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Cryopreservation of Kluai Namwa (*Musa* × *paradisiaca* 'Kluai Namwa')

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ABSTRACT

Cryopreservation encapsulated-vitrified meristem of K.Namwa stored in liquid nitrogen with various loading solutions were investigated. A mixture of 2M glycerol and 0.4 M sucrose could produce 85 percent survival of meristem while 2M ethylene glycol without sucrose gave only 5 percent survival of meristem with pale green colour. After treating the encapsulated-vitrified meristem with loading solution in liquid nitrogen for 1 hour and then transferred to modified MS medium for two weeks, they returned to green colour. All of them were recultured in MS media and proliferation occurred within two months.

The study on shoot development of encapsulated-vitrified meristems, which were dehydrated in PVS2 at various times of 0 10 15 20 25 30 35 and 40 min. at 25°C, before cryopreservation was conducted. The treated encapsulated-vitrified meristems were kept in liquid nitrogen for 1 hour, and transferred to modified MS medium for two months. The result showed that meristems treated with PVS2 for 25 min. gave the highest shoot proliferation of 85 percent followed by 30 min. of 80 percent. The shoot proliferation decreased with increasing times of dehydration which was similar to the one that was not kept in liquid nitrogen.

Key words: cryopreservation, Kluai Namwa, Pisang Awak, *Musa* × *paradisiaca*

INTRODUCTION

Kluai Namwa (K.Namwa) or Pisang Awak, an ancient Thai banana, is a herbaceous pereninal plant belonging to Family Musaceae, Order Zingiberales or Sciataminaceae (Simmonds, 1966) which has been cultivated throughout the country. It is often used as the first solid food fed to infants. K.Namwa provides a more balanced diet than any other fruit and vegetable. It is filling, easy to digest, nearly fat free, rich source of carbohydrate with calorific value of 67/100gm. Flesh of banana is free of sodium, contains various vitamins and has therapeutic value for the treatment of many diseases.

K.Namwa is quite tolerant to arid soil and some diseases. The serious disease of K.Namwa is Fusarium wilt race1 caused by *Fusarium oxysporum* cubense. (Daniells, and Bryde, 2001). It is a soil borne disease destroying many orchards of K.Namwa. Several controls have been employed, including field sanitation, soil fumigation and liming but without success. Crop rotation with paddy has been shown effectiveness in controlling the disease only for 1 to 2 years in the same field. The disease is under controlled by replacing the susceptible cultivar with the resistant one(Hwang,1985). There is an urgent need for conserving K.Namwa *in vitro* because widespread disease of Fusarium wilt is serious threat to germplasm in nature or in the field conservation Method of *in-vitro* storage of germplasm includes development of micropropagation system, slow growth storage and

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cryopreservation.

Cryopreservation is a long-term germplasm storage *in vitro* using cryogenic methods. Storage in liquid nitrogen (liquid N) at –196°C will overcome the problem of repeated subculturing, contamination of cultures and avoiding somaclonal variation (Banerjee and de Langhe, 1985). The success of cryopreservation of *Musa* germplasm using cell suspension and meristem have been reported by several authors. (Panis *et al.,* 1990; Panis and Swenen, 1993). Glycerol, ethylene glycol (EG) and dimethyl sulphoxide (DMSO) were used as loading solution and PVS2 (plant vitrification solution no.2) as dehydrating solution before storing in liquid N for apple, pear, and wasabi (Sakai and Nishiyama, 1978; Sakai *et al* 1990; Matsumoto and Sakai, 1995). The use of meristem encapsuled in a gel and dehydrated using osmoticum were also done. The 3% Na-alginate was reported for encapsulated shoot tip of Basrai Banana (Gunapathi *et al*., 1992). Those beads were directly plunged in liquid N and thus the meristem could theoretically be stored for an indefinite period of time. The plants were recovered by thawing the beads rapidly and placing them on recovery medium. This technique of cryopreservation has also been applied for long-term storage of seed of *Musa balbisiana* (Bhat *et al.,* 1994).

The studies were conducted with the various loading solutions and dehydrating times before storing in liquid N for cryopreservation of K.Namwa.

MATERIALS AND METHODS

One mm. size meristem of K.Namwa sword suckers were used as explants. The explants were precultured in 1/2MS (1962) medium supplemented with 0.3M sucrose for one day. Then the explants were loaded in various solutions having 3%Na-alginate, with and without sucrose. There were 7 treatments and 20 replications (5 explants for one replication) in the experiment.

