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CHALLENGES IN SUNFLOWER BREEDING FOR COLD TOLERANCE

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Cold tolerance of sunflower lines and hybrids in the laboratory and their tolerance in the field were evaluated; promising combinations were identified; the capacity of a laboratory test to predict tolerance of sunflower in the field was assessed; and relationships of cold tolerance with ripeness group and yield were verified.

Key words*: sunflower, cold tolerance, line, hybrid, linear regression*

Introduction. Sunflower (*Helianthus annuus L.*) is grown as an oilseed crop worldwide in temperate and subtropical climates, being the most popular oilseed crop in Europe and North America. Globally, it is grown in more than 80 countries [1] and worldwide over 56,000,000 tones of sunflower is produced per year [2]. In 2021/2022, sunflower was harvested on 30,152,000 hectares [3]. Ukraine is among the world's top producers and exporters of sunflower seed and oil. *H. annuus* is a strategically important oilseed crop in Ukraine, playing a key role in the agricultural sector due to high profitability of this crop [4]. As of 2021, the planted acreage of sunflower in Ukraine reached 6,622,000 hectares, with 16,392,410 tones of gross collection [5].

In the Left-Bank Forest-Steppe of Ukraine, sunflower is usually sown in late April or in early May, when the soil at a depth of 10 cm warms up to 8-12˚C. It should be noted that plant cells mitotically divide at >+7°C, therefore, for good growth and development of shoots, optimal temperature is a crucial factor. However, recently temperature on the soil surface has often decreased below the optimal values soon after the sunflower sowing timeframe, negatively affecting the germination of sunflower seeds as well as initial growth and development of sunflower plants. Early sowing is especially associated with risk of cold shock and chilling injury, since temperature can drop to -4°C at night. Crops are even at risk of freezing, since spring frosts on the territory of Ukraine are possible in March and even in April. Thus, it is vital to improve cold tolerance in the early stages of growth and development, that is, at germination, emergence, and the stage of two to three leaf pairs, so as to enable successful early sowing. Cultivation of low temperature-resistant sunflower will ensure intensive initial growth of plants, including at early sowing, avoidance of heat and drought stresses during anthesis, as it is shifted to earlier timeframes, and more complete fulfillment of the genetic potential [6]. Availability of cold-tolerant sunflower will potentially extend its growing season, allowing earlier planting and harvesting. In addition, a northward shift of southern crops, including sunflower, is likely to occur as temperature steadily raises [7]. It is admitted that the area suitable for the crop cultivation may shift northward as far as by 180 km per 1°C increase in the annual mean temperature [8].

Therefore, sunflower breeding for cold tolerance seems to be urgent and feasible, so a number of researchers focused on assessments of cold tolerance in sunflower species [9 – 11]. In the context of possible spring frosts, effects of freezing temperature on sunflower were also investigated [12]. In hope to find solutions to cold tolerance issues, species related to *H. annuus* were studied [13 – 14]. Researchers strive to elucidate molecular mechanisms of cold tolerance in sunflower [15].

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For sunflower production, it is vital to have cold-tolerant hybrids. An appropriate method to assess cold tolerance is a prerequisite for successful breeding of cold-tolerant sunflower. Time-saving screening of sunflower accessions for cold tolerance could immensely boost the breeding efficiency. Researchers use various approaches to quickly assess cold tolerance of plants. V. Bozhanova and T. Petrova [16] suggested a simple express-method to assess tolerance to a stress by growth depression in stems and roots of seedling under stressful conditions. Duration of stress varied from 1 four to several days in different publications [17, 18]. T. Hewezi et al. [15] applied quite a long test: they germinated seeds at 15 °C until seedlings reached a two-leaf stage (about 50 days after sowing), then the temperature was decreased to 7 °C until plants reached a four-leaf stage (about 70 days after sowing). To assess tolerance to freezing temperatures, plants with 6–8 fully developed leaves were exposed to –3°C for 10 hours and then chlorophyll fluorescence and relative electrolyte leakage were measured [12]. Other researchers acclimated two-leaf sunflower seedlings at 15 °C for 4 days and then at 4 °C for 12 days in combination with a short-day photoperiod and reduced light intensity. Afterwards seedlings were chilled at a rate of 2.5 °C per hour to −3 °C, −4 °C, or −5 °C and their freezing tolerance was evaluated by electrolyte leakage after freezing. [15].

