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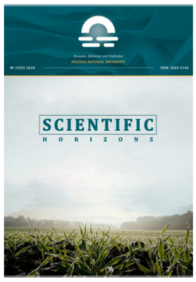

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Determining the germinative qualities of red clover seed samples with varying biological status

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Abstract. The development of forage crop varieties that adapt to changing environmental conditions is an effective strategy to mitigate the impact of climate change with minimal losses. Temperature influences both the timing and rate of seed germination, which in turn ensures uniform seedling emergence, compatibility of red clover with other crops, and the ability to exploit optimal sowing conditions. This study aimed to determine the temperature and time required to achieve maximum germination,

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as well as the range within which germination occurred, for four red clover samples of varying biological status (cultivars, wild and local populations). Germination capacity and energy of red clover samples were assessed according to the standards outlined in DSTU 4138 2002 and DSTU 2949-94 at temperatures ranging from 3°C to 33°C in 10°C increments. It was established that the optimal temperature for the germination of red clover seeds among the four studied samples ranges from 13°C to 33°C on filter paper and from 13°C to 23°C on sand. The research identified the Vytis cultivar as outperforming other samples in all measured parameters when germinated on filter paper, while the Truskavchanka cultivar demonstrated superior traits when germinated on sand. The best germination rates were observed in the wild population at a temperature of 13°C. These findings explain the uneven emergence of red clover samples of varying biological status in collection and breeding nurseries, as observed under field conditions. The results of this study can inform the development of sowing technologies for red clover under various temperature regimes

Keywords: *Trifolium pratense* L.; cultivar; wild population; local population; temperature; germination

INTRODUCTION

Red clover (*Trifolium pratense L.*), a perennial legume belonging to the Fabaceae family, thrives in a wide range of climates and is widely distributed in temperate and subtropical regions of both the Northern and Southern Hemispheres. It is easily cultivated on acidic and moist soils, both in pure stands and in mixtures with cereal species. Red clover plays a crucial role in sustainable forage production through biological nitrogen fixation, soil fertility restoration, efficient use of phosphorus and potassium fertilisers, and reducing the environmental impact of agriculture. With the changing climate, there is a growing need for new cultivars with high adaptive potential. The foundation for developing new cultivars lies in the establishment and study of germplasm collections, which include cultivars, wild, and local populations from various eco-geographic origins. The Precarpathian Department of Scientific Research of the Institute of Agriculture of the Carpathian Region of the National Academy of Sciences has established a red clover collection comprising 330 samples with diverse biological statuses.

The value of red clover in agriculture and the study of its genetic resources have been the subject of numerous studies by prominent scientists. B. Sawicka *et al.* (2023) demonstrated that *Trifolium* species effectively counteract agricultural degradation, restore soils, and support forage production. C. Harris and F. Ratnieks (2022) argue that concerns over the rising costs and environmental impact of high inorganic nitrogen (N) inputs have led to a renewed interest in legumes, particularly clover, for maintaining soil fertility and improving natural hay meadows. A major limitation of red clover in cultivation, as highlighted by the authors, is its low persistence due to high plant mortality caused by a complex of biotic and abiotic factors.

In studying red clover samples of diverse ecological and geographical origins, researchers such as G. Petrauskas *et al.* (2020) and M. Tucak *et al.* (2021) identified superior red clover populations, which they recommend for improving the nutritional value of forage crops in breeding programmes aimed at developing new cultivars. According to I. Radinovic *et al.* (2022), to assess the level of genetic diversity and identify the best genotypes for developing new cultivars, it is necessary to obtain information about the germplasm, which is the primary basis for breeders. Therefore, the evaluation of genetic resources is based on morphological, phenological, and agronomic traits. Since breeders use different forms when studying the initial material, it is advisable to know the response of their seeds to temperature during germination.

