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Effect of Liquefied Gases and Aqueous Biostimulant Solutions on Seed Germination and Subsequent Seedling Development of Meadow Clover

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Abstract—After treating seeds with liquid nitrogen and other liquefied gases with additional exposure to aqueous biostimulant solutions, several experimental variants display the preservation (as in control groups) or improvement of seed germination and seedling development in cultivated meadow clover cultivars. The cryotreatment of wild meadow clover seeds (in contrast to cultivated varieties) results in cotyledon detachment in some seedlings and delays their development.

Keywords: meadow clover, seeds, liquefied gases, cryotreatment, germination, seedling development

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INTRODUCTION

For many species in cultivated meadow clover cultivars is the principal means of reproduction in both natural ecosystems and cultivated plantings. With higher plants, seeds are extensively employed to conserve and sustainably utilize genetic resources (plant gene pools). Ways of conserving gene pools vary, and the cryopreservation of seeds at ultralow temperatures (77 K in liquid nitrogen) is especially important and long established [1, 2]. Studies of the cryotreatment and cryopreservation of seeds in liquefied gases, including various legume species and cultivars, have revealed key observations and patterns. These findings pertain mainly to alterations in seed structural components and the emergence and subsequent growth of seedlings following different means and regimes of cryotreatment [3]. Data indicate that prolonged storage of diverse plant seeds in liquid nitrogen, followed by thawing and germination, does not impair and can even enhance their rates of germination and the subsequent development of seedlings [4–6]. For many legume species, cryopreservation synergizes effectively with cryotreatment to overcome hardseededness (seed coat impermeability) [7]. Hardseededness markedly impedes the propagation of seeds in legume species. Treatment with liquefied gases, particularly liquid nitrogen, is a promising strategy for overcoming hardseededness, [1–10]. Research continues to identify effective means of treatment for seeds of diverse legume species to maximize their rates of germination and support normal seedling development. This is especially critical for seeds of wild meadow clover (from natural populations) and certain cultivated varieties used in breeding and large-scale propagation for land reclamation [9–11].

Improving the pre-sowing preparation of seeds of leguminous plants (particularly meadow clover) is of great importance. Given meadow clover's great value as a forage and medicinal crop [12], and its role in soil reclamation, preserving its gene pool via seed collections in cryobanks at ultralow temperatures is essential. It is therefore vital to determine which seed and emerging seedling traits can be altered, and whether seed performance changes after cryopreservation. The effectiveness of other established pre-sowing treatments for these seeds must be assessed.

The aims of this work were to compare germination characteristics and seedling development in samples of wild and cultivated meadow clover after cryotreatment in liquefied gases, and to develop ways of enhancing the germination and seedling growth of cultivated meadow clover in soil planting by combining liquefied gas pretreatment with the subsequent application of an aqueous solutions of biostimulants.

EXPERIMENTAL

A variety of meadow clover seed samples and means of treatment were used in this work. A laboratory experiment was performed to assess cotyledon detachment (breakage) in germinating meadow clover seeds after preliminary cryotreatment with liquefied gases. The experiment employed two familial seed samples from wild meadow clover (derived from two individuals of meadow clover) in a natural population from Sverdlovsk Oblast. These seeds were subjected to cryotreatment in liquid argon for 1 h prior to germination, as described in [9]. Seedlings obtained from these seeds under laboratory conditions after cryotreatment were compared to those of Dobryak cultivar, also obtained after treatment consisting of 15 min in liquid oxygen, slow thawing at 25°C, and 3 days in liquid argon with more slow thawing at room temperature (25°C).

Cultivated varieties of meadow clover (cultivars Orion, Drakon, and Dobryak), bred at the Ural Scientific Research Institute of Agriculture and recommended for planting in the Middle Urals and other Russian regions, were selected for this work [13]. New insights into ways of preserving and enhancing the sowing qualities of these clover cultivar seeds, along with improving seedling development after a variety of pre-sowing treatments, could be of practical value and contribute to our body of knowledge in seed biophysics. Our experiments used cultivars that differed qualitatively in morphogenesis (the formation of individual morphological structures), and seeds from the two studied cultivars in the double-cut group had varying lengths of storage. Based on first-year ontogenetic traits of meadow clover plants (seedlings) from seeds, Orion is classified as a single-cut (late-maturing) cultivar, while Drakon and Dobryak are double-cut (early-maturing) cultivars [11].

