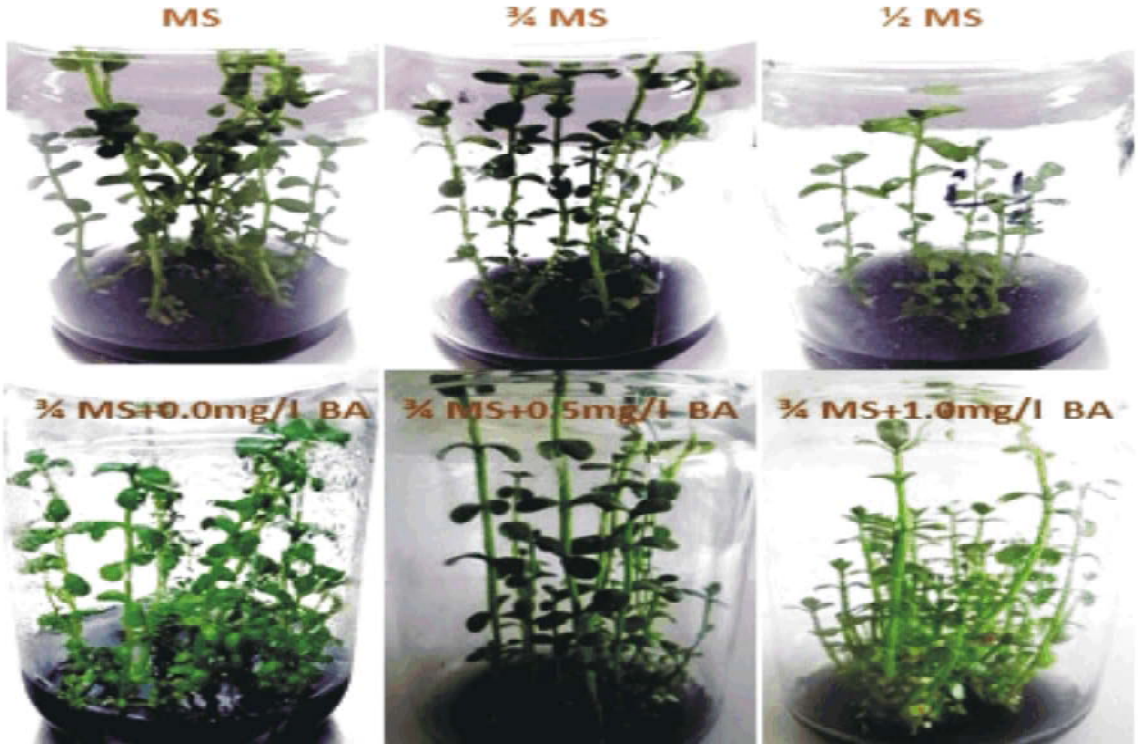

Fig. 1: Effect of MS strength and BA concentrations on the multiplication and growth parameters of *Bacopa monnieri* during multiplication stage

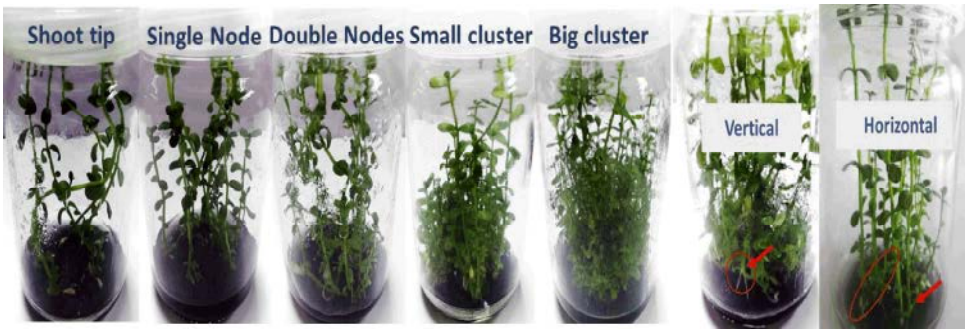

Fig. 2: Effect of the types of explant as well as explant orientation on the growth parameters of *Bacopa monnieri* during multiplication stage

