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Effect of cryopreservation on essential oil of Satureja spicigera

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A B S T R A C T

Conservation of genetic resources of S. spicigera is important. Because the life of this valuable species has been threatened by biotic and abiotic stresses. Seed storage techniques at very low temperature (-196 °C) using liquid nitrogen is one way of maintaining germplasm with much less cost and without loss of viability on storage and it is useful when it does not lead to changes in the chemical composition of essential oil in the desired plant. In order to investigate the phytochemical stability and compare the type and amount of identified compositions, the treated seeds were transferred to the liquid nitrogen at –196 °C for 1 week as compared to the control treatment. The aerial parts of S. spicigera were collected at flowering stage and dried in the laboratory. The essential oil was isolated by hydrodistillation and analyzed by a combination of capillary GC and GC-MS. Eleven compounds were recognized in the essential oil of mentioned plant in the cryopreservation. The main constituents in the essential oil included thymol (41.1%), γ-terpinene (21.1%), p-cymene (17.2%) and carvacrol (11.9%). Results indicated that regarding the number, type and percentage of compounds existing in the essential oils, there was no difference between the control and cryopreservation treatment so by using the cryopreservation technique, the seeds of this valuable endangered species can be preserved for a long period.

Introduction

Preservation in the cryopreservation has been considered as an efficient strategy in order to preserve the fine samples of endangered plants in the plant gene banks and it is of high importance to have the regenerated plants with genetic, morphologic and phytochemical stability after getting out of the cryopreservation. Since the savory is one of the endangered medicinal native plants having essential oil in Iran, it is of considerable significance to assess its phytochemical stability after the cryopreservation as compared to the control plant. Lots of researches on the preservation of various plant seeds in the cryopreservation have been conducted and their phytochemical, genetic and morphologic stability has been studied. For example, lack of clear and valid evidence for genetic, cytological and morphologic changes related to cryopreservation (Panis and Lambardi, 2005), lack of genetic variety in the regenerated potato plants after cryopreservation (Harding and Benson, 2000), lack of disgenin changes in the cryopreservation of *D. bulbifera* and *D. floribund* (Ahuja *et al*., 2002; Dixit et al., 2003), preservation of genetic stability of two species of *Rubus* in the liquid nitrogen for a long time (Rosa *et al*., 2010), proof of genetic stability of *Prunus* using molecular RAPD and AFLP indices after the cryopreservation (Wang *et al*., 2013) have been evaluated.

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Phytochemical investigation of *Thymus moroderi* after the cryopreservation indicated that the genetic evaluation and stability investigation of all types of phytochemical materials are more likely to be done and the preservation of plant material properties is necessary after the storage when the aromatic plants are to be studied and preserved in the cryopreservation; the findings have confirmed the suitability of cryopreservation protocol (Marco-Medina and Casas, 2013). In this relation, the genetic stability evaluation of *Wsabi* plant which has been put in the cryopreservation for a long period was performed using the molecular, morphologic and biochemical indices and no significant difference was seen with respect to the number of shoots, leaf area index and petiole length (Matsumoto *et al*., 2013).

Effects of cryopreservation on the gene expression and transfer in *Arabidopsis thaliana* have been studied. Results have shown that after the cryopreservation, no considerable increase was seen in the efficiency of gene transfer but the freezing can improve the exogen expression (Li *et al*., 2013). Choudhary *et al*. (2013) conducted some studies on *Mulberry* plant and concluded that the cryopreservation could preserve the genetic stability of germplasm.

Molecular indices of RAPD and AFLP have been used to investigate the genetic stability of *Humulu slupulus L.* in the cryopreservation and results demonstrated that there was no genetic variety between the control and cryopreservation treatments (Peredo *et al*., 2008).

Although some studies on the genetic stability of cryopreserved plants have been conducted worldwide, no investigations on *S. spicigera* and the cryopreservation impact on essential oil composition with respect to the genetic stability have been done.

Materials and Methods

Plant material

S. spicigera seeds were collected from Gilan Province, located in North of Iran (latitude: N 36º 50' 58.8", longitude: E 049º 28' 02.2", altitude: 191 m). The aerial parts of *S. spicigera* were harvested during flowering stage from control and cryopreserved seed.

Cryopreservation

Before transferring seed samples in liquid nitrogen (temperature -196 °C), protocol-Desiccation was tested for cryopreservation.

Protocol-Desiccation

For determination of water contents, seeds 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). In order to reduction moisture, seeds were placed in an airtight desiccator containing silica\gel and transferred in an oven at 28 °C for 19 hours (Table 1). So seeds were immediately submerged in liquid nitrogen for 1 week. Non-cryopreserved seeds were stored at +4 °C temperature for 1 week as a control.

Afterward, the cryotubes were rapidly thawed in a warm water bath at 40 ºC for 100 sec (Engelmann, 1990). The seeds were then transferred to MS medium supplemented with 1.2 M sucrose for 20 min at 25 °C.

