# Cryopreservation of the vulnerable medicinal plant *Nothapodytes nimmoniana* (J. Graham) Mebb.'s shoot tips using the vitrification technique: effects of the vitrification procedure and recovery media

# 藥用植物青脆枝玻璃化法超低溫保存之研究：玻璃化程序及培養基對存活率的影響

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# Abstract

*Nothapodytes nimmoniana* (J. Graham) Mebb. is a highly valuable anticancer medicinal plant and a near-threatened species on the Red List of Taiwan Plants. This study aims to study the influence of the vitrification technique's treatment procedures and recovery media on the survival of *in vitro* shoot tips in cryopreservation. To prepare the explants for a suitable state before cryopreservation, the pretreatment involved comparing sucrose concentrations ranging from 0.3 M to 0.7 M and durations ranging from one to seven days following the 0.7 M concentration. Additionally, to ensure reliable cryopreserved results, we tested the cryoprotectant (PVS2) dehydration period and the recovery medium after post-thawing. The results of sucrose pretreatment after non-cryopreservation showed that the highest survival rates, 72.6% and 73.3%, were achieved either with a 0.7 M sucrose concentration or after a three-day sucrose pretreatment. These results are based on the following procedure: intact plantlets underwent different pretreatment processes, in which dissected shoot tips were treated with a loading solution containing 2.0 M glycerol and 0.4 M sucrose for 60 minutes at 25°C, and the shoot tips were subsequently dehydrated in PVS2 for 90 minutes at 0°C before being plunged into liquid nitrogen. The optimum cryoprotectant dehydration for 90 minutes resulted in a 68.8% survival rate after cryopreservation. Rewarming was conducted in a water bath for 30 seconds at 40°C, and PVS2 was replaced with a 1.2 M sucrose solution for 30 minutes at 25°C. Shoot tips transferred to WPM medium supplemented with 1.0 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$ IBA achieved a 73.3% survival rate after four weeks of recovery. The procedure provided another high survival rate of *in vitro* preservation of *N. nimmoniana*.


**Keywords:** *Nothapodytes nimmoniana*, cryopreserved, sucrose pretreatment,

PVS2

## 摘要

青脆枝（*Nothapodytes nimmoniana*）為臺灣植物紅皮書列名接近威脅的物種，也是極具價值的抗癌藥用植物。本研究利用玻璃化技術，探討超低溫處理程序及回復培養基，對保存後莖頂存活之影響。首先分別比較培植體在 0.3 M ~ 0.7 M 蔗糖濃度下及 0.7 M 蔗糖濃度 1~7 天兩種預處理，對存活率產生之影響；除此也比較抗凍劑 PVS2 的處理時間和 2 種不同回復培養基對保存結果產生的差異。結果顯示，完整培植體經 0.7 M 蔗糖浸泡或預處理 3 天，接著在 25℃ 下切下莖頂浸入含有 2.0 M glycerol 及 0.4 M 蔗糖溶液 60 分鐘後在 0℃ 下透過 PVS2 脫水 90 分鐘，未放入液態氮的存活率分別為 72.6% 和 73.3%，如經超低溫保存實驗，最佳的的抗凍脫水處裡時間為 90 分鐘，莖頂存活率可達 68.8%。除此，解凍程序則是將冷凍管置於 40℃ 水浴 30 秒後吸出 PVS2，在 25℃ 條件下加入 1.2 M 蔗糖溶液，靜置 30 分鐘以置換 PVS2，再將莖頂轉入含 1.0 $mg{\cdot}L^{-1}$ BA and 0.1 $mg{\cdot}L^{-1}$ IBA 的 WPM 最佳回復培養基，4 周後存活率可達 73.3%，本結果提供青脆枝種原保存的一種高存活率方法。

