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Effect of Temperature, Sugar and Vessel Closure on *in Vitro* Growth of *Gypsophila paniculata*

Mohamed Rady* • **Shawky Bekheet**

Plant Biotechnology Department, Genetic Engineering and Biotechnology Division, National Research Centre, El-Bohos St., P.O. Box 12311, Dokki, Giza Cairo, Egypt

Corresponding author: * **drrady1@yahoo.com**

ABSTRACT

Shoot cultures of *Gypsophila paniculata* L. 'Bristol fairy' were established on MS medium supplemented with 2.69 µM α-naphthaleneacetic acid (NAA) and 2.22 µM ^{6}N-benzyl amino purine (BAP). After three subcultures, shoots were grown in media with different concentrations of mannitol and incubated at 25 or 5°C for 6 months. To evaluate the role of type of enclosures for culture tubes and sucrose level in the culture medium on preservation of shoot cultures of gypsophila, four different enclosures namely, polypropylene caps, cotton, one and two layers of aluminum foil were used. Shoot cultures were incubated at 25°C for 6 months. The presence of mannitol in the culture retarded growth and proliferation with highest survival rates at 4% mannitol. Relatively slower growth was observed at 5°C than at 25°C. Mannitol (4%) and incubation at 5°C can be used successfully to preserve gypsophila shoot cultures for up to 6 months or more. Moreover, the absence of sucrose in the media during preservation retarded the growth of gypsophila shoot culture in all treatments with all caps used. In contrast, the addition of high levels of sucrose (2 or 3%) improved the growth (number of proliferated shoots and shoot length) of shoot cultures and was considered the best condition for *in vitro* preservation. Also, polypropylene caps and two layers of foil were the best covers for the proliferation of shoot cultures. Although this study does not seek to find the appropriate conditions to preserve gypsophila plantlets, the results indicate that physical and chemical treatments affect survival percentage and growth characters. In this study, gypsophila plantlets were resistant to low temperature and water deficit conditions but sensitive to the type of closure system and carbon source.

Keywords: cut flower plant, mannitol, sucrose, temperature variation, tissue culture
Abbreviations: BAP, ^{6}N-benzyl amino purine; **NAA**, α-naphthaleneacetic acid

INTRODUCTION

Seeds offer a reliable means for storage of plant germplasm. However, seed storage is sometimes impractical and in some cases impossible. Also, some species are seedless and vegetative propagated, leading to preservation problems (Withers 1989). *In vitro* technology offers feasible storage alternatives for difficult-to-store categories of germplasm e.g. recalcitrant seeds and vegetative propagated plants (Grout 1990; Keller *et al*. 2006).

In vitro preservation of shoot cultures provides a source of explants protected from contamination and permanently available for micropropagation. A preservation system with high growth rates is not ideal for many objects as it requires frequent attention and large proportion of available culture space. In this respect, extensive research has been done to reduce growth rates by decreasing culture medium components and modifying the physical environments. Even though modification of the gaseous environment by control of the gas balance is one way to retard growth, the most practical and effective slow growth methods to date involve reducing the incubation temperature (reviewed by Cha-Um and Kirdmanee 2007) and/or adding osmotic retardant to the culture medium. Cultures of many species can be maintained in this way from six months to two years without subculturing (Withers 1987, 1992). In this connection, several attempts have been achieved to preserve tissue cultures of some important plant species by slow growth methods (Wanas 1992; Bekheet *et al*. 2001; Winarto *et al*. 2004; Islam *et al*. 2005; Divakaran *et al*. 2006; Cha-Um *et al*. 2007; Bekheet 2007).

Gypsophila paniculata L. is one of the most important cut flowers in commercial floriculture. It is a vegetatively propagated plant because it is naturally sterile or seedless (Shillo 1985). Thus, its germplasm can not be stored or handled easily using conventional method. *In vitro* propagation of different *Gypsophila* cultivars has been developed by several researchers (Zamorano-Mendoza and Mejia-Munoz 1994 on 'Perfecta'; Ahroni *et al*. 1997 on 'Arbel'; Lee and Bae 1999 on 'Bristol fairy'). The objective of this work was to study the effect of temperature and mannitol level as well as sucrose level and capping type on *in vitro* shoot preservation of *G. paniculata*.

