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Preservation of Rare Apricot and Walnut Germplasms Grown in Saint Catherine Valleys by Encapsulation Dehydration Technique.

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Abstract: This study was conducted during the period 2009 and 2012 using apricot and walnut germplasms grown in Saint Catherine Valleys in Sinai Peninsula, Egypt. The encapsulation-dehydration technique has led to successful results with shoot tips of numerous temperate and tropical plant species. Shoot tips, 1-3 mm long have been widely used due to their high genetic stability, high survival and regrowth percentages. Shoot tips are characterized by their small dense and actively dividing cells and it would insure rapid multiplication rates after thawing also, the low water content of shoot tip cells would propose a strong reason for choosing them as basic plant material for cryopreservation. The survival rates of encapsulated shoot tips of apricot (*P. armeniaca* L.) and walnut *(Juglans regia* L.) after pregrowth procedure, varied depending on the sucrose content in the preculture medium. Preculture for 1 day with 0.75, 1.0 and 1.5M sucrose was detrimental to survival. By contrast, shoot tips could withstand extended preculture duration (up 10 day) in media with lower sucrose concentrations (0.5 M sucrose). It was concluded that preculture for 1 day with 0.75, 1.0 and 1.5µ sucrose was detrimental to survival. By contrast, shoot tips of walnut could withstand extended preculture duration (up 10 day) in media with lower sucrose concentrations (0.5 M sucrose). It was found that the highest survival rate with 0.5 M sucrose (90%) after 10 day. Cold acclimation was essential for successful cryopreservation of apricot and walnut. All cold-acclimated shoot tips had significantly more regrowth following liquid nitrogen (LN) exposure than the non-acclimated control. Growth recovery of cryopreserved apricot and walnut shoot tips occurred directly without callus formation. This allowed to gain most cells of the shoot tips with only slight or not all damaged during the cryopreservation process in liquid nitrogen. Survival of desiccated encapsulated shoot tips decreased in line with decreasing bead moisture content (MC) after cryopreservation, and decreased with 1.0 M sucrose. The highest survival was obtained after slow freezing with beads dehydrated to 23or 28% MC.

Key words: *In vitro* preservation, encapsulation, acclimation, dehydration, germplasms, apricot and walnut.

INTRODUCTION

Many economic and Valuable germplasms are available in Egypt especially in very remote or distant areas. These germplasms have been threatened by distinction due to some stressful conditions or pests, even though; they are characteristics with many desired traits. Rare germplasms, in general, represent a wealth or source of fortune to the field of agriculture. It is very important to pay more attention to the available germplasm especially those growing under arid conditions. The valleys of Saint Catherine are a rich source of fruit trees germplasms. The utilization of available techniques such as encapsulation-dehydration would provide a valuable mean to preserve such invaluable plants. To be able to achieve mass production of such rare germplasms while maintaining their traits, it is very important to establish an *in vitro* production system that could be adapted and implemented on a commercial scale.

Cryopreservation is the storage of biological specimens at ultra low temperature (-196°C) in a cryogenic medium such as liquid nitrogen (LN) (Withers and Engelmann, 1997). At this ultra – low temperature, all cellular division and metabolic processes were ceased, allowing conservation for a theoretically unlimited period of time (Engelmann, 2004).

Long-term conservation of plant genetic resources using cryopreservation relies on freezing embryo and / or shoot tips. Embryo and shoot tips are complex structures with a heterogeneous cellular composition. Thus, they require cryogenic- protective treatments to origin. Specific treatments are required to artificially induce cold tolerance, because tropical plants do not develop cold – tolerance mechanisms, and are thus highly sensitive to low temperatures (Engelmann, 2000).

The state of water and osmotic equilibrium related to movements of water into and out of the cells are parameters of particular importance for cryopreservation (Mazur, 2004), water removal plays a central role in

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preventing freezing injury and in maintaining postthaw viability. There are two types of cryopreservation protocols, which differ, based on their physical mechanisms. In the so-called classical or conventional cryopreservation, freezing is performed in the presence of ice, while in the encapsulation- based protocols, freezing normally takes place without ice formation.

Cryopreservation for germplasm storage is increasingly important for national germplasm collection (Withers, 1991) clonally propagated crops such as fruit , nut, and many root and tuber vegetables cannot be stored as seed and are especially suited for cryopreserved storage.

Storage methods for plants kept as seeds, pollen, dormant bud and shoot tips are actively developed (Withers, 1991), although storage of genetic resources has been practiced for many years. Germplasm conservation is becoming increasingly important due to the loss of diversity in agriculture systems as formerly isolated forming communities begin replacing old and race varieties with new cultivars (Tanksley and McCouch, 1997). The genetic diversity in wild relatives is very important for future crop improvement. Thus, all unique accessions could be collected and preserved (Engelmann, 1991).

