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Mass propagation, Cultivation, Phytochemical and Fingerprint of *Bacopa monnieri* L. in Egypt

Mohamed F. Mohamed[1] and Ebtsam M. Hamza[2*]
[1]Medecinal and aromatic Plants Department, Horticulture Research Inistitute, Agriculture Research Center, Ministry of Agriculture and Lands Reclamation, Egypt.
[2]Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Egypt.

Bacopa monnieri (BM) is an endangered plant with great pharmaceutical uses. This work aims to improve the protocol of mass propagation, establish a cultivation system in the newly reclaimed lands and estimate the quantitative phytochemical and fingerprint of the cultivated plants. Numerous factors affected micropropagation; *i.e*, MS strength, growth regulators concentrations and types of explant. The highest shoot number and growth vigor were observed on 3/4 MS strength supplemented with 0.5 mg/l BA and big cluster as an explant. The highest roots number was obtained from 2.0 mg/l IAA and 0.1%AC. The acclimatization systems significantly affected the acclimatization of *B. monnieri*. The culture mills system possessed the highest acclimatized number followed by pots. The cultivation distance affected plant fresh and dry weight through 80 days of cultivation, 60cm cultivation distance was the best. The fingerprint of the cultivated plants was determined through eleven RAPD and thirteen ISSR primers. The number of AF differed according to the implemented molecular marker. The RAPD primers produced 34 AF, while ISSR gave 24 AF. The RAPD and ISSR were effective to determine the BM fingerprint. The quantitative analysis using HPLC stated that the main phenolic components in the ethanol/water extract were Chlorogenic acid and Gallic acid (330.36 µg/g and 309.86 µg/g, respectively). The Quercetin concentration was 42.99 µg/g. While the main flavonoid compound was rutin with a concentration of 506.36µg/g.

Keywords: *Bacopa*, Acclimatization system, Cultivation distance, RAPD, ISSR, Chlorogenic, Gallic, Rutin.

Introduction

Bacopa monnieri L. is a creeping herbal plant, known as Brahmi or jalabrahmi (Rajan et al., 2015). It belongs to Family Scrophulariaceae. It is growing in India, the Eastern Mediterranean coastal and North Sinai, Egypt (Hegazi, 2016). It is commonly used in folk medicine as an anti-aging, anti-inflammatory, anti-epileptic agent, nerve relaxing and memory enhancing, anticancer and antioxidant activities, analgesic and antipyretic (Vijay et al., 2016, Bhatia et al., 2017 and Pandey, 2022). Also, *Bacopa monniera* contains plentiful alkaloids like brahmine, nicotine, herpestine and bacosides A, B, C and D, which are triterpenoid principles known as "Memory chemicals" (Sivaramakrishna et al., 2005). In addition, *B. monnieri* is used to remove heavy metals in phytoremediation programs. In nature, the plant needs water for its growth and development (Jauhari et al., 2017). *B. monnieri* is not propagated through seeds because of their short viability (two months) and seedling death frequently. Vegetative propagation using stem cuttings produces a poor performance of propagules and it is slow growing (Ali et al., 2021). Micropropagation is an efficient method for rapid clonal propagation, a constant supply of plant material, and sustainable conservation of rare plants (Hamza, 2013 and 2019b).

The micropropagation of *Bacopa* was affected by the composition of the culture medium as well as salt strength. Different explants were implemented in micropropagation, such as shoot tip, nodal and leaf. The number of shoots, roots

Corresponding author: Ebtsam M. Hamza, E-mail:ebtsam.hamza@gebri.usc.edu.eg, Tel. +20 01009763467
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and callus formation varied according to the types and combinations of growth regulators (Koul et al., 2014). For shoot proliferation, BA was implemented and possessed a greater response than other cytokinins in earlier studies on *B. monnieri* (Mehta, 2017). MS fortified with 3.0 mg^{-1}BA maximized the shoot induction from nodal explant, which showed great responses than the shoot tip (Behera et al., 2015). Another report stated that the greatest multiplied number of plantlets (10.00 ± 2.58) was obtained from MS medium with 1 mg^{-1} BA and 0.5 mg^{-1} IAA (Haque et al., 2015). A positive response of increasing cytokinin concentration from 0.5 to 2 mg/l, was observed (Jain et al., 2014). Direct shoots proliferation was observed on MS supported with 2.0 mg^{-1} BA (Mohapatra and Rath, 2005). On the contrary, Asha et al. (2013) stated that basal MS medium possessed the tallest shoot length and enhanced internodes length. On the other side, the increase in BA concentration (2.5 and 3.0mg^{-1}) decreased the number of shoots (Tiwari et al., 2001). The explant type affected shoot proliferation, nodal explants augmented the shoots number more than shoot tips in the same medium (18.0 and 15.0 shoot/explant, respectively) (Kumari et al., 2014).

For the formation of roots on the proliferated shoots, the auxin was needed in the culture medium. The maximum number of *Bacopa monnieri* roots was obtained from 1/2 strength of MS medium supplemented with 100 mg^{-1} activated charcoal. Also, well-formed roots were observed on MS medium with 0.5 mg^{-1} NAA and 1 mg^{-1} IBA and the surviving percent of the acclimatized plantlets increased to 96% of the total plantlets (Kumari et al., 2014). In another study, for reducing the time and cost of micropropagation, root induction and acclimatization are implemented in one step which escapes the *in vitro* rooting step (Pandiyan and Selvaraj, 2012 and Phulwaria et al., 2012). Healthy roots were observed on the half and the full MS basal media strength (Tiwari et al., 2001).

Bacopa cultivation management is a major challenge. Where Bacopa cultivation is time-consuming, requires a labor team, and needs a great effort to maintain the quality of growth parameters which are affected by environmental factors such as soil, water, temperature, climate, pests, and pathogens (Panda et al., 2020).

