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Vitrification of Dikaryotic Mycelial Cells from *Lignosus rhinocerus*

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ABSTRACT

In Malaysia, *Lignosus rhinocerus* is one of the few important traditional medicinal mushrooms being used by indigenous communities to treat diseases. Currently, this rare mushroom can be found in the deep forest in Peninsular Malaysia, but its number is insufficient to meet the increasing local demand. Therefore, a vitrification technique previously used in the cryopreservation of actinomycetes was adapted in this study to preserve and maintain the commercially potential *L. rhinocerus* strain in a viable state. In this study, combinations of different sucrose concentrations and exposure time were experimented without serial washing phase after thawing. In addition, electron microscopy and comet assay were applied to study the cryoinjury and genotoxity of vitrified mycelial cells. Mycelial cells incubated for 10 minutes in 1.6 M sucrose of Plant Vitrification Solution 2 (PVS2) yielded largest radial mycelial growth with 100% survival rate. Scanning electron microscopy results indicated the swelling of mycelial cells due to osmotic shock which occurred from thawing procedure, while transmission electron microscopy findings revealed fusion of two nucleus membranes of dikaryotic mycelium. Comet assay suggested insignificant differences ($p > 0.05$) of comet formation between the normal and vitrified mycelial cells, suggesting cryoprotectants used in vitrification will

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not cause genotoxity to mycelial cells of *L. rhinocerus*. In conclusion, the current vitrification technique is suitable to cryopreserve the dikaryotic mycelial cells of *L. rhinocerus* with 100% regeneration and without trace of genotoxicity.

Keywords: Lignosus rhinocerus, vitrification, electron microscopy, comet assay

INTRODUCTION

Various organisms in nature are found to be able to survive at low temperature (Cannon & Block, 1988; Storey *et al*., 1988). Those that have adapted to the subzero temperatures are generally classified as either freeze tolerant or freeze avoidant organisms (Cannon & Block, 1988). Freeze avoidant organisms are able to prevent their body fluid from freezing together whilst freeze tolerant counterparts can survive body fluid freezing (Duman, 2001). The cold tolerance strategy raise the interest among cryobiologists to understand these mechanisms which are important for the cryopreservation of mammalian tissues and organs, environment security, as well as the sustainable use of biological resources.

Maintenance of active cultures is expensive, time consuming and prone to contamination for many types of biological samples. Thus, the most practicable way is to cryopreserve the cells. Cryopreservation has long been developed since 50 years ago from the studies of cell freezing to the application in living plant cells. Sakai (1960) first initiated the plant cryopreservation by studying the response of dormant plant buds to liquid nitrogen, and subsequently, freezing of callus tissues in liquid nitrogen was done by Quatrano (1968). This was followed by the first storage of differentiated plant tissues, such as the shoot tips of strawberry (Kartha *et al.,* 1980) and potato (Schafer-Menuhr, 1996). Vitrification (Langis *et al.,* 1990; Sakai *et al*., 1991) was developed and sharply increased the number of species cryopreserved over the years (Sakai *et al.,* 1990, 1991; Sakai & Engelmann, 2007).

Vitrification happens when a highly concentrated cryoprotective solution supercools to very low temperatures (at or below glass transition temperature, T_g) and eventually molecular motion ceases and a glassy solid is formed without undergoing crystallization (Fahy *et al.,* 1984). As a glass, it is extremely viscous, thus at this stage, all chemical reactions that require molecular diffusion will stop and lead to metabolic inactivity, and stability can be sustained over prolonged time (Burke, 1986). Vitrification is generally applied for cryopreservation of cultured plant materials (Sakai *et al.,* 1990, 1991; Niino *et al.,* 1990). Normally, serial dilution is not often practised in vitrification of plant materials such as shoot tips during rewarming procedure, but it still gives promising growth and survival rate (Martin & González-Benito, 2005; Panis *et al.,* 2005; Kami *et al.,* 2009). Therefore, Engelmann's (2009) vitrification protocol for shoot tips in combination with vitrification protocol for Actinomycetes by Stalper and Tan (1996) were adapted to cryopreserve the dikaryotic mycelial cells of Tiger's Milk Mushroom (*Lignosus rhinocerus*).

