EFFECTS OF PVS2 EXPOSURE ON VIABILITY OF APICAL MERISTEMS OF VATICA BELLA, AN ENDEMIC SPECIES OF PENINSULAR MALAYSIA

Nashatul Zaimah, N. A.
Forest Research Institute Malaysia (FRIM),
Forest Biotechnology Division, 52019 Kepong Selangor.
Email: nashatul@frim.gov.my

Nadiah Salmi, N.
Forest Research Institute Malaysia (FRIM),
Forest Biotechnology Division, 52019 Kepong Selangor.
Email: nadiah@frim.gov.my

Nor Asmah, H.
Forest Research Institute Malaysia (FRIM),
Forest Biotechnology Division, 52019 Kepong Selangor.
Email: norasmah@frim.gov.my

Noraliza, A.
Forest Research Institute Malaysia (FRIM),
Forest Biotechnology Division, 52019 Kepong Selangor.
Email: noraliza@frim.gov.my

Nor Rashidah, M.
Forest Research Institute Malaysia (FRIM),
Forest Biotechnology Division, 52019 Kepong Selangor.
Email: norrashidah@frim.gov.my

ABSTRACT

Vatica bella (resak keluang) a member of Dipterocarpaceae is endemic to Peninsular Malaysia and has been categorised as nearly threatened according to the Malaysia Plant Red List v.3.1 (2010). The aim of the first part of this study was to obtain effective method and sterilising agents for surface sterilisation of different V. bella explant parts (apical meristems and axillary buds). The apical meristems showed better viability (60%) than axillary buds (20%) when treated with 5% chlorox for 15 min. Therefore, apical meristems were selected as an explant for further study. In the second part of the study, the objective was to evaluate the respond of apical meristems to cryopreservation, where they were exposed to different concentrations (0-100%) of plant vitrification solution 2 (PVS2) followed by rapid plunging in liquid nitrogen. Generally, after cryopreservation, the percentage of viability of V. bella apical meristems reduced with an increase of PVS2 concentrations. Exposure to 50% PVS2 showed the highest viability (80%) compared to the others. This study demonstrates the feasibility to cryopreserve V. bella apical meristems for long-term purposes.

Keywords: Cryopreservation, plant vitrification solution 2 (PVS2), forest species, endemic

INTRODUCTION

The tropical forest of Malaysia is rich in various plant species and the mixed forest is dominated by the dipterocarp species from different families. The dipterocarps trees especially species from the Dipterocarpus, Hopea, Shorea, and Vatica play an important role in the international tropical timber market for many Southeast Asian countries as they provide valuable hardwood. In addition to that, they also produce other non-timber products such as resins, oils, camphor, and turpentine (Anne-Mette Hüls Dyrmose et al. 2017). Conservation of Dipterocarpaceae is becoming more important due to the changes in land-use patterns hence increasing the demands on forestry resources and Malaysian commitment to sustainable utilisation of the forest resources (Saw & Sam, 2000).

Among the important dipterocarp species that are important for the conservation purpose are those belonging to the threatened and near-threatened species. Long-term preservation of this category of species is crucial to avoid the loss of essential genetic resources in the future. A native plant to Malaysia, *Vatica bella* known as resak keluang to the locals is classified as near threatened by the Malaysia Plant Red List version 3.1 (2010) and critically endangered by the IUCN Red List of Threatened Species (2000) due to habitat degradation, habitat loss, and over-exploitation for timber. The medium to the large tree can grow up to 50 m tall and is commonly found in lowland dipterocarp forests on undulating land below 250 m. The *V. bella* fruits are large up to 25 mm long indicating that they belong to the recalcitrant group. The term 'recalcitrant' was introduced by Roberts in 1973 which means the seeds are sensitive to desiccation and low temperature, germinate easily and wet storage is limited from a few weeks to three months in *Shorea roxburghii* and *Hopea odorata* (Corbineau & Côme, 1989) and 2 months for *Mesua ferrea* when stored at room temperature (Lestari, 2018). Propagation by seed is common method for most plant species; however, most dipterocarp trees flower irregularly (Sasaki, 2008) at an interval of every 2-10 years (Appanah, 1993). Thus, delaying the conservation effort of this species. To overcome this, cryopreservation is a promising option for the long-term conservation of recalcitrant seeds. Plant

cryopreservation is a technique where plant tissues are preserved at ultra-low temperatures (-196°) without losing viability (Benson, 2008). Among the methods available are encapsulation/dehydration, vitrification, desiccation, pre-growth desiccation (Englemann 1999) and droplet-freezing (2005).

