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# *IN VITRO* SHORT-TERM GERMPLASM PRESERVATION OF *Myrtus communis* L

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## ABSTRACT

Shoot tips were obtained from *Myrtus communis* tissue cultured shootlets and *in vitro* preserved either by cryopreservation protocols (through vitrification, encapsulation-dehydration) or by cold preservation (4°C), as well as control storage at culture room conditions, however, nodal stem was involved in dehydration protocol in order to be cryopreserved. Preservation was adopted for 1, 2 and 3 months durations; yet, control treatment was prolonged for one year. Several cryoprotection trials were adopted, involving DMSO and/or PEG and glycerol, prior to vitrification protocol. Cryopreserved explant recovery was tested, as well as shootlets regeneration following preservation processes underwent several investigations. Shoot tips precultured on 0.18 M sucrose recorded higher recovered survival, shootlets number, length and leaves number than on a higher concentration and than on mannitol, either for 2 or 4 days, however, vitrification treatment induced no shootlets proliferation. Regenerated shootlets growth characters (survival %, shootlets number and dry weights) induced after preservation under growth chamber conditions (control) for one year, recorded the highest observed results. Similarly, chemical composition (chlorophyll a, total chlorophylls, carotenoids, total indoles, total free amino acids, total sugars and indoles/phenols ratio) as well as essential oil content and components were augmented due to preservation under control conditions; however, fresh weight, chlorophyll b, as well as total soluble phenols were low. RAPD analysis proved that preserved shootlets maintained their genetic stability through germplasm preservation methods.

**Keywords:** *Myrtus communis*, cryopreservation, dehydration, encapsulation-dehydration, vitrification, essential oil, RAPD.

## INTRODUCTION

*Myrtus communis* is considered as an aromatic and medicinal as well as ornamental shrub, and its berries are used in producing alcoholic liquor (Mulas *et al.*, 1996).

Conservation of plant genetic resources has become an issue of common global concern due to the growing awareness of rapid erosion of plant genetic diversity and the enormous potential value of genetic resources. Traditionally, genetic resource conservation involves field planting (field gene banks) as for vegetatively propagated plants. But, this method usually takes up too much space and requires extensive maintenance. Variation can also occur in the use of tissue culture and slow growth of myrtle and oak germplasm. Besides, reports of genetic changes in plant material from tissue cultures have been described for many species such as: apple, alfalfa,

horseradishes and pecan trees (Piccioni *et al.*, 1997; Rostiana *et al.*, 1999 and Liu *et al.*, 2004).

Cryopreservation is the storage of living cells and tissues at ultra-low temperature in liquid nitrogen (LN, -196°C) and it is a useful method for long-term storage of germplasm using minimum space and maintenance (Bajaj, 1995 and Benson, 1999). The development of techniques to successfully store shoot tips in LN is needed for preservation of plant genetic resources.

Genetic stability of plants derived from cryopreserved tissues has long been an issue of debate. Recent studies on the assessment of genetic stability reveals that plants obtained from cryopreserved tissues remain stable in all the conditions tested (Ahuja *et al.*, 2002; Dixit *et al.*, 2003; Gagliardi *et al.*, 2003 and Zhai *et al.*, 2003). Many papers (Gonzales-Arnao *et al.*, 1999 and Harding and Benson, 2000) have investigated somaclonal changes of cryopreserved plant material including potato, scots pine, cassava and sugarcane. None of them found genetic differences in regenerated plantlets following LN immersion. However, no papers, to our knowledge, have reported and analyzed genetic alteration in myrtle (*Myrtus communis*).

The aim of this work is to investigate the most efficient method of *in vitro* short-term preservation of *Myrtus communis* germplasm, which fulfills post preservation optimum growth and chemical characters, as well as genetic stability of preserved germplasm.

## MATERIALS AND METHODS

**\- Plant material**

The upper microcuttings (including the shoot tip + 2-3 nodes) of myrtle (*Myrtus communis* L.) used in the present study were obtained from *in vitro* stocks derived from field-collected shoots of the mother plant located at the Faculty of Agriculture, Cairo University, Giza, Egypt and maintained at the Biotechnology and Bioengineering Laboratory, at the same faculty during the period between 2003-2006.

Stock cultures of myrtle plantlets were maintained in glass jars (ca. 200 ml) containing 25 ml of MS-medium (Murashige and Skoog, 1962) at full strength supplemented with 0.1 mg $l^{-1}$ NAA, 1.0 mg $l^{-1}$ BA, 3% sucrose and solidified by 7% agar during establishment as well as multiplication stages, as previously recommended in Hanafy *et al.* (2002). Cultures were subjected to room temperature 25±2°C, relatively humidity 75-85% RH and photoperiod of 16 h/8 h (light/dark) provided with white fluorescent light tubes and light intensity of 40 $\mu Em^{-2}s^{-1}$, following of 7 days of complete darkness (to overcome explant browning problem caused by phenolic compounds exudates). *In vitro* sub-cultures were adopted every 4 weeks intervals.

**2-Preservation Protocols**

**2-1. Cryopreservation protocols**

Three cryopreservation procedures were performed in the present study to perceive the ideal cryopreservation protocol for myrtle.

**2-1.1. Vitrification procedure**

The vitrification procedure was performed according to the protocol developed by Yamada *et al.* (1991), with some modifications in the preculture and cryoprotection steps.

**Explant selection**

Shoot tips were selected from shootlets of the 3rd subculture.

**Preculturing**

Shoot tips were precultured at 4°C in the recommended medium containing either 0.18 or 0.26 M of mannitol or sucrose for 2 and 4 days.

**Cryoprotection and freezing**

Vitrification solution (used as a protectant against freezing hazards) consisting of dimethylsulphoxide (DMSO) and/or glycerol, polyethylene glycol (PEG) was prepared as the following:

5% (w/v) DMSO, 5% (w/v) DMSO + 5% (w/v) PEG 6000, 5% (w/v) DMSO + 5% (w/v) PEG 8000, 5% (w/v) DMSO + 10% (w/v) glycerol, 5% (w/v) DMSO + 5% (w/v) PEG 6000 + 10% (w/v) glycerol and 5% (w/v) DMSO + 5% (w/v) PEG 8000 + 10% (w/v) glycerol. Each protectant was added (as %) to the recommended liquid medium containing 0.15 M sucrose as a stock solution. Each of the previously mentioned stock treatments was added to the recommended liquid medium (supplemented with 0.4 M sucrose) to form 60% and 40%, respectively, of a stock solution volume. A volume of 1.0 ml of this solution was added to a cryovial (ca. 2 ml) containing the shoot tip for 5 minutes at 25°C and sealed. Cryovials were divided into two groups, one group was plunged into liquid nitrogen (+LN) and the other group was kept under growth chamber conditions (-LN) to be stored for periods of 1, 2 and 3 months.

**Warming and washing**

Stored explants were removed from liquid nitrogen and warmed at 25°C. Vitrification solution was drained from the cryovials and replaced twice by the recommended liquid MS medium containing 1.2 M sucrose. The explants were then kept in this solution at 25°C for 30 min.

**Recovery**

Explants were transferred to a sterilized filter paper (Whatmann 5.0 cm Ø) overlaying the recommended solid medium and daily-transferred into a new filter paper until regeneration is evident and then transferred to standard growth conditions as described prior to cryopreservation process.

**2-1.2. Dehydration procedure**

This procedure was performed according to the protocol developed by Uragami *et al.* (1990), with some modifications in preculture process.

**Explant selection and preculturing**

Nodal stems were selected from the 3rd subculture and precultured as in vitrification procedure.

**Desiccation and freezing**

Nodes were transferred to a sterilized filter paper (Whatmann 9.0 cm Ø) and exposed to sterile air from laminar air flow for desiccation for 1, 2, 3 and 4 hours. Nodes were transferred to cryovials, sealed and a group was

plunged directly into liquid nitrogen (+LN) and the other group was kept under growth chamber conditions (-LN) for 1, 2 and 3 months.

**Warming and recovery**

Explants were thawed at 25°C and recultured on the recommended growth medium for regeneration.

**2-1.3. Encapsulation-Dehydration procedure**

This procedure was performed according to the protocol developed by Fabre and Dereuddre (1990) and Niino *et al.* (1992), with some modifications in preculture process.

**Explant selection and preculturing**

These processes were the same as in vitrification procedure.

