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Analysis of the Effects of Cryopreservation Conditions on Germination Ability of *Tanacetum Ulutavicum* Seeds.

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ABSTRACT

Storing in liquid nitrogen is one of promising methods of preservation of biological material, allowing depositing it for an unlimited time period. Conservation of endemic plants is a relevant, top-priority task. *Tanacetum ulutavicum* Tzvel. is a perennial plant, endemic of the Ulytau mountains. The seeds were frozen in liquid nitrogen in a variety of ways – rapid freezing, two-step method, using different containers and cryoprotective agents; then the effects of different thawing techniques on viability of *Tanacetum ulutavicum* seeds were studied. We examined the biological features of germination under normal conditions and after deep freezing; no changes in the morphology of seedlings and duration of ontogenetic stages were observed. Our experiments allowed us to develop optimal freeze-thawing conditions – freezing in glycerin, followed by slow thawing at room temperature. These results can be used for creation of seed gene banks.

Keywords: *Tanacetum ulutavicum,* cryopreservation, cryoprotector, germination biology, germination capacity.

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INTRODUCTION

Studying the methods of preservation of species with limited habitats and medicinal value is an important issue that will provide the opportunities to create proper conditions for restoration of natural populations and for supplying the Kazakhstan pharmaceutical companies with raw plant materials. It should be noted that storing seeds at room temperature leads to reduced germination capacity due to accumulation of mutations and germ damage. Deep freezing of seeds (at the temperature of liquid nitrogen) is currently considered a promising method of storing plant genomes, as in theory it allows one to maintain germination capacity and genetic consistency of seeds for an unlimited time period. The current research includes the experiments on optimization of freeze-thawing conditions for *Tanacetum ulutavicum* seed material. Cryopreservation of *Tanacetum ulutavicum* seeds has never been studied before. This research has been conducted as a part of the scientific grant project "Examination of the current state of endemic plant populations in North and Central Kazakhstan and the development of methods for preservation of genetic material".

MATERIALS AND METHODS

Tanacetum ulutavicum Tzvel. (*Asteraceae* fam.) is a perennial endemic plant, inhabiting rocks and rocky slopes of the Ulytau mountains. Flavonoids, obtained from inflorescences, exhibit choleretic action [1-3]. We used seeds collected in different years for the experiments. The seeds had not been selected in any specific way and seed coats had not been peeled. Pest-damaged heads were excluded.

Germination capacity and energy of germination were assessed, as described in the guidelines by M.S. Zorina and S.P. Kabanov [4], M.V. Mal'tseva [5].

Seeds were sown in the laboratory in Petri dishes, on 2 layers of blotter paper, soaked in distilled water, in 4 replicates. Petri dishes with seeds were incubated in climate-control chambers at +24 °C. Seeds had not been selected for the experiments, yet damaged, hollow or ones of altered color were excluded.

We performed statistical analysis of the results, according to N.L. Udol'skaya guidelines [6].

Seeds were frozen in two different ways. The first method included gradual cooling down to -(48-50) °C in the MDF – U 442(T) Sanyo Medical Freezer, in two stages, with the initial rate of 1-2 °C per half an hour, until the temperature of seeds decreased to -30 °C. During the second stage, the freezing speed was increased up to 4-5 °C per half an hour, the seeds were cooled down to -50 °C and loaded into large cryotanks for nitrogen-vapor storage at -183-185 °C [7]. Another technique included rapid freezing, when seeds in various containers were directly submerged in liquid nitrogen at -196 °C, with glycerin used as cryoprotector [8-10].

RESULTS AND DISCUSSION

We conducted a set of experiments on freezing the seeds of the examined species. *Tanacetum ulutavicum* seeds were immersed in liquid nitrogen (-196 °C) in different containers: cloth bags, plastic tubes ("Nunc" cryotubes), foil packets. Seeds were thawed according to various techniques – slow thawing at room temperature; rapid hot-water bath thawing at 80 °C; seeds were sown two days after air thawing.

Initial germination capacity of *Tanacetum ulutavicum* seeds was 33±2%.

