INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2007/09–3–404–407
http://www.fspublishers.org

In vitro Conservation of Globe Artichoke (*Cynara scolymus* L.) Germplasm

BEKHEET SHAWKY AND USAMA IBRAHIM ALY[1]
Department of Plant Biotechnology, National Research Centre, El-Behooth St., Dokki, Cairo, Egypt
[1]Corresponding author's email: usamajp@yahoo.com

ABSTRACT

An effective method for *in vitro* mid-term storage of globe artichoke cultures was recognized. Aseptically, shoot bud and callus cultures of globe artichoke were successfully stored for twelve months at 6ºC in dark. At these conditions, high percentage of cultures remained viable without serious signs of senenscence. Although the rate of regrowth slightly decreased, the recovery percentage was enough to obtain high frequency of healthy globe artichoke tissue cultures. Mannitol and sorbitol were also examined as osmotic stress agents by slowing down the rate of growth of globe artichoke tissue cultures. The storage at cold and dark conditions was more effective compared with that caused by osmotic stress. Shoot bud cultures also showed higher viability percentages than callus cultures. According to RAPD analysis, plantlets derived from preserved shoot bud cultures were genetically identical to the control, while callus cultures showed genetic variation.

Key Words: Globe artichoke; Germplasm; Cold storage; Osmotic stress; RAPD analysis

INTRODUCTION

True seeds offer a reliable mean for storage of plant species that produce orthodox propagule. However, seed storage is impossible for other species (Chin & Roberts, 1980). Many plants produce recalcitrant seeds that are viable for only short periods, other species are vegetatively propagated, which pose preservation problems (Withers, 1989). Because of the fact that sexual propagation produces high level of heterogeneity, globe artichoke is conventionally propagated vegetatively by offshoots and crown segments, which however shows low rate of multiplication and transmision of diseases. Accordingly, tissue culture propagation offers an alternative approach of producing large, homogeneous and disease-free population of globe artichoke (Ancora *et al.,* 1981; Pecaut & Dumas de Vaulx, 1983; Rossi & Paoli, 1992) and can also allow the *in vitro* storing of selected genotypes.

According to Bajaj (1983) a variety of cells and tissues, such as protoplast, single cells and organized tissues as well as meristems and somatic embryos could be stored. *In vitro* storage offers several advantages over maintained field collections, because of spatial and maintenance requiremennts are minimal. Moreover, propagation potential of cultures is greater and genetic erosion, pests and diseases are avoided. There are two approaches to storing vegetatively propagated germplasm. The first is to slow the growth rate of the callus or plants and the second is to suspend or stop the growth alltogether by cryopreservation (Withers, 1991). This paper describes a method for preservation of globe artichoke cultures for short and mid-term period through *in vitro* storage of shoot buds and callus cultures by slow growth method.

MATERIAL AND METHODS

Establishment of tissue cultures. Offshoots of globe artichoke (*Cynara scolymus* L. cv. Balady, an Egyptian local cultivar) were isolated and their basal parts were removed to a length of about 5 cm. 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% sodium hypochlorite (NaOCl) for 20 min. The explants were then rinsed thrice with sterile distilled water. The meristem tips (2 - 3 mm) with a small part of submeristematic tissues were isolated and cultured on Murashige and Skoog 1962 (MS) medium supplemented with 10 mg/L Kinetin and 50 mg L^{-1} sodium dihydrogen phosphate as described by Ancora *et al.* (1981). The differentiated shoot buds were multiplicated on the same culture medium except of Kinetin (Kin) from 10 to 5 mg L^{-1}. To obtain callus cultures, leaf segments, taken from *in vitro* grown shoots were subcultured on MS-medium contained 2 mg L^{-1} benzyladenine (BA) + 5 mg L^{-1} naphthaleneacetic acid (NAA) according to El-Bahr *et al.* (2001). The pH of the media was adjusted to 5.8 ± 0.1 before the addition of 0.8% agar. Media were autoclaved at 121ºC and 15 Ib m^{-2} for 20 min before explants inoculation.

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 incubated at 6ºC in dark condition. Percentage of survival and healthy cultures were recorded after 3, 6, 9 and 12 months of storage using ten replicates. The number of proliferated shoots were also recorded after four weeks of transferring the green shoots from different treatments onto normal growth conditions (recovery potential). For callus

storage, equal inocula (250 mg) of callus were transferred to callus maintaining medium and incubated at 6ºC in dark condition.