**Encapsulating and freezing**

Shoot tip explants were added to a conical flask (ca. 50 ml) containing ≈ 25 ml alginate solution (calcium-free of the recommended liquid medium containing 3% (w/v) sodium alginate). Conical flask (ca. 100 ml) containing 50 ml $CaCl_2$ liquid medium (the recommended medium containing 20% $CaCl_2$) was adopted. Using a micropipette withdraw 3 ml of the alginate solution and take up several shoot-tips then the micropipette tip was placed over the flask of $CaCl_2$ medium and dispense drops containing 1-2 shoot tips into $CaCl_2$ medium; beads form as the alginate solidifies on contact with the $Ca^{+2}$ ions. The alginate beads were picked up by means of a forceps tip to polymerize for 30 min over a filter paper and desiccated in sterile air flow for 4 hours. A group of these dried beads was transferred to a cryovial and plunged directly into LN and the other group was kept under growth chamber conditions (-LN) for 1, 2 and 3 months.

**Warming and recovery**

Explants were thawed at 25°C and recultured on the recommended growth medium for regeneration.

**2-2. Storage at 4°C procedure**

Shoot tips were selected from the 3$^{rd}$ subculture, cultured on the recommended medium and incubated at 4°C and 6 $\mu Em^{-2}s^{-1}$ illumination for 1, 2 and 3 months.

**2-3. Growth chamber conditions procedure**

Shoot tips were selected and cultured as mentioned in storage at 4°C treatment, however, cultures were incubated under growth chamber temperature (25°C ± 2°C) and 6 $\mu Em^{-2}s^{-1}$ illumination, for a period extended to one year.

After culture recovery, the survived explants were subcultured three times, with 4 weeks intervals, for regeneration .

**3- Investigated parameters:**

**3-1. Morphological characters:**

Survival percentage, shootlets length (cm), shootlets number, leaves number were investigated following to explant recovery as well as to shootlets regeneration, and fresh and dry weights (g/replicate) were recorded after regeneration.

**3-2. Chemical analysis:**

Regenerated shootlets were subjected to the following investigations after the third subculture.

**Photosynthetic pigments:** Fresh leaves and shootlets were extracted with dimethylformamide (DMF) solution as described by Nornai (1982) and placed overnight at 5°C. Chlorophylls a, b and carotenoids were measured by spectrophotometer at wavelengths 663, 647 and 470 nm, respectively. Chlorophylls and carotenoids were calculated according to the following equations:

Chl. a = 12.70 $A_{663}$ – 2.79 $A_{647}$

Chl. b = 20.76 $A_{647}$ – 4.62 $A_{663}$

Total Chl. = 17.90 $A_{647}$ + 8.08 $A_{663}$

Total carotenoids = 1000x$A_{470}$ – 3.72 Chl. a – 104 Chl. b / 229

**Ethanol extract**: 2.5 grams of Fresh leaves and shootlets were crushed in a porcelain mortar using 12.5ml of 80% boiling ethanol for 10 min, then filtrated through a sintered glass funnel ($G_3$). The residue was re-extracted and filtered twice with 80% boiling ethanol, and then the volume was adjusted to 50 ml with 80% ethanol for the determination of:

Total sugars were determined in the ethanolic extract by using the phenol sulphuric acid reagent according to Dubois *et al.* (1956). Total free amino acids were determined by using ninhydrin reagent according to Moore and Stein (1954). Total soluble phenols were determined by the colorimetric method of Folin-Denis as described by Swain and Hillis (1959). Total indoles were determined by using para-dimethyl amino benzaldhyde (PDAB) as a reagent, according to Larsen *et al.* (1962). Spectrophotometer was used at wavelength 530 for samples and standard solution readings.

**3-3. Essential oil content and components:**

Plant material (50 g) were subjected to hydrodistillation (3 hours) using "Clevenger-type" apparatus to determine the essential oil content, according to the Egyptian Pharmacopoeia (1984). Pro-GLC Pyeunicam gas chromatography mass spectrum apparatus was used to investigate the principle components of essential oil. Initial temperature was 70°C, final temperature 190°C and final time 20 minutes. The injector was maintained at 250°C and the detector at 300°C. Chart speed was 2 min/cm and flow rate of gases were 30, 33 and 330 ml/min for nitrogen, hydrogen and air, respectively.

**3-4. RAPD analysis:**

Isolation of DNA was performed using CTAB method according to Proebski *et al.* (1997). PCR amplification was performed in 0.25 ml reaction mixture containing 20 ng template DNA, 0.5 unit Taq polymerase, 200 µM each of dATP, dCTP, dGTP, dTTP, 10 p mol random primer (10 mer) and appropriate amplification buffer. Fifteen 10 mer oligonucleotide DNA primers of arbitrary sequences from Operon Kit (Operon Tech. Inc., USA) were independently used in the PCR reactions according to Williams *et al.* (1990). Only three primers with the following sequences were able to detect reproducible polymorphism in the generated DNA profiles.

| Primer | Sequence | GC% |
|---|---|---|
| OPC-16 | 5`- CACACTCCAG -3` | 60 |
| OPC-19 | 5`- GTTGCCAGCC -3` | 70 |
| OPC-20 | 5`- ACTTCGCCAC -3` | 60 |

Amplification was preformed for 45 cycles at 92°C for 3 min., then for 30 sec. for denaturation, 35°C for 60 sec. for annealing and 72°C for 2 min. for extension. Reaction was finally incubated at 72°C for 10 min. and further 10 min. at 62°C.

The amplification products were analyzed by electrophoresis in 2% agarose in TAE buffer, stained with 0.2 µg $cm^{-3}$ ethidium bromide and photographed under UV light.

**Data analysis:**

Data obtained were subjected to analysis of variance according to Steel and Torrie (1980) assuming a complete randomized design. Mean separation was made using least significant difference (L.S.D. at 5% level of significance), according to Snedecor and Cochran (1982).

## RESULTS

### A- Effect of preculture and cryopreservation treatments on shoot tips recovery

#### A- 1- Shoot tip survival percentage

Data represented in Fig. (1) reveal that, increasing the concentration of either osmotic substances (sucrose and mannitol) prior to preservation treatments, as well as extending the preculture duration (from 2 to 4 days), except with sucrose (0.18 M), generally, had a depressive effect on explant survival. It is observed that sucrose, in both concentrations, surpassed mannitol by giving more survived explants values.

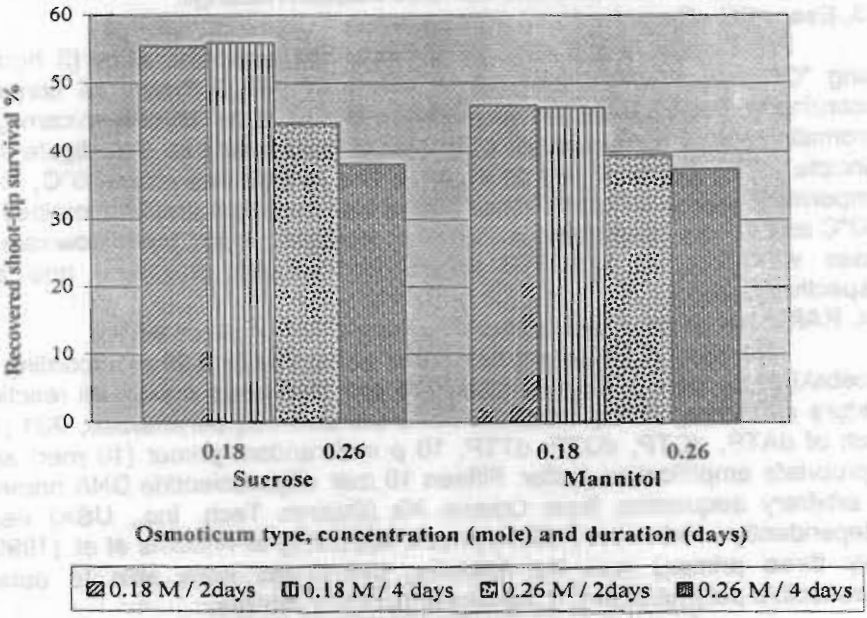



**Fig. (1): Effect of preculture osmoticum (sucrose and mannitol), its concentration (Mole) and duration on recovered shoot tip survival %.**