**關鍵詞：**青脆枝、超低溫保存、蔗糖預處理、PVS2

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## Introduction

*Nothapodytes nimmoniana* (J. Graham) Mebb., a member of the Icacinaceae, is a vulnerable tree species. It is recommended as an ornamental greening plant in urban Taiwan for its various adaptable capacities (Chen and Wu 2012). This species is distributed across an extensive range, including the Western Ghats of India, Sri Lanka, North Sumatra, southeast Asia, southern China, and the Philippines, as well as Orchid Island and Green Island in Taiwan (Chang 1993; Kårehed 2001). The tree contains abundant sources for extracting camptothecin (CPT) and 9-methyl camptothecin (9-Met CPT) in higher quantities than other natural sources (Isah and Mujib 2015). Previous studies have demonstrated that CPT exhibits excellent antitumor activity; therefore, the species is considered a medicinally valuable tree (Padmanabha *et al*. 2006; Beretta *et al*. 2013; Yang *et al*. 2022). CPT can be isolatable from different parts of the whole plant, but the stem and bark have the highest accumulation content and yield quantity (Isah and Mujib 2015). The most common way to utilize *N. nimmoniana* involves cutting it down and stripping the bark to extract alkaloids. Consequently, natural populations of *N. nimmoniana* have been overexploited and destroyed due to the increasing market demand for natural sources of CPT, which is used to produce high-quality anticancer drugs. This has led to the species becoming extinct or extremely rare, resulting in its classification as a red-listed endangered species (Swamy *et al*. 2021). In Taiwan, the species is reported as near-threatened (NT) in its native habitat, and similarly in many other countries, it is considered endangered or threatened by extinction under IUCN categories (Isah and Mujib 2015; Prakash *et al*. 2016; Editorial Committee of the Red List of Taiwan Plants 2017).

Based on the reasons above,

conserving this endangered and highly valuable medicinal species has become a significant issue. Traditional *ex situ* conservation methods for such forest trees typically involve the use of seed banks or field collections. However, conserving plant species through these methods is generally challenging due to their high heterozygosity, making it difficult to preserve extraordinary genotypes (Engelmann 2011). In addition, seed banks often rely on storage conditions that may cause some seeds to lose viability and require frequent renewal to maintain seed vitality (van Treuren *et al*. 2017). Plants remaining in field gene banks are subjected to abiotic and biotic stress, necessitate costly labor for maintenance, face space constraints, and are vulnerable to human errors, among other challenges (Hu *et al.* 2015). Cryopreservation has advantages in terms of safety, low contamination risks, cost savings in maintenance, and stability for long-term storage (Engelmann 2011; Hu *et al*. 2015). Moreover, selecting superior individuals from the wild population is essential to achieve a high yield of phytoextraction alkaloids in *N. nimmoniana*. Maintaining genetic consistency and stability emerges as a crucial objective in this process. Among the various types of explants, shoot tips stand out as differentiated vegetative organs that, lacking genetic heterogeneity, are established to be the most practical choice for the cryopreservation of an economically valuable crop (Benelli *et al.* 2013; Isah and Mujib 2015; Coelho *et al*. 2020).

A previous study reported on the cryopreservation of *N. nimmoniana* using embryonic axes, which were heterozygous explants in a storage situation where the future growth was unclear (Radha *et al*. 2010). This study is the first report on using *in vitro* shoot tips of *N. nimmoniana* to examine cryopreservation by vitrification, including various processes such as

the effect of sucrose pretreatment and cryoprotectant dehydration durations on survival of shoot tips after cryopreservation (+LN) or non-cryopreservation (-LN). This study also investigated the effect of the recovery medium by comparing various plant growth regulators involved in the survival of shoot tips.

## Materials and methods

### Plant material

Seeds of *N. nimmoniana* were collected from the Botanical Garden of the National Museum of Natural Science, Taichung, Taiwan, and seedlings were cultivated in the nursery of the Department of Life Sciences at National Chung-Hsing University in Taichung under a 60% shading greenhouse. Shoots, approximately 1-2 cm in length with two to three nodes, were collected from two-year-old seedlings. They were initially rinsed in running tap water for 30 minutes to remove surface dirt, then sterilized with 1.5% sodium hypochlorite (NaOCl) supplemented with one drop of Tween 20 for ten minutes, and rinsed three times in sterile distilled water under a laminar flow hood. Aseptic shoots were incubated in 617-mL Erlenmeyer flasks, each containing 125 mL of woody plant medium (WPM) (Lloyd and McCown 1981) supplemented with 0.5 mg · $L^{-1}$ BA, 0.05 mg · $L^{-1}$ IBA, 3% (w/v) sucrose, and 0.7% (w/v) agar (Fei Kuan Agar Co.) for multiplication. The pH of all media was adjusted to 5.8 with 0.1 M KOH before autoclaving at 121°C for 15 minutes. The experimental explants were maintained in a culture room at 25 ± 2°C for 60-90 days under a photosynthetic photon flux density of 60 μmol·$m^{-2}$·$s^{-1}$ provided by cool white fluorescent tubes, following a 14/10 day/night photoperiod (China Electric Manufacturing Corporation, Taipei, Taiwan).