Seeds represent a crucial stage in a plant's life cycle, serving as the vital link for the continued existence of a species. They encapsulate the genetic information necessary for the dispersal, establishment, development, and reproduction of the next generation. Seeds possess the ability to maintain life in a dormant state when dry and to resume metabolic activity under favourable environmental conditions. Climatic factors are significant determinants, playing a pivotal role in influencing various stages of a plant's life cycle, from seed germination to development (Baxter *et al.,* 2019; Talwar *et al*., 2023). Temperature, light, and moisture are critical factors influencing seed germination. These abiotic factors are major limiting factors that significantly affect the timing and rate of clover seed germination, ultimately impacting its compatibility with other crops, ability to suppress weeds, and capacity to capitalise on optimal sowing conditions.

J. Yu *et al.* (2020) assert that the germination of beggarticks (*Bidens alba* L.) seeds decreases with reduced moisture availability. The seeds germinated more effectively in the presence of light than in continuous darkness at temperatures ranging from 15°C to 35°C. However, the optimal germination temperature for beggarticks seeds was 15°C under light conditions. Better seedling emergence was observed when seeds were sown on the soil surface. The number of germinated seeds decreased sharply when the sowing depth was 1 cmor more. Identifying the minimum, maximum, and optimal temperatures for clover germination enables researchers to finetune sowing recommendations for successful establishment. A. Szczerba *et al.* (2021) emphasised the importance of analysing the characteristics of cultivars, not only in terms of the length of the growing season but also their ability to germinate at lower temperatures. This would allow for earlier sowing, resulting in earlier seed harvest, as confirmed by their studies on four soybean samples.

Therefore, temperature has a significant impact on the maximum percentage, uniformity, and speed of germination. Consequently, clover should be sown under optimal environmental conditions to avoid soil and climatic factors that negatively affect seed germination and seedling emergence. There is a need to evaluate the impact of various factors on the germination of red clover seeds and seedling emergence in the early stages of breeding programs when studying the initial material. Thus, this study aimed to investigate the seed quality of red clover samples with different biological statuses (cultivar, local and wild populations) under various temperature regimes.

MATERIALS AND METHODS

The study utilised red clover seeds from the cultivars Vytis (Lithuania) and Truskavchanka (Ukraine), as well as wild and local populations. These seeds, harvested in 2021, were stored under uncontrolled conditions (*ex situ*) at the Precarpathian Department of Scientific Research. Germination and germination energy were determined according to the Ukrainian State Standards (DSTU 4138-2002, 2004; DSTU 2949-94, 1996). Samples (32 per batch, each containing 100 seeds) were drawn from the breeding material to evaluate seed quality, including germination energy and laboratory germination rates. For the experiment, 100 seeds of each red clover sample were evenly distributed in 9 cm Petri dishes lined with filter paper saturated with 4 mL of distilled water. The dishes were then placed in a dry-air thermostat TS-80 (Ukraine, 2019). To determine the impact of temperature on seed germination, the Petri dishes were maintained in complete darkness at constant temperatures of 3°C, 13°C, 23°C, and 33°C with a relative humidity of 70%. The experiments were conducted in four replicates. Seeds were considered germinated once the radicle was visibly protruding.

The germination of seeds from the four aforementioned samples was also assessed on sand. The sand was prepared as a substrate for seed germination by sieving it through a 1 mm mesh, washing, and heating until a piece of paper placed within it charred. The following method was applied: "on sand" (oS) – seeds were pressed into the sand's surface to a depth of 1 mm. For the experiment setup, a 1.5 cmlayer of sand was added to the base of a Petri dish and moistened with 7 mL of distilled water, after which 100 seeds were evenly distributed on the surface. On the fourth day, an additional 2 mL of distilled water was added. The Petri dishes were kept in complete darkness at constant temperatures of 3°C, 13°C, 23°C, and 33°C with a relative humidity of 70%. The experiments were conducted in four replicates.

