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Evaluation of the *Dracocephalum ruyschiana* L. and *Salvia sclarea* seed material cryopreservation effectiveness.

Cryopreservation of plant material of valuable plants is an effective way to preserve biological diversity in conditions of active and widespread anthropogenic pressure on natural ecosystems. Introduction to cryocollections is justified not only for food agricultural plants, but also for medicinal plants that have valuable secondary metabolites and essential oils. These include *Dracocephalum ruyschian* and *Salvia sclarea*. To effectively restore the viability of seeds after freezing in liquid nitrogen, we examined the influence of various cryoprotectants: glycerin, dimethyl sulfoxide and polyvinypyrrolidone, as well as various methods of their application — at room temperature and in the cold. It was found that for effective cryopreservation of *Dracocephalum ruyschiana* seed material, the use of cryoprotectors is justified; however, the method of their application does not matter. When cryopreserving *Salvia sclarea* seed material, it is important to use cryoprotectors and introduce them into the cooling system, preferably at the crystallization temperature of water; in this case, the energy of seed germination increases significantly.

Keywords: Dracocephalum ruyschiana, *Salvia sclarea*, cryoprotectors, cryopreservation, seed material, germination, germination energy.

Introduction

Human economic activities, especially the mining industry, have a significant degrading impact on ecosystems. Biological diversity is constantly declining, populations, and then species, may disappear. The loss of any species has a negative impact, however, the loss of a species that is of practical importance for the national economy leaves an indelible imprint. *Dracocephalum ruyschiana* and *Salvia sclarea* — essential oil and medicinal plants of the Lamiaceae family [1–3], are valuable representatives of the flora of Kazakhstan and their conservation is an urgent task.

Preservation of the gene pool of plants can currently be carried out in a variety of ways. Depending on the chosen method, storage times will vary. Cryopreservation is an innovative, popular, effective and inexpensive way to preserve biological material for a long time without loss of genetic stability [4–9]. The stages of freezing and thawing of the material are critical, therefore optimization of the conditions for freezing seed material of the studied species in liquid nitrogen is the subject of discussion in this publication. Among the factors influencing the degree of preservation of the viability of biological material during cryopreservation are endocellular and exocellular cryoprotectants, which have different mechanisms of action on the cell and therefore contribute to different degrees of survival, the method of introducing the cryoprotectant — at room temperature and in the cold, which leads to a decrease in the negative impact of cryoprotectants substances on cell metabolism. The addition of cryoprotectants in the cold can be considered as a two-stage freezing, since the biological material is cooled to the crystallization temperature of water before cryopreservation.

Materials and Methods

The object of the study was the seed material of *Dracocephalum ruyschiana* and *Salvia sclarea*. The studied species are valuable representatives of the Lamiaceae family, are included in the list of medicinal plants of Kazakhstan, are essential oil plants, and contain a large number of secondary metabolites with biological activity. They are widely used, for example, clary sage essential oil is used in medicine, cooking, winemaking, it has a pleasant aroma, and it is often used in the composition of expensive perfumes [10–14].

Seed material was frozen in plastic tubes in liquid nitrogen using the direct immersion method. Cryoprotectors were used in various concentrations, endocellular — glycerin and dimethyl sulfoxide (DMSO), exocellular — polyvinylpyrrolidone (PVP). Two methods of introducing cryoprotectants into the

freezing system were used: at room temperature and in the cold. For the second application method, the seed material was pre-cooled in an ice bath.

After cryopreservation, cryoprotectants were washed three times with distilled water, then placed in Petri dishes on two layers of filter paper to assess viability.

The viability of seed material was determined by germination and germination energy [15–17]. All experiments were performed in triplicate.

Results and Discussion

The initial growth characteristics of *Dracocephalum ruyschiana* seeds were determined; germination was 84.33±7.72 %, germination energy was 42.33±3.09 %. Dry seeds were frozen in liquid nitrogen without cryoprotectants, germination in this experiment was 67±11.22 %, germination energy was 12±4.24 %. Note that after cryopreservation the energy of germination of seed material decreased significantly. Damage to the embryo occurs, which slows down the process of exiting the dormant state. These initial data were used in the comparative analysis as Control 1 and Control 2.