We studied germination biology of *Tanacetum ulutavicum* seeds. Seed germination was observed at 2-3 days, white root with well-marked root caps appeared at the tapered parts of the seeds. At 3rd day we could observe emergence of white hypocotyls. In a 24 hours, hypocotyls were 6-6-7 mm long, up to 0.5-0.8 mm in diameter, root were elongated up to 5 mm. Light-green hypocotyls slightly elongated, curved, and cotyledons were expanded. Cotyledon expansion was observed at 4th day, with folded leaves, unfolding in 3 days. Root length increased up to 10 mm, the length of hypocotyl – to 13 mm, up to 1 mm in diameter. Cotyledons were green, shiny, smooth, had an elliptical shape, up to 2.5 mm long, up to 1.5 mm wide, with well-pronounced midribs in the middle of the leaf, petiolar length was 1-1.5 mm. Seedling length on the 10th day of germination was 23 mm, up to 1 mm in diameter, roots were approximately 10 mm long, up to 0.8 mm

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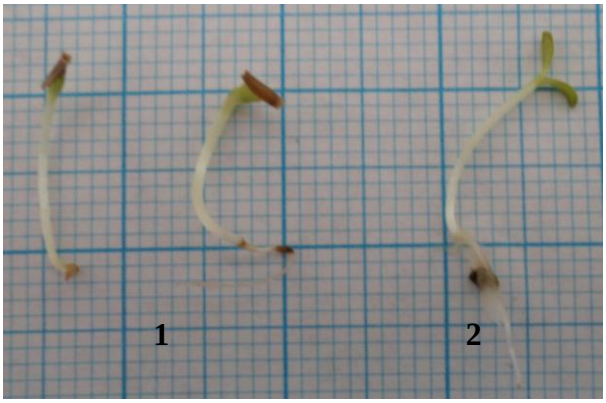
in diameter. Length of light-green hypocotyles was 16.4 mm, diameter – up to 1 mm. Cotyledons were elliptical, up to 2.5-2.6 mm long, 1.2-1.5 mm wide (Figure 1).



1 – elongation of hypocotyl, expansion of cotyledons, 2 – unfolding of cotyledons

Figure 1 – Biology of *Tanacetum ulutavicum* germination

We also examined the biology of *Tanacetum ulutavicum* seed germination after cryopreservation, in order to assess the influence of ultralow temperature. The seedlings developed similarly to the control group, undergoing all the major ontogenetic stages.

It is well known that seed humidity is one of the most important factors of preservation of seed germination ability during cryopreservation, and the optimal level is 5-6%. We conducted experiments on freezing *Tanacetum ulutavicum* seeds with different moisture content (14%, 7%) in liquid nitrogen [11]; the seeds were immersed in nitrogen in various containers: foil packages and plastic tubes. We did not observe any significant differences in germination capacity and energy of germination – germination capacity of seeds with 7% moisture was 39±2%, whereas in case of 14% moisture – 28±1.5%. Insignificant discrepancy in germination capacity of seeds with different moisture content are, probably, related to small size of seeds and small differences in moisture content. *Tanacetum ulutavicum* is a arid plant; therefore, its seeds can be described as orthodox ones, containing little moisture.

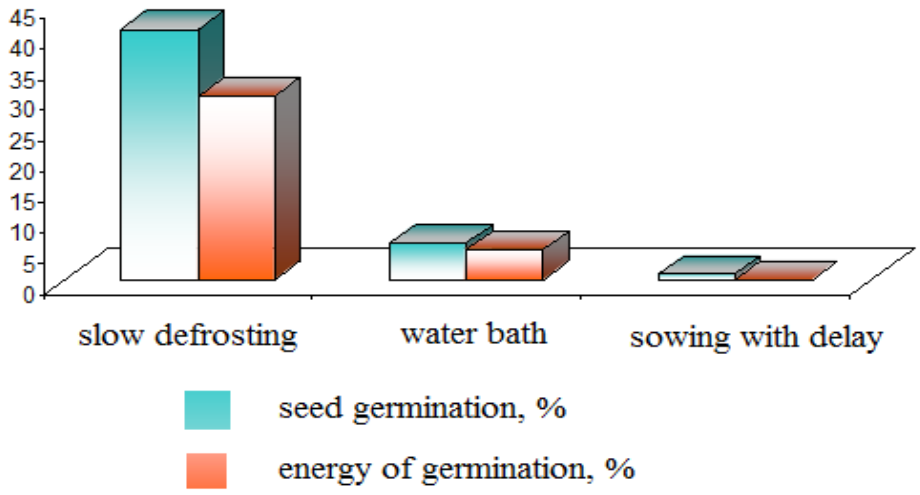


Figure 2 – *Tanacetum ulutavicum* seed germination capacity after freezing and thawing in different ways

The data present in literature indicate that the containers for nitrogen immersion have influence on preservation of biological material. We used foil, plastic tubes, and fabric containers in pour experiments. Seeds, frozen in foil packages, had the highest level of preservation of germination capacity and energy of germination – 38±2%. Plastic and fabric containers lead to a reduction in seed viability down to 20% and 10%

respectively, which is 60% and 30% of the initial germination capacity. Therefore, we recommend using foil packages for cryopreservation of *Tanacetum ulutavicum* seeds.

