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Cryopreservation of in vitro-grown axillary shoot-tip meristems of mint (Mentha spicata L.) by encapsulation vitrification

Abstract Alginate-coated meristems from in vitro-grown axillary buds of mint (Mentha spicata L.) were successfully cryopreserved by vitrification. Excised meristems from nodal segments cold hardened at 4 °C for 3 weeks were encapsulated and osmoprotected by a mixture of 2 M glycerol plus 0.4 M sucrose. These meristems were dehydrated with a highly concentrated vitrification solution (PVS2 solution) for 3 h at 0 °C prior to a plunge into liquid nitrogen. Successfully encapsulated vitrified meristems developed shoots within a week after plating without intermediary callus formation. The average rate of shoot formation amounted to nearly 90%. This procedure was successfully applied to other Mentha species. It was also confirmed that encapsulated vitrified meristems produced a much higher rate of shoot formation than the encapsulated dried meristems. Thus, this revised encapsulation vitrification method appears promising for the cryopreservation of mint and other germplasm.

Key words Cryopreservation · Encapsulation-vitrification · Meristems · Mint · Vitrification

Abbreviations MS Murashige and Skoog medium · PVS2 Plant vitrification solution · LN Liquid nitrogen · PEG Polyethylene glycol · EG Ethylene glycol · DMSO Dimethyl sulfoxide

Introduction

Mint germplasm is commonly preserved in field gene banks as growing plants. Thirty-five Mentha species are maintained in a field of the Hokkaido Prefectural Plant Genetic Resources Center. The maintenance of these plants in the field is a major drain on time, manpower, and space aside from problems related to diseases and environmental stresses. Cryopreservation is an important tool for the long-term storage of germplasm using a minimum of space and maintenance. Recently, some simple and reliable cryogenic procedures such as vitrification (Matsumoto et al. 1994, 1995; Niino and Sakai 1992; Takagi et al. 1997; Tannoury et al. 1991; Yamada et al. 1991), encapsulation-drying (Bachiri et al. 1995; Bouafia et al. 1996; Fabre and Dereuddre 1990; González-Arnao et al. 1996), and encapsulation-vitrification (Hirai et al. 1998) have been developed, and the number of species or cultivars cryopreserved has sharply increased during the last few years.

Towill was the first to report the cryopreservation of mint by vitrification (1990). In his method, precultured meristems are gradually dehydrated using a mixture of EG, DMSO, and PEG-8000 over three steps. The dehydrated meristems are placed on a small folded piece of tissue paper (85×16 mm) and ultra-rapidly cooled in LN (cooling rate: 75 °C/s). Towill achieved a considerable survival, ranging from 32% to 75% among experiments. Some callus formation was observed. However, this vitrification procedure is complicated and time-consuming, and the rate of shoot formation seems to be unstable. As will be shown, encapsulated mint meristems which were dehydrated with a vitrification solution (PVS2 solution) at 0 °C produced high levels of shoot formation without intermediary callus formation after cooling to −196 °C. An effective cryogenic procedure for mint meristems, the encapsulation-vitrification method, and the advantage of this method compared with the encapsulation-drying method is described here.
Materials and methods

Plant materials

Mint (spearmint, *Mentha spicata* L.) cv ‘Spearmint common’ was used in the present study. Apical meristems excised from plants growing in the field were cultured on MS medium (Murashige and Skoog 1962) with 0.5 g/l casein acids, 30 g/l sucrose, 2.5 g/l gellan-gum in tissue culture tubes (25 x 120 mm) at 23°C under a 16-h photoperiod at a light intensity of 96 mol m⁻² s⁻¹ for periods of 3 weeks. This medium was adjusted to pH 5.8 prior to autoclaving at 121°C for 10 min and used as the basal medium.

Apical buds of the plantlets were excised and subcultured every 3 weeks (each plantlet had three nodes and an apical bud after a 3-week incubation, see Fig. 1) as stock culture. The nodal segments, consisting of a pair of leaves and an approximately 8-mm-long stem, were transferred to the basal medium in plastic dishes (90 x 20 mm) and cultured for 24 h under the conditions described above to induce axillary buds. These segments were then cold-hardened at 4°C for 1–3 weeks under a 12-h photoperiod at a light intensity of 20 mol m⁻² s⁻¹. Non-hardened nodal segments were cultured for 3–7 days under the conditions mentioned above. Axillary shoot-tip meristems with two leaf primordia (about 0.8 mm in size) were then excised for experiments. Other *Mentha* species, *M. arvensis* L. var ‘piperascens’ Malinvaud, cv ‘Shubi’ and *M. arvensis* L. var ‘agrestis’ (Sole) Smith, cv ‘Ezo’ were also used in the present study.

