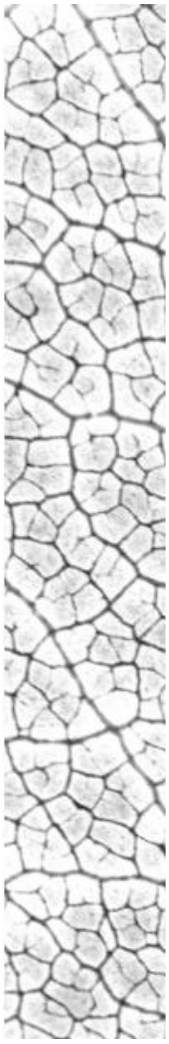

EurAsian Journal of BioSciences
Eurasia J Biosci 14, 501-505 (2020)

Cryopreservation of *Calendula officinalis* seeds

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Abstract
The article presents the results of cryopreservation of seeds of the medical plant *Calendula officinalis*. Cryopreservation is a highly promising direction of biology, allowing deep freezing of objects with full preservation of life properties. Calendula seeds have different size and weight. The best survival rates are observed for large seeds (germination rate - 77.5%, energy of germination - 55.0%). Assessment of seed survival in the storage rate showed a linear decrease in germination rate and energy of germination during storage. After 1.5 years of storage, germination decreased to 30.0%.As part of the research, the container was optimized for cryopreservation of *Calendula officinalis* seeds, defrosting conditions, seed humidity and the use of different cryoprotectors. The results showed that the optimal container for freezing in liquid nitrogen was plastic cryotubes; defrosting should be carried out at room temperature; the best survival rates were found at seed humidity 5-6%; the use of cryoprotectors was not feasible. The results of the studies can be used to organize long-term storage of *Calendula officinalis* seeds at extra low temperatures (liquid nitrogen).

Keywords: *Calendula officinalis*, cryopreservation, germination, liquid nitrogen, seed material

Ishmuratova MY, Tleukenova SU, Atikeyeva SN, Auelbekova AK, Zhuzbayeva GO, Zhumagaliyeva ZZ (2020) Cryopreservation of *Calendula officinalis* seeds. Eurasia J Biosci 14: 501-505.

INTRODUCTION

Studying the different methods of preservation of seeds species with medical, food, feed and technical value is an important and actual goal of botany and agriculture of Republic of Kazakhstan. Research on medicinal plants (herbs) is highly relevant. This area is currently limited by insufficient production of vegetative raw materials (Grudzinskaya et al. 2014, Pharmaceutical Market of Kazakhstan 2015), especially for species which do not grow in natural flora.

Calendula officinalis L. (*Asteraceae* family) is one of most popular crops in Kazakhstan used for the manufactory of medical preparations and cosmetic products (Ishmuratova 2015, Loseva 2008).

Calendula officinalis (marigold) has many pharmacological properties. Preparations from raw material are used as antibacterial, antiseptic, antifungal, anti-inflammatory and anti-allergenic cures; in cosmetic practice for improving skin condition (Alsaraf et al. 2019, Butnariu et al. 2012, Goktas et al. 2015, Jan et al. 2017, Kurkin et al. 2016, Muley et al. 2009, Preethi et al. 2009, Shafeie et al. 2015, Ukiya et al. 2006, Voskresenskaya et al. 2016)

Seed material of *Calendula officinalis* has a limited shelf life, losing viability during 3-5 years (Arjenali et al. 2011, Ishmuratova et al. 2010). As a modern method of long storage of genetic resources will use the cryopreservation in liquid nitrogen (González-Benito et al. 2009, Nesterova 2004, Nilishina et al. 2007, Reed 2002).

Cryopreservation allows freezing plant seed at the critical low temperatures with maintaining viability for a long period (Babu et al. 2012, Gonzales-Benito et al. 2009).

The aim of present research is to determine peculiarities of cryopreservation of seeds of *Calendula officinalis* for introduction in cryobank (Ansari et al 2015).

MATERIALS AND METHODS

The collection of seeds of *Calendula officinalis* varies “Calta” was made from the collection of local flora of the botanical garden of Ye.A. Buketov Karaganda State University in 2016-2019.