Nowadays, medicinal plants have been applied in many nutritional, pharmaceutical, medicinal and health-promoting fields; this ethnopharmacological usage confidently warrants their compatibility and human biosafety. *Bacopa monnieri* has been used in many countries all over the world as complementary and alternative medicines, particularly for the treatment of various neurological disorders. *Bacopa monnieri* is used as a potent "tonic for the human brain," which utilizes as a memory enhancer. Numerous studies indicated that Bacopa contains an excess of potential bioactive, phytochemical compounds and has synergistic properties as well as helps in the management of neurological disorders, also it has anti-sickling, anti-paralytic and anti-venomous properties (Banerjee et al., 2021 and Pandey, 2022). It is known that 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 (Emara et al., 2019); therefore, these are the main reasons for screening plants as potential sources for antioxidant agents.

This work aims to improve the protocol of masspropagation, establish a cultivation system in the newly reclaimed lands and estimate the quantitative phytochemical and fingerprint of the cultivated plants.

Materials and Methods

Places of implementation experiments

The tissue culture and molecular investigations were implemented in the Laboratories of Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, University of Sadat City. While the field experiments were implemented in the Horticulture research station, Horticulture Research Institute, and Agriculture Research Center.

Plant materials

The explants of the investigations were taken from *in vitro* germplasm of the Department of Plant Biotechnology. The explants were shoot tips, single nodes, double nodes and clusters of shortened shoots.

Composition of culture medium and method of sterilization

Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) was implemented in all investigations. MS nutrient medium was supplemented with 30g^{-1} sucrose and 6 % phytoagar. The value of the pH of the media was adjusted to 5.8. The medium was distributed into

350 ml glass culture jars; 50 ml medium for each jar, before the medium sterilization process at 121°C and pressure of 1.2 Kg/cm^2 for 20 min.

Culture incubation conditions

The experiments were incubated at 26±2°C, 2000 lux light intensity and 16/8 light and dark as photoperiod.

Shoot multiplication

Response of Bacopa to the combination between MS strength and the concentrations of BA during the multiplication stage.

Different strengths of MS medium (Full, 3/4 and 1/2 MS strength) combined with different benzyl adenine (BA) concentrations (0.0, 0.5, 1.0 and 1.5 mg^{-1}) was implemented. Each treatment included ten jars as replicates with three explants in each jar. After 30 days, shoots number/explant and shoot length (cm) were recorded. Also, growth vigor was determined as described by Pottino (1981)

Response of Bacopa growth parameters to different types of explant during multiplication stage

Different types of explants were employed and examined during the multiplication stage, i.e., shoot tips, single nodes, double nodes, small clusters (about 3 shortened shoots) and big clusters (about six shortened shoots). Ten jars for treatment. The jar contained one type of explant. After one month, growth parameters; *i.e*., shoot length (cm) and growth vigor were recorded.

Root formation of Bacopa shoots

Estimate the effects of Indol Acetic Acid (IAA) concentrations and activated charcoal on the root formation and characters of Brahmi

The *in vitro* shoots were cultivated into a rooting medium. MS (3/4 medium strength) fortified with different concentrations of indole-3-Acetic acid (IAA) (0.0, 0.5, 1.0, or 2.0 mg/l) and the presence of 1 g/l activated charcoal were examined against the absence of AC.

Acclimatization stage

Estimate the effects of two acclimatization systems and different explant types on the acclimatization success of Bacopa

Produced plantlets from different explant types, were harvested from culture jars and were washed using tap water to remove any residual of *in vitro* medium and soaked in antifungal solution (0.1% Rizolex for 7 min). Plantlets were transplanted into two different acclimatization systems as the following:

- Pots: a 10cm pots-filled culture medium; consisting of 1:1:1 v/v sand, peat moss, and perlite were used. The plantlets resulting from the various explant types were transplanted into the pots. To provide a suitable relative humidity around plantlets, pots were covered with transparent polyethylene bags then, the bags were removed gradually.
- Cultural mills: 96 wells of culture mills were filled with culture medium, consisting of 1:1:1 v/v sand, peat moss, and perlite. The various explant types were transplanted in the wells, watered and covered with a polyethylene bag, which was gradually removed.

In each acclimatization system, ten explants of each explant type were planted as replicates. After 30 days, the number and percent of survival plantlets were calculated for each treatment. Shoot length (cm) of plantlets was measured (cm). The acclimatized plantlets were cultured into a sandy soil field with a drip irrigation system.

Effect of cultivation distance on the growth of Bacopa under drip irrigation system

The acclimatized plantlets were transplanted in the field in south El Tahrir Horticulture Research Station. The plots contained three drip hoses about four meters with different drippers distance (cultivation distances) (30, 60 and 90cm) in each one, which was considered a replicate. The experiments were designed in complete randomized plots. The data were recorded after 30 , 45 and 60 days of cultivation. The investigation included three replicates for each treatment. Plant fresh weight (g), plant dry weight (g), fingerprint and phytochemicals composition of cultivated plants were determined.

Statical analysis

The investigations were designed in a complete randomized design. The investigations included one or two factors. Results were analyzed by using the MSTAT software program ver. 2.2. Differences between the recorded data were compared according to the least significant difference (LSD) (Steel et al., 1997).

The fingerprint of cultivated plants of Bacopa

To determine the fingerprint of *Bacopa monnieri***,** the cultivated plants were genetically characterized after being planted in the field using both random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) PCR-based techniques.

DNA extraction

About 200 mg of meristematic tissues were grounded with aid of liquid nitrogen to a fine powder. Mini Kit of i-genomic Plant (protocol A), from iNtRON Biotechnology Co., was used to extract DNA. The quality of the isolated DNA was detected through the electrophoresis separation gel of DNA in agarose gel (1%).

RAPD and ISSR techniques

RAPD and ISSR primers (Table, 6) were obtained from Bio Basic Inc. The PCR reaction contained 100 ng of DNA, 12.5 µl master mix (i-TaqTM, iNtRON Biotechnology), 2 µl RAPD or ISSR primer and PCR buffer (4 µl) with $MgCl_2$ (1.5 mM). The final reaction volume was 25 µl. The PCR program was implemented as described by Hamza et al. (2017).

Electrophoresis of the DNA

The electrophoresis was implemented in 1.5% agarose gel for both RAPD and ISSR techniques. The agarose gel was stained with ethidium bromide as described by Sambrook and Russel (2001). The DNA ladder (1-Kb plus blue DNA Ladder) from GeneOne.Co. was used to determine the molecular weight of the DNA fragments. The stained agarose gel was photographed with a UV transilluminator after electrophoresis.