We conducted an experiment in which seeds were frozen in different types of cryoprotectants: endocellular (glycerol and dimethyl sulfoxide) and exocellular (polyvinylpyrrolidone) in different concentrations. In this case, two methods of introducing the cryoprotector were used — at room temperature and in the cold (0 °C).

The results obtained are presented in Table 1. When considering the germination rate, we do not see significant differences in the effect of cryoprotectors of different types. And the germination energy indicator turned out to be more labile and informative. For clarity of the analysis, we calculated the proportion of surviving seeds and their germination energy from the initial indicators (control 1). These calculations are presented in the form of diagrams in Figure 1.

Analysis of the presented diagram shows that the seed material of *Dracocephalum ruyschiana* does not lose its viability after freezing; the germination rate from the original practically does not fall below 0.8. It is worth noting that the use of high concentrations of cryoprotectors, for example, 50 % glycerol and its cold introduction into the system even leads to a stratification effect and an increase in the number of sprouted seeds. The energy of germination in almost all variants of the experiment was greater than one, that is, after cryopreservation, the seed material sprouted more efficiently than the original seeds. We cannot see a definite trend in the effectiveness of adding cryoprotectants at 0 °C. However, we see the best options for preserving, even increasing the growth performance of *Dracocephalum ruyschiana* seed material in the variant of cold application of glycerol.

Table 1

Growth parameters of *Dracocephalum ruyschiana* seed material after cryopreservation in various cryoprotectants

Cryoprotector (mass fraction in %)	Germination energy (%)	Germination (%)	Proportion of the initial germination energy	Proportion of initial germination	Share of the germination energy of frozen seeds without cryoprotectors	Share of the germination rate of frozen seeds without cryoprotectors
1	2	3	4	5	6	7
Glycerol 50	43,33±12,47	70±8,16	1,02	0,83	3,6	1,04
Glycerol 25	43,33±4,71	71,67±2,36	1,02	0,84	3,6	1,07
Glycerol 12	53,33±9,43	73,33±17	1,25	0,86	4,4	1,09
Glycerol 50 (cold application)	76,67±12,47	93,33±4,71	1,81	1,1	6,3	1,39
Glycerol 25 (cold application)	56,67±12,47	83,33±9,43	1,33	0,98	4,7	1,24
Glycerol 12 (cold application)	60±8,15	70±0	1,41	0,83	5	1,04
DMSO 15	70±16,33	70±16,33	1,65	0,83	5,8	1,04
DMSO 10	60±24,49	73,33±12,47	1,41	0,87	5	1,09
DMSO 5	60±14,4	80±0	1,41	0,95	5	1,19
DMSO 15 (cold application)	53,33±4,71	73,33±4,71	1,26	0,86	4,4	1,09

Continuation of Table 1

1	2	3	4	5	6	7
DMSO 10 (cold application)	56,67±20,55	66,67±12,47	1,34	0,79	4,7	0,99
DMSO 5 (cold application)	60±8,16	73,33±9,43	1,42	0,87	5	1,09
PVP 5	47,33±0,47	72,67±9,10	1,12	0,86	3,9	1,08
PVP 3	35,67±6,8	65,33±2,49	0,84	0,77	2,9	0,98
PVP 1	67,67±3,3	80,67±0,94	1,6	0,96	5,6	1,2
PVP 5 (cold application)	38,67±3,86	70,67±6,13	0,91	0,84	3,2	1,05
PVP 3 (cold application)	47,33±3,77	75±4,08	1,12	0,89	3,9	1,12
PVP 1 (cold application)	47,33±0,47	84,67±4,71	1,12	1,0	3,9	1,26
Control 1 (initial indicators)	42,33±3,09	84,33±7,72				
Control 2 (freezing without cryoprotectants)	12±4,24	67±11,22	0,28	0,79		