Table 3: Determination the effects of IBA concentrations and activated charcoal on rooting parameters of *Bacopa monnieri*

	Root Number/plantlet			Root length (cm)			Plantlet height (cm)		
IBA conc. (mg/l)(A)	With AC	Without AC	Mean (A)	With AC	Without AC	Mean (A)	With AC	Without AC	Mean (A)
0.0	3.50	3.14	3.34	7.32	8.24	7.52	8.24	7.32	7.78
0.5	6.02	3.92	4.90	8.9	9.32	9.17	9.32	8.9	9.11
1.0	7.24	6.74	7.60	7.64	10.38	8.99	10.33	7.46	8.92
2.0	7.76	7.04	7.65	7.63	9.72	8.71	9.72	7.36	8.54
Mean (B)	6.19	5.34		7.63	9.21		9.42	7.76	
LSD at 5%	A: 0.74	B: 0.52	AxB:1.04	A: 0.2	B: 0.2	AxB: 0.7	A: 0.5	B: 0.35	AxB: 0.7

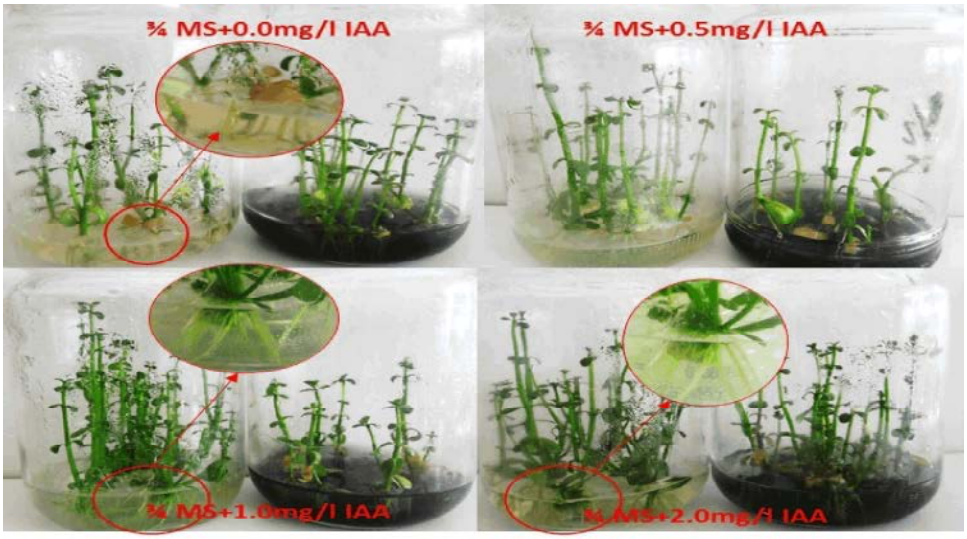

Fig. 3: Some effects of IBA concentrations and activated charcoal on the root induction and parameters of *Bacopa monnieri*

Determination the Effects of the Explant Types on Root Induction and Parameters of *Bacopa monnieri*: Roots number significantly affected by different types of explant, big cluster showed the highest roots number followed by small cluster and double nodes (54.4, 27.00, 9.69 roots/ explant) (Table 4 and Fig. 4). It seemed that presence of multi shoots in the same explant supported root induction through the production of the natural auxin (IAA) in the tips of shoots, so, the explant which included a high number of shoots produced a high number of roots. Also, root length was affected by different types of the explant. The big cluster produced the tallest roots (8.52 cm). Growth vigor slightly affected by the explant types, there were no significant differences among big cluster, small cluster, double nodes and single node.