**Table (1): Effect of preculture osmoticum (sucrose and mannitol), its duration (2 and 4 days) and following cryopreservation treatments on recovered shoot tip survival percentage of *Myrtus communis* L.**

| Cryopreservation treatment and duration (months) (A) | Preculture osmoticum treatments (B): duration and type. | | | | | |
|---|---|---|---|---|---|---|
| | Two days | | | Four days | | |
| | Sucrose | Mannitol | Mean (A) | Sucrose | Mannitol | Mean (A) |
| Enc.-deh. (+LN) for 1 month | 33.33 | 27.77 | 30.55 | 33.33 | 27.77 | 30.55 |
| Enc.-deh. (+LN) for 2 months | 33.33 | 27.77 | 30.55 | 27.78 | 22.22 | 25.00 |
| Enc.-deh. (+LN) for 3 months | 33.33 | 22.22 | 27.78 | 27.77 | 22.22 | 25.00 |
| Mean (B) | 33.33 | 25.92 | | 29.63 | 24.07 | |
| L.S.D. 5% | A = N.S. | B= N.S. | A × B = N.S. | A = N.S. | B= N.S. | A × B = N.S. |
| Enc.-deh. (-LN) for 1 month | 50.00 | 33.35 | 41.68 | 44.45 | 33.33 | 38.89 |
| Enc.-deh. (-LN) for 2 months | 44.45 | 33.33 | 38.89 | 44.45 | 33.33 | 38.89 |
| Enc.-deh. (-LN) for 3 months | 55.56 | 38.88 | 47.22 | 66.67 | 38.88 | 52.78 |
| Mean (B) | 50.00 | 35.19 | | 51.86 | 35.18 | |
| L.S.D. 5% | A= N.S. | B=9.939 | A × B=17.220 | A = 13.370 | B=10.920 | A × B = 18.910 |
| Vitrific. (+LN) for 1 month | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Vitrific. (+LN) for 2 months | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Vitrific. (+LN) for 3 months | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Mean (B) | 100.00 | 100.00 | | 100.00 | 100.00 | |
| L.S.D. 5% | A = N.S. | B= N.S. | A × B = N.S. | A = N.S. | B= N.S. | A × B =N.S. |
| Vitrific. (-LN) for 1 month | 23.34 | 10.00 | 16.67 | 10.00 | 16.67 | 13.34 |
| Vitrific. (-LN) for 2 months | 16.67 | 13.33 | 15.00 | 6.67 | 10.00 | 8.34 |
| Vitrific. (-LN) for 3 months | 6.67 | 10.00 | 8.34 | 3.34 | 3.34 | 3.34 |
| Mean (B) | 15.56 | 11.11 | | 6.67 | 10.00 | |
| L.S.D. 5% | A = N.S. | B= N.S. | A × B=13.690 | = 7.790 | B= N.S. | A×B=11.020 |

Enc.-deh. = Encapsulation-dehydration
+ LN = with immersion in liquid nitrogen
- LN = without immersion in liquid nitrogen  Vitrific. = Vitrification

Concerning cryopreservation treatments, results indicate that dehydration procedure resulted in the death of all preserved shoot tips. However, it is clear from Table (1) that vitrification procedure with keeping shoot tips in liquid nitrogen (+LN) resulted in 100% survival all along the three periods of preservation and under all preculture conditions. On the other hand, vitrification (-LN) brought about the least survival values. Moreover, enc.-deh. (-LN) procedure resulted in shoot tips higher survival values compared to enc.-deh. (+LN) and vitrification (-LN) procedures. It is obvious that increasing the period of preservation resulted in shoot tip survival reduction, with significant difference only in vitrification (-LN) treatment. However, increasing the period of preservation from 1 to 3 months on enc.-deh. (-LN) resulted in a non-significant increase (except after 4 days on sucrose pregrowth) in survival value. The highest interaction significant value (66.67%) after 3 months of preservation with enc.-deh. and subjected to sucrose preculture treatment for 4 days, however the lowest value (3.34%) was observed due to 3 months of preservation with vitrification (-LN) treatment precultured for 4 days on both of sucrose and mannitol.

**A- 2- Shootlets number**

Data illustrated in Table (2) show that, increasing the concentration of either osmotic substances (sucrose and mannitol) prior to preservation treatments had a depressive effect on recovered shootlets number. In this concern, significant difference was observed between concentration values in sucrose preculture with the highest recovered shootlets number (2.11) in enc.-deh. (-LN) treatment after 2 days of preculture on 0.18 M of sucrose, while non-significant difference was recorded by preculturing on mannitol. However, it is clear that sucrose, at low concentration, surpassed mannitol by inducing higher shootlets number.

**Table (2): Effect of preculture osmoticum (sucrose and mannitol) and encapsulation-dehydration treatments on recovered shootlets number of preserved *Myrtus communis* L. shoot-tips.**

| Cryopreservation Treatmen and duration (months) (A) | Preculture osmoticum treatments (B): duration, type and concentration (Mole) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Two days | | | | | | Four days | | | | | |
| | Sucrose | | | Mannitol | | | Sucrose | | | Mannitol | | |
| | 0.18 | 0.26 | Mean (A) | 0.18 | 0.26 | Mean (A) | 0.18 | 0.26 | Mean (A) | 0.18 | 0.26 | Mean (A) |
| enc.-deh. (+LN) for 1 month | 1.33 | 0.67 | 1.00 | 1.00 | 0.67 | 0.84 | 1.33 | 1.00 | 1.17 | 0.67 | 0.33 | 0.50 |
| enc.-deh. (+LN) for 2 month | 1.33 | 0.67 | 1.00 | 1.00 | 0.67 | 0.84 | 2.00 | 0.67 | 1.34 | 1.00 | 0.67 | 0.84 |
| enc.-deh. (+LN) for 3 month | 2.00 | 1.00 | 1.50 | 1.67 | 1.33 | 1.50 | 1.67 | 0.67 | 1.17 | 0.67 | 0.33 | 0.50 |
| Mean (B) | 1.55 | 0.78 | | 1.22 | 0.89 | | 1.67 | 0.78 | | 0.78 | 0.44 | |
| L.S.D. 0.05 | A = N.S.<br>B = 0.507<br>A×B = 0.878 | | | A = 0.651<br>B = N.S.<br>A×B = 0.921 | | | A = N.S.<br>B = 0.531<br>A×B = 0.921 | | | A = N.S.<br>B = N.S.<br>A×B = N.S. | | |
| enc.-deh. (-LN) for 1 month | 2.00 | 1.00 | 1.50 | 1.33 | 1.00 | 1.17 | 1.33 | 1.00 | 1.17 | 1.00 | 0.67 | 0.84 |
| enc.-deh. (-LN) for 2 month | 2.00 | 1.00 | 1.50 | 2.00 | 1.00 | 1.50 | 1.33 | 0.67 | 1.00 | 1.33 | 1.00 | 1.17 |
| enc.-deh. (-LN) for 3 month | 2.33 | 1.33 | 1.83 | 2.00 | 1.33 | 1.67 | 1.33 | 1.00 | 1.16 | 1.33 | 1.00 | 1.17 |
| Mean (B) | 2.11 | 1.11 | | 1.78 | 1.11 | | 1.33 | 0.89 | | 1.22 | 0.89 | |
| L.S.D. 0.05 | A = N.S.<br>B = 0.457<br>A×B = 0.791 | | | A = N.S.<br>B = N.S.<br>A×B = N.S. | | | A = N.S.<br>B = N.S.<br>A×B = N.S. | | | A = N.S.<br>B = N.S.<br>A×B = 0.635 | | |

Regarding the cryopreservation treatments, vitrification procedure resulted the highest shoot tip in survival but had no ability to proliferate into shootlets. Also, it is clear that enc.-deh. (-LN) resulted in the highest shootlets number values compared to enc.-deh. (+LN) procedure. It is clear from data that, increasing the preservation period, generally, resulted in non-significant increase in recovered shootlets number values. The highest values (1.83 and 1.67) recorded by enc.-deh. (-LN) for 3 months, precultured for 2 days on sucrose and mannitol, respectively.

Concerning the highest interaction value (2.33) was observed due to enc.-deh. (-LN) procedure for 3 months precultured on 0.18 M of sucrose for 2 days.

**A- 3- Shootlets length**

Results represented in Table (3) demonstrate that, increasing the osmotic substances concentration (sucrose and mannitol), generally, had a depressive effect, as well, on recovered shootlets length. In this respect,

significant difference was obtained between concentration values with the highest shootlets length value 12.90 mm after 2 days of culture on 0.18 M of mannitol by enc.-deh. procedures (-LN), followed by 9.67 mm due to enc.-deh. (+LN) procedure after 4 days of preculture on 0.18 M of sucrose. However, it was observed that mannitol, in both concentrations, surpassed sucrose by giving higher shootlets length after 2 days of preculture, while, sucrose surpassed mannitol after 4 days of preculture.

