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Short-Term preservation of *Nepeta septemcrenata via* Production of Synthetic Seeds

Ebtsam M. Hamza

Plant Biotechnology Department, Genetic Engineering
and Biotechnology Research Institute (GEBRI), University of Sadat City (USC), Egypt

Abstract: *Nepeta septemcrenata* Benth is an Egyptian medicinal plant exhibition of extinction. Biotechnology has an important role in protection plant flora conservation. The aim of this work was employment biotechnology to avoid the extinction of *Nepeta septemcrenata* plant through the optimizing of a protocol for producing synthetic seeds as a method of short-term preservation. In addition to the estimation of the genetic stability of the regenerated plantlets after preservation. Abscisic acid (ABA) may reduce the plant growth and prevent the acceleration of plant injury *in vitro*. So, the effects of MS medium fortified with 0.0, 0.1, 0.2 or 0.3 µg/l ABA on *Nepeta* preservation were examined. All ABA concentrations retard the growth parameters. The shoots maintained healthy on the most ABA concentrations for six months; except for 0.1 µg/l ABA which began to be injured because of physiological disorders. MS+0.2 µg/l ABA was recommended to be included in the composition of synthetic seeds. The synthetic seeds formation was optimized through examined the effect of Na-alginate percentage on shape and texture of synthetic seeds. The optimum formation resulted from 3 and 4% Na-alginate, which produced round, good texture and easy to be operated. The percentage of Na-alginate affected short term preservation through retarding the shoot germination. Well-formed synthetic seeds (3 or 4% Na-alginate) could preserve the shoots for 8 or 9 months without subculture, respectively. The regenerated shoots from preserved synthetic seeds were 3.5 shoots/synthetic seed. The roots appeared on the bases of shoots and plantlets showed high acclimatization ability. The genetic characterization of the preserved plantlets and the mother plants were determined using ISSR and SSR molecular markers. Gel electrophoresis of PCR products of eight ISSR primers showed 23 and 24 TAF for the mother plant and the regenerated plants after the preservation. Monomorphism was 96.4%. Also, gel electrophoresis of eight primers of SSR resulted in 16 TAA with 100% monomorphism. Finally, based on the used primers of ISSR and microsatellite molecular markers, the preservation of *Nepeta septemcrenata* through synthetic seeds is a safe technique for *Nepeta septemcrenata* preservation which resulted in the production of true to type plantlets.

Key words: *Nepeta septemcrenata* · preservation · synthetic seed · ABA · ISSR · SSR

INTRODUCTION

The chances of the extinction of medicinal plants are increasing due to the expanding of urban crawling and great demand of pharmaceutical and cosmetics industries, which depend on plants harvest especially natural habitat ones [1]. *Nepeta septemcrenata* Benth is the only species of genus *Nepeta* which recorded as endemic for Sinai, Egypt [2-5]. It is a medicinal plant belongs to family Lamiaceae. It faces extinction because of its widely uses in folk medicine for their high margin of safety and promising pharmacological and therapeutic effects like sedative, relaxant, cholesterol lowering, higher antioxidant activity, antispasmodic, expectorant, diuretic, antiseptic, antitussive, anti-asthmatic and several enzyme inhibitions as well as promising uses in food industry [6-9].

To prevent the extinction of *Nepeta septemcrenata*, the biotechnology techniques especially micropropagation and germplasm preservation techniques should be implemented. Micropropagaton provided a constant supply of plant material which covers the industrial needs and considers a valuable method for

Corresponding Author: Ebtsam M. Hamza, Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City (USC), Egypt.
E-mail: ebtsam.hamza@gebri.usc.edu.eg; ebtsamhamza2000@yahoo.com.

sustainable conservation [10, 11]. Micropropagation is a method for ex situ conservation of rare plant species [12]. Micropropagation protocol of *Nepeta septemcrenata* is established, but there was difficult to maintain shoots *in vitro* for long period because of shoot tip necrosis and the possibility of somaclonal variation occurrence [13].