In vitrification, the most common vitrification solution used is Plant Vitrification Solution 2 (PVS2), a highly concentrated cryoprotective solution developed by Sakai et al. (1990). It consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide in Murashige and Skoog (MS) medium supplemented with 0.4 M sucrose. This solution partially dehydrates the tissues thus, enabling the formation of a stable glass-like state in the plant tissues prior to immersion in liquid nitrogen. This technique is simple, does not require sophisticated equipment, and has been studied in several tropical tree species including the shoot tips of *Nephelium ramboutan-ake* (Chua & Normah, 2011), embryonic axes of a tropical forest tree *Elateriospermum tapos* (Nashatul et al., 2007), shoot-tips of *Garcinia cowa* (Yap et al., 2011), and *Parkia speciose* zygotic embryos (Sinniah et al., 2013).
The objectives of this study are firstly to identify and establish an effective surface sterilisation method for *V. bella* explants and secondly, to investigate the effects of PVS2 exposure on the apical meristems of *V. bella.*

MATERIALS AND METHODS

PLANT MATERIALS AND SURFACE STERILISATION

Explants were obtained from new twigs from field-grown mature trees of *V. bella* from an arboretum at the Forest Research Institute Malaysia. Before sterilisation, the attached leaves were removed leaving the apical meristems and the axillary buds to the size of 5-10 mm. These two types of explants were separated into two different perforated polythene bags and were washed under running tap water for one hour to remove impurities and dirt. Further washing was performed in a laminar air-flow hood in which the explants were washed and shaken vigorously in sterile distilled water. Three sterilising agents were applied to both types of explants in order to evaluate the sterilizing effect. For a better sterilising agent, an explant contact, a few drops of Tween 20 detergent were added while disinfecting. Comparison was made between 0.1% boric acid (5 min), 200mg/L streptomycin sulphate (10 min), and Clorox bleach (containing 5-6% sodium hypochlorite) in the presence and without ethanol (5%, 10% and 20% v/v) for 15 min. A drop of Tween 20 was added to the sterilising agents to function as a wetting agent to reduce surface tension and allow better surface contact. The sterilising solutions were removed by rinsing them with sterile distilled water three to five times. The surface sterilised explants were placed on Murashige and Skoog medium (Murashige & Skoog, 1962) where data on survival (uncontaminated) was recorded. A total of 30 explants were used in each treatment with 10 explants and 3 replications. The data were then converted into a percentage.

VITRIFICATION

Apical meristems (5 to 10 mm in size) were placed in a cryovial containing 2 ml of loading solution (2M glycerol and .4 M sucrose) for 20 min at room temperature (25 ºC). The loading solution was then replaced by pre-chilled PVS2 solution [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide, and 0.4M sucrose] that was prepared at different concentrations (10 to 100%) and the explants were dehydrated for 10 min at 25 ºC. PVS2 was decanted and 1 ml of fresh PVS2 was then added and rapidly immersed in liquid nitrogen. After liquid nitrogen storage, the cryovials were removed from the tank and thawed in a 40ºC water bath for 90 s. PVS2 solution was drained and replaced by an unloading solution (0.4 M sucrose) and incubated for 20 min at 25 ˚C. The apical meristems were rinsed with sterile distilled water twice, blotted on filter paper, and transferred onto recovery medium (MS medium) in which the viability was observed. The cultures were then placed on culture shelves provided with white fluorescent tubes (3000 lux) in a 25 ± 2 °C culture room. Treatments were replicated thrice with 10 apical meristems in each replication. Results were expressed as mean ± one standard error (SE) of the three replicates.

