A synthetic seed method through encapsulation of *in vitro* proliferated bulblets of garlic (*Allium sativum* L.)

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ABSTRACT

A synthetic seed protocol for conservation of garlic as asexual propgated plant was established using in vitro rgenerated bulblets. Shoot cultures were obtained directly from excised shoot apices. Bulblets were formed in vitro on shoot cultures in high frequencies using MS medium containing 2 mg /l benzyladenine (BA) + 1 mg /l gibberellic acid (GA3) or 2 mg/l Jasmonic acid. Among four tested concentrations of sodium alginate used as gel matrix, 3 % gave the highest percentages of survival and conversion of encapsulated bulblets to plantlets. Also 30 min was the best duration of exposure to calcium chloride for hardening of sodium alginate capsules. For plantlets recovery from encapsulated bulblets, different combinations of growth regulators were examined. The highest number of proliferated shoots and their growth parameters (shoot length and fresh mass) were obtained using MS medium containing 2 mg /l BA +2 mg /l naphthaleneacetic acid (NAA). Randomly Amplified Polymorphic DNA (RAPD) analysis has been used to study the genetic stability of convered plantlets of garlic. According to data obtained, plantlets derived from capsulated cultures as well as normally in vitro propagated cultures were generally similar to those the in vivo grown plants.The developed plantlets were successfully adapted to the free living conditions after phase of simple acclimatization.

***Key words**: Garlic, in vitro, bulblet, encapsulation, RAPD analysis.*

INTRODUCTION

Some crop species have genotypes which do not produce seeds and others , have either sterile genotypes or produce orthodox seeds which are hihghly heterozygous and are therefore of limited interest for the conservation of particular gene combinations. These species are mainly propagated vegetatively to maintain clonal genotypes. At present, the common methods to preserve the genetic resources of these problem crop species are as tuber, bulbs and corms. There are, however, several serious problems with these methods (Withers and Engels, 1990). The collections are exposed to natural disasters and attack by pests and pathogens; moreover, labour costs and the requirment for technical personnel are very high. In addition, distribution and exchange from field genebank is difficult because of the vegetative nature of the material and the greater risks of disease transfer. *In vitro* techniques are of great interest for the collecting, multiplication and storage of plant germplasm (Engelmann, 1991). Moreover, establishment of synthetic seeds have multiple advantage including ease of handling, potential long-term storage and low cost of production and supsequent propagation (Ghosh and Sen, 1994). In this respect, natural unipolar

propagule like microbulbs, rhizomes, protocorms (Standardi and Piccioni, 1998 ; Datta *et al*., 1999 ; Saiprasad and Polisetty, 2003), nodal cuttings (Danso and Ford, 2003) and shoot buds (Uozumi and Kobayashi, 1995 ; Micheli *et al*., 2002) besides bipolar somatic embryos (Gary and Purohit, 1991 ; Patel *et al*., 2002) have been subjected to encapsulation for storage, easy handling, transport, delivery and their establishment under *in vitro* and/or *extra vitrum* conditions. Although a variety of natural and synthetic polymers are available for encapsulation, sodium alginate is the most commonly used gel-matrix because of its easy gelling properties, non-toxicity and low cost. Different concentrations of sodium alginate ranging from 1.5 % to 6 % have been used for different systems (Bapat *et al*., 1987; Ahuja *et al*., 1989; Sakamoto *et al*., 1995, Datta *et al*., 1999; Vij *et al*., 2001; Mondal *et al*., 2002). Because garlic is sterile, it is vegetatively propagated by cloves and air bulbils. Development of efficient *in vitro* techniques to ensure its safe conservation is therefore of paramount importance. Due to its ease of handling and quick of conversion to plantlets, *in vitro* regenerated bulblet seems to be the sutiable explant for germplasm preservation of garlic. *In vitro* bulblets formation of garlic largely dependent on growth regulators and sucrose in culture media (Matsubara and Chen, 1989 ; Nagakubo *et al*., 1993), as well as other conditions such as cultivar, photoperiod and temperature. In this respect, BA and Jasmonic acid are reported more effective factors promoting *in vitro* bulblet of garlic shoot, despite a stimulatory role of the higher concentration of sucrose on *in vitro* bulblet formation (Nagakubo *et al*., 1993). The objective of this investigation is to establish a protocol for mid-term preservation of garlic germplasm through encapsulation of *in vitro* regenerated bulblets