**Effect of various sucrose concentrations or 0.7 M sucrose pretreatment**

**duration on shoot tips survival after cryo- and noncryopreservation**

(1) Effect of sucrose concentrations: After 60-90 days of multiplication, the *in vitro* stock plants had their roots removed, and then six to eight plantlets were transferred to 617-mL Erlenmeyer flasks, each containing 125 mL of WPM medium supplemented with 0.3, 0.5, or 0.7 M sucrose for three days. After pretreatment with various sucrose concentrations, shoot tips were dissected from the pretreated plantlets and placed in a cryovial with a loading solution (Matsumoto *et al.* 1994) for 60 minutes. They were then dehydrated with plant vitrification solution 2 (PVS2) (Sakai *et al.* 1990) for 90 minutes before immersion in liquid nitrogen (LN). (2) Effect of 0.7 M sucrose treatment durations: The experimental materials were the same as in the previous paragraph. The plantlets with their roots eliminated were placed in Erlenmeyer flasks containing 125 mL of WPM medium supplemented with 0.7 M sucrose for one, three, or seven days. After various durations of 0.7 M sucrose pretreatment, shoot tips were dissected from the pretreatment plantlets and placed in a cryovial with a loading solution for 60 minutes. They were then dehydrated with PVS2 for 90 minutes before immersion into LN.

**Effect of PVS2 dehydrated treatments on survival after cryo- and noncryopreservation**

After 60-90 days of 2-3 cm multiplication, *in vitro* stock plants were transferred, with six to eight plantlets in each 617-mL Erlenmeyer flask. Each flask contained 125 mL of WPM medium supplemented with 0.7 M sucrose for a three-day pretreatment. The shoot tips underwent the same process described in the previous paragraph and were dehydrated with PVS2 for 60, 90, or 120 minutes before immersion in LN.

**Cryopreservation protocol and effect**

**of recovery media**

(1) Cryopreservation basic protocol: The method was performed based on the protocols from our previous studies (Hu *et al*. 2015; 2021). As mentioned above, experimental plantlets underwent the pretreatments, osmoprotection during loading, and PVS2 dehydration steps. The cryovials for cryopreserved were immersed in LN and stored for at least 24 hours (Table 1). Regardless of cryopreservation (+LN) or non-cryopreservation (-LN), the cryovials were warmed in a water bath at 40 °C for 30 seconds. PVS2 was replaced with an unloading solution (1.2 M sucrose), and incubated at 25°Cfor 30 minutes. After being treated with the unloading solution, the shoot tips were transferred onto sterile Petri dishes with filter paper on the surface of the recovery medium to remove any residual cryoprotectant solution. The culture medium and filter paper were replaced daily in the dark for one week, and then the shoot tips were transferred to fresh recovery medium under dim light conditions (5 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for three weeks. (2) Effect of recovery media: The stock explants underwent a three-day pretreatment with 0.7 M sucrose. The excised shoot tips were subjected to cryopreservation using the vitrification procedure, which included osmoprotection for 60 minutes and dehydration with PVS2 for 90 minutes. After cryostorage, they were thawed using the unloading process mentioned above. The recovery medium was prepared using WPM medium supplemented with either 1.0 mg · $L^{-1}$ BA and 0.1 mg · $L^{-1}$ IBA (Nn) or 2.0 mg · $L^{-1}$ BA and 0.1 mg · $L^{-1}$ IBA (P). In each experiment, survival rates were recorded by observing the shoot tips turning green compared to the total number of tests after four weeks of rewarming (Table 1 and Fig. 5)

## Statistical analysis

All experiments involved ten shoot

tips in each treatment and were repeated three times. Statistical analysis was performed using SPSS software. The significance of the differences between mean values was evaluated using Fisher's protected least significant difference test. (LSD, $p \leq 0.05$).

## Result

### Effect of various sucrose concentrations or pretreatment duration on shoot tips survival after cryo- and noncryopreservation

After subjecting *in vitro* stock plants to 0.3, 0.5, and 0.7 M sucrose pretreatments for three days, the survival rates of shoot tips were 90.0%, 93.3%, and 93.3%, respectively, after being placed in cryopreservation with loading solution for 60 minutes and dehydrated with PVS2 for 90 minutes. The results showed no significant differences in the non-cryopreservation group. In contrast, the survival rates in the cryopreservation group were 61.3%, 64.2%, and 72.6%, respectively, and displayed significant differences among the various sucrose concentration pretreatments (Fig. 1). The survival rates got higher when the sucrose concentration reached 0.7 M.