RESULTS AND DISCUSSIONS

SURFACE STERILISATION

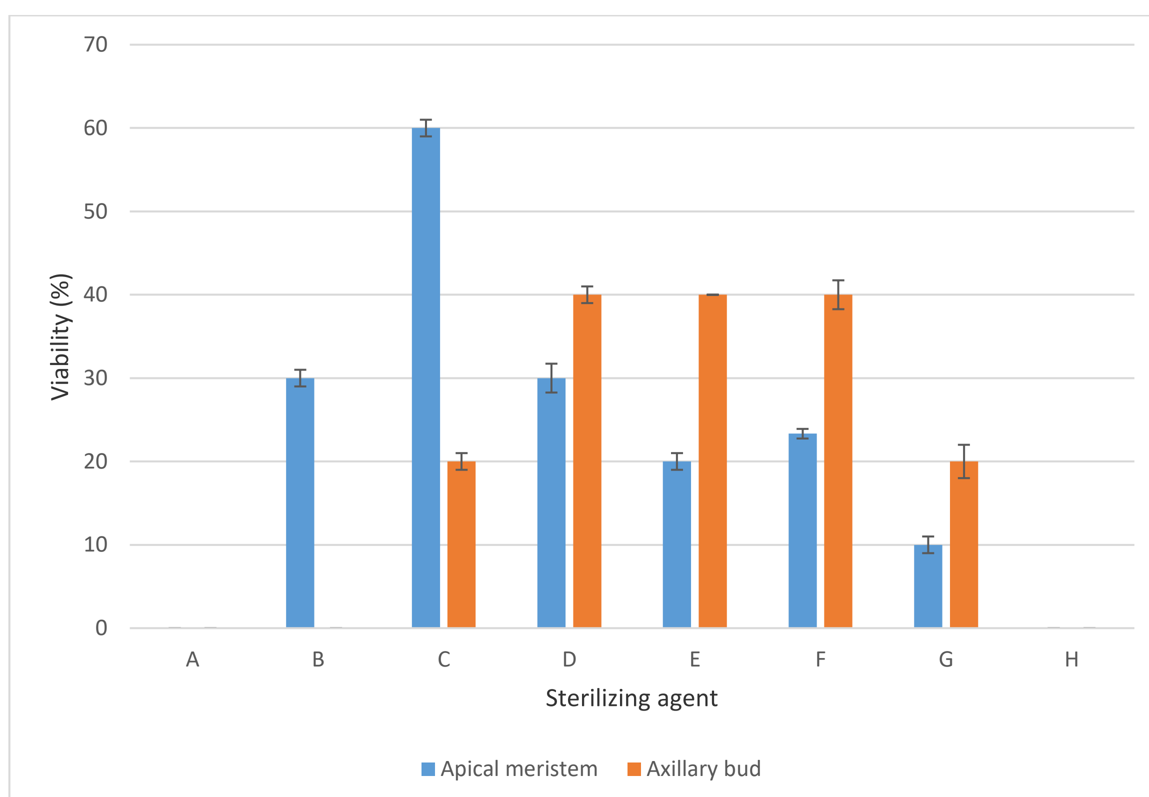
The results showed that 5% (v/v) sodium hypochlorite (NaOCl) for 15 min is the best sterilising agent (Figure 1) exhibiting 60% clean and viable apical meristems. The same treatment applied to the axillary bud only gave 20% uncontaminated explants. At a higher concentration of NaOCl (20%), the viability reduced to 20%. In a study by Ervin & Wetzel (2002), the researchers concluded that surface sterilisation of *Juncus effuses* explants beyond 15 min with 0.5% chlorinated local bleach leads to phytotoxicity. Similar findings were obtained by Pratiwi et al. (2021) where application of sodium hypochlorite at higher concentration increased browing to oil palm explants. Sodium hypochlorite is commonly used for surface sterilisation of explants despite of the availability of other chemicals such as mercuric chloride, calcium hypochlorite and hydrogen peroxide; due to its low cost, safety and its effectiveness in sterilising tissue culture explants. Its has a strong oxidising property and very effective against all kinds of bacteria, fungi, and viruses (Yildiz et al. 2012).

