Cryopreservation Of Sour Orange (Citrus Aurantium) Shoot Tips By Encapsulation-Dehydration

Karaputugala P.N, Edirisinghe E.S.C, Fonseka D.L.C, Samarasinghe W.L.G

Abstract: Ex-situ conservation of the Citrus has been considered as problematic and early reports have found that Citrus was a sub orthodox species and cannot be stored at low temperature. Field genebanks are difficult to maintain due to pest, disease and climate hazards. In-vitro culture methods also provide only short-term storage but this is difficult and time-consuming. For a long-term storage of plant germplasm, cryopreservation is currently the best option. Therefore a cryopreservation protocol needed to be developed to conserve Citrus germplasm for long-term. 2, 3, 5-Triphenyl tetrazolium (TTC) test was used for an assessment of cell survival after cryopreservation at 490nm. The shoot tips were encapsulated and osmoprotectant on a shaker at 100 rpm with two sucrose concentrations (0.5M & 0.75M). Subsequently, encapsulated beads were dehydrated under laminar air flow and silica gel for 6 hours. The encapsulated beads were plunged directly into liquid nitrogen for a minimum of 48 hours. Encapsulated beads were thawed at 40^0C for 2 minutes and rehydrated using liquid MS for 10 minutes. The beads were transferred to re-culture media optimized in experiment 2. Then cultures were kept in dark for 2 days and 1 day in semi light condition to avoid photo-oxidative stress. High viability could be seen when used mature shoots & encapsulated using 4% sodium alginate. The best condition for the encapsulation-dehydration of Citrus aurantium was obtained when beads were pretreated with the osmoprotection medium for 20 hours and dehydrated for 6 hours. Beads were pretreated with 0.75M sucrose and dehydrated under laminar air flow recorded the maximum survival (21.6%). MS medium supplemented with 2mg/l BAP was used as a re-culture medium with 56.5% survival. Finally, beads were pretreated with 0.75M sucrose and dehydrated under laminar air flow method was appropriate to cryopreservation of Citrus aurantium within the tested range.

Index Terms: Beads**,** Citrus, Cryopreservation, Encapsulation-dehydration, Liquid nitrogen, Re-culture, 2, 3, 5-triphenyl tetrazolium

1 INTRODUCTION

Conservation of genetic diversity in existing species is essential in term of keeping up biodiversity and sustainable future crop production. For that, there are two ways to deal with preserve plant genetic resources. They are ex-situ conservation and in-situ conservation. In-situ approaches incorporate on-farm techniques, home garden conservation and genetic reserves while ex-situ conservation includes seed preservation, in-vitro conservation, DNA conservation, field genebanks and botanical gardens [1]. Cryopreservation is the part of in-vitro conservation method and it has been introduced as a new technique for long-term preservation of plant genetic resources at an ultra-low temperature (-196^{0}C) in liquid nitrogen. At this temperature all the biological and physical reactions are practically haltered. So that planting material can be preserved for unlimited time of period with low cost and little space [2].

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Sour Orange (Citrus aurantium L) is mainly preferred as a rootstock over commercially utilized seedling rootstocks. As it shows resistance to several viral diseases and a change of fruit quality of grafted species. Sour orange is in danger of extinction and the use of other rootstocks will result in a decline in performance of sour orange rootstock with time due to unfavourable environmental conditions, especially dry spell and salinity [3].Therefore, their preservation is essential for future use and improvement programs. Citrus genetic resources are presently preserved as entire plants in field genebanks or screen houses where they continue exposed to pest and diseases and other natural risks. For example dry spell, climate and human activities. Field genebanks are expensive to keep up and it limits the level of repetition of accessions and the superiority of maintenance. Indeed, even under the best situation field genebanks require impressive contributions to the type of land, labour, supervision and materials [4]. Seeds of numerous Citrus species show recalcitrant or intermediate storage behaviour and therefore cannot be stored at low temperature in seed genebanks. Sometimes seeds are heterozygous and certain gene arrangement cannot be preserved. A few cultivars are seedless and are hence proliferated vegetatively. The only current option for long-term preservation of problematic crops is cryopreservation. Cryopreservation procedure has been produced for more than 100 species. To date, cryopreservation of Citrus germplasm has been effectively connected to seeds [5], embryos [6], ovules [7], embryonic axes [8] and embryonic cultures [9].However, evidence on the use of cryopreservation to shoot tips is still extremely constrained [4]. 2,3,5-triphenyltetrazolium chloride (TTC) test and vital staining with fluorescein diacetate (FDA) [10] are methods frequently used to determine cell viability [11], showing their response to stress factors such as cold, salinity and heat [12]. While FDA staining can be used to evaluate the viability of protoplasts and small callus fragments or suspension aggregates of no more than 30-100 cells, the