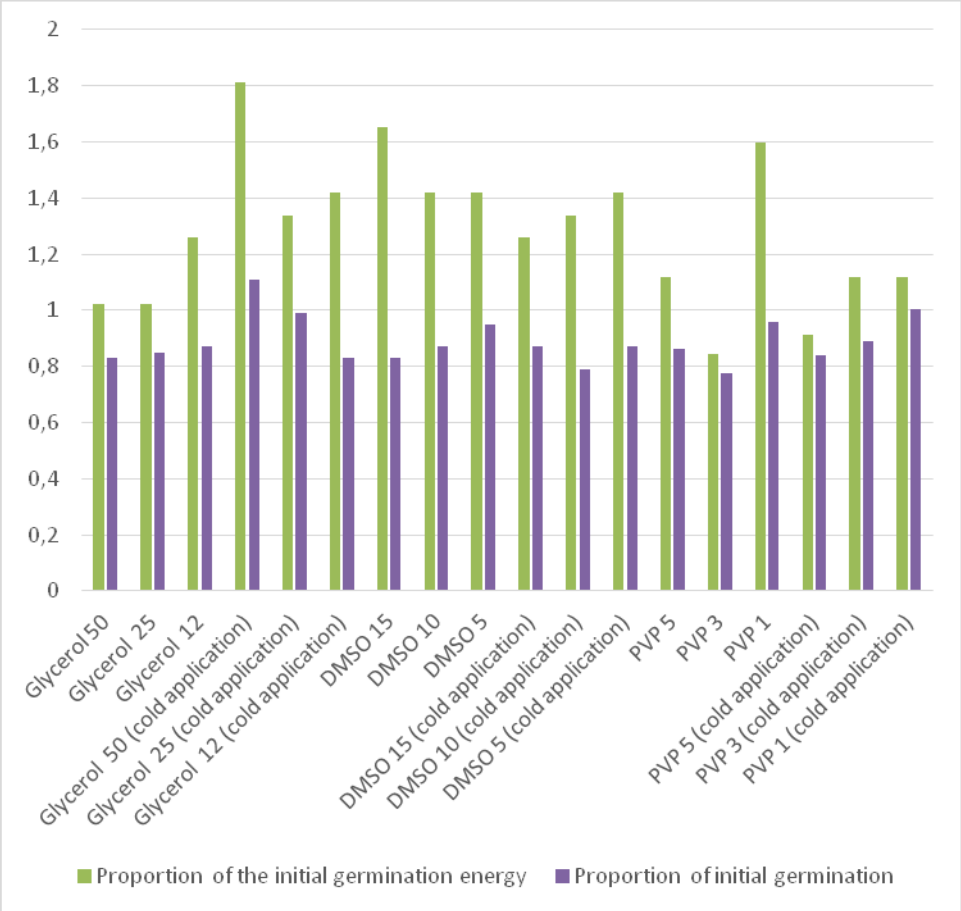

Figure 1. Proportion of growth characteristics of *Dracocephalum ruyschiana* seeds after cryopreservation from the initial indicators

To compare the effectiveness of using cryoprotectants compared to freezing biological material without using them, we calculated the proportion of surviving seeds compared to similar indicators for seeds frozen without cryoprotective solutions. The obtained data are presented in Figure 2.

We see that the germination rate has not changed significantly; in almost all variants of the experiment it is slightly more than one.

However, the germination energy increased several times, in some variants of the experiment by 5–6 times. Germination energy is an important factor that demonstrates the vigor of seedlings and the activation of the state of the entire biochemical arsenal of the embryo, its exit from the dormant state. High rates of germination energy demonstrate the good adaptive qualities of the seed material and its ability to withstand extreme environmental factors.

It is interesting to note that the maximum increase in germination energy was achieved with cold addition of glycerol. Probably, this method of administration reduces its pseudotoxic effect and its inhibition of biochemical processes. In experiments with DMSO and PVP, the maximum performance was achieved in the variants of adding cryoprotectants at room temperature, i.e. the reaction to the method of administration depends not only on the type of seed material, but also on the type of cryoprotector.