Measurement of callus growth. Callus growth, expressed as fresh and dry weights and growth value were determined after 3, 6, 9 and 12 months of cultivation. The callus cultures were washed thrice with deionized water, filtered and weighed as fresh weight then dried at 60ºC overnight in an oven for dry weight measurement. Ten replicates of either shoot buds or callus at each storage period were used in this experiment. The growth value was calculated as follows:

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

Osmotic stress storage. To evaluate the role of mannitol and sorbitol as osmotic agents, shoot buds of globe artichoke were grown on shoot multiplication medium supplemented with 40 g L^{-1} of either mannitol or sorbitol and incubated under normal growth conditions. Survival and healthy culture percentages were calculated from ten replicates after 3, 6, 9 and 12 months of storage. For callus storage, pieces of callus tissues (250 mg each) were subcultured on callus maintaining medium containing mannitol or sorbitol and then stored at normal growth conditions. Culture media were adjusted to pH 5.8 before autoclaving at 121ºC and 15 Ib m^{-2} for 20 min. The cultures were stored at 25 ± 2ºC under 16 h photoperiod with light irradiance of 45 µmol m^{-1} s^{-1} (Phillips white fluorescent tubes). Ten replicates were collected after 3, 6, 9 and 12 months storage. Fresh and dry weights were measured and growth value was calculated.

Randomly amplified polymorphic DNA (RAPD) analysis. DNA isolation was performed using the Cetyl Trimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle (1987). Half grams of fresh samples was ground to powder in liquid nitrogen with a pre-chilled pestle and mortar, suspended in 5 mL preheated CTAB buffer and incubated at 65°C for 1 h 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 precipitated by centrifugation. The pellet was washed carefully twice with 70% ethanol, dried at room temperature and re-suspended in 0.5 mL TE buffer. The enzyme, RNAse (20 µg), was added to the re-suspended 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 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. Thermocycler programmed for 45 cycles as follow: 94ºC/5 min (1 cycle), 94ºC/30 sec, 36ºC/30 sec, 72ºC/2 min (45 cycle) and 72ºC/7 min (1 cycle), then hold at 4ºC. The amplified DNA fragments were separated on 2% agarose gel and stained with ethidium bromide. Three oligonucleotide primers 10-base (Operon technologies Inc., Alameda, California) were randomly selected and used in RAPD analysis. The primers are K1 (TGGCGACCTG), K2 (GAGGCGTCGC) and K4 (TCGTTCCGC). 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.

Statistical analysis. Experiments were run in completely randomized design and data were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

Storage at low temperature. Table I shows the percentage of survival and the healthy shoot bud cultures and the mean number of proliferated shoots retrieved from storage. After three months storage at 6ºC in dark, shoot buds showed high percentage of viability (100%) and healthy shoots (90%). A decline in both was recorded thereafter with increasing the duration of storage to 6, 9 and 12 months. The number of proliferated shoots after recovery also followed the same trend (Table I). Although the lowest percentage of survived shoot buds (50%) was recorded after 12 months of storage, most of these buds were viable enough to convert into healthy shoots on recovery (Table I, Fig. 1a, b).

The results of globe artichoke callus cultures reveal that stored cultures remained healthy without any serious signs of senescence during different storage periods. Callus growth presented as fresh and dry cell weight as well as growth value increased with storage duration until the ninth month. A deterioration in all parameters tested (Table II), with slight browning of the preserved callus, was then noticed after 12 months of storage. Engelmann (1991) reported that growth reduction could be achieved by modifying the environmental conditions and/or the culture medium. He added that, the most widely applied technique is temperature reduction, which can be combined with a decrease in light intensity or culture in dark. The present results are also in accordance with those reported by Bekheet (2000), in his study on *Asparagus officinalis*. He mentioned that tissue cultures remained viable after eighteen months of storage at 5ºC. In addition, 80% of pear and 85% of apple shoot bud cultures grown *in vitro* remained alive after eighteen months storage at 4ºC and 8ºC, respectively (Wanas *et al*., 1986; Wanas, 1992). In this respect, strawberry (*Fragaria ananassa*) plantlets have been stored at 4ºC in the dark and remained viable for 6 years with the regular addition of a few drops of fresh liquid medium (Mullin & Schlegel, 1976). Moreover, Dodds (1988)