Analysis of DNA Electrophoresis

For RAPD and ISSR primers, the amplified fragments (AF) were recorded as found (1) or not found (0) fragments. Data were analyzed as described by Rohlf (2005).

The phytochemicals analysis of cultivated plants

Bacopa extract (BE) preparation

Bacopa herp was air predried, then fully dried in the oven at 45 °C for 24 h. The dried materials were powdered and sieved to get ~ a 60 mesh particle size. Bacopa powder (3g) was soaked in 25 ml of 70% ethanol and shaked using a rotary shaker at 230 xg for six h. Brahmi extraction was filtered through filter papers (Whatman No. 41) by a Buchner funnel. Then, the filtrated plant materials were re-extracted with 25 ml of the solvent, then filtered again. The total extracts were collected and subjected to flash evaporation, which was exposed to reduced pressure at 40 °C to discard about 90% of solvent and reached the constant weight. In a desiccator and under vacuum, the final dry extract was well dried, weighed and grounded into a powder. 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 as described by Chan et al., (2007).

Quantitative determination of Bacopa Ethanol/ Water extract (BE/WE) phytochemicals

The phytochemical analysis of Ethanol/Water extract (BE/WE) was carried out in the Central Lab. of the Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI) and the Central Lab. of the Medicinal and Aromatic Plants Department, Horticulture Research Institute, Agriculture Research Cente. The phenolic contents quantification of BME/WE were carried out according to the method described by Spigno et al., (2006), whereas flavonoid contents were illustrated according to Mattila et al., (2000). HPLC analysis was implemented using an Agilent 1260 series. the Eclipse Plus C18 column (4.6 mm x 250 mm i.d., 5 µm) was used for The separating. 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 stable at 35°C.

Results

Response of Bacopa to MS strength and concentrations of BA during multiplication stage

Data in Fig. 1 and Photo 1 estimated that MS medium strength significantly affected the number of shoots, 3/4 MS medium maximized shoots number compared with full MS and 1/2 MS. Concentrations of BA also positively enhanced the shoots multiplication, and the number of shoots was maximized with 1.0mg^{-1} BA followed by 0.5 and 0.0 mg^{-1} BA (5.9, 5.8 and 5.8 shoots/ shoot, respectively). Anyway, the differences between BA concentrations were not significant. The interaction data cleared that the highest shoot proliferation has resulted from 3/4 MS strength and 0.5 mg^{-1} BA (6.5 shoots/explant), followed by the other MS strength with 1.0 mg^{-1} BA, with no significant difference among them. Shoot length was augmented by the high MS strength insignificant way. While there was a negative relationship between BA concentrations and shoot length of *B. monnieri*. The interaction results

showed that 3/4 MS strength supplemented with 0.5 mg^{-1} BA and full MS strength fortified with 0.5 mg^{-1} BA significantly ascending the shoot length (11.8 and 11.2 cm, respectively). *Bacopa* growth vigor was affected by MS strength, while, the effect of the BA concentrations was not significant. Data of the interaction showed a significant increase in growth vigor as a result of full and 3/4 MS with all concentrations of BA.

Response of Bacopa growth parameters to different types of explant during multiplication stage

Different types of explant were examined (Table 1 and Photo, 2). Big cluster significantly enhanced shoot number and cleared high proliferation shoots than all examined explant i.e., small cluster, double nodes, single node and shoot tip (66.3, 47.0, 22.4, 11.8, 5.6 shoots/jar, respectively). On the contrary, the negative resonance of shoot length was observed as a result of high shoot proliferation. Where, small and big clusters inhibited the shoot length (8.3 and 7.8 cm, respectively) compared with other examined explants. The shoot length reduction of the small and big clusters may be attributed to the high shoot proliferation of these explants compared with other explants. All explants possessed high growth vigor with exception of the big cluster (3.6).

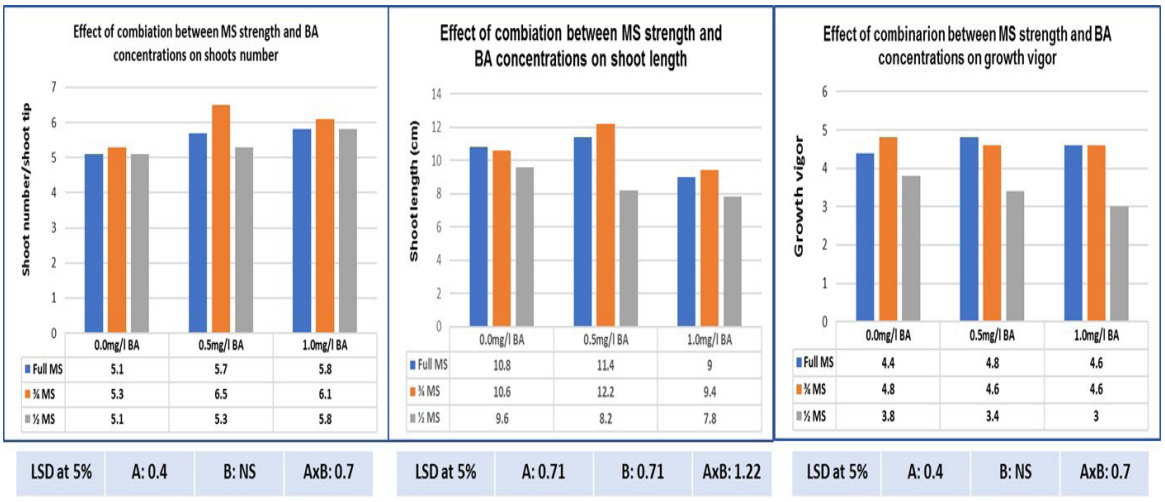

Fig. 1. Response of *Bacopa* to the combination between MS strength and BA concentrations during the multiplication stage.