Growth retardant may play a role in plant preservation through deceleration growth and inhibition of cell metabolism. Abscisic acid (ABA) plays an important role in the regulation of many physiological processes in the plant, *i.e*., the induction of primary dormancy through regulation of the gene expression which control the transitions from dormancy to germination and the opposite as well as the enhancement of shoot or bud proliferation. The exogenous ABA develops *in vitro* conservation through control the response of plant cell [14].

Utilization the encapsulation technology as a method for propagation and plant preservation provides multi-potential attributes like the effective alternative for micropropagation with high ability of regeneration, exchange of germplasm between laboratories in the form of microcuttings alginate encapsulated, which include easy handling and saving elite, rare and endangered plant species. Also, synthetic seeds are effective for avoiding human errors, diseases and environmental injuries which may occur in conventional conservation [15-17]. In addition, synthetic seeds technique has a great advantage which is "genetically, identical materials", this means it can utilize as a method for micropropagation [18-20].

The formation of synthetic seeds (hardness or rigidity) depends on the ions number of sodium which exchanged with calcium ones. The ions exchange is affected by the concentrations of both sodium alginate and calcium chloride as well as the duration period of ions exchange [21, 22]. In addition, the encapsulated micro shoots germination is affected by the matrix composition of synthetic seeds (hardness and nutrient availability) especially sodium alginate concentration, molarity of calcium chloride and duration of exposure to $CaCl_2.2H_2O$ solution as well as the ability of micro shoots to uptake nutrient substances in the sodium alginate solution [23].

Recently, genetic stability is one of the most important aspects of plant biotechnology techniques. Molecular markers; which based on DNA electrophoretic techniques, provides great markers for the study of various aspects in the most biological fields including detection of somaclonal variations which reflect any occurrence of cryptic genetic interruption and genetic relationships of plant species [24, 22].

Fingerprinting profiles demonstrated genetic uniformity amongst the examined samples to ensure the quality of the produced plantlets. The effect of synthetic seeds composition on genetic stability was examined depending on PCR based techniques like the random amplified polymorphic DNA (RAPD) and the inter simple sequence repeats (ISSR) [11, 21, 25-27].

The aim of this work was employment biotechnology to avoid plant extinction of *Nepeta septemcrenata* through the optimization of a protocol for producing synthetic seeds as a method of short-term preservation. Estimation of the genetic stability of encapsulated plantlets after short-term preservation.

MATERIAL AND METHODS

Plant Materials: *In vitro* plant materials were obtained from the Tissue Culture Laboratory of Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Egypt. Shoot tips and nodal cuttings were multiplied as described by Hamza [13] with minor modification.

Media Composition: Murashige and Skoog medium, 1962 (MS) was implemented in all investigations. MS was instituted with 30g/l sucrose, 6% agar and either 2mg/l 6, benzylaminopurine (BAP) in multiplication or indole-3-butyric acid (IBA) in rooting, Hamza [13]. The pH (5.8±0.2) was adjusted prior to the media were steam-sterilized in the autoclave at 121°C and 1.2 Par for 20min.

Effect of Abscisic Acid (ABA) on Short-term Preservation and Shoot Regeneration of *Nepeta septemcrenata*: Different concentrations of abscisic acid (ABA) (0.0, 1.0, 2.0 or 3.0 µg/l) were added to MS media. After autoclaving, media were distributed to Petri dishes and let to be solidified before being cultured. Nodes were used as explant; six nodes were cultured in each dish and five dishes were employed as replicates in each treatment. Cultures were observed after two, four and six months and the following data were recorded: survival percentage, shoot proliferation number/shoot tip, shoot length (cm) and nodes number/shoot.

The Formation of Synthetic Seeds: Different concentrations (2, 3, 4 and 5%) of sodium alginate were dissolved in the recommended medium composition of the previous experiment; MS medium contained 2mg/l BAP, 0.2 µg/l ABA and 3% sucrose. Also, one litter of 100mM Calcium Chloride solution ($CaCl_2$) was prepared.