MATERIALS AND METHODS

Establishment of aseptic cultures

The Egyptian cultivar (Balady) of garlic was used as plant material. The cloves were taken and the protective leaf sheaths were removed. Then the naked cloves were washed by tap water and surface sterilized using 70 % ethanol for 3 min followed by 50 % commercial Clorox (contained 5.25 % NaOCl) for 20 min. Under aseptic conditions of laminar flow cabinet, shoot tips with basal part of bullbs were excised and cultured on Murashige and Skoog, 1962 (MS) medium supplemented with growth regulators described by Bekheet (2004) for direct shoot proliferation.

In vitro bulblets formation

The cluster of multiple shoots of garlic were taken and divided into single shoots and then cultured on MS medium contained several concentrations of cytokinins i.e. kinetin (Kin) or BA in combinations with GA3. Also, different concentrations of Jasmonic acid i.e. 0.5 ,1.0, 2.0, 3.0, 4.0 and 5.0 mg /l added to cultute medium were examined for their potential for *in vitro* bulblet formation of garlic. The percentage of bulblet formation, number of bulblets per culture and bulblet fresh and dry masses were recorded after six weeks of culturing.

Table (1): Primers used and their annealing temperatures.

Primer	Sequence 5'- 3'	Annealing Tm °C / Sec
K1	TGGCGACCTG	
K3	GAGGCGTCGC	36
K3	CCCTACCGAC	

Bulblets encapsulation

In two separate experiments, the effect of gel matrix concentrations and duration of exposure to calcium chloride on encapsulation of *in vitro* regenerated bulblets of garlic were tested. In the first experiment, sodium alginate was examined at 1, 2, 3, and 4 % (w/v). Bulblets in uniform size were taken and blot dried using filter paper and then mixed well with the defferent level of sodium alginate (from Sigma) prepared in distilled water. Activated charcoal (0.2%) was added to the matrix to absorb phenols and other compounds exudate to the encapsulated bulblets. To avoid bacterial contamination, antibiotic mixture was used as described by Ganapathi *et al.* (1992). The bulblets were picked up and immersed into calcium chloride solution (2.5 %) and stirred continuously up to 20 min on a magnetic stirrer. Then the encapsulated beads were washed in autoclaved distilled water and transferred to autoclaved glass vessel for storage at 15 or 25°C. For the second experiment, bulblets were coated by 3 % sodium alginate and then immersed in 2.5 % calcium chloride solution for 10, 20, 30, 40 and 50 min for hardening of alginate capsules. All other conditions were as described for the first experiment mentioned above. The percentage of survival and conversion to plantlets were recorded after 60 days of storing

Plantlet recovery from encapsulated bulblets

After the storing at the two temperature conditions, the encapsulated bulblets of garlic were washed using steriled water and sodium alginate was thawed. Then the bulblets were cultured on MS-medium supplemented with different combinations of BA and NAA. The number of proliferated shoots shoot length and shoot fresh mass were recorded after six weeks of culturing on recovery media.

Culture conditions and statistical analysis

All culture media were solidfied with 0.7 % agar and adjusted to pH 5.8 before autoclaving at 121°C and 1.5 Ib/M^2 for 25 min. Cultures were normally incubated at 25°C and 16 hr photoperiod provided by philips white fluroescent tubes. Each experiment was set up as a separate completely randomized design with 20 replicates per treatment. Data were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1967).

