Plant Tissue Cult. & Biotech. **17**(1): 1-9, 2007 (June)

PTC&B

In vitro Preservation of Globe Artichoke Germplasm

S. A. Bekheet

Plant Biotechnology Department of National Research Centre, Dokki - 12622, Cairo, Egypt

Key words : Globe artichoke, Germplasm, *In vitro* preservation

Abstract

Aseptically, shoot bud and undifferentiated cultures of globe artichoke were successfully stored for 12 months at 5°C in the dark. Under these conditions, high percentage of cultures remained viable without perceptable serious signs of senenscence. Although the rates of regrowth slightly decreased, the recovery percentages were enough to obtain high frequencies of healthy globe artichoke (= *Cynara scolymus* L.). The storage at cold and dark conditions was strongly effective compared with storage in the medium under osmotic stress. Also, the differentiated cultures registered a higher viability compared to undifferentiated cultures. RAPD analysis suggests that plantlets derived from the preserved cultures were identical to those derived from nonstored differentiated tissues culrtures. However, undifferentiated (callus) cultures showed relatively less genetic variation.

Introduction

True seeds offer a reliable means for storage of plant specimens that produce orthodox propagules. However, seed storage is sometimes impractical, and in some cases impossible, for other species (Chin and Roberts 1980). Many plants produce recalcitrant seeds that are viable for only short periods. Also, some species are vegetatively propagated, and which pose preservation problems (Withers 1989). Since sexual propagation produces high level of heterogeneity, globe artichoke (*Cynara scolymus* L.) is conventionally propagated vegetatively by offshoots and crown segments. The disadvantages of this type of propagation are: the low rate of multiplication and transmision of diseases. Thus, globe artichoke germplasm cannot be effectively stored using conventional means. Considering limitations of the two methods of propagation, tissue culture propagation offers an alternative method of producing large, homogeneous and disease-free population of globe artichoke (Ancora et al. 1981, Pecaut and Dumas de Vaulx 1983, Rossi and Paoli 1992) enabling *in vitro* storage of selected genotypes. A variety of cells and tissues could be stored such as protoplasts, single cells and differentiated tissues as well as meristems and somatic embryos

(Bajaj 1983). *In vitro* storage offers several advantages over field collections because of minimal spatial and maintenance requiremennts. Moreover, propagation potential of cultures is greater and genetic erosion, pests and diseases are avoided. There are two methods for in vitro storage of plant cultures by slowing down or suspending their growth. Slow growth is achieved for short and mid-term storage by modifying the culturre medium or reducing temperature requirements (Withers 1991). A further application, to use in vitro techniques to store specific genotypes was recognized by several scientists as a way of conserving the genetic resources of such problem crops (Kartha et al. 1981, Wanas et al. 1986, Fletcher 1994, Bekheet 2000, Bekheet et al. 2005).

This paper describes a method for preservation of globe artichoke tissue cultures for short- and mid-term periods through in vitro storage of shoot buds and callus cultures by slow growth method.

Materials and Methods

Establishment of tissue cultures: Offshoots of globe artichoke cv. Balady (Egyptian local cultivar) were taken and their basal parts were removed to a length of about 5 cm. Then the outer leaves were discarded during washing by running tap water. Shoot tips (2 - 3 cm) were sterilized by 70% ethanol for 5 sec, followed by immersion in 1.25% NaOCl for 20 min. The explants were then rinsed three times using sterile distilled water. The meristem tips (2 - 3 mm) were isolated with a small part of submeristematic tissues and cultured on nutrient medium described by Ancora et al. (1981). The differentiated shoot buds were multiplicated on same medium but with reduction of Kn from 10 to 5 mg/l. To obtain callus cultures, leave segments taken from in vitro grown shoots were subcultured on medium contained 2 mg/l BA + 5 mg/ l NAA according to El-Bahr et al. (2001).