MATERIALS AND METHODS

Establishment of *in vitro* culture

G. paniculata L. 'Bristol fairy' shoot cuttings (3.5 ± 0.5 cm in length), were obtained from maturated plants from the greenhouse, surface sterilized with 70% ethanol for 1 min, followed by 20% commercial Clorox® [5.25% (w/v) active ingredient, sodium hypochlorite] for 20 min. The explants were then rinsed three times using sterile distilled water. Shoot tips (2.5 ± 0.5 mm in length) were aseptically excised and then cultured in glass vessels containing 40 mL of MS medium (Murashige and Skoog 1962) supplemented 2.69 µM α-naphthaleneacetic acid (NAA) and 2.22 µM ^{6}N-benzyl amino purine (BAP) according to Rady and Hanafy (2004) who also used 'Bristol fairy'. The MS basal medium was supplemented with 3% sucrose and solidified with 0.7% agar. The pH was adjusted to 5.8 before autoclaving. Culture vessels containing shoot tips were incubated in a growth chamber at 25 ± 2°C under 40 µmol m^{-2} s^{-1} photosynthetic photon flux (FFP) provided by cool white fluorescent lamps (Philips, 40 W Champion AR) with a 16 h photoperiod for 4-5 weeks then subcultured three times on fresh medium to generate plant materials (**Fig. 1**).

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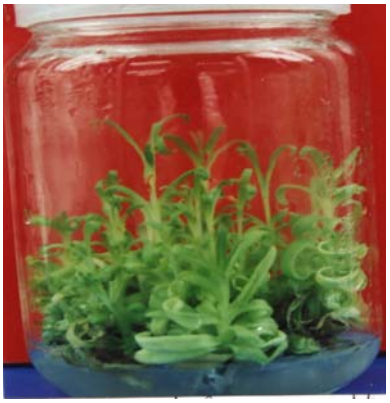

Fig. 1 Shoot cultures of gypsophila after three subcultures on MS-medium supplemented with 2.69 µM α-naphthaleneacetic acid (NAA) and 2.22 µM ^{6}N-benzyl amino purine (BAP).

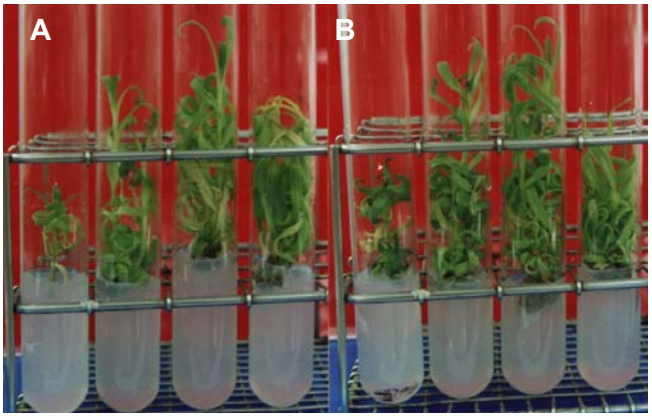

Fig. 2 Shoot cultures of gypsophila after six months of preservation on MS-medium contained 0, 2, 4 and 8% mannitol and incubated at 25°C (A) and 5°C (B).

Osmotic stress and low temperature

To investigate the effect of temperature in combination with osmotic stress (mannitol) on preservation of shoot cultures of *G. paniculata*, proliferated shoots (2.0 ± 0.5 cm in length) were transferred into fresh medium and supplemented with 0, 2, 4 and 8% mannitol. The plantlet cultures were then incubated at 25 or 5°C for 6 months. Survival percentage, healthy shoot percentage, number of shoots and shoot height (cm) were recorded.

Sucrose levels in media and type of enclosures

To evaluate the role of sucrose level in culture medium and type of enclosures on preservation of shoot cultures of gypsophila, sucrose was added at 0, 1, 2 and 3% and the culture tubes were enclosed with polypropylene caps, cotton, one or two layers of aluminum foil. After 6 months of incubation at 25°C, survival percentage, healthy shoot cultures percentage, number of shoots and shoot height (cm) were registered.

Statistical analysis

The results presented are the mean values ± standard errors obtained from at least five replicates. Statistical significance between mean values was assessed using the F-test according to Steel and Torrie (1960). Analysis of variance was determined and the value of least significant difference was calculated at 5% level to compare different treatment. All experiments were designed in completely randomized design and repeated twice.

RESULTS AND DISCUSSION

Effect of osmotic stress and low temperature preservation

Data of *in vitro* storage of gypsophila tissue culture presented as survival percentage, healthy cultures percentage, and number of shoots and shoot height were illustrated in **Table 1** and **Fig. 2**.