Table 4: Determination the effects of the explant types on root induction and parameters of *Bacopa monnieri*

Treatments		Roots number/explant	Root length (cm)	Growth vigor*
Explant types	Shoot tip	3.0	4.46	11.20
	Single Node	3.2	4.42	13.48
	Double Nodes	9.6	5.44	13.98
	Small cluster	27	7.44	12.70
	Big cluster	54.4	8.52	12.80
LSD at 5%		4.39	0.23	0.90

• *Growth vigor was determined as described by Pottino [46], (5=Excellent, 4=very good, 3=good, 2=moderate and 1=less than moderate)

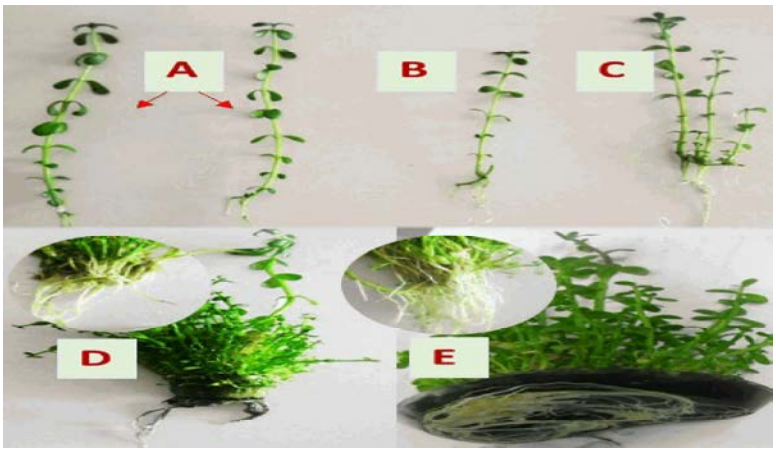

Fig. 4: The effects of the explant types on root induction and parameters of *Bacopa monnieri*
A: Shoot tip B: Single node C: Double nodes D: Small cluster E: Big cluster

Effect of Several Acclimatization Systems and Different Explant Types on the Success of the Acclimatization and the Mass Production of *Bacopa monnieri*: The success of the acclimatization of *Bacopa monnieri* significantly affected by the examined acclimatization system (Table 5 and Fig. 5 A & B). The system which included culture jars with 150 ml water (Fig. 5C) possessed the highest acclimatized number and percent (9.95 vital plantlets and 99.5% success of acclimatization) followed by acclimatization using culture mill (Fig. 5B) and pots (Fig. 5A) filled with garden culture medium (9.55 or 9.00 survival plantlets). Also, the explant type which produced the plantlet affected the success number and percent of the acclimatization, all the explant types possessed 100.0% vital plantlets with an exclusion for shoot tip and single node (84.2 and 90.8% vitality, respectively). Analysis of interaction revealed that all acclimatization system possessed 100.0% success of acclimatization when double nodes, small cluster or big cluster was the source of plantlets. Also, the single node showed 100.0% success in acclimatization when culture jars with water were the acclimatization system. It seems that the system which provided a rich humidity environment possessed the successful acclimatization system. The same trend was observed in the case of mass production which may be due to the high ability of early acclimatization which enhanced growth and resulted in high mass production. Indeed, water system incubation with big cluster resulted in the highest mass production (7.18 g). The acclimatized plantlets were followed up in the same systems until flowering and seed production (Fig. 6 A). The colour of flower was varied according to the used system; in culture jars with water, flowers were light purple, while in pots with garden cultivated plantlet in the field (Fig. 6 B), flowers were white. It is possible that the colour of flowers may be affected by environmental conditions around the plants especially the enrichment of relative humidity.

Conservation *via* Synthetic Seeds

The Effect of Calcium Chloride Concentrations and the Duration of Ions Exchanged on Synseeds Formation and the Conservation: Calcium chloride concentrations and allowed ions exchange duration affected the quality of shape and texture of synseeds. The low $CaCl_2.2H_2O$ and the small period for ions exchange produced very soft and poor formation of synthetic seeds. Actually, the good texture and shape formation of synseeds were obtained from 100 mM of $CaCl_2.2H_2O$ when the ions exchange period was 15 - or 20 min (Table 6 and Fig. 7). Synseeds affected survival number of the conserved explants.

