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Development of *in vitro* propagation by node culture and cryopreservation by V-Cryo-plate method for *Perilla frutescens*

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Abstract: A clonal propagation method by node culture of perilla (*Perilla frutescens* L. Britton) was investigated. Nodes were plated on solidified 1/2 MS medium with BA and optimum shoot elongation and propagation was obtained at BA 0.05 and 0.1 mg l^{-1}. Cryopreservation using an aluminum cryo-plate was successfully applied to *in vitro*-grown perilla shoot tips. Excised shoot tips from nodes were precultured on 1/2 MS medium with 0.3 M sucrose and embedded on an aluminum cryo-plate with alginate gel. The cryo-plate with shoot tips was osmo-protected with LS solution and dehydrated in PVS2 for 20 min at 25°C prior to immersion into liquid nitrogen. The recovery growth after cryopreservation was found to be about 80%. This new V-Cryo-plate method has many advantages and may facilitate the cryo-storage of other medicinal plants.

1. Introduction

Perilla (*Perilla frutescens* L. Britton) belongs to the *Lamiaceae* family and is cultivated in China and Japan (Pandey and Bhatt, 2008). The seeds yield oil and the leaves are used for medicine or garnish for fish (Nitta *et al.*, 2005; Hossain *et al.*, 2010). Dried red *Perilla* leaves are also used as 'Soyou' in Kampo medicine and it is one of the components of 'Saibokuto,' which is used to treat bronchial asthma (Homma *et al.*, 1992; Ueda *et al.*, 2002). The seeds of Perilla have been used for food for birds or humans, oil as a fuel or a cooking oil, and also the leaves are used as a potherb for medicine or food coloring and the foliage to produce an essential oil for flavoring (Brenner, 1993). Perilla has a variable chromosome complement (Brenner, 1993); a haploid chromosome count of fourteen plus zero to two beta chromosomes (Vij and Kashyap, 1976) and chromosome counts of both n=20 and 2n=38, and three distinguished chromosome sizes (Yamane, 1950). As the traditional crop landraces are facing danger of complete extinction in some areas, some of *Perilla frutescens* species are at the verge of extinction in the Central Himalaya (Negi *et al.*, 2011). Usually *Perilla* species are conserved in genebanks as plant genetic resources (Arora, 1997). Biotechnological tools are important to conserve the critical genotypes of medicinal plants like *Perilla* species. However, previous reports of plant regeneration for *Perilla* used seedlings segments such as the cotyledon and hypocotyl as material (Hou and Jia, 2005; Zhang *et al.*, 2005; Hossain *et al.*, 2010). Moreover, there are few reports about effective clonal propagation of *Perilla*. Cryopreservation techniques are now used for plant germplasm storage at several institutes around the world (Niino, 2006) and this method has become an important tool for the long-term storage of plant germplasm and of experimental materials that possess unique attributes, minimizing space and maintenance requirements without causing genetic alterations (Sakai, 1997; Matsumoto *et al.*, 2013). Recently, a vitrification protocol using the aluminum cryo-plate method (V-Cryo-plate method) has been reported (Sekizawa *et al.*, 2011; Yamamoto *et al.*, 2011 a, b; 2012). Niino *et al.* (2013) reported that the V-Cryo-plate method has two main advantages: a user-friendly procedure and very high cooling and warming rates of treated samples. As a result, very high regrowth was obtained after cryopreservation of the tested materials (Niino *et al.*, 2013).

In this study, we have developed effective clonal propagation by node culture and cryopreservation of *in vitro* grown shoot tips using the V-Cryo-plate method for *Perilla*.

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2. Materials and Methods

In vitro *propagation by node culture*

The material, seeds of *Perilla frutescens* L. Britton, was obtained from Tsukuba Division, Research Center for Medicinal Plant Resources, National Institution of Biomedical Innovation (Tsukuba, Japan). The seeds were surface sterilized in 70% ethyl alcohol for 30 sec then in 1% NaOCl for 15 min. After rinsing three times in sterile distilled water, they were placed on hormone-free half-strength solidified MS medium (Murashige and Skoog, 1962; termed 1/2 MS) and incubated at 25°C with 16 h light/8 h dark photoperiod under a light intensity of 50 µmol m^{-2} s^{-1} for germination. After one month, the axillary buds were placed on solidified 1/2 MS medium with 0, 0.01, 0.05, 0.1 and 0.5 mg l^{-1} BA and incubated. The shoot length, number of leaves, number of shoots, number of roots and longest root length were measured. Ten buds were used for each treatment with three replicates.