Results indicate that presence of mannitol in the culture media had a retardant effect on growth and tissue culture differentiation, and gradually decreased growth as mannitol level increased in the medium, although only extremely high levels (6 and 8% mannitol) affected growth parameters negatively. From the obtained data it could be observed that number of shoots declined from 4 and 3.50 at 0.0% mannitol to 2 at 8% mannitol with the two different temperatures used as well as shoot height declined from 4.60 and 4.30 cm at 0.0% mannitol to 3.30 and 3.00 cm at 8% mannitol with 25 and 5°C respectively.). However, the best survival rates (100%) were at 4% mannitol in the culture medium at both temperatures tested. Mannitol was reported to inhibit the growth of micropropagated asparagus (Conner and Fallon 1993) and chrysanthemum (Teixeira da Silva 2004). One of the most pronounced results of this study is that low temperature storage did not inhibit the growth of *in vitro* grown shoot cultures of gypsophila, and this may be because gypsophila is a temperate plant species, which grows well at cool temperatures and is able to resist low temperatures and water deficit (Cha-um, pers. comm.). A relatively slower growth was observed at normal temperature compared with those incubated at low temperature, since healthy shoots percentage values were 70, 80, 90 and 75% at 25°C and 80, 90, 100 and 95% at 5°C.

The highest percentage of survival and healthy shoots (100%) was recorded with medium amended with 4% mannitol and their cultured incubated at 5°C. However, in spite of the lowest growth rates observed with 8% mannitol containing medium which incubated at 5°C, some evidences of hyperhydricity and senescence were noticed (**Table 1, Fig. 2**). Bekheet (2000) found that 90% of shoot cultures of asparagus grown *in vitro* remained healthy and green on medium contained 4% mannitol up to 6 months at normal culture conditions.

Statistical analysis revealed no significant differences between treatments of cultures which where incubated at 5°C. Thus, the significant change in survival and growth of gypsophila cultures is caused by incubation at 25°C and this may be due to the changes in the concentration of mannitol add to the culture media that affect the nature of plant growth. It is obvious that the addition of mannitol to the culture media in different levels significantly affects healthy shoots and the number of shoot in the treatments. From the above results we can conclude that the addition of mannitol to the culture medium with incubation at 5°C can be used successfully for preservation of gypsophila shoot cultures and sub-culturing schedule can be extended to 6 months or more. In this respect, Son *et al*. (1991) stated that low tem-

Table 1 Effect of different mannitol concentrations in culture medium and temperature on survival and growth of gypsophila cultures *in vitro* for 6 months.

Mannitol (%)	Incubation at 25°C				Incubation at 5°C			
	Survival rate (%)	Healthy shoots (%)	№ of shoots	Shoot length (cm)	Survival rate (%)	Healthy shoots (%)	№ of shoots	Shoot height (cm)
0	90 a	70 b	4.00 ± 0.05 a	4.60 ± 0.10 a	100 a	80 a	3.50 ± 0.17 a	4.80 ± 0.09 a
2	90 a	80 a	3.00 ± 0.11 a	4.00 ± 0.20 a	100 a	90 a	3.00 ± 0.22 a	4.70 ± 0.08 a
4	100 a	90 a	2.10 ± 0.15 b	3.90 ± 0.22 a	100 a	100 a	2.90 ± 0.18 a	3.80 ± 0.12 a
8	90 a	75 b	2.00 ± 0.13 b	3.30 ± 0.10 a	100 a	95 a	2.00 ± 0.21 a	3.00 ± 0.08 a

Numbers in the same column followed by the same letter are not significantly different at $P \leq 0.05$.

Table 2 Effect of sucrose concentration and type of caps on survival and growth of gypsophila shoot cultures *in vitro* for 6 months.