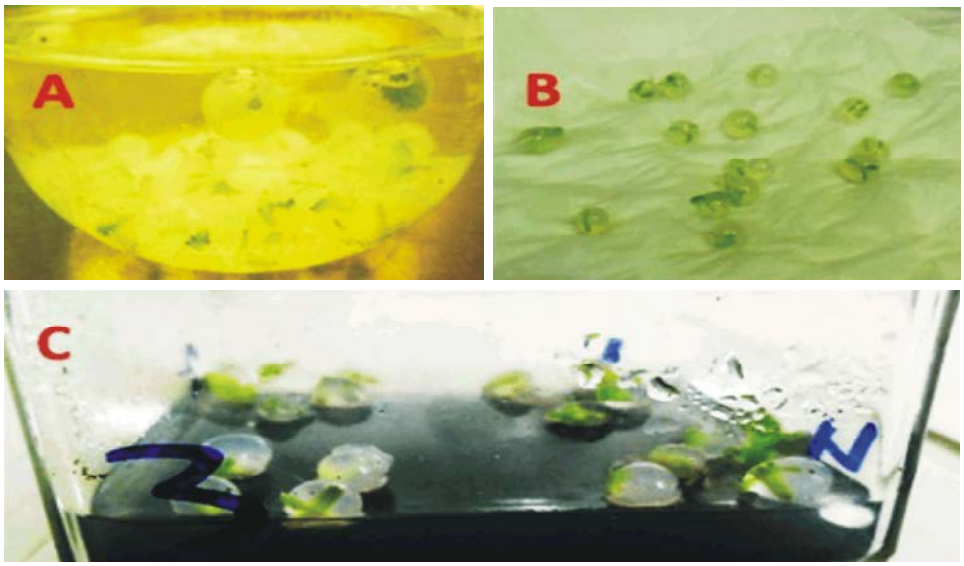

Fig. 1: The synthetic seeds formation procedure of *Nepeta septemcrenata* microcuttings.
A: The drops of sodium alginate including shoot tips were soaking in $CaCl_2$ solution to allow ion exchange and formation of Ca-alginate polymer, *B*: Air dry of synthetic seeds on sterilized tissues and *C*: Synthetic seeds cultured on MS medium

Then media including sodium alginate as well as $CaCl_2$ solution were distributed into ten jars and sterilized in the autoclave at 121°C and 1.2 Par. for 20 min. Shoot tips were coated by sodium alginate then drops of sodium alginate including shoot tips were soaked in $CaCl_2$ solution for 15min to allow the occurrence of ion exchange between Na and Ca form a polymer of Ca-alginate (synthetic seed) (Figure, 1A). The synthetic seeds were collected and washed twice in sterilized distilled water to remove any residual of $CaCl_2$, then, synthetic seeds were air dry on sterilized tissues (Figure, 1B) as described by Hamza [22]. Synthetic seeds (16 synthetic seed/ jar) were cultured on jars contained MS medium fortified with 2mg/l IBA, 1g/l Activated charcoal, 30g/l sucrose and 6% Agar (Figure 1C). Each treatment included five jars as replicates. The effects of Na-alginate concentrations on synthetic seed shape and texture, the period of conservation, the germination ability and the root formation were recorded. For acclimatization, the produced plantlets were planted in 5cm pots contained culture medium (sand and peat moss, 1:1, v/v) and coated by transparent polyethylene bags, which were gradually removed. The number of success acclimatized plantlets and acclimatization success percent was calculated after one month.

Data Analysis: All experiments were complete randomized designed. Each treatment contained five replicates and each replicate was an average of culture jar. Data were statically analyzed by using SPSS v22.0, SPSS Inc. Chicago, USA, software. Significant differences were compared among the recorded results (mean ± standard error) according to Duncan's multiple range test (DMRT), Duncan [28] at a 0.05 level.

Genetic Characterization of *Nepeta septemcrenata* Mother Plant and the Regenerated Plantlets Preserved *Via* Synthetic Seeds: Two techniques based on PCR; *i.e.,* inter-simple sequence repeats (ISSR) and simple sequence repeats (SSR) or microsatellite markers, were used to determine genetic stability of synthetic seeds.

Plant Samples: Meristematic leaves of the mother plant of *Nepeta septemcrenata* as well as plants resulted from preservation were collected for ISSR and SSR analysis.