Application of 0.1% (v/v) boric acid for 5 min and 20% (v/v) NaOCl with 70% ethanol for 15 min were unsuccessful when applied to both explants in which 100% death was observed for both types of explants. At higher concentration of 80-90% ethanol can be lethal to oil palm explants (Pratiwi et al, 2021) but in this study the use of 70% ethanol was harmful to *V. bella* apical meristems and axillary bud when exposed for 15 minutes. Ethanol is known as a powerful sterilising agent but it is also phytotoxic. Its application to soft and tender tissues such as apical meristems and axillary bud will damage them. Usually, explants were exposed for only a few minutes; thus it is suggested that exposure time of ethanol should be further explored.

A work performed by Kuppusamy et al. (2019) with *Eucalyptus* hybrid species showed that the best sterilisation method was the application of 0.1% $HgCl_2$ for 3 min and rifampicin (1 mg/mL) for 5 min along with 1% of NaOCl and 70% ethanol. The purpose of surface sterilisation is to obtain clean explants prior to culturing and eliminating all kinds of contaminants. This is the most important step in plant tissue culture and by developing a suitable protocol, higher success rates can be achieved (Wendling et al, 2014). These observations showed that the choice of sterilising agents plays an important role in producing uncontaminated cultures and different types of explants require a different and specific type of sterilising agent and exposure time (Oyebanji et al., 2009).

From the results obtained, it can be concluded that sterilisation with 5% (v/v) NaOCl for 15 min showed more effective decontamination and survival (60%) of apical meristems but with lower survival (20%) for the axillary bud. At 10% and 20% v/v concentration of NaOCl, the apical meristem survival reduced to 30% and 20%, respectively. Other sterilisation agents did not show effectiveness in controlling contamination of *V. bella* apical meristems and axillary buds.