Seeds were cryotreated using the methodology described in [9, 10]. Germination in Petri dishes was assessed under controlled conditions (20°C, 12 h light/12 h dark). Field trials were performed in 2023 at the botanical garden of Kurgan State University using three samples of cultivar seeds of meadow clover:

— Orion cultivar, 2022 harvest, 95% laboratory germination, treated with liquid nitrogen for 3 h; untreated controls;

— Drakon cultivar, 2021 harvest, 95% laboratory germination, treated with liquid nitrogen for 24 h; untreated controls;

— Dobryak cultivar, 2015 harvest, 40% laboratory germination; untreated controls. There were two versions of cryotreatment: (a) with 24 h in liquid nitrogen and (b) with liquid oxygen and argon (as above).

Liquid nitrogen treatment of cultivar seeds consisted of immersing them in the cryogen for 3 or 24 h inside gauze bags, with gradual thawing on cardboard at 30°C under ambient conditions.

Pre-sowing preparation of both experimental and control seeds of all cultivars followed established agricultural protocols [14, 15]. On sowing day, seeds of all cultivars were soaked in distilled water and then treated for 2 h at room temperature in each of the below solutions:

— distilled water with air bubbling at 7 L/min (not applied to Orion);

— 1% hydrogen peroxide solution (excluding Orion);

— 3% hydrogen peroxide solution (all cultivars);

— 10% Biomaster potassium humate solution (all cultivars).

Seeds were then dried and sown the same day (late-summer sowing). Sowing was done in block/line form: 100 seeds per block, with 3 cm between seeds in a row. Blocks were arranged in a randomized linear design with 50 cm between rows. Three replications were sown for Orion cultivar, while Drakon and Dobryak each had two replications per variant of treatment. A total of 6600 seeds were sown across 66 blocks covering 0.2 ha.

Germination was assessed on the 10th day after sowing. Parameters of growth and development were recorded at the end of the growing season (October 18–20, 2023). Because juvenile meadow clover plants produce only a single juvenile-type leaf, the rate of individual development was measured as the proportion of plants still bearing a juvenile leaf when examined. The greater the proportion of plants retaining a juvenile leaf when examined, the slower the rate of ontogenetic development for that cultivar. A juvenile leaf senesces upon transitioning to a more mature (virginal) ontogenetic state, leaving the plant with only adult leaves (long-petioled trifoliate). Vegetative vigor was quantified for each assessed clover plant according to the number of fully formed mature trifoliate leaves. Vegetative potential was measured by counting the primordia of incompletely expanded mature trifoliate leaves per plant.

Results were analyzed by means of variational statistics and studying dispersion was studied using the MS Excel and Statistica 10 computer programs [16]. Differences between experimental and control samples were examined using ANOVA. Statistical significance was set at the conventional level for biological studies ($p = 0.05$). Statistical analysis included means, errors, of ranges variation, and variances for each parameter. One- and two-way ANOVA designs were used to identify significant factors affecting plant development parameters. Fisher's F values and p-levels were reported only for strong effects on plant development ($p < 0.05$). The arithmetic means of the studied parameters are presented in Tables 1–4 without indications of error.

RESULTS AND DISCUSSION

As in [9], the proportion of hard seeds fell markedly in the studied samples of wild meadow clover seeds treated with liquefied gases. However, cotyledons detached from the axial part in some seedlings. In this

Table 1. Field germination of meadow clover seeds, arithmetic mean (limits of variation (%) are in parentheses)