For experiments the seeds are divided into batches of 100 pieces, rejected damaged and slow-developed achenes; dried to different humidity (Dodonova et al 2017, Kushnarenko et al. 2008, Pavlov et al. 2016, Sakai 2000, Zhimulyev 2014). Humidity of seeds is determined

Received: January 2019
Accepted: October 2019
Printed: March 2020

Table 1. Energy of germination and germination rate of *Calendula officinalis* seeds depending on size and weight

Size of seeds	Weight of 1000 pieces, gram	Germination rate, %	Energy of germination, %
Large	16.6±0.5	77.5±3.1	55.0±2.2
Medium	12.3±0.6	61.3±2.8	45.0±1.6
Small	6.7±0.4	55.0±2.4	37.5±0.9
Control (non separated seeds)	14.6±3.9	71.4±2.3	62.5±1.8

using a moisture meter MS-2000. The seeds are controlled without freezing. Calendula has different-quality seeds, so they are divided by party size. Germination is evaluated separately for each batch.

Part of samples is stayed in paper package for determination of germination during storage at the room temperature.

Packaging of seeds for experiments is carried out in 2 types of containers: plastic cryotubes and foil bags. Seeds samples are placed in liquid nitrogen in Dewar Cryo Vessels CDC 20 (CryoMash).

Defrosting after freezing is carried out in 2 versions:

(a) Rapid defrosting in water bath (70 °C) for 15 minutes;

(b) Slow defrosting at room temperature (22-23 °C) for 4-5 hours.

In second series of experiments it was used different cryoprotectors: sucrose – 10 and 40%; glucose – 20 and 40%; glycerin – 20 and 40%. Seed materials are soaked in various solutions of cryoprotectors during 8-10 minutes; after replaced in liquid nitrogen. As a control is used seeds frosted without cryoprotectors. These seeds are washed from cryoprotectors 3-4 times with distilled water before checking of their viability.

To the test of viability experimented seeds and control samples are sown in Petri dishes on two-layer filter paper moistened with distilled water (Baskin & Baskin 1998). The energy of germination (on 6th day) and germination (on 15th day) are noted.

Statistic treatment is conducted according recommendation of G.F. Lakin (1990) using program package EXCEL-2010.

RESULTS AND DISCUSSION

Preservation of viability of plant seed material depends on a number of factors, including packaging, moisture content of seed material, size and weight of seeds, period of their maturation, cryopreservation conditions. Different seed groups require another freezing and defrosting conditions (Dodonova et al. 2017, Nesterova 2004). Thus, seeds with deep physiological rest can be frozen without prior drying; and seeds with shallow physiological rest are more sensitive to cryopreservation conditions.

However, there is no unique algorithm for freezing seeds, so it is necessary to individually select cryopreservation conditions for each species.

On the first stage of experiments is analyzed energy of germination and germination of *Calendula officinalis* seeds depending on the size and weight (**Table 1**).

It is noted the difference in germination between separated and non-separated seeds. The results showed that the maximum germination and germination energy values were observed for large seeds - 77.5 and 55.0%. In small seeds germination made 55.0%, in medium 61.3%, which is lower than the control parameters. The results show in favor of selection of large seeds of *Calendula* for further use and storage, as having maximum germination rate and energy of germination.

Indicators of viability of *Calendula officinalis* seeds are studied during storage. Thus, it is found that after a year of storage germination rate of *Calendula* was 34,2 %, after 1,5 years was 30,0 % (**Fig. 1**).

On both indicators of quality of seeds we can observe linear dependence between viability of seeds, energy of germination and a period of storage. That is, we can observe that the seeds of herb are rapidly losing their sowing properties, so, it is necessary to optimize their storage conditions and methods of increasing germination.