TTC test is suitable for large cell aggregates [11, 12,13]. The TTC test, in view of the enzymatic activity of living plant cells, demonstrates the level of respiration in tried examples [11]. Active dehydrogenases in mitochondria diminish colorless TTC to red triphenylformazan [13, 14,15]. Living parts of tissue or single cells recolor red. This test was at first used to assess seed viability and freezing made harm plants [14]. Plant Genetic Resources Centre (PGRC) introduced the plant cryopreservation technique to Sri Lanka recently. Therefore this study is to initiate work on development of a cryopreservation protocol for sour orange. The published methods for Citrus shoot tips taken from cultivars in tissue culture conditions. Establishment of in-vitro plantlets for woody cultivars are difficult. This research project aims to cryopreservation of Citrus aurantium using shoot tips excised directly from field genebanks as explant for the cryopreservation project.

2 METHODOLOGY

This research was conducted at the in-vitro conservation laboratory, Plant Genetic Resources Centre, Gannoruwa, Sri Lanka.

2.1 Planting material and explant preparation

Shoot tips of Sour orange (Citrus aurantium) were collected from healthy mature, pruned tree in field genebank at Plant Genetic Resources Centre, Gannoruwa, Peradeniya. Mature shoot tips of actively growing explants of sour orange were taken. Leaves removed and 2-3cm section of the stem with a bud were excised. These explants were soaked in teepol for 30 minutes. After that explants were washed in running tap water for about an hour and taken in the laminar flow (Hitachi-clean bench). They were then washed thrice in sterilized distilled water and kept in fungicide solution (Topsin 1g/100ml) for half an hour in rotary shaker (180rpm). Then they were washed thrice in sterilized distilled water and further sterilized with 10% v/v NaOCl (Chlorox) with 2-3 drops of Tween 20 by shaking for 20 minutes. Then the explants were washed 3 times in sterilized distilled water. The explants were cut in to 3-5mm pieces with auxiliary bud and also all browned parts of the stem removed.

Experiment 1

2.2 Encapsulation of Sour orange shoot tips

Excised shoot tips were then suspended in calcium free liquid MS medium added with sodium alginate solution and 0.4M sucrose. Using a micro pipette individual shoot tips were captured with some sodium alginate solution and distributed as drops into a calcium chloride solution (100mM). Then allowed to harden for 30 minutes to form beads approximately of 4-6 mm in diameter.

2.3 Osmotic dehydration

To study the effect of two concentrations of sucrose dehydration solutions, 0.5M and 0.75M sucrose solutions were prepared. The encapsulated shoot tips were taken out aseptically one by one softly using sterilized forceps and were placed into a conical flask which contained sucrose solution with different concentrations ranging from 0.5 and 0.75 M. Then conical flask was kept in a rotary shaker (100rpm) culture room (25±2°C, 16 hour photoperiod under cool white florescent lamps)

2.4 Air desiccation

To study the effect of two desiccation methods, laminar flow dehydration and silica gel methods were used. The beads were allowed to blot on sterile filter paper and desiccated for 6 hours in a glass petri dishes under laminar flow at ambient temperature of 25^0C. To study the effect of silica gel method, encapsulated shoot tips were placed on sterile filter paper and dehydrated in para-film sealed petri dishes containing 17g of silica gel for 6 hours

2.5 Freezing, thawing and growth recovery

Dehydrated encapsulated shoot tips were placed in 1.2 ml sterilized cryovials (15 beads per each) and directly plunge in to liquid nitrogen for minimum two days. Cryovials were removed from LN and warmed in a water bath (Jelotech-BS11) at 40^0C for 10 minutes. The beads from the cryovial were taken out and rehydrated using liquid MS for 10 minutes. Then beads were transferred aseptically into Petri dishes containing growth recovery medium and were put in dark in culture room condition for a 2 days followed by semi-light for another 1 day and transfer in to growth room For the control (non-cryopreserved) samples, the sucrose dehydration, air dehydration, cryostorage and thawing steps were omitted and they were transferred to hormone free MS medium. The beads were stored in culture room at 25±2°C with 16 hours photoperiod under cool white fluorescent lamp