Hence, our purposes were to evaluate cold tolerance of sunflower lines and hybrids in the laboratory and their tolerance in the field, to identify promising combinations, to assess the capacity of a laboratory cold germination test to predict tolerance of sunflower in the field and to verify relationships of cold tolerance with ripeness group and yield.

Materials and Methods. Sunflower was grown in compliance with standard farming techniques [19]. The record plot area was 10.15 m^2 in three replications. Sunflower was sown within the optimal timeframe. Lines Mkh845A, Skh1002A, Skh1006A, Skh1010A, Skh1012A, Skh146A, Skh51A, Skh777A, Skh808A, Skh908A, and Skh93A were taken as female forms. Lines Kh 15-107 V, Kh 15-113 V, Kh 57-13 V, Kh 15-146 V, Kh 15-80 V, Kh 15-75 V, Kh 15-157 V, Kh06134V, Kh06135V, Kh114V, Kh276V, Kh4413V, Kh4713V, Kh4913V, Kh526V, Kh5613V, Kh720V, and Kh785V were taken as male forms. Crossings were performed in 2018-2019. The crossing design was 11x18 using group mesh tents for each male line. In total, 192 F_1 hybrid combinations were obtained, and 18 of them (the most promising ones) were screened both for laboratory cold tolerance and field tolerance.

The lines and hybrids were screened for ability to germinate at above-zero low temperature by laboratory test [20]. All seeds were presterilized in 1.0% $KMnO_4$ for 10 min, rinsed several times with water and then placed on wetted filter paper, which was rolled up (25 seeds per roll, 4 rolls for each line/hybrid). Four lines and four hybrids were tested at three temperatures. The rolls were incubated in a cold thermostat at 4°C, 5°C or at 6°C. The control seeds were germinated at 25°C, which is considered to be the optimal temperature. The germination period was 10 days, as after longer periods no germination occurred. After 10 days, seedlings were counted. The seed was considered germinable if a seedling of minimum length was recorded. At 4°C, seeds could hardly germinate, at 5°C the number of germinated seeds ranged from 17 to 61%, and at 6°C - from 39 to 75%. Thus, the clearest differentiation of the lines and hybrids by cold tolerance can be achieved at 5°C. So, further experiments were carried out at 5ºC. Cold tolerance was computed as germinability at 5ºC related to germinability at 25ºC in percent.

At the initial stages of sunflower development, tolerance in the field is a complex feature combining cold tolerance (to suboptimal positive temperatures and subzero temperatures), resistance to pathogens and parasites, and resilience to water deficit. The field tolerance score was determined with a scale in 2019-2020. The scale was developed from the tolerance scale for corn [21], where 1 – the condition of a plot is bad, < 50% of the sown seeds have germinated, the seedlings are weak; 3 – the plant density is about 50% of the planned value, 5 – the plot condition is satisfactory, the plant density is about 70% of the planned value; 7 – the condition is good, the plant density is about 80%; 9 – the condition is excellent, the plant density has reached the planned value. During germination and emergence, the temperature on the soil surface ranged 0ºC to 9ºC, thus the seedlings did not face freezing temperatures that year.

The laboratory cold tolerance and field tolerance were evaluated after similar periods of seed dormancy.

Relationships between the investigated parameters were analyzed by linear regression at website Statistics Kingdom [22].

Results and Discussion. The highest tolerance (7 points) in the field was recorded for Kh4713V, Skh777A and Skh808A lines. They were considered as sources and possible donors of this trait. However, they only conferred tolerance to some of their hybrids: Skh808A/Kh15113V (excellent germinability of 9 points), Skh808A/X5613V (7 points), and Skh777A/Kh4713V (7). At the same time, several more hybrids were fairly tolerant in the field: Skh93A/Kh720V (7), Skh93A/Kh4413V (7), Skh146A/Kh15113V (7), and Skh1006A/Kh15157V (7), though the tolerance scores of their parental lines ranged 1 (Kh15157V) or 3 (Skh1006A, Kh15113V) to 5 points (Skh93A, Skh146A, Kh720V, Kh5613V, Kh4413V).

Regression lines were constructed to estimate relationships between laboratory cold tolerance and field tolerance. They are presented in Figure 1. The negative correlation coefficients even indicate a moderate/weak inverse relationship between cold tolerance of the sunflower accessions under laboratory conditions and tolerance in the field, though P-values of ≥0.10 mean that it is not statistically significant.