Thus, the objectives of this research were to preserve apricot and walnut germplasms by encapsulation – dehydration and to provide with a standardized procedure to conserve rare apricot and walnut clones.

MATERIALS AND METHODS

1.Cryopreservation of Apricot and Walnut:

Used plant materials were shoot tips (1.5 cm) cut in early spring from apricot (*Prunus armemiaca* L.) and walnut *(Juglans regia* L.), plants grown in Saint Catherine Valleys. Shoot tips were washed in running tap water for 30 minutes, surface sterilized shoot tips were placed vertically on initiation medium consisted of MS medium supplemented with 2 mg BA/L, the pH of the medium was adjusted to 5.6 before the addition of 2.5% phytagel. Media were sterilized by autoclaving for 15 minutes at 121°C and plant materials were incubated six weeks at 27°C and 16h photo period, then plant materials were transferred to MS media (free-hormones) containing sucrose ranging from (0.5 M to 1.5 M). Apical and axillary buds (3 mm) were excised from micro-shoots produced and used as explants for encapsulation.

2. The Encapsulation Procedure:

Encapsulation used different concentration (1, 2, 3, 4 and 5%) sodium alginate (Duchefa Biochemie BV, Netherlands) dissolved in $^1/_2$ strength MS medium containing 2g/L sucrose was dropped into a sterile solution of 100 mM $CaCl_2$. Explants were immediately immersed into the formed beads. Beads were left for 20 second during which Na^+ and Ca^{++} ions exchanged (Kinoshita and Saito, 1990). Beads containing shoot tips and buds were then picked up and washed three times with sterile 1/2 MS liquid medium –encapsulated shoot tips and buds were then transferred to 250 ml –quick-fit jars or tubes containing 100ml substrates (1/2 MS medium (free-hormones) and 1% sucrose). All substrates were gelled with 2.5% phytagel and sterilized before use, or without phytagel.

3. Encapsulation–Dehydration:

Encapsulation dehydration included encapsulation of plant material in calcium alginate beads, followed by pregrowth treatment in a medium containing high levels of sucrose ranging from 171.15, 256.73, 342.30 and 513.45 g/L or (0.5, 0.75, 1.0 and 1.5 M) for different durations (from 1 to 10 days). The alginate beads were then dehydrated before freezing using either air-drying in a Laminar Flow Hood (from 1-8 h).

Then, after 1-4 weeks of transfer to fresh multiplication medium, plantlets were cold acclimated in a growth chamber at -1 ^{o}C or deep freezer at -20 and16 h dark/22 ^{o}C 8h with light. Fresh weight was taken at each time intervals and beads were dried by heating in an oven at 103 ^{o}C for 16 h for dry weight. Moisture content was determined as the difference between dry weight (DW) and fresh weight (FW) calculated as: $[(FW - DW)/FW] * 100\%$.

4. Freezing or Storage:

Beads containing shoot tips and buds were transferred to liquid nitrogen (at -196 ^{o}C) directly for different durations or before transferred to LN, they were stored in different temperatures for different durations.

5. Thawing and Regrowth:

After storage in liquid nitrogen, beads samples were taken and tested for regrowth every three months. Cryopreserved shoot tips were rapidly thawed in a water bath at 37 °C for 2-3 minutes. After warming up, shoot tips were washed by liquid MS medium and thawed shoot tips were cultured on a MS medium supplemented with 1.0 mg/L BA in the presence of 100 mg/L ascorbic acid and 150 mg/L citric acid and 3% sucrose at 25 °C in the dark. After 3 days, shoot tips were transferred to fresh medium under the light conditions described above.

Data Analysis:

The experiments were subjected to completely randomized design. Variance analysis of data was carried out using Anova program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan´s multiple range. Means followed by the same letter are not significantly different at $P \leq 0.05$. Analysis of variance ANOVA was done using SAS, 2000 software program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan, 1955 new multiple range. Means followed by the same letter are not significantly different at $p\leq 0.05$.

RESULTS AND DISCUSSION

1. Cryopreservation of Apricot and Walnut:

Plant Material:

Shoot tips represented the material of choice for apricot (*Prunus armeniaca* L.) and walnut (*Juglans regia* L.) germplasm conservation, since plants regenerated from shoot tips of adult cultivars would not present juvenility characteristics and would be true - to type, in contrast to plants produced from any other type of material.

The encapsulation-dehydration technique had led to successful results with shoot tips of numerous temperate and tropical plant species. Shoot tips, 1-3 mm long were widely used due to their high genetic stability, high survival and regrowth percentages. Shoot tips have been characterized by their small dense and actively dividing cells and it would insure rapid multiplication rates after thawing. The low water content of shoot tip cells would also propose a strong reason for choosing them as basic plant material for cryopreservation ,as indicated by Ashmore, 1997.