2.6 Determination of viability 2, 3, 5-triphenyltetrazolium chloride (TTC assay)

The viability of alive cells in both cryopreserved and non-cryopreserved of sour orange was determined through spectrophotometrical analysis which is constructed on the metabolic activity of alive cells. Colorless triphenyltetrazolium chloride was reduced by dehydrogenases in alive plant cells to yield triphenylformazon, a reduced TTC which colored red. The procedure in this study was adapted from Verleysen et al. (2004) [16]. Shoots were taken out from each bead and washed three times with 5 mL distilled water. After removal the distilled water, shoots were soaked in 5 mL TTC solution (1g/100ml of distilled water) overnight in dark condition. After 18 hours, TTC solution was discarded and shoots were rinsed with 7 mL distilled water thrice. Then shoots were re-suspended in a universal bottle containing 7 mL 95% ethanol and triphenyl formazon was extracted from shoots by boiling in 80ºC water bath for an hour. The formazan extracts were cooled to room temperature, topped up to 7 ml with 95% ethanol. Analysis was then carried out using spectrophotometer (Jenway-6305) where absorbance was taken at 490 nm (Mubbarakh et al., 2014). The formazon content was expressed as a percentage of the control calculated as (absorption of thawed tissue/ absorption of control) × 100%. Formazon content thus calculated will be termed viability [17].

2.7 Determination of Moisture content

Moisture content was measured using infra-red moisture determination balance The experiment was carried out

under completely randomized design and consists of 5 replicates. Each replicate consist of 15 beads.

Experiment 2

2.8 Develop a re-culture medium for direct shoot regeneration of sour orange (Citrus aurantium)

To found a regeneration medium for cryopreserved beads, regeneration medium for direct shoots were optimized. The treatment consist of MS medium supplemented with three level of BAP including 1, 2, 3 mg/l respectively. Each treatment consist of 3 replicates and each replicate consist of 30 shoots. The experiment was carried out using completely randomize design. Control was carried out using hormone free MS medium**.**

3 RESULTS & DISCUSSION

Experiment 1

3.1 Influence of maturity of explants on the viability

Effect on explant type on the viability of cryopreserved shoots were investigated using spectrophotometric-TTC assay. According to the **Figure 1**, higher survival% could be seen in cryopreserved mature shoots than immature or semi-mature. To acquire a higher viability percentage mature shoots were used in all further experiments. Immature tissues derived from in-vitro germinated seeds are the regular explants used for in-vitro propagation. These in-vitro propagated shoots were used as planting material for the cryopreservation. But this research project aims to use shoot tips excised directly from the field genebank as explant. For the in-vitro propagated cultures adult material are the greatest tissues for the cryopreservation because redeveloped plants will not have juvenile characteristics and will be readily accessible for the breeding [18]. Lately, the successful cryopreservation of shoot tips from immature plants of Poncirus trifoliate (Trifoliate orange) using encapsulation-dehydration method has been reported. However, this could not be practicable to other species because the following freezing and thawing only a portion of the shoot tip may live. But the use of mature shoots have been limited due to their lower regeneration [18].

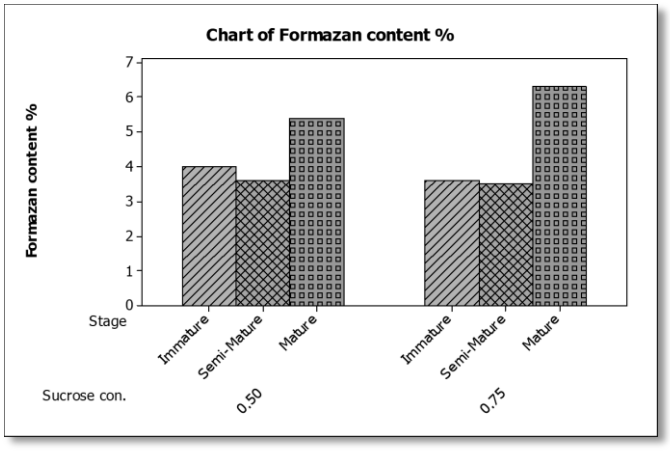

Figure 1: *Influence of maturity of explant on the viability*

3.2 Determine the suitable sodium alginate matrix concentration.

Alginate matrix containing 4% sodium alginate has seemed suitable for the creation of firm isometric alginate beads than alginate matrix containing 3% sodium alginate.