Two types of assessments were conducted: the first to evaluate germination energy and the second to measure germination rates and determine the root, hypocotyl, and total length of 25 randomly selected seeds. Results were expressed as percentages, rounded to the nearest whole number, for each observed category. The accuracy of the analysis was established by comparing the extreme values of the replicates with the arithmetic mean. The result was deemed reliable if the difference between the extreme values and the arithmetic mean, rounded to a whole number, did not exceed the permissible deviations. Statistical analysis was performed using analysis of variance (ANOVA). Throughout the study, the authors adhered to the standards established by the Convention on Biological Diversity (1992) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (1979).

RESULTS AND DISCUSSION

Germination energy refers to the proportion of seeds that germinate within the first four days, expressed as a percentage. Seeds with high germination energy exhibit rapid germination, leading to uniform seedling emergence. Such seedlings are less suppressed by weeds and exhibit greater resilience to adverse conditions. The germination energy of red clover seeds on filter paper under varying temperature regimes ranged from 61% in the Truskavchanka cultivar (at 3°C) and the local population (at 33°C) to 78% in the Vytis cultivar (at 33°C). The highest germination energy was observed in all studied samples at 13°C, with an $LSD_{0.05} = 0.02$ (Table 1).

***Table 1**. Germination energy, germination rate, and seedling length of red clover seeds germinated on filter paper under varying temperature regimes*

Sample	Temperature regime (°C)				
	3	13	23	33	Average
Germination energy, % (Day 4)					
Local population	64	73	62	61	65
Truskavchanka	61	70	66	67	66
Vytis	68	75	68	78	72
Wild population	62	71	70	63	67

Table 1. Continued

Sample	Temperature regime (°C)				
	3	13	23	33	Average
Average	64	72	67	67	68
$LSD_{0.05}$	0.01	0.02	0.02	0.02	
Germination, % (Day 10)					
Local population	86	93	81	86	87
Truskavchanka	84	91	86	90	88
Vytis	89	96	90	96	93
Wild population	87	92	90	92	90
Average	87	93	87	91	90
$LSD_{0.05}$	13.32	11.15	14.09	10.67	
Root length, mm (Day 10)					
Local population	3.5	5.0	4.9	3.9	4.3
Truskavchanka	3.1	4.8	4.6	3.7	4.1
Vytis	3.6	5.3	4.8	4.1	4.5
Wild population	3.4	5.6	5.1	5.3	4.9
Average	3.4	5.2	4.9	4.3	4.5
$LSD_{0.05}$	0.01	0.38	0.49	0.02	
Hypocotyl length, mm (Day 10)					
Local population	4.1	6.2	7.0	5.9	5.8
Truskavchanka	3.8	5.9	7.2	6.3	5.8
Vytis	3.5	6.4	7.7	6.1	5.9
Wild population	4.0	6.9	7.1	7.0	6.3
Average	3.9	6.4	7.3	6.3	6.0
$LSD_{0.05}$	0.01	0.03	0.49	0.07	
Total length, mm (Day 10)					
Local population	7.6	11.2	11.9	9.8	10.1
Truskavchanka	6.9	10.7	11.8	10.0	9.9
Vytis	7.1	11.7	12.5	10.2	10.4
Wild population	7.4	12.5	12.2	12.3	11.1
Average	7.3	11.5	12.1	10.6	10.4
$LSD_{0.05}$	0.38	0.43	0.81	0.12	

Source: *created by the authors*

When germinating the studied seeds on the sand, germination energy ranged from 53% in the local population (at 3°C) to 87% in the Vytis cultivar (at 13°C). The highest germination energy was observed across all samples at 23°C, with an $LSD_{0.05}$ = 0.02 (Table 2).

Germination rate, a critical indicator of seed quality, was determined by counting the number of normal seedlings after 10 days. On filter paper, germination ranged from 81% (local population) to 96% (cultivar Vytis) with $LSD_{0.05}$ = 10.67-14.09. The best germination (91-96%) was observed at 13°C. On sand, germination was lower compared to filter paper, with the highest values (92-98%) at 23°C. The Vytis cultivar consistently showed the highest germination on filter paper across all temperatures (89-96%), while the Truskavchanka cultivar performed best on sand at all temperatures (76-96%).