2. Effect of Sucrose Treatment:

The survival rate of encapsulated shoot tips of apricot (*P. armeniaca* L.) after pregrowth varied depending on the sucrose content in the preculture medium (Table 1). Preculture for 1 day with 0.75, 1.0 and 1.5 M sucrose was detrimental to survival. On contrast, shoot tips could withstand extended preculture duration (up 10 day) in media containing lower sucrose concentrations (0.5 M sucrose).

Table 1: Effect of preculture duration and sucrose concentration on the survival (%) of encapsulated apricot (*P. armeniaca*).

Preculture duration (days)	Sucrose concentration (M)			
	0.5	0.75	1.0	1.5
0	0.0^{f*}	0.0^{e}	0.0^{c}	0.0
1	20^{e}	30^{a}	10^{a}	-
2	30^{d}	20^{b}	5^{ab}	-
3	40^{cd}	10^{c}	0.0^{c}	-
4	40^{cd}	5.0^{cd}	-	-
5	70^{c}	0.0^{e}	-	-
6	80^{b}	-	-	-
7	80^{b}	-	-	-
8	90^{ab}	-	-	-
9	100^{a}	-	-	-
10	100^{a}	-	-	-

* Values, within a column, of similar letters were not significantly different according to the least significant difference (LSD) at 0.05 levels.

Table 2: Effect of preculture duration and sucrose concentration on the survival (%) of encapsulated walnut (*Juglans regia* L.).

Preculture duration (days)	Sucrose concentration (M)			
	0.5	0.75	1.0	1.5
0	0.0f *	0.0c	0.0b	0.0
1	10e	10b	10a	-
2	20d	20a	0.0b	-
3	20d	0.0c	-	-
4	30c	-	-	-
5	30c	-	-	-
6	50b	-	-	-
7	60b	-	-	-
8	80ab	-	-	-
9	80ab	-	-	-
10	90a	-	-	-

* Values, within a column, of similar letters were not significantly different according to the least significant difference (LSD) at 0.05 level.

It could be concluded from Table 1 that inhibition of growth observed with sucrose concentrations at 1.0 and 1.5 M, achieved the highest survival percentage (100 %) with 0.5 M sucrose after (up 10 day).

The data in Table 2 showed that Preculture for 1 day with 0.75, 1.0 and 1.5M sucrose was detrimental to survival. On contrast, shoot tips of walnut could withstand extended preculture duration (up 10 day) in media with lower sucrose concentrations (0.5 M sucrose) noticed that the highest survival rate with 0.5 M sucrose (90%) after 10 day.

The established cryopreservation protocol for shoot tips of apricot (*Prunus armeniaca*) and walnut (*Juglans regia*) (Tables 1 and 2) comprised a preculture after (up 10 day) in liquid or solidified media containing 0.5 M sucrose or in medium with progressively increasing sucrose concentrations (up to 0.75M) ,desiccation to 20-25 % MC followed by slow freezing. Shoot tips displayed high sensitivity to sucrose since exposure to a concentration of 0.75, 1.0 and 1.5M sucrose during preculture was tolerated only after progressive increase in sucrose concentration, and used 3% sodium alginate and 100 mM calcium chloride for formation or creating capsules includes encapsulation of shoot tip, followed by pregrowth treatment.

These results of Swan *et al*., 1998 reported that with the use of encapsulation –dehydration technique, sucrose pretreatment played a major role in the tolerance of apices to dehydration and further freezing. Meanwhile, Shatnawi *et al*., 1999 used calcium chloride and sodium alginate for formation capsules, includes encapsulation of plant material in calcium alginate beads, followed by pregrowth treatment in a medium containing high level of sucrose ranging from 0.3 M to 1.5 M for at least one day.

The data reported in Table 3 also, showed that Preculture durations longer than 2 weeks resulted in survival rates of around 80% after desiccation. After slow freezing, the survival was achieved for all experimented durations, whereas after rapid freezing, survival was not noted. The highest survival rate with slow freezing was achieved after 2 and 3 weeks of preculture.

Slow freezing achieved better results than rapid freezing, suggesting that not all-freezable water were extracted from beads /shoot tips during desiccation down to 20-23 %. Moreover, further freeze-induced dehydration during slow freezing was necessary to achieve optimal survival. Similar results were noticed with encapsulated grape apices for which slow freezing was necessary to achieve optimal survival according to Gnanaprogasam and Vasil, 1992. Who studied effective factors on encapsulation-dehydration such as slow cooling, during slow freezing, cooling to very low temperature or prolonged exposure to low temperature before transferring the plant material to LN may be injurious to cells because of excessive cellular dehydration and the formation of damagingly large crystals. Moreover, Zhao *et al*., 2006 using shoot tips that were excised from healthy *in vitro* plantlets that were subculture for 30 days, hardened at 5° C for 3-4 weeks, precultured with 0.7mol/liter sucrose for 2 days and dehydrated with PVS3 for 100 minutes before direct plunging in liquid nitrogen.