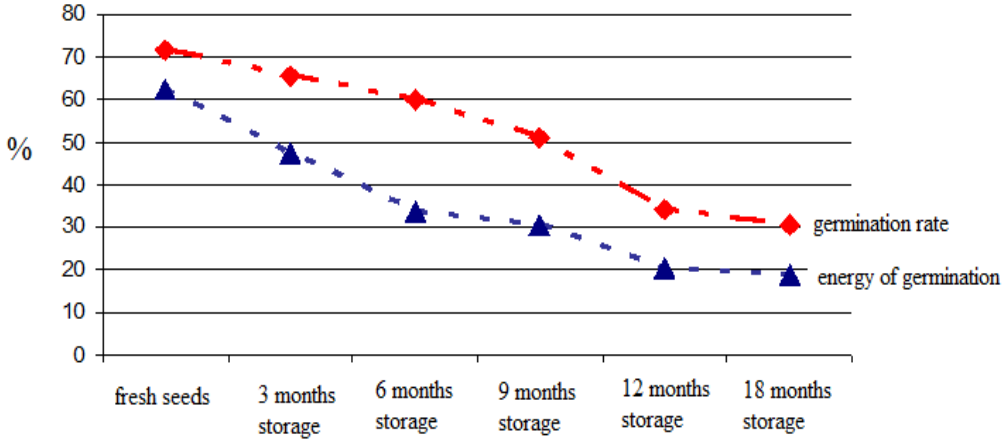

Fig. 1. Germination rate and energy of germination of *Calendula officinalis* seeds in process of storage

Table 2. Germination rate and energy of germination of *Calendula officinalis* seed after cryopreservation in different containers and using different methods of defrosting

Variant of experiment	Germination rate, %	Energy of germination, %
Control, storage for 6 month in room temperature, paper packs	47.0±1.0	44.0±1.0
Cryopreservation in foil pack, defrosting in water bath	1.0	1.0
Cryopreservation in plastic tubes, defrosting in water bath	63.3±1.4	56.7±1.2
Cryopreservation in foil pack, defrosting in room temperature	70.0±1.5	65.0±1.4
Cryopreservation in plastic tubes, defrosting in room temperature	71.7±1.6	68.3±1.5

Table 3. Germination rate and energy of germination of *Calendula officinalis* seeds of different humidity after cryopreservation (fresh seeds)

Variant of experiment	Germination rate, %	Energy of germination, %
Constrol, without cryopreservation	47.0±1.0	44.0±1.0
Humidity 5-6%	72.0±3.2	68.5±2.4
Humidity 10-11%	54.8±1.4	48.9±0.7
Humidity 15%	20.5±0.2	15.6±0.1

At the second stage, the effects of package and defrosting conditions on viability of seeds after storage in liquid nitrogen (6 months) are analyzed (**Table 2**).

The results shew that there was a difference in defrosting in a rapid or slow manner. Thus, the germination of *Calendula officinalis* seeds after freezing in foil and defrosting in a water bath was significantly lower, than the control values and defrosting at the room temperature - 1.0%. Freezing in plastic containers and defrosting in water bath are slightly better. The germination rate of plant seeds in this test variant was 63,3%, energy of germination – 56,7%; which was higher the control parameters on 16,3 and 12,7%. The best result was noted for variant preservation in plastic tubes and defrosting in room temperature: germination rate was 71,7%, energy of germination – 68,3%. So, we can recommend these conditions for cryopreservation of *Calendula officinalis* seeds.

The effect of seed humidity on cryopreservation survival rates is analyzed. Free moisture is able to form ice crystals, which can damage plant cells. Therefore, low moisture content can reduce crystal formation, thereby increasing survival of plant objects.

Seeds of different humidity (5-6; 10-11 and 15%) are frozen in plastic containers. Results showed that when humidity decreased, the survival rate of *Calendula officinalis* seeds increased (**Table 3**).

Thus, we can observe that the maximum values of germination rate and germination energy of *Calendula officinalis* seeds are noted at the humidity 5-6%, the minimum - at the seed humidity 15%. That is, freezing of marigold seeds should be carried out after preliminary drying.

It is known (Reed 2002) that in cryobiology one of the ways to increase germination is the use of special chemicals - cryoprotectors. These substances are able to penetrate cells by binding free water, or to be embedded in the structure of cell membranes, thereby making it stronger and more resistant for freezing in liquid nitrogen. The disadvantages of many cryoprotectors are their toxicity, so it is necessary to thoroughly wash the seeds from cryoprotectors after defrosting, as well as to strictly dose the concentration.