Randomly Amplified Polymorphic DNA (RAPD) analysis

DNA isolation was performed using the Cetyl Trimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle (1987). Half gram of fresh samples of shoot buds developed from encapsulated bulblets, *in vitro* grown shoot buds and *in vivo* growing cultures were ground to powder in liquid nitrogen with a prechilled pestle and mortar, suspended in 5 ml preheated CTAB buffer, and incubated at 65°C for 1 hour with occasional shaking. The suspension was then mixed with 1/3 volume of chloroform, mixed gently, centrifuged and the upper phase was transferred to a new sterilized tube. Extraction was repeated with an equal volume of chloroform. The aqueous layer was transferred to a new tube, 2/3 volume of isopropanol was added and nucleic acids were either spooled using a Pasteur pipette or sedimentated by centrifugation. The pellet was washed carefully twice with 70% ethanol, dried at room temperature and resuspended in 0.5 ml TE buffer. The enzyme, RNAse a (20 μg) was added to the resuspended mixture to digest any contaminating RNA and the tube was incubated at 37 °C for 30 min. To remove the enzyme and other contaminating proteins, phenol/chloroform extraction was performed.

The polymerase chain reaction (PCR) mixture (25 ul) consisted of 0.8 units of Taq DNA polymerase, 25 pmol dNTPs, and 25 pmol of random primer, and 50 ng of genomic DNA. The reaction mixture was placed on a DNA thermal cycler. The PCR programme included an initial denaturation step at 94°C for 2 mins followed by 45 cycles with 94°C for 1 min for DNA denaturation, annealing as mentioned with each primer, extension at 72°C for 30 seconds and final extension at 72 °C for 10 minutes were carried out. The amplified DNA fragments were separated on 2% agarose gel and stained with ethidium bromide. Three 10-mer primers (Operon technologies Inc., Alameda, California) randomly selected were used in RAPD analysis (Table 1). A 100 bp DNA ladder (Promga) was used as a Marker with molecular size of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. The amplified pattern was visualized on a UV transilluminator and photographed

Development of plantlets and acclimatization

Shoots were elongated and rooted *in vitro* using medium contained 1 mg/l indole-3-acetic acid (IAA). Complete plantlets were washed with tap water and disinfected by soaking in benlate solution (1g /l) for 20 min. Then plantlets were transplanted into plastic pots containing peatmoss and perlite (1:1). The pots were covered with clear polyethylene bags which sprayed with water to maintain a high relative humidity. Humidity was gradually reduced and covers were completely removed within four weeks of transplanting.

RESULTS AND DISCUSSION

***In vitro* bulblets formation**

Influence of growth

The role of different combinations of growth regulators on *in vitro* bulblet formation of garlic from shoot cultures was investigated. As shown in Fig. (1-A), bulblets were formed on the basis of shoots after six weeks of culturing. The frequency of bulblet formation was varied dependent on type and concentration of cytokinin added to culture medium (Table 2). The highest percentage of bulblet formation and number of bulblets per culture were found with MS medium containing 2 mg /l BA + 1 mg /l GA3. However, the highest values of fresh and dry masses were observed when MS medium contained 2 mg /l Kin + 1 mg /l GA3. This may be due to the large size of bulblets formed on the last medium. These results are in line with those reported by Kahane *et al.* (1992) on garlic. They mentioned that, bulblet formation was regularly obtained in garlic after shoot induction using 2 mg /l BA and it happened however, at erratic frequencies and rarely with kinetin (2mg/l). In this connection, cytokinins were generally reported to improve bulb formation in garlic plantlets by Matsubara and Chen (1989). On the other hand, Nagakubo *et al.* (1993) observed the *in vitro* bulblet formation of garlic on hormone-free medium. Moreover, Kim *et al.* (2003) mentioned that, garlic bulblet formation *in vitro* was not only induced by plant growth regulators, but also largely dependent on the size and quality of regenerated shoots.

Influence of jasmonic acid

A range of jasmonic acid concentrations has been experimented for *in vitro* bulblet formation in garlic. Results generally indicated that, incorporation of culture medium by Jasmonic acid had great effects on bulblet formation. The frequencies of bulblet formation increased with increasing of Jasmonic acid till 2 mg/l and then decreased. Also, the highest number of bulblets per culture as well as bulblet fresh and dry masses were registered when using 2 mg/l Jasmonic acid (Table 3).