Table 3: Effect of preculture duration in medium with 0.5 M sucrose on the survival (%) of encapsulated apricot and walnut after slow freezing and rapid freezing.

Preculture duration (days)	Survival (%)	
	Slow freezing	Rapid freezing
2	0.0^{e*}	0.0^{c}
4	10^{d}	10^{a}
6	20^{d}	5.0^{ab}
8	30^{cd}	-
10	30^{cd}	-
12	40^{c}	-
14	60^{b}	-
16	80^{a}	-
18	80^{a}	-
20	80^{a}	-

* Values, within a column, of similar letters were not significantly different according to the least significant difference (LSD) at 0.05 level.

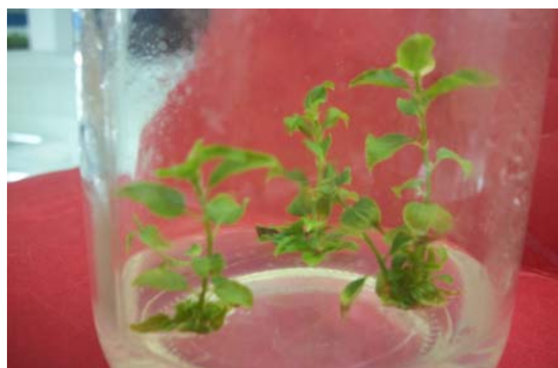

Photo 1: Effect of sucrose concentration and dehydration duration on survival and regrowth percentages of conserved encapsules.

3. Cold Acclimation and Encapsulation Dehydration:

Shoot tips responded well to increasing dehydration. Regrowth of shoot tip dehydrated, LN exposed shoot tips was best at 6 h drying time and with 0.75M sucrose Tables 4 and 5 which corresponds to 20 to 23 % moisture content. Hours of dehydration correlated well both bead moisture content and shoot tip regrowth moisture content of beads declined from 33% for dried for 4 h to 19% after 7-8 h dehydration. Optimum bead moisture content for shoot tips preserved by the encapsulation dehydration technique is normally about 20-23% (Chang *et al*., 2000 and Dereuddre *et al*., 1990). Moreover, shoot tips dried to 20-23% moisture (4-7 h dehydration), and cryopreserved also recovered better than those with 4 h dehydration (Chang *et al*., 2000). Dehydration tolerance is required for successful cryopreservation by encapsulation dehydration because the cells must have no freezable water, thus vitrifying and avoiding ice crystal formation when exposed to LN (Benson, 1999). Scanning differential Calorimetric studies showed that alginate beads dried to 20-23% moisture vitrify on exposure to LN and form stable glasses that do not form ice crystals on rewarming (Dumet *et al*., 2000). This stability is reflected in the high regrowth of viable shoot tips in beads dried to approximately 20-23% moisture. Alginate encapsulated pear shoot tips also recovered best at moisture content near 20% (Scottez *et al*., 1992). In this study, *Prunus armeniaca* and *Juglans regia* shoot tips beads with 20-23% moisture content had the best survival and regrowth.

It could be concluded that cold acclimation was essential for successful cryopreservation of apricot and walnut. All cold acclimated shoot tips had significantly more regrowth following liquid nitrogen (LN) exposure than the non-acclimated control.

After one week of cold acclimation, all accessions significantly produced more regrowth. Regrowth following 3 or 4 week of cold acclimation was significantly better than 1 week. This cold acclimation response resembles the results for many other genera tested with this technique such as Rubus, Ribes and Pyrus that exhibited low regrowth without cold acclimation and all exhibited regrowth from cryopreservation with appropriate acclimation but the optimum varied with genotype (Chang and reed, 1999 and 2000; Reed and Yu, 1995 and Reed *et al*., 1998). In cold hardiness tests with pear, shoots survived at temperatures 4 ^{o}C lower following one week of cold acclimation and 7 ^{o}C lower following two week of cold acclimation compared with non-cold acclimation shoots.

Low water content results in dehydration damage to cells, often resulting in membrane alterations. However, low moisture content is necessary for successful grass cryopreservation. Cold acclimation treatment not only increased freezing tolerance, but also increased the dehydration tolerance of apricot and walnut shoot tips. Cold acclimated shoot tips dried to 20-23 % moisture content retained high viability while, non-acclimated shoot tips died at bead moisture contents of 33 %. Shoot tips of apricot and walnut for 4 week of cold acclimation and dehydrated to 20% moisture content all had good to excellent regrowth following LN exposure.