Sucrose concentration (%)	Survival rate (%)	Healthy shoots (%)	№ of shoots	Shoot length (cm)
Polypropylene caps				
0	100 a	90 a	0.0 b	2.00 ± 0.02 b
1	100 a	95 a	3.50 ± 0.05 a	5.00 ± 0.20 a
2	100 a	100 a	4.30 ± 0.09 a	4.80 ± 0.11 a
3	100 a	95 a	5.00 ± 0.20 a	4.20 ± 0.03 a
Cotton wool				
0	100 a	95 a	2.20 ± 0.04 b	3.00 ± 0.11 b
1	100 a	100 a	3.00 ± 0.01 a	6.00 ± 0.30 a
2	100 a	100 a	3.30 ± 0.08 a	7.00 ± 0.20 a
3	100 a	100 a	4.00 ± 0.10 a	3.30 ± 0.05 b
One layer foil				
0	100 a	80 a	0.0 c	3.00 ± 0.13 b
1	100 a	90 a	2.00 ± 0.04 b	4.50 ± 0.22 a
2	100 a	95 a	4.80 ± 0.20 a	5.20 ± 0.17 a
3	100 a	90 a	5.30 ± 0.13 a	3.90 ± 0.20 b
Two layer foil				
0	70 b	50 c	0.0 b	2.30 ± 0.02 b
1	100 a	80 a	4.50 ± 0.25 a	4.90 ± 0.15 a
2	100 a	90 a	5.80 ± 0.14 a	8.00 ± 0.19 a
3	90 a	80 a	3.80 ± 0.15 a	4.90 ± 0.25 a

Numbers in the same column followed by the same letter are not significantly different at $P \leq 0.05$.

perature (about 4°C) has been widely applied for short-term, minimal growth storage of cultured plant cells, tissues and organs. To prepare for nursery planting during the optimal growing season, low-temperature storage was used successfully for large-scale synchronization of transplanting propagules.

Slow growth involves osmotic stress, which may be a problem in culture prone to somaclonal variation. Therefore, slow growth is not generally recommended for disorganized cultures such as callus tissues. In this respect, several authors claimed that osmotic medium and low temperature increased survival percentage of *in vitro* stored cultures (Dodds 1988; Arora and Bhojwani 1989; Bekheet 2001).

Effect of sucrose level and type of enclosure on preservation

The role of sucrose level in culture medium and type of enclosures on preservation of shoot cultures of *G. paniculata* were studied. Sucrose was added to the culture medium from 0 to 3% and the culture tubes were covered with polypropylene caps, cotton, and one layer of aluminum foil and two layers of aluminum foil.

Data in **Table 2** demonstrates the influence of sugar added to culture media and type of enclosures on survival and growth of gypsophila shoot cultures preserved *in vitro* for 6 months. Data presented in **Table 2** shows that absence of sucrose in the culture media caused a decrease in healthy shoot percentage with all caps used. The greatest decrease in the percentage of healthy shoots (50%) was observed when culture vessels were covered with two layers of foil. There was a highly significant difference between all treatments with and without sucrose. When shoot culture tubes were covered with polypropylene caps, the longest shoots (5 cm) were obtained with a low concentration of sucrose (10%), while the highest percentage (100%) of healthy and proliferated shoots were obtained with high levels of sucrose (2 and 3%) in the media. When shoot cultures tubes were covered with cotton, the longest shoots (7 cm) were obtained when 2% sucrose was added to the culture medium, while the highest survival percentage, healthy shoot percentage (100%) and number of shoots (4%) were obtained when 3% sucrose was added to the media (in many ways 3% sucrose is a fairly standard level in tissue culture and this treatment could also be considered as a control).

When shoot culture tubes were covered with one layer of foil the highest survival rate (100%), healthy shoot percentage (95%) and shoot length (5.20 cm) were observed when 2% sucrose was added to the culture medium, while the highest number of proliferated shoots (5.30) was obtained when 3% sucrose was added to the media. When shoot cultures tubes were covered with two layers of foil the highest survival rate (100%), healthy shoot percentage (90%), number of shoots (5.80) and shoot height (8 cm) were obtained when 2% sucrose was added to the culture medium (**Fig. 3**).