Low temperature storage: For cold storage of globe artichoke tissue cultures, shoot buds (2 cm length) were transfferred into jars (80 × 40 mm) containing multiplication medium and then incubated at 6^{o}C in complete dark. Survival and healthy culture percentages were recorded for ten replicates after 3, 6, 9 and 12 months of storage. The number of proliferated shoots were also observed after four weeks of transferring the green shoots from different treatments onto normal growth conditions (recovery potential). On other side, equal inoculum (250 mg) of callus were transferred on callus induction medium and then incubated at 6^{o}C in complete dark. Fresh and dry weights and growth value were recorded after 3, 6, 9 and 12 months of storage. Ten replicates of each treatment (storage period) were used in this experiment.

$$\text{Growth value} = \frac{\text{Initial fresh weight} - \text{Final fresh weight}}{\text{Initial fresh weight}}$$

Osmotic stress storage: To evaluate the role of mannitol and sorbitol as osmotic agents in storage of globe artichoke tissue cultures, 40 g/l of both mannitol and sorbitol were added separately to storage media. Shoot buds were cultured on shoot multiplication medium containing the additions of mannitol or sorbitol and incubated at normal growth conditions. Survival and healthy cultures percentages were calculated from ten replicates after 3, 6, 9 and 12 months of storage. The number of proliferated shoots were recorded after four weeks of culturing of viable cultures on fresh medium lacking mannitol or sorbitol. Pecies (250 mg) of callus were subcultured on callus incubation medium containing both of mannitol or sorbitol and then stored at normal growth conditions. Ten replicates were taken after 3, 6, 9 and 12 months and fresh and dry weights and growth value were assessed.

Culture conditions and statistical analysis: The media used contained 30 g/l sucrose and 7 g/l agar and were adjusted to pH 5.8 before autclaving. The normal incubation conditions were : temperature of 25 ± 2°C photoperiod 16 hr, irradiance of 45 µmol m^{-2} s^{-1} (Philips white fluroescent tubes). Experiments were designed in completely randomized design and obtained 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 a gram of fresh sample was 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 one hr 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 protein, phenol/chloroform extraction was performed.

The polymerase chain reaction (PCR) mixture (25 µl) 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.

Table 1. Primers used and their annealing temperatures.

Primer	Sequence 5′- 3′	Annealing Tm °C/Sec
K1	TGGCGACCTG	36
K 2	GAGGCGTCGC	
K4	TCGTTCCGC	

Results and Discussion

Storage at low temperature : Table 2 shows the percentages of survival and the healthy shoot bud cultures and the mean number of proliferation of the stored shoot cultures. One hundred per cent of survival was observed after three months of storage. Also, the highest percentage of healthy cultures as well as the highest rate of proliferation were recorded within the first three months of storage at low temperature and complete dark conditions. Then, all the survival parameters measured were decreased as storage duration increased. It is important here to notice that 50% of survival of shoot bud cultures were registered after 12 months of storage. Two fifth of this percentage was vitrified and the rest was healthy (Fig. 1A) and was suitable for recovery and proliferation into new cultures in high frequencies (Fig 2B). On the other hand 50% of stored cultures suffered mortality and then death after 12 months of storage at low temperature and complete dark conditions.

The results of undifferentiated (callus) cultures of globe artichoke reveal that stored cultures remain healthy without serious signs of senescence within the different storage periods. Callus growth presented as fresh and dry weight increased as storage duration increased till the ninth month and then decreased (Table 3). Also, growth value took same trend. Callus cultures remained viable with slight browning after 12 months of storage at low temperature of 6^{o}C in complete dark. Growth reduction is achieved by modifying the environmental conditions and/or the culture medium. The most widely applied technique is

Table 2. Survival and proliferation of globe artichoke shoot bud cultures during storage at low temperature of 6 °C or under osmotic stress induced by 40 g/l of mannitol or sorbitol. Means ± SE, n= 10.

Storage duration (month)	Survival (%)			Healthy shoots (%)			No. of proliferated shoots of recovery cultures		
	6°C	Mannitol	Sorbitol	6°C	Mannitol	Sorbitol	6°C	Mannitol	Sorbitol
3	100	100	100	90	80	80	5.50 ± 0.25	4.30 ± 0.15	4.00 ± 0.20
6	80	70	60	60	50	50	4.00 ± 0.33	3.70 ± 0.18	3.20 ± 0.18
9	60	50	50	50	30	30	2.50 ± 0.50	2.10 ± 0.35	1.75 ± 0.25
12	50	30	20	30	20	10	1.80 ± 0.30	1.50 ± 0.40	1.10 ± 0.50

Table 3. Growth dynamics of globe artichoke callus cultures during storage at low temperature of 6 °C or under osmotic stress induced by 40 g/l mannitol or sorbitol. Mean ± SE, n= 10, Initial fresh weight = 250 mg.