However, the *in vitro* bulblet formation of garlic was significantly supressed by addition of high concentration of Jasmonic acid to culture medium. In similar study, Kim *et al.* (2003) mentioned that, single treatment of 2 mg /l Jasmonic acid formed bulblets at rate of 77%. They added that fresh weight of bulblets was markedly increased by addition of 1 mg /l gibberelic acid to jasmonic acid treated medium. In this respect, Jasmonic acid was reported to be an effective factor for promoting *in vitro* bulbing of garlic shoots (Nagakubo *et al.*, 1993.

Table (2): Effect of different combinations of growth regulators on in vitro bulblet formation of garlic.

Growth regulators (%)	Bulblets formation (%)	No bulblets /culture (g)	Bulblets fresh mass (mg)	Bulblets dry mass (mg)
1 mg/l kin +1 mg/l GA3	10	2.50 ± 0.05	1.25 ± 0.50	138.80 ± 6.00
2 mg/l kin +1 mg/l GA3	20	3.00 ± 0.09	1.80 ± 0.33	189.40 ± 5.50
1 mg/l BA+1 mg/l GA3	35	4.00 ± 0.08	1.65 ± 0.40	169.00 ± 6.30
2 mg/l BA+1mg/l GA3	55	4.00 ± 0.03	1.75 ± 0.55	160.50 ±7.00

± SE =Standard Error. Each treatment is the average of 20 replicates.

Table (3): Effect of Jasmonic acid concentrations on in vitro bulblet formation of garlic.

Jasmonic acid conc. (mg/l)	Bulblet formation (%)	No bulblet/culture	Bulblet fresh mass (mg)	Bulblet dry mass (mg)
0.5	30	2.00 ± 0.04	1.20 ± 0.05	109.00 ± 4.00
1.00	40	3.50 ± 0.08	1.75 ± 0.09	166.80 ± 5.30
2.00	75	6.00 ± 0.05	3.00 ± 0.10	315.70 ± 7.50
3.00	65	4.00 ± 0.03	2.00 ± 0.08	210.50 ± 5.00
4.00	35	3.00 ± 0.06	1.50 ± 0.05	142.80 ± 4.50
5.00	20	1.50 ± 0.03	0.75 ± 0.02	83.30 ± 4.40

± SE =Standard Error. Each treatment is the average of 20 replicates

Bulblets encapsulation

Effect of gel matrix concentration

In this study, 1, 2, 3 and 4 % of sodium alginate were investigated as gel matrix for encapsulation of garlic bulblets formed *in vitro*. The results presented in Fig. (1-B) and Fig. (2) Indicated that increasing of sodium alginate from 1 up to 3 % increased percentage of survival and conversion of encapsulated bulblets since smooth texture is appropriate for the storage as well as for survival and conversion was observed with 3 g/l sodium alginate solution. At low concentration (1%) of sodium alginate, capsules were weak and bulblets dried and turned brown. However, at high level (4 %) of sodium alginate, capsules were so hard and prevented bulblets proliferation. Results also revealed that, storage of encapsulated bulblets at 15 ºC was more effective than storage at 25 ºC. At low temperature, about 90 percent survival and 80 percent conversion were observed. In the concern of sodium alginate level for capsulation, the use of 3 % for capsulation of shoot tips of banana was reported by Ganapathi *et al.* (1992). In this connection, Castillo *et al.* (1998) mentioned that, beads of uniform size and shape were obtained when 2.5 % sodium alginate was used for encapsulation of somatic embryos of papaya. Ghosh and Sen (1994) achieved a maximum conversion of encapsultaed somatic embryos of *Asparagus cooperi* with 3.5 % sodium alginate. They added, higher or lower levels of sodium alginate reduced the conversion frequency.

However, Rady and Hanafy (2004) in their study on *Gypsophila paniculata* reported that, high percent of vitrification was observed in shoots derived from shoot tips coated by 2 % sodium alginate.