Due to reduction ice crystal, after thawing, seeds were sterilized by 1.5% hypochlorite (V/V) for 5 min. Seeds were washed three times with sterile distilled water and placed on sterile filter paper in a covered Petri dish and kept at 25 ºC under a 16 h photoperiod for 30 days. For cultivating the plants, the 1:3 peat moss-perlite mixtures were used, and then, the soil package was sterilized and used by an oven.

Table 1. Seed characteristics of *S. spicigera*

Species	Geographical location	Moisture content (After desiccation)	Moisture content (Before desiccation)	1000-grain weight (gr)
S. spicigera	Gilan Province, located in North of Iran	2.25	4	0.6

Drying method

The flower shoots were harvested in the flowering stage and dried at 25 °C under shadow.

Essential oil isolation

The powdered plant parts were hydrodistilled using a Clevenger type apparatus. The resultant essential oil was dried over anhydrous sodium

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sulfate and stored at 4 °C until analyzed and tested.

Essential oil analysis and identification procedure

In order to separate and identify the essential oil compositions, gas chromatography and gas chromatography attached to mass spectrometer (GC/MS) were used. UFM Thermo GC equipped with a Ph-5 capillary column with the length of 10 m and inner diameter of 0.1 mm along with the diameter of stationary phase layer as 0.4 µm was used. Column heat planning has increased from 60 °C to primary temperature of 285 °C with the speed of 80 °C. Temperature of injection chamber was regulated at 280 °C. The used detector in the GC was a FID and applied helium as the carrier gas with the speed of 0.5 mm min^{-1}.

GC/MS device characteristics

Varian 3400 ion trap GC/MS equipped with a DB-5 column with the length of 30 m and diameter of 0.25 mm along with the stationary phase layer diameter of 0.25 mm was used. Column heat planning was similar to that of GC device. Temperature of injection chamber was regulated at 10 °C more than the final temperature of column and helium was applied as the carrier gas with the speed of 31.5 cm s^{-1}. Scanning time was 1 s, the ionization energy was 70 eV and mass area was 40-340.

Identification of essential oil compositions

After injecting the essential oils to GC device and finding the most suitable column heat planning, the resultant essential oils were diluted with dichloromethane to achieve the best separation and then, injected to GC/MS device to obtain the mass spectra and related chromatograms. Afterwards, using the retention time, and Kovats index, and studying and comparing the mass spectra and the standard compositions, the essential oil compositions were qualitatively and quantitatively identified.

Results

Identification results of S. spicigera essential oil compositions

The goal of cryopreservation plant genetic resources is not only to store germplasm but also to limit genetic and phytochemical variation. Essential oil compositions of *S. spicigera* species were specified in the control and cryopreservation treatments after achieving the chromatograms and mass spectra through calculating the retention index. Studying the phytochemical stability and comparing the type and value of identified essential oil compositions of *S. spicigera* species after the cryopreservation as compared to the control indicated that 11 compositions were specified.

Fig.1 show the chromatograms resulting from the separated compositions in the control treatments demonstrating that the most primary identified compositions included thymol (38.2%), α-terpinene (22.6%), p-cymene (17.5%), and carvacrol (11.5%). The constituents of the essential oil of *S. spicigera* with their IR are presented in Table 2. In the essential oil extraction of mentioned species with the seeds which were put in the liquid nitrogen for 1 week at -196 °C, 11 compositions were identified. Fig. 2 display the chromatograms of separated compositions. The most primary identified compositions included thymol (41.1%), α-terpinene (21.1%), p-cymene (17.2%) and carvacrol (11.9%). The constituents of the essential oil of *S. spicigera* with their IR are presented in Table 2.

Comparing the obtained compositions indicated that putting the seeds in the liquid nitrogen had no effects on the type of essential oil composition and no important difference was observed.

Thymol was regarded as the most primary component in the control treatment with the highest amount given as 38.2% and had no significant difference with the essential oil of seeds in the liquid nitrogen at -196 °C.

The value of α- terpinene was given as 22.6% to be the second component in the control treatment and 21.1% in the liquid nitrogen treatment at -196 °C; in addition, p-cymene was computed as 17.5% in the control and 17.2% in the liquid nitrogen treatment and carvacrol was computed as 11.5% in the control and 11.9% in the liquid nitrogen treatment.

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Studying the cryopreservation of *S. spicigera* species, the seeds after being taken out of liquid nitrogen like the control samples were able to germinate and produce seedlings and with respect to the health, no symptoms were seen; therefore, it can be concluded that the cryopreservation of seeds and plant organs did not lead to changes in the chemical compositions.