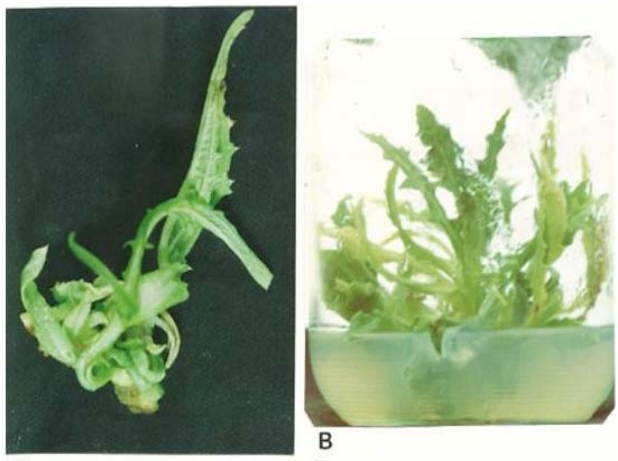
Table I. Percentage of survival and healthy shoots and number of proliferated recovered shoots afterstorage for 3, 6, 9 or 12 months under 6ºC or osmotic stress induced by 40 g/L mannitol or sorbitol. Each value is the mean ± S.E. of 10 replicates

Storage duration	Survival (%)			Healthy shoots (%)			No. of proliferated shoots after recovery		
(months)	6 ºC	Mannitol	Sorbitol	6 ºC	Mannitol	Sorbitol	6 ºC	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 II. Growth dynamics of globe artichoke callus cultures during storage for 3, 6, 9 or 12 months at low temperature of 6ºC or osmotic stress induced by 40 g/L mannitol or sorbitol. Each value is the mean ± S.E. of 10 replicates. Initial fresh weight is 250 mg

Storage duration	Fresh weight (g)			Dry weight (mg)			Growth value		
(month)	6 ºC	Mannitol	Sorbitol	6 ºC	Mannitol	Sorbitol	6 ºC	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

Fig. 1a. Shoot bud of globe artichoke after twelve months of storage at 6ºC in dark, (b) Proliferated shoots after transferred to normal growth conditions for recovery



reported that at temperature lower than 3ºC, *in vitro* potato cultures experienced frost damage, but 6ºC seem to be well tolerated.

Osmotic stress storage. Storage by changing in the composition of the culture medium involves the addition of osmotica. In the present study, modification of storage medium by incorporating 40 g L^{-1} of mannitol or sorbitol was investigated. Results indicate that the presence of mannitol or sorbitol in culture medium had a retardant effect on the growth and development of all cultures. Data of reveal that high percentage of survival (100%) and healthy shoot bud cultures (80%) were recorded after the first three months of storage on medium containing 40 g L^{-1} mannitol or sorbitol at normal growth conditions (Table I). However, survival and healthy shoot cultures were sharply decreased after six months of storage. After twelve months, high percentage of cultures suffered from etiolation. Proliferation rates of recovered shoots decreased as storage duration increased (Table I). No clear difference between mannitol and sorbitol in their effect on survival of stored shoot cultures of globe artichoke was observed. However, mannitol showed slightly positive effect on the proliferation rate of recovered shoot cultures.

Growth dynamics of callus cultures of globe artichoke stored on modified medium supplemented with 40 g L^{-1} of mannitol or sorbitol presented as fresh and dry weights and growth value were increased with increasing the storage duration until the ninth month then decreased after 12 months storage (Table II). Raising the osmolarity of the medium leads to less dramatic results, but might, in combination with a reduced temperature, help to prolong storage period (Kartha *et al.,* 1981). The results of the present investigation agree with those reported by Bekheet *et al*. (2001), who mentioned that at normal temperature, shoot and callus cultures of date palm survived for nine and six months under osmotic stress on medium containing 40 mg L^{-1} sorbitol. They also found that low temperature and dark conditions were more effective for the storage of date palm tissue cultures compared with osmotic stress storage. However, *in vitro* shoot cultures of asparagus survived for 20 months when stored on 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).

RAPD analysis. RAPD analysis was used in this study to determine the genetic stability of preserved tissue cultures of globe artichoke. 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), sugar beet (Sabir *et al.,* 1992) and wheat (Brown *et al.,* 1993). Three randomly selected primers were used and only one (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 reveals

Fig. 2. RAPD profile of shoot bud cultures grown in vitro under standard conditions, (lane 1), *in vitro* cold preserved plantlets, (lane 2) and un-differentiated callus cultures of globe artichoke, (lane 3). As (M) represents the DNA marker, tow random primers were used, K1 at the left and K2 at the right

that the differentiated tissue cultures either *in vitro* under standard conditions or preserved under low temperature were identical. However, some bands were absent in the preserved callus tissues. This may be due to somaclonal variation or to the growth of callus under harsh *in vitro* conditions. It is particularly important to confirm that preserved cultures of globe artichoke produce plantlets genetically similar their controls. The present results are in line with those of Saker *et al.* (2000), who mentioned that no significant variations were observed in tissue cultures derived date palm plantlets. RAPD analysis showed genetic variation in only 4% of analyzed plants (70 regenerants), which were incubated for 6 - 12 months under 25˚C.