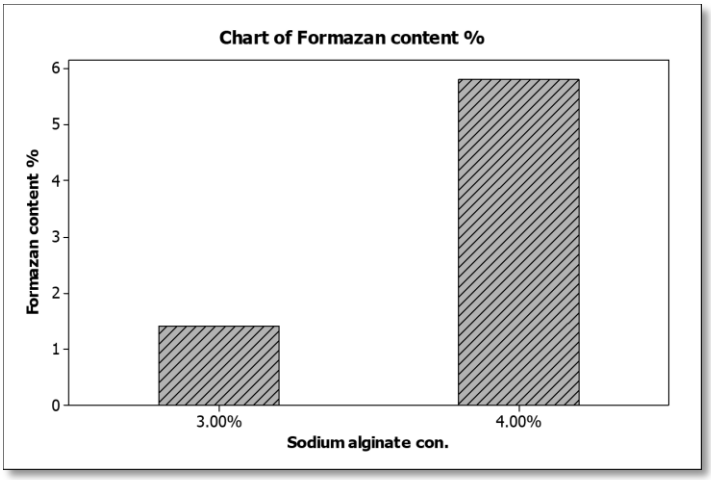

Figure 2*: Survival of different sodium alginate encapsulated shoots*

The significant aspect of the successful production of synthetic seeds and capsule quality is to use the precise composition of sodium alginate and calcium chloride. The diameter and shape of the alginate beads were depended on that factors [19]. This results correspond to the findings by Kavyashree et al, 2006, [20] Sundararaj et al, 2010 [21] and Gholami et al, 2013 [19] who reported that 4% sodium alginate and 100mM calcium chloride was the optimum concentration for bead creation in Gypsophila paniculata,, Morus alba, Zingiber officinale and Cirtus limon respectively. Gholami et al, 2013 [19] found that 3% of sodium alginate concentration was inappropriate because the alginate beads were irregular, transparent and could not be easily manipulated. Because of the irregular formation of beads shoot tips were damaged during cryopreservation. Moreover Gholami et al, 2013 [19] reported that higher concentration of sodium alginate (5%) also not appropriate because it made hard beads and it impossible for the shoot emerges. Therefore alginate beads encapsulated in 4% sodium alginate and 100mM calcium chloride were used for encapsulating shoot tips in all further experiments.

3.3 Determine suitable duration for pretreated with sucrose

There was no significant interaction effect of sucrose concentration and pretreated days on survival and moisture content of cryopreserved shoot tips. The lowest moisture content was exhibited by shoot tips that were pretreated with 0.75M sucrose for 20 hours. Meanwhile, the higher viability was exhibited by shoot tips that were pretreated for 20 hours. Increase sucrose concentration leads to a reduced moisture content of the encapsulated beads but increase the time not leads to reduce the moisture content. There was no significant difference of sucrose concentration (0.5M & 0.75M) for viability. However, there was no correlation between moisture content and survival.

***Table 1**: Determine suitable duration for pretreated with sucrose*

Sucrose concentration (mol/dm^3)	Pre - treated hours	Moisture content %	Survival % After cryopreservation
0.5	20	79.4 ± 0.46	8.1 ± 0.41
	40	83.2 ± 2.81	4.4 ± 0.16
0.75	20	70.8 ± 2.08	7.2 ± 0.55
	40	76.6 ± 1.77	2.75 ± 0.37