Table 5: Effect of several acclimatization systems and different explant types on the success of acclimatization and the mass production of *Bacopa monnieri*

Treatments		Acclimatization systems(A)											
		Pots*	Mills*	Jars**		Pots*	Mills*	Jars**		Pots*	Mills*	Jars**	
Parameters		Survival number			Mean (B)	Survival %			Mean (B)	Mass production/plantlet (g)			Mean (B)
Explant types (B)	Shoot tip	7.00	8.50	8.75	8.42	70.0	85.0	87.5	84.2	2.00	3.00	3.13	2.71
	Single Node	8.000	9.25	10.00	9.08	80.0	92.5	100.0	90.8	1.80	3.30	3.70	2.93
	Double Nodes	10.00	10.00	10.00	10.00	100.0	100.0	100.0	100.0	4.10	5.15	5.23	4.83
	Small cluster	10.00	10.00	10.00	10.00	100.0	100.0	100.0	100.0	5.50	6.43	6.78	6.23
	Big cluster	10.00	10.00	10.00	10.00	100.0	100.0	100.0	100.0	5.85	6.78	7.18	6.57
Mean (A)		9.00	9.55	9.95	---	90.0	95.5	99.5	---	3.85	4.91	5.20	---
LSD at 5%		A:0.30	B: 0.23	AxB: 0.46	---	---	---	---	---	A: 0.39	B: 0.31	AxB: 0.69	---

*pots and mills filled with garden culture medium (sand, peatmoss and perlite)

**350ml culture jar contained 150ml water and cup of foam

Fig. 5 A: The first acclimatization system; Pots filled with garden culture medium (PGM):

Fig. 5B: The second acclimatization system; culture mill filled with garden culture medium

Fig. 5C: The third acclimatization system; culture jars contained water and fome cup

Fig. 6: Follow up of the acclimatized plantlets until flowering
A: culture Jar with water system (light purple flowers) and pot with garden soil (white flower) B: Flowering (white flower) after planting in the field

Table 6: The effect of Calcium Chloride concentrations and the duration of ions exchange on synseeds formation and germination along the conservation periods

Treatments	Duration of ions exchange (min)															
	10	15	20		10	15	20		10	15	20		10	15	20	
$CaCl_2$ Conc.	Survival number after 60 days			Mean	Survival number after 150 days			Mean	Germinated synseeds % after 60 days			Mean	Germinated synseeds % after 150 days			Mean
50 mM	10	10	10	10	4.43	5.47	6.33	5.41	97.00	98.33	98.67	98.00	42.00	52.33	64.00	52.78
75 mM	10	10	10	10	5.40	6.40	7.30	6.37	92.33	92.33	86.67	90.44	53.00	64.33	74.33	63.89
100 mM	10	10	10	10	7.50	8.53	9.8	8.61	91.33	81.00	72.67	81.67	73.67	85.67	97.00	85.44
Mean	10	10	10	10	3.78	6.80	7.81	---	93.55	90.55	86.00	---	56.22	67.44	78.44	---
LSD at 5%	A: Ns	B: Ns	AxB:Ns		A: 0.4	B: 0.4	AxB: 0.7	----	A: 2.18	B: 2.18	AxB: 3.78	---	A: 2.16	B: 2.16	AxB: 3.74	---

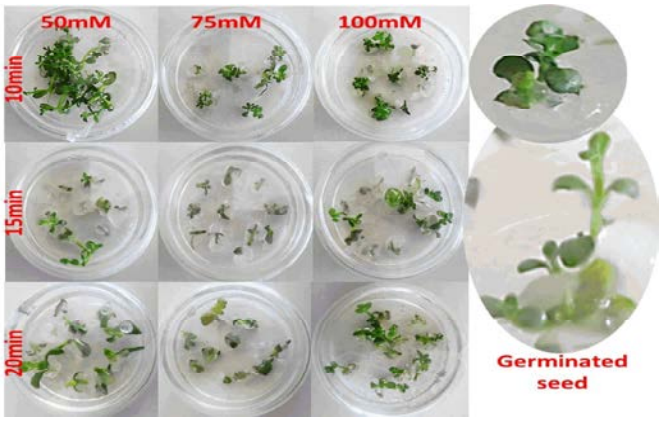

Fig. 7: The effect of Calcium Chloride concentrations and the duration of ions exchange on synseeds formation and germination along the conservation periods