*Where: Growth vigor was determined as described by Pottino 1981, (5=Excellent, 4=very good, 3=good, 2=moderate and 1=less than moderate)

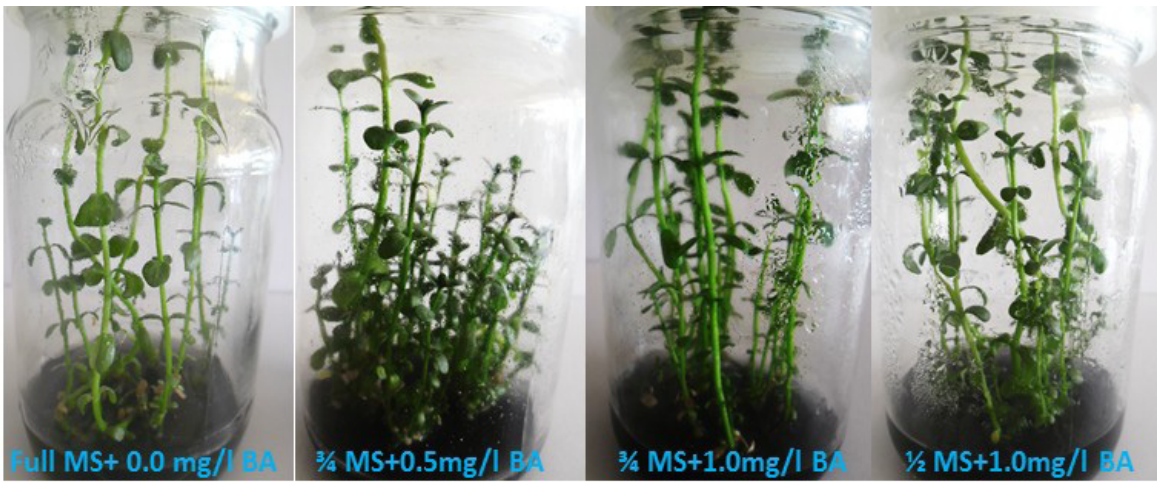

Photo. 1. Response of Bacopa to the combination between MS strength and BA concentrations during the multiplication stage

TABLE 1. Response of *Bacopa* growth parameters to different types of explant during multiplication stage

Explant types	Shoot number/jar	Shoot length (cm)	Growth vigor*
Shoot tip	5.6	12.4	4.6
Single Node	11.8	12.8	4.8
Double Nodes	22.4	11.4	4.4
Small cluster	47.0	8.3	4.4
Big cluster	66.3	7.8	3.6
LSD at 5%	1.8	1.43	0.8

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

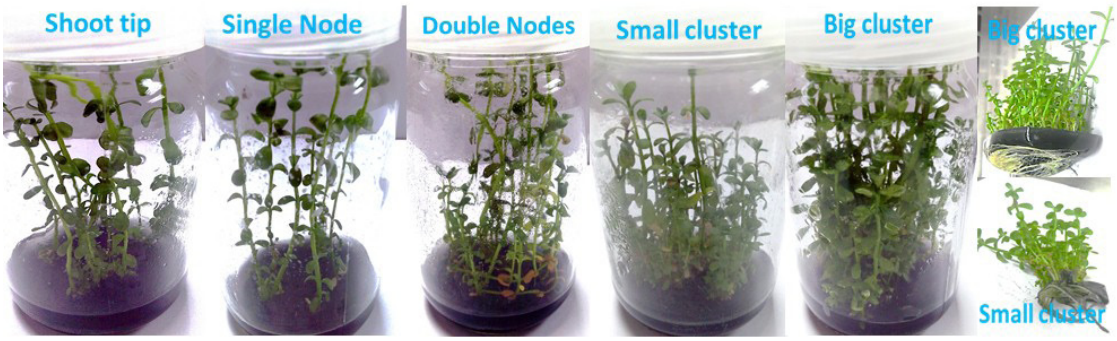

Photo 2. Response of *Bacopa* growth parameters to different types of explant during multiplication stage.

Estimated the effects of Indol Acetic Acid (IAA) concentrations and activated charcoal on the root formation and characters of Bacopa monnieri

Data in Table 2 and Photo 3 stated that increasing IAA concentrations lead to increase roots formation and growth parameters of *B. monnieri*. Also, the presence of activated charcoal augmented the root formation as well as growth parameters. Interaction between the concentrations of IAA and activated charcoal (AC) resulted in a significat effect on root formation. The highest roots number (7.79 roots/plantlet) was obtained from 2.0 mg^{-1} IAA in presence of AC. While the tallest root length (10.35 cm) has resulted from MS+1.0 mg/l IAA without AC. The highest plantlet (10.53 cm) was observed on MS+1.0 g^{-1} IAA in presence of AC.

Estimated the effects of two acclimatization systems and different explant types on the acclimatization success and mass production of Bacopa monnieri

Different responses to the examined acclimatization system were observed. The acclimatization system significantly affected the success of the *Bacopa monnieri* acclimatization (Table 3 and Photo, 4 A, B & D, E). The culture mill acclimatization system (Photo, 4A) was significantly superior in survival numbers when compared with the pots system (Photo, 4 D) filled with garden culture medium (9.60 or 9.10 survival plantlets, respectively). Also, the explant type which produced the plantlet affected the successful number and percent of the acclimatization, shoot tip, and single node gave the lowest values (73.0 and 86.3% vitality, respectively), while double nodes, small and big clusters possessed 100% vital plantlets. Analysis of interaction revealed that all acclimatization system possessed 100% success of acclimatization when double nodes, small cluster, or big cluster was the source of plantlets. Also, the single node showed 100% success in acclimatization when culture jars with water were the acclimatization system. It seems that the system which provided a rich humidity environment success in stay plantlets vital and ensures the success of acclimatization. Also, mass production appeared to the same trend which may

be due to the high ability of early acclimatization which increased growth and resulted in high mass production. It could be concluded that water system incubation with a big cluster resulted in the highest mass production (6.78 g). The acclimatized plantlets were followed up in the same systems for one month after the success of acclimatization (Photo, 4 C, F).