DNA Extraction: Leaves (200mg) were grounded in liquid nitrogen to obtain a fine powder. To isolate DNA, i-genomic Plant DNA Extraction Mini Kit (protocol A for Lyophilized leaf), (iNtRON Biotechnology Co.) was used. The isolated DNA concentration was 50 ng/µl. To ensure the isolated DNA quality, 10 µl of each sample was separated electrophoresis (5 V/cm) in 1% agarose gel depending on the charge.

ISSR and SSR Techniques: Eight ISSR primers (OP-A08, OP-A09, OP-A10, OP-Amic02, OP-Amic03, OP-Amic06 and OP- Mic07) and eight SSR primers CAC15, CAT01, TAA27, Org23, AMB03, CT19, Ag14 and

Table 1: Effect of abscisic acid (ABA) concentrations on short-term preservation and shoot regeneration of *Nepeta septemcrenata.*

	Observed responses*					
ABA (µg/l)	After two months	After four months	After six months	Shoot length (cm)*	Nodes NO./shoot*	Recommendation
0.0	4 shoot proliferation/ shoot tip Beginning of verification	Dieback and death for all shoots	Totally death	3.2±0.09^{ns}	3.5±0.24^{b}	Shoots should be subcultured after 3weeks in multiplication stage to avoid verification
0.1	Compact and dwarf shoots	8±0.15^{c} shoot proliferation/ shoot tip & Grow of dwarf shoots	25 ±1.53^{b} shoot proliferation Grow of healthy shoots	3.1±0.35^{ns}	4.3±0.21^{a}	Explants could be conserved for six months then begin to damage
0.2	swelling &compact organogenesis	15±0.19^{a} shoot proliferation/ shoot tip	30±2.52^{a} healthy shoot proliferation/shoot tip	2.9±0.15^{ns}	4.3±0.32^{a}	Explants could be conserved for six months without any injury
0.3	swelling &compact organogenesis	10±0.35^{b} shoot proliferation/ shoot tip	25±4.04^{b} healthy shoot proliferation/ shoot tip	2.3±0.25^{ns}	3.1±0.12^{b}	Explants could be conserved for six months without any injury

*Results are presented as mean ± standard error

-Different superscripts in the same row indicate significant different (P<0.05), according to Duncan?s test difference

CIR-16 (Table, 1) were obtained from Bio Basic Inc. used in PCR reaction, which contained 100ng of DNA template, 12.5µl master mix solution (i- TaqTM, iNtRON Biotechnology), 2 µl of primer for ISSR and 1µl of each forward and reverse primers for SSR and 4 µl PCR buffer with 1.5mM of $MgCl_2$ in a 25µl as a final volume. PCR program conditions were implemented as described by Hamza *et al.* [29].

DNA Electrophoresis: The PCR products were charge dependent separated (5V/cm) in 1.5% agarose gel for ISSR primers and 3% agarose gel for SSR primers. The DNA fragments were stained with ethidium bromide as described by Sambrook and Russel [30]. The DNA ladder (1-Kb plus blue DNA Ladder, GeneOne.Co.) was used to estimate the molecular weight of the DNA fragments. The agarose gel photo-record was taken using UV transilluminator.

DNA Electrophoresis Analysis: The DNA amplified fragment was recorded as present (1) or absent (0) fragment or allele for ISSR or SSR primers, respectively. Data were analyzed according to Rohlf [31]. The genetic relationship was determined by unweighted pair group's method arithmetic (UPGMA) with Jaccard similar coefficient.

RESULTS

Effect of abscisic acid (ABA) on the short-term Preservation of *Nepeta septemcrenata*: ABA affected preservation and shoot proliferation of *Nepeta septemcrenata* (Table 1 and Figure 2). After two months, MS medium fortified with 0.1 µg/l ABA resulted in swelling and producing compact few dwarf shoots. While just swelling explants and compact organogenesis appeared at 0.2 or 0.3 µg/l ABA compared with MS medium free of ABA which produced shoots with tip necrosis. After four months, the free MS resulted in the death of necrosis shoots. On the other hand, MS+0.2µg/l ABA produced the highest number of healthy shoots (15 shoot/shoot tip) followed by MS+0.3 or 0.1 µg/l ABA (10 or 8 shoot/shoot tip, respectively). The same trend maintained after six months, but shoots which resulted from 0.1µg/l ABA began to be injured. Using ABA helped in maintaining plant preservation for six months without damage but shoot length was slightly affected for a period (one month later). The nodes number was positively affected as a result of ABA presence compared with free ABA produced shoots.