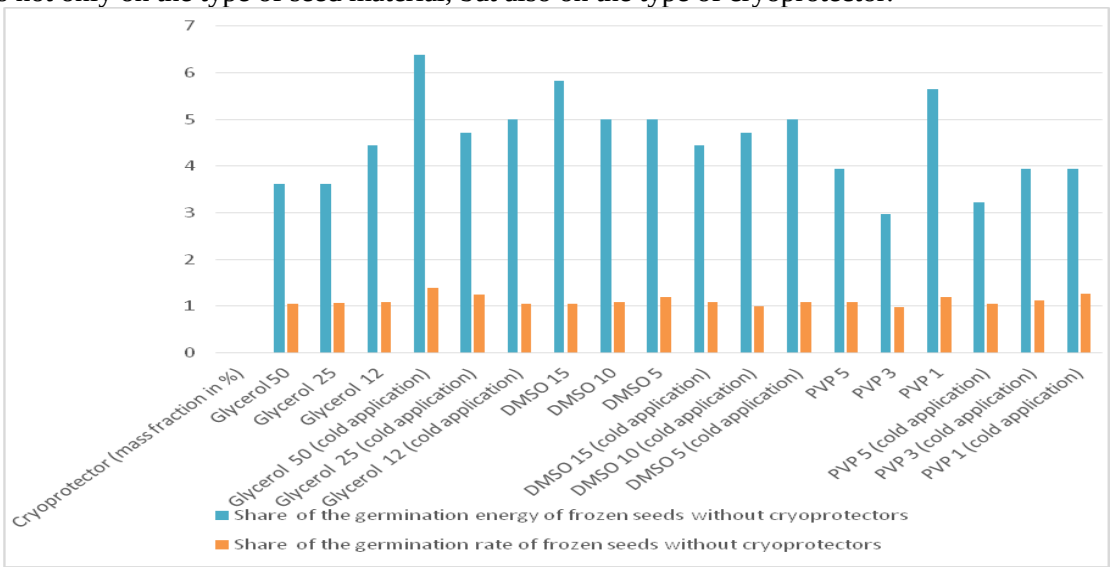

Figure 2. Ratio of growth characteristics of Dracocephalum ruyschiana seed material after freezing in liquid nitrogen to similar indicators of seeds frozen without cryoprotectants

Based on the analysis of the data obtained, we can conclude that the use of cryoprotectants for cryopreservation of *Dracocephalum ruyschiana* seed material is justified, while the method of applying the cryoprotectant is not particularly important.

At the next stage, a similar experiment was conducted to study the influence of various cryoprotectors and methods of their introduction on the preservation of the viability of *Salvia sclarea* seed material during cryopreservation. The obtained data are presented in Table 2. The initial growth characteristics of *Salvia sclarea* seeds were determined, germination was 83.33±17 %, germination energy was 16.67±4.71 %. Dry seeds were frozen in liquid nitrogen without cryoprotectants, germination in this experiment was 73.67±3.4 %, germination energy was 6.67±4.71 %. It can be seen that when frozen in liquid nitrogen, seeds lose 10 % in terms of germination and germination energy, which can be assessed as a slight decrease. However, if we look at it in absolute numbers, then the germination energy of 7 % means very slowly and unharmonious germinating seeds.

Table 2

Growth parameters of Salvia sclarea seed material after cryopreservation in various cryoprotectants

Cryoprotector (mass fraction in %)	Germination energy (%)	Germination (%)	Proportion of the initial germination energy	Proportion of initial germination	Share of the germination energy of frozen seeds without cryoprotectors	Share of the germination rate of frozen seeds without cryoprotectors
1	2	3	4	5	6	7
Glycerol 50	16,67±4,71	46,67±12,47	1,02	0,83	3,61	1,04
Glycerol 25	9,83±11,11	25,5±11,4	1,02	0,84	3,61	1,06
Glycerol 12	13,33±4,71	73,33±7,45	1,25	0,86	4,44	1,09
Glycerol 50 (cold application)	0	43,33±20,55	1,33	0,98	4,72	1,24
Glycerol 25 (cold application)	31±0,82	65,67±4,92	1,33	0,98	4,7	1,24
Glycerol 12 (cold application)	10±8,16	56,67±4,71	1,47	0,83	5	1,04