Data were expressed as the mean ± standard error

This protocol is based on osmotic regulation of cellular water. Excised shoot tips were encapsulated in calcium alginate mixture dissolved in MS medium with added sucrose. The alginate beads protects biological materials against osmotic shock which may occur when directly exposed to high sucrose concentration [22]. Also the beads help achieve partial desiccation of the cell through osmosis due to the initial higher solute concentration of the beads compared to those of the cytosol [23]. Reduced moisture content is reported to be essential for cryopreservation).Encapsulation-dehydration technique used osmotic dehydration and air dehydration to reduce moisture content. Normally sucrose is loaded in the beads for 18-24 hours during pre-culture in sucrose solution. But Al-ababneh & Karam, 2002 [3] found that 83% survival and 47% regrowth were exhibited by shoot tips were pre-cultured with 0.5M for 40 hours. Meanwhile, 47% survival and 23% regrowth were exhibited by shoot tips were pre-cultured with 0.75M sucrose for 40 hours.

3.4 Determine the duration for air desiccation / Silica gel dehydration

There was no significant interaction effect of sucrose concentration (0.5M & 0.75M), dehydration method (Laminar floor / Silica gel) and dehydration duration (4 hour and 6 hour) on survival and moisture content of cryopreserved shoot tips. Lowest moisture content was exhibited by shoot tips that were dehydrated by laminar floor than silica gel. After 6 hour of dehydration in the current study, moisture content was declined than 4 hours. The survival was increased with the laminar floor method for the 6 hour period. There was no difference between sucrose concentration (0.5M/0.75M) for survival and moisture content. However, there was a negative correlation between survival and moisture content. It indicate that when increased the moisture content survival percentage was reduced

***Table 2:** Determine the duration for laminar floor / silica gel dehydration*

Sucrose Con. Mol/dm3	Dehydration method	Dehydration time (Hours)	Moisture content %	Survival % After cryopreservation
0.5	Laminar	4	41.1 ± 4.1	4.6 ± 0.1
	Laminar	6	29.6 ± 3.6	5.9 ± 0.5
0.5	Silica	4	58.6 ± 0.6	4.4 ± 0.2
	Silica	6	44.2 ± 3.3	4.9 ± 0.2
0.75	Laminar	4	39.3 ± 1.3	4.7 ± 0.1
	Laminar	6	29.1 ± 1.6	5.4 ± 0.3
0.75	Silica	4	49.4 ± 1.1	4.0 ±0.0
	Silica	6	43.4 ± 1.5	5.4 ±0.2

Data were expressed as the mean ± standard error

Dehydration of encapsulated tissue significantly reduce cell's water but not to the level of reaching willing point, thus making tissues immersed in LN to survive (Al-ababneh et al, 2002) [3]. Dehydration step was essential to avoid the development of intracellular ice crystals even though increasing dehydration time led to damage of encapsulated shoot tips. The low values of regrowth may result from severe dehydration and occur from uneven distribution of water in tissue. Thus uneven spreading of water result in diverse freezing responses among cells in the same tissue [24].