Optimization of the Synthetic Seeds Formation: Data in Table 1 clear that synthetic seeds formation (shape and texture) was affected by Na-alginate concentrations. The texture of synthetic seeds varied from very soft, soft, good and hard as a result of increasing Na-alginate concentrations from 2 to 5%. Indeed, Na-alginate at concentration 2% resulted in very soft seeds which difficult to be operated, while increasing the Na-alginate concentrations lead to the good formation of synthetic seeds and easy to be handled.

Preservation Ability of the Synthetic Seeds and Shoot Regeneration: Results presented in Table 1 show that the synthetic seeds which included 0.2µg/l ABA (the recommended concentration of the previous

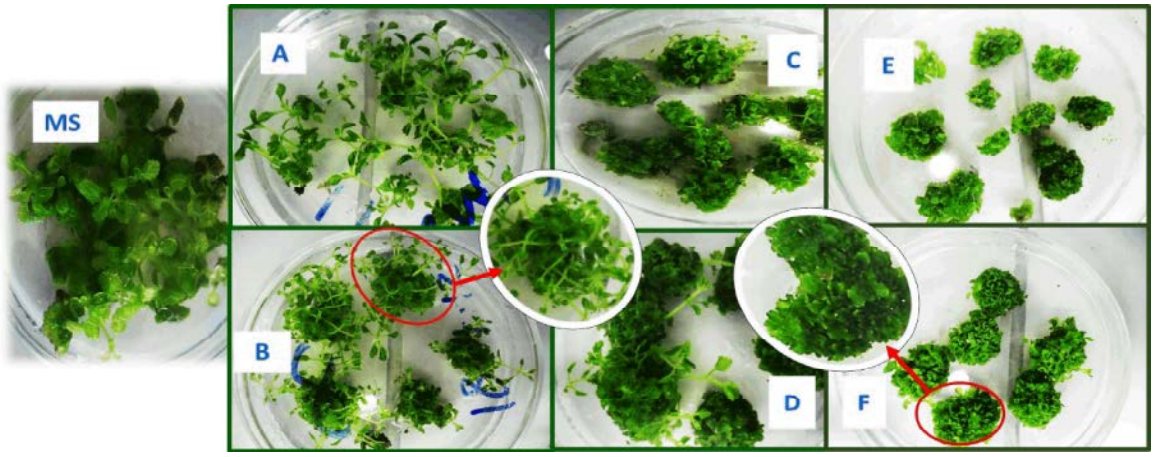

Fig. 2: Effect of abscisic acid (ABA) on short-term preservation and shoot regeneration of *Nepeta septemcrenata*
MS: Free of ABA after two months, *A and B*: MS+0.1ABA after four and six months, *C and D*: MS+0.2ABA after four and six months and *E and F*: MS+ 0.3ABA after four and six months

Fig. 3: The preservation ability and shoot regeneration of *Nepeta septemcrenata* synthetic seeds.
A: Shoot regeneration at 2% Na-alginate after 5months, *B*: Shoot regeneration at 2% Na-alginate after 7months, *C*: Shoot regeneration at 3% Na-alginate after 8months, *D*: Shoot regeneration at 4% Na-alginate after 9months, *E and F*: Root formation of shoots regenerated from synthetic seeds