Table 4. Germination rate and energy of germination of *Calendula officinalis* seeds after cryopreservation in different proprotectors

Variant of experiment	Germination rate, %	Energy of germination, %
Control, cryopreservation without cryoprotectors	65.0±1.4	55.0±1.2
Sucrose, 10%	45.0±1.0	40.0±0.7
Sucrose, 40%	10.0±0.2	5.0±0.1
Glycose, 20%	15.0±0.3	10.0±0.2
Glycose, 40%	15.0±0.3	10.0±0.2
Glycerin, 20%	5.0±0.1	5.0±0.1
Glycerin, 40%	5.0±0.1	5.0±0.1

At the third stage of research is tested the effects of cryoprotectors on viability of *Calendula officinalis* seeds (**Table 4**).

The results showed that *Calendula officinalis* seeds had lower germination rate and energy of germination after using of cryoprotectors. Thus, the control parameters were 65,0 and 55,0%, whereas the use of cryoprotectors showed a germination rate from 5 to 45%; energy of germination was from 5 to 40%.

The last results show that it is not recommended to use cryoprotectors during cryopreservation of *Calendula officinalis* seeds.

CONCLUSION

Thus, *Calendula officinalis* has the large perspectives of using as vegetative raw materials in Kazakhstan for medicine and cosmetic industry. Marigold seeds have different quality and limited shelf life.

Conducted research is shown that the seed material of *Calendula officinalis* could be successfully freezing at extremely low critical temperatures (in condition of liquid nitrogen), and the selection of optimal conditions allowed not only to preserve the viability of the seeds, but also increased the germination rate and energy of germination.

The optimal container for freezing in liquid nitrogen was plastic cryotubes; defrosting was necessary to spend at room temperature. The best survival results are obtained after using seeds with humidity 5-6%.

It was determined that the use of cryoprotectors did not have a positive effect on the increase in germination rate and energy of germination of *Calendula officinalis* seeds. Viability of seeds after cryoprotectors treatment was lower than for seeds frozen in liquid nitrogen without cryoprotectors. Therefore, the use of cryoprotectors is not appropriate.

The results of the studies can be used to organize long-term storage of *Calendula officinalis* seeds at extra low temperatures (liquid nitrogen).

ACKNOWLEDGEMENTS

The studies were carried out within the framework of the internal grant project of Ye.A. Buketov Karaganda State University 218-B-19 “Cryopreservation of plant and animal biological materials to develop the algorithm of preservation viability and creating a bank of frozen samples.” The research team would express their gratitude to the Rector of Ye.A. Buketov Karaganda State University for the financial support.

REFERENCES

Alsaraf KM, Abbas IS, Hassan EF (2019) Extraction and Clinical Application of Calendula officinalis L. Flowers Cream. IOP Conf. Series: Materials Science and Engineering 571. https://doi.org/10.1088/1757-899X/571/1/012082

Ansari A, Hanief A (2015) Microbial degradation of organic waste through vermicomposting. International Journal of Sustainable Agricultural Research, 2(2): 45-54.

Arjenaki FG, Dehaghi MA, Jabbari R (2011) Effects of priming on seed germination of marigold (*Calendula officinalis*). Advances in Environmental Biology (2): 276-280.

Babu KN, Yamuna G, Praveen K, Minoo D, Ravindran PN, Peter KV (2012) Cryopreservation of Spices Genetic Resources. In book: Current Frontiers in Cryobiology: 16-484. https://doi.org/10.5772/35401

Baskin CC, Baskin JM (1998) Seeds: ecology, biogeography, and evolution of dormancy, and germination. London: Academic Press. 666 p.

Butnariu M, Coradini CZ (2012) Evaluation of biological active compounds from *Calendula officinalis* flowers using spectrophotometry. Chemistry Central Journal, 6: 35.

Dodonova Ash, Ishmuratova My, Gavrilkova EA, Tleukenova SU (2017) Recommendation on cryopreservation of medicinal and endemic plants species. Karaganda: Polygraphist. 120 p.

Goktas FM, Sahin B, Yigitarslan S (2015) Production of Sterilizing Agents from Calendula officinalis Extracts Optimized by Response Surface Methodology. International Journal of Analytical Chemistry, 2015 (5), Article ID 789732: 7. https://doi.org/10.1155/2015/789732

González-Benito ME, Aguilar N, Avila T (2009) Germination and embryo rescue from Passiflora species seeds post-cryopreservation. CryoLetters, 30 (2): 142-147.