Effect of cultivation distance on the growth of Bacopa monnieri (BM) under drip irrigation system

The acclimatized plantlets were transplanted into the field in sandy soil (Table 4 and Photo, 5). In the first three weeks, all plants in different treatments slowly grew, then, the different drippers distance (cultivation distances) affected the growth of plants, the 30cm distance enhanced the growth and fresh weight compared with 60 and 90cm cultivation distances after 40 days(300, 250 and 200 g, respectively). While the growth of the plants, as well as the fresh weight, improved after 60 days in both 30 and 60 cm (660 and 600 g, respectively) while the 90 cm gave the lowest growth and fresh weight (380g). The same trend was observed for plant dry weight, plants cultivated on 30cm cultivation the distance was superior after 40 and 60 days of cultivation (85 and 215g, respectively). While 60cm cultivation distance gave the best dry weight after 80 days (586g). it seems that the low cultivation distance saves a suitable humidity for BM plants during the first 60days of cultivation then the plants grew and cover a big area which helps to save humidity for the 60cm cultivation distance. On the other hand, the 90cm cultivation distance failed in saving suitable humidity which led to slow growth of BM.

TABLE 2. Estimated the effects of Indol Acetic Acid (IAA) concentrations and activated charcoal on the root formation and characters of *Bacopa monnieri.*

IAA conc. (mg/l)(A)	Root Number/plantlet			Root length (cm)			Plantlet height (cm)		
	With AC	Without AC	Mean (A)	With AC	Without AC	Mean (A)	With AC	Without AC	Mean (A)
0.0	3.40	3.12	3.26	6.92	8.54	7.73	8.44	7.52	7.98
0.5	6.00	4.12	5.06	8.54	9.25	8.90	9.42	9.00	9.21
1.0	7.33	6.84	7.09	7.54	10.35	8.95	10.53	7.85	9.19
2.0	7.79	7.10	7.45	6.75	9.62	8.19	9.85	7.96	8.91
Mean (B)	6.13	5.29		7.43	9.44		9.56	8.08	
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

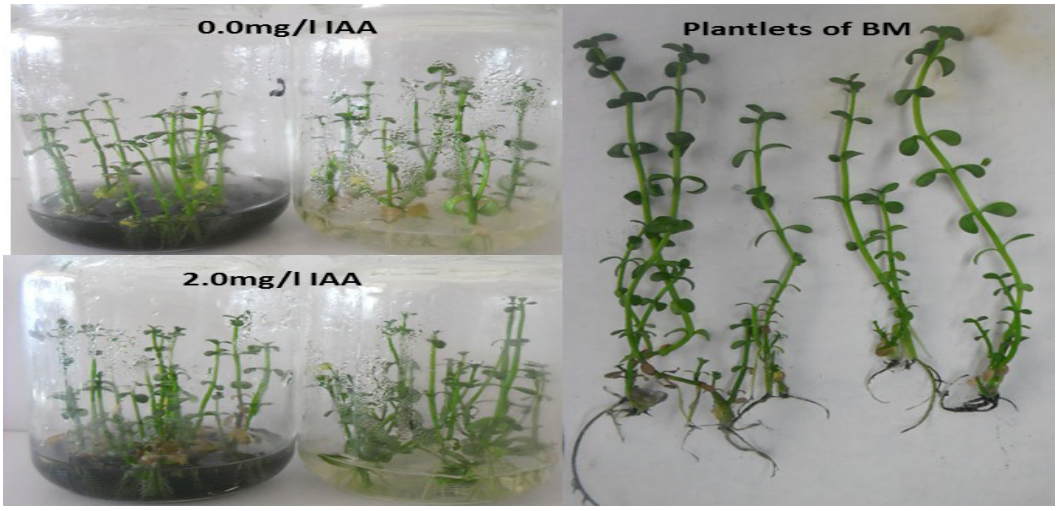

Photo 3. Estimated the effects of Indol Acetic Acid (IAA) concentrations and activated charcoal on the root formation and characters of *Bacopa monnieri.*

TABLE 3. Estimated the effects of two acclimatization systems and different explant types on the acclimatization success and mass production.

Treatments		Pots*	Mills*	Mean	Pots*	Mills*	Mean	Pots*	Mills*	Mean
Parameters		Survival number			Survival %			Mass production/ plantlet (g)		
Explant types(B)	Shoot tip	7.2	8.7	7.95	60.0	86.0	73.0	2.00	3.00	2.50
	Single Node	8.1	9.3	8.7	80.0	92.5	86.3	1.80	3.30	2.55
	Double Nodes	10.0	10.0	10.0	100.0	100	100	4.10	5.15	4.63
	Small cluster	10.0	10.0	10.0	100.0	100	100	5.50	6.43	5.97
	Big cluster	10.0	10.0	10.0	100.0	100	100	5.85	6.78	6.29
Mean		9.10	9.60		88.0	95.7		3.85	4.93	
LSD at 5%		A:0.3	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).