After 60 days of capsulation, the survival number was 100% for all examined treatments. While, after 150 days, high concentrations of $CaCl_2.2H_2O$ positively affected survival number. Also, increasing the allowed period for ions exchange to 15 or 20 min led to maximize the survival number after 150 days. The interaction between concentrations of $CaCl_2.2H_2O$ and ions exchange durations revealed that 100 mM $CaCl_2.2H_2O$ with 20 min for ions exchange significantly enhanced survival number of synseeds (9.8 synseeds). Concerning the synseeds germination of the capsulated explants after 60 days, increasing both $CaCl_2.2H_2O$ concentrations and ions exchange period retarded the germination percent of encapsulated explants. Generally, the high concentration of $CaCl_2.2H_2O$ resulted in decreased synseeds germination percent to 81.67%. and the long period of ions exchange (20 min) negatively affected the percent of synseeds germination of encapsulated explants (86.0%). The interaction between the two factors indicated that 100 mM $CaCl_2.2H_2O$ and 20 min ions exchange period gave

Table 7: Genetic characterizations of *Bacopa monnieri* regenerated plants of after conservation using RAPD and ISSR

No.	RAPD Primer Name	Sequences	Number of Amplified Fragments	ISSR primer Name	Sequences	Number of Amplified Fragments
1	OPA-09	GGGTAACGCC	5	ISSR-2	(AC)8T	3
2	OPA-14	TCTGTGCTGG	4	ISSR-4	(GA)8 T	1
3	OPA-19	CAAACGTCGG	3	ISSR-7	(TC)8 C	2
4	OPA-20	GTTGCGATGC	3	ISSR-9	(TG)8 A	2
5	OPAF-14	GGTGCGCACT	5	ISSR-10	(CTC)6	3
6	OPAT	CAGTGGTTCC	2	ISSR-11	(AGG)5 CC	3
7	OPE-01	CCCAAGGTCC	3	ISSR15	(AC)8GA	4
8	OPE-20	AACGGTGACC	4	A12	(GA) 6 CC	5
9	OPM-01	GTTGGTGGCT	4	UBC855	(AC)8CT	3
10	OP-G6	GTGCCTAACC	4	UBC859	(TG)8GC	1
11	OPH-13	GACGCCACAC	4	RAMP-GAC	G(AC)9	1
12	---	---	---	Amic-05	CGGC (AC)6 A	2
13	---	---	---	A08	(AGC)4 GC	3
Total number of amplified fragments*			41	Total number of amplified fragments*		33

*The summation of amplified fragments of the used primers

Fig. 8: Gel electrophoresis of *Bacopa monnieri* regenerated plants after conservation using RAPD and ISSR

the lowest germination percent (72.67%) which referred to a high possibility of preservation ability of this combination. After 150 days, the vitality of the most germinated synseeds was decreased. The high concentration of $CaCl_2.2H_2O$ and the long period of ions exchanged minimized the deterioration of germinated explants. The highest germinated and vital synseeds (97.00%) was obtained when the concentration of $CaCl_2.2H_2O$ was 100 mM and duration of ions exchange was 20 min.

Genetic Characterization of Conserved Plants: Genetic characterizations of regenerated plants after conservation were determined through both the random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) PCR based techniques. Eleven RAPD primers were used to amplify the isolated DNA of *B. monnieri* (Table 7 an Fig. 8). The number of amplified DNA fragments (AF) varied according to the primer and ranged from 2 to 5 AF. The highest number of amplified fragments was obtained from OPA-09 and OPAF-14. The total number of amplified fragments produced by using RAPD primers was 44 AF. Regarding the ISSR primers, thirteen primers were used for amplified DNA fragments of *B. monnieri* (Table 7 and Fig. 8). The number of amplified fragments ranged from 1 to 5 AF. OPA-12 produced the highest amplified DNA fragments number (5AF). The total number of amplified fragments of the used ISSR primers was 33AF.