Table 4: Effect of sucrose concentration and dehydration duration on survival and regrowth percentages of encapsulated non-cold acclimation (-CA) and cold acclimation (+CA) shoot tips of apricot (*Prunus armeniaca* L.).

Sucrose concentration(M)	Moisture content	Dehydration duration (h)	Survival (%)		Regrowth (%)	
			-CA	+CA	-CA	+CA
	73.68	0	0.0^{e*}	0.0^{d}	0.0^{d}	0.0^{e}
0.5	55.21	2	5^{d}	50^{c}	10^{b}	40^{d}
	50.72	4	10^{c}	60^{b}	25^{c}	50^{c}
	40.31	6	30^{a}	70^{a}	40^{a}	80^{a}
	33.92	8	20^{b}	50^{c}	30^{b}	60^{b}
	73.68	0	0.0^{e}	0.0^{c}	0.0^{e}	0.0^{d}
0.75	40.75	2	20^{d}	50^{b}	40^{c}	70^{c}
	32.92	4	40^{c}	50^{b}	50^{b}	90^{a}
	23.23	6	60^{a}	80^{a}	60^{a}	90^{a}
	18.95	8	50^{b}	0.0^{c}	50^{d}	80^{b}
	73.68	0	0.0^{c}	0.0^{b}	0.0^{b}	0.0^{b}
1.0	18.7	2	10^{a}	5.0^{a}	0.0^{b}	10^{a}
	15.5	4	5.0^{ab}	0.0^{b}	5.0^{a}	0.0^{b}
	12.1	6	0.0^{c}	0.0^{b}	0.0^{b}	0.0^{b}
	10.5	8	0.0^{c}	0.0^{b}	0.0^{b}	0.0^{b}

* Values, within a column, of similar letters were not significantly different according to the least significant difference (LSD) at 0.05 levels.

4. Thawing and Rregrowth:

Data in Table 6 showed the highest survival and regrowth of encapsulated shoot tips of apricot and walnut after 3 month in LN (Photos 2 and 3). On the other hand, survival and regrowth of encapsulated shoot tips decreased in line with increased the storage period in (LN)

It could conclude that the growth recovery of cryopreserved apricot and walnut shoot tips occurred directly without callus formation. It allowed to assume that most cells of the shoot tips region were only slightly or not all damaged during the cryopreservation process in liquid nitrogen.

Survival of desiccated encapsulated shoot tips increased in line with decreasing bead moisture content after cryopreservation, and decreased with 1.0 M sucrose. The highest survival was obtained after slow freezing with beads dehydrated to 23 or 28% moisture. These results agreed with Wang *et al*., 1998 who found that the viability of dehydrated cells of grape (*Vitis vinifera* L.) was 96 % at the beginning of dehydration. Moreover, it slowly decreased to 84 % as the water content fell from 67.7 to 20.6 % and then declined sharply to 42% at a water content of 16.2 % after 10 h of dehydration under Laminar -Air Flow. The highest viability (78 %) was recorded with 20.6 % water content after 6 h of dehydration. Moreover, Hirai *et al*., 1999 investigated dehydration as another factor which affected on sensitivity of the cryopreserved plant material to freezing with liquid nitrogen (-196 ºC) partial dehydration is usually achieved by using osmoticum or cryoprotectants in the medium before or during cryopreservation.

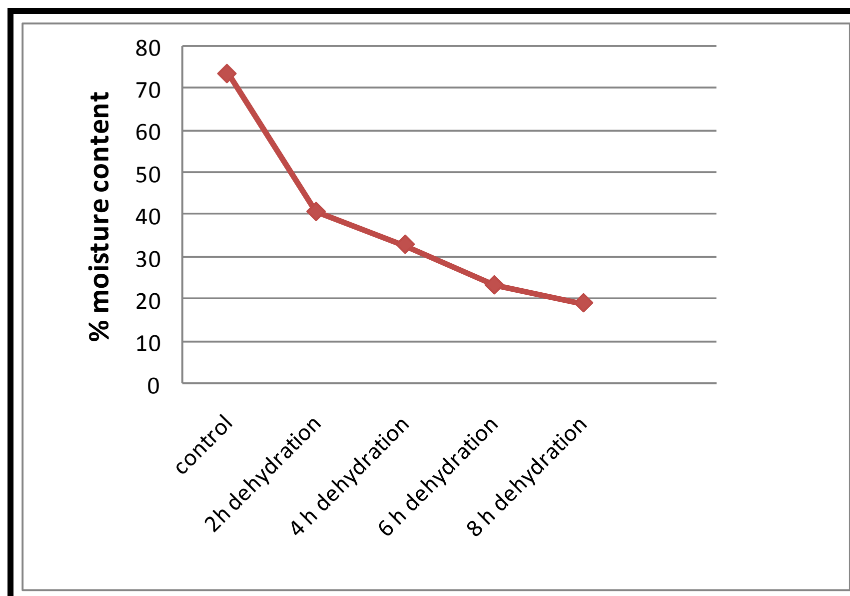

Fig. 1: Effect of dehydration duration on moisture content.