Experiment 1 Suitable loading solution

The loading solutions were: control or no loading solution, 2M glycerol with and without 0.4M sucrose, 2M ethylene glycol (EG) with and without 0.4M sucrose and 2M dimethyl sulphoxide (DMSO) with and without 0.4M sucrose. After 20 min. in loading solution, the explants were transferred to liquid MS medium with 0.1M $CaCl_2$ and 0.4M sucrose for 30 min. The explants which would be covered with clear gel, was called encapsulated vitrified meristem. Then, the encapsulated-vitrified meristems were soaked in PVS2 which composing of 30% glycerol, 15% ethylene glycol, and 15% DMSO for 20 min. (Sakai *et al.*,1990). The capsules were then plunged directly in liquid nitrogen for 1 hour. The freezed capsules in 40°C water would be thawed and unloaded by soaking in 1.2 M sucrose for 20 min. for 2 times and cultured in semi solid MS medium.

Experiment 2 Proper time for dehydration with PVS2

The procedure of this experiment was similar to experiment1. The best loading solution in experiment1 was used for encapsulation in experiment 2. The encapsulated vitrified meristems were then soaked in PVS2 as dehydration at 25°C for 0,10,15,20,25,30,35, 40 min. and kept in liquid nitrogen for 1 hour. Thawing and unloading were the same as experiment 1.

After cultured in semi solid MS medium, the number of green meristems or survival meristems and number of shoot were recorded.

The experiment was conducted at Department of Horticulture, Faculty of Agriculture, Kasetsart University. Bangkok, Thailand.

RESULTS AND DISCUSSIONS

Experiment 1

When the meristems of K.Namwa were precultured with 0.3M sucrose for 1 day, and then the encapsulated vitrified meristems were made in

Na-alginate having different loading solutions, it was found that the mixture of 2M glycerol and 0.4M sucrose gave the highest survival of 85% followed by 2M DMSO and 2M ethylene glycol (both with sucrose) respectively. The loading solution giving the lowest survival of 5% was noticed in 2M ethylene glycol without sucrose (Table1). The results, which were similar to those reported by Sakai and Nishiyama (1978), Sakai *et al*. (1990) and Matsumoto and Sakai (1995), showed the loading solution with no sucrose to have less survival than those with sucrose. The meristems, when taken out of liquid nitrogen, became pale green, while when cultured in MS media, for 1 day the colour of meristems changed to yellow brown and later to black, the sign of dead tissue. However, the meristems in the loading solution with sucrose especially the one with the mixture of 2M glycerol, when cultured in MS media for 2 weeks was found to regain the greeness (Figure 1). The encapsulated -vitrified meristems transferred to be recultured in the previous formula media were also noticed to be able to grow into normally physiological plants again in 2 months (Figure 2). The use of cryoprotectant, as the loading solution, alone revealed the low survival since the cryoprotectant which acted in dehydrating water from cells, caused them wilted before soaking in liquid nitrogen. If the appropriate concentration was employed, the cell would then be protected from ice-crystal damage. Besides, cell membrane would be protected while the denaturing of protein and nucleic acid would be prevented (Towill,1991). Still, if too much concentration was applied, the chemical toxicity might occur resulting in low survival. It should, thus, be used with other cryoprotectants especially sucrose of low concentration, which would increase greater meristem survival of carnation than the application of cryoprotectant alone (Uema and Sakai,1980). Sucrose, the energy source for cells, its carbon is used in cell wall forming and maintaining osmotic pressure. It is the osmotic active substance which confers vigor to plant, enabling it to tolerate any stress such as dehydration, coldness etc. In comparison of chemical toxicity, glycerol was found to be solution of the least toxicity (Sakai *et al.*, 1990). Therefore, when glycerol is used with low concentrated sucrose, together they enable the plant to tolerate to dehydration as well as freezing. Cryoprotectant will cause changing in cell size making cells to be more flexible. In addition, the application with sugar or other cryoprotectants will prevent the toxicity of the added substances resulting in the highest survival. It was also found that the soaking of meristem in loading solution was of necessity in increasing survival of cells and tissue by giving cells to have dehydration tolerance.

Experiment 2

When the meristems of K.Namwa which went through preculturing and then were made into encapsulated-vitrified meristems in loading solution with the mixture of 2M glycerol and 0.4M sucrose for 30 minutes, after which, the encapsulated-vitrified meristems obtained were soaked in PVS2 solution at 25°C for various different times before being stored in liquid nitrogen and without liquid nitrogen, it was found that the encapsulated-vitrified meristems soaking in PVS2 for 25 minutes gave the highest shoot tip developing of 85% followed by 30

Table 1 Loading solutions affecting the survival of encapsulated -vitrified meristem stored in liquid nitrogen after which transfer to be cultured in MS media for 2 weeks.