Lignosus is a macrofungal genus that belongs to Polyporaceae family in Basidiomycota phylum. *Lignosus* comprises 5 species, namely, *L. dimiticus, L. ekombitii, L. goetzii, L. rhinocerus,* and *L. sacer* (Douanla-Meli & Langer, 2003; Núñez & Ryvarden, 2001; Ryvarden & Johansen, 1980), until just recently, a new species called *L. hainanensis* was discovered in the tropical forest of Hainan Province, southern China (Cui *et al.*, 2010). In Malaysia, *L. rhinocerus* is singled out as one of the most popular medicinal mushrooms sought by indigenous communities upon request by herbalists (Lee *et al.*, 2009). The local community has been using *L. rhinocerus* for medicinal purposes since 1930 as described by a local in Pahang, Tuan Haji Mat Yusop (Corner, 1989). *L. rhinocerus* is believed to have therapeutic properties and is used as treatment for asthma, breast cancer, cough, fever, food poisoning and wound healing, as well as a tonic to maintain health (Chang & Lee, 2001).

However, *L. rhinocerus* is rarely found in nature and the amount of wild mushrooms is not sufficient for commercial exploitation and research purposes. Thus, vitrification of mycelial cells for this mushroom was studied. In addition, electron microscopy observation and comet assay were employed to study the effect of vitrification to the ultra-structure and genetic materials of mycelial cells.

MATERIALS AND METHODS

Mycelial Growth

The fruit bodies and tubers of *L. rhinocerus* were collected at the state of Pahang, Malaysia, in June 2009. Pure cultures of *L. rhinocerus* were obtained from the sterilized surface of small pieces of pileus, stipe, and tuber, which were then innoculated to Potato Dextrose Agar (PDA) supplemented with streptomycin (200 µg/L) and incubated in the dark condition for 15 days at 25°C to be used as inoculums. The radial mycelial growth was observed and measured after 10 days. All experiments in this study were conducted in triplicates.

Vitrification

Vitrification experiment was designed using Design-Expert version 6 (DX6) with conduction of two parameters (sucrose concentration and exposure time) and one response (mycelia diameter) as shown in Table 1. Vitrification procedure was applied to a modified procedure of Tan and Stalper (1996). The mycelia from the solid media were placed into 2 mL cryovials followed with loading solution (2 M glycerol and 0.4 M sucrose) for 20-30 min at 25°C. Two millilitres of Plant Vitrification Solution 2 (PVS2) solution containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) and different concentrations (0.4, 1.0, and 1.6 M) of sucrose at pH 5.8 was added and gently mixed vigorously for 5 min after removing the loading solution. Replacement of another 2 mL fresh PVS2 was held at

25°C for different lengths of time without shaking followed by reducing the PVS2 until 0.5 mL in the cryovials, which was subsequently plunged directly into the liquid nitrogen (LN, -196°C) for at least 1 h. Cryovials were rapidly transferred to sterile distilled water in a water bath (37°C – 40°C) for 1.5 min rewarming with vigorous shaking. Immediately, the PVS2 solution was drained from the cryovials and replaced with a 2-mL basal culture medium (Potato Dextrose Broth) supplemented with 1.2 M sucrose which was then left for 20 min. The basal medium was used for 10 min washing at room temperature prior to the growth performance analysis by measuring the mycelia diameter.

TABLE 1
Vitrification experiment design using Design-Expert version 6 (DX6)

Design	Sucrose concentration (M)	Exposure time (min)
1	0.40	10
2	1.00	10
3	1.60	10
4	0.40	20
5	1.00	20
6	1.60	20
7	0.40	30
8	1.00	30
9	1.60	30
10	1.00	20
11	1.00	20
12	1.00	20
13	1.00	20

Electron Microscopy of Vitrified Mycelia

Revived mycelial cells with optimal radial growth were submitted to electron microscopy study. Starting with primary fixation, 1 mm^3 and 1 cm^3 slides of tissues were stored in separate vials for transmission electron microscopy (LEO 912AB-TEM) and scanning electron microscopy (JEOL JSM-6400-SEM), and then they were fixed in fixative (4% glutaraldehyde) for 2 days at 4°C. The slides were washed 3 times with 0.1 M sodium cacodylate buffer at 30 min. Post-fixation was done in 1% osmium tetraoxide for 2 hr at 4°C, and the same washing step was repeated once. Dehydration was performed by a series of acetone with 35%, 50%, 75%, and 95% for 30 min each followed by three (3) changes of 100% at 1 hr.