Table 2*. Germination energy, germination rate, and seedling length of red clover seeds germinated on sand under varying temperature regimes*

Sample	Temperature regime (°C)				
	3	13	23	33	Average
Germination energy, % (Day 4)					
Local population	53	71	80	64	67
Truskavchanka	71	85	82	68	77
Vytis	69	87	84	61	75
Wild population	58	81	79	69	72
Average	63	81	81	66	73
$LSD_{0.05}$	0.01	0.03	0.02	0.02	
Germination, % (Day 10)					
Local population	66	77	92	74	77

Table 2. Continued

Sample	Temperature regime (°C)				
	3	13	23	33	Average
Truskavchanka	79	99	96	76	88
Vytis	74	92	96	69	83
Wild population	63	89	92	77	80
Average	71	89	95	74	82
$LSD_{0.05}$	21.32	25.13	19.34	21.17	
Root length, mm (Day 10)					
Local population	2.6	5.4	6.7	2.3	4.3
Truskavchanka	2.9	5.6	6.9	2.5	4.5
Vytis	3.1	5.8	7.1	2.9	4.7
Wild population	2.9	6.1	7.6	3.4	5.0
Average	2.9	5.7	7.1	2.8	4.6
$LSD_{0.05}$	0.02	0.45	0.38	0.01	
Hypocotyl length, mm (Day 10)					
Local population	3.2	7.1	7.9	3.5	5.4
Truskavchanka	3.4	7.5	8.3	3.1	5.6
Vytis	3.0	6.9	8.1	3.7	5.4
Wild population	3.3	7.8	7.7	4.0	5.7
Average	3.2	7.3	8.0	3.6	5.5
$LSD_{0.05}$	0.01	0.03	0.29	0.08	
Total length, mm (Day 10)					
Local population	5.8	12.5	14.6	5.8	9.7
Truskavchanka	6.3	13.1	15.2	5.6	10.1
Vytis	6.1	12.7	15.2	6.6	10.2
Wild population	6.2	13.9	15.3	7.4	10.7
Average	6.1	13.1	15.1	6.4	10.2
$LSD_{0.05}$	0.47	0.67	0.08	0.24	

Source: *created by the authors*

The root length of the studied samples germinated on filter paper was greater at 13°C (4.85.6 mm), while the shortest roots were observed at 3°C (3.1-3.6 mm). The hypocotyl length was longest at 23°C (7.0-7.7 mm) and shortest at 3°C (3.5-4.1 mm). The overall seedling length was greatest at 23°C (11.8-12.5 mm) and smallest at 3°C (6.9-7.6 mm), with $LSD_{0.05}$ = 0.12-0.81. When germinated on sand, root length was greatest at 23°C (6.7-7.6 mm) and shortest at 33°C (2.3-3.4 mm). The hypocotyl length was longest at 23°C (7.7-8.3 mm) and shortest at 3°C (3.0-3.4 mm). The overall seedling length was greatest at 23°C (14.6-15.3 mm) and smallest at 3°C (5.8-6.3 mm), with $LSD_{0.05}$ = 0.08-0.67. A comparison of the seed quality (germination energy and rate) and seedling traits (root and hypocotyl length, total seedling length) across samples under different temperature regimes highlighted the Vytis cultivar as superior across most parameters when germinated on filter paper. The Truskavchanka cultivar, however, excelled in most traits when germinated on sand. These findings indicate varying levels of germination energy and germination rates among the samples of different biological status (cultivar, wild and local populations).

Therefore, understanding the biology of clover seed germination about environmental factors is crucial for achieving good seedling emergence. Several authors have conducted various studies to determine the seed quality under controlled and uncontrolled conditions, in different environments, and at varying temperatures and stress levels across a range of agricultural crops. For instance, L. Chu *et al.* (2022) carried out a series of experiments to investigate the impact of light, temperature, sowing depth, drought, and salt stress on the germination of white clover (*Trifolium repens* L.) seeds. The seed germination rate was on average 63-66% under both light and complete darkness, measured four weeks after sowing. At temperatures of 15-25°C, the authors observed a significantly higher germination rate compared to lower temperatures of 5 and 10°C. The results indicate that the germination rate of white clover decreased with increasing sowing depth. Therefore, the authors recommend sowing seeds on the soil surface or burying them to a shallow depth of ≤1 cm to achieve optimal germination, which was taken into account in the sand germination experiments.