Table 2- Identified essential oil Components of *S. spicigera* control and cryopreservation treatment (in liquid nitrogen for 1 week at -196 °C)

Peak No.	Components	RI	% of the oil (Cryopreservation treatment)	% of the oil (Control)
1	α-Thujene	944	1.3	1.6
2	α-Pinene	957	0.8	0.9
3	β-Pinene	989	1.6	1.8
4	α-Terpinene	1044	1.6	1.7
5	P-Cymene	1049	17.2	17.5
6	γ-Terpinene	1081	21.1	22.6
7	Methyl ether carvacrol	1269	1.3	1.9
8	Thymol	1310	41.1	38.2
9	Carvacrol	1319	11.9	11.5
10	E-Caryophyllene	1488	1.3	1.2
11	Germacrene D	1533	0.4	0.4

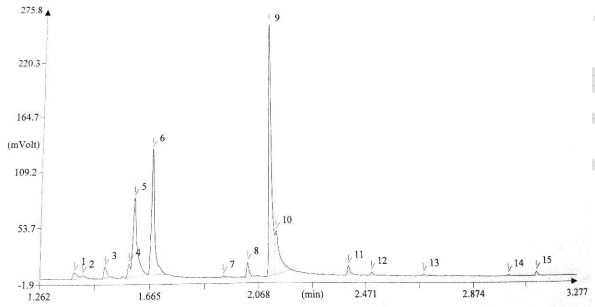


Fig. 1- Essential oil S. spicigera chromatograms (Control)

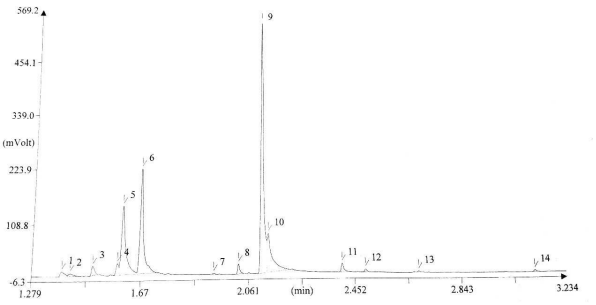


Fig. 2- Essential oil S. spicigera chromatograms (Cryopreservation treatment)

Discussion

Some investigations have been conducted to report that at very low temperatures, plant metabolism and cell division activities were delayed and the materials remained with no changes for a long period. Thus, the freezing in the cryopreservation led to guarantee the genetic stability of germplasm (Panis and Lambardi, 2005). Regeneration and genetic stability of plants are maintained for a long period (Al-Ababneh *et al*., 2003).

Evidence of morphologic, cytological and biochemical or molecular changes might not be found in the plants in the cryopreservation (Harding, 2004). Plenty of reports exist concerning the genetic stability of plants in the cryopreservation (Scocchi *et al*, 2004; Zhai *et al*., 2003; Choudhary *et al*., 2013; wang *et al*., 2013). The first report for in vitro conservation or genetic assessment of *Ziziphora tenuior* L. which grows wild in Jordan was conducted to study the possibility of long-term conservation of *Ziziphora tenuior* L. by cryopreservation using encapsulation-dehydration and encapsulation-vitrification methods.

The genetic stability of cryopreserved plants were studied by AFLP. For genetic stability study on *Ziziphora tenuior* L., tissues cryopreserved and non-cryopreserved tissues showed no clear differences when studied by AFLP (Al-Baba *et al*., 2015). There were not genetic changes in the cryopreserved shoots as assessed using several morphological and molecular markers, thereby demonstrating that cryopreservation using two-step freezing of dormant buds is a practical method for the long-term storage of apple germplasm (Yi *et al*., 2015). There were no morphological differences between cryopreserved greenhouse-grown *Rubus grabowskii* when compared with the control mother plants (Castillo *et al*., 2010).

The epigenetic stability throughout the encapsulation-dehydration cryopreservation protocol, with or without the addition of ascorbic acid, was evaluated with MSAP markers from 33 samples. no significant differences were found due to the addition of ascorbic acid (Ibaneza, 2019).

Although some genotype changes of cryopreserved plants and control ones were

observed, it does not mean that these changes are related to the cryopreservation process which can be attributed to the plant tissue culture and regeneration stages (Harding, 2004). Problems like genetic instability and risk of loss biological materials can be attributed to contamination or human error during subculture (Kaviani, 2011). Due to some genotype changes of cryopreserved plants and control, there was a need to accredit plant genetic uniformity of the cryopreserved plant material after cryopreservation (Harding, 2004; Micula *et al*., 2011). To detect any possible genetic variation in plants material, several molecular techniques have been used (Harding, 2004). DNA (RAPD) and amplified fragment length polymorphisms (AFLP) were the most popular markers used in this domain (Micula, *et al*., 2011).

Investigations of phytochemical stability of mentioned species have reported that lack of phytochemical stability changes has been obvious and the cryopreservation had no impact on the phytochemical properties of desired plant.

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