Cryopreservation by Encapsulation-vitrification of Embryogenic Callus of Wild Crocus (*Crocus hyemalis* and *Crocus moabiticus*) (Research Note)

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ABSTRACT

Crocus is asexually propagated plant by corms and it is susceptible to a wide range of pathogens and environmental stresses. It cannot be effectively stored for long periods using conventional methods. Cryopreservation by encapsulation-vitrification was studied as a long-term conservation method. Callus fragments were encapsulated in 4 mm calcium alginate beads containing hormone-free MS medium. Encapsulated beads were dehydrated with the Plant Vitrification Solution 2 (PVS2) [(w/v) 30% glycerol, 15% DMSO and 15% EG] for 10, 20, 30, 60 and 90 minutes and dipped in liquid nitrogen for at least 1 h. Dehydration of cryopreserved encapsulated callus fragments with PVS2 for 20 minutes resulted in 100% survival and regrowth prior to cryopreservation for both species. After cryopreservation, the highest survival (55.6% and 75.0% for *C. moabiticus* and *C. hyemalis*, respectively) and highest regrowth (66.7% for both species) were obtained for 20 minutes dehydration with PVS2.

Keywords: *Crocus hymalis*, *Crocus moabiticus*, Encapsulation, Verification, PVS2.

INTRODUCTION

Cultures from ancient times to the present day have thoroughly exploited biodiversity (Zedan, 2000). It is the responsibility of countries which still have a significant genetic and species diversity, to conserve it and make it available for use (Rao, 1998). Many important varieties of field, horticultural and forestry species are either difficult or impossible to conserve as seeds (i.e. having recalcitrant seeds) or reproduce vegetatively. Vegetative propagules (tubers, tuber roots, bulbs, corms, rhizomes… etc) can be stored at low temperatures but must be regenerated often because they can lose viability easily.

Crocus species are one of these vegetatively-grown plants, and unique species are found in the wilds of Jordan. They propagate by corms (Bhagyahauakshmi, 1999; Rangahau, 2003) and are susceptible to a wide range of pathogens and environmental stresses (Plessner et al., 1990). The corms cannot be effectively stored for long time using conventional methods. The autotriploid nature of the cultivated crocus species also prevents improvement by plant breeding programs (Plessner et al., 1990; Rangahau, 2003). Therefore, this germplasm

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Received on 19/5/2009 and Accepted for Publication on 24/2/2010.

must be conserved and protected from loss to ensure its availability for future plant improvement.

An effective way to conserve such plant material is by using *in vitro* preservation techniques which provide pathogen-free plants, thereby facilitating safer distribution, and the cultures are not subjected to environmental disturbances (Rotach, 1999; Rao, 2004). In general, *in vitro* conservation techniques fall under two categories (Rao, 2004):

1. Slow growth procedures: where germplasm accessions are kept as sterile plant tissues or plantlets on nutrient gels providing short- and medium-term storage options.

2. Cryopreservation: where plant material is stored in liquid nitrogen enabling long-term storage of the plant material.

There are several cryopreservation techniques available for germplasm storage. Encapsulation-vitrification is a combination of the encapsulation-dehydration and vitrification techniques that has the advantage of ease of handling of plant material and decreased time needed for dehydration (Sakai and Engelmann, 2007; Shatnawi, 2004). Samples are encapsulated in alginate beads, and subjected to very fast cooling resulting in vitrification (glass formation rather than crystals) (Shibli et al., 2006). This might reduce toxicity of vitrification solution on explants and provides higher and earlier recovery growth than encapsulation-dehydration (Shatnawi, 2004); depending on species.

Various techniques are used to recognize viable plant cells in thawed material; of these 2,3,5-Triphenyltetrazolium Chloride (TTC) reduction assay which distinguishes between surviving and dead plant cells, tissues and organs (Lutts et al., 1996; Shibli et al., 2004), on the basis of relative respiration rate (Shibli et al., 2006). The test utilizes the activity of the dehydrogenase enzyme as an index of respiration rate of viable plant material. Regrowth of the cryopreserved plant material is another indicator of plant viability (Shibli et al., 2004).

Cryopreservation by encapsulation-vitrification of crocus embryogenic callus was investigated in this study as one of the most important and effective preservation methods for such plant species.

Materials and Methods

The experimental work was conducted in the Plant Tissue Culture Laboratory, Agriculture Center for Research and Production at Jordan University of Science and Technology (Irbid, Jordan).