Loading solution	Survival(%)
Control	37
2M glycerol+0.4M sucrose	85
2M glycerol	32
2M EG+0.4M sucrose	52
2M EG	5
2M DMSO+0.4M sucrose	63
2M DMSO	12

min. of 80% when stored in liquid nitrogen (Table2). As for the encapsulated-vitrified meristems which had not gone through PVS2 soaking, the meristems turned black after transferred to be cultured in MS media for 1 day, whereas the encapsulated-vitrified meristems which had not gone through storage in liquid nitrogen and was not dehydrated with PVS2, acquired the highest shoot development of 100%. The shoot development decreased with the increasing times of going through dehydration with PVS2. The results were similar to those maintained in liquid nitrogen, that was, the dehydration with PVS2 for longer than 25 min., causing the reduction in shoot tip development.

The 25-30 min. period was appropriate for PVS2 to make balance in cell dehydration and making replacement in plant cells. The nature of PVS2 was that when being stored in liquid nitrogen, instead of forming ice crystal like water, it would create clear crystal covering meristem, preventing cell damage, thus resulting in the highest survival. In less than 25 min. period, the exchange of substance might not be balanced resulting in rather great amount of water remaining in cells. When stored in liquid nitrogen, such water in cell would form ice crystals with greater volume than cell volume, making cell disrupted, followed by death.

As for those soaked in PVS2 longer than 25 min., there would be tendency in shoot developing to decrease, which was quite similar to encapsulated-vitrified meristems that were not stored in liquid nitrogen. It might be due to the chemical toxicity and osmotic pressure, which was harmful to cells, resulting in percent decreasing of shoot development. Therefore, the success in maintaining by encapsulated-vitrification of cryopreservation will depend on cautious cell dehydration, permeability of cryoprotectant into cells and drainage prevention from too much osmotic pressure (Rall,1987).

Figure 1 Encapsulated-vitrified meristems after going through storage in liquid nitrogen and culturing in MS media for 2 weeks.

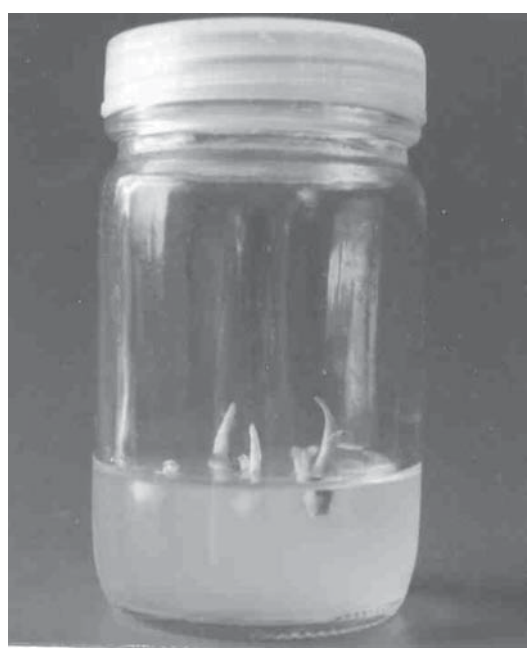

Figure 2 Plantlets developed from encapsulated-vitrified meristems after transferred to be recultured in MS media for 2 months.

Table 2 Dehydration with PVS2 through various times at 25°C with and without going through liquid nitrogen storage affected shoot development after recultured in MS media for 2 months.

Time (min.)	Percentage of shoot	
	Liquid nitrogen	Without liquid nitrogen
0	0	100
10	25	95
15	40	92
20	72	90
25	85	92
30	80	73
35	60	60
40	45	54

CONCLUSIONS

1. The appropriate loading solution for storage of meristem of K.Namwa in liquid nitrogen was the one with the mixture of 2M glycerol and 0.4M sucrose which gave the highest survival rate of 85% followed by 2M DMSO and 2M EG (both with sucrose) respectively.

2. The soaking of encapsulated-vitrified meristems in PVS2 at different periods of time found the 25-30 min.to be the most appropriate in storing the meristems of K.Namwa due to the highest shoot development of 80-85% obtain.

3. The kind of loading solution and the most suitable period in dehydration with the essential factors in the success of storing meristems of K.Namwa in liquid nitrogen was found to be the employing encapsulated vitrification of cryopreservation.

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