HPLC Biochemical Analysis of Phytochemical Constituents in *Bacopa monnieri* Extract: The biochemical analysis of BME content from phytochemical constituents (Table 8 and Fig. 9) revealed that the extract was very rich in its contents of phenolic compounds. The main phenolic compound in the BME Chlorogenic acid, with a concentration of 329.49 µg/g followed Gallic acid with a concentration of 310.88 µg/g. On the other hand, the lowest concentration of BME phenolic constituents was recorded for cinnamic acid. The main flavonoid compounds in BME was Rutin with a concentration of 517.58 µg/g, whereas the lowest concentrations of flavonoid compounds were for Querectin with 44.89 µg/g.

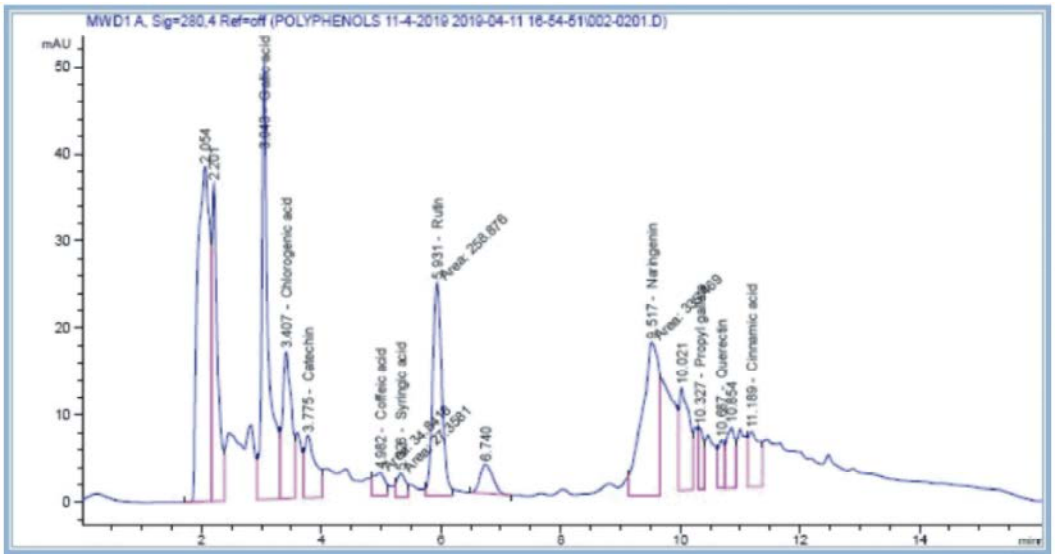

Fig. 9: HPLC biochemical analysis of phytochemical constituents in *Bacopa monnieri* extract.

Table 8: Biochemical analysis of phytochemical constituents in *Bacopa monnieri* extract

Sample (Conc.= 60 mg Plant / ml)			
	Area	Conc. (µg/ml = µg/60 mg)	Conc. (µg /g)
Gallic acid	384.89	18.65	310.88
Chlorogenic acid	177.64	19.77	329.49
Catechin	94.11	20.57	342.90
Coffeic acid	34.84	0.97	16.18
Syringic acid	27.36	0.95	15.88
Rutin	258.88	31.06	517.58
Ellagic acid	0.00	0.00	0.00
Coumaric acid	0.00	0.00	0.00
Vanillin	0.00	0.00	0.00
Ferulic acid	0.00	0.00	0.00
Naringenin	335.47	7.51	125.23
Propyl Gallate	44.03	0.95	15.84
Querectin	37.22	2.69	44.89
Cinnamic acid	84.09	0.75	12.49