Continuation of Table 2

1	2	3	4	5	6	7
DMSO 15	10±0	46,67±12,47	1,65	0,83	5,89	1,04
DMSO 10	13,33±4,71	40±0	1,41	0,86	5	1,09
DMSO 5	10±0	43,33±4,71	1,42	0,94	5	1,19
DMSO 15 (cold application)	20±0	36,67±4,71	1,25	0,87	4,44	1,09
DMSO 10 (cold application)	33,33±9,43	63,33±4,71	1,34	0,79	4,72	0,99
DMSO 5 (cold application)	6,67±0,93	56,67±17	1,42	0,86	5	1,09
PVP 5	12,33±5,56	25,67±13,82	1,11	0,86	3,94	1,08
PVP 3	9,33±0,94	34,33±4,19	0,84	0,77	2,97	0,97
PVP 1	29,33±8,99	45±4,08	1,59	0,95	5,64	1,2
PVP 5 (cold application)	0	44±17,48	0,91	0,83	3,22	1,05
PVP 3 (cold application)	33,33±12,47	64,33±24,3	1,11	0,89	3,94	1,11
PVP 1 (cold application)	22±2,16	49±5,89	1,11	1,01	3,94	1,26
Control 1 (initial indicators)	16,67±4,71	83,33±17				
Control 2 (freezing without cryoprotectants)	6,67±4,71	73,67±3,4				

Analysis of the growth performance of Salvia sclarea seed material after cryopreservation showed that exposure to extreme temperatures leads to a decrease in germination rate (Fig. 3). In all variants of the experiment, we observe the value of the indicator under consideration at a level less than one. This indicates that low temperatures lead to, albeit minor, damage to the seed embryo in the species Salvia sclarea.

However, when considering the share of germination energy from a similar indicator in intact seeds, we see in some variants of the experiment an increase in this characteristic in seeds subjected to cryopreservation by almost 2 times: when using glycerol 12 %, DMSO 10 % and PVP 3 % introduced into the system in the cold.

This indicates the activation of metabolic processes in the tissues of the seed embryo and the stratification effect, which lead to more uniform seed germination.

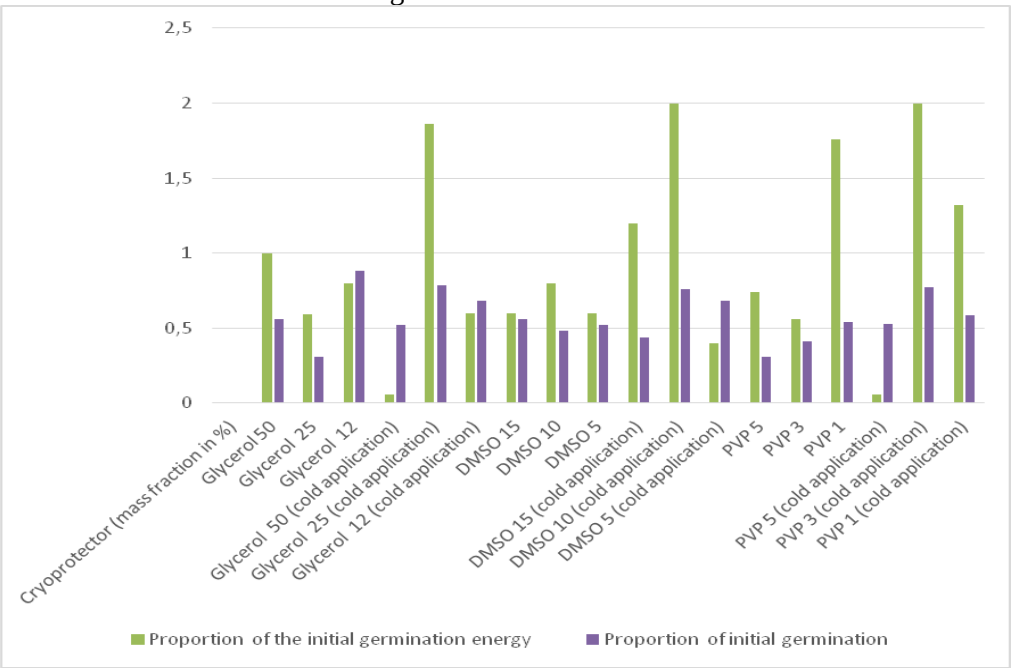

Figure 3. Proportion of growth characteristics frozen in liquid nitrogen Salvia sclarea seeds from the original

When comparing the obtained data with the indicators of germination and germination energy of *Salvia sclarea* seed material, which was frozen without the use of cryoprotectants, we see that the germination indicators did not change or became less than one (Fig. 4).