Survival rates of non-cryopreserved explants after one, three, and seven days of 0.7 M sucrose pretreatment were consistently above 90% and did not show any significant differences after dehydration with PVS2 for 90 minutes (Fig. 2). However, the survival rates of cryopreserved samples showed significant differences after one (55.1%), three (73.3%), and seven (51.5%) days of 0.7 M sucrose pretreatment. Explants subjected to a three-day 0.7 M sucrose pretreatment exhibited a higher survival rate compared to those with shorter or longer pretreatment times (Fig. 2).

### Effect of PVS2 dehydrated treatments and loading osmoprotection duration

After subjecting *in vitro N. nimmoniana* explants to 0.5 M

sucrose pretreatments for three days, no significant differences in survival rates were observed in the non-cryopreservation group, regardless of the varying PVS2 treatment duration (Fig. 3). After 24 hours of cryopreservation and four weeks of recovery, the cryopreservation group showed significant differences in shoot tip survival rates. Specifically, cryoprotection with PVS2 for 90 minutes resulted in a higher survival rate (68.8%) compared to shorter (32.9%) or longer (40.0%) PVS2 exposure periods of 60 and 120 minutes (Fig. 3).

**Effect of recovery media**

After four weeks of re-warming incubation, cryopreserved explants were examined using Nn and P recovery medium, resulting in survival rates of 73.3% (Nn) and 44.8% (P), respectively, which exhibited a significant difference (Fig. 4). In the Nn medium, reduced plant growth regulator concentrations were observed, which led to a higher recovery rate. The growth and greening of the cryopreserved shoot tips indicated that the recovery medium with the appropriate concentration of plant growth regulators effectively promoted explant regrowth.

## Discussion

The first attempt to cryostore *N. nimmoniana* germplasm was conducted by the team of Radha *et al.* (2010), using embryonic axes and a dehydration method under laminar airflow to minimize ice crystal damage during the cryopreservation process. Notably, *N. nimmoniana* is a high-profile medicinal tree producing the alkaloid camptothecin, a popular anticancer drug. This species is widely distributed from India to Taiwan and is characterized by high genetic diversity (Shrivastava *et al.* 2021). The search for abundant natural chemical extracts from high-yielding individuals to produce substances through a clonal

multiplication system with a high camptothecin yield represents a future application trend (Isah and Mujib 2015). Therefore, our cryopreservation approach, focusing on shoot tips, aligns more closely with current needs. It marks the first successful application of the cryopreservation by vitrification protocol to this species.

Pretreating explants is a crucial step because it can enhance the dehydration tolerance of plants and subsequently improve survival rates after cryopreservation. This approach has been successfully employed in various crops, including root and tuber crops (Hu *et al*. 2021; Zhang *et al*. 2023), raspberry (Palonena and Junttila 1999), papaya (Tsai *et al*. 2009), cherry (Barraco *et al*. 2012), and pineapple (Hu *et al*. 2015). Sucrose-enriched pretreatments play a pivotal role in enhancing the physiological tolerance of donor plantlets and improving their survival during cryopreservation (Dumet *et al*. 2000; Sulong *et al*. 2018; Bettoni *et al*. 2021b). Pretreating plantlets with the right sucrose concentration has been shown to effectively reduce osmotic potential, leading to a decrease in water content. This, in turn, helps prevent cryoprotectant toxicity and the crystallization of cells when exposed to liquid nitrogen (El Merzougui *et al*. 2023). However, previous studies have reported that plantlets overexposed to sucrose can cause toxicity to plant cells (Quain *et al*. 2009; Oliveira *et al*. 2017). This study examined the appropriateness of sucrose concentrations and pretreatment durations in the recovery of shoot tips after cryopreservation using vitrification. We found that a steadily increasing sucrose concentration of 0.7 M and a three-day duration in the pretreatment medium contributed to an increase in the survival of shoot tips after cryopreservation (Fig. 1, 2). Furthermore, our study used intact plantlets instead of the traditional excised apical shoot

tips, allowing for hardening through pretreatments before dehydration. This approach can withstand higher concentrations of sucrose (0.7 M) and a longer treatment duration (three days). A similar method has also been successfully applied in cryopreservation studies for pineapple and sweet potato within our team (Hu *et al*. 2015, 2021).