In the SEM preparation, the specimens were allowed for critical point drying (CPD) by transferring them into a specimen basket, and then they were put into a critical dryer for about half (½) day. Before coating with gold coating in a sputter coater for SEM viewing, the specimens were stuck onto a stub using a double-sided tape or colloidal silver. For TEM viewing, a mixture of acetone and resin was used to infiltrate the specimens before placing them into beam capsules which were then filled up with resin. Polymerization was allowed in an oven set at 60°C for 1-2 days before going through the thick and ultra-thick sectioning. The section was stained with uranyl acetate for 10 min.

Comet Assay of Vitrified Mycelia

In addition to electron microscopy study, the revived mycelial cells with optimal radial growth were also submitted to comet assay. Approximately 0.03 g of untreated and cryopreserved mycelia from the agar medium was added into 1 mL of Y1 buffer (1 M sorbitol, 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 7.4 and 0.1% beta-mercaptoethanol) before the addition of 0.25 mg/mL enzyme chitinase (Torres-Bauza & Riggsby, 1980) and incubated at 37°C with mild shaking for 2 hr.

Comet assay was performed with minor modification from Singh *et al.* (1988). A base layer of 1.0% agarose on a microscope slide was added with 50 µL of the cell agarose mixture (50 µL of 0.7% agarose mixed with 10 µL of spheroplasts in Y1 buffer) and covered with a cover slip. The slides were then allowed to dry on a flat surface on ice for 2 min before the addition of the last layer of 0.7% agarose. Again, the agarose was allowed to solidify on ice for 2 min. The uncovered slides were then immersed in a freshly made lysing solution composed of a 100-mL stock solution (1.25 M NaCl, 50 mM EDTA, 100 mM Tris base, adjusted to pH 10 and topped up with 0.1% sodium dodecyl sulphate). Subsequently, 1.5 mg/mL proteinase K was added and incubated at 37°C overnight. After lysis, the slides were transferred to the electrophoresis unit, filled up with a fresh electrophoresis solution (300 mM NaOH, 1 mM EDTA, 0.2% DMSO and 0.1% hydroxyquinoline) and left for 20 min. A current of 12 V (0.4 V/cm, 250 mA) was applied for 20 min before the slides were immerged in a neutralization buffer (50% ethanol, 20 mM Tris-HCl, pH 7.4) for 30 min. Followed by another 30 min in a new buffer, they were then oven dried at 50°C. Lastly, the slides were stained with the SYBR-Green dye and evaluated at 20x magnification using a ZeissAxiovert 200M fluorescence microscope.

DNA damage on the untreated and cryopreserved mycelial cells was evaluated using the Tritek Comet Score™ (Ver. 1.5) software. During evaluation, the percentage of DNA in the tail of each cell was recorded. The comets were categorized into four classes (0, 1, 2, and 3), namely; (0) tail size with no tail, (1) short tail length smaller than the diameter of the head (nucleus), (2) tail length between 1 and 2 times the head diameter, and (3) long tail more than twice the diameter of the head. The comets with no head and very wide tail observed were excluded from the analysis which represented dead cells (Hartmann & Speit, 1997).

RESULTS AND DISCUSSIONS

Radial Growth of Vitrified Mycelial Cells

The current results suggest that the mycelial cells exposed to PVS2 at 1.6 M sucrose and 10 min yielded the largest radial mycelial growth diameter, while those treated with 0.4 M sucrose at 30 min yielded the smallest radial mycelial growth diameter (Table 2). PVS2 plays an important role in obtaining a sufficient level of dehydration, which allows growth after cryopreservation (Yamada *et*

al., 1991) and protects the mycelia from damaging effects due to freezing. Higher sucrose concentration is ideal because the hydroxyl group present in sucrose will interact with phospholipid membrane by hydrogen bonding and membrane stabilization imparting during dehydration (Crowe & Crowe, 1986). In addition, shorter incubation period is ideal because PVS2 is potentially harmful due to phytotoxicity or osmotic stress damages (Rajnesh *et al.*, 2008). This current study indicates that higher sucrose concentration in combination with shorter incubation period is ideal for the revival of vitrified mycelia cells of *L. rhinocerus*.

Electron Microscopy Observation of Vitrified Mycelia from L. rhinocerus

Cryopreserved mycelial cells can be regenerated with no significant growth difference compared to normal mycelial cells. However, it is unclear on the effects of vitrification without serial dilution washing to the ultra-structure of cryopreserved mycelial. Hence, SEM and TEM experiments were conducted to study the ultra-structure of cryopreserved mycelial cells with the optimal regrowth rate (design 3, treatment of PVS2 containing 1.6 M sucrose at 10 min).