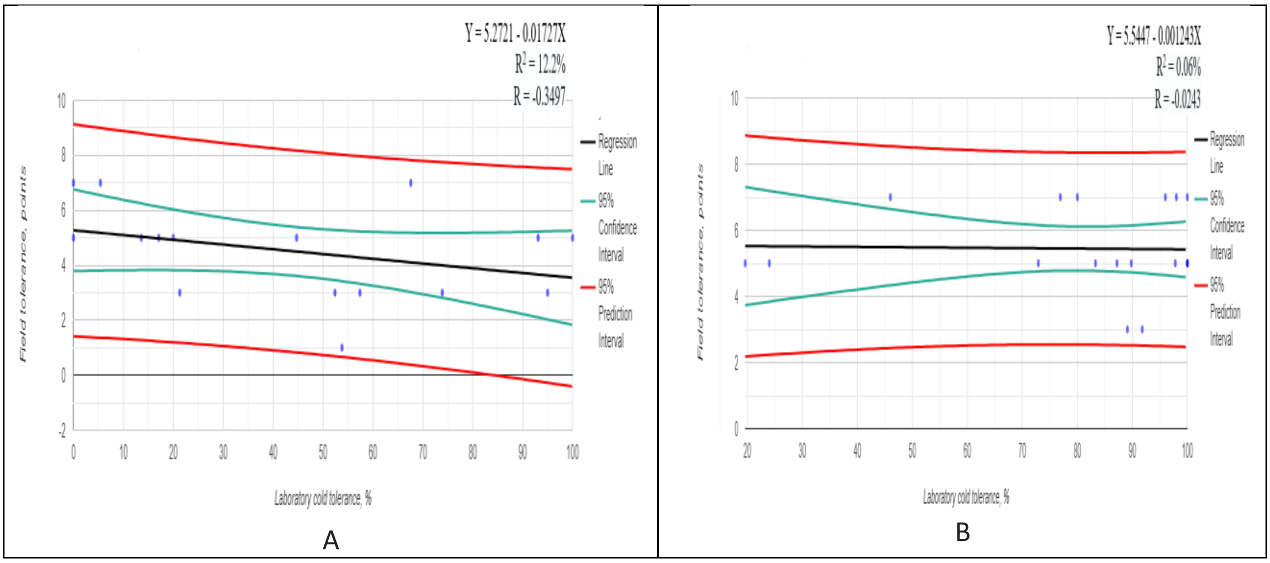

Fig 1. Linear regression of field tolerance on laboratory cold tolerance. A – lines, B – hybrids.

Relatively low values of R^2 and the wide prediction intervals mean that there is a great deal of uncertainty associated with predicting tolerance of sunflower lines and hybrids in the field from their cold tolerance assessed by laboratory test. We think this may be attributed to the following issues:

1. In Lakhanov's laboratory test [20], low temperature is the only factor, while in the field, there are other factors, including pathogens, water deficit, etc.
2. In Lakhanov's laboratory test [20], seeds are exposed to stable temperature, whereas there may be rather sharp fluctuations of temperature in the field, which are thought to be rather detrimental to plants [23, 24].

So, the laboratory cold germination test might inadequately reflect tolerance of sunflower seedlings under real conditions in the field.

At first we thought that early-ripening sunflower accessions would be more cold-tolerant. However, our observations refuted this assumption. There was no correlation between ripeness group and cold tolerance (Figure 2).

From Figure 2, it can be seen that there were weak correlations (oppositely directed and statistically insignificant; P≥0.10) between the 'emergence – anthesis' period and cold tolerance of the sunflower lines and hybrids under investigation. Relatively low values of R^2 and the wide prediction intervals mean that there is a great deal of uncertainty associated with predicting cold tolerance of sunflower lines and hybrids from their growing periods. This is in line with other researchers' findings [14]. The authors found that early- and late-flowering genotypes responded similarly to low-temperature exposure.