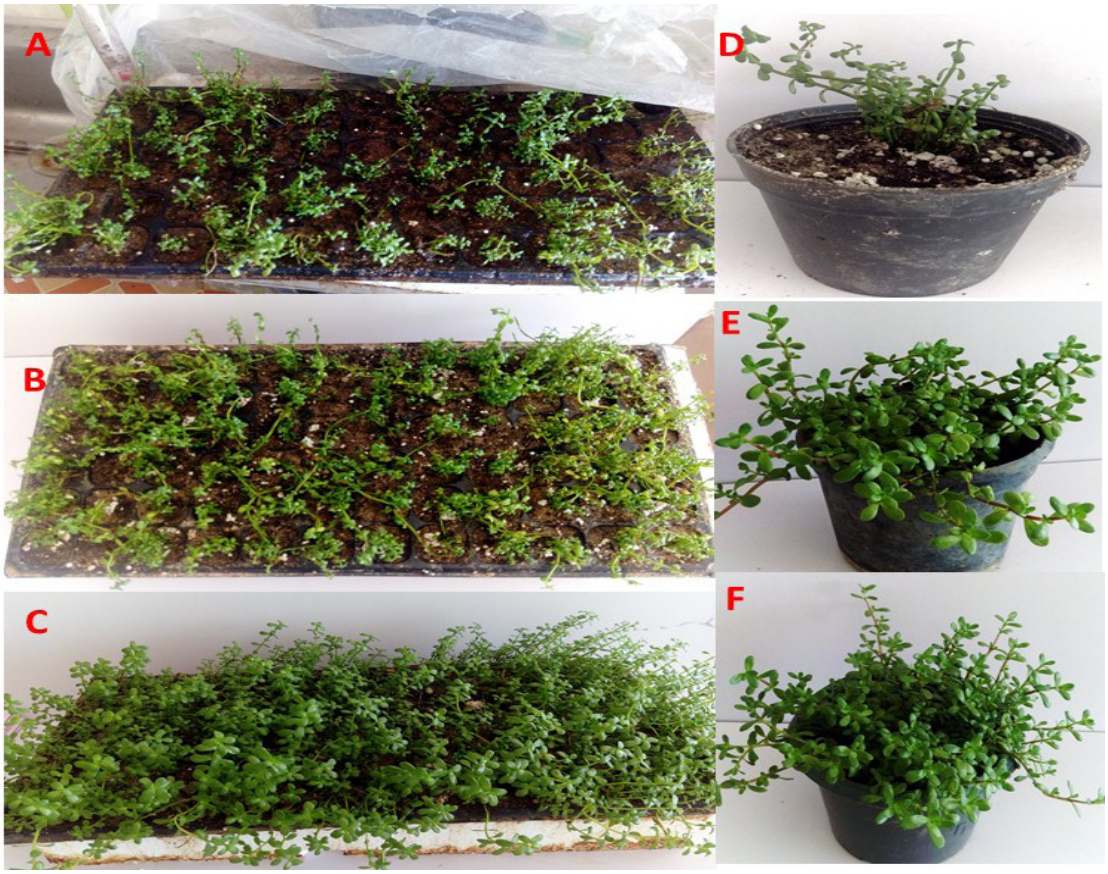

Photo 4. A, D: The acclimatization systems; culture mill filled with garden culture medium and Pots filled with garden culture medium (PGM), B, E: both acclimatization systems after removing the poly ethylene Bags, C, F: both acclimatization system after one month of acclimatization.

TABLE 4. Effect of cultivation distance on the growth of *Bacopa monnieri* (BM) under drip irrigation system .

Cultivation distance(A)	Plant fresh weight (g)				Plant dry weight (g)			
	The period after cultivation (day)			Mean (B)	The period after cultivation (day)			Mean (B)
	40	60	80		40	60	80	
30cm	300	660	950	636.7	85	215	494	264.0
60cm	250	600	1200	683.3	81	213	586	293.3
90cm	200	380	580	386.7	65	123	162	116.7
Mean	250.0	546.7	910.0		77.0	183.7	414.0	
LSD at 5%	**A:58.6**	**B:125.4**	**AxB:254.3**		**A:43.5**	**B:51.6**	**AxB:96.4**	

Photo, 5. Effect of cultivation distance on the growth of *Bacopa monnieri* (BM) under drip irrigation system in sandy soil.

The fingerprint of cultivated plants of Bacopa monnieri

After three months of cultivation, the genetic characterizations of cultivated plants were estimated via two molecular markers; the RAPD and ISSR PCR-based techniques. For amplifying the extracted DNA of *B. monnieri*, eleven RAPD primers were used (Table 6 and Fig. 6). The total number of amplified DNA fragments (TAF) for each primer varied according to the used molecular marker. TAF ranged from 2 to 5 AF. The primers OPA-09, OPA-14 and OPAF-14 gave the highest number of amplified fragments (5,4 and 4 AF, respectively). The eleven RAPD primers produced 34 AF as a total number of amplified fragments. While the thirteen primers of ISSR (Table 7 and Fig. 8) amplified 24 AF. the highest number (4AF) resulted from UBC855.

HPLC chemical analysis of Bacopa monnieri phytochemical constituents in Ethanol/water extract

The chemical analysis of the content of phytochemical constituents of Bacopa moniera Ethanol/water Extraction (BME/WE) (Table, 6 and Figure, 2) revealed that the extract contained high contents of phenolic compounds. Chlorogenic acid was the main phenolic compound in the BMW/EE at a concentration of 330.36 µg/g, followed by Gallic acid (309.86 µg/g). On the other hand, the lowest concentration of BME/WE phenolic constituents was measured for cinnamic acid. Rutin was the main flavonoid compound in BMW/EE with a concentration of 506.36µg/g, whereas Quercetin with a concentration of 42.99 µg/g possessed the lowest concentrations of flavonoid compounds of BME phytochemical contents.

TABLE 5. Fingerprint of *Bacopa monnieri* cultivated plants according to RAPD and ISSR molecular markers

No.	RAPD Primer Name	Sequences	Number of Amplified Fragments	ISSR primer Name	Sequences	Number of Amplified Fragments
1	OPA-09	GGGTAACGCC	4	ISSR-2	(AC)8T	1
2	OPA-14	TCTGTGCTGG	5	ISSR-4	(GA)8 T	2
3	OPA-19	CAAACGTCGG	1	ISSR-7	(TC)8 C	2
4	OPA-20	GTTGCGATGC	3	ISSR-9	(TG)8 A	2
5	OPAF-14	GGTGCGCACT	4	ISSR-10	(CTC)6	2
6	OPAT	CAGTGGTTCC	2	ISSR-11	(AGG)5 CC	2
7	OPE-01	CCCAAGGTCC	2	ISSR15	(AC)8GA	2
8	OPE-20	AACGGTGACC	3	A12	(GA) 6 CC	2
9	OPM-01	GTTGGTGGCT	4	UBC855	(AC)8CT	4
10	OP-G6	GTGCCTAACC	3	UBC859	(TG)8GC	1
11	OPH-13	GACGCCACAC	3	RAMP-GAC	G(AC)9	1
12				Amic-05	CGGC (AC)6 A	1
13				A08	(AGC)4 GC	2
Total number of amplified fragments*			34	Total number of amplified fragments*		24