The SEM images indicated that the ultra-structures of cryopreserved hyphaes were bigger compared to normal hyphaes (Fig.1A and Fig.1B). This phenomenon could be due to osmotic shock and ice-crystal formation during freezing (Karlsson & Toner, 1996; Farrant, 1980). Throughout cooling process, ice formation will lead to the occurrence of osmotic imbalance or shock in which water migrates across cell membrane and cell wall (Ferrant, 1980). In addition, lipid is potentially deleted from

TABLE 2
Survival rates of cryopreserved mycelial cells based on mycelia diameter in triplicate measurement

Design	Sucrose concentration (M)	Exposure time (min)	Mycelium diameter		Mycelia regrowth on plate (%)	Survival rate (% ± S.E)
			Actual	Predicted		
1	0.40	10	2.0	2.005172	67	67 ± 0.004
2	1.00	10	2.7	2.656322	100	100 ± 0.031
3	1.60	10	2.9	2.938506	100	100 ± 0.192
4	0.40	20	1.6	1.622989	100	100 ± 0.163
5	1.00	20	2.4	2.324138	67	67 ± 0.054
6	1.60	20	2.7	2.656322	67	67 ± 0.031
7	0.40	30	1.4	1.371839	67	67 ± 0.020
8	1.00	30	2.1	2.122989	100	100 ± 0.515
9	1.60	30	2.5	2.505172	100	100 ± 0.004
10	1.00	20	2.3	2.324138	67	67 ± 0.017
11	1.00	20	2.4	2.324138	67	67 ± 0.054
12	1.00	20	2.3	2.324138	67	67 ± 0.017
13	1.00	20	2.2	2.324138	67	67 ± 0.088

Mean±SD (n= 3)

membrane during osmotic dehydration, which reduces the ability for the cell to return its isotonic volume during rehydration (Steponkus *et al.,* 1983).

Complimentary to SEM, TEM was conducted to study the cell wall, membrane plasma, nucleus and cytoplasmic content of the cryopreserved mycelial cells. The TEM observation of both normal and cryopreserved mycelial cells (Fig2A and Fig.2B) suggested that the cell wall of vitrified mycelium was ruptured. The current findings could be due to the formation of cell wall made from chitin which possesses priority in terms of strength and rigidity and can withstand mechanical stress. This

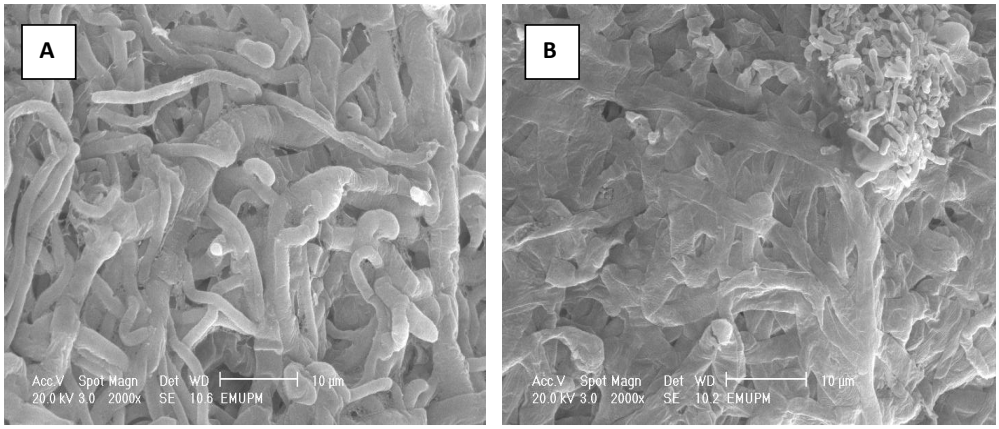

Fig.1: Ultrastructure of mycelial cells: (A) normal mycelial cells (control); (B) cryopreserved mycelial cells with enlarged and swollen hyphae due to cryoinjury. Magnification: 2000x (A, B).