Cryopreservation by V-Cryo-plate method

Axillary shoot tips (1 mm size) were excised from *in vitro* grown plants of *Perilla* and used for cryopreservation by V-Cryo-plate method (Yamamoto *et al.*, 2011 a, b) with some modification. Figures 1 and 2 show the schematic diagram of the aluminum cryo-plate used and the V-Cryo-plate procedure. Cryo-plates used in this study were obtained from the National Institute of Agrobiological Sciences (Tsukuba, Japan). The following steps were performed:

1) Excised shoot tips were precultured on solidified 1/2 MS medium with 0.3 M sucrose for 1 day at 25°C to induce the osmo-protection.
2) An aluminum cryo-plate (Fig. 3) was placed in a petri-dish and 2.0-2.5 µl of 2% (w/w) Na-alginate solution with 0.4 M sucrose in 1/2 MS medium was poured in a well.
3) The precultured shoot tips were positioned in each well and 100 mM $CaCl_2$ solution with 0.4 M sucrose in 1/2 MS were added to the aluminum plate for 15 min for polymerization (Fig. 3).
4) After removing $CaCl_2$ solution, the cryo-plate with shoot tips was treated with LS solution (2 M glycerol + 0.6 M sucrose) (Matsumoto *et al.*, 1994; Yamamoto *et al.*, 2011 b) in a petri dish (8 cm in diameter) for 20 min at 25°C for osmo-protection.

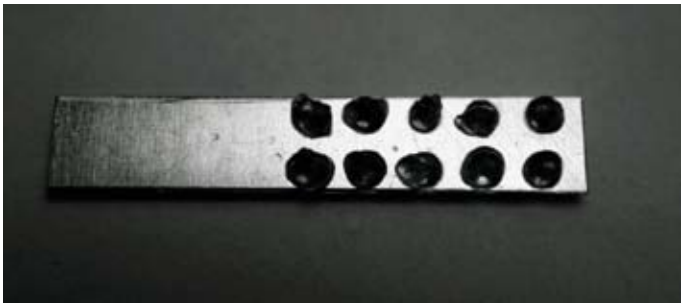

Fig. 1 - Aluminum cryo-plate with embedded shoot tips. Size: 7 mm × 37 mm × 0.5 mm with ten wells (diameter 1.5 mm. depth 0.75 mm).

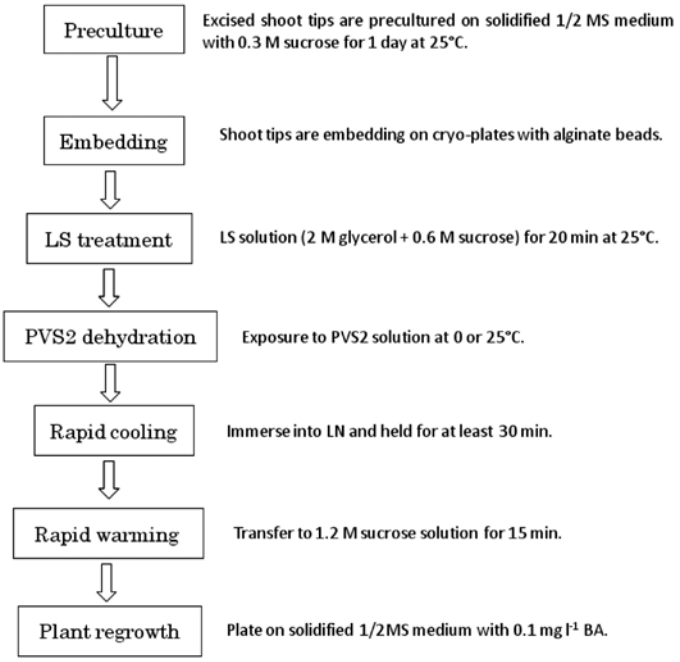

Fig. 2 - Procedure of V-Cryo-plate for cryopreservation of *Perilla* shoot tips.

Fig. 3 - Shoot formation by node culture after 2 months 1/2MS + BA 0.1 mg l^{-1}. Bar = 10 mm.

5) After LS treatment, the cryo-plate with shoot tips was dehydrated with PVS2 solution (Sakai *et al*., 1990) for 0 to 40 min at 0 C and for 0 to 20 min at 25°C.
6) The cryo-plate was then transferred to an uncapped 2 ml cryotube held on a cryo-cane and directly plunged into liquid nitrogen (LN) for at least 30 min.
7) For plant regeneration, the cryo-plate with shoot tips in LN was transferred to 1.2 M sucrose solution with 1/2 MS in a petri dish (8 cm in diameter) for 15 min at 25°C for rapid warming and unloading. Shoot tips were then plated on solidified 1/2 MS medium with 3% sucrose and incubated at 25°C under standard conditions.

Three replicates of 10 shoot tips were tested in each experiment. Statistical analyses were performed using Tukey's test and significant differences ($P<0.05$) were determined.

3. Result and Discussions

In vitro *propagation by node culture*

After incubation for 30 days on 1/2MS medium with different concentrations of BA, all nodes of *Perilla* formed shoots without callus formation. The optimum shoot elongation and propagation was obtained at BA 0.05 and 0.1 mg l^{-1} (Table 1). In previous reports, plant regeneration of *Perilla* was obtained from hypocotyl segment including apical bud (Hossain *et al.*, 2010), cotyledon, and hypocotyl (Zhang *et al.*, 2005). The shoot recovery rate of this node culture (100%) is higher than that of the previous reports (65 to 91%). Moreover, regenerated plants from cotyledon and hypocotyl segments are not genetically the same and the propagation systems are not suitable for clonal propagation. In this experiment, 1.6 shoots with 5.8 leaves (average of BA 0.05 and 0.1 mg l^{-1}) were obtained from one node after 30 days of incubation. Nodes were located basal respect to each petiole, and we found that one node produced 9.28 nodes (5.8 × 1.6) after 30 days incubation. This propagation efficiency (9.28) is considered to be as high as clonal propagation. Furthermore, no abnormalities were observed in the regenerated shoots (Fig. 3) indicating that this method can be considered a suitable propagation method for the material of cryopreservation.