When biological material is introduced to a gene bank, one should develop an optimal thawing regime for the object. We examined preservation of *Tanacetum ulutavicum* seed viability after rapid freezing by immersion in liquid nitrogen and different thawing techniques – slow thawing at room temperature, rapid thawing in hot-water bath, sowing with 3-days delay (Figure 2).

We determined that the best thawing technique for *Tanacetum ulutavicum* seeds was slow defrosting at room temperature, providing 41% seed germination, which was higher than in the control group. Rapid water-bath thawing and sowing with 3-days delay were fatal for seed germs and led to a significant decrease in seed viability down to 6% and 1%, respectively; therefore, we did not recommend using these techniques. Apparently, no recrystallization occurs during seed defrosting, which is why slow thawing allows one to preserve germination capacity of seed material to the greatest extent possible.

We implemented the programmable freezing method, utilized in N.I. Vavilov All-Russian Research Institute of Plant Industry (Saint Petersburg), in our study. The seed material was subjected to two-stage freezing – slow cooling to -30 °C and -50 °C, followed by rapid immersion in nitrogen vapor at -183-185 °C. Seed defrosting was performed slowly, at room temperature. Our results (Figure 3) indicate that slow cooling should continue until the temperature reaches -50 °C, as in this case seed germination is 106.6% of the initial level. Preliminary cooling to -30 °C leads to a significant decrease in germination characteristics down to 9.6%, which could be related to insufficient withdrawal of intracellular free water and germ damage with forming crystals.

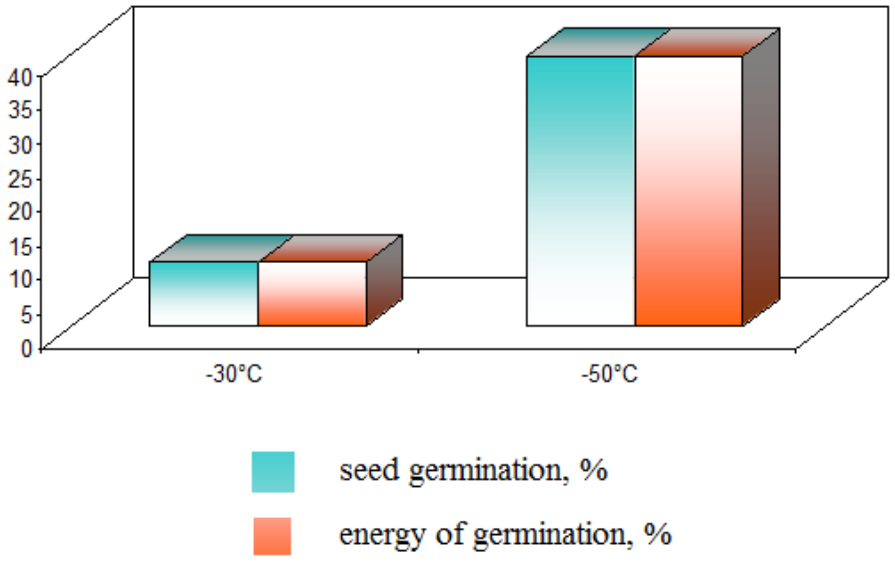


Figure 3 – Preservation of viability of *Tanacetum ulutavicum* seed material in the course of two-stage freezing

Table 1. Influence of cryoprotective agents on preservation of *Tanacetum ulutavicum* seed viability

Cryoprotector		Energy of germination, %	Energy of germination,% of initial level	Germination capacity, %	Preservation of viability, % of initial level
Glycerin	A	20	64.5	22	66.6
	B	38	122.5	44	**133.3**
10% sucrose	A	62	200	72	**232.2**
	B	-	-	-	-
20% sucrose	A	24	77.4	30	90.9
	B	-	-	-	-
10% sucrose, 50% glycerin	A	4	12.9	44	**133.3**
	B	12	38.7	36	**109**
A – slow thawing at +22 °C; B – rapid defrosting in water bath					

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In spite of the fact that *Tanacetum ulutavicum* seeds have low moisture content and are orthodocal, we have studied preservation of seed viability during fast freezing with cryoprotective agents (Table 1). We used two thawing techniques – slow defrosting at room temperature and rapid defrosting in hot water bath.

Application of various cryoprotectors significantly increased seed viability. The best results were observed in case of 10% sucrose solution used as cryoprotector and slow thawing, leading to almost 2.5-fold rise in germination capacity, compared to the initial level. Increasing sucrose concentration to 20% led to lower proportion of viable seeds. We observed intriguing results of application of pure glycerin and glycerin-sucrose mixture – seeds better survived rapid thawing. Water bath defrosting of *Tanacetum ulutavicum* seeds, frozen in sucrose solutions, led to absence of germination; therefore, we recommend slow thawing of seed material, if sucrose is used as cryoprotector. Stratifying effect of deep freezing is frequently observed, but in *Tanacetum ulutavicum* seeds it only manifests itself, if cryoprotective agents are used.

