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CRYOPRESERVATION OF *IN VITRO*- SHOOT TIPS OF TWO LOCAL ORANGE *(CITRUS SINENSIS)* CVS.

[11]

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ABSTRACT

This investigation was a part of a study to conserve the germplasm of some orange cultivars. It was performed through establishing a protocol for high propagation rate, the application of cryopresevation method with the exploration of the best cryoprotectants to achieve high survival during long-term storage of orange shoot tips. Addition of cryoprotectant led to increasing the survival % and recovery of shoots especially with using glycerol at low concentration. Exposure to all cryoprotectants treatment (T1-T10) for 5-10 min was enough to achieve 100% survival for the orange shoot tips. However, the survival percentage was decreased with increasing exposure duration to 15 min upwards especially for T1 (0.4 M sucrose + 15% PEG + 30% glycerol + 15 % DMSO), T3 (0.4 M sucrose + 30% glycerol) and T4 (0.4 M sucrose +15% DMSO). Exposure to treatments T2, T8 & T10 for up to 110 min continued to record 100% survival but recovery was decreased after the exposure to T5, T6, and T7 &T9 for 80-90 min. The results of cryopreservation experiment showed that the recovery of frozen shoot tips reached 100% after one day post culture when the exposure to PVS2 was 5-40 min for Succari cv. shoot tips and 5-60 min for Ahmar Bedamo cv. There was a gradual insignificant decrease in the survival of both cultivars of oranges by the increasing exposure time for 40 min. The decrease became significant after 50-100 min exposure time and also by increasing post culture time to 15-20 days. The survival percentage reached 40 and 50 % after 20 days of post culture for the shoot tips exposed to PVS2 for 5 min while it was 10 and 0.0% when the exposure time was 100 min, respectively for Succari and Ahmar Bedamo cvs and the differences were insignificant.

Key words: *Citrus*, Cryopreservation, Cryoprotectants, Germplasm, In-*vitro*, Shoot tips, Vitrification method.

INTRODUCTION

The function of any technique designed for the conservation of plant germplasm is to preserve the greatest possible genetic diversity of a particular plant for future use. This diversity may be found at the species level, and into other

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groupings such as varieties, cultivars or discrete local populations (**Grout, 1990**). *In vitro* storage technique includes the medium-term storage option utilizing slow growth procedures (**Wanas *et al* 1986 and Wanas 1992 & 1999**), and long-term storage using cryopreservation of plant material (**Wanas 1987 & Withers and Engelmann, 1997**).

Cryopreservation of plant material is the only viable option for the long-term storage of germplasm of vegetatively propagated species. This involves storage at ultra low temperatures, usually that of liquid nitrogen (-196°C). At this temperature, cell division and metabolic processes stop, and plant material can thus be stored without modification or alteration in genetic make up for unlimited periods of time (**Ashmore, 1997**). In most cases the freezing of plant tissues has been performed in the presence of cry protective chemicals such as dimethyl sulfoxied (DMSO), glycerol, sugars, large molecular weight polymers, or combinations of these . These compounds protect plant cells from damage by freezing even at very cold temperatures, so that growth can continue after thawing (**Finkle and Ulrich, 1979**).

Since cryopreservation involves essentially three steps, specimen treatments and freezing, storage at ultra-low temperature, and thawing an ideal cryoprotectants should protect cells from all the factors which would affect the viability of the frozen biological sample during all these stages. Such cryoprotectants can lower the temperature at which freezing first occurs and can alter the crystal habit of ice when it separates. It is to be borne in mind that exposure duration of cryoprotectants to cells needs to be such that the concentration applied does not cause sudden plasmolsis, which in itself could be a major cause of injury to the osmotic responsiveness of the cells (**Kartha and Engelmann, 1994**).

Sakai (1995) used vitrification method for cryopreservation of shoot tips of navel orange. He Obtained high survival (80-90%) when treated with PVS2 solution contains (w/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in 0.4M sucrose solution at 25°C for 3 min after cooling to -196°C but the survival decreased rapidly after longer exposure. The same method was successfully applied to *in vitro* –grown shoot tips of 5 apple cultivars, 8 pear cultivars and 13 mulberry species or cultivars (**Niino *et al* 1992**). Also, shoot tips of cherry recorded high survival (80%) when treated with PVS2 at 25°C for 105min (**Niino *et al* 1997**) and in banana the survival reached to 70% (**Takagi *et al* 1998**) while almond rootstock M 51 apices achieved limited survival (10%) when treated with PVS2 for 120-180 min (**Shatnawl *et al* 1999**). In another work, shoot tips of two almond cvs. recorded high survival and the recovery after thawing was 87.5, 60 and 72.5%, respectively (**Channuntapipat *et al* 2000**). High rate recovery growth reached 85-100% by *Vitis vinifera* and 65-75% by *Rubus idaeus* (**Wang *et al* 2003 & 2005**).