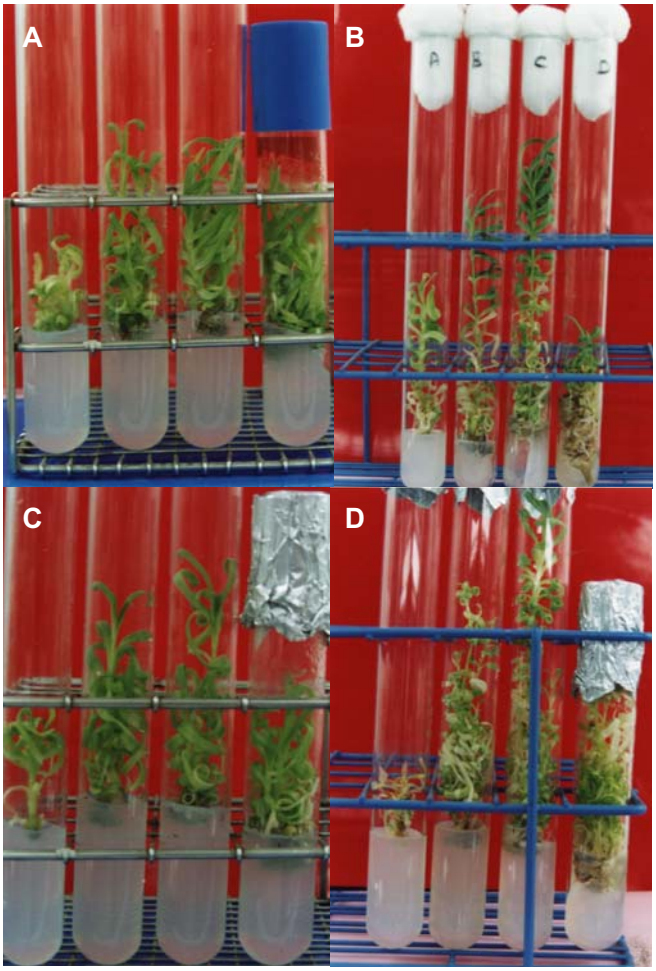

Fig. 3 Shoot cultures of gypsophila after six months of preservation on MS-medium supplemented with 2.69 µM α-naphthaleneacetic acid (NAA) and 2.22 µM ^{6}N-benzyl amino purine (BAP) and covered with polypropylene caps (A), cotton (B), one layer aluminum foil (C) and two layers aluminum foil (D).

When comparing the different enclosures it was observed that covering shoot cultures tubes with polypropylene caps and cotton gave best healthy shoot percentage and percentage survival (100%), while covering shoot cultures with two layers of foil gave the longer shoots (8 cm) and highest number of shoots (5.80) with 2% sucrose in the media. On the other hand, covering shoot culture tubes with two layers of foil resulted in the lowest survival percentage (70%) and healthy shoots percentage (50%) while covering with polypropylene caps and two layers of foil gave the shortest shoots (2 cm) and number of proliferated shoots when shoots were grown in sucrose-free medium. Statistical analysis showed that although an increased concentration of sucrose in the culture media did not significantly affect the survival percentage and healthy shoot percentage, except with two layers of foil, there was an increase in survival rate and percentage healthy shoots as the concentration of sucrose increased. However, increased sucrose concentration in the culture media also significantly affected shoot length with all caps used.

In this respect, Woo and Joo (1999) reported that aera-

tion treatment on cap and agar concentration did not affect vitrification, but promoted the elongation of adventitious shoots of *G. paniculata* 'Bristol fairy' and 'Red sea' cultures. Also, Dillen and Buysens (1989) reported that a simple adaptation of the plastic lids that are commonly used in micropropagation of *G. paniculata* L. shoot cultures allowed controlled and contamination-free transpiration.

In conclusion, the absence of sucrose in the media during preservation retarded the overall growth of *Gypsophila* shoot cultures in treatments where caps were used while the addition of high levels of sucrose (2 or 3%) improved growth (number of proliferated shoots and shoot length) of shoot cultures. As for the capping type, polypropylene caps and two layers of foil were the best for the proliferation of shoot cultures. The main reason for prolonged survival of plants in tubes with polypropylene and two layers of foil appears to be due to the slow loss of water from the tubes. Moreover, it could be concluded that addition of mannitol to the culture medium with incubation at 5°C resulted in better growth of gypsophila shoots. From the present study we can recommend that this method may provide an economical means to preserve germplasm and could be useful in exchange of sterile material between commercial laboratories due to the small size and relative ease in handling, transportation, or in conservation of elite germplasm.

REFERENCES

Ahroni A, Zuker A, Rozen Y, Shejtman H, Vainstein A (1997) An efficient method for adventitious shoot regeneration from stem segment explants of *Gypsophila*. *Plant Cell, Tissue and Organ Culture* **49**, 101-106

Arora R, Bhojwani S (1989) *In vitro* propagation and low temperature storage of *Saussurea lappa* C.B Clarke – an endangered medicinal plant. *Plant Cell Reports* **8**, 44-47

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

Bekheet SA (2007) *In vitro* preservation of Globe artichoke germplasm. *Plant Tissue Culture and Biotechnology* **17 (1)**, 1-9

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

Cha-Um S, Kirdmanee C (2007) Minimal growth *in vitro* culture for preservation of plant species. *Fruit, Vegetable and Cereal Science and Biotechnology* **1**, 13-25

Cha-Um S, Kirdmanee C, Huyen PX, Vathany T (2007) Disease-free production and minimal-growth preservation of *in vitro* banana (*Musa* spp.) *Acta Horticulturae* **760**, 233-240

Conner AJ, Fallon PG (1993) Osmotic versus nutritional effects when rooting *in vitro* asparagus minicrowns on high sucrose media. *Plant Science* **89**, 101-106

Dillen W, Buysens S (1989) A simple technique to overcome vitrification in *Gypsophila paniculata* L. *Plant Cell, Tissue and Organ Culture* **19**, 181-188

Divakaran M, Nirmal Babu K, Peter KV (2006) Conservation of *Vanilla* species *in vitro*. *Scientia Horticulturae* **110**, 175-180

Dodds JH (1988) Tissue culture technology: practical application of sophisticated methods. *American Potato Journal* **65**, 167-180

Grout BW (1990) *In vitro* conservation of germplasm. In: Bhojwani SS (Ed) *Plant Tissue Culture Applications and Limitations*, Elsevier, Amesterdam, pp 394-411

Islam MDT, Dembele PD, Keller ERJ (2005) Influence of explant, temperature and different culture vessels on *in vitro* culture for germplasm maintenance of four mint accessions. *Plant Cell, Tissue and Organ Culture* **81**, 123-130

Keller ERJ, Senula A, Leunufnas S, Grübe M (2006) Slow growth storage and cryopreservation – tools to facilitate germplasm maintenance of vegetatively propagated crops in living plant collections. *International Journal of Refrigeration* **29**, 411-417

Lee SW, Bae JJ (1999) Improvement of efficiency for multiplication of vigorous seedling by shoot cultured *in vitro* in *Gypsophila paniculata* L. *Korean Journal of Horticulture and Science Technology* **17**, 768-769

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Rady MR, Hanafy MS (2004) Synthetic seed technology for encapsulation and regrowth of *in vitro*-derived *Gypsophila paniculata* L. shoot-tips. *Arab Journal of Biotechnology* **7 (2)**, 251-264

Shillo R (1985) *Gypsophila paniculata*. In: Halevy AH (Ed) *Hand-Book of Flowering* (Vol 3), CRC Press, Inc., Boca Raton, Florida, pp 83-87

Son SH, Chun YW, Hall RB (1991) Cold storage of *in vitro* cultures of hybrid poplar shoots (*Populus alba* L. x *P. grandidentata* Michx.). *Plant Cell, Tissue and Organ Culture* **27**, 161-168

Steel RCD, Torrie JH (1960) *Principles and Procedures of Statistics*, McGraw Hill, New York, 134 pp

Teixeira da Silva JA (2004) The effect of carbon source on the *in vitro* organogenesis of chrysanthemum thin cell layers. *Bragantia* **63 (2)**, 165-177

Wanas WH (1992) *In vitro* storage proliferated apple rootstock shoot-tip cultures. *Annals of Agriculture, Ain Shams University, Cairo* **37 (2)**, 501-510

Winarto B, Aziz MA, Rashid AA, Ismail MR (2004) Effect of permeable vessel and gelling vessel on reduction of hyperhydricity in *in vitro* culture of carnation. *Indonesian Journal of Agriculture Science* **5 (1)**, 11-19

Withers LA (1987) Long-term preservation of plant cells, tissue and organs. *Plant Molecular and Cell Biology* **4**, 221-272

Withers LA (1989) *In vitro* conservation and germplasm utilization. In: Brown HD, Marshall DR, Williams JT (Eds) *The Use of Plant Genetic Resources*, Cambridge University Press, Cambridge, pp 309-334

Withers LA (1992) *In vitro* conservation. In: Hammerschlag F, Litz RE (Eds) *Biotechnology of Perennial Fruit Crops*, CABI, Wallingford, UK, pp 169-200

Woo LS, Joo BJ (1999) Improved of efficiency for multiplication of vigorous seedling by shoot cultured *in vitro* in *Gypsophila paniculata* L. *Korean Journal of Horticulture and Science Technology* **17**, 768-769

Zamorano-Mendoza JJ, Mejia-Muñoz JM (1994) *In vitro* propagation of gypsophila (*Gypsophila paniculata* L.) cv. Perfecta. *Revista Chapingo*. *Serie Horticultura* **1**, 67-71