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Investigations on various methods for cryopreservation of callus of the medicinal plant *Satureja spicigera*

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ABSTRACT

Satureja spicigera callus initiated from young leaves was successfully cryopreserved by vitrification. Four protocols desiccation, vitrification with PVS2, vitrification with PVS3 and DMSO freezing were tested for suitability. According to the results of the analysis of variance, significant differences were found among four protocols used for callus cryopreservation. The regrowth percentage of cryopreserved callus was significantly highest after treatment with PVS3 (98.7%). Based on this result, vitrification with PVS3 is recommended as the most suitable protocol for cryopreservation of *S. spicigera* callus. In this protocol, the callus excised from leaves of in vitro-grown plants was precultured on MS medium supplemented with 0.4 M sucrose, 0.5 mg l^{-1} 2,4-D, 0.6 mg l^{-1} BAP and 6 g l^{-1} agar under a 16 h photoperiod at 25 °C for two days. After preculture, the callus was transferred to a Petri dish with 2 ml loading solution and loaded at 25 °C for 25 min. Then, the callus was dehydrated with 2 ml PVS3 at 25 °C for two hours. After transferring to a 1.8 ml cryotube containing 1 ml PVS3, the cryotube was directly immersed into liquid nitrogen. The fast rewarming procedure consisted of plunging the cryotubes into a water-bath at 40 °C for 2 min. After removing PVS3, the contents of the cryotubes were poured into a Petri dish with 4 ml of a 1.2 M sucrose solution. Callus clumps were incubated in this solution at 25 °C for 20 min. After that, the callus was transferred to recovery medium. Results of measurements by differential scanning calorimetry (DSC) have shown that callus treated by desiccation and vitrification no freezing and melting peaks occurred. These cryopreservation protocols would be useful for the ex-situ conservation of *Satureja spicigera* callus at very low temperatures.

1. Introduction

The genus *Satureja* (Lamiaceae) has 15 species in Iran. Many species of the genus *Satureja* are reported to have aromatic and medicinal properties. For the treatment of different diseases for example cramps, muscle aches, nausea and infectious diseases can be leaves, flowers, stems of the plant used (Eminagaoglu et al., 2007).

One of these species is *S. spicigera* that grows wildly in North-west of Iran (Rechinger, 1986; Mozaffarian, 1996). Reports on the composition of essential oils from the aerial flowering parts of *S. spicigera* from Iran also showed the list of major components as thymol (35.1%), *p*-cymene (22.1%), γ-terpinene (13.75%) and carvacrol (4.0%) (Sefidkon and Jamzad, 2004). Antibacterial activity of the oil of *S. spicigera* has been reported against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* (Eftekhar et al., 2009).

Cryopreservation has the advantage to enable long-term storage without performing frequent subcultures. This would possibly endanger the genetic stability by risk of somaclonal variation (Jain, 2011).

Efficient cryopreservation protocols have been designed for numerous crop and medicinal plant cell cultures like *Ginkgo biloba* (Popova et al., 2009), *Anemarrhena asphodeloides* (Hong and Yin, 2012), grapevine (Wang et al., 2004), *Dioscorea bulbifera* (Hong et al., 2009). Callus induced from anthers of *Hevea brasiliensis* was successfully cryopreserved in liquid nitrogen by vitrification and subsequently regenerated into plants (Zhou et al., 2012). *Kalopanax*

Abbreviations: MS, medium after Murashige and Skoog (1962); 2,4-D, 2,4-Dichlorophenoxyacetic acid; BAP, benzylaminopurin; PVS, plant vitrification solution; GA_3, gibberellic acid; DMSO, dimethyl sulfoxide.

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embryogenic callus was efficiently cryopreserved also using a vitrification procedure (Shin et al., 2012).

However, no information is available on cryopreservation of *S. spicigera* germplasm. Preculture of callus in presence of osmotically active compounds, e.g. proline, sugars and sugar alcohols contributes to plant cell dehydration and chilling tolerance and is thus considered important for achieving regrowth after cryopreservation.

In method vitrification, using a highly concentrated cryoprotective solution forbids detrimental intracellular ice formation because of dehydrating (Sakai and Engelmann, 2007).

In this study, we present results of investigating four different methods of cryopreservation of *S. spicigera* callus culture.

2. Material and methods

2.1. Plant material

Satureja spicigera seeds were collected from Gilan Province, located in North of Iran, in fall of 2009 (latitude: N 36° 50′ 58.8″, longitude: E 049° 28′ 02.2″, altitude: 191 m). Seeds were immersed in 1.5% sodium hypochlorite solution for 5 min, then washed three times with sterile distilled water. Seeds were transferred to MS medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar and kept at 25 °C under a 16 h photoperiod for two weeks. Leaves were cut (size of fragment was 3–4 mm) and transferred on MS supplemented with 0.5 mg l^{-1} 2,4-D, 0.6 mg l^{-1} BAP and 8 g l^{-1} agar to induce callus formation (six explants were cultured per Petri dish). Cultures were kept at 25 °C under a 16 h photoperiod for periods of six weeks. During this time, green calli developed on the leaves. These calli were selected for cryopreservation and further multiplication of source material.