**Table (3): Effect of preculture osmoticum (sucrose and mannitol) and encapsulation-dehydration treatments on recovered shootlets length (mm) of preserved *Myrtus communis* L. shoot-tips.**

| Cryopreservation Treatment and duration (months) (A) | Preculture osmoticum treatments (B): duration, type and concentration (Mole) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Two days | | | | | | Four days | | | | | |
| | Sucrose | | | Mannitol | | | Sucrose | | | Mannitol | | |
| | 0.18 | 0.26 | Mean (A) | 0.18 | 0.26 | Mean (A) | 0.18 | 0.26 | Mean (A) | 0.18 | 0.26 | Mean (A) |
| Enc.-deh. (+LN) for 1 month | 3.00 | 1.70 | 2.34 | 6.33 | 2.33 | 4.33 | 5.33 | 2.00 | 3.67 | 4.67 | 1.33 | 3.00 |
| Enc.-deh. (+LN) for 2 months | 5.33 | 3.00 | 4.17 | 6.33 | 3.00 | 4.67 | 10.00 | 4.00 | 7.00 | 4.67 | 2.33 | 3.50 |
| Enc.-deh. (+LN) for 3 months | 9.00 | 3.00 | 6.00 | 13.00 | 4.33 | 8.67 | 13.67 | 3.33 | 8.50 | 3.00 | 2.67 | 2.84 |
| Mean (B) | 5.78 | 2.56 | | 8.55 | 3.22 | | 9.67 | 3.11 | | 4.11 | 2.11 | |
| L.S.D. 0.05 | A = 2.301<br>B = 1.879<br>A×B = 3.254 | | | A = 2.397<br>B = 1.957<br>A×B = 3.389 | | | A = 2.437<br>B = 1.990<br>A×B = 3.447 | | | A = N.S.<br>B = N.S.<br>A×B = N.S. | | |
| Enc.-deh. (-LN) for 1 month | 4.67 | 1.67 | 3.17 | 9.00 | 6.70 | 7.90 | 4.30 | 3.00 | 3.83 | 7.33 | 2.00 | 4.67 |
| Enc.-deh. (-LN) for 2 months | 8.67 | 3.00 | 5.84 | 13.00 | 5.70 | 8.90 | 13.70 | 5.70 | 9.67 | 8.33 | 3.67 | 6.00 |
| Enc.-deh. (-LN) for 3 months | 12.33 | 7.00 | 9.67 | 16.70 | 5.70 | 11.20 | 10.30 | 7.70 | 9.00 | 9.33 | 4.33 | 6.83 |
| Mean (B) | 8.56 | 5.84 | | 12.90 | 6.00 | | 9.44 | 5.56 | | 8.33 | 3.33 | |
| L.S.D. 0.05 | A = 1.143<br>B = 0.933<br>A×B = 1.616 | | | A = N.S.<br>B = 2.696<br>A×B = 4.670 | | | A = 3.997<br>B = 3.264<br>A×B = 5.653 | | | A = N.S.<br>B = N.S.<br>A×B = 4.638 | | |

Concerning cryopreservation treatments, enc.-deh. (-LN) resulted in the highest shootlets length compared to enc.-deh. (+LN) procedures. It is obvious that, increasing the period of preservation, generally, resulted in significant increase in shootlets length values. Yet, non-significant values were recorded after 4 days of mannitol preculture. However, the highest value (11.20 mm) was recorded due to enc.-deh. (-LN) for 3 months after 2 days of preculture on mannitol.

Regarding the highest interaction value (16.70 mm) was obtained by enc.-deh. (-LN) treatment for 3 months with preculture on 0.18 M of mannitol for 2 days.

**A- 4- Leaves number**

Data tabulated in Table (4) reveal that, in general, increasing the concentration of either osmotic substances (sucrose and mannitol) prior to

preservation treatments similarly had a depressive effect on leaves number of recovered shootlets.

In this concern, significant difference was observed with the highest leaves number values 1.97 and 1.93 after 2 and 4 days, respectively of preculture on sucrose lower concentration (0.18 M) in enc.-deh. procedures (-LN). However, it was clear that sucrose, in both concentrations, surpassed mannitol by giving higher leaves number.

**Table (4): Effect of preculture osmoticum (sucrose and mannitol) and encapsulation-dehydration treatments on recovered leaves number of preserved *Myrtus communis* L. shoot-tips.**

| Cryopreservation Treatment and duration (months) (A) | Preculture osmoticum treatments (B): duration, type and concentration (Mole) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Two days | | | | | | Four days | | | | | |
| | Sucrose | | | Mannitol | | | Sucrose | | | Mannitol | | |
| | 0.18 | 0.26 | Mean (A) | 0.18 | 0.26 | Mean (A) | 0.18 | 0.26 | Mean (A) | 0.18 | 0.26 | Mean (A) |
| Enc.-deh. (+LN) for 1 month | 0.87 | 0.50 | 0.69 | 0.50 | 0.23 | 0.35 | 1.43 | 0.67 | 1.05 | 0.40 | 0.27 | 0.34 |
| Enc.-deh. (+LN) for 2 months | 1.50 | 1.00 | 1.25 | 0.67 | 0.23 | 0.45 | 1.77 | 0.93 | 1.35 | 0.40 | 0.37 | 0.39 |
| Enc.-deh. (+LN) for 3 months | 2.10 | 0.77 | 1.44 | 1.23 | 0.60 | 0.92 | 1.93 | 0.47 | 1.20 | 0.30 | 0.30 | 0.30 |
| Mean (B) | 1.49 | 0.76 | | 0.80 | 0.35 | | 1.71 | 0.69 | | 0.37 | 0.31 | |
| L.S.D. 0.05 | A = 0.588<br>B = 0.480<br>A×B = 0.832 | | | A = 0.247<br>B = 0.202<br>A×B = 0.350 | | | A = N.S.<br>B = 0.386<br>A×B = 0.668 | | | A = N.S.<br>B = N.S.<br>A×B = N.S. | | |
| Enc.-deh. (-LN) for 1 month | 1.17 | 0.67 | 0.92 | 0.97 | 0.30 | 0.64 | 1.17 | 1.00 | 1.09 | 0.40 | 0.40 | 0.40 |
| Enc.-deh. (-LN) for 2 months | 2.33 | 0.90 | 1.62 | 1.63 | 0.47 | 1.05 | 2.37 | 0.93 | 1.65 | 0.73 | 0.43 | 0.58 |
| Enc.-deh. (-LN) for 3 months | 2.40 | 1.30 | 1.85 | 1.80 | 0.47 | 1.14 | 2.17 | 1.20 | 1.69 | 0.87 | 0.50 | 0.69 |
| Mean (B) | 1.97 | 0.96 | | 1.47 | 0.41 | | 1.93 | 1.04 | | 0.67 | 0.44 | |
| L.S.D. 0.05 | A = 0.320<br>B = 0.262<br>A×B = 0.453 | | | A = 0.442<br>B = 0.361<br>A×B = 0.625 | | | A = N.S.<br>B = 0.760<br>A×B = 1.316 | | | A = 0.244<br>B = 0.199<br>A×B = 0.345 | | |

Regarding cryopreservation procedures, enc.-deh. (-LN) resulted in the highest leaves number compared to enc.-deh. (+LN) treatments. It was obvious that, increasing the period of preservation, generally, resulted in significant increase in leaves number. The highest values (1.85 and 1.69) were recorded by enc.-deh. (-LN) for 3 months after 2 and 4 days on sucrose. While, increasing the period from 1 to 3 months on enc.-deh. (+LN) resulted in significant enhancement in leaves number after 2 days on both osmoticum (sucrose and mannitol), however, non-significant values were observed after 4 days.

Concerning the highest interaction value (2.40) was observed due to enc.-deh. (-LN) procedure for 3 months precultured on 0.18 M of sucrose for 2 days.

**B- Effect of preservation procedures on shootlets regeneration characters**

It worths to indicate that despite vetrification procedure resulted in the highest recovery survival (100%), however, no shootlets regeneration was observed.