*The summation of amplified fragments of the used primers

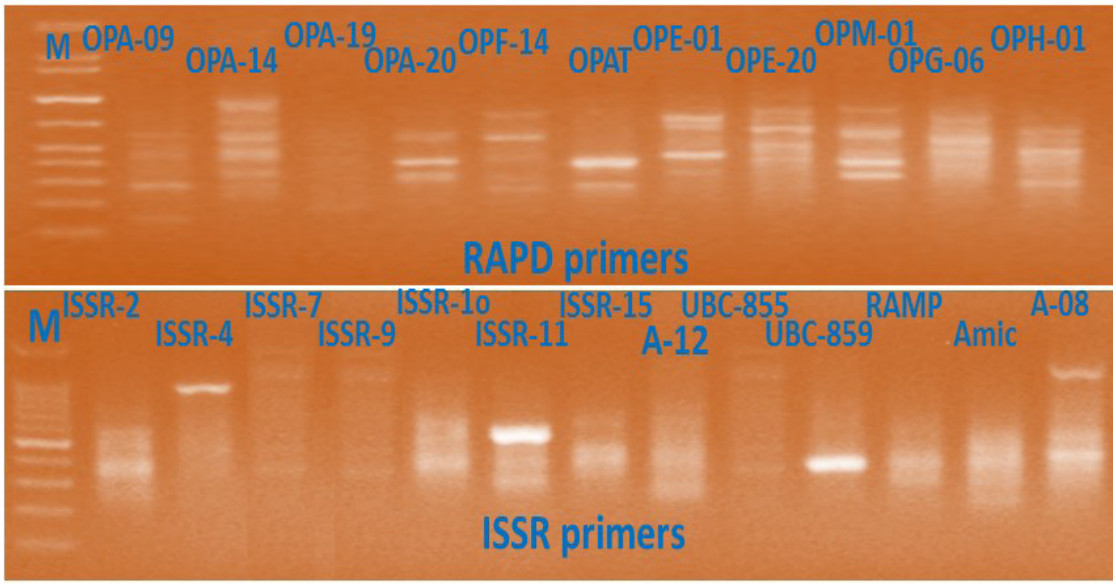

Photo 6. Gel electrophoresis of *Bacopa monnieri* cultivated plants after three months of cultivation using RAPD and ISSR

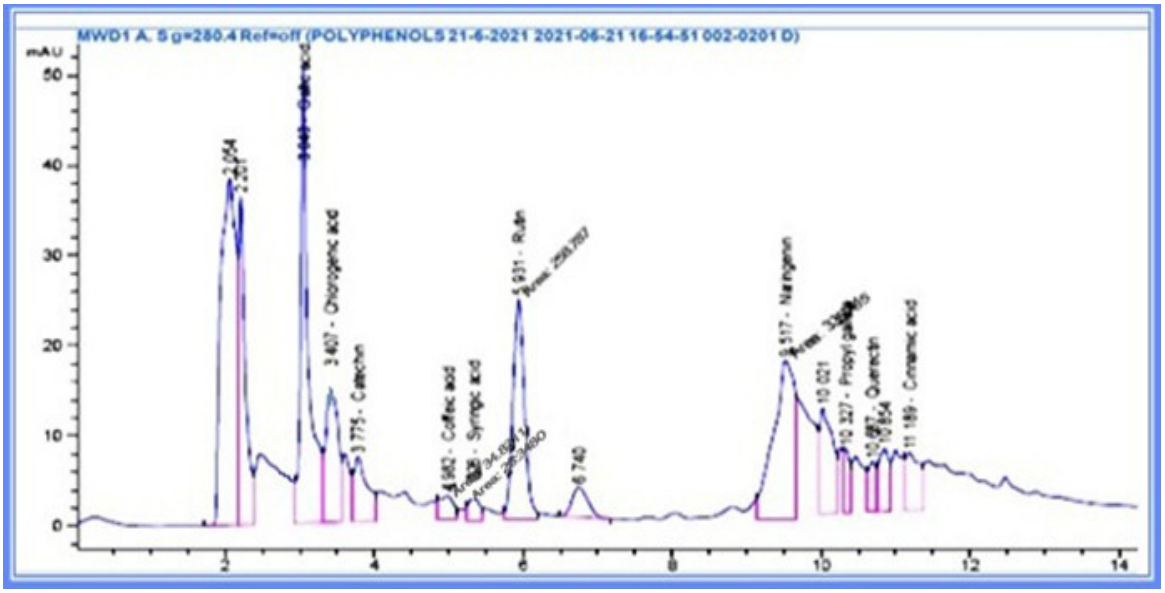

Fig. 2. HPLC chemical analysis of *Bacopa monnieri* phytochemical constituents in Ethanol/water extract.

TABLE 6. HPLC chemical analysis of *Bacopa monnieri* phytochemical constituents in Ethanol/water extract

Sample (Conc.= 60 mg Plant / ml)			
	Area	**Conc. (µg/ml = µg/60 mg)**	**Conc. (µg /g)**
Gallic acid	382.89	18.69	309.86
Chlorogenic acid	176.81	19.87	330.36
Catechin	93.99	20.63	343.10
Coffeic acid	34.84	0.97	16.18
Syringic acid	27.36	0.95	15.88
Rutin	218.68	31.06	506.36
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	36.21	2.69	42.99
Cinnamic acid	84.09	0.75	12.49

Discussion

Plant mass propagation of *B. monnieri* is a great demand because it is a rare plant and has promising pharmaceutical uses, which give it great importance among medicinal plant requirements. It may be utilized as a major source of national income if it is well managed. MS salt strength and concentrations of BA affected the number of shoots multiplication and growth parameters of *B. monnieri*. The highest shoots number resulted from 3/4 MS strength + 0.5 or 1.0 mg/l BA (6.5 and 6.1 shoots /explant, respectively), followed by 1/2 MS + 1.0 mg/l BA (5.8 shoots /explant) with no significant difference among them. Also, full and