Numerous studies have been conducted to investigate the germination characteristics of various perennial grass species. For instance, A. Lina and A. Escobar-Gutiérrez (2022) studied the germination of eight samples of perennial ryegrass (*Lolium perenne* L.) of different biological origins at temperatures ranging from 5 to 40°C. They found that wild populations exhibited higher germination rates at higher temperatures, while local populations performed better at lower temperatures. Similarly, V. Oleksiak *et al.* (2023) researched perennial ryegrass, focusing on germination and germination energy of five samples. The authors also concluded that the germination of seed varieties was higher than that of local and wild populations. A. López *et al.* (2019) corroborated these findings, suggesting that the optimal germination temperature for different populations is influenced by the environmental conditions in which they evolved. Furthermore, W. Chaleb *et al.* (2021) examined 38 alfalfa (*Medicago sativa* L.) samples and observed that cultivars exhibited high germination rates at 5 and 34°C, while wild populations showed lower germination rates, likely due to adaptations to their specific geographic origins. P. Vivanco *et al.* (2021) highlighted the challenges in assessing the viability of wild perennial grass populations due to seed dormancy and high variability among populations. They determined that the optimal germination temperature for wild populations was 15°C. These results were confirmed by current studies, where the best germination of wild population seeds was observed at 13°C.

G. Herman *et al.* (2022) claim that the temperature during the initial phase of germination affects both the germination of seeds and seedlings of crimson clover. The best results for absorbent capacity and electrical conductivity were obtained at 22°C, while the highest germination energy, seedling emergence, root length, and total seedling length were observed at the lowest temperature of 10°C. Authors Ç. Bayram *et al.* (2024) conducted studies in controlled conditions to examine the effect of salt stress on flax germination, a key industrial plant with diverse uses worldwide. The experiment involved three flax varieties (Somme, Midin and Norman) and four salt concentrations (control, 50 mm, 100 mm, and 200 mm). They investigated the germination rate, radicle length, plumula length, seedling length, and seedling fresh weight characteristics of seedlings under salt stress conditions. The study showed that the control group (pure water) had the highest values for all examined traits, but these values significantly decreased as the salinity intensity increased. Similar studies were conducted by S. Büyükyıldız *et al.* (2023) on bird's-foot trefoil (*Lotus corniculatus* L.) and S. Shiade and B. Boelt (2020) on tall fescue (*Festuca arundinacea* Schreb). The authors assert that increased salinity negatively impacts seed germination.

I. Buranji *et al.* (2019) studied the effect of pH levels (4.5, 5.5, 6.5, 7.5, and 8.5) and temperature (10, 15, and 20°C) on the germination of flaxseed and the morphological parameters of the seedlings. The experiment was conducted under controlled conditions on rolled filter paper with four repetitions over seven days. The highest germination rate (88%) was observed at 20°C, which was statistically significant ($P < 0.01$) compared to the germination rate at 10°C, which was 9% lower. The highest percentage of normal seedlings (70%) was recorded at pH 5.5, while the lowest (59%) occurred at pH 8.5. The effect of pH on the morphological characteristics of the seedlings (root length, stem length, and total length) was statistically insignificant. In contrast, temperature significantly affected seedling length: at 20°C, after seven days, the average length was 14.4 cm, whereas at 10°C, it was only 2.1 cm($P < 0.01$). Overall, the lowest (4.5) and highest (8.5) pH values resulted in a reduction in the proportion of normal seedlings (63% and 59%, respectively), as well as less development of the root and stem.