Storage duration (month)	Fresh weight (g)			Dry weight (mg)			Growth value		
	6°C	Mannitol	Sorbitol	6°C	Mannitol	Sorbitol	6°C	Mannitol	Sorbitol
3	1.25 ± 0.13	1.20 ± 0.10	1.00 ± 0.19	131.25 ± 14.00	126.30 ± 10.50	105.20 ± 20.00	4.00	3.80	3.00
6	1.80 ± 0.30	1.50 ± 0.15	1.22 ± 0.30	180.00 ± 20.00	155.20 ± 15.00	120.00 ± 29.40	6.20	5.00	3.88
9	2.00 ± 0.20	1.60 ± 0.23	1.45 ± 0.10	190.00 ± 19.00	150.80 ± 21.90	138.00 ± 9.50	7.00	5.40	4.80
12	1.75 ± 0.25	1.10 ± 0.25	0.90 ± 0.30	166.70 ± 22.70	104.80 ± 22.70	81.80 ± 18.00	6.00	3.40	2.60

temperature reduction, which can be combined with a decrease in light intensity or culture in dark (Engelmann 1991). The present results also are in accordance with those reported by Bekheet (2000) in his study on *Asparagus officinalis*. He mentioned that tissue cultures remained viable after 18 months of storage at 5°C. Also, 80% of pear and 85% of apple shoot bud cultures grown in vitro remained alive after 18 months storage at 4 and 8°C, respectively (Wanas et al. 1986, Wanas 1992). In this respect, strawberry (*Fragaria* × *Ananassa*) plantlets have been stored at 4°C in the dark and kept viable for six years with the regular addition of a few drops of liquid medium (Mullin and Schlegel 1976). Moreover, Dodds (1988) reported that at temperature lower than 3°C, potato *in vitro* cultures experiences frost damage, but 6°C seem to be well tolerated.

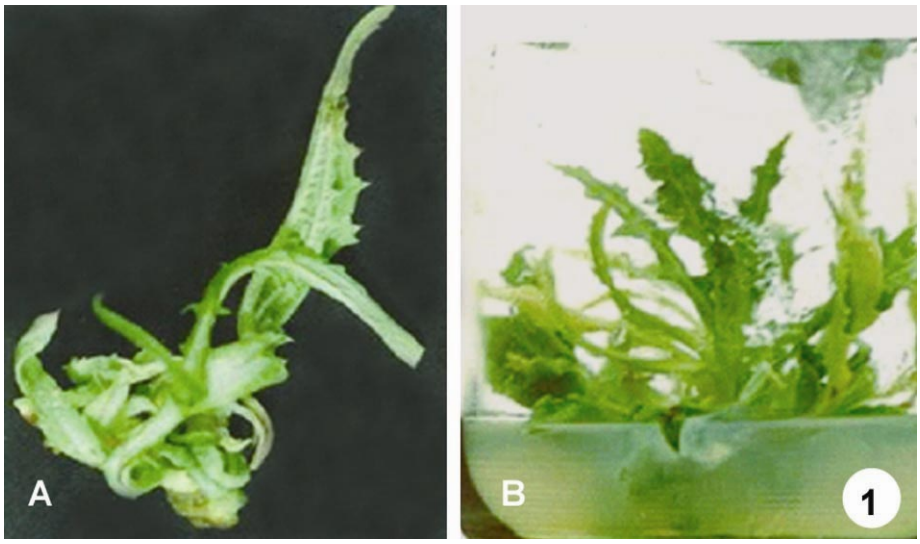
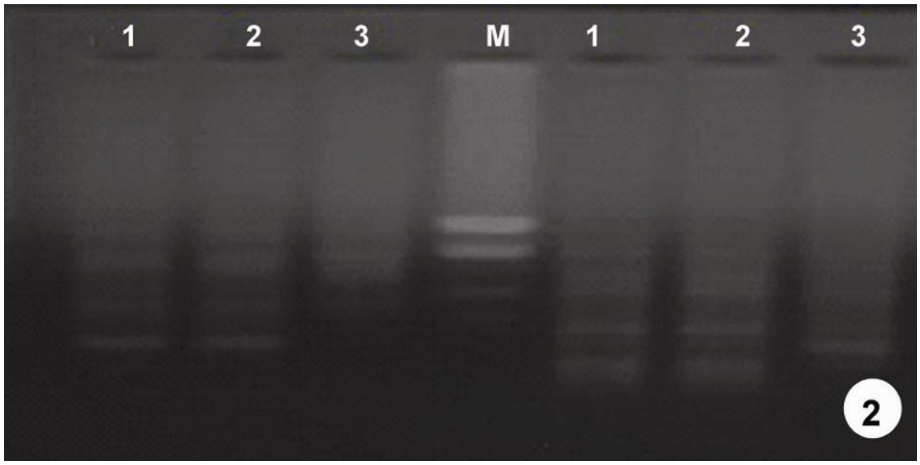


