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Cryopreservation of in vitro-grown shoot tips of papaya (*Carica papaya* L.) by vitrification

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Abstract. Shoot tips from 4- to 6-week-old in vitro grown plantlets of six different cultivars of papaya were cryopreserved by vitrification. Shoot tips were treated in a 2 ml cryo-tube filled with a solution of 2 M glycerol and 0.4 M sucrose at 25°C for 20 min, then dehydrated with 1 ml pre-cooled vitrification solution at 4°C for 60 min. Treated samples were kept in liquid nitrogen for at least one week. The recovery percentages of six cultivars' shoot tips after vitrification were between 48% and 93%. Cryopreserved shoot tips after being warmed resumed growth within 7 days and developed shoots directly without intermediate callus formation. Prolonged storage in the liquid nitrogen up to 2 years had no affect on regeneration. This study has successfully developed a simple and effective protocol for the cryopreservation of papaya shoot tips.

Keywords: *Carica papaya*; Cryopreservation; Papaya; Vitrification.

Abbreviations: **BA,** 6-Benzylaminopurine; **DMSO,** Dimethylsufoxide; **IBA,** Indole-3-butyric acid; **LS,** Loading solution.

Introduction

Promoting sustainable utilization of biological resources is critical to the development of modern biotechnology. Because plant germplasm is indispensable for breeding new cultivars that enable the maintenance of genetic diversity and ecological environment and the avoidance of genetic erosion (Lambardi et al., 2000), germplasm conservation plays an important role in the sustainable utilization of biological resources.

Traditional methods of preserving plant germplasm are seed storage and maintaining plants in the field (Thinh et al., 1999). Those ways apply to most cultivars but have many disadvantages. Field collections require a large space and considerable labor, and the quantity of the species that can be preserved is limited (Hirai and Sakai, 1999; Lambardi et al., 2000; Pennycooke and Towill, 2000; Matsumoto et al., 2001). Field collections are also subject to environmental stress, climate change, and various plant diseases (Hirai and Sakai, 1999; Pennycooke and Towill, 2000; Matsumoto et al., 2001).

In vitro culture techniques supplement plant conservation and have been applied to germplasm collection, preservation, and rapid clonal multiplication. Due to factors such as the methods and duration of storage and the characteristics of the species, in vitro culture systems provide only short-medium term storage. Short-medium term storage achieves conservation by delaying growth and increasing the time interval between culture generations, but this is laborious and time consuming (Engelmann, 1991; Escobar et al., 1997; Charoensub et al., 1999). In addition, genotypes could possibly be lost due to contamination or human error during the process (Engelmann, 1991; Thinh et al., 1999; Vandenbussche et al., 2000). Therefore, in vitro culture cannot be efficiently applied to long-term plant germplasm conservation.

For long-term conservation of plant germplasm, cryopreservation is currently the only option (Engelmann, 1991; Takagi et al., 1997; Thinh et al., 1999; Tsukazaki et al., 2000; Sakai, 2000; Matsumoto et al., 2001; Helliot et al., 2002). Storing plant seed, organs, tissues and cells in liquid nitrogen (-196°C) halts metabolic activities yet maintains their viability. In theory, this method enables plant materials to be stored indefinitely without physiological alteration or generation of somaclonal variation, and it requires minimal storage space and maintenance (Charoensub et al., 1999; Hirai and Sakai, 1999; Thinh et al., 1999; Sakai, 2000; Matsumoto et al., 2001; Touchell et al., 2002).

Traditional cryopreservation often uses a slow cooling to avoid intracellular ice formation, a common cause of lethal cell damage (Thinh et al., 1999; Lambardi et al., 2000; Touchell et al., 2002). However, equipment is costly, and the method is not effective for low temperature sensitive species (Pennycooke and Towill, 2000). Recently, approaches to plant germplasm cryopreservation involving direct plunging into liquid nitrogen have been explored,

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and vitrification procedures proved to be the most promising among them (Matsumoto et al., 1994; Takagi et al., 1997; Hirai and Sakai, 1999; Lambardi et al., 2000; Tsukazaki et al., 2000).