Seed treatment	Seed pre-sowing preparation				
	soaking in water	barbotage	1% peroxide	3% peroxide	10% potassium humate
	Orion cultivar, 2022 harvest				
Liquid nitrogen	25.3 (4–43)	–	–	30.0 (18–42)	25.7 (10–42)
Control	21.3 (8–40)	–	–	13.3 (6–27)	19.7 (7–37)
	Drakon cultivar, 2021 harvest				
Liquid nitrogen	17.0 (17–17)	16.0 (16–16)	10.5 (7–14)	19.0 (17–21)	18.5 (15–22)
Control	16.5 (11–22)	7.0 (7–7)	16.0 (16–16)	12.5 (12–13)	13.5 (13–14)
	Dobryak cultivar, 2015 harvest				
Liquid oxygen and argon	5.0 (4–6)	5.0 (4–6)	3.0 (2–4)	3.5 (3–4)	8.0 (7–9)
Liquid nitrogen	6.0 (5–7)	3.5 (2–5)	5.0 (2–8)	2.5 (2–3)	7.5 (2–13)
Control	6.5 (3–10)	4.5 (4–5)	6.5 (3–10)	3.5 (3–4)	3.0 (2–4)

work, cotyledon detachment (producing cotyledonary leaves) was observed in 10% of seedlings in one sample and 80% in another. This delayed the development of seedlings even under laboratory conditions of germination. Formation of the first true leaves was delayed. Similar effects were noted in [3] for seeds from another wild clover species exposed to liquid nitrogen prior to germination. The cited authors attributed the damage (cotyledon detachment) to different rates of the contraction and expansion of embryo tissues (embryo including cotyledons) while cooling to 77 K and subsequent warming to room temperature.

Laboratory experiments showed that cultivar meadow clover seeds retained great viability after freezing in liquid nitrogen and thawing at room temperature (high rates of laboratory germination corresponding to results from pre-cryotreatment analysis obtained at the Ural Scientific Research Institute of Agriculture) with nearly no hard seeds. The germination of Dobryak seeds after cryotreatment (with liquid oxygen and then argon) was 36%. This was close to the pre-treatment rate of 40%, with no cotyledon damage or detachment. Similar resistance to liquid nitrogen, with high germination and no cotyledon damage, was observed in a Far Eastern legume species lacking seed hardness [6]. The authors of that work, having studied plant development from seeds after cryopreservation in liquid nitrogen, concluded that this technique is advisable for long-term preservation of high seed germination and the studied species capability of normal plant development. Our results (see below) indicate this type of cryopreservation is also suitable for cultivar meadow clover seeds.

Field rates of germination for cultivars were considerably (but proportionally) lower than laboratory rates. In certain field treatments (cryoprocessing with 3% hydrogen peroxide and potassium humate), cultivar germination improved relative to controls (Table 1).

By day 10, 822 seedlings had emerged in our soil plots, yielding an average germination of 12.5%. Seedlings were tagged for monitoring, and 27 of them (3.3% of those that emerged) died during the season.

Table 1 shows the field germination (%) of cultivar seeds after treatment with liquefied gas and a number of pre-sowing preparations.

Field germination declined consistently with longer durations of seed storage. The greatest field germination was observed in Orion harvested in 2022. Drakon (2021 harvest) had less germination, while Dobryak (2015 harvest) had the lowest. Considerable variation in this parameter among replicates across treatments was observed and considered in subsequent data analysis.

Compared to other pre-sowing treatments, soaking with water enhanced germination among control seeds and therefore became our standard.

Liquid nitrogen treatment with subsequent pre-sowing soaking in 3% hydrogen peroxide and 10% potassium humate improved the field germination of fresh seeds of Orion and Drakon (1–2 years of storage). Aerating the seeds and soaking them in 1% hydrogen peroxide revealed no beneficial effects.

For aged Dobryak seeds (8 years of storage), positive effects were observed only after pre-sowing soaking in 10% potassium humate, regardless of whether the seeds were treated previously with liquid nitrogen, oxygen, or argon. Other pre-sowing treatments for this cultivar revealed no beneficial effects.

Ontogenetic (Individual) Rates of Plant Development

Table 2 presents data on individual rates of development of clover plants from treated seeds.