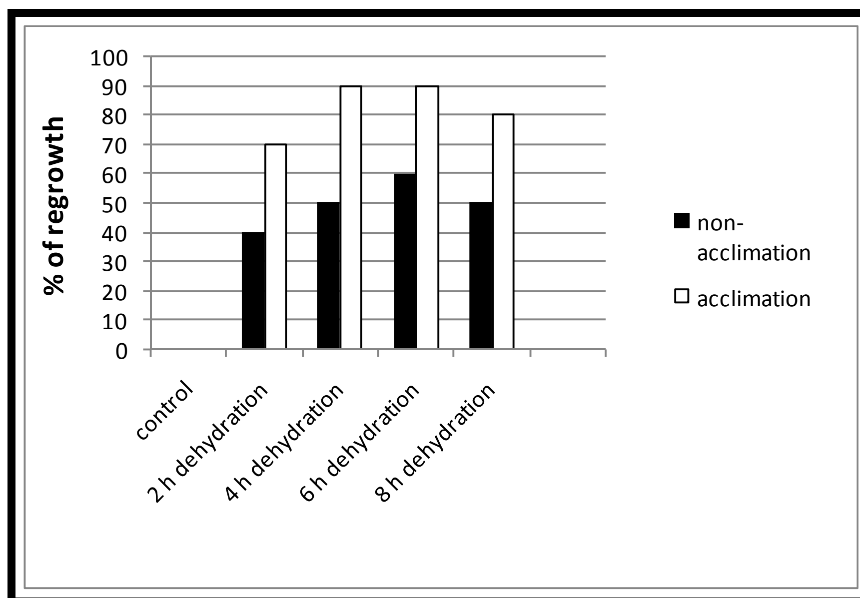

Fig. 2: Percentage of regrowth of 4- week cold acclimation and non-acclimation with 0.75 M sucrose, following encapsulation in alginate beads, air drying for 0 to 8 h and exposure in liquid nitrogen.

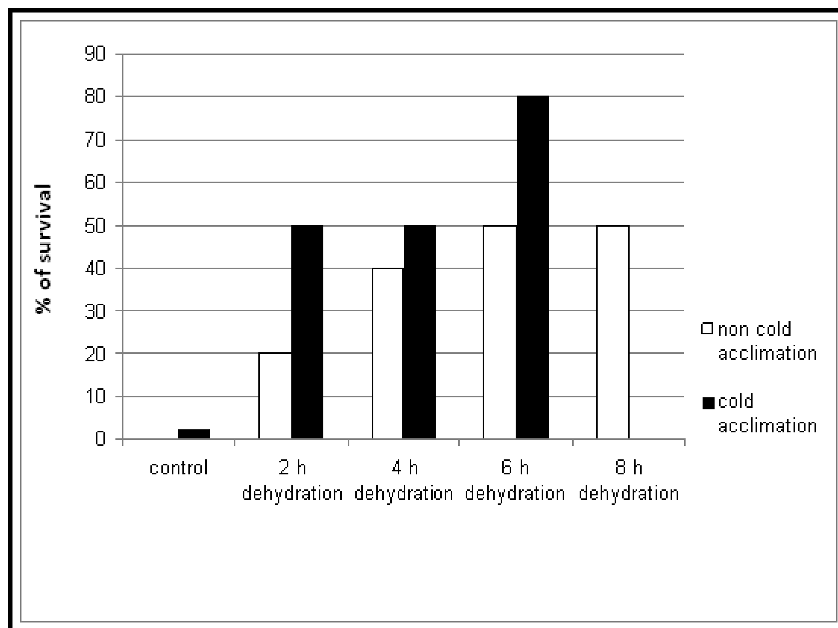

Fig. 3: Percentage of survival for apricot of 4- week cold acclimation and non-acclimation with 0.75 M sucrose, following encapsulation in alginate beads, air drying for 0 to 8 h and exposure to liquid nitrogen.

Table 5: Effect of sucrose concentration and dehydration duration on survival and regrowth percentages of encapsulated non-cold acclimation (-CA) and cold acclimation (+CA) shoot tips of walnut *(Juglans regia* L.).