Effect of duration of exposure to calcium chloride

The encapsulated bulblets (3 % sodium alginate) were tested for different periods of exposure to 2.5 % calcium chloride solution.The duration of exposure to calcium chloride during the hardening process strongly affected the frequency of germination from encapsulated bulblets of garlic (Fig.3). The highest percentage of survival and conversion to plantlets were obtained when coated beads were submerged in calcium chloride (2.5 %) for 30 min for hardening. There was no dehydration and desiccation of beads during the storage of encapsulated bulblets hardened with this period. Following more than 30 min of exposure to calcium chloride a very hard bead was formed and a lower percentage of regeneration into plantlets was achieved. Capsules were made for most of the synthetic seeds production researches either by mixing the explants in sodium alginate followed by dropping into calcium salt solution or by inserting an explant into a drop of sodium alginate just as it was falling into the calcium solution. Calcium alginate beads form as a result of the ion exchange between Na+ and Ca++ ions (Redenbaugh *et al.*, 1987). The present results are in line with those obtained by Ganapathi *et al.* (1992). They followed 30 min duration of exposure to calcium chloride for hardening of encapsulated shoot tips of banana. However, Dave *et al.*(2004) mentioned that four millimeter long shoot buds of *Chloroohytum borivilianum* encapsulated in 3% sodium alginate matrix polymerised for 40 min by CaCl2 2H2O yielded the best results of regrowth. On the other hand, Castillo *et al.* (1998) obtained the highest germination frequency of encapsulated somatic embryos of papaya when coats were hardened for only 10 min

Plant recovery

Shoot regeneration from encapsulated bulblets

After thawing the capsules, the garlic bulblets were cultured on medium containing different combinatios of BA and NAA for plantlets recovery. Normal growth was achieved in encapsulated bulblets and the shoots proliferated normally without any morphological aberrations (Fig.1-C). Results of Table (4) show that, growth paramaters presented as number of shoots, shoot length and fresh mass varied depending on the balance of BA to NAA.The highest value of shoot number (6.30), shoot length (6.00 cm) as well as shoot fresh mass (3.80 g) were obtained when using medium containing 2 mg /l BA + 2 mg /l NAA (Fig. 1-D). These results are accordance with that obtained by Changeup *et al.*(1995). They mentioned that, supplementation of MS culture medium with 1-2 mg /l BA and 0.1 mg /l NAA increased the frequency of *in vitro* regenerated shoot buds of garlic. In this respect, Masuda *et al.* (1994) in their study on micro propagation of garlic reported that, basal segments of the bulblets regenerated multiple shoots on MS medium containing BA and NAA

RAPD analysis

RAPD analysis has been used successfully for measuring diversity in plants, and the patterns of variation observed have been shown to be closely resemble to those obtained using more classical characters (Howell *et al.*, 1994). In the present investigation, RAPD-DNA analysis was used to compare the shoot buds developed from

encapsulated bulblets of garlic to those grown *in vitro* and their source which was growing *in vivo*. Three randomly selected primers were used in this investigation. The three primers gave sufficient and reproducible amplification products (Fig. 4). Six polymorphic bands (900, 780, 650, 550, 400, and 100 bp) were detected with primer k1. Most of bands were similar in the three types of cultures except one band (100 bp) which was absent in the encapsulated bulblet-derived plantlets. However, the polymorphic bands of primer k3 were identical in the three types of cultures. The results of banding reveal that the three types of cultures were similar and there is no effective variation and for this reason we did not investigate more primers. It is particularly important to confirm that encapsulated bulblet cultures of garlic produce plantlets genetically similar to both nontreated and plant grown in free-living conditions. In this respect, DNA-based analysis was applied to study the genetic stability of plant tissue cultures (Williams *et al.*, 1990). Other genetic marker analysis has been used to study the degree of genetic change in plants regenerated *in vitro* such as pea (Cecchini *et al.*, 1992), sugarbeet (Sabir *et al.*, 1992) and wheat (Brown *et al.*, 1993).

Table (4): Effect of different combinations of BA and NAA on plantlets recovery from encapsulated bulblets of garlic.