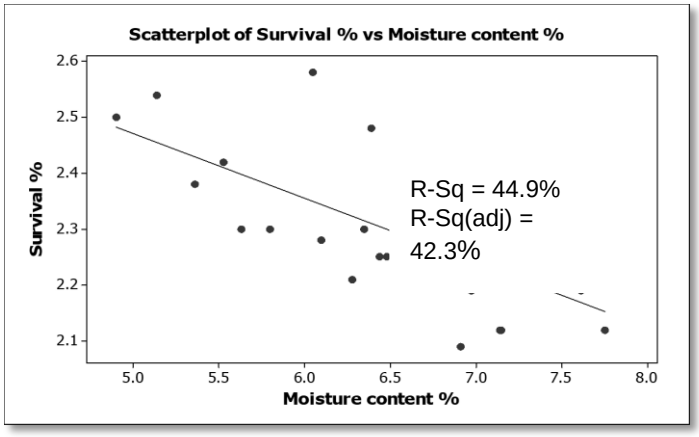

***Figure 3**: Relationship between moisture content and survival*

3.5 Regeneration of cryopreserved beads

Alginate beads pretreated with 0.75M sucrose and dehydrated under silica gel (0.75M / silica gel), pretreated with 0.5M sucrose and dehydrated under laminar air floor (0.5M / Laminar) and pretreated with 0.5M sucrose and dehydrated under silica gel (0.5M / silica) were not significantly differ over a alginate beads pretreated with 0.75M sucrose and dehydrated under laminar air floor cabinet. It showed maximum survival (21.6%) after cryopreservation. Cryopreserved shoots were transferred to bead re-culture medium for the regrowth. The culture media for the cryopreserved material is usually same as unfrozen material. In this study, direct shoot regeneration medium (2BAP mg/l + MS) optimized in experiment 2 was used as a re-culture medium. An effective cryopreservation procedure produces visible signs of regrowth within few days of re-culture. In the case of three year old cryopreserved Arabidopsis suspension cells encapsulated in alginate matrix, regrowth was observed in less than 7 days following re-culture [25].Greening is typically the first observable sign of regrowth with shoot cultures is the best indicator of a successful cryopreservation technique [26]. Plant physiological status, type of plant material and the cryopreservation protocol determines the recovery after cryopreservation. In this study, alginate beads pretreated with 0.75M sucrose and dehydrated under laminar air floor was showed greening after 7 days of re-culture. It was a positive sign of regrowth but couldn't be observed the regeneration. This may be happening because of several reasons. To gain a higher viability mature shoots were taken as planting material for this study. Mature shoots have low regeneration capacity than juvenile phase tissues. Meanwhile, small shoot tips of Citrus do not regenerate in-vitro (with the exception of some species such as p. trifoliata) and it is essential to regenerate plants by shoot tip grafting, which cannot be done with only a portion of the shoot tip [18]. Moreover, initiation of woody species has been difficult with many plant species. Browning after re-culture occurred due to cryoinjury.0.75M / silica, 0.5M / silica and 0.5M /lamina resulted in browning after one day of re-culture. Cryoinjury results from excessive intracellular dehydration or ice crystal formation [27]. Tissue browning is one of the early detectable sign and occurs as a result of low temperature induced damage to plant tissues. This is commonly seen with woody plant species. Tissue browning caused by stress or cold, leads to loss of cellular compartmentation, leaking phenolics and formation of insoluble brown complexes upon oxidation [28].In some preservation centers 20% recovery is considered sufficient for cryopreservation experiments. Other authors consider that survival should be higher than 40% [29].

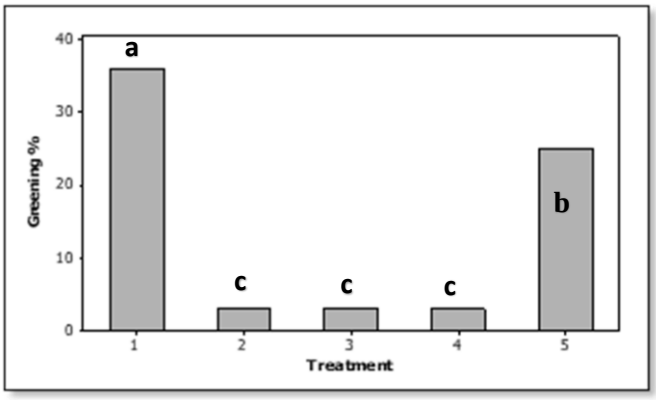

Figure 4: Survival percentage of cryopreserved beads one week after post-culture

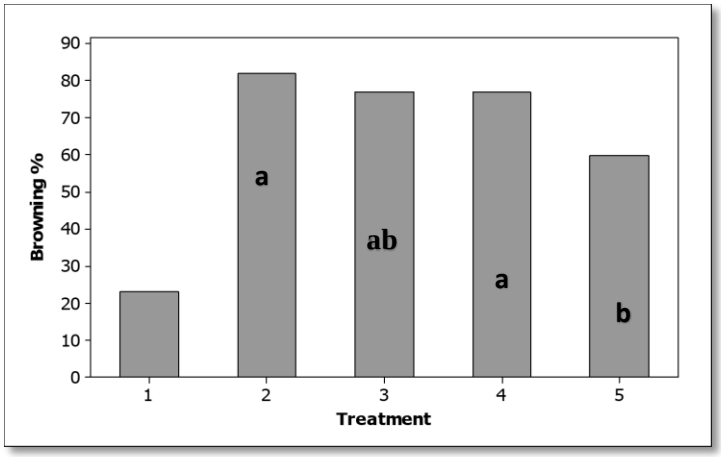

Figure 5: *Browning percentage of cryopreserved beads*

*Same letters indicate that no significant difference at 0.05 probability level. Means were separated using least significant difference test (LSD)