During this investigation the effect of different cryoprotectants on the survival of orange cvs. shoot tips prior to freezing was explored. Also the vitrification method with PVS2 was applied to *in-vitro* grown shoot tips of orange cvs.

MATERIALS AND METHODS

This study was achieved through the period from year 2000 until 2005 in the

Tissue Culture Laboratory, Horticulture Department, Faculty of Agriculture, Ain Shams University, Cairo. The different steps for this investigation will be explained as follow:-

Establishment of orange *Citrus sinensis* stem node cultures

Two cultivars of orange (*Citrus sinensis*) were used through out this study namely; Acid less orange "Succari", Blood orange "Ahmar Bedamo", orange. Shoots at 10-15 cm in length were collected during the growing season (March-May) from adult trees of citrus located in the orchard of the Faculty of Agriculture, Ain Shams University. The stem nodes were surface sterilized and grown for four weeks in basal salts and vitamins of free hormones Murashige and Skoog 1962 (MS) plus 30 gl^{-1} sucrose and 7 gl^{-1} agar and maintained in the culture room for establishment.

The explants from established cultures were transferred to jars (85 x 50 mm) filled with 35 ml MS salts and vitamins at full strength plus (as mgl^{-1}) (0.2) benzyl amino purine (BAP) and (0.5) indole-3-butyric acid (IBA) 30 gl^{-1} sucrose, and kept in the culture room as a stock for the cryopreservation experiments.

Cryopreservation experiments

Effect of culturing period in different cryoprotectants on shoot tips survival and regrowth of shoots

In this experiment, different combinations of cryoprotectants were used, the types and concentrations were listed in Table (1).

Table 1. Different concentrations and combinations of cryoprotectants

Treatment	Sucrose	Polyethylene glycol (PEG) %	Glycerol (%)	Dimethyl sulfoxide (DMSO)%
1(control)	0.4M	15%	30%	15%
2	0.4M	15%	0.0	0.0
3	0.4M	0.0	30%	0.0
4	0.4M	0.0	0.0	15%
5	0.4M	10%	0.0	0.0
6	0.4M	0.0	20%	0.0
7	0.4M	0.0	0.0	10%
8	0.4M	0.0	10%	0.0
9	0.4M	0.0	0.0	5.0%
10	0.4M	0.0	5.0%	0.0

Four weeks old cultures were cold hardened at 7°C for 2 weeks. Shoot tips were dissected and cultured in full strength MS medium supplemented with the previously mentioned concentrations of the cryoprotectants at 25°C for different periods (5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 150, 180 and 210 min). Four shoot tips were tested per each of two replicates for each treatment. And the data on survival percentages were collected after 7 days of transfer to normal culture medium.

Effect of exposure time to PVS2 solution during cryopreservation on the survival of orange cvs.

The main aim of this experiment was the determination of the optimal time of exposure to PVS2 at 25°C when using vitrification method for cryopreservation.

Vitrification procedure

Shoot tips 3mm (in length) were dissected from four weeks old cold hardened cultures. Cold hardened shoot tips were precultured at 7°C for 24 h on full strength MS supplemented with 0.7M sucrose.

Following preculture treatments, each treatment consisted of 2 replicates, five shoot tips in each replicate were transferred to 1.0ml of Plant vitrification solution 2(PVS_2) in a 2 ml plastic cryotube at 25°C .The vitrification solution (PVS_2) contains at (w/v) 15% DMSO, 30% glycerol and 15% PEG in MS basal medium plus 0.4 M sucrose (pH5.8). After 30 min treatment, the PVS_2 was removed using a Pasteur pipette, and replaced twice with fresh PVS_2 and held at 25°C for different periods of time (5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 min.). The cryotubes in which shoot tips were finally suspended in 0.7 ml of fresh PVS_2 were immediately strained before being directly plunged and held in LN. Shoot tips stored in Liquid nitrogen (LN) overnight from were rapidly warmed in a water bath at 37-40°C.

After rapid warming, PVS_2 was drained from cryotubes and replaced twice with liquid MS medium containing 1.2M sucrose and held for 30 min. The thawed shoot tips were transferred to filter paper for 10 min for desiccation of the media. After that, shoot tips were cultured onto 0.7% agar MS medium excluding NH_4NO_3 and contain (as mgl-1) 0.2 BA + 0.5 IBA, for recovery the shoot tips incubated for 48 h in dark, then transferred to standard conditions (Light 3000lux for 16h at 27°C). The survival of shoot tips after freezing and thawing procedures was judged by their green color after 2 weeks of post culture.