experiment) and different concentrations of Na- alginate affected the store ability by affecting the germination ability. The period for starting synthetic seeds germination increased by increasing the Na-alginate concentration, it's important to know that 5% Na-alginate failed to germinate because it was too hard. Anyway, the period to start germination ranged from 4 to 6 months. Synthetic seeds consuming about two months to reach full germination with 2, 3 and 4% Na-alginate. Synthetic seeds conservation maintained for 7 months at 2% Na-alginate, 8 and 9 months for 3 and 4% Na-alginate. Germination percentage adversely related to Na-alginate concentrations; 2 and 3% Na-alginate resulted in 100% germination while 4% Na-alginate gave 90% germination (Figure, 3 A, B, C and D). After synthetic seeds germination, shoots grew for one month and each synthetic seed gave 3.55shoot/synthetic seed. Also, roots appeared on bases of shoots. Roots number ranged from 3.43 to 4.41 root/shoot (Figure, 3 E and F). Finally, synthetic seeds could conserve *Nepeta septemcrenata* shoots for about 10 months without subculture and without injury or appearance of physiological disorders. Plantlets resulted from conservation via synthetic seeds success to be acclimatized. The percentage of acclimatization success was affected by Na-alginate concentrations as well as the ability of shoot preservation. The highest acclimatization percentage resulted from 3 and 4% Na-alginate (Table 2 and Figure 4).

Fig. 4: Acclimatization of *Nepeta septemcrenata* plantlets after conservation for nine months using synthetic seeds, A: After one month, B and C: After two months

Table 2: Optimization of the synthetic seeds formation and its preservation ability and shoot regeneration of *Nepeta septemcrenata*

Synthetic seeds										
				Germination*						
				Period (month)						
Na-alginate	Shape	Texture	Observation	Start	Final	%	Conservation period (month)*	Shoot No/ synseed*	Root NO/ synseed*	Acclimatization success percent (%)
2%	Fragile	Very soft	Difficult to be operate	4±0.20 ^{b}	6±0.12 ^{b}	100	7±0.15 ^{b}	3.55±0.11 ^{ns}	4.41±0.15 ^{a}	50
3%	Rounded with tail	Soft	Easy to be operated	5±0.15 ^{ab}	7±0.15 ^{ab}	100	8±0.21 ^{ab}	3.50±0.10 ^{ns}	3.45±0.13 ^{b}	95
4%	Rounded with shot tail	Good	Easy to be operated	6±0.26 ^{a}	8±0.07 ^{a}	90	9±0.35 ^{a}	3.50±0.15 ^{ns}	3.43±0.09 ^{b}	95
5%	Round	Hard	Fall to germinate	==	==	==	==	==	==	==

*Results are presented as mean ± standard error

-Different superscripts in the same row indicate significant different (P<0.05), according to Duncan?s test difference

Genetic Characterization of the *Nepeta Septemcrenata* Mother Plant and Regenerated Plantlets Preserved *Via* Synthetic Seeds: Determination of the genetic characterization and uniformity of both the *Nepeta septemcrenata* mother plant and the regenerated plantlets; after short-time conservation using synthetic seeds, was conducted based on the inter-simple sequence repeats (ISSR) and the simple sequence repeats (SSR) or microsatellite markers. The analysis of PCR products based on eight primers of the ISSR molecular marker revealed that only seven primers amplified the DNA of the mother plant and the regenerated plantlets after preservation. The two examined plant samples gave minor dissimilatory in the number of total amplified fragments (23 and 24 TAF, for the mother and the conserved plants, respectively). Each primer resulted in producing TAF ranged from two to five fragments. The most ISSR primers produced 100% monomorphism with one exception with OP-A08 which showed 75% monomorphic fragments. The total average of monomorphism was 96.43% (Table 3 and Figure 5A).

Also, analysis of PCR products based on microsatellite markers (SSR) showed that all primers produced the same number of total amplified alleles (TAA) (2 amplified alleles), where each plant sample produced 16 TAA. The monomorphism was 100%. Based on the used primers of ISSR and microsatellite molecular markers, it could be concluded that the preservation of *Nepeta septemcrenata* through synthetic seed is a safe method for preservation without any expectation of somaclonal variation. this means the synthetic seed treatments did not affect the genetic characterization of preserved plants and the produced plantlets are true to type (Table 3 and Figure 5B).

DISCUSSION

Short-term preservation *via* synthetic seeds is an important technique deal with reducing the cost, the human efforts and protection the plants which exhibition of extinction. ABA affected the preservation and shoot proliferation of *Nepeta septemcrenata*.