Table 1 - Effect of BA concentration on shoot regrowth from nodes in Perilla

BA (mg l^{-1})	Shoot length (mm)	No of leaves	No of shoots	No of roots	Longest root (mm)	Re-growth (%)
0	6.7 ab	5.4 a	1.0 b	1.9 b	26.5 ab	100
0.01	10.1 a	5.1 ab	1.0 b	1.4 ab	30.7 a	100
0.05	11.3 a	6.0 a	1.5 a	2.2 b	16.3 ab	100
0.1	8.2 a	5.6 a	1.7 a	1.3 ab	10.8 ab	100
0.5	5.1 b	3.7 b	1.0 b	0.7 a	6.2 b	100

Cryopreservation by V-Cryo-plate method

In our previous reports of cryopreservation using vitrification (Matsumoto *et al.*, 1994; 1995), we demonstrated that the osmo-protection treatments (preculture of high sucrose medium, LS treatment of 2 M glycerol + 0.4 M sucrose solution) were necessary to produce high regrowth before PVS2 dehydration for shoot tips of most plant species. The V-Cryo-plate procedure was carried out according to Yamamoto *et al.* (2011 a, b) with these two osmo-protection treatments. Figure 4 shows the regrowth rates with different exposure times to PVS2 at 0 and 25°C. The highest regrowth rates obtained were about 55% for 30 min at 0°C and about 70% for 15 min at 25°C. In the vitrification protocol, direct exposure of less tolerant cells and meristems to highly concentrated PVS2 at 25°C was found to possibly be harmful due to osmotic stress or chemical toxicity (Matsumoto and Sakai, 2003). In this experiment, the regrowth rate at 0°C was 15% lower than that at 25°C. The reason of this result was not clear, but might be related to problems associated with sensitivity to PVS2 and/or dehydration. The high regrowth rate after cryopreservation by V-Cryo-plate method is due to rapid cooling (4,000-5,000°C min^{-1}) and warming (3,000-4,500°C min^{-1}) (Niino *et al.*, 2013). In vitrification-based procedures, damage can be caused by chemical toxicity of PVS2 and osmotic stress by excessive duration of PVS2 treatment (Engelmann, 1997; Sakai *et al.*, 2008). Our results suggest that this high regrowth rate with exposure to PVS2 at 25°C may be due to the rapid cooling and warming. For practical use for cryopreservation, the time for the cryogenic procedure should be short and the regrowth rate after cooling should be high (at least 70%). It is worth noting that no abnormalities were found in shoots developed from

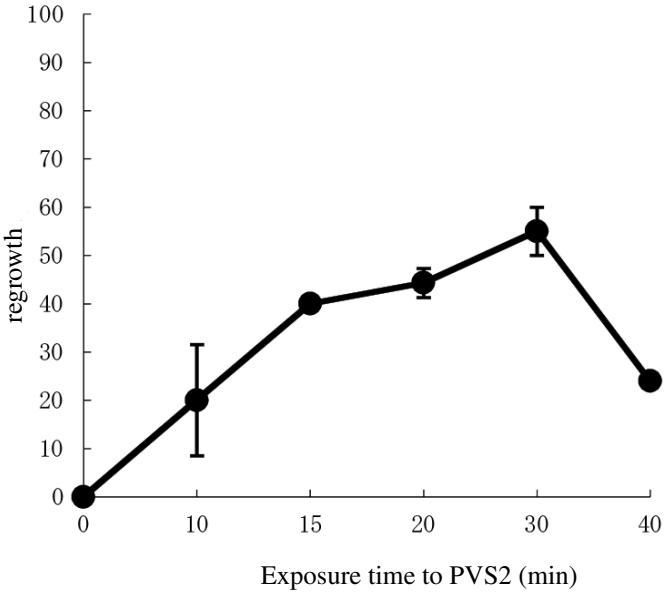

Fig. 4 - Effect of different exposure times and temperatures of PVS2 solution on regrowth of cryopreserved *Perilla* shoot tips using V-Cryo-pate method.

cryopreserved shoot tips using V-Cryo-plate method treated at 25°C. Thus, the PVS2 dehydration at 25°C in this V-Cryo-plate method is suitable for use with *Perilla*.

In conclusion, an efficient clonal propagation method for *Perilla* using node culture was successfully established. In addition, we demonstrated that the V-Cryo-plate procedure led to about 70% regrowth and this procedure was thus a very efficient and practical method for cryopreservation of *Perilla* germplasm. This protocol appears promising for cryopreservation of other medicinal plants, as well as other plant species and/or cultivars of horticultural interest after marginal modifications to the procedure.

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