The dynamics of germination rate of seed material is of particular interest – are there any changes in this parameter after deep freezing? We conducted comparative analysis of *Tanacetum ulutavicum* seeds germination in the control group, after rapid freezing, and after freezing with cryoprotector (Figure 4).

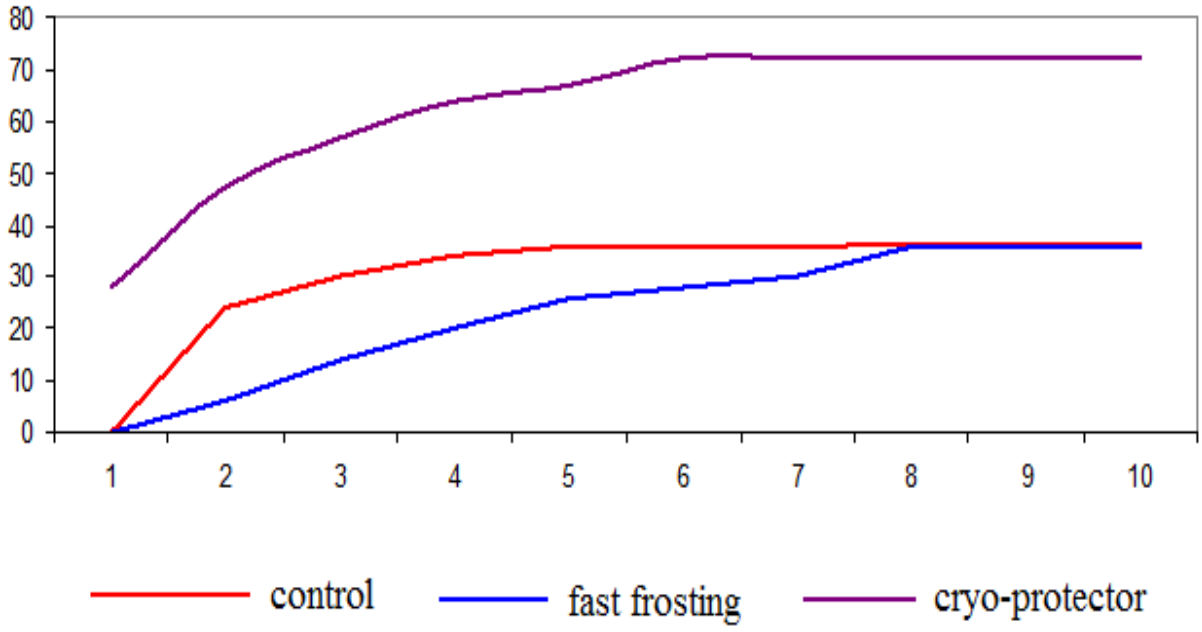

Figure 4 – Germination rates of *Tanacetum ulutavicum* seed material

Deep-frozen seeds without cryoprotector exhibit preservation of initial germination rate with less even germination of the biological material. Application of cryoprotector significantly increases germination rate, while the shape of the curve is slightly changed; seeds germinate less evenly than those of the control group, yet better than in case of fast freezing without cryoprotection.

We should note faster ontogenetic development of seedlings; cotyledon unfolding occurred 1-2 days earlier, and the seedlings appeared to be stronger and more viable, which is additional evidence in favor of this freezing technique.

Our results can be used for creation of a gene bank of endemic or medicinal plants. Given initially low germination capacity of *Tanacetum ulutavicum* seeds, one should use an optimal cryopreservation mode to maintain viability to the greatest possible extent. According to our data, *Tanacetum ulutavicum* seeds should be frozen with cryoprotective agents, such as glycerin which provides 2.5-fold increase in germination capacity compared to the initial values. Thawing should be performed slowly, at room temperature. We did not observe any changes in germination biology of the seeds after deep freezing, although the seedlings emerging from the seed material exposed to ultralow temperatures turned out to be stronger and more viable, in spite of the decrease in certain morphometric characteristics.

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CONCLUSION

Summarizing our experiments, we come to the conclusion that the optimal freezing conditions of *Tanacetum ulutavicum* seed material include freezing in 10% solution of sucrose, used as cryoprotector; seeds should be thawed slowly, at room temperature. These techniques provide 2.3-fold increase in germination capacity, compared to the control.

Recommendations on cryopreservation of *Tanacetum ulutavicum* seeds, suggested in current paper, can be used for creation of a gene bank of endemic plants of Central Kazakhstan.

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