In practice, plant materials are gradually dehydrated by application of a vitrification solution, with an aim to reduce water content to levels where ice crystal formation does not occur or is non-lethal during immersion in liquid nitrogen. The vitrification can let the cell solidify into a non-crystalline, glassy-state (Fahy et al., 1984; Hirai and Sakai, 1999; Lambardi et al., 2000; Sakai, 2000; Touchell et al., 2002). The vitrification procedure is easy to perform and often has a high recovery percentage, which makes it widely applicable, particularly to the conservation of plant species sensitive to low temperature (Takagi et al., 1997; Thinh et al., 1999).

Carica papaya is a plant native to tropical America and a member of the *Caricaceae*. Owing to its fast growth, easy cultivation, long fruiting season, high yield, and high nutrition content, papaya has become an economically important fruit crop, widely cultivated in tropical and subtropical regions around the world. Papaya, as a typical cross-pollinated and seed-propagated plant, appears genetically unstable in subsequent generations when seed is the only means of propagation. It is also susceptible to viral infection and environmental stress under conventional field maintenance, which result in loss of germplasm and worsened genetic erosion. In addition, seeds cannot be used to preserve the germplasm of transgenic lines. Therefore, in order to maintain biological resources and sustain genetic diversity, a cryopreservation method for vegetatively propagated lines is needed.

Materials and Methods

Plant Material

Shoots were obtained from six cultivars (Tai-nung No. 1, Tai-nung No. 2, Tai-nung No. 5, Tai-nung No. 6, Red Lady and $C_{4k-7} \times C_{4k-2}$ [cv. Florida]) of *Carica papaya* L. Shoot tips from in vitro cultures of Tai-nung No. 2 were used to establish the cryopreservation protocol. Mother plants were cultivated on a semi-solid MS medium (Murashige and Skoog, 1962) containing 0.1 mg l^{-1} 6-benzylaminopurine (BA), 0.05 mg l^{-1} indole-3-butyric acid (IBA), and 30 g l^{-1} sucrose at pH 5.8 (Yie and Liaw, 1977). All cultures were incubated under light intensity of 60 μmol s^{-1} m^{-2} (white fluorescent lamps) with a 14-h photoperiod at 25 ± 1°C. For cryopreservation experiments, 4- to 6-week-old in vitro plants were used. Shoot tips (about 0.8 to 1.2 mm) were dissected from plants cultured on semi-solid MS medium without any plant growth hormone for one week.

Vitrification Procedure

Shoot tips were put into 2 ml cryotubes containing 1 ml of loading solution (LS) of 2 M glycerol and 0.4 M sucrose (Matsumoto et al., 1994) at 25°C for 20 min. Subsequently, shoot tips were dehydrated with 1 ml of vitrification solution at 25°C for 0-100 min or ice-cold vitrification solution at 4°C for 0-100 min. The cryotubes were directly immersed into liquid nitrogen and stored for at least one week. This vitrification solution (modified from PVS2 solution; Sakai et al., 1990) consists of 3% (w/v) polyethylenglycol 6000, 0.02 M proline, 20% (v/v) glycerol, 13.6% (v/v) ethylene glycol, 10% (v/v) dimethylsufoxide, and 0.4 M sucrose dissolved in liquid MS medium.

Thaw and Regrow

After storage in liquid nitrogen for 1 week, vials were thawed rapidly in a water bath at 40°C for 90-120 seconds. After warming, shoot tips were washed by liquid MS medium in a sucrose concentration series of 1.2 M, 0.8 M and 0.4 M for 15 min each. Thawed shoot tips were cultured on a semi-solid MS medium containing 0.1 mg l^{-1} 6-benzylaminopurine (BA), 0.05 mg l^{-1} indole-3-butyric acid (IBA), and 30 g l^{-1} sucrose at 25°C in the dark. After 3 days, shoot tips were transferred to fresh medium under the light conditions described above. Initial survival was scored after 14-21 days and was defined as greening and active growth of the apical region. Regeneration shoots were assessed under the microscope to check the regeneration of the meristem.

Statistical Analysis

In the cryopreservation experiments, 20 shoot tips were used in each treatment. No liquid nitrogen was used in the control group. In vitrification trials, each experiment was performed at least three times, and data were calculated using the Microsoft Excel program.

Results

Shoot tip survival decreased with exposure duration of the vitrification solution (Figure 1). At room temperature when the vitrification solution treatment lasted more than 20 min the survival decreased to 50% or lower. When samples were treated at 4°C for more than 40 min, the survival dropped below 50%.