In Vitro Culture of Plant Material

Callus of *Crocus hyemalis* Bioss. and Blanche. and *C. moabiticus* Bornm. and Dinsmore ex Bornm. were originally obtained from the Plant Tissue Culture Laboratory/National Center for Agricultural Research and Extention (NCARE) (Baqa, Jordan).

The seeds of both species were collected from their natural habitat in Jordan: *C. hyemalis* was collected from Rhaba region in Irbid district; at longitude of 354853.3 E, latitude 322430.1 N longitudes and elevation of 943 m above sea level, while *C. moabiticus* was collected from Al-Khanasri region in Mafraq district; at longitude of 360344.5 E and latitude of 322445.6 N with elevation of 576.40 m above sea level (Nazzal, 2007). Both species were identified according to Flora-Palestina (Zohari and Feinburn-Dothan, 1986).

The embryogenic callus (Fig. 1) was induced from culturing the sterilized seeds (Fig. 2) of both species as explants on full strength Murashige and Skoog (MS) medium (1962) with 4.4 µM 6-benzylaminopurine (BA), 5.4 µM α-naphthaleneacetic acid (NAA). Callus was subcultured on full strength MS with 4.4 µM 6-benzylaminopurine (BA), 5.4 µM NAA) and 0.2 $g.l^{-1}$

polyvinylpyrrolidone (PVP). The subculturing medium used for multiplication of mother cultures consisted of solid MS medium with 0.09 M sucrose, 4.4 µM BA, 5.4 µM NAA and 0.5 $g.l^{-1}$ PVP. Subculturing was performed every three weeks using a fresh growth medium to establish sufficient callus stock for experimentation. The cultures were incubated in the growth room at 24 ± 2 ºC, with complete darkness.

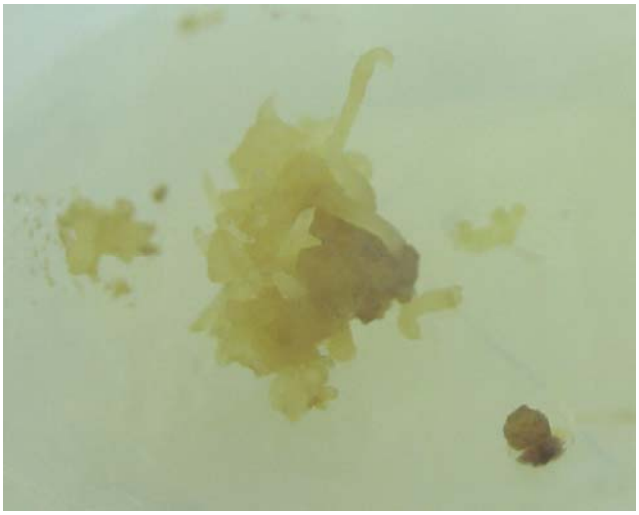

Figure1. Embryogenic callus of *Crocus moabiticus*.

Figure2. Embryogenic callus induced from sterilized seed of *Crocus moabiticus*

Cryopreservation by Encapsulation-Vitrification

Callus clumps were precultured on hormone-free MS with 0.1 M sucrose for 3 days. Precultured callus was then suspended in calcium-free and hormone-free liquid MS with 3% (w/v) sodium alginate, 2.0 M glycerol and 0.4 M sucrose (Subaih, 2005; Wang et al., 2000). The callus was picked up using a 10 ml sterile pipette with some alginate solution and then dispensed into a hormone-free liquid MS medium containing 100 mM calcium chloride and 2.0 M glycerol plus 0.4 M sucrose

and held for 30 minutes for polymerization.

Beads (Fig. 3) were dehydrated with Plant Vitrification Solution (PVS2) [(w/v) 30% glycerol, 15% DMSO and 15% EG] for 10, 20, 30, 60 and 90 minutes. at 25° C. The cryovials with the beads and the vitrification solution were plunged directly in LN and held for at least 1 h. Cryovials were then rewarmed in a water bath at 38 ºC for 2-3 minutes.