Trt1- Control **Trt2**- 0.5M / Laminar **Trt3**- 0.5M / Silica **Trt4**- 0.75M/ Silica **Trt5**- 0.75M/ Laminar

3.6 Change of moisture content and survival during encapsulation-dehydration

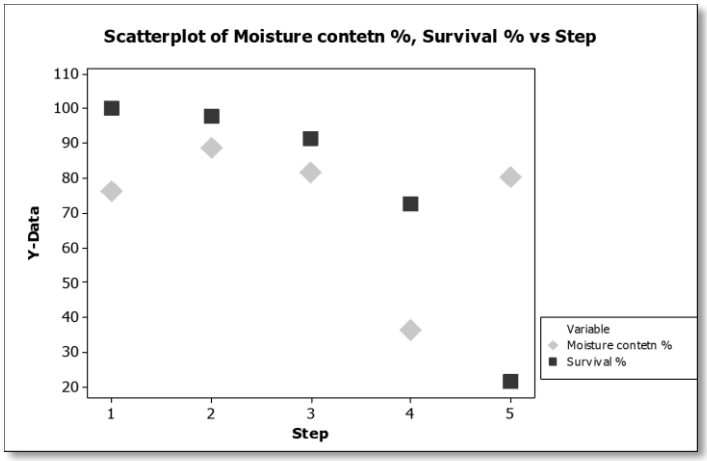

Figure 6*: Change of moisture content and survival (0.75M / Laminar floor method)*