Table 2. Proportion of plants in juvenile ontogenetic state (at the initial stage of individual development) at the time of assessment, arithmetic mean

Seed treatment method	Method of seed pre-sowing preparation				
	soaking in water	barbotage	1% peroxide	3% peroxide	10% potassium humate
	Orion cultivar, 2022 harvest				
Liquid nitrogen	0.68			0.84	0.78
Control	0.84			0.77	0.93
	Drakon cultivar, 2021 harvest				
Liquid nitrogen	0.41	0.25	0.38	0.23	0.36
Control	0.18	0.57	0.31	0.20	0.42
	Dobryak cultivar, 2015 harvest				
Liquid oxygen and argon	0.50	0.40	0.00	0.57	0.50
Liquid nitrogen	0.17	0.71	0.00	0.00	0.33
Control	0.54	0.56	0.58	0.14	0.50

Across treatments, individual rates of development were lowest for single-cut Orion, and considerably slower than the double-cut cultivars (Drakon and Dobryak) characteristic of these distinct clover groups. Among double-cut cultivars, accelerated development of seedlings versus controls was observed after treatment with liquid nitrogen and soaking in water before sowing ($F = 2.77$; $p = 0.018$). Other pre-sowing treatments showed no differences from controls in juvenile plant proportions. Response to cryotreatment and pre-sowing preparation differed strongly between double-cut cultivars Drakon and Dobryak. Plants from relatively fresh Drakon seeds (2 years of storage) showed maximum rates of development in water-soaked controls, while treatment with liquid nitrogen, aeration, and potassium humate inhibited development ($F = 2.01$; $p = 0.022$). However, plants from aged Dobryak seeds (8 years of storage) displayed significantly accelerated development when liquid nitrogen treatment was combined with water and hydrogen peroxide soaking. The accelerated development of seedlings was also observed for this cultivar after treatment with liquid oxygen and argon, accompanied by soaking in 1% hydrogen peroxide, and without cryotreatment when seeds were soaked in 3% hydrogen peroxide. The importance of seed treatment and pre-sowing preparation for regulating rates of clover development thus depends on both the cultivars' characteristics (single- vs. double-cut) and the duration of seed storage.

Plant Vegetative Vigor

Table 3 presents data on the vegetative vigor of clover plants (seedlings). The vegetative vigor of double-cut cultivars Drakon and Dobryak was several times higher than in single-cut Orion, which showed minimal variation in this parameter. Vegetative vigor grew in Drakon, when liquid nitrogen treatment was combined with either aeration or 3% hydrogen peroxide soaking ($F = 2.13$; $p = 0.028$). No other treatments had any positive effect. Vegetative vigor rose in aged Dobryak (8 years old), only when its seeds were treated with liquid oxygen and argon, accompanied by soaking in 10% potassium humate ($F = 2.77$; $p = 0.001$). No other types of exposure yielded positive effects.

Plant Vegetative Potential

Table 4 presents data on the vegetative potential of clover plants. Data characterizing the variability of clover plant vegetative potential depending on the cultivar and types of seed treatment were isomorphic in regard to the variability of their vegetative vigor. The vegetative potential of single-cut Orion cultivar plants was several times lower than that of double-cut cultivars Drakon and Dobryak and varied minimally. The vegetative potential of Drakon grew when liquid nitrogen treatment was combined with aeration or soaking in 3% hydrogen peroxide ($F = 3.25$; $p = 0.001$). No other treatments yielded positive effects. In aged Dobryak (8 years old), vegetative potential also rose when seeds were treated with liquid oxygen and argon, accompanied by soaking in 10% potassium humate ($F = 2.63$; $p = 0.002$). A slight increase in vegetative potential versus controls was seen upon soaking untreated seeds in hydrogen peroxide, and with liquid nitrogen treatment plus soaking in water.