At 25°C if a loading solution was used, survival dropped below 50% at 30 min of exposure to the vitrification solution (Figure 1). However, with vitrification solution application at 4°C, survival did not drop below 50% until 50 min of exposure.

Figure 2 shows the results of survival after vitrification solution application and liquid nitrogen exposure. Survival with vitrification solution application at 4°C was superior to 25°C exposure. Furthermore, pretreatment with a loading solution increased survival. The best result was obtained when the vitrification solution treatment was between 50-65 min (survival was 68%). After culture, some part of the tissue appeared yellow or white, but after one week, some green coloration was observed. This green region contained a meristem that grew into a seedling of 10 mm after 8-10 weeks. This growth did not involve callus formation but direct shoot elongation (Figure 3). No morphological abnormalities were observed in the plants developed from cryopreserved shoot tips.

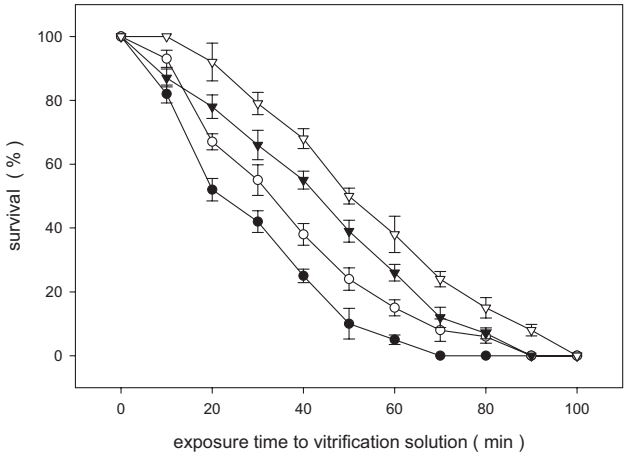

Figure 1. Effect of exposure times to vitrification solution on percent survival of papaya (Tai-nung No. 2) shoot tips. Shoot tips were excised and treated with (○,▽) or without (●, ▼) loading solution for 20 min at 25°C before dehydration with vitrification solution at 25°C (○, ●) or 4°C (▽, ▼) for 0-100 min. Samples were not exposed to liquid nitrogen. Data are presented as means ± standard error.

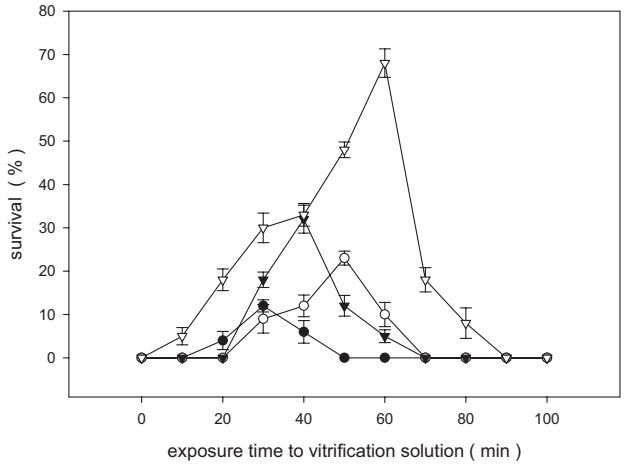

Figure 2. Effect of exposure times to vitrification solution on percent survival of papaya (Tai-nung No. 2) shoot tips after liquid nitrogen storage for one week. Shoot tips were excised and treated with (○, ▽) or without (●, ▼) loading solution for 20 min at 25°C before dehydration with vitrification solution at 25°C (○, ●) or 4°C (▽, ▼) for 0-100 min, followed by immersion into liquid nitrogen. Data are presented as means ± standard error.