Fig. 1. A. shoot bud cultures of globe artichoke after 12 months of storage at low temperature of 6°C. B. Proliferation of cold stored globe artichoke bud cultures on recovery medium.

Modified medium storage : In the present study, modification of storage medium by incorporating 40 g/l of mannitol or sorbitol was investigated. Results indicate that presence of mannitol or sorbitol in culture medium had a retardant effect on the growth and development of all cultures. Data of Table 2 show that high percentage (80) of healthy shoot bud cultures was obtained after storage for three months in the medium containing 40 g/l mannitol or sorbitol under normal growth conditions. However, survival and healthy shoot cultures sharply decreased after six months of storage. After 12 months, high per cent of cultures suffered etiolation. Also, proliferation rates of shoots decreased as storage duration increased (Table 2). It was observed that there is no clear cut difference between mannitol and sorbitol in their effects on survival of stored shoot

cultures. However, mannitol showed slightly positive effect on the proliferation rate of recovering shoot cultures. Growth of callus cultures of globe artichoke stored in the modified medium supplemented with 40 g/l of mannitol or sorbitol expressed in terms of fresh and dry weight showed an increase as the storage time extended till the ninth month and thereafter it decreased (Table 3). Raising the osmolarity of the medium leads to less dramatic results, but might, in combination with a reduced temperature, help prolong storage period. The results are similar with those reported by Bekheet et al. (2001). They mentioned that at normal temperature, shoot and callus cultures of date palm survived for nine and six months, respectively under osmotic stress on a medium containing 40 mg/l sorbitol. They added that low temperature and dark conditions were more effective on storage of date palm tissue cultures compared to osmotic stress storage. However, in vitro grown shoot cultures of asparagus survived for 20 months, when stored on a medium containing 3% sucrose and 4% sorbitol (Fletcher 1994). In this respect, replacement of sucrose by ribose allowed the conservation of banana plantlets for 24 months (Ko et al. 1991).



2. RAPD profile of normally *in vitro* grown bud cultures (lane 1), *in vitro* preserved plantlets (lane 2), undifferentiated (callus) cultures of globe artichoke (lane 3) and the DNA marker (M) from left to right using random primers; i.e., K1 and K2.

RAPD analysis: RAPD-DNA analysis was used to determine the genetic stability of preserved tissue cultures of globe artichoke. Three randomly selected primers were used in this investigation. Only one of them (K4) did not give reproducible and sufficient amplification products. As shown in Fig. 2, DNA fragments varied in numbers and sizes depending on the primers used. The banding pattern reveals that the differentiated tissue cultures both in normal *in vitro* grown or preserved cultures were identical. However, some bands were absent in the preserved undifferentiated tissue (callus). This may be due to

somaclonal variation or to the growth habit of callus. It is particularly important to confirm that preserved cultures of globe artichoke produce plantlets that are genetically similar to those in non-treated cultures. The present results are in agreement with those reported by Saker et al. (2000). They mentioned that no significant variation was observed among tissue culture derived plantlets. RAPD analysis showed genetic variation in only 4% of the analyzed plants (70 regenerants) which were grown for 6 - 12 months at 25ºC. It may be mentioned here that in the past genetic marker analysis has been used to study the degree of genetic changes in plants regenerated *in vitro* such as pea (Cecchini et al. 1992), sugar beet (Sabir et al. 1992) and wheat (Brown et al. 1993).

References

Ancora G, Bell-Donini ML and **Cuozzo L** (1981) Globe artichoke plants from shoot apices through rapid *in vitro* micropropagation. Scientia Horticult. **14** : 207-213.

Bajaj YPS (1983) Cryopreservation and international exchange of germplasm. *In:* Plant Cell Culture in Crop Improvement. Sen, S.K. and Giles, K.L. (Eds). pp. 19-41, New York, Plenum Press.