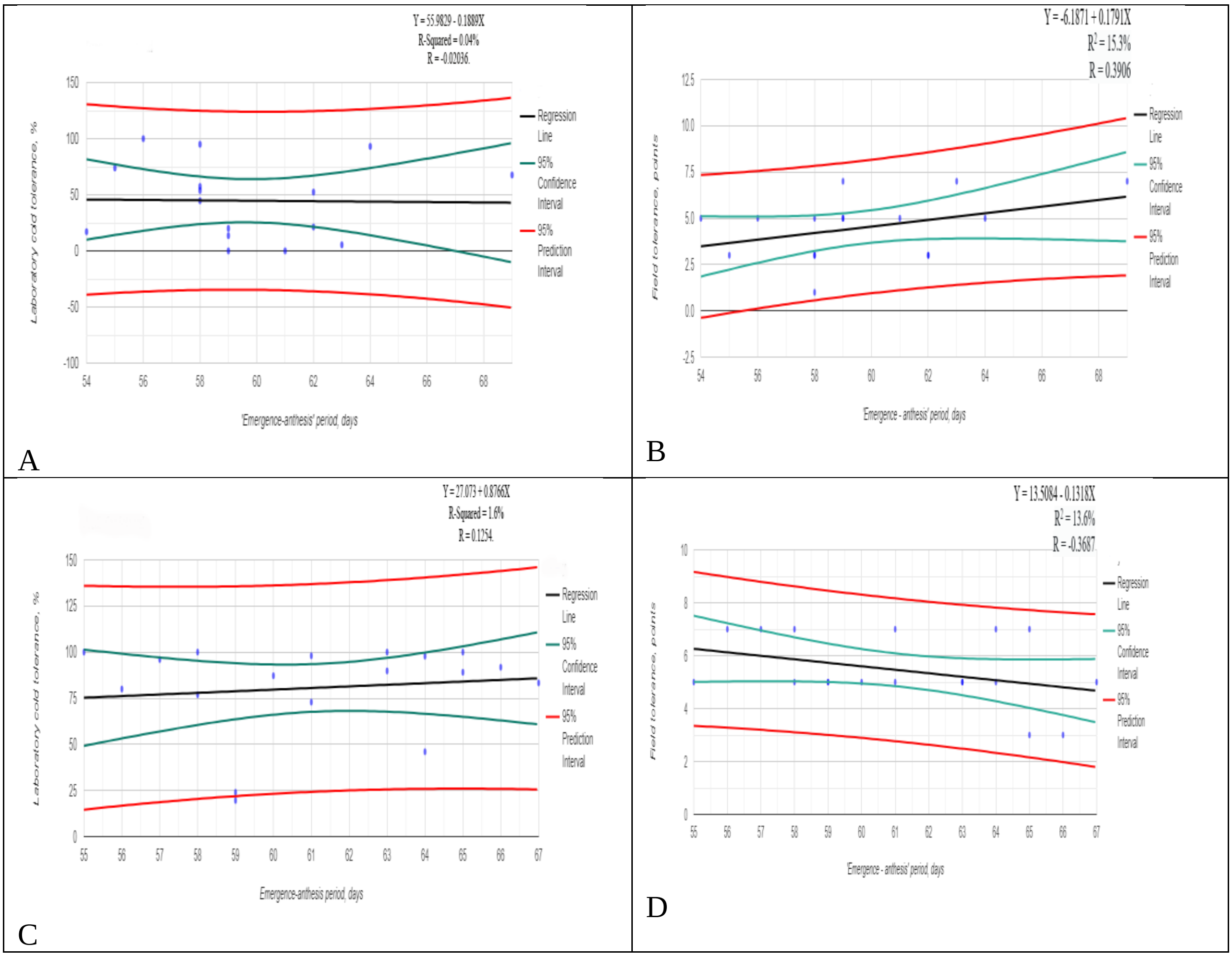

Fig 2. Linear regression of tolerance (A, C – laboratory cold tolerance, B, D – field tolerance) on 'emergence – anthesis' period. A, B – lines, C, D – hybrids.

Tolerance to non-freezing temperatures is of multi-factor nature. Extensive inhibition of transcription was reported to take place during sunflower plant development at sub-optimal temperatures [14]. Cold-shock proteins, which regulate transcription and translation at low temperatures, are common in bacteria. Similar proteins were also found in barley [25], winter wheat [26], rice [27], Arabidopsis [28 - 31], velvet bentgrass [32], soybean, sorghum, grapes, and maize [33]. Heat-shock proteins are known to be induced in response to different stresses, including cold, and act as molecular chaperones for polypeptides [34 – 36]. Some enzymes that were up-regulated in responses to cold treatment included methionine synthase and serine hydroxymethyltransferase [32]. Serine hydroxymethyl transferase was also demonstrated to be induced in response to cold exposure in *H. annuus*, suggesting cold-induced activation of amino acid metabolism [37]. Expression of energy generation enzymes, such as aconitase, UDP-D-glucuronate decarboxylase, was also enhanced by low temperatures [32]. Plant cells can improve membrane fluidity and, consequently, cold tolerance via increasing unsaturated/saturated fatty acid ratio by activation of fatty acid desaturases, which introduce double bonds into fatty acids [38 – 40]. A cold-induced rise in cytosolic calcium activates a lot of enzymes, such as phospholipases and calcium-dependent protein kinases [41].