Table 3: Genetic characterization of mother plant and regenerated plantlets preserved *via* synthetic seeds of *Nepeta septemcrenata*

Inter-Simple Sequence Repeat Primers (ISSR)		TAF			Microsatellite Primers (SSR)			TAA		
Names	Sequences	MP	RS	Mm%	Names	Motif	Sequences	MP	RS	Mm%
A-08	AGC AGC AGC AGC GC	3	4	75	CAC15	CAC	F:TAAATCTCCACTCTGCAAAAGC R: GATAGGAAGCGTCGTAGACCC	2	2	100
A-09	AGCAGCAGCAGCAC	3	3	100	CAT01	CAT	F: GCTTTCGATCCCTCCACATA R: GATCCCTACAATCCTTGGTCC	2	2	100
A-10	GCTGCTGCTGCTC	=	=	=	TAA27	TAA	F: GGATGAAAAATGCTCAAAATG R:TAGTACCCACAGGGAAGAGAGC	2	2	100
AMIC02	GAC GAT AGA TAG ATA GATA	4	4	100	Org23	TG	F: AGGTCTACATTGGCATTGTC R:ACATGCAGZTGCTATAATGAATG	2	2	100
Amic03	AGA TAG ATA GAT AGA TA	2	2	100	AMB03	TC	F: AACACACACACTCGCCTCAC R: CAGCCAAATGTGGAGAGACC	2	2	100
Amic06	GGCCACACACACACACA	2	2	100	CT19	CT	F:CGCCAAGCTTACCACTCACTAC R: GCCACGATTTGTAGGGGATA	2	2	100
Amic07	CGACGACAGCAGCAGCAG	4	4	100	AG14	AG	F:AAAGGGAAAGCCCTAATCTCA R: CTTCCTCTTGGAGTGTTG	2	2	100
Mic08	CGA CGA CGA CGA CGA	5	5	100	CIR16	ATG	F: AGC GGG AAA TGA AAA GGT AT R: ATG AAA ACG TGC CAA ATG TC	2	2	100
	Total	23	24	96.43			Total	16	16	100

MP: mother plant, *RS*: plantlets derived from synthetic seeds, *TAF*: the total number of amplified fragments, *TAA*: total amplified alleles, *Mm*%: the percent of monomorphic fragments

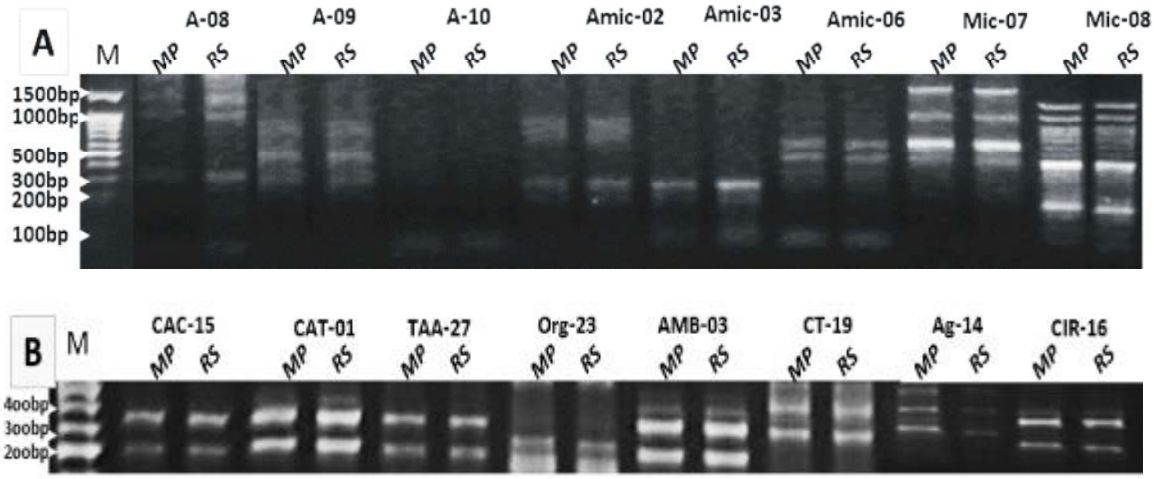

Fig. 5: Gel electrophoresis of *Nepeta septemcrenata* PCR products
A: ISSR primers *B*: Microsatellite (SSR) primers, *M*: DNA ladder, *MP*: Mother plants and *RS*: Regenerated plantlets from synthetic seeds