Bekheet SA (2000) *In vitro* preservation of *Asparagus officinalis*. Biologia Plantarum **43**(2): 179-183.

Bekheet SA, Taha HS and **Saker MM** (2001) *In vitro* long term storage of date palm. Biologia Plantarum **45**(1): 121-124.

Bekheet SA, Taha HS and **El-Bahr MK** (2005) Preservation of date palm cultures using encapsulated somatic embryos. Arab J. Biotech. **8**(2): 309-328.

Brown PH, Lange FD, Kranz E and **Lorz H** (1993) Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Mol. Gen. Genet. **237**: 311-317.

Cecchini E, Natali N, Cavallini A and **Durante M** (1992) DNA variation in regenerated plants of pea (*Pisum sativum* L.). Theor. Appl. Genet. **84**: 874-879.

Chin HF and **Roberts EH** (1980) Recalicitrant crop seed. Kuala. L (Ed.), Malaysia, Tropical Press, SDN, BDH.

Dodds JH (1988) Tissue culture technology: Practical application of sophisticated methods. Amer. Potato J. **65**: 167-180.

Doyle JJ and **Doyle JL** (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin **19**: 11-15.

El-Bahr MK, Okasha Kh A and **Bekheet SA** (2001) *In vitro* morphogenesis of globe artichoke (*Cynara scolymus* L.) Arab J. Biotech. **4**(1): 119-128.

Engelmann F (1991) *In vitro* conservation of horticultural species. Acta Horticulturae **29**: 327-334.

Fletcher PJ (1994) *In vitro* long term storage of asparagus. New Zeal. J. Crop Hort. Sci. **22**: 351-359.

Kartha KK, Mroginski LA, Pahl K and **Leung NL** (1981) Germplasm preservation of coffee (*Coffea arabica* L.) by *in vitro* culture of shoot apical meristems. Plant Sci. Lett. **22**: 301-307.

Ko WH, Hwang SC and **Ku FM** (1991) A new technique for storage of meristem-tip cultures of Cavendish banana. Plant Cell, Tissue and Organ Cult. **25** : 179-183.

Mullin RH and **Schlegel DE** (1976) Cold storage maintenance of strawberry meristem plantlets. Horti. Sci. **11**: 101-110.

Pecaut P and **Dumas de vaulx R** (1983) Virus-free clones of globe artichoke (*Cynara scolymus* L.) obtained after *in vitro* propagation . Acta Horti. **131**: 303-309.

Rossi V and **Paoli De** (1992) Micropropagation of artichoke (*Cynara scolymus*). *In :* Biotechnology in Agriculture and Forestry, Vol, 19. High-Tech and Micropropagation III (Ed. By Bajaj, Y.P.S.). Springer- Verlag Berlin, Heidelberg.

Sabir A, Newbury HJ, Todd G, Catty J and **Ford-Lloyd BV** (1992) Determination of genetic stability using isozymes and RFLP in beet plants regenerated *in vitro*. Theor. Appl. Genet. 84:113-117.

Saker MM, Bekheet SA, Taha HS, Fahmy AS and **Moursy HA** (2000) Detection of somaclonal variation in tissue culture-derived date palm plants using isoenzyme analysis and RAPD fingerprints. Biologia Plantarum.

Snedecor GW and **Cochran WG** (1967) Statistical Methods. 6th Edition.- Iowa State. University Press , Iowa. 1967.

Wanas WH (1992) *In vitro* storage of proliferated apple rootstock shoot-tip cultures. Annala Agric.Sci. Ain Shams Univ. **37** : 501-510.

Wanas WH, Callow JA and **Withers LA** (1986) Growth limitation for the conservation of pear genotypes. *In :* Plant Ttissue Culture and its Agricultural Application.(Eds.) Withers, L. and Alderson, P.G., pp.285-291, Cambridge Univ. Press., Cambridge.

Withers LA (1989) *In vitro* conservation and germplasm utilization. *In :* The Use of Plant Genetic resources. Brown, a. H.D. Marshall, D.R. and Williams, J.T. (Eds.). pp. 309-334., Cambridge Univ., Cambridge Press.

Withers LA (1991) Biotechnology and plant genetic resources conservation. *In :* Plant Genetic Rresources, Conservation and Managemennt Concepts and Apptoaches. Paroda, R.S. and Arora, R.K. (Eds.). pp. 273-297. International Board for Plant Genetic Resources, New Delhi.