Cryoprotectant solutions are another critical factor that influences the survival rate after cryopreservation. In the vitrification method of cryopreservation, high concentrations of cryoprotectant solutions, such as PVS2, are used to reduce the water content of explants and increase the solute concentration. This prevents damage from ice crystals, allowing the explants to be directly plunged into liquid nitrogen (Sakai *et al*. 1990; Panis and Lambardi 2005; El Merzougui *et al*. 2023; Zhang *et al*. 2023). PVS2 has been widely employed as a cryoprotectant to induce a vitrified state in plant cells and has made significant contributions to cryopreservation studies in various genera (Coutinho Silva *et al*. 2013; Vollmer *et al*. 2014; Bettoni *et al*. 2021a; El Merzougui *et al*. 2023). Nevertheless, PVS2 contains several toxic chemicals, such as glycerol, DMSO, and ethylene glycol. Overexposure of explants to PVS2 can be harmful (Yi *et al.* 2012; Merhy *et al*. 2014; Rafique *et al*. 2015). Therefore, the duration of explant exposure to PVS2 for dehydration is another critical factor affecting the recovery rates after cryopreservation. Our results observed the highest survival rate in the treatment of PVS2 for 90 minutes on ice after cryopreservation (Fig. 3). According to previous studies, the optimal exposure time can vary tremendously, ranging from 20 minutes to over 200 minutes. This variation may depend on plant species, explant conditions, treatment temperature, and additional factors such as pretreatment and preculture procedures (Bettoni *et al.*

2021a; El Merzougui *et al*. 2023).

To evaluate the success of cryopreserved rare plant species or elite genotypes, it is essential to consider not only the post-thaw survival rate of shoot tips but also the likelihood of somaclonal variation in the recovery medium. An appropriate tissue culture system for shoot tip recovery after cryopreservation is necessary (Bettoni *et al*. 2021 a). The base medium commonly used for *in vitro* multiplication of woody plants is WPM medium, which was also employed in this study. Besides the base medium, medium supplements with different concentrations and types of plant growth regulators play another key role in shoot tip recovery after cryopreservation. Chang *et al*. (2008) reported the MS medium supplement 0.5 ml · $L^{-1}$ TDZ obtained 100% multi-shoots in *N. nimmoniana* shoot tip *in vitro* multiplication. However, the genetic stability of the cryopreserved material is a vital target for the long-term conservation of plant genetic resources (Engelmann 2011). Many studies have reported that inappropriate use of TZD may induce abnormalities in plant growth and development, cause poor elongation, and weaken rooting capacity, ultimately affecting the overall health and productivity of the plant (Dewir *et al*. 2018; Faisal *et al*. 2023). In our experiment, the best survival performance was obtained with the WPM medium containing 1.0 ml · $L^{-1}$ BA and 0.1 ml · $L^{-1}$ IBA after four weeks of recovery incubation (Fig. 4). Prakash *et al*. (2016) showed that the positive combined effect of BA and IBA was significantly proved in *N. nimmoniana*'s multiple shoot induction. On the basis of a previous similar study, a WPM medium containing 2 ml · $L^{-1}$ BA and 0.2 ml · $L^{-1}$ IBA induced a higher average number of shoots from axillary buds (Ju 2010). Ju's (2010) report also mentions that the medium raised the BA concentration and simultaneously

encouraged callus formation. In our study, we intensified the synergistic auxin/cytokinin fitting ratio, so that lower auxin and higher cytokinin concentration were conducive to shoot tip survival after cryopreservation recovery.

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Table 1 Experimental scheme of *Nothapodytes nimmoniana* cryopreservation by vitrification
表 1 青脆枝玻璃化法超低溫保存實驗步驟

| Cryo-step | Treatment | Categories |
|---|---|---|
| Pretreatment | 1. Pretreatment sucrose concentration varied | 0.3, 0.5, 0.7 (M) (each pretreatment for 3 days) |
| | 2. Pretreatment duration (at 25℃) | 1, 3, 7 (day) (pretreatment in 0.7 M sucrose medium) |
| Cryoprotectant | Dehydration period (duration) (PVS2[a] at 0℃) | 60, 90, 120 (min) (each treatment with LS[b] for 60 min) |
| Recovery Post-thaw | Recovery media for four weeks(Filter paper on top of recovery medium and change filter papers daily in first week) | Nn[c] medium P[d] medium |

[a] PVS2: plant vitrification solution (Sakai *et al.* 1990).

[b] LS: Loading solution (Matsumoto *et al.* 1994).