Encapsulation and osmoprotection

Excised axillary shoot-tip meristems with or without cold hardening were suspended in calcium-free MS inorganic medium supplemented with 2% (w/v) Na-alginate and 0.4 M sucrose. The mixture, including meristems, as dispensed with a sterile pipette supplemented with 2% (w/v) Na-alginate and 0.4 M palmitic acid was then used as the basal medium. Apical buds of the plantlets excised and subcultured every 3 weeks (each plantlet had three nodes and an apical bud after a 3-week incubation, see Fig. 1) as stock culture. The nodal segments, consisting of a pair of leaves and an approximately 8-mm-long stem, were transferred to the basal medium in plastic dishes (90 x 20 mm) and cultured for 24 h under the conditions described above to induce axillary buds. These segments were then cold-hardened at 4°C for 1–3 weeks under a 12-h photoperiod at a light intensity of 20 mol m⁻² s⁻¹. Non-hardened nodal segments were cultured for 3–7 days under the conditions mentioned above. Axillary shoot-tip meristems with two leaf primordia (about 0.8 mm in size) were then excised for experiments. Other *Mentha* species, *M. arvensis* L. var ‘piperascens’ Malinvaud, cv ‘Shubi’ and *M. arvensis* L. var ‘agrestis’ (Sole) Smith, cv ‘Ezo’ were also used in the present study.

Vitrification procedure

The encapsulated axillary shoot-tip meristems were dehydrated with PVS2 solution in a 50-ml plastic tube at 0°C for different lengths of time. PVS2 solution was used at 1–2 ml per bead. PVS2 solution contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) DMSO in MS medium supplemented with 0.4 M sucrose (pH 5.8). The mixture of beads and PVS2 solution was gently shaken (15 rpm) in a water bath and shaker during dehydration. After dehydration, 10–15 beads were suspended in 1 ml PVS2 solution in a 1.8-ml cryotube and then plunged into LN (cooling rate: about 200°C/min). The cryotubes were held there for at least 1 h.

Dilution process

After storing, cryotubes were rapidly warmed in a water bath at 38°C (rewarming rate: about 200°C/min). PVS2 solution was drained from the cryotubes and replaced twice at 10-min intervals with 1 ml of 1.2 M sucrose solution.

Encapsulation-drying procedure

Meristems with or without cold hardening were suspended in calcium-free MS inorganic medium supplemented with 2% (w/v) Na-alginate and 0.4 M sucrose. The mixture, including the meristems, was encapsulated in 0.1 M CaCl₂ solution containing 0.4 M sucrose. To increase dehydration tolerance before air-drying (Fabre and Dereuddre 1990), these encapsulated meristems were treated in MS medium supplemented with 0.8 M sucrose for 16 h at 30 rpm on a rotary shaker at 25°C. They were subjected to air-drying for 3 h in petri dishes (12 cm diameter) containing 50 g of dried silica-gel. The water content of the beads after air-drying for 3 h was about 25% on a fresh-weight basis. Dried, encapsulated meristems were placed in a 1.8-ml cryotube and directly plunged into LN and held there for at least 1 h. Cryotubes were warmed in a water bath at 38°C for 3 min.

Viability and plant regrowth

The cryopreserved meristems were plated on basal medium in plastic dishes and then cultured under the conditions described above. The rate of shoot formation was expressed as a percentage of the total number of meristems forming normal shoots 2 weeks after plating. In all experiments approximately ten meristems were treated in each of three replicates.

Results

In the preliminary experiments, little or no difference was observed with respect to cryopreservability among nodal segments (1st to 3rd node from the top of apical meristems, Fig. 1) and in the performance of segments over a culture period of 3–7 days.

Excised axillary shoot-tip meristems from segments with or without cold hardening were precultured on MS medium supplemented with 0.3 M sucrose for 16 h at 4°C (for cold-hardened meristems) or 23°C (for non-hardened meristems). This preculture resulted in a considerable decrease in the rate of shoot formation of vitrified meristems cooled to −196°C (data not shown).

**Fig. 1** The scheme adapted for the subculturing and preconditioning of nodal segments of mint