Sucrose concentration (M)	Moisture content	Dehydration duration (h)	Survival (%)		Regrowth (%)	
			-CA	+CA	-CA	+CA
0.5	75.68	0	0.0[d*]	0.0[d]	0.0[d]	0.0[c]
	60.23	2	10[c]	40[b]	10[c]	40[b]
	55.11	4	20[b]	50[a]	10[c]	50[a]
	48.32	6	30[a]	50[a]	40[a]	50[a]
	40.31	8	20[b]	40[b]	20[b]	50[a]
0.75	75.68	0	0.0[d]	0.0[e]	0.0[d]	0.0[e]
	40.44	2	20[c]	40[c]	10[c]	30[c]
	33.91	4	30[b]	80[b]	20[b]	50[b]
	24.99	6	50[a]	100[a]	40[a]	80a
	19.33	8	20[c]	30[d]	10[c]	10[d]
1.0	75.68	0	0.0[c]	0.0[c]	0.0[c]	0.0[b]
	33.51	2	20[a]	10[a]	20[a]	10[a]
	19.91	4	10[b]	5.0[b]	10[b]	0.0[b]
	15.21	6	0.0[c]	0.0[c]	0.0[c]	0.0[b]
		8	0.0[c]	0.0[c]	0.0[c]	0.0[b]

* Values, within a column, of similar letters were not significantly different according to the least significant difference (LSD) at 0.05 levels.

Table 6: The effect of storage time on survival and regrowth of apricot and walnut after shoot tips cryopreservation.

Storage period	Survival		Regrowth	
	Apricot	Walnut	Apricot	Walnut
3 month	92.72[a*]	93.5[a]	91.22[a]	91.78[a]
6 month	91.53[a]	91.75[a]	89.77[a]	90.02[a]
12 month	81.75[b]	79.58[b]	79.80[b]	75.55[b]
18 month	80.35[b]	79.23[b]	78.03[b]	74.79[b]
24 month	68.37[c]	57.92[c]	65.32[c]	55.87[c]

* Values, within a column, of similar letters were not significantly different according to the least significant difference (LSD) at 0.05 levels.

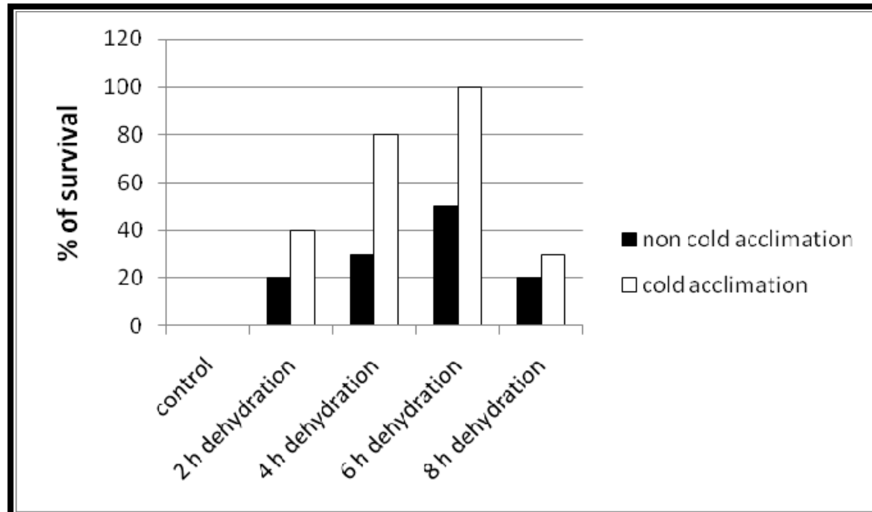

Fig. 4: Percentage of survival for *Juglans regia* of 4- week cold acclimation and non-acclimation with 0.75 M sucrose, following encapsulation in alginate beads, air drying for 0 to 8 h and exposure to liquid nitrogen.