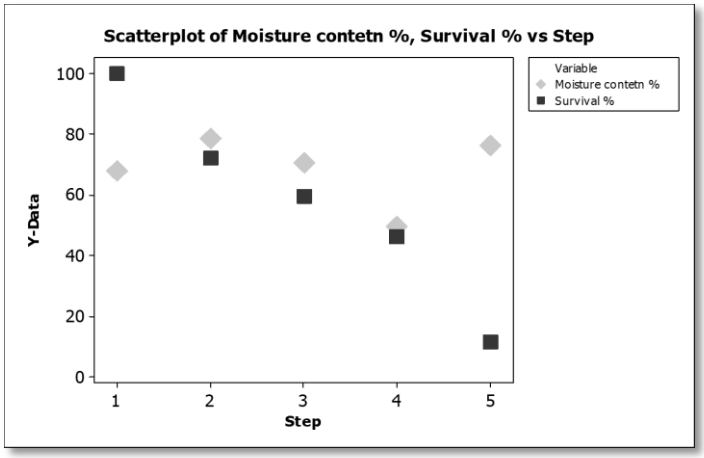

***Figure 7**: Change of moisture content and survival (0.5M / Laminar floor method)*

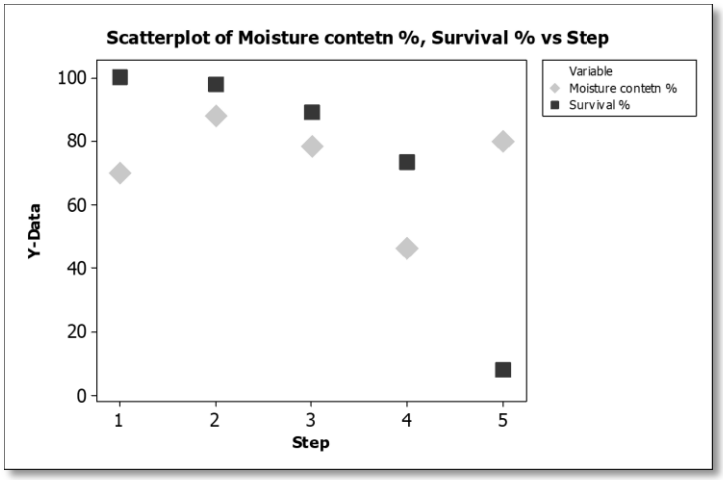

***Figure 8:** Change of moisture content and survival (0.5M / Silica gel method)*

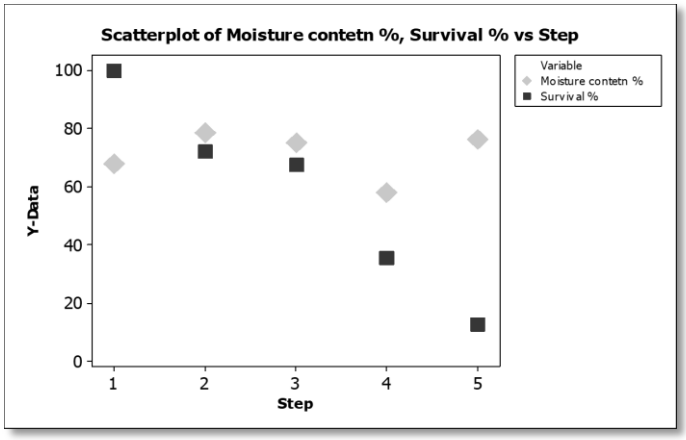

***Figure 9**: Change of moisture content and Survival (0.75M / Silica gel method*

***Step 1**- Initial **Step 2**- Encapsulation **Step 3**- Osmotic dehydration **Step 4**- Dehydration **Step 5-** After cryopreservation These graphs showed that variation of moisture content and survival according to the treatments during encapsulation-dehydration procedure. It recorded that higher viability 21.6 % after cryopreservation and 36.2% moisture content during desiccation. The cellular water content optimal for survival following encapsulation-dehydration and liquid nitrogen exposure 20% for most shoot tips, but depending on the dehydration tolerance of individual genotypes, some alterations may be needed. A 30% water content yielded regrowth in the range of 70-90% for encapsulated apple cultivar [30]. A 20% moisture content was best for shoot tips of Vistis vinifera [31]. 20% water content resulted in 60-100% recovery of Rubus species [32]. A range of 15.6-17.6% water content was best for cryopreservation of some grape cultivars [33]. While 10% water content yielded 78-90% survival Gentian auxiliary buds [34]. Although these moisture content was recorded at in-vitro propagated and cryopreserved cultures. For the direct shoots 29% moisture content also did not record the significant viability than the 21.6%.

Experiment 2

3.7 Development of direct shoot regeneration medium

Shoot induction was occurred in all media with all levels of BAP (1-3 mg/l). The results showed that best treatment for shoot induction of Citrus aurantium was 2mg/l BAP, as the average number of shoot induction response was 56.5%. The lowest shoot regeneration response (16.5%) was observed on MS medium supplemented with BAP 1mg/l. MS supplemented with 2mg/l BAP showed a significant difference over MS supplemented with 1mg/l BAP and 3mg/l BAP.

***Table 3:** Survival of Citrus aurantium shoots*

Treatment	Medium	Shoot Induction %
Control	MS	22 ± 2.0^{b}
1	MS + 1 BAP mg/l	16.5 ± 2.9^{b}
2	MS + 2 BAP mg/l	56.5 ± 2.9^{a}
3	MS + 3 BAP mg/l	22 ± 2.0^{b}

Data were expressed as the mean $\pm$ standard error

*Same letters indicate that no significant difference at 0.05 probability level. Means were separated using least significant difference test (LSD)**.** Initiation of tissue culture is one of the most problematic stage in the plants in-vitro cultivation especially for perennial woody species including citrus [35]. During the development of cryopreservation protocols the composition of the growth recovery medium is of vital importance in order to obtain fully viable structures without callus formation. Senanayaka & Edirisinghe, 2004 [36] showed that the treatment 2mg/l BAP, significantly increased the number of in-vitro shoots formed in Sweet orange var.Bibile. Moreover, it clearly indicate that BAP alone is sufficient to increase the rate of multiplication of shoots.

4 CONCLUSION

This study has shown that encapsulated mature shoots using 4% sodium alginate achieved the highest viability after cryopreservation based on spectrophotometric TTC assay. Cell dehydration is an essential step in cryopreservation.20 hours pretreated with an osmoprotection solution and 6 hours air desiccation period recorded the higher viability after cryopreservation. Maximum survival percentage (21.6%) was recoded the alginate beads were pretreated with 0.75M sucrose and dehydrate under laminar air flow. MS supplemented with 2mg/l BAP was used as a re-culture medium and it has a significant shoot induction capacity. However, additional studies required to increase the survival and recovery rate. Moreover, this study was performed with direct shoots only. This results should be tested using in-vitro generated ex-plants.

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