3/4 MS in combination with all BA concentrations enhanced growth vigor which ranged from 4.6 to 4.8. Results supported the finding of Jain et al. (2014) and Asha et al. (2013), who reported that decreasing MS strength in combination with low concentrations of BA improved *B. monnieri* multiplication. The same results were obtained by Tiwari et al., (2001), who reported that the higher BA concentration (2.5 and 3.0 mg/l) decreased the number of shoots. Also, shoots proliferation was affected by explant types. Big cluster explant enhanced shoot number followed by small cluster, double nodes, while, single node and shoot tip gave the lowest response. On the other hand, shoot tip and single and double nodes ascending shoot length. Results support the obtained results of Ali et al. (2021), Hamza and Abd El-Maksoud (2019), Kumari et al. (2014) and Asha et al. (2013) who stated that nodal explants were the most enhancer for shoots number. The rooting stage cleared that roots number, root length and growth vigor significantly enhanced by IAA concentrations as well as adding AC to the rooting medium; the best roots number and parameters were obtained at 2.0 mg/l IAA in presence of AC. Results agree with those obtained by Mehta (2017), Vijay et al. (2016), Haque et al. (2015), and Jain et al., (2014) who reported that auxin and MS strength, as well as AC, affected rooting in *B. monnieri*. The acclimatization success of *B. monnieri* was ascended by the two examined acclimatization systems, the system which included culture mills possessed the highest acclimatized number. All acclimatization system possessed 100% success of acclimatization when double nodes, small cluster, or big cluster was the sources of plantlets. This result proved that the system which provided a suitable humidity environment possessed a high and early acclimatization which enhanced growth and gave high mass production. Results came in line with Pandey (2022) and Pandiyan and Selvaraj, (2012) 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. Cultivation distance affected the growth of BM plants in the field as well as fresh and dry weight 60cm cultivated distance gave the highest fresh and dry weight after 60days.

Genetic characterizations (fingerprint) of cultivated plants after 60 days of cultivation were determined through eleven RAPD and thirteen ISSR primers**.** The total number of amplified fragments produced by using eleven RAPD primers was 34AF, while, thirteen ISSR primers produced 24AF. RAPD and ISSR are successful molecular markers to give a sufficient Fingerprint for BM. Results consciences with Panda et al., (2020), Hamza (2019a), Hamza and Abd El-Maksoud (2019) and Muthiah et al. (2013) and. For the extraction of bioactive compounds in *Bacopa monnieri*, ethanol (70%) and water (30%) were used as a solvent in this study. Flavonoids are the major compounds in BE, and where it is soluble in water, the used solvent had a portion of water to dissolve the high amounts of total flavonoid contents. The extract was very rich in its contents of phenolic compounds; Chlorogenic acid and Gallic acid were the main BE phenolic components (329.49 µg/g and 310.88 µg /g, respectively.). While the main BME flavonoid compound was rutin with a concentration of 517.58 µg/g. The results supported by Pandey (2022), Banerjee et al. (2021), Hamza and Abd El-Maksoud (2019), Bhatia et al. (2017), Spigno et al. (2006) and Mattila et al. (2000) who stated that Bacopa extraction is rich with multi-active constituents, especially alkaloids and antioxidant.

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Conflicts of interest

The authors declare that there are no conflicts of interest related to the publication of this study.

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الإكثار الدقيق والاستزراع والصفات الوراثية والفيتوكيميائية لنبات الباكوبا مونييرا المنزرع في الأراضى المصرية

محمد فريج محمد[١] و ابتسام مبارك حمزة[٢]

[١] قسم النباتات الطبية والعطرية - معهد بحوث البساتين - مركز البحوث الزراعية - وزارة الزراعة واستصلاح الاراضى - مصر.

[٢] قسم البيوتكنولوجيا النباتية - معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية - جامعة مدينة السادات - مصر.

الباكوبا مونيرا نبات معرض للانقراض، ذو استخدامات دوائية عظيمة. تهدف الدراسة الى تأسيس بروتوكول فعال للاكثار المعملى للنبات، وكذلك دراسة نظم استزراع الباكوبا وتحديد البصمة الوراثية للنباتات الناتجة وكذا دراسة المركبات الفعالة بالنبات تحت ظروف الزراعة في الأراضى الرملية. حيث تؤثر العديد من العوامل على الاكثار الدقيق للباكوبا، منها قوة بيئة التضاعف (بيئة موراشيج وسكوج)، تركيز منظمات النمو، ونوع المنفصل النباتى. اثناء مرحلة تضاعف النبات، أوضحت النتائج أن أعلى عدد للفروع لوحظ عند استخدام ثلاثة ارباع قوة الأملاح بالإضافة إلى ٠,٥ مليجرام /لتر من البنزيل أدينين واستخدام الكلاستر الكبير(Big cluster) كمنفصل نباتى. بينما نتتج أفضل مجموع جذرى عند استخدام اندول حمض الخليك بتركيز ٢ملليجرام/لتر في وجود ٠,١٪ فحم نشط. كما أثرت النظم المستخدمة للأقلمة على نسبة نجاح الأقلمة، حيث حققت الاقلمة في صوانى الزراعة أعلى عدد للنباتات الناجحة في الاقلمة تلتها النباتات المنزرعة في قصارى. وفى الحقل، أثرت مسافات الزراعة على الوزن الطازج والجاف للنبات ، حيث حققت مسافة الزراعة ٦٠ سم بين النباتات أفضل قراءات. تم تحديد البصمة الوراثية للنباتات الناتجة باستخدام اثنان من المعلمات الوراثية المعتمدة على الPCR وهى RAPD، ISSR ، حيث استخدم أحد عشر بادئ (Primers) من RAPD وثلاثة عشر بادئ من ISSR . أختلف عدد القطع المتضاعفة من الدنا طبقا للمعلم الوراثى المستخدم، حيث نتج عدد ٤٤ قطعة متضاعفة من الدنا من بادئات RAPD و ٣٣قطعة متضاعفة من الدنا من بادئات ISSR. تعد المعلمات الوراثية RAPD، ISSR معلمات فعالة لتحديد الصفات الوراثية لنبات الباكوبا. أظهرت التحاليل الكمية أن المركبات الفينولية الرئيسية في مستخلص الماء والايثانول هما حمض الكلوروجينك وحمض الجاليك (٣٣٠,٤٩ و ٣٠٩,٨٦ميكروجرام/جرام، على التوالي). بينما كان الفلافونويد الاساسى في المستخلص هو الراتين بتركيز ٥١٧,٣٨ميكروجرام/جرام.