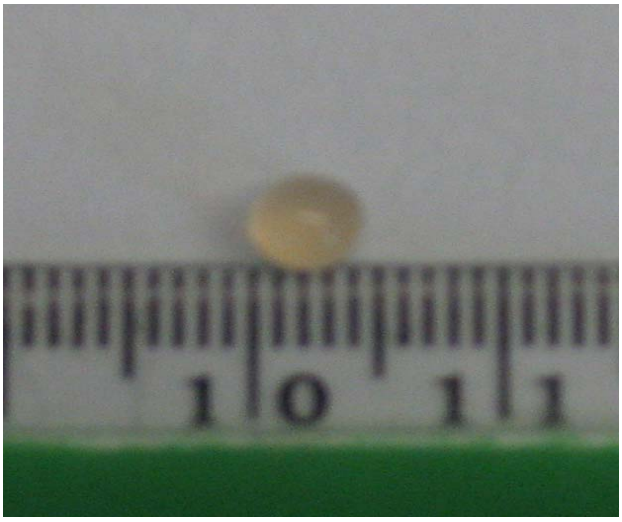

Figure3. Encapsulation beads of 4mm width containing small pieces of callus.

The PVS2 solution was removed and replaced with unloading solution (hormone-free liquid MS medium with 1.2 M sucrose), which was changed three times for 10 minutes each. Half the number of the untreated (-LN) and treated (+LN) beads were transferred to a solid MS recovery medium of hormone-free MS medium with 0.09 M sucrose, 4.4 µM BA, 5.4 µM NAA and 0.5 $g.l^{-1}$ PVP for regrowth determination. Regrowth % = [No. of regrown callus clumps / total No. of callus clumps] X 100. Survival was determined using the other half of the beads for the TTC test. Survival % = [No. of TTC positive clumps (red) / total No. of callus clumps] X 100.

TTC Test

TTC test was performed by incubating callus in test tubes with 5 ml of 0.5% (w/v) TTC salt dissolved in 50 mM K_2HPO_4 at pH 7.0 for 15 h at 30 ºC in complete darkness. Tetrazolium salt solution is reduced to formazen by hydrogen ions released with respiration of viable plant cells and results in a diagnostic red color formation (Lutts et al., 1996). The callus fragments were then rinsed in deionized water and formazen was extracted with 5 ml ethanol 94% for 5 minutes at 80 ºC. After the removal of the callus, absorbance was measured at 487 nm and the viability index was calculated as: Viability index (I_v) = Absorbance / 50 mg fresh weight (Lutts et al., 1996).

Experimental Design

Treatments were arranged in a Completely Randomized Design (CRD). Each treatment was replicated three times with five callus clumps per replicate. The collected data were statistically analyzed using SAS (Statistical Analysis System, Cary, NC, 2001). Means were separated according to the Least Significance Difference (LSD) at 0.05 probability level.

Results and Discussion

Complete survival and regrowth of the encapsulated

non-cryopreserved crocus callus was obtained when callus was exposed to PVS2 at 25° C for 20 minutes in both species (Tables 1-2). Induction of dehydration and freezing tolerance was achieved through preculture on 0.1 M sucrose, encapsulation in beads with 2.0 M glycerol and 0.4 M sucrose followed by exposure to PVS2. Reduced PVS2 exposure increased the survival of the non-cryopreserved callus due to reduced osmotic shock (Subaih, 2005).

Table1. Survival percentage, viability index (I_v) and regrowth percentage of encapsulated non-cryopreserved (-LN) and cryopreserved (+LN) callus of *C. moabiticus* as influenced by dehydration duration with PVS2.

Duration (minutes)	Survival %	I_v	Regrowth %
	Non-cryopreserved (-LN)		
10	88.9 b*	0.077 b	66.7 b
20	100 a	0.275 a	100 a
30	50.0 d	0.054 c	50.0 c
60	25.0 e	0.027 d	16.7 d
90	13.3 f	0.009 e	0.0 f
	Cryopreserved (+LN)		
10	13.3 f	0.012 e	6.0 e
20	55.6 c	0.090 b	66.7 b
30	25.0 e	0.027 d	13.3 d
60	13.3 f	0.009 e	0.0 f
90	0.0 g	0.000 e	0.0 f

*** Means within column having different letters are significantly different according to LSD at P ≤ 0.05.**

Table 2. Survival percentage, viability index (I_v) and regrowth percentage of encapsulated non-cryopreserved (-LN) and cryopreserved (+LN) callus of *C. hyemalis* as influenced by dehydration duration with PVS2.