These findings confirm a familiar pattern: longer dry storage of cultivar seeds reduces their field germination substantially. Cryopreserving these seeds in liquid nitrogen is an effective way of maintaining their long-term viability and strong germination [1, 2]. The ultralow temperatures (~77 K) of liquid nitrogen and

Table 3. Number of fully formed trifoliolate (mature) leaves in clover plants at the time of assessment, arithmetic mean (pieces)

Seed treatment method	Method of seed pre-sowing preparation				
	soaking in water	barbotage	1% peroxide	3% peroxide	10% potassium humate
	Orion cultivar, 2022 harvest				
Liquid nitrogen	2.73			2.56	2.75
Control	2.69			2.44	2.80
	Drakon cultivar, 2021 harvest				
Liquid nitrogen	7.97	11.31	6.90	10.31	8.11
Control	8.88	8.29	7.91	7.56	6.77
	Dobryak cultivar, 2015 harvest				
Liquid oxygen and argon	4.20	4.40	4.00	3.86	11.19
Liquid nitrogen	6.50	4.00	3.90	4.75	4.60
Control	6.31	3.67	6.08	5.43	5.00

Table 4. Number of rudiments of not fully expanded trifoliolate leaves in clover plants, arithmetic mean (pieces)

Seed treatment method	Method of seed pre-sowing preparation				
	Soaking in water	Barbotage	1% peroxide	3% peroxide	10% potassium humate
	Orion cultivar, 2022 harvest				
Liquid nitrogen	0.88			0.82	0.86
Control	0.90			0.79	0.92
	Drakon cultivar, 2021 harvest				
Liquid nitrogen	3.29	5.00	3.43	5.46	4.08
Control	4.18	3.14	3.72	3.56	3.42
	Dobryak cultivar, 2015 harvest				
Liquid oxygen and argon	1.90	2.40	2.25	2.00	6.94
Liquid nitrogen	3.58	1.00	2.00	2.00	2.40
Control	3.31	1.67	3.92	3.57	3.00

other liquefied gases do not damage embryos or cause cotyledon detachment. Seed germination and seedling development proceed normally after gentle thawing. Liquefied-gas treatments of cultivar seeds, especially liquid nitrogen (often containing dissolved oxygen), can enhance field germination when followed by appropriate pre-sowing preparation. Pre-sowing soaking in 10% potassium humate is particularly beneficial, while soaking in water or 3% hydrogen peroxide also shows positive effects for fresh seeds. The effects of low-temperature liquid gases (nitrogen, oxygen, and argon) on seed germination are similar, suggesting the key factor is low temperature and not gas chemistry. Three hours of exposure are enough for this effect.

Double-cut cultivars exhibit faster individual development, accelerating juvenile organ (cotyledon and juvenile leaf) senescence compared to single-cut types. Extended seed storage likely slows plant development, but specific combinations of hydrogen peroxide soaking and liquid gas treatment can mitigate this inhibition in aging seeds. This hypothesis requires dedicated experimental validation.

Double-cut clover cultivars show higher vegetative vigor and potential. These parameters vary little in single-cut Orion, and treatments do not differ from controls. In Drakon, both vegetative vigor and potential increase under identical liquid nitrogen treatments combined with aeration or soaking in 3% hydrogen peroxide. Distinct effects appear in aged Dobryak (8 years old), e.g., maximum vegetative vigor and potential upon treatment with liquid oxygen and argon, accompanied by soaking in 10% potassium humate. Such varied effects indicate that both the duration of seed storage and the chemistry of the liquid gas are important factors.

CONCLUSIONS

The liquefied gas treatment of meadow clover seeds from natural and cultivar populations has different effects on their embryo state and characteristics germination. Cryotreatment (down to 77 K) of wild clover seeds caused cotyledon detachment from the embryonic axis in 10–80% of our seedlings, slowing their laboratory development by sharply reducing the surface area (cotyledonary leaves) of photosynthesis. Seedlings developed normally after the cryotreatment of cultivar seeds, with no cotyledon damage being observed. The cryopreserving of such seeds is therefore a viable strategy for the long-term preservation of their viability and high rates of germination.

When combined with different pre-sowing preparations in aqueous biostimulant solutions, the physicochemical effects of liquefied gases (particularly liquid nitrogen) affect the germination, growth, and development of cultivar seedlings differently under field conditions. For cultivar seeds, the outcomes depend strongly on biological traits (single- vs. double-cut), length of storage (fresh vs. long-term), and the combination of cryogenic treatment and traditional pre-sowing techniques. The patterns identified in this work can guide the targeted development of ways to enhance the sowing quality of meadow clover seeds; improve their germination and the growth and development of seedlings; and overcome problems related to seed aging.

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CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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