- **Growth characters**

**1. Regenerated Shootlets survival percentage**

Data illustrated in Table (5) reveal that, the highest regenerated shootlets survival (48%) after two subcultures was significantly induced due to shoot tip preservation under growth chamber conditions for one year, followed by (32%) survival of regenerated shootlets with no significant difference as a result to preservation by enc.-deh. (-LN) procedure for 3 months. Meanwhile, it is obvious that prolonged enc.-deh. (-LN) from 1 to 3 months resulted in enhancing regenerated shootlets survival from 24% to 36%. However, adverse result was obtained due to prolonging preservation duration at 4°C from 1 to 3 months.

**Table (5): Effect of preservation treatments on shootlets regeneration growth characters.**

| Preservation treatment | Shootlets survival % after 2 subcult. | Shootlets number | | Shootlets length (cm) | | Fresh weight (g) after 3 subcult. | Dry weight (g) after 3 subcult. |
|---|---|---|---|---|---|---|---|
| | | After 2 subcult. | After 3 subcult. | After 2 subcult. | After 3 subcult. | | |
| Enc.-deh. (+LN) for 1 month | 20 | 1.20 | 7.45 | 1.48 | 13.03 | 4.62 | 0.64 |
| Enc.-deh. (+LN) for 2 months | 16 | 1.00 | 6.49 | 2.36 | 12.15 | 5.28 | 0.69 |
| Enc.-deh. (+LN) for 3 months | 28 | 1.60 | 8.18 | 2.54 | 7.16 | 4.72 | 0.61 |
| Enc.-deh. (-LN) for 1 month | 24 | 1.20 | 8.97 | 1.46 | 12.74 | 5.68 | 0.68 |
| Enc.-deh. (-LN) for 2 months | 32 | 1.80 | 8.57 | 1.76 | 10.19 | 4.99 | 0.72 |
| Enc.-deh. (-LN) for 3 months | 36 | 2.00 | 9.47 | 2.34 | 18.50 | 5.51 | 0.77 |
| Storage at 4°C for 1 month | 24 | 1.80 | 7.27 | 2.14 | 13.15 | 5.26 | 0.74 |
| Storage at 4°C for 2 months | 16 | 1.80 | 6.87 | 1.85 | 15.95 | 6.03 | 0.70 |
| Storage at 4°C for 3 months | 16 | 1.20 | 6.94 | 1.76 | 11.17 | 6.08 | 0.72 |
| Growth chamber conditions | 48 | 2.80 | 11.64 | 2.95 | 8.26 | 5.30 | 0.85 |
| L.S.D. 0.05 | 23.97 | N.S. | 0.93 | N.S. | N.S. | 1.28 | 0.08 |

**2. Regenerated Shootlets number**

It is clear from Table (5) that, the highest regenerated shootlets number (2.8 and 2.0) was resulted from preservation under growth chamber conditions and enc.-deh. (-LN) for 3 months, respectively, with no significant difference after 2 subcultures. However, the same results were observed after three subcultures, but with significant differences (11.64 shootlets) due to preservation under growth chamber conditions for one year and (9.47 shootlets) as a result to enc.-deh. (-LN) for 3 months. On the other hand, preservation by enc.-deh. (+LN) for 2 months brought about the lowest regenerated shootlets number (1.0 and 6.49) after 2 and 3 subcultures, respectively.

Prolonging the period of preservation induced various responses. After 2 subcultures, shootlets number raised from 1.2 into 2.0 shootlets due to prolonging enc.-deh. (-LN) period from 1 to 3 months, while shootlets

number after 3 subcultures fluctuated (8.97, 8.57 and 9.47) due to extending the period (1, 2 and 3 months) by the same preservation procedure.

**3. Regenerated shootlets length**

Shootlets length was influenced by the preservation procedure, since the longest shootlets after 2 subcultures (2.95 cm) were resulted from preservation under growth chamber conditions. The longest shootlets after 3 subcultures (18.5 cm) was resulted from enc.-deh. (-LN) for 3 months. The shortest shootlets (1.46 and 7.16 cm) were induced as a result of enc.-deh. (-LN) for 1 month and after 2 subcultures and enc.-deh. (+LN) for 3 months after 3 subcultures, respectively. Only enc.-deh. (-LN) and (+LN) showed a positive effect on shootlets elongation by prolonging preservation period, after 2 subcultures, while a depressive effect is observed after 3 subculture by preserving in enc.-deh. (+LN) on shootlets length. Shootlets length was fluctuated by prolonging 4°C preservation procedure.

**4. Regenerated shootlets fresh and dry weights**

Fresh and dry weights of regenerated shootlets are significantly influenced by the preservation procedures adopted prior to regeneration process as exhibited in Table (5). The highest observed fresh weight (6.08 and 6.03 g) with no significant difference as a result of preservation at 4°C for 3 and 2 months, respectively, while preservation under growth chamber conditions for one year and enc.-deh. (-LN) for 3 months significantly induced the highest dry weights (0.85 and 0.77 g) with no significant different between their values. However, the lowest fresh and dry weights (4.62 and 0.61 g) were resulted from the preservation of enc.-deh. (+LN) for 1 and 3 months, respectively. Moreover, extending the preservation period from 1 to 3 months brought about augmentation in fresh weight (from 5.26 to 6.08 g) due to preservation at 4°C and in dry weight (from 0.68 to 0.77 g) as a result of enc.-deh. (-LN) preservation procedure, however, the other preservation methods showed fluctuations in regenerated shootlets fresh and dry weights as a result to prolonging the preservation duration.

- **Chemical composition**

**1. Photosynthetic pigments**

Data illustrated in Table (6) exhibit that, preservation procedure and duration significantly influenced regenerated shootlets photosynthetic pigments. Values were compared with those obtained from the mother plant, which showed the highest values.

The highest chlorophyll a value (0.64 mg/g) was observed as a result of preservation under growth chamber conditions for one year, with no significant difference compared to the mother plant. Moreover, concerning chl. b, no significant difference was noticed between preservation procedures compared to the mother plant, however, enc.-deh. (+LN) demonstrated a significant reduction in both chl. a and b values. Moreover, the highest significant values of total chlorophylls and carotenoids (0.92 and 0.45 mg/g) were observed as a result of preservation under growth chamber conditions, while the lowest values were due to enc.-deh. (+LN) during the three periods of preservation.

On the other hand, extending the preservation period from 1 to 3 months resulted in enhancing chl. a due to enc.-deh. (+LN), while the

increase in chl. b, as well as total chls. are influenced by enc.-deh. (-LN) and 4°C preservation procedures. However, diminishing in chl. a and carotenoids was observed because of enc.-deh. (-LN), as well, chl. b was reduced by prolonging enc.-deh. (+LN) duration.

**Table (6): Effect of preservation procedures on photosynthetic pigments (mg/g F.W.) in comparison to mother plant.**

| Preservation treatment | Photosynthesis pigments | | | |
|---|---|---|---|---|
| | Chlorophyll a | Chlorophyll b | Total chlorophylls | Carotenoids |
| Mother plant (Control) | 0.83 | 0.46 | 1.29 | 0.45 |
| Enc.-deh. (+LN) for 1 month | 0.27 | 0.18 | 0.45 | 0.22 |
| Enc.-deh. (+LN) for 2 months | 0.29 | 0.17 | 0.46 | 0.21 |
| Enc.-deh. (+LN) for 3 months | 0.30 | 0.15 | 0.45 | 0.19 |
| Enc.-deh. (-LN) for 1 month | 0.37 | 0.24 | 0.61 | 0.19 |
| Enc.-deh. (-LN) for 2 months | 0.36 | 0.28 | 0.64 | 0.16 |
| Enc.-deh. (-LN) for 3 months | 0.34 | 0.31 | 0.65 | 0.16 |
| Storage at 4°C for 1 month | 0.39 | 0.18 | 0.57 | 0.22 |
| Storage at 4°C for 2 months | 0.36 | 0.26 | 0.62 | 0.16 |
| Storage at 4°C for 3 months | 0.39 | 0.28 | 0.67 | 0.16 |
| Growth chamber conditions | 0.64 | 0.28 | 0.92 | 0.45 |
| L.S.D. 0.05 | 0.25 | 0.22 | 0.30 | 0.17 |

**2. Total sugars**

Chemical composition of regenerated shootlets were obviously exceeding the relevant values of the mother plant with few exceptions.

The highest total sugars concentration (4.26 mg/g) was recorded because of preservation under growth chamber conditions for one year, however, with no significant difference except with enc.-deh. (-LN) for one month, which exhibited the lowest value (2.42 mg/g). There was a general augmenting tendency by increasing preservation period from 1 to 3 months, except for enc.-deh. (-LN), since values were fluctuated from one month to another.