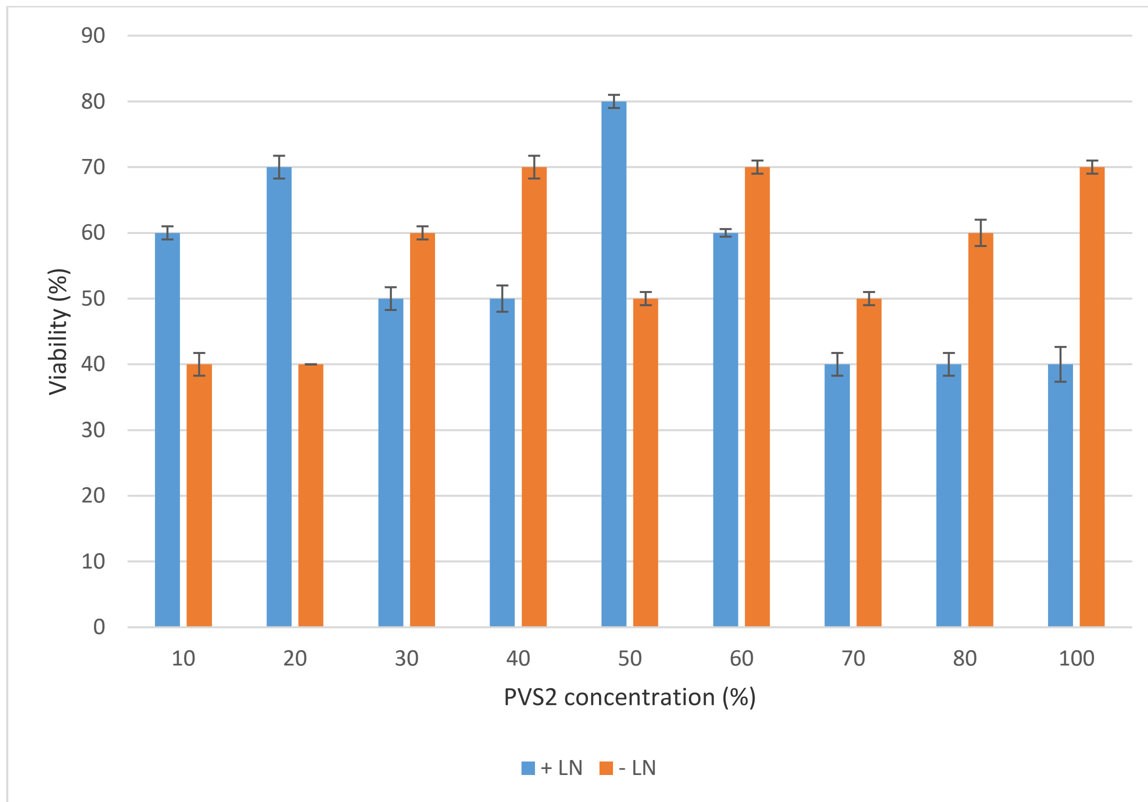
Figure 1. Viability of apical meristem and axillary bud of *V. bella* treated with different types of sterilising agents. A: 0.1% (v/v) Boric acid (5 min), B: 200 mg/L Streptomycin sulphate (10 min), C: 5% (v/v) Sodium hypochlorite (15 min), D: 10% (v/v) Sodium hypochlorite (15 min), E: 20% (v/v) Sodium hypochlorite (15 min), F: 5% (v/v) Sodium hypochlorite + 70% ethanol (15 min), G: 10% (v/v) Sodium hypochlorite + 70% ethanol (15 min), and H: 20% (v/v) Sodium hypochlorite + 70% ethanol (15 min)



VITRIFICATION

In these trials, apical meristems of *V. bella* were treated with different concentrations of PVS2 solution. It was found that *V. bella* apical meristems withstand cryopreservation at all PVS2 concentrations (Figure 2). These results suggest that the PVS2 solution is not highly toxic to the apical meristem of *V. bella.* The highest viability (80%) was obtained when these explants were treated with 50% PVS2. Similar findings were observed in a study conducted by Norafarain et al. (2018) whereby stepwise PVS2 pretreatment at 50% PVS2 for 15 min followed by 10 min exposure in 100% PVS2 exhibited 41.67% survival to the shoot-tips of *Gacinia hombroniana.* However, the application of 50% PVS2 to a tropical and recalcitrant seed species of *Nephelium ramboutan-ake* shoot-tips did not reduce the toxicity effect (Chua & Normah, 2011). At 60% to 100% concentration, the viability reduced gradually from 60% to only 40%. These observations indicate that PVS2 solution can be lethal at a high concentration (Volk et al. 2014). The toxicity of PVS2 is widely recognised. However, the application of cryoprotectant treatments is important prior to cryopreservation as these are a standard step in ensuring the success of vitrification methods for plant species (Kim et al., 2009). A previous study by our group on a tropical forest tree of *Elateriospermum tapos* showed that the embryos exhibited high viability when the application of PVS2 solution was combined with desiccation prior to immersion in liquid nitrogen (Nashatul et al., 2007). This study concludes that the application on 50% of PVS2 solution is recommended for cryopreservation of *V. bella* apical meristems using the vitrification technique.

Figure 2. Viability of cryopreserved (+ LN) and non-cryopreserved (- LN) *V. bella* apical meristem exposed to different PVS2 concentrations



CONCLUSION

In the first part of this study, we have established the surface sterilisation method for apical meristem of *V. bella* by immersion in 5% (v/v) NaOCl for 15 min. Besides, based on our findings, long term *ex situ* preservation of *V. bella* is feasible with the application of vitrification technique. However, further studies are required to increase the rate of regeneration. We also believe that this technique can be applied to apical meristem of other forest tree species.

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