[c] Nn medium：WPM containing 1.0 mg·$L^{-1}$ BA and 0.1 mg·$L^{-1}$ IBA

[d] P medium：WPM containing 2.0 mg·$L^{-1}$ BA and 0.1 mg·$L^{-1}$ IBA

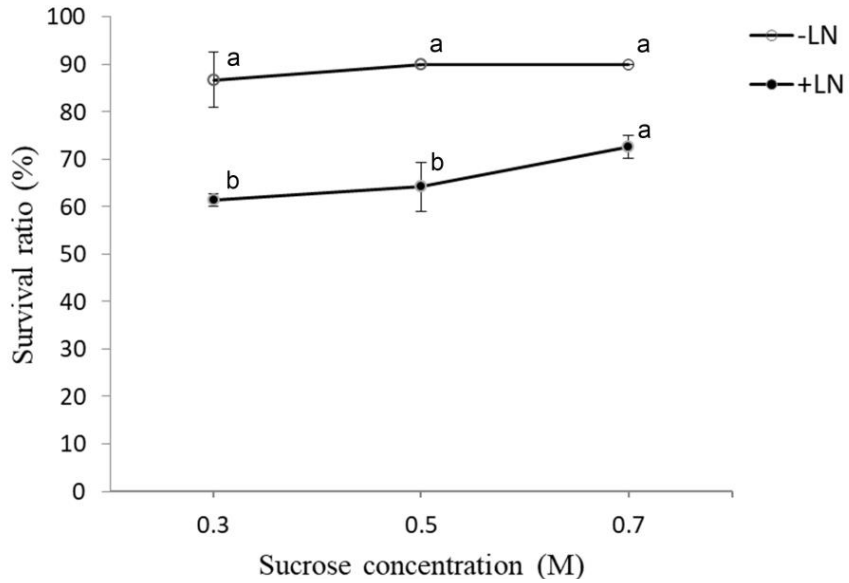


Fig. 1 Effect of sucrose pretreatment for 3 days with 60-min LS and 90-min PVS2 dehydration treatments, with subsequent cryopreservation (+LN) or non-cryopreservation (-LN), on the survival (%) of shoot tips of *Nothapodytes nimmoniana*.
Bars represent the standard error of the mean (n = 3). The letters indicate significant differences among the treatments based on Duncan's multiple range tests at $p \leq 0.05$.

圖 1 青脆枝莖頂於不同蔗糖濃度預培養 3 天，再進行 60 分鐘 LS 及 90 分鐘 PVS2 的脫水處理後，比較超低溫保存和未經超低溫保存的存活率。
每個處理重復三次，圖上線段代表標準誤差（n=3），不同英文字母代表各處理間經鄧肯氏法測驗後之顯著差異情形（$p \leq 0.05$）。

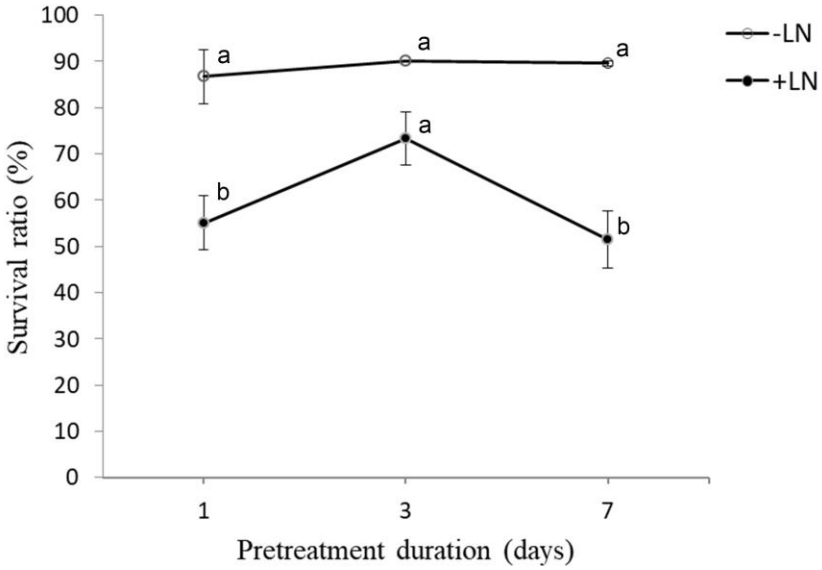


Fig. 2 Effect of 0.7 M sucrose pretreatment for different periods with 60-min LS and 90-min PVS2 dehydration treatments, with subsequent cryopreservation (+LN) or non-cryopreservation (-LN), on the survival (%) of shoot tips of *Nothapodytes nimmoniana*.

Bars represent the standard error of the mean (n = 3). The letters indicate significant differences among the treatments based on Duncan's multiple range tests at $p \leq 0.05$.