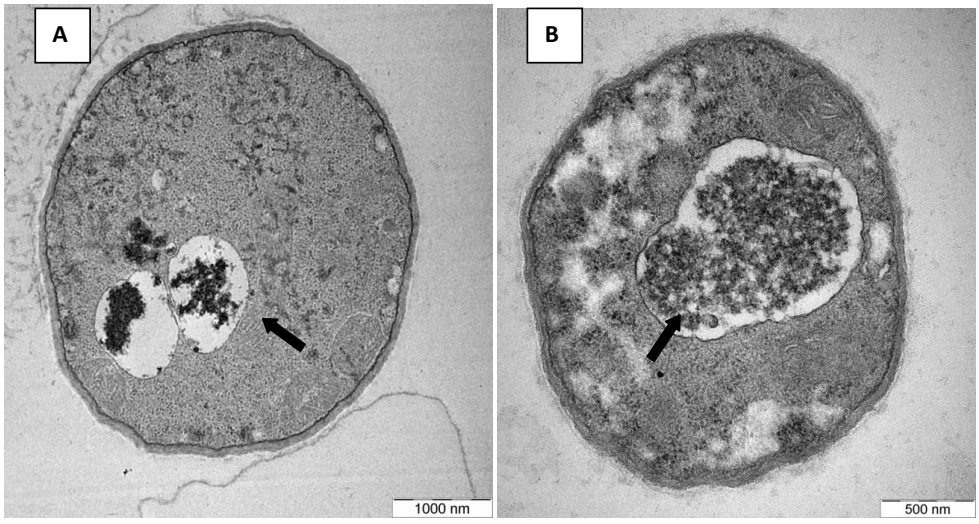

Fig.2: Cross section of mycelium: (A) normal mycelium (control); arrow shows two nucleus (n + n); (B) cryopreserved mycelium with ruptured cell wall and arrow shows fusion of two nucleus membranes. Scale bars = 1000 nm (A), 500 nm (B).

could be due to branched polysaccharide linked by α-1,4 and β-1,4 glycosidic bond in chitin that is composed of amino sugar N-acetyl-D glucosamine [GlcNAc] (Ibrahim *et al.,* 2000), which is not in cellulose that can strengthen the bond and cell wall structure. Thus, the cell wall is strong enough to withstand the osmotic pressure from the difference in solute concentration between the cell interior and distilled water (Howland, 2000), which in turn makes the cell wall remain intact.

In addition, Fig.2B illustrates the fusion of nuclear membrane from two nucleuses, and the occurrence of this phenomenon was unclear. The white region probably represented non-dehydrated zone where water still remained in the cell after vitrification. Farrant (1980) suggested that if too much water remains inside the cell, damage due to ice crystal formation and recrystallization during warming might occur.

Comet Assay of Vitrified Mycelia from L. rhinocerus

Table 3 summarizes the percentage of comet tails of the untreated and cyropreserved *L. rhinocerus* mycelial cells with 10.506 ± 0.898 and 10.836 ± 0.807, respectively. Statistical analysis demonstrated insignificant differences ($p > 0.05$) of comet formation between the untreated and cryopreserved *L. rhinocerus* mycelial cells, indicating that the DNA damage inflicted by cryopreservation to the mycelial cells was minimal.

Vitrification is an alternative cryopreservation method for those organisms that cannot stand at slow cooling as studying species. The cooling rate is important for a period of time before cooling to liquid nitrogen temperatures. This process enhances the dehydration of the cytoplasm prior to freezing. The noxious effects of toxic vitrification solution are minimized by performing the exposure towards PVS2 as brief as possible (Tan & Stalper, 1996). The choice of a cryoprotective agent is dependent upon the type of cell to be preserved. Cryoprotectants work as an antifreeze to prevent formation of ice crystals and aid in the process of vitrification. As in PVS2 solution, a mixture of cryoprotectants (glycerol, ethylene glycol, and DMSO) was used. When used together, cryroprotection was enhanced as much as double than when used alone, possibly because of a decrease in toxic effect (Ulrich *et al*., 1979). They work by diffusing into cells and replacing much of the cells' water. This indicates that PVS2 solution is an applicable cryoprotectant for vitrification of *L. rhinocerus*.

TABLE 3
The percentage of DNA in comet tail of the untreated (control) and cryopreserved *L. rhinocerus* mycelial cells

Samples	Percentage of DNA in tail (%) Mean ± SD
Untreated (control)	10.506 ± 0.898
Cryopreserved	10.836 ± 0.807

Mean±SD (n= 3).
*$p > 0.05$; t test.

CONCLUSION

The current study elucidates the efficiency of vitrification technique and subsequent impact towards dikaryotic mycelial cells of *L. rhinocerus*. Although serial dilution during the washing stage was not applied in the thawing phase, all vitrified samples were successfully regenerated. The current findings suggest that vitrification method remains an easy and inexpensive method for long-term preservation of mycelial cells from Basidioamycetes or more specifically Polyporaceae.

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