Combinations of BA and NAA	No. proliferated Shoots	Shoot length (cm)	Shoots fresh mass (g)
1mg/l BA + 0.5 mg/l NAA	3.90 ± 0.12	1.30 ± 0.12	2.50 ± 0.10
1mg/l BA + 1 mg/l NAA	3.75 ± 0.20	2.10 ± 0.20	2.30 ± 0.15
1mg/l BA + 2 mg/l NAA	3.20 ± 0.25	3.75 ± 0.25	2.60 ± 0.14
2 mg/l BA + 0.5 mg/l NAA	4.80 ± 0.17	4.00 ± 0.17	2.75 ± 0.20
2 mg/l BA + 1 mg/l NAA	5.60 ± 0.30	4.80 ± 0.30	3.50 ± 0.50
2 mg/l BA + 2 mg/l NAA	6.30 ± 0.32	6.00 ± 0.30	3.80 ± 0.24

± SE =Standard Error. Each treatment is the average of 20 replicates.

Plantlets development and acclimatization

Shoots of garlic derived from encapsulated bulblets were cultured on MS medium containing 1 mg /l IAA for elongation and rooting. On this medium suffecient root system was initiated without bulb formation. Within short period of acclimatization, plantlets were successfully adapted to free-living conditions when strong rooted shoots were transplanted into pots containing perlite and peatmoss (1:1) (Fig. 1-E).

Finally, we can conclude that encapsulation of *in vitro* grown bulblets of garlic is a suitable system for mid-term storage of garlic tissue cultures since encapsulation saves space, time and resources and it demonstrates advantages over conventional method is of shoot multiplication repeated by subculturing. Also this method considered a very good tool to exchange the garlic germplasm between countries and international plant genebanks.

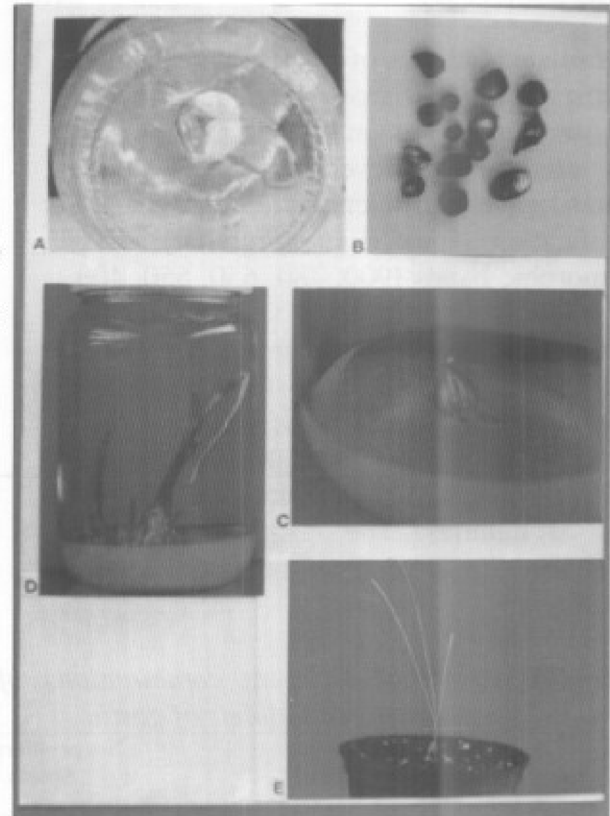

Fig. (1) : A- Bulblets formation on the basis of shoots of garlic cultured on MS medium containing 2 mg/l Kin + 1 mg/l GA3.
B- Encapsulated bulblets of garlic using 3 % sodium alginate .
C- Culturing of garlic bulblets after thawing of sodium alginate capsules.
D- Emerged shoots from recovery encapsulated bulblets of garlic using MS medium containing 2 mg /l BA + 2 mg /l NAA.
E- Adapted plantlet of garlic transplanted onto pots containing perlite and peatmoss (1:1).