Such a complex nature of cold tolerance makes creating cold-tolerant sunflower hybrids a difficult problem (though in this season sunflower seedlings did not suffer from freezing temperatures, cold tolerance both to non-freezing and to freezing conditions may be of even more complex nature, as mechanisms of tolerance to freezing temperatures are thought to differ from the above-described ones). Previously, our attempts to create cold-tolerant sunflower hybrids by traditional crossing cold-tolerant lines were not always successful (in press). There was no correlation between the cold tolerance of the hybrids and their parental lines.

Hence, more advanced approaches are required to achieve this goal. D. Škorić believes that "Sources of cold resistance should be sought exclusively in the wild *Helianthus* species that are found growing wild in the mountains where winters are harsh and springs are cold" [9]. For example, H. Tetreault et al. [13] examined cold tolerance in three natural populations of the perennial sunflower species *Helianthus maximiliani*. 'Texas' and 'Kansas' population were cold susceptible, while 'Manitoba' population showed tolerance. However, the authors failed to obtain a cold tolerant hybrid, as cold tolerance of 'Manitoba' × 'Texas' F_1 hybrids did not statistically differ from that of the 'Texas' population.

D. Škorić also thinks that, apart from wild *Helianthus* species, induced mutations can be used as sources of resistance to low temperatures [10].

During early growth stages, sunflower plants can withstand temperatures of −3.3 to −3.8 °C for short periods [42]. As plants develop (V2 to V6), they become progressively more susceptible to frost. At the V2 stage, the lowest temperature plants can withstand is −2.7 °C to −3.3 °C but, for the V4–V6 stages, −1.6 to −2.2 °C is the lower limit. C. Alline et al. [43] confirmed this information: at cotyledon stage, sunflower plants could survive at −5 to −7 °C, but as soon as the first leaves appear, −3 °C could induce severe necrosis of plant tissues. K. Houmanat et al [44, 45] presented data on sunflower accessions sown on 2 January 2014 in Morocco that survived the minimum temperature -5°C, though they did not provide evidence on seedling survival at subzero temperatures. It was dormant seeds, not seedlings, might overwinter and survive at −5°C.