That is, the use of cryoprotectors does not lead to better preservation of seeds when frozen in liquid nitrogen.

But if we turn to the germination energy indicator, we see that the use of cryoprotectants contributes to more friendly seed germination; the germination energy exceeds that of seeds frozen without cryoprotectors several times, in some experimental variants — 5 times — when using 12 % glycerol, DMSO 10 % and PVP 3 % with the addition of cryoprotective substances in the cold.

Thus, we believe that the use of cryoprotectants and their cold application is justified during cryopreservation of *Salvia sclarea* seed material.

Conclusion

Application of cryoprotectants in the cold is often more effective due to several factors. This is a slowdown in cell metabolism, a decrease in the toxicity of the cryoprotector, and a decrease in osmotic stress caused by cryoprotectants [18]. In addition, at low physiological temperatures, membranes are stabilized, which reduces the penetration of the cryoprotector into the cell and additionally protects against the toxic effects of these substances.

The study determined that seed material of different species, even within the same family, reacts differently to extremely low temperatures, the type and concentration of the cryoprotectant, and the method of its introduction into the cooling system.

We recommend freezing the seed material of *Dracocephalum ruyschiana* using cryoprotectants, and the type and concentration do not seriously matter; the growth performance of the seeds will be higher than in the case of freezing without cryoprotectants. When cryopreserving *Salvia sclarea* seeds, we recommend using low concentrations of cryoprotectants and introducing it into the cooling system at the crystallization temperature of water.