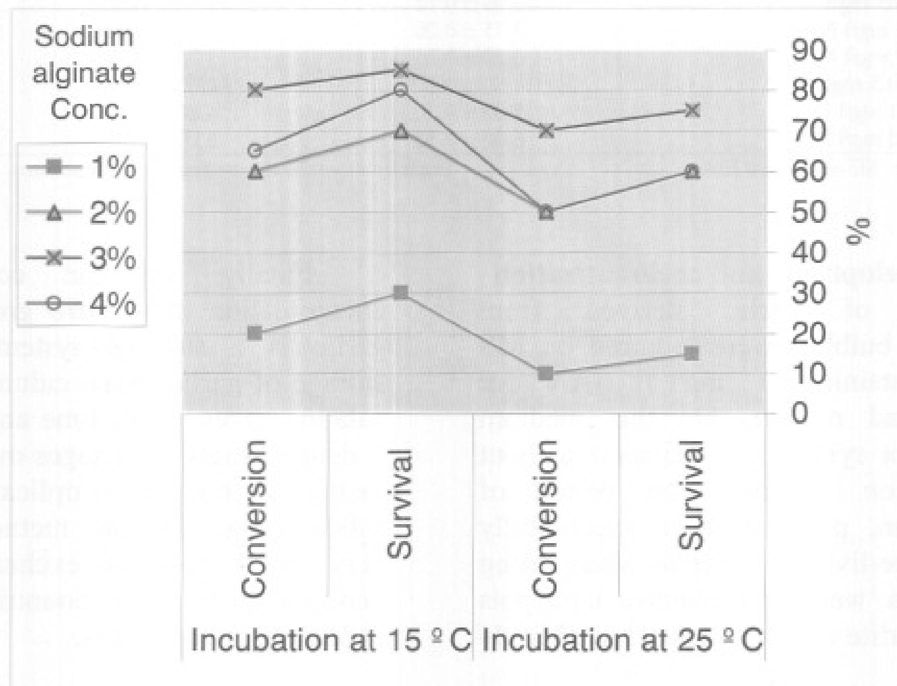

Fig. (2): Effect of gel matrix concentration on survival and conversion of encapsulated bulblets of garlic.

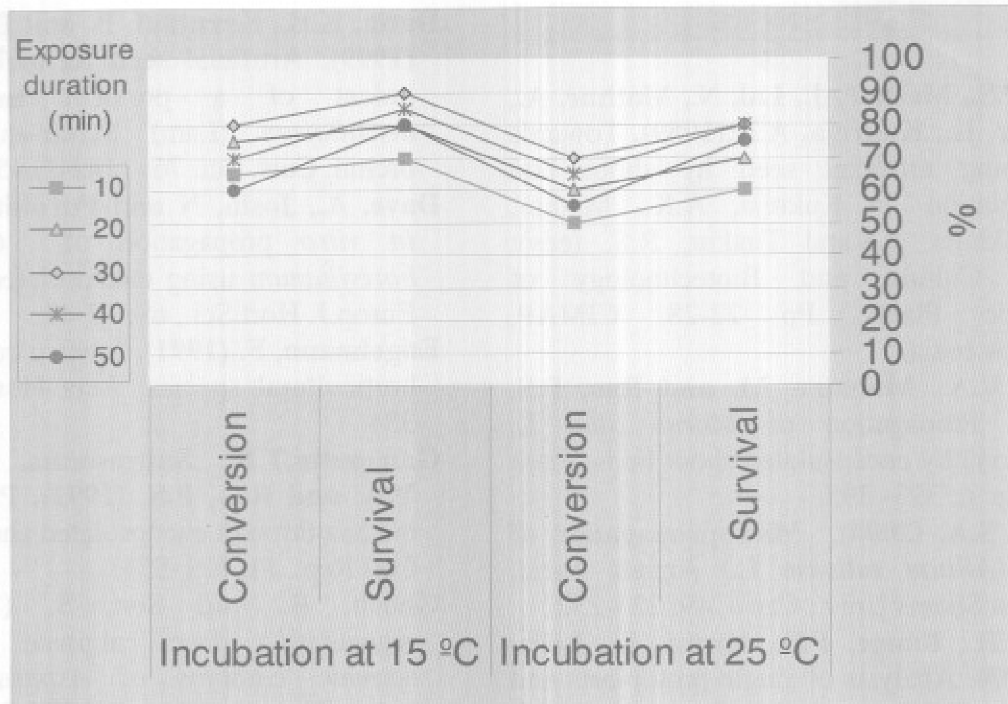

Fig. (3): Effect of duration of exposure to calcium chloride on survival and conversion of encapsulated bulblets of garlic.