Duration (minutes)	Survival %	I_v	Regrowth %
	Non-cryopreserved (-LN)		
10	75.0 c*	0.165 b	66.7 c
20	100 a	0.247 a	100 a
30	87.8 b	0.149 c	83.3 b
60	50.0 e	0.066 de	33.3 e
90	0.0 h	0.000 g	0.0 h
	Cryopreserved (+LN)		
10	50.0 e	0.08d	44.4 d
20	75.0 c	0.165 b	66.7 c

Duration (minutes)	Survival %	I_v	Regrowth %
30	66.7 d	0.052 e	66.7 c
60	13.3 g	0.025 f	5.6 g
90	0.0 h	0.000 g	0.0 h

*** Means within column having different letters are significantly different according to LSD at P ≤ 0.05.**

In the current study, after cryopreservation the greatest survival of *C. moabiticus* (55.6%) and *C. hyemalis* (75.0%), and regrowth (66.7%) of both species were obtained when encapsulated callus was dehydrated with PVS2 solution at 25° C for 20 minutes (Tables 1-2). Viability index indicated that only *C. hyemalis* maintained good viability after a 30 minute exposure of PVS2 at 25° C. Subaih (2005) reported that high survival (73.3-80) of encapsulated non-cryopreserved date palm callus was obtained with PVS2 at 25 ˚C irrespective of the duration of dehydration. Similarly, Moges et al. (2004) found that the greatest survival (80-85%) and regrowth (70-80%) of cryopreserved African violet shoots with pale green or yellow color were obtained when encapsulated shoot tips were dehydrated with PVS2 at 25 ˚C for 5 to 30 minutes. Furthermore, Al-Ababneh et al. (2002) also found maximum recovery of sour orange shoot tips after dehydration with PVS2 at 0 ˚C for 2-3 h. Although the mentioned plants above cannot tolerate more than 30 minutes but they had a range of exposure time while *Crocus* species did not gave high regrowth after 20 minutes.

The survival and regrowth of encapsulated cryopreserved callus declined with increased exposure duration with PVS2 (Tables 1-2). Both survival and regrowth ability were lost after 90 minutes in both species (Tables 1-2). The appearance of yellow or brown color in the regrowth callus was observed for the frozen callus, which may be attributed to cellular changes of some tissues in the cryopreserved *Crocus* callus (Subaih, 2005).

Conclusions

From this study, it can be concluded that dehydration of *Crocus* callus encapsulated and cryoprotected with PVS2 at 25° C for 20 minutes resulted in high viability after cryopreservation. Furthermore, *C. hyemalis* callus can maintain good viability after cryoprotection with PVS2 at 25° C for 30 minutes. In addition, increasing the duration of exposure decreased the viability. Further studies should be initiated to enhance regrowth of the survived callus and to study genetic stability after cryopreservation.

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حفظ الكالوس الجنيني لنبات الزعفران البري بالتجميد بالنيتروجين السائل بوساطة الكبسلة والإحلال بالمحاليل الأسموزية

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ملخـص

يعد الزعفران البري مصدراً مهماً من المصادر الوراثية النباتية المنتشرة في برية الأردن. النبات يتكاثر خضريا وهو معرض للعديد من الأخطار الحيوية والبيئية مما يوجب الاهتمام به والمحافظة عليه. تمت دراسة إمكانية حفظ نوعين من أنواع الزعفران البري *C. hyemalis* و*C. moabiticus* باستخدام التجميد بالنيتروجين السائل (Cryopreservation) بعد الكبسلة والإحلال بالمحاليل الأسموزية (Encapsulation-vitrification). استخدمت أنسجة الكالوس الجنيني (Embryogenic callus) التي تم إكثارها داخل الأنابيب في هذه الدراسة. تمت كبسلة أنسجة الكالوس الجنيني لنبات الزعفران البري داخل وسط غذائي خالٍ من الهرمونات يحوي 3% ألجينات الصوديوم. ثم تم تجفيف الكبسولات بمحلول إحلال نباتي عالي التركيز (100% PVS2) لفترات مختلفة؛ حيث أعطى التجفيف لمدة 20 دقيقة نسب 55.6% نجاة و66.7% إعادة نمو لنبات الزعفران المؤابي *C. moabiticus* ونسب 75.0% نجاة و66.7% إعادة نمو للزعفران الهايمالي *C. hyemalis*. زيادة فترة التجفيف أدت إلى تناقص في قدرتي النبات للنجاة وإعادة النمو. يوصى بإجراء مزيد من التجارب حول تحسين نسب إعادة النمو والنجاة ولدراسة الثبات الجيني (Genetic stability) بعد التجميد بالنيتروجين السائل.

الكلمات الدالة: الكالوس الجيني، التجميد بالنيتروجين، الكبسلة، الإحلال بالمحاليل الأسموزية.

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تاريخ استلام البحث 2009/5/19 وتاريخ قبوله 2010/2/24.