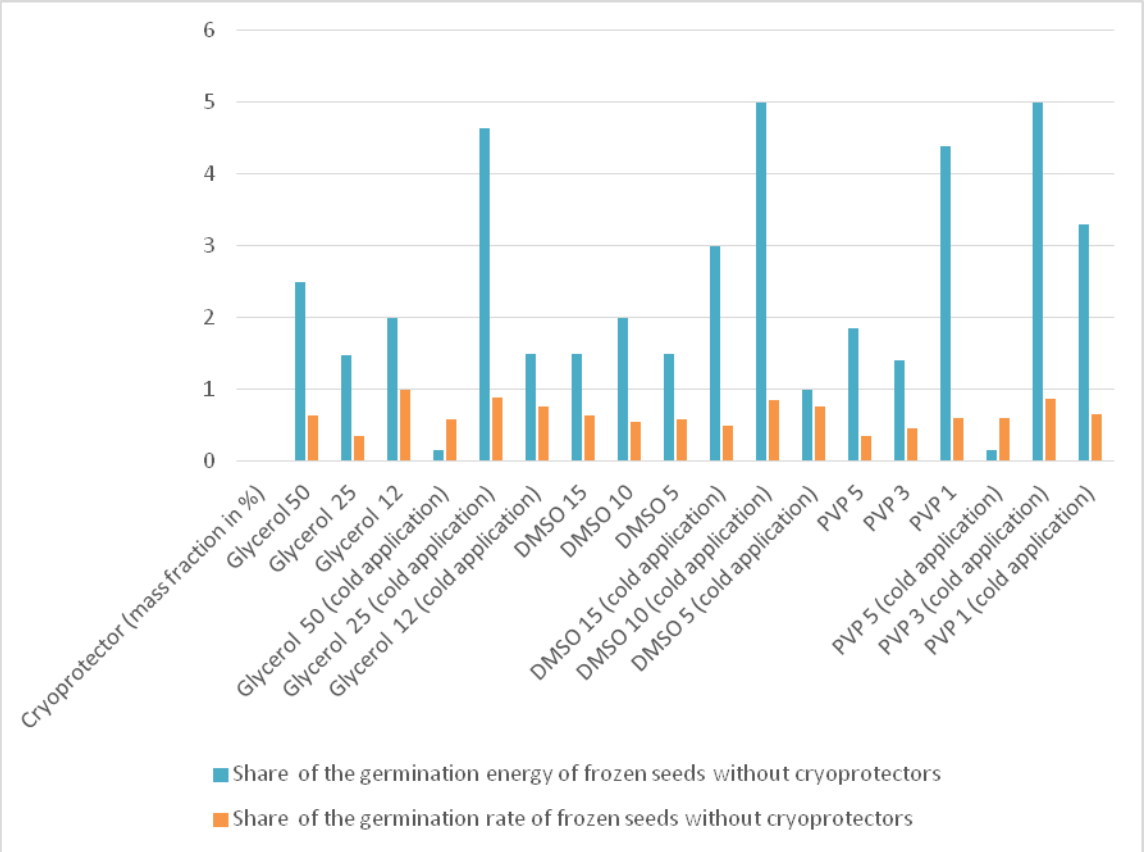

Figure 4. Ratio of growth parameters of Salvia sclarea seeds after cryopreservation with the use of cryoprotectants to those after freezing without cryoprotectors

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Dracocephalum ruyschiana L. және *Salvia sclarea* тұқымдық материалдарын криоконсервациялау тиімділігін бағалау

Бағалы өсімдіктердің өсімдік шикізатын криоконсервациялау табиғи экожүйелерге белсенді және кең таралған антропогендік қысым жағдайында биологиялық әртүрлілікті сақтаудың тиімді әдісі болып табылады. Криоколлекцияларға кіріспе азық-түлік ауылшаруашылық өсімдіктеріне ғана емес, сонымен қатар бағалы екіншілік метаболиттері мен эфир майлары бар дәрілік өсімдіктерге де негізделген. Оларға *Dracocephalum ruyschian* және *Salvia sclarea* жатады. Сұйық азотта мұздатылғаннан кейін тұқымның өміршеңдігін тиімді қалпына келтіру үшін әртүрлі криопротекторлардың әсері зерттелген: яғни, глицерин, диметилсульфоксид және поливинипирролидон, сондай-ақ оларды қолданудың әртүрлі әдістері — бөлме температурасында және суықта. *Dracocephalum ruyschiana* тұқымдық материалды тиімді криопросервациялау үшін криопротекторларды қолдану негізделген, алайда оларды қолдану әдісі маңызды емес. *Salvia sclarea* тұқымдық материалды криоконсервациялау кезінде криопротекторларды қолдану және оларды салқындату жүйесіне енгізу маңызды, бұл жағдайда тұқымның өну энергиясы айтарлықтай артады.

Кілт сөздер: *Dracocephalum ruyschiana, Salvia sclarea*, криопротекторлар, криоконсервация, тұқым материалы, өну, өну энергиясы.

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Оценка эффективности криоконсервации семенного материала *Dracocephalum ruyschiana* L. и *Salvia sclarea*

Криоконсервация растительного материала ценных растений — эффективный способ сохранения биологического разнообразия в условиях активного и повсеместного антропогенного давления на природные экосистемы. Введение в криоколлекции оправдано не только для пищевых сельскохозяйственных растений, но и лекарственных, обладающих ценными вторичными метаболитами и эфирными маслами. К таковым можно отнести *Dracocephalum ruyschian* и *Salvia sclarea*. Для эффективного восстановления показателей жизнеспособности семян после замораживания в жидком азоте рассмотрели влияние различных криопротекторов: глицерина, диметилсульфоксида и поливинипирролидона, а также различные способы их внесения — при комнатной температуре и на холоде. Обнаружено, что для эффективного криосохранения семенного материала *Dracocephalum ruyschiana* применение криопротетокров оправдано, однако способ их внесения не имеет значения. При криоконсервации семенного материала *Salvia sclarea* важно использовать криопротекторы и вводить их в систему охлаждения лучше при температуре кристаллизации воды, в этом случае значительно вырастает энергия прорастания семян.

Ключевые слова: Dracocephalum ruyschiana, Salvia sclarea, криопротекторы, криоконсервация, семенной материал, всхожесть, энергия прорастания.

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