圖 2 青脆枝莖頂於 0.7 M 蔗糖經不同時間預培養，再進行 60 分鐘 LS 及 90 分鐘 PVS2 脫水處理後，比較超低溫保存和未經超低溫保存處理的存活率。

每個處理重復三次，圖上線段代表標準誤差（n=3），不同英文字母代表各處理間經鄧肯氏法測驗後之顯著差異情形（$p \leq 0.05$）。

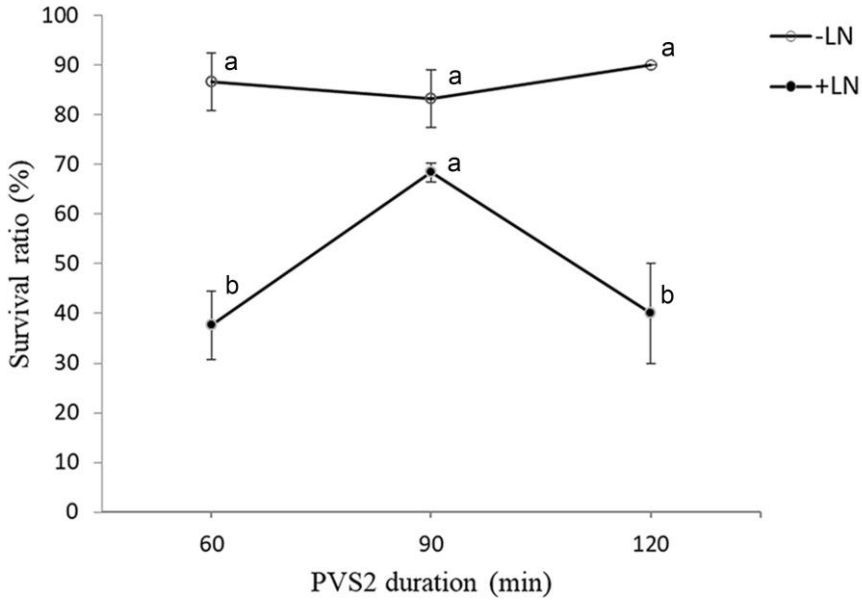


Fig. 3 Effect of 0.5 M sucrose pretreatment for 3 days with 60-min LS and PVS2 dehydration treatments, with subsequent cryopreservation (+LN) or non-cryopreservation (-LN), on the survival of *Nothapodytes nimmoniana* shoot tips.

Bars represent the standard error of the mean (n = 3). The letters indicate significant differences among the treatments based on Duncan's multiple range tests at $p \leq 0.05$.