Recovering of the shoot tips was observed for 20-30 days. Shoot formation was recorded as percent of total number of shoot tips forming shoots 30 days after plating .Ten shoot tips were tested for each replicate, three replicates were used for each treatment in a completely randomized design and means comparisons were done according to Snedecor (1980).

RESULTS AND DISCUSSION

In a trial to increase the survival percentage, different cryopotectants were used as shown in Table (2). Exposure time starting from 5 min was enough to achieve 100 % survival with all the tested cryopotectants. The survival percentages decreased with increasing exposure duration especially for T_1 and T_3 at 20 min

Table 2. Effect of exposure times to different cryoprotectants on shoot tips survival.

Time (min)	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
5	100	100	100	100	100	100	100	100	100	100
10	100	100	100	75	100	100	100	100	100	100
15	75	100	100	75	100	100	100	100	100	100
20	75	100	75	100	100	100	100	100	100	100
30	75	100	100	75	100	100	100	100	100	100
40	75	100	50	50	100	100	75	100	75	100
50	75	100	75	50	100	100	75	100	75	100
60	75	100	75	75	100	100	75	100	75	100
70	50	100	75	50	100	100	75	100	75	100
80	50	100	50	50	100	100	50	100	75	100
90	50	100	50	25	100	75	50	100	50	100
100	50	100	25	25	75	75	50	100	50	100
110	25	100	25	25	75	75	25	100	50	100
120	25	25	50	25	0.0	75	75	25	100	50
150	25	25	25	0.0	75	50	25	100	25	75
180	25	25	25	0.0	75	50	25	100	50	50
210	25	25	25	0.0	50	50	25	75	25	50

	Sucrose	PEG(%)	Glycerol(%)	DMSO(%)
T1	0.4 M	15	30	15
T2	0.4 M	15	0.0	0.0
T3	0.4 M	0.0	30	0.0
T4	0.4 M	0.0	0.0	15
T5	0.4 M	10	0.0	0.0
T6	0.4 M	0.0	20	0.0
T7	0.4 M	0.0	0.0	10
T8	0.4 M	0.0	10	0.0
T9	0.4 M	0.0	0.0	5
T10	0.4 M	0.0	5	0.0

and for all the exposure between periods 20-210 min and all cryoprotectants except for T_2, T5, T6, T8 and T_{10}. Similar case was noticed by **Druart** (1985) on Prunus, Malus and Cydonia clones when DMSO and glycerol at 5, 10 and 15 % were added to the multiplication medium.

Table (3) showed the effect of cryopreservation using different exposure times to PVS2 solution on the survival percentage for orange cvs. shoot tips. After one-day post culture for the cryopreserved shoot tips, the survival reached 100% when the time of exposure to PVS2 was 5-50 min in case of Succari cv or 5-60 min in case of Ahmar Bedamo cv. There was a gradual decrease in the survival of both cultivars by increasing of the exposure time from 40 or 60 to 100 min and also by increasing of post culture time from 15-20 days for Succari shoot tips and for 20 days in Red Bedamo shoot tips. The survival % reached 40 and 50 % after 20 days of post culture for the shoot tips exposed to 5 min PVS2, while it was 10 and 0.0 % when the exposure time was 100 min, respectively for Succari and Ahmar Bedamo.

The green survived tips continue to differentiate and elongate the leaf primordia within 7-28 days in the recovery medium. Increasing exposure time to PVS2 turned out to be toxic and reduced survival percentage.

Table 3. Effect of cryopresrvation (using different exposure times to PVS2 solution) on the survival % of orange shoot tips cvs Succari and Red Bedamo after 1-20 days post culture.

Time (min)	Days after recovery of Succari shoot tips						Days after recovery of Red Bedamo shoot tips					
	1	3	7	10	15	20	1	3	7	10	15	20
5	100a	100a	100a	100a	60a	40a	100a	100a	90a	70a	70a	50a
10	100a	90a	80a	70a	50a	50a	100a	90a	70a	50a	20b	10b
15	100a	90a	80a	70a	60a	50a	100a	90a	80a	50a	30a	20b
20	100a	80a	70a	70a	50a	40a	100a	90a	90a	70a	70a	50a
30	100a	80a	80a	60a	50a	40a	100a	90a	90a	70a	50a	30a
40	100a	90a	80a	60a	40a	50a	100a	90a	80a	70a	50a	50a
50	90a	70a	50a	20b	10b	0.0b	100a	90a	90a	70a	40a	20b
60	90a	60a	50a	50a	10b	0.0b	100a	80a	70a	60a	30a	10b
70	80a	80a	80a	60a	30a	0.0b	90a	80a	70a	50a	30a	10b
80	80a	80a	80a	70a	40a	20b	80a	80a	70a	40a	20b	0.0b
90	80a	80a	70a	70a	30a	10b	80a	70a	70a	70a	50a	20b
100	70a	70a	70a	60a	20b	10b	70a	70a	70a	50a	30a	0.0b