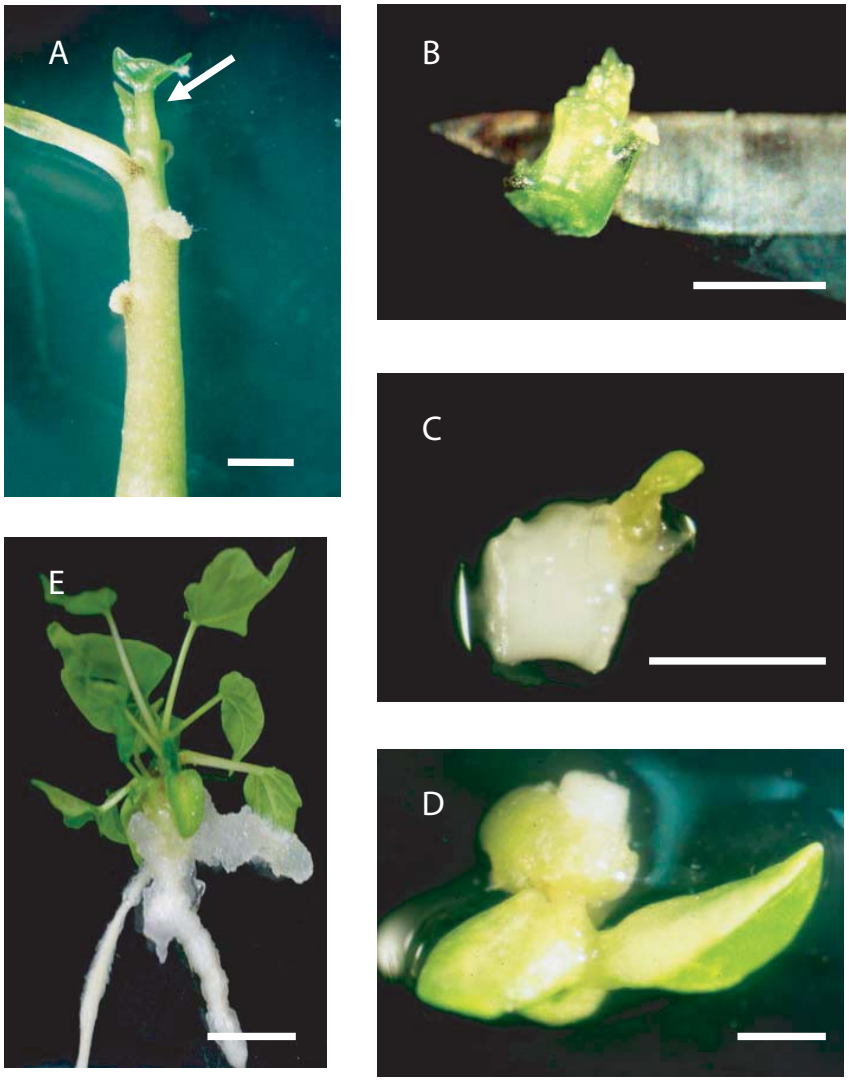

Figure 3. Shoot development and rooting from successfully cryopreserved meristems. A, The shoot tip of papaya (arrows) from plantlet was excised from an *in vitro* plant (bar: 1 mm); B, Excised shoot tips utilized for cryopreservation (bar: 1 mm); C, After vitrification and warming, shoot tip remained green during culture and resumed growth within 1 week without forming callus (bar: 1 mm); D, Shoot tip elongated after 4 weeks (Bar: 1 mm); E, Rooting of elongated shoot occurred after 8 weeks (bar: 1 cm).

Papaya shoot tips were stored for different durations (Table 1). The survival percentage of the papaya shoot tips did not differ with storage duration. This procedure was then applied to six different lines, and the survival of shoot tips from these cultivars ranged from 48 to 93% (Table 2).

Discussion

The treatment of vitrification solution subjects cells to osmotic stress, making it very likely for some constituents to enter into cells and resulting in an in vivo toxication phenomenon (Matsumoto et al., 1994; Escobar et al., 1997; Takagi et al., 1997; Pennycooke and Towill, 2000; Matsumoto et al., 2001; Helliot et al., 2002; Touchell et al., 2002). Therefore, careful vitrification solution exposure is critical. Damage to tropical and subtropical plants can occur with short exposure periods to a vitrification solution. This damage may be due to chemical toxicity or osmotic stress (Matsumoto et al., 1994; Takagi et al., 1997; Charoensub et al., 1999; Thinh et al., 1999; Sakai, 2000). Therefore, delaying permeation of some components of vitrification solution but still allowing time for adequate dehydration is critical (Charoensub et al., 1999; Tsukazaki et al., 2000).