A. Costa *et al.* (2019) obtained somewhat different results in their study of the impact of seed colour on the sowing qualities of red clover. The seeds of this species exhibit a heteromorphic nature, with two visually distinguishable morphs: light purple and yellow, which are determined by heterozygosity and recessive homozygosity at two loci. The study analysed seed germination and the initial growth of seedlings in distilled water, sodium chloride solution, and nutrient medium. It was found that seed sensitivity to treatment increased at later stages of development, indicating a cumulative effect. No differences in water absorption between the morphs or treatments were observed. However, at the seedling growth stage, treatments proved effective, with differences between the morphs only observed when using the nutrient medium. At the intermediate stage of germination, differences between the morphs were also evident in distilled water and sodium chloride solution. The yellow morph, which had an advantage at the initial germination stages, was overtaken by the light purple morph during the active seedling growth phase.

Overall, all researchers highlight the importance of studying the sowing qualities of agricultural crops, including red clover, especially in the context of climate change. The results obtained should be considered when establishing field conditions for nursery studies of the initial material and improving the elements of red clover cultivation technology. During sowing, temperature plays a crucial role in achieving rapid germination and successful establishment of the crop. The mid-summer period in the Western region of Ukraine is characterised by high temperatures and a soil moisture deficit. Therefore, determining the optimal temperatures for sowing red clover is of critical importance, particularly in the breeding process, when the amount of seed from the initial material is limited.

CONCLUSIONS

The study examined the germination characteristics of four samples of red clover with different biological statuses (cultivars Truskavchanka (Ukraine) and Vytis (Lithuania), and wild and local populations) under various temperature regimes of 3, 13, 23, and 33°C, and different substrate types (filter paper and sand). The results indicated that the optimal temperature for seed germination on filter paper ranged from 13°C to 33°C, while on sand it ranged from 13°C to 23°C. Seeds of the red clover cultivars Truskavchanka and Vytis generally demonstrated higher germination rates compared to the local and wild populations. These results should be considered when studying initial material of various biological statuses and eco-geographical origins under field conditions, as well as when improving cultivation techniques in breeding nurseries for red clover. Research on the sowing qualities of red clover seeds will continue, taking into account the obtained results, under different temperature regimes, and will assess the impact of soil acidity on seed germination and energy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Визначення посівних якостей насіння зразків конюшини лучної різного біологічного статусу

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Анотація. Процес створення сортів кормових культур, які адаптуються до мінливих умов навколишнього середовища, є ефективним способом подолання зміни клімату з мінімальними втратами. Температура впливає на час і швидкість проростання насіння, а це в свою чергу забезпечує рівномірність сходів та сумісність конюшини при вирощуванні з іншими культурами, здатність скористатися ідеальними умовами посіву. Метою дослідження було визначення температури та часу, необхідних для досягнення максимальної схожості, та діапазону, в якому проростання відбувалося для чотирьох зразків конюшини лучної різного біологічного статусу (сорти, дикоросла та місцева популяція). Визначення схожості та енергії проростання зразків конюшини лучної проводили відповідно до діючих методик ДСТУ 4138-2002 та ДСТУ 2949-94. при температурі від 3 до 33 °C з кроком 10 °C. Встановлено, що оптимальна температура для проростання насіння конюшини лучної чортирьох досліджуваних зразків на фільтрувальному папері становить від 13 °C до 33 °C, на піску – від 13 °C до 23 °C. В результаті проведених досліджень виділився сорт Vytis, який за всіма показниками перевищив інші зразки при пророщуванні на фільтрувальному папері, та сорт Трускавчанка, який виділився за досліджуваними ознаками при пророщуванні на піску. Кращі показники схожості відмічено в дикорослої популяції при температурі 13 °C. Отримані висновки дають пояснення нерівномірних сходів при посіві зразків конюшини лучної різного біологічного статусу в колекційних та селекційних розсадниках, що спостерігається в польових умовах. Результати, отримані в цьому дослідженні, можуть бути використані для розробки елементів технології посіву конюшини лучної за різних температурних режимів

Ключові слова: *Trifolium pratense* L.; сорт; дикоросла популяція; місцева популяція; температура; схожість