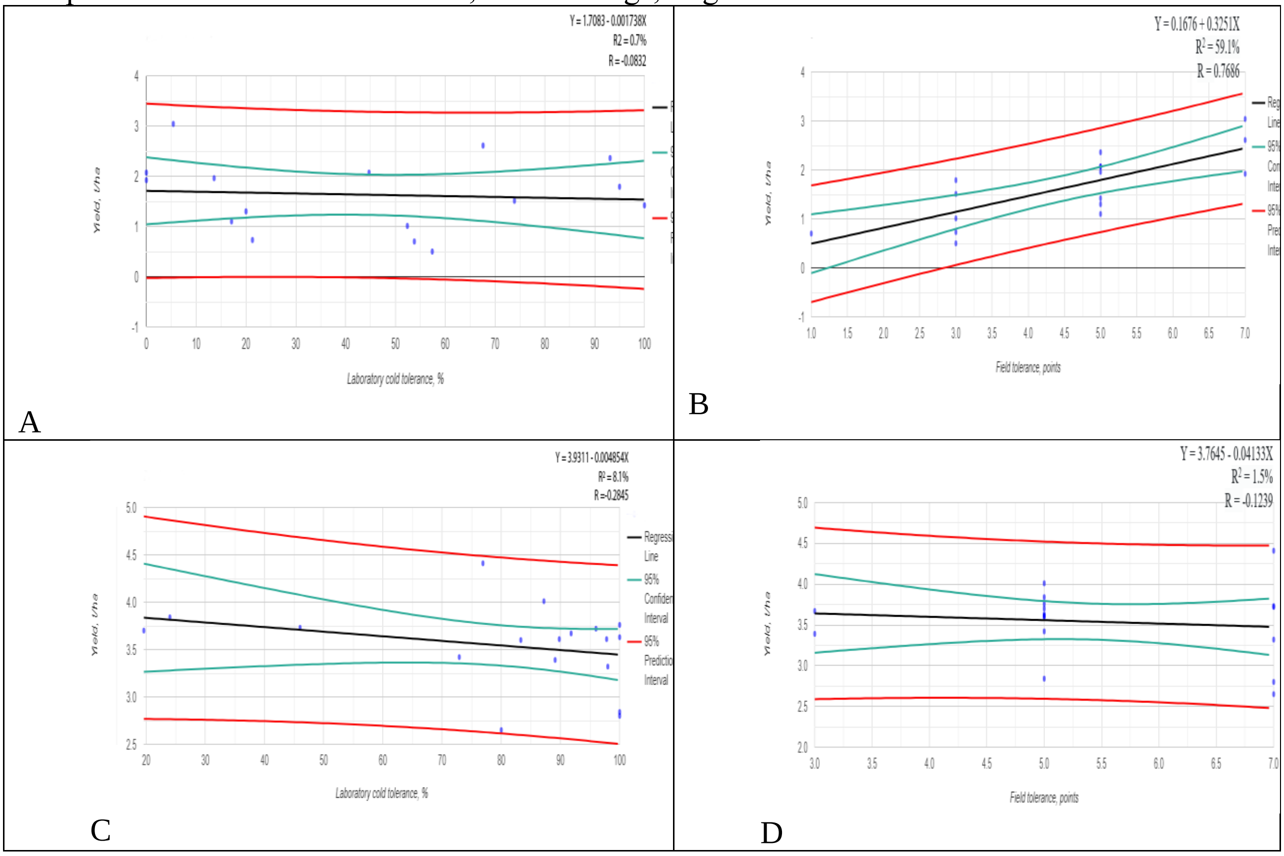

Fig 3. Linear regression of sunflower yield on tolerance (A, C – laboratory cold tolerance, B, D – field tolerance). A, B – lines, C, D – hybrids.

We believe that molecular genetics-based approaches are needed to develop cold-tolerant sunflowers. For example, in France, 15 partners, including laboratories with multi-disciplinary expertise, sunflower breeding and biotech companies, and an institute in charge of oil-protein crops joined within the SUNRISE consortium and launched a project to identify physiological, molecular and genetic components of tolerance of sunflower hybrids to environmental stressors (with focus on water deficit stress) [11]. Sunflower breeders hope that the project will accelerate sunflower hybrid creation. We can expect a similar project to be launched for cold tolerance too.

Since in agriculture the final benchmark of the crop's value is its yield, we constructed regression lines of the yields of the sunflower lines and hybrids on the laboratory cold tolerance and field tolerance (Figure 3).

It is obvious that the laboratory cold germination test cannot be used to predict yielding capacity of either sunflower lines or sunflower hybrids. The correlation coefficient (R = - 0.0832; P =0.7594 and R = -0.2845; P= 0.2525) indicated a very weak or weak inverse (insignificant) relationship for the tested sunflower lines and hybrids, respectively. We can hypnotize that, while in Lakhanov's cold germination test, the ability of seeds to GERMINATE at low temperatures is assessed, later stages of initial growth and development may be crucial for survival in the field.

As to the relationship between the field tolerance and sunflower yield, the situation was more complicated: there was a strong positive correlation (R = 0.7886, P =0.0005) in the lines, but the correlation coefficient of -0.1239 (P = 0.6242) for the hybrids indicated no significant correlation. It is noteworthy that the confidence and prediction intervals were also considerably narrower for the regression of the line yield on the field tolerance. The slope (b_1) of 0.3251 meant that a 1-point increment in the field tolerance increased the yield by 0.3251 t/ha. This finding can be attributed to the fact that inbred lines' were more susceptible to stressful factors; therefore, the negative impact of an unfavorable environment during the early stages of plant growth and development had long-term effects, i.e., the yields of lines to a large extent depended on their tolerance in the field shortly after germination. However, heterotic hybrids, being more adapted, were able to recover from the negative impact of external stressors during the early stages, so their yields were not directly associated with the conditions during the initial stages of plant growth and development. It should be noted that this pattern was recorded for the hybrids under investigation in 2020, when germinating seeds did not face freezing temperatures. If conditions were harsher, the tolerance at the initial stages of plant growth and development might have