2.2. Cryopreservation of callus

Four protocols were tested for cryopreservation

- Protocol 1 - Desiccation
- Protocol 2 - Vitrification with PVS2
- Protocol 3 - Vitrification with PVS3
- Protocol 4 - DMSO freezing

All protocols begin with a preculture. Excised calli (size of callus clumps 3–4 mm) were transferred to MS medium supplemented with 0.5 mg l^{-1} 2,4-D, 0.6 mg l^{-1} BAP, and 6 g l^{-1} agar containing either 3% (w/v) sucrose, (P1) or 13.7% sucrose (P2) for two days under continuous light at 25 °C in a growth room. For regrowth solid MS medium with 3% (w/v) sucrose, 0.5 mg l^{-1} 2,4-D, 0.6 mg l^{-1} BAP, 1 mg l^{-1} GA_3 and 5 g l^{-1} agar was used (recovery medium).

2.2.1. Protocol 1: desiccation

2.2.1.1. Determination of water contents. Calli were weighed and dried in an oven at 103 °C for 17 h (ISTA, 1996). Water content was calculated on a fresh weight basis (FW). Three measurements were performed for each point of desiccation curves; about 10 callus clumps were taken for each measurement.

Recording the water contents during desiccation: After blotting the precultured calli on sterile filter paper for removing residual water, they were placed on sterile filter paper in an uncovered Petri dish and dehydrated under a sterile air flow in a laminar flow cabinet for seven time periods (0, 30, 60, 90, 120, 150, 180 min). Temperature and relative air humidity during desiccation were controlled to 22–24 °C and 38–40%, respectively, as determined with a data logger Testostor 175 (Testo AG, Lenzkirch, Germany). This was recorded for the two preculture media. Additionally, the regrowth rates of the calli were determined.

To determine regrowth at a certain water content, the desiccated calli were transferred to MS medium with 3% (w/v) sucrose, 0.5 mg l^{-1} 2,4-D, 0.6 mg l^{-1} BAP and 6 g l^{-1} agar (P1) and kept at 25 °C under a 16 h photoperiod. Regrowth was evaluated as the number of callus clumps showing formation of new tissues four weeks after treatment, and expressed in percent of the total number of callus pieces.

2.2.1.2. Cryopreservation. Calli precultured on medium P2 were desiccated for 120 min as determined from the preliminary desiccation experiments. Following desiccation, one half of the callus clumps were transferred to 1.8 ml empty cryotube and rapidly frozen in liquid nitrogen for at least one hour, while the other half served as controls without freezing. The cryotubes with callus clumps were rapidly thawed in a warm water bath at 40 °C for 90 s. Both the control and cryopreserved callus clumps were then transferred to MS medium supplemented with 41.1% sucrose for 20 min. After 20 min, the callus clumps were transferred to recovery medium and kept at 25 °C under a 16 h photoperiod.

2.2.2. Protocol 2: vitrification with PVS2

The complete vitrification procedure involves (a) preculture on medium with 13.7% sucrose (b) loading of the calli with a mixture containing 2 M glycerol and 13.7% sucrose for 25 min; (c) dehydration of the cells by exposing them to PVS2 (Sakai et al., 1990) for 45 min at 25 °C; (d) transferring the calli to a 1.8 ml cryotube (containing 1 ml PVS2) (e) the cryotubes were immersed in liquid nitrogen (f) removal of the cryotubes from liquid nitrogen and warming them in a water bath at 40 °C for 2 min; (g) unloading PVS2 by transferring the callus to liquid MS medium containing 41.1% sucrose for 20 min. Then, the callus clumps were transferred to recovery medium and kept at 25 °C under a 16 h photoperiod.

2.2.3. Protocol 3: vitrification with PVS3

It was like protocol 2, but the vitrification was performed by PVS3 (Nishizawa et al., 1993) for 120 min at 25 °C.

2.2.4. Protocol 4: DMSO freezing

The calli were transferred to 1.8 ml cryotubes (containing 1 ml cryoprotectant solution) and kept for one hour. The cryoprotectant solution consisted of MS medium supplemented with 13.7% sucrose, 0.5 mg l^{-1} 2,4-D, 0.6 mg l^{-1} BAP and 10% DMSO (filter-sterilized). The cryotubes were directly immersed in liquid nitrogen. After one hour, the cryotubes were rapidly rewarmed in a water bath at 40 °C for 2 min. The callus clumps were then transferred to MS medium supplemented with 41.1% sucrose for 20 min. After 20 min, the callus clumps were transferred to recovery medium and kept at 25 °C under a 16 h photoperiod.

2.3. Regrowth

A number of 15 clumps were transferred to 9 cm Petri dish each containing recovery

medium and placed at 25 °C under a 16-h photoperiod. Regrowth was evaluated as the number of callus clumps showing formation of newly developed tissues four weeks after treatment and expressed in percent of the total number of callus pieces.

2.4. Thermal analysis

Thermal analyses were undertaken using a differential scanning calorimeter (DSC) Q2000 with a liquid nitrogen (LN) cooling system (TA Instruments – Waters, New Castle, Delaware, USA). Samples were weighed on a Sartorius microbalance ME235S (accuracy ± 10 μg; Sartorius, Göttingen, Germany) and placed in 40 μl aluminum pans, which were hermetically sealed. The samples were

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