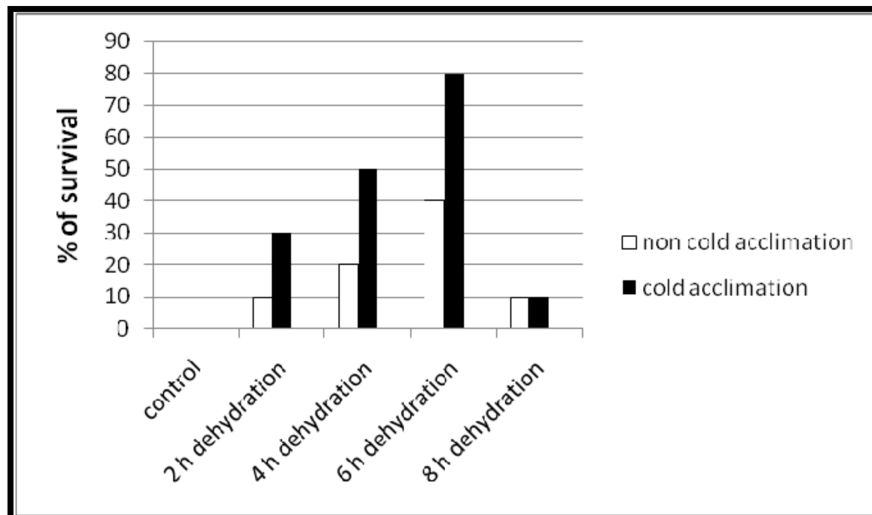

Fig. 5: Percentage of regrowth for *Juglans regia* of 4- week cold acclimation and non-acclimation with 0.75 M sucrose, following encapsulation in alginate beads, air drying for 0 to 8 h and exposure to liquid nitrogen.

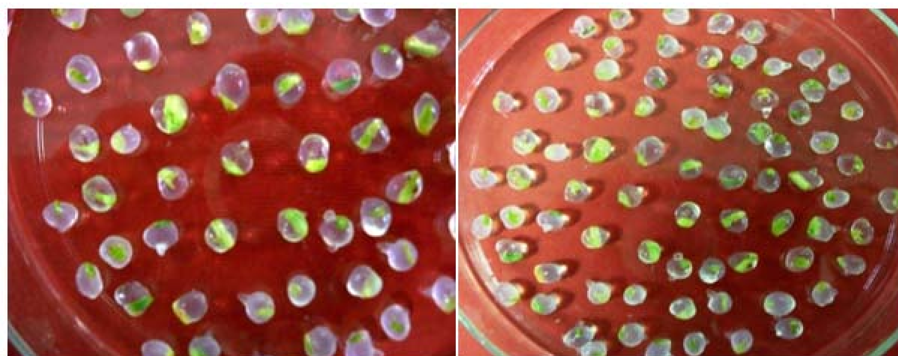

Photo 2: Shoot tips cryopreservation of apricot (*Prunus armeniaca*).

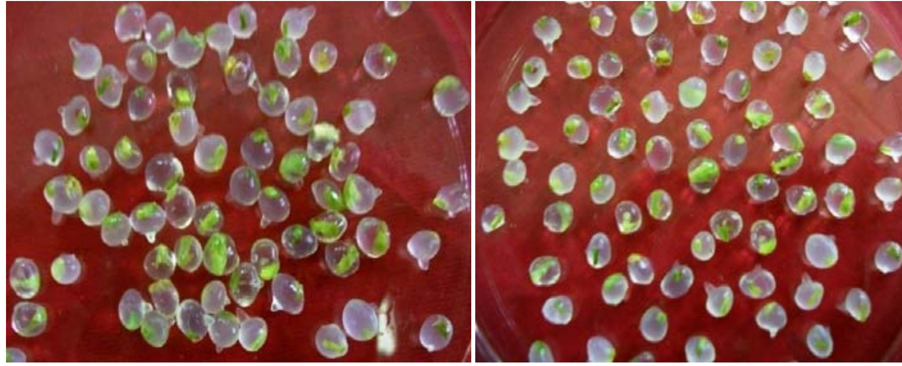

Photo 3: Shoot tips cryopreservation of walnut (*Juglans regia*).

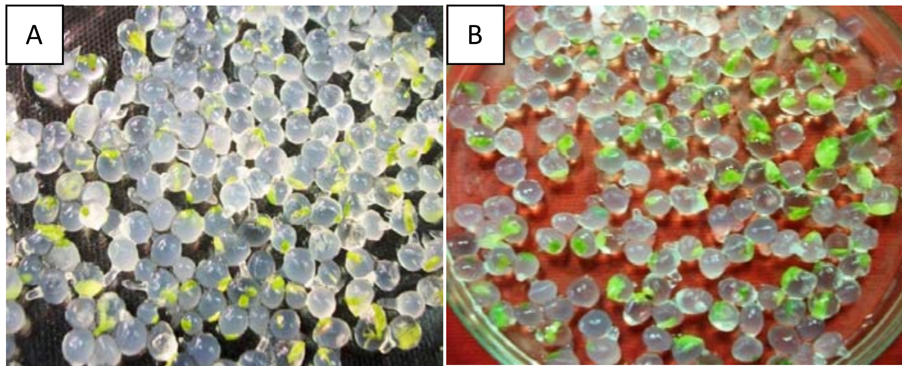

Photo 4: Shoot tips encapsulation after cryopreserved in liquid nitrogen (LN), (A) apricot and (B) walnut.

Photo 5: Regrowth shoot tips beads of apricot.

Photo 6: Regrowth shoots tips of walnut.

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