These results in papaya demonstrate that the vitrification solution used caused damage to papaya shoot tips. However this damage was minimized by means of reducing the application temperature. Reducing the temperature from room temperature to 4°C has improved survival in other cases (Thinh et al., 1999; Lambardi et al., 2000; Helliot et al., 2002).

Applying a loading solution pretreatment has also been proven useful in reducing damage during dehydration (Matsumoto et al., 1994; Takagi et al., 1997; Charoensub et al., 1999; Thinh et al., 1999; Lambardi et al., 2000; Pennycooke and Towill, 2000; Tsukazaki et al., 2000; Vandenbussche et al., 2000; Matsumoto et al., 2001). The use of a loading solution and application of vitrification solution at 4°C improved survival of the cryopreservation of papaya shoot tips, and the results strongly indicated that both were beneficial.

The issue of whether storage in liquid nitrogen affects the viability of the preserved material is controversial. Many studies have shown no viability decline with short-term storage (Dereuddre et al., 1991). However, in this study, the storage was extended to 2 years. Viability did not decline over this duration. Similar results have been presented for carrot (Dereuddre et al., 1991) and sugarcane (Martínez-Montero et al., 1998; González-Arnao et al., 1999).

This cryopreservation procedure was effective for six papaya cultivars but also demonstrated that survival was dependent upon genotype. Similar genotype dependence results have been reported for banana (Thinh et al., 1999), black spruce (Touchell et al., 2002), sugar beet (Vandenbussche et al., 2000), and taro (Takagi et al., 1997). These results demonstrate that this method may be feasible as a standard method for cryopreservation. With the advantages of easy operation, fewer expenditures on lab equipment, and a short processing time, this method should be useful for long term germplasm conservation.

Table 1. Effect of storage duration on percent survival of papaya shoot tips after a loading solution pretreatment and liquid nitrogen storage.

Store duration	Survival rate (%)	
	Tai-nung No. 2	Tai-nung No. 5
1 day	72 ± 4	70 ± 3
1 week	68 ± 3	75 ± 4
1 month	65 ± 8	78 ± 2
3 month	74 ± 6	69 ± 8
6 month	72 ± 4	78 ± 6
1 year	70 ± 3	76 ± 4
2 year	76 ± 4	79 ± 3

Note: Shoot tips were excised and pretreated with loading solution for 20 min at 25°C, then dehydrated with vitrification solution at 4°C for 60 min before immersion into liquid nitrogen. Data are presented as means ± standard error.

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Table 2. Effect of exposure times to vitrification solution on percent survival of shoot tips from six cultivars of papaya after loading solution pretreatment and liquid nitrogen storage for one week.

Exposure times to vitrification solution (min)	Survival rate (%)					
	Tai-nung No. 1	Tai-nung No. 2	Tai-nung No. 5	Tai-nung No. 6	Red Lady	$C4_{k-7} \times C4_{k-2}$
50	55±9	67±5	80±5	48±4	75±9	83±5
60	52±7	75±4	74±6	52±8	68±5	87±6

Note: The papaya shoot tips were excised and treated with loading solution for 20 min at 25°C, then dehydrated with vitrification solution at 4°C for 50 or 60 min before immersion into liquid nitrogen. Data are presented as means ± standard error.

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使用玻璃化法進行番木瓜莖頂的超低溫冷凍保存

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六個不同種品種的番木瓜莖頂，成功地以玻璃化法進行超低溫冷凍保存。番木瓜莖頂取自繼代培養 4-6 週大的植株。番木瓜莖頂先以低濃度冷凍保護劑（含 2 M 甘油及 0.4 M 蔗糖）在室溫下處理 20 分鐘，接著用預冷過的高濃度冷凍保護劑（vitrification solution）在 4°C 下處理 60 分鐘後，直接置入液態氮中保存至少一週。六個品系的番木瓜莖頂在冷凍保存後，存活率分別介於 48% 和 93% 之間。冷凍保存之莖頂在回溫後，約一週內即可觀察其明顯生長，且可直接生長發根為一完整植株，而未見癒傷組織分化。實驗結果顯示番木瓜莖頂可成功地在液態氮中保存 2 年，其存活再生能力並不受保存時間延長之影響。實驗結果證實本研究成功地發展一個簡單、有效率的番木瓜種原保存流程，能有效地達成番木瓜種原長期保存的目的。

關鍵詞：番木瓜；冷凍保存；玻璃化法。