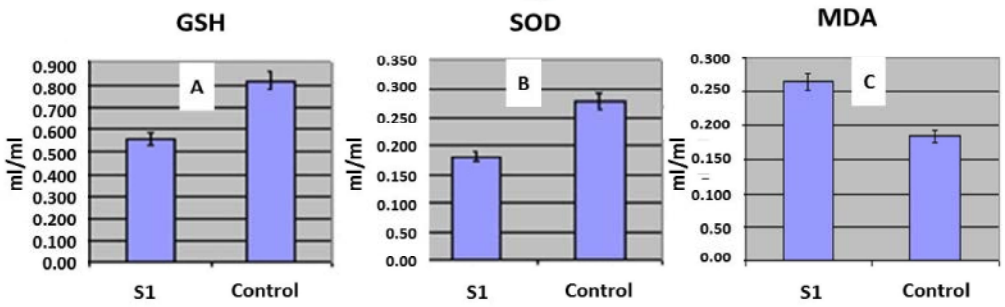

Fig. 10: Bacopa monnieri extract (BME) diminished the antioxidant defense system of lung carcinoma (A549) cells after treated with 100 µg/ml BME for 24 hours. At the end of treatment, glutathione (GSH) level and antioxidant enzyme activity were determined. (A) GSH, (B) Superoxide dismutase (SOD). (C) Bacopa monnieri extract (BME) induced oxidant generation in human lung carcinoma(A549) cells treated with 100 µg/ml BME for 24 hours. At the end of treatment malondialdehyde (MDA) levels were determined. Data represented are the mean ± standard deviation of three identical experiments made in triplicate

Oxidative Stress and Antioxidants Biomarkers: Figure (10, A) shows the Glutathione of the treatment of A549 cells at 100µg decreased compared with control cells that indicated BME has an effective scavenger against hydroperoxides. Due to GSH peroxidase with treated cell via BME decreased with 32% in compared with control. Also, after A549 cells were exposed to BME there was a decrease in the level of SOD-1 after 24 h (Fig. 10, B). The blockage may be an indicator of reduced intracellular oxidative stress supplied by BME and therefore, the expression of SOD-1 was decreased concordantly. MDA is considered to be a convenient biomarker for lipid peroxidation of omega -3 and omega -6 fatty acids due to their reaction with thiobarbituric acid (TBA). The percentage of MDA of A549 cells treated with 100µg BME is higher than controls as shown in Fig. 10, C.

DISCUSSION

Micropropagation is the first step in plant biotechnology improvement. For involving modern plant improvement techniques in plant breeding programs, an efficient micropropagation protocol should be established. Plant micropropagation of *B. monnieri* is a critical demand because it is a rare plant and has promising pharmaceutical uses which put it in the top of medicinal plant requirement and could be utilized as a major source of national income if it well manages. MS strength and BA concentrations affected the number of shoots proliferation and growth parameters of *B. monnieri*. The highest shoots number obtained from 3/4 MS strength + 0.5 mg/l BA (6.6 shoots /shoot tip), followed by 3/4 MS + 1.0 mg/l BA and 1/2 MS + 1.0 mg/l BA (6.2 and 6.0 shoots /shoot tip, respectively) with no significant difference among them. Also, full and 3/4 MS in combination with all BA concentrations ascending growth vigor which ranged from 4.40 to 4.80. Results are supported with the finding of Mehta *et al*. [23], Asha *et al*. [24], Kaur *et al*. [22], Jain *et al*. [21] and Mehta [25], who indicated that reduction MS strength and presence of low concentrations of BA in medium improved *B. monnieri* propagation. The same results obtained by Tiwari *et al*. [29], who reported that the higher BA concentration (2.5 and 3.0 mg/l) inhibited the number of shoots. Also, Explant types affected shoots proliferation; big cluster explant maximized shoot number (62.6 shoots) followed by small cluster, double nodes, single node and shoot tip. While, shoot tip and single and double nodes augmented shoot length. The orientation of explant on the medium revealed that horizontal orientation maximized the growth vigor. Results came in line with Mehta *et al*. [23] and Kumari *et al*. [30] who proved that nodal explants more enhanced shoots number than shoot tips in the same medium composition. In rooting stage, roots number, root length and growth vigor significantly affected by IBA concentrations as well as adding AC to the rooting medium; the highest roots number and the best root parameters were observed at 2.0 mg/l IBA in presence of AC. Also, different types of explant affected root induction of *B. monnieri*, the big cluster was superior in the most measured parameters. It was possible that presence of multi shoots in the same explant supported root induction through the production of the natural auxin (IAA) in the tips of shoots, so, the explant which included a high number of shoots produced the high number of roots. Results came in line with those obtained by Sharma *et al* [20]; Jain *et al*. [21]; Haque *et al*. [31] and Vijay *et al*. [5] who reported that IBA and MS strength, as well as AC, affected rooting in *B. monnieri*. The success of acclimatization of *B. monnieri* was enhanced by the three examined acclimatization systems, the system which included culture jars with 150 ml water possessed the highest acclimatized number followed by culture mills and pots. All acclimatization system possessed 100% success of acclimatization when double nodes, small cluster or big cluster was the sources of plantlets. Also, the single node showed 100% success in acclimatization when the culture jars with water were the acclimatization system. This result may be attributed to the system which provided a rich humidity environment possessed a high ability of early acclimatization which enhanced growth and resulted in high mass production. Results are supported by Pandiyan and Selvaraj [32] who possessed high survival percent of hardening (96%). Plantlets were followed up until flowering in the water system and pots, but plantlets of culture mills were transplanted on the field. The colour of the flowers was white in the pots and the field while it was light purple in the water system. It seems that the colour of the flower may be affected by the enrichment of relative humidity around the plants. Results disagreed with Asha *et al*. [24], who stated that there was no morphological variation were observed in regenerated plants from synseeds.