圖 3 青脆枝莖頂於 0.5 M 蔗糖預培養 3 天，再進行 60 分鐘 LS 及不同時間 PVS2 脫水處理後，比較超低溫保存和未經超低溫保存的存活率。

每個處理重復三次，圖上線段代表標準誤差（n=3），不同英文字母代表各處理間經鄧肯氏法測驗後之顯著差異情形（$p \leq 0.05$）。

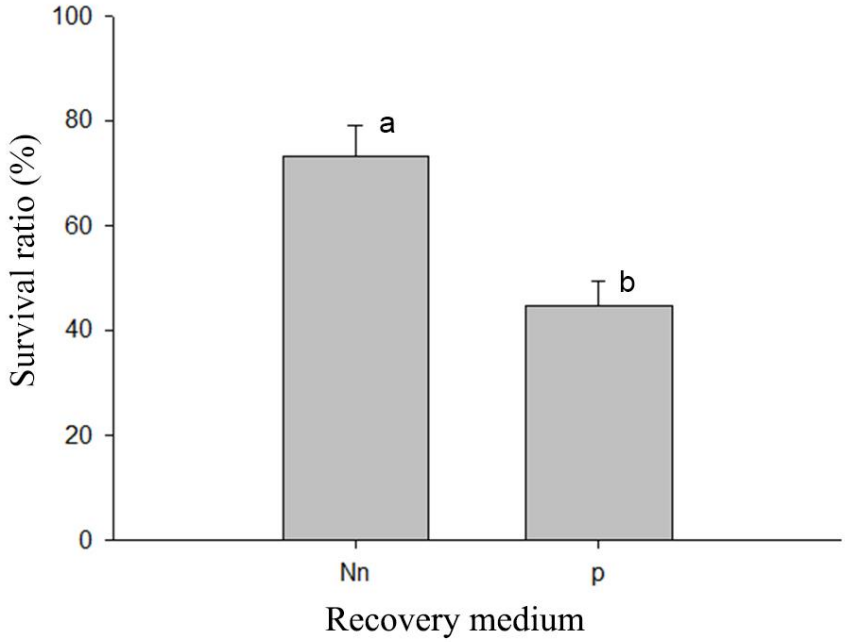


Fig. 4 Shoot tips survival rates of *Nothapodytes nimmoniana* on Nn and P medium after cryopreservation by vitrification. Explants through 3 days of 0.7 M sucrose pretreatment, excised shoot tips under 60-min LS and 90-min PVS2 dehydrated.
Bars represent the standard error of the mean (n = 3). The letters indicate significant differences among the treatments based on Duncan's multiple range tests at $p \leq 0.05$.

圖 4 青脆枝莖頂於 0.7 M 蔗糖預培養 3 天，再進行 60 分鐘 LS 及 90 分鐘 PVS2 脫水處理後，經玻璃化法超低溫保存，比較 Nn 和 P 兩種培養基的存活率。
每個處理重復三次，圖上線段代表標準誤差（n=3），不同英文字母代表各處理間經鄧肯氏法測驗後之顯著差異情形（$p \leq 0.05$）。

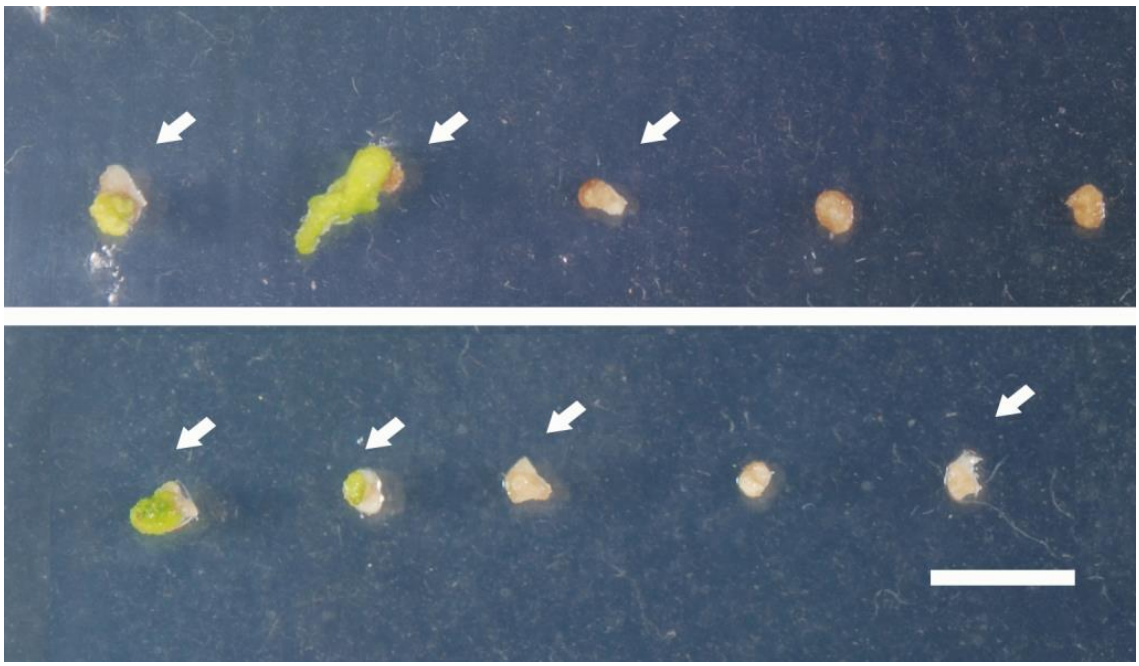

Fig. 5 Cryopreservation of *Nothapodytes nimmoniana* shoot tips after four weeks of culture on recovery media. Cryopreservation explants through three days of 0.7 M sucrose pretreatment, excised shoot tips under 60-min LS and 90-min PVS2, dehydrated, then plunge into LN for 24 hr. The arrows indicated survival shoot tips. The scale bar indicated 5 mm.

圖 5 青脆枝莖頂經超低溫保存處理，於回復培養基培養 4 周後存活狀況。培植體於 0.7 M 蔗糖預培養 3 天後切下莖頂，再將莖頂進行 60 分鐘 LS 及 90 分鐘 PVS2 脫水處理後，置入液態氮 24 小時。箭頭顯示經超低溫保存後莖頂存活情形；比例尺代表 5 公釐。