In the current study, longer exposure to PVS2 (100 min) led to rapid significant decrease in survival (0 and 10%) after cooling to −196°C. Similarly, almond rootstock M51 apices achieved limited survival (10%) when treated with PVS2 for 120-180 min (Shatanwi *et al* 1999). On the contrary cherry shoot tips recorded high survival (80%) when treated with PVS2 for 105 min at 25°C (Niino *et al* 1997).

It is obvious that 5-40 min. exposure time offers significant survival after 20 days recovery (40-50%) for orange cvs. Comparative higher survival percentages were recorded by Sakai (1995); Niino *et al* (1997) and Wang *et al* (2003& 2005). Further studies would be suggested to increase survival and recovery of Citrus germplasm. Such improvement would be achieved through the application of sorbitol in the pregrowth medium as well as the use of different cooling rates and transfer temperatures suggested by Wanas (1987).

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الحفظ بالتجميد لقمم الأفرع الناتجة بالمعمل لصنفين من البرتقال

[١١]

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هذا البحث كان جزءاً من دراسة أجريت على حفظ الجرمبلازم لبعض أصناف البرتقال وأجرى من خلال تأسيس بروتوكول لإيجاد معدل عالي من الإكثار في المعمل ثم تطبيق طريقة الحفظ بالتجميد لقمم الأفرع لهذه الأصناف ومحاولة التعرف على أفضل المواد التي تحمي من التجمد لتحقيق أعلى معدل من البقاء أثناء التخزين طويل المدى.

وأوضحت نتائج التجربة الأولى أن التعرض لمعاملات المواد لتي تحمى من التجمد وهي من معاملة رقم ١ حتى معاملة رقم ١٠ لمدة ٥-١٠ دقائق تكون كافية للحصول على معدل بقاء ١٠٠% لقمم أفرع البرتقال السكري ولكن تناقص هذا المعدل بزيادة مدة التعرض إلى ١٥ دقيقة فيما فوق خاصة عند استخدام معاملة رقم واحد (٠,٤ مولر سكروز + ١٥% بولى ايثيلين جليكول + ٣٠% جليسرول + ١٥% داى ميثيل سلفوكسيد DMSO) والمعاملة رقم ٣ (٠,٤ مولر سكروز + ٣٠%جليسرول) والمعاملة رقم ٤ (٠,٤ مولر سكروز + ١٥% داى ميثيل سلفوكسيد) ولكن استمرار التعرض للمعاملات رقم ٢، ٨ ، ١٠ لمدة تصل إلى ١١٠ دقيقة أعطى ١٠٠% نسبة بقاء بينما تناقص هذه النسبة عند التعرض للمعاملات رقم ٥ ، ٦ ، ٧ ، ٩ عند التعرض لحاميات التجمد اكثر من ٨٠- ٩٠ دقيقة.

وأظهرت تجربة الحفظ بالتجميد في النيتروجين السائل أن قمم الأفرع التي تعرضت للتجمد تتعافى بنسبة ١٠٠% بعد يوم واحد من الزراعة في بيئة مناسبة والتي تكون قد تعرضت لحاميات التجمد المعروفة ب PVS2 لمدة من ٥-٤٠ دقيقة بالنسبة للصنف السكري والى مدة ٥-٦٠ دقيقة بالنسبة للصنف أحمر بدمه. وكان هناك تناقص تدريجي غير معنوي في نسبة البقاء لكلا الصنفين للبرتقال بزيادة مدة التعرض لل PVS2 إلى ٤- دقيقة واصبح هذا

التناقص معنوي بعد ٥٠ – ١٠٠ دقيقة وأيضا زيادة المدة بعد التعافي من التجمد إلى ١٥-٢٠يوما . ووصلت نسبة البقاء إلى ٤٠%، ٥٠% بعد ٢٠ يوماً من إعادة الزراعة بعد التجميد بالنسبة لقمم الأفرع التي تعرضت لل PVS2 لمدة ٥ دقائق بينما كانت صفر ، ١٠% عندما وصلت مدة التعرض لل PVS2 إلى ١٠٠ دقيقة على التوالي بالنسبة لصنف السكري وصنف البرتقال أبو دمه.

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