REFERENCES

Ancora, G., M.L. Bell-Donini and L. Cuozzo, 1981. Globe artichoke plants from shoot apices through rapid *in vitro* micropropagation. *Sci. Hort*., 14: 207–13

Bajaj, Y.P., 1983. Cryopreservation and international exchange of germplasm. *In*: Sen, S.K. and K.L. Giles (eds.), *Plant Cell Culture in Crop Improvement,* pp: 19–41. New York

Bekheet, S.A., 2000. *In viro* preservation of *Asparagus officinalis*. *Biol. Plant.*, 43: 179–83

Bekheet, S.A., H.S. Taha and M.M. Saker, 2001. *In vitro* long-term storage of date palm. *Biol. Plant.*, 45: 121–4

Brown, P.H., F.D. Lange, E. Kranz and H. Lorz, 1993. Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. *Mol. Gen. Genet*., 237: 311–7

Cecchini, E., N. Natali, A. Cavallini and M. Durante, 1992. DNA variation in regenerated plants of pea (*Pisum sativum* L.). *Theor. Appl. Genet*., 84: 874–9

Chin, H.F. and E.H. Roberts, 1980. *In*: Kuala, L. (ed.), *Recalcitrant Crop Seed*. Malaysia, Tropical Press, SDN. BDH

Dodds, J.H., 1988. Tissue culture technology: practical application of sophisticated methods. *American Pot. J*., 65: 167–80

Doyle, J.J. and J.L. Doyle, 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull*., 19: 11–5

El-Bahr, M.K., K.A. Okasha and S.A. Bekheet, 2001. *In vitro* morphogenesis of globe artichoke (*Cynara scolymus* L.) *Arab J. Biotech*., 4: 119–28

Engelmann, F., 1991. *In vitro* conservation of horticultural species. *Acta Hort*., 29: 327–34

Fletcher, P.J., 1994. *In vitro* long-term storage of asparagus. *New Zealand J. Crop Hort. Sci*., 22: 351–9

Kartha, K.K., L.A. Mroginski, K. Pahl and N.L. Leung, 1981. Germplasm preservation of coffee (*Coffea arabica* L.) by *in vitro* culture of shoot apical meristem. *Pl. Sci. Lett*., 22: 301–7

Ko, W.H., S.C. Hwang and F.M. Ku, 1991. A new technique for storage of meristem-tip cultures of Cavendish banana. *Pl. Cell Tiss. Organ Cult*., 25: 179–83

Mullin, R.H. and D.E. Schlegel, 1976. Cold storage maintenance of strawberry meristem plantlets. *HortSci*., 11: 101–10

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.,* 15: 473–97

Pecaut, P. and R. Dumas de Vaulx, 1983. Virus-free clones of globe artichoke (*Cynara scolymus* L.) obtained after *in vitro* propagation. *Acta Hort*., 131: 303–9

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

Sabir, A., H.J. Newbury, G. Todd, J. Catty and B.V. Ford-Lloyd, 1992. Determination of genetic stability using isozymes and RFLP in beet plants regenerated *in vitro*. *Theor. Appl. Genet*., 84: 113–7

Saker, M.M., S.A. Bekheet, H.S. Taha, A.S. Fahmy and H.A. Moursy, 2000. Detection of somaclonal variation in tissue culture-derived date palm plants using isoenzyme analysis and RAPD fingerprints. *Biol. Planta*, 43: 347–51

Snedecor, G.W. and W.G. Cochran, 1967. "*Statistical methods*," 6th edition, Iowa Stat University Press, Iowa, USA

Wanas, W.H., 1992. *In vitro* storage of proliferated apple rootstock shoot-tip cultures. *Anal. Agric.Sci. Ain Shams University*, 37: 501–10

Wanas, W.H., J.A. Callow and L.A. Withers, 1986. Growth limitation for the conservation of pear genotypes. *In*: Withers, L. and P.G. Alderson (eds.), *Plant Tissue Culture and its Agricultural Application,* pp: 285–91, Cambridge University, Press, Cambridge

Withers, L.A., 1989. *In vitro* conservation and germplasm utilization. *In*: Brown, H.D., D.R. Marshall and J.T. Williams, (eds.), *The Use of Plant Genetic Resources,* pp: 309–34. Cambridge University, Cambridge Press

Withers, L.A., 1991. Biotechnology and plant genetic resources conservation. *In*: Paroda, R.S. and R.K. Arora, (eds.), *Plant Genetic Rresources, Conservation and Managemennt Concepts and Approaches,* pp: 273–97. International Board for Plant Genetic Resources, New Delhi

(Received 02 February 2007; Accepted 25 April 2007)