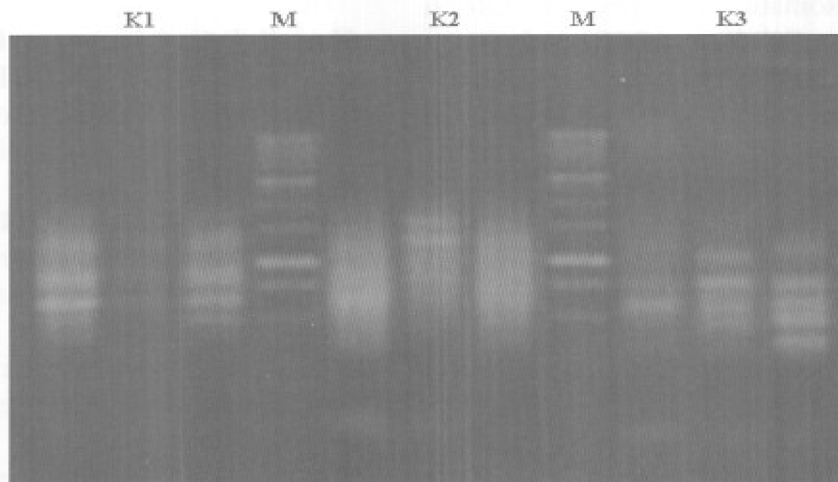

Fig (4): RAPD profile of in vivo grown plant (lane 1), normally in vitro propagated plantlets (lane2), encapsulated bulblets-derived plantlets (lane 3) and the DNA marker (M) from left to right using random primers i.e.K1, K2, and K3.

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المستخلص العربي

طريقة للبذور الصناعية من خلال تغليف بصيلات نبات الثوم المستولدة معمليا

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تم ترسيخ بروتوكول لحفظ الأصول الوراثية للثوم كأحد النباتات الغير جنسية التكاثر باستخدام البصيلات المستولدة معمليا. تم الحصول على مزارع السيقان فى الأنابيب بعد زراعة القمم المرستيمية. تكونت البصيلات بكثافة عالية على مزارع السيقان باستخدام بيئة احتوت على 2 ملجم/لتر بنزيل أدينين + 1 مجلم/لتر حمض الجبريللين وكذلك على بيئة احتوت 2 ملجم/لتر حمض الجاسمونيك. من بين أربعة تركيزات تم استخدامها من مادة ألجينات الصوديوم كمادة مغلفة للبصيلات، كان التركيز 3% قد أعطى أعلى نسبة لبقاء البصيلات حية وكذلك أعلى نسبة لتحولها بعد ذلك الى نباتات فى الأنابيب. أيضا ثلاثون دقيقة كانت أفضل فترة لتعريض البصيلات عند تغليفها الى مادة كلوريد الكالسيوم اللازم لتصلب كبسولات الجينات الصوديوم. تم اختبارتأثيراضافة توافيق مختلفة من منظمات النموالى بيئة الزراعة على تطور النموات من البصيلات المغلفة. أعلى معدل لتفريخ النموات وكذلك مقاييس نموها (الوزن الطازج والجاف) تم الحصول عليها باستخدام بيئة احتوت على 2 ملجم/لتر بنزيل أدينين + 2 مجلم/لتر نفثالين حمض الخليك. تم استخدام تحليل التكبير العشوائى لمقاطع الحمض النووى المتباينة (RAPD) لدراسة الثبات الوراثى للنباتات المتطورة من البصيلات المغلفة معمليا. بالاستناد الى البيانات المتحصل عليها ، النباتات المتحولة من البصيلات المغلفة و كذلك المستولدة مباشرة فى الانابيب كانت متشابهة ومشابهة لتلك الزراعات النامية فى الظروف البيئية الحرة. النباتات المتحولة تم أقلمتها بنجاح للنمو خارج الأنابيب بعد المرور بمرحلة بسيطة من اجراءات الاقلمة.