Conservation via Synthetic Seeds: The short-term preservation *via* synthetic seeds is an important technique concern with reducing the cost of plant conservation by reducing the need for subculture, economize the available area and save the human efforts. Synthetic seeds texture and shape varied according to

calcium chloride concentrations and the ions exchanged duration. The round shape and good texture obtained from 100 mM of $CaCl_2.2H_2O$ when the ions exchange period was 15- or 20 min. After 150 days, 100 mM $CaCl_2.2H_2O$ with 20 min for ions exchange significantly enhanced survival number of synseeds (9.8 synseeds). The high concentration of $CaCl_2.2H_2O$ and the long period of ions exchange minimized the deterioration of germinated explants. The highest germinated and vital synseeds (97.00%) was obtained when the concentration of $CaCl_2.2H_2O$ was 100 mM and duration of ions exchange was 20 min. The results agree with Dhir and Shekhawat [39]; Haque *et al*. [31] and Hamza [15, 17] who reported that alginate matrix composition affected formation and germination of synthetic seeds. Also, Hegazi [2] conducted that there was a monthly decline of the regeneration ability of synthetic seeds during storage duration. Genetic characterizations of regenerated plants after conservation were determined through eleven RAPD and thirteen ISSR primers. The total number of amplified fragments produced by using RAPD primers was 44 AF, while, ISSR produced 33 AF. Results consciences with Muthiah *et al*. [38].

The applied solvent in this study for the extraction of bioactive compounds in *Bacopa monnieri*, contained 70% ethanol and 30% water. As flavonoids are the major compounds in BME, which is soluble in water, the used solvent had a portion of water to dissolve the high amounts of total flavonoids contents. The extract was very rich in its contents of phenolic compounds; Chlorogenic acid and Gallic acid was the main phenolic components (329.49 µg/g and 310.88 µg /g, respectively.). While Rutin with a concentration of 517.58 µg/g was the main of flavonoid compounds. The results supported with Tayel *et al*. [51] and Spigno *et al*. [52].

The percentage of MDA of A549 cells treated with 100 µg BME is higher than controls. This indicates that the lipid peroxidation occurs due to increased the reactive Oxygen species (ROS) because of BME. The molecular mechanism involved is a much stronger oxidant than the superoxide anion – radical and could initiate the oxidation chain of polyunsaturated phospholipids, leading to impairment of membrane function Schneider *et al*. [44] and Skipper *et al*. [57]. Treated cell via BME decreased GSH with 32% in compared with control due to GSH peroxidase. The result agrees with Marklund *et al*. [43] who stated that this enzyme catalyzes the reduction of other hydroperoxides and thus it has a broader protective spectrum. The percentage of MDA of A549 cells treated with 100µg BME is higher than controls. This indicates that the lipid peroxidation occurs due to increased ROS because of BME. The result harmony with Skipper *et al*. [57] who concluded that the molecular mechanism involved is a much stronger oxidant than the superoxide anion – radical and could initiate the oxidation chain of polyunsaturated phospholipids, leading to impairment of membrane function.

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