Grudzinskaia LM, Gemedzhieva NG, Nelina NV, Karzhaubekova JJ (2014) The annotated list of medicinal plants of Kazakhstan. Almaty. Vol. 20: 200 p.

Ishmuratova My (2015) Introduction of medicinal plants in the Central Kazakhstan. Monograph. Karaganda: Bolashaq-Baspa: 170 p.

Ishmuratova My, Nashenova GZ, Nashenov ZhB (2010) Atlas of seeds of medicinal and essential oil plants. Zhezkazgan: Er Mura. 57 p.

Jan N, Andrabi KI, John R (2017) *Calendula officinalis* – an important medicinal plant with potential biological properties. Proc Indian Natn Sci Acad., 83(4): 769-787. https://doi.org/10.16943/ptinsa/2017/49126

Kurkin VA, Dubishchev AV, Afanasyeva PV, Kurkina AV, Zaitceva EN (2016) Investigation of diuretic activity of phytopreparations of *Calendula officinalis* L. flowers. Bulletin of Siberian Medicine, 15(2): 51–57. https://doi.org/10.20538/1682-0363-2016-2-51-57

Kushnarenko SV, Kovalchuk IYu, Romadanova NV (2008) Cryopreservation of apical meristems of fruit and berry cultures. Криосохранение апикальных меристем плодовых и ягодных культур. Methodical recommendations. Almaty. 58 p.

Lakin GF (1990) Biometry. Moscow: Science. 352 p.

Loseva IV (2008) Raw material base of medicinal plants of Kazakhstan and its rational use. Karaganda: Karaganda State Medical University. 110 p.

Muley BP, Khadabadi SS, Banarase NB (2009) Phytochemical Constituents and Pharmacological Activities of *Calendula officinalis* Linn (Asteraceae). Trop J Pharm Res., 8: 455-465.

Nesterova S.V. (2004) Cryopreservation of seeds of wild plants of Premorsky Krai. Thesis. Vladivostok. 150 p.

Nikishina TV, Popova E, Vakhrameeva MG, Popov AS (2007) Cryopreservation of seeds and protocorms of rare temperate orchids. Russian Journal of Plant Physiology, 54(1): 121-127. https://doi.org/10.1134/S1021443707010189

Pavlov AV, Porotnikov IV, Verzhuk VG, Vorobeykov GA (2016) Storage of selection materials of fruit and berry cultures at the extra low temperatures. Research Journal of National Investigation University. Series processes and apparatus of food industries., 1: 55-60.

Pharmaceutical Market of Kazakhstan: history, general directions of development and current state (2015). Almaty: AEQUITA. 36 c.

Preethi KC, Kuttan G, Kuttan R (2009) Anti-inflammatory activity of flower extract of *C. officinalis* Linn. and its possible mechanism of action. Ind. J. Exp. Biol., 47: 113-120.

Reed BM (2002) The basics of in vitro storage and cryopreservation. National Clonal Germplasm Repository, Corvallis: O.R. USA: P. 34-46.

Sakai A (2000) Development of cryopreservation techniques // Cryopreservation of tropical plant germplasm. Current research progress and application. Eds. Engelmann F& Takagi H., JIRCAS, Tsukuba & IPGRI, Rome. 215 p.

Shafeie N, Naini AT, Jahromi HK (2015) Comparison of different concentrations of *Calendula officinalis* gel on cutaneous wound healing. Biomedical and Pharmacology Journal, 8(2): 979-992. https://doi.org/10.13005/bpj/850

Ukiya M, Akihisa T, Yasukawa K, Tokuda H, Suzuki T, Kimura Y (2006) Anti-inflammatory, anti-tumor-promoting, and cytotoxic activities of constituents of marigold (*C. officinalis*) flowers. J. Nat. Prod. 69: 1692-1696.

Voskresenskaya ML, Plekhanov AN, Dorzhiev BD, Tsyrempilov SV (2016) Prospects of phytopreparations based on *Calendula officinalis* in complex treatment of chemical burn of the esophagus. Acta Biomedica Scientifica, 1(5): 175-180. https://doi.org/10.12737/23420

Zhimulyev IF (2014) Cryopreservation of seeds: results and perspectives. Novosibirsk: Siberian Department of Russian Academy of Science. 110 p.