Using ABA concentrations helped in maintaining the plant preservation for six months without damage, but it may affect the shoot length for one month later. Moreover, the nodes number was positively affected as a result of ABA presence compared with free ABA produced shoots. These results may be attributed to the inhibitory effect of ABA which may lead to slow down of the cell metabolism and decelerate the cell growth and division. These effects of ABA retard the growth and physiological injury of the plant cell and elongate the preservation period. The results were supported with those obtained by Kermode [14] who concluded that ABA plays an important role in the gene expression which regulates the dormancy manipulation in the plants. Also, results agree with Rai *et al*. [32] who stated that ABA enhances the shoot or the bud proliferation.

Synthetic seeds formation (shape and texture) was affected by Na-alginate concentrations. Actually, a positive relationship was observed between Na-alginate concentrations and both the shape and the texture of synthetic seeds. The good synthetic seeds formation was obtained at 3% Na-alginate, which resulted in producing

round synthetic seeds with good texture and easy to handle. These results reflect the effect of high concentration on ions exchange as well as the good formation of the polymer of Ca-alginate. These results were supported with the findings of Hamza [22], Anis and Ahmad [33], Alatar *et al*. [1] and Khan *et al*. [27] who reported that Na-alginate at low concentration is unsuitable for encapsulation, because low concentration may reduce its gelling ability, after exposure to autoclaving which lead to the difficulty to handle. While, the results came in contrary with the finding of Kundu *et al*., [11], Sharma *et al*. [34] and Lata *et al*. [35] where they reported that the optimum percent of Na-alginate were 2.5, 4 and 5%, respectively. The difference in the value of the optimum Na-alginate concentrations may attribute to the plant species and the type of explant as well as the source of used chemicals. Indeed, the good formation is not only depended on shape and texture but also the ability of explant to germinate.

Synthetic seeds formation affected the store ability by affecting the germination ability of *Nepeta* microshoots. Germination percentage adversely related to Na-alginate concentrations; 2 and 3% Na-alginate resulted in 100% germination, while 4% Na-alginate gave 90% germination and 5% Na-alginate failed to germinate. Synthetic seeds could conserve *Nepeta septemcrenata* shoots for about 10 months without subculture and without injury or appearance of the physiological disorders and the high ability to shoot preservation. The acclimatization percentage resulted from 3 and 4% Na-alginate. The results were in consonance with those obtained by Anis and Ahmad [33] and Khan *et al*. [27].

Based on the ISSR molecular marker, the two examined plant samples gave minor genetic dissimilatory in the total number of amplified fragments. The total average of monomorphism was 96.43%. Also, genetic analysis based on microsatellite markers (SSR) cleared that the monomorphism of produced alleles was 100%. Results proved that the preservation of *Nepeta septemcrenata* through synthetic seeds is a safe technique for preservation without any expectation of somaclonal variation, which means the produced plantlets are true to type. The obtained results corroborated with Daud *et al*. [21], Saha *et al*. [25]; Gantait and Kundu [26], Kundu *et al*. [11] who demonstrated that ISSR and SSR are valuable and accurate molecular markers which could determine the occurrence of somaclonal variation and the genetic stability of the plant genomes. Also, results were supported with Saha *et al*. [25] and Khan *et al*. [27] who reported that all the generated plants after preservation gave monomorphic bands like the mother plants and they also reported that no variants have crept as a result of the synthetic seeds process and all the produced plantlets were true to type.

CONCLUSION

Short-term preservation through synthetic seeds technology is a promising branch of the plant biotechnology. It allocates multi-advantages like providing an ideal solution to prevent the plant extinction, a tool to the germplasm exchange between labs or countries and a method for producing true to type plants. The technique needs more research because the synthetic seeds optimization differs according to the used explant as well as the examined species. This work success in the establishment of a short-term preservation protocol for *Nepeta septemcrenata* through the encapsulation of microcuttings in a step to prevent this valuable medicinal species from extinction.

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