**3. Total free amino acids**

Total free amino acids represented in Table (7) indicate that, regenerated shootlets out of growth chamber conditions preservation treatment recorded the highest significant value (2.68 mg/g), while the lowest value (0.33 mg/g) was obtained from the mother plant leaves. Moreover, extending preservation duration from 1 to 3 months resulted in augmentation in total free amino acids from (1.05 to 1.30 mg/g) as a result of enc.-deh. (+LN) preservation procedure, however, storage at 4°C brought about an adverse result by reducing the values from (1.99 to 1.69 mg/g) due to prolonging the duration from 1 to 3 months.

**4. Total soluble phenols**

Data illustrated in Table (7) indicate that, enc.-deh. (+LN) resulted in inducing regenerated shootlets with the highest total soluble phenols values (5.56, 5.08 and 4.81 mg/g) for 3, 2 and 1 months with no significant difference between the duration of preservation. Yet, storage at 4°C for 1, 2 and 3 months significantly brought about the least phenol values (3.87, 3.15 and 3.71 mg/g) after 1, 2 and 3 months of preservation in regenerated

shootlets, with no significant difference between the duration of preservation, as well. Increasing the duration of preservation of enc.-deh. (+LN) resulted in an obvious increase in total soluble phenols values in regenerated shootlets, however, there was no clear trend in concern with the other preservation treatments.

**Table (7): Effect of preservation procedures on regenerated shootlets chemical composition (mg/g F.W.) after the third subculture in comparison to mother plant.**

| Preservation treatment | Chemical composition | | | | |
|---|---|---|---|---|---|
| | Total sugars | Total free amino acids | Total soluble phenols | Total indoles | Indoles:Phenols ratio |
| Mother plant (Control) | 3.15 | 0.33 | 3.93 | 1.01 | 0.26 |
| Enc.-deh. (+LN) for 1 month | 3.43 | 1.05 | 4.81 | 2.11 | 0.44 |
| Enc.-deh. (+LN) for 2 months | 3.56 | 1.27 | 5.08 | 2.11 | 0.42 |
| Enc.-deh. (+LN) for 3 months | 3.60 | 1.30 | 5.56 | 2.28 | 0.41 |
| Enc.-deh. (-LN) for 1 month | 2.42 | 1.34 | 3.97 | 2.80 | 0.71 |
| Enc.-deh. (-LN) for 2 months | 3.62 | 1.58 | 4.03 | 2.18 | 0.54 |
| Enc.-deh. (-LN) for 3 months | 3.55 | 1.43 | 3.80 | 2.75 | 0.72 |
| Storage at 4°C for 1 month | 3.46 | 1.99 | 3.87 | 2.13 | 0.55 |
| Storage at 4°C for 2 months | 3.42 | 1.78 | 3.15 | 2.02 | 0.64 |
| Storage at 4°C for 3 months | 3.69 | 1.69 | 3.71 | 2.25 | 0.61 |
| Growth chamber conditions | 4.26 | 2.68 | 3.81 | 3.77 | 0.99 |
| L.S.D. 0.05 | 1.74 | 0.49 | 0.91 | 0.63 | 0.17 |

**5. Total indoles**

The highest significant observed value (3.77 mg/g) of total indoles in regenerated shootlets was related to the prior preservation under growth chamber conditions, compared to the lowest (1.01 mg/g) which resulted from the mother plant. However, values of other treatments and preservation periods had no significant difference between each other.

Moreover, there was a significant increase in total indoles/total phenols ratio by preservation under growth chamber conditions (0.99) compared to the lowest value (0.26) of the mother plant, as well as with the other preservation treatments. Meanwhile, a gradual non-significant decrease (from 0.44 to 0.41) due to prolongation of enc.-deh. (+LN) duration from 1 to 3 months, was observed.

- **Essential oil content and its components**

Essential oil content represented in Fig. (1) was significantly influenced by preservation method and period. The highest recorded essential oil content (120 mg/50 g)corresponding to 0.24% of fresh sample weight due to preservation under growth chamber conditions. On the other hand, the lowest observed value (62 mg/50 g) correlating to 0.124% of sample, was influenced by enc.-deh. (-LN) preservation for 1 month. Generally, there was augmentation tendency in essential oil content by increasing preservation duration throughout all the preservation treatments. Moreover, data illustrated in Table (8) reveal that, ten essential oil components were determined. It is clear that, the major components in mother plant are: β-pinene, α-pinene, thujone, thymol, caryophellene, carvacrol, camphore and linalool.

Encapsulation-dehydration preservation procedure influenced regenerated shootlets essential oil components by inducing disappearance of some components and increasing some others, compared to mother plant components. In enc.-deh. (+LN) preservation procedure, the components of mother plant appeared again (except myrcene) and some of them exceeded the value of mother plant, i.e. thymol, caryophellene, linalool, carvacrol and cineol (6.60, 5.15, 4.62, 4.95 and 3.72%), respectively after 1 month.

Moreover, α- pinene, camphore, thujone, caryophellene, carvacrol, cineol and myrcene (8.20, 2.50, 7.90, 4.54, 3.5, 1.01 and 5.82%), respectively were increased after 2 months, while α- pinene, thujone, thymol, caryophellene, linalool and carvacrol (8.20, 7.88, 8.22, 6.20, 5.74 and 5.95%) were augmented while cineol and myrcene were disappeared again after 3 months of preservation.

**Table (8): Effect of preservation procedures on essential oil components (%) of regenerated shootlets after three subcultures in comparison to mother plant.**

| Essential oil components (%) / Preservation treatment | α-pinene | β-pinene | Camphore | Thujone | Thymol | Caryophellene | Linalool | Carvacrol | Cineol | Myrcene |
|---|---|---|---|---|---|---|---|---|---|---|
| lother plant (control) | 7.90 | 8.30 | 2.25 | 7.50 | 4.65 | 3.21 | 1.20 | 2.50 | 0.90 | 0.62 |
| nc.-deh. For 1 month (+ LN) | 3.40 | 0.00 | 1.12 | 6.60 | 6.60 | 5.15 | 4.62 | 4.95 | 3.72 | 0.00 |
| nc.-deh. For 2 months (+ LN) | 8.20 | 8.20 | 2.50 | 7.90 | 0.00 | 4.54 | 1.14 | 3.50 | 1.01 | 5.82 |
| nc.-deh. For 3 months (+ LN) | 8.20 | 8.00 | 2.14 | 7.88 | 8.22 | 6.20 | 5.74 | 5.95 | 0.00 | 0.00 |
| nc.-deh. for 1 month (- LN) | 0.00 | 0.00 | 4.03 | 2.50 | 0.00 | 0.00 | 4.72 | 0.00 | 0.00 | 2.33 |
| nc.-deh. For 2 months (- LN) | 0.00 | 0.00 | 0.00 | 7.20 | 0.00 | 0.00 | 0.00 | 4.22 | 0.00 | 4.01 |
| nc.-deh. For 3 months (- LN) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 6.20 | 0.00 | 0.00 | 5.10 | 4.30 |
| torage at 4°C for 1 month | 8.25 | 5.22 | 2.70 | 7.92 | 4.00 | 0.00 | 2.12 | 3.50 | 0.00 | 0.00 |
| torage at 4°C for 2 months | 7.90 | 2.50 | 0.00 | 3.62 | 2.42 | 6.20 | 5.30 | 6.05 | 4.52 | 0.00 |
| torage at 4°C for 3 months | 6.50 | 7.50 | 0.00 | 1.40 | 4.20 | 5.12 | 3.90 | 3.25 | 7.52 | 3.72 |
| rowth chamber conditions | 8.90 | 8.90 | 6.50 | 4.25 | 8.90 | 7.46 | 6.02 | 6.30 | 4.72 | 1.22 |

However, the major constituents resulted from enc.-deh. (-LN) with observed increase than in mother plant after 1 month of preservation are: linalool, camphor and myrcene (4.72, 4.03 and 2.33%), respectively. After two months of preservation, thujone, carvacrol and myrcene (7.20, 4.22 and 4.01%), respectively are increased while, after 3 months of preservation, caryophellene, cineol and myrcene increased (6.20, 5.10 and 4.30%), respectively.

Regarding regenerated shootlets following to preservation at 4°C, their essential oil content are characterized as follows: after 1 month of preservation, α- pinene, camphore, thujone, linalool and carvacrol (8.25, 2.70, 7.92, 2.12 and 3.50%) are increased, while after 2 months α-pinene is stable, caryophellene, linalool, carvacrol and cineol (6.20, 5.30, 6.05 and 4.52%) are increased. After 3 months, caryophellene, linalool, carvacrol, cineol and myrcene (5.12, 3.90, 3.25, 7.52 and 3.72%) are increased.

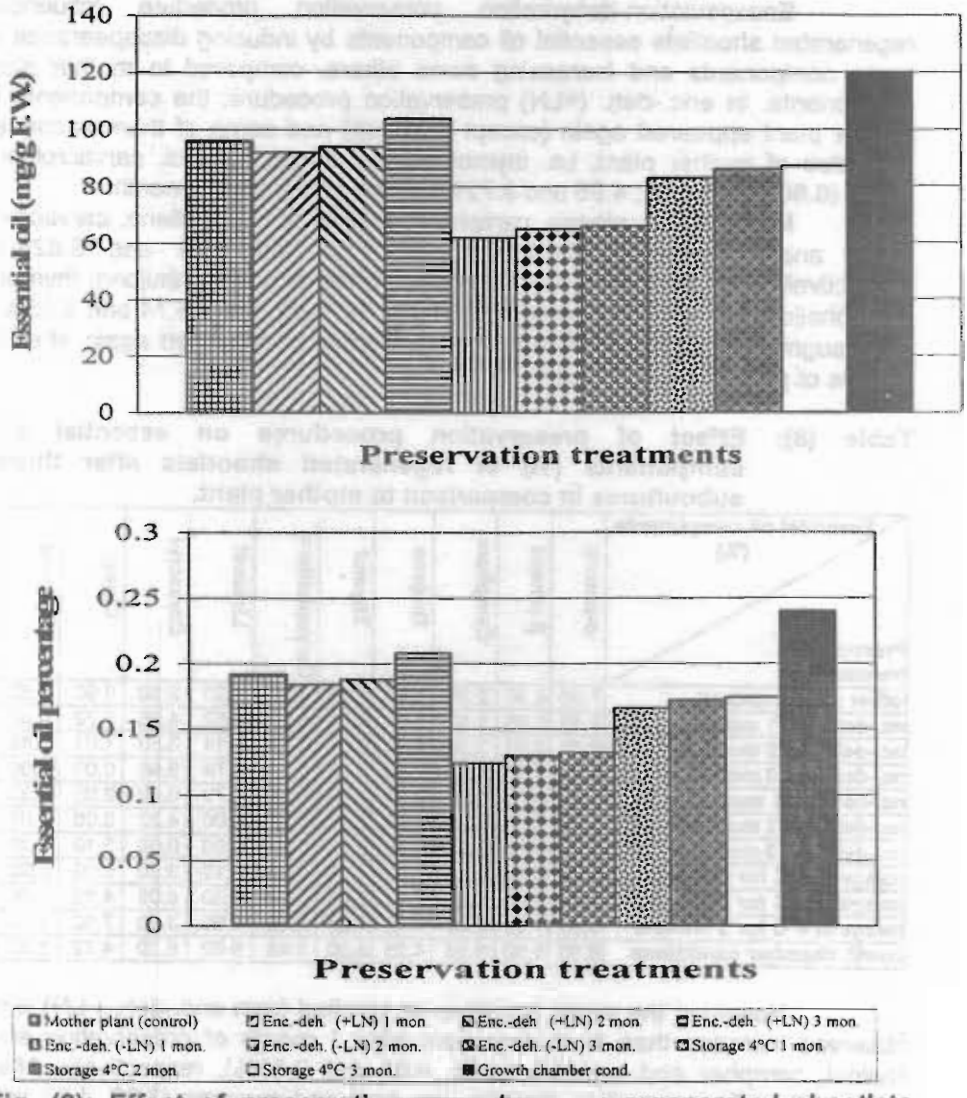



**Fig. (2): Effect of preservation procedures on regenerated shootlets essential oil content and percentage of regenerated shootlets after three subcultures compared to the mother plant.**

Concerning preservation under growth chamber conditions, all the essential oil components are increased, except thujone, compared to mother plant components.

**Molecular genetic identification**

**Randomly amplified polymorphic DNA (RAPD)**

Out of fifteen primers screened, three primers produced clear reproducible bands. The three primers produced 39 total amplified bands with a total polymorphic % 18.16. Data of the amplified fragments using those

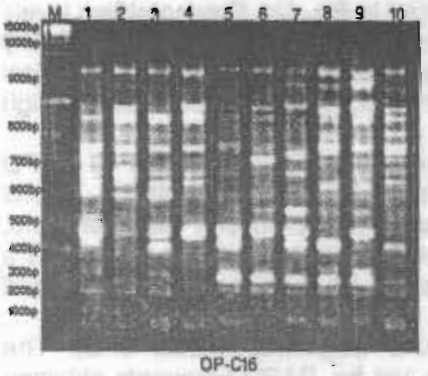
three 10-mer arbitrary primers, regenerated shootlets following to preservation treatments succeeded in amplifying DNA fragments by primers OP-C16, OP-C19 and OP-C20, as illustrated in Fig. (2). Polymorphism levels differed from one primer to another. Only OP-C20 primer did not show any polymorphism among treatments, while primer OP-C16 exhibited low polymorphism (6.7%). On the other hand, primer OP-C19 exhibited high levels of polymorphism (50%).

It is clear from Table (9 a) that, primer OP-C16 produced 15 RAPD fragments. The amplification product ranged from 1200 to 145 bp. RAPD fragments obtained from preservation treatments produced identical band, however lane 5 [enc.-deh. (-LN) for 1 month] exhibit the disappearance of a unique fragment of 1130bp which is present in other preserved shootlets and control plants.

Primer OP-C19 produced 12 RAPD fragments (Table 9 b). The amplification product ranged from 1170 to 194 bp. RAPD fragments obtained from preserved shootlets produced identical bands. Three unique fragments were observed in control shootlets, two of them were absent in other preserved shootlets with molecular weights 1170 and 405 bp. However, there is absent fragment in control plantlets and present in other preserved shootlets with molecular weight 680 bp. On the other hand, in lane 4 (4°C preserved shootlets after 3 months) three unique fragments with 1120, 980 and 620bp, respectively, were absent and these fragments were present in other preservation treatments, as well as control plantlets.

Primer OP-C20 produced 12 RAPD fragments (Table 9 c). The amplification products ranged from 1024bp to 190bp. All the preserved shootlets and control appeared homozygous to all the fragments.

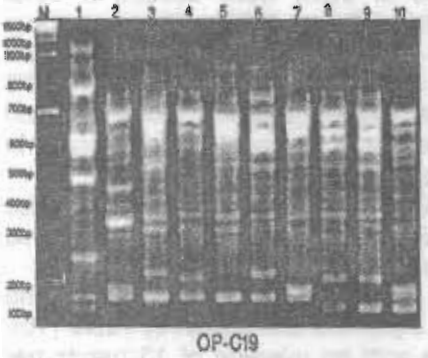
The number of total amplified bands, polymorphic bands and polymorphic % for each primer using the 3 primers are shown in Table (10). The 3 primers yielded 39 scorable bands with an average of 13 bands per primer. The total number of bands are calculated [number of shootlets in each treatment (10×10) × number of bands with 3 primers] with a result of 3900 bands, while the total scored polymorphic bands were 7 as exhibited, as well.




OP-C16

| Band No. | M.W. (bp) | Treatments 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1200 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | 1130 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| 3 | 945 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 4 | 880 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | 790 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 6 | 760 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 7 | 600 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 8 | 515 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 9 | 480 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 10 | 395 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 11 | 305 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 12 | 280 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 13 | 210 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 14 | 180 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 15 | 145 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Total | | 15 | 15 | 15 | 15 | 14 | 15 | 15 | 15 | 15 | 15 |

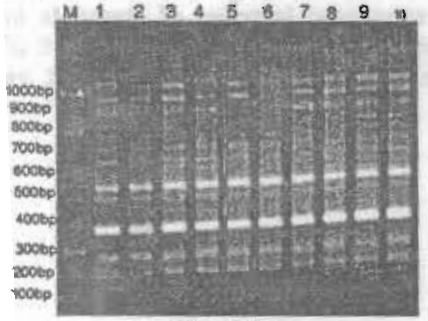
a- OP-C16




OP-C19

| Band No. | M.W. (bp) | Treatments 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1170 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 1120 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| 3 | 980 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| 4 | 890 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | 800 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 6 | 680 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 7 | 620 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| 8 | 570 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 9 | 405 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 341 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 11 | 240 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 12 | 194 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| Total | | 11 | 10 | 10 | 7 | 10 | 9 | 10 | 9 | 10 | 10 |

b- OP-C19




OP-C20

| Band No. | M.W. (bp) | Treatments 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1024 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | 990 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 3 | 800 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 4 | 700 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | 580 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 6 | 545 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 7 | 510 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 8 | 489 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 9 | 420 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 10 | 370 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 11 | 260 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 12 | 190 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Total | | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |

c- OP-C20

Fig. (3) Table (9)

RAPD fingerprints of preserved shootlets using 3 random primers (a, b and c):

(1) = Growth chamber conditions (control).
(2-4) = Storage at 4°C (for 1, 2 and 3 months, respectively).
(5-7) = Enc.-deh. -LN (for 1, 2 and 3 months, respectively).
(8-10) = Enc.-deh. +LN (for 1, 2 and 3 months, respectively).

**Table (10): Number of amplified bands, monomorphic bands, polymorphic bands and % of polymorphic bands of preserved shootlets based on RAPD-PCR analysis using three primers.**

| Primer Name | Polymorphic Bands | Monomorphic Bands | Total Amplified Bands | Polymorphic % |
|---|---|---|---|---|
| OP-C-16 | 1 | 14 | 15 | 6.7 |
| OP-C-19 | 6 | 6 | 12 | 50.0 |
| OP-C-20 | -- | 12 | 12 | -- |
| Total | 7 | 32 | 39 | 17.95 |

## DISCUSSION

Concerning explant cryopreservation preculture recovery, results indicated that increasing osmotic substances concentration and duration had a depressive effect on explant survival, shootlets number, shootlets length and leaves number. Sucrose at 0.18 M for 2 days exhibited explant recovery characters enhancement. This might be ascribed to increasing cell osmotic potential of plant cell sap to the limits that led to cell flaccidity and death due to the active accumulation of the osmotic active substances supplemented with higher concentration to the medium; i.e. sucrose and mannitol, as previously found by Salem *et al.* (2006).

Besides, shootlets regeneration characters were significantly influenced by preservation methods. Dehydration method resulted in a complete death, while vitrification (+LN) induced 100% of recovered explant survival with no shootlets regeneration. However, with vitrification (-LN), low survival was observed. The highest obtained survival of recovered explant, as well as shootlets number and length and leaves number was shown by encapsulation-dehydration (-LN).

Moreover, shootlets regeneration characters were progressively enhanced by preservation under growth chamber conditions. Control treatment may have maintained cell wall turgor in the ambient atmosphere of the culture (Salem *et al.*, 2006). However, fresh and/or dry weights were improved by preservation under 4°C, and encapsulation-dehydration (-LN) methods. These results are supported by those obtained by Saleh (2002). As well, soluble phenols increment which is considered as a growth retardants indicator was noticed as a result of encapsulation-dehydration (+LN) method. In this concern, Devlin (1975) reported that, IAA oxidase activity is increased by monophenols and reduced by diphenols.

Essential oil content and components are pronouncly enhanced by growth chamber conditions (control), this may be explained on basis of that monoterpenes may appear simply to be byproducts of metabolism which accumulate due to prolonged storage (Taiz and Zeiger, 1998).

Concerning genetic stability of preserved plantlets, results indicated that the total number of RAPD bands scored in this study (3900) seems to be fairly high to allow detection of polymorphism. The obtained RAPD bands were mostly monomorphic, however, only total 7 bands detected from

regenerated shootlets were polymorphic out of total 3900, this indicates that shootlets maintained their genetic stability through germplasm preservation methods. The obviously stable RAPD pattern could be a certain indication that genetic properties of preserved shootlets are not influenced by preservation methods. These results are in agreement with Hao *et al.* (2004). RAPD analysis was carried out as it is relatively simple, fast and it can provide cheap way of randomly screening large part of the genome (Lowe *et al.*, 1996). Assessing genetic stability of tissue culture-regenerated plants, Munthali *et al.* (1996) detected 3 polymorphism after scoring a total of 5607 bands, respectively. There is relatively little documentation on the molecular genetic analysis of shootlets regenerated from cryopreserved meristems or shoot tips. However, increasing numbers of studies are indicating that plants recovered from storage *in vitro* are genetically stable if the storage procedures minimize the production of somaclonal variations, mainly by avoiding the use of high concentration of growth inhibitor and osmotic materials (Hao *et al.*, 2004).

Conclusively, slow growth could be more widely used to provide safer alternatives to the field gene bank for species that are readily propagated *in vitro* by low-risk methods. Cryopreservation is not yet as advanced stage of development as slow growth, particularly for organized cultures. Room temperature storage may be interesting for tropical, cold-sensitive species because it allows growth reduction at the normal growth temperature. However, it still has to be tested with additional species and over longer storage periods. One of the most effective ways of minimizing risks of instability both at the outset and during slow growth storage is through control of the culture system. If the cultures are maintained in a highly organized state, as shoots, plantlets, or embryos, the risk of somaclonal variation is much lower than if they were in the form of cells or calluses (Withers and Engelmann, 1998).

There are differences from species to species in their susceptibility to somaclonal variation. Thus there is a pressing need for controlled experiments to test the genetic integrity of cultures stored by various methods of preservation, in comparison with controls maintained under normal growth conditions.

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## الحفظ قصير الأجل للاصل الوراثي لشجيرة الآس معملياً

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خضعت القمة النامية لنباتات الميرسين الناشئة من مزرعة للانسجة الي تجربة حفظ باستخدام تقنية الحفظ فائق التجميد (سابقة التعرض لمعاملات التزجج و الكبسلة و التجفيف) او باستخدام الحفظ البارد (لدرجة ٤°م) و كذلك معاملة الكنترول (الشاهد)؛ حيث تم حفظ النبتات تحت ظروف غرفة الزراعة العادية. كذلك تم حفظ العقيدات الساقية باستخدام تقنية التجفيف و ذلك قبل تعرضها للحفظ باستخدام التجميد الفائق (- ١٩٦°م). تم تعرض النبتات لتقنيات الحفظ المختلفة لمدة شهر, شهرين و ثلاثة أشهر, فيما عدا الحفظ في ظروف الحضانة فإمتد الحفظ لمدة عام. تم تعريض القمة النامية لعدة تجارب حماية من التجميد و التي تشمل التعرض لمواد كيماوية مختلفة مثل الداي ميثيل سالفوكسيد DMSO بالاشتراك مع او بدون بولي ايثيلين جليكول PEG او الجليسرول و ذلك قبل التعرض لتقنية التزجج. تم اختبار شفاء العزلات النباتية من التعرض لتقنية الحفظ فائق التجمد و كذلك اختبار اعادة تنشيط و تكوين النبتات و ذلك بعد الانتهاء من وسائل الحفظ المختلفة. و قد دلت النتائج علي ان القمة النامية التي تم تعرضها لتركيز ٠,١٨ مول سكروز أعطت افضل نتائج شفائية من نسبة البقاء حية و عدد للافرع و طولها و عدد الاوراق و ذلك بقيم اكبر من التركيز الاعلي و كذلك تفوق السكروز علي المانتول في هذا الشأن سواء كان التعرض لمدة يومين او اربعة ايام. و علي الرغم ان تقنية التزجج اعطت اعلي نسبة بقاء إلا انها لم ينتج عنها اعادة تكوين اي فرع نباتي. و قد نتج عن الحفظ في ظروف غرفة الزراعة لمدة عام اعلي صفات نمو (اعلي نسبة بقاء و عدد افرع و وزن جاف) و بالمثل فإن المكونات الكيماوية (كلورفيل أ, الكلورفيلات الكلية و الكاروتنويدات و الاندولات الكلية و الاحماض الامينية الحرة الكلية و السكريات الكلية و النسبة بين الاندولات و الفينولات, و كذلك محتوي و مكونات الزيوت الطيارة اعطت اعلي نتائج علي نفس معاملة الحفظ). إلا ان الوزن الطازج و كلورفيل ب و الفينولات الكلية الذائبة كانت منخفضة نتيجة هذه الوسيلة من الحفظ. و قد دلت نتائج اختبار الثبات الوراثي باستخدام تكنيك سلسلة تفاعل البلمرة العشوائية (RAPD ) ان النباتات التي تم حفظها بالمعمل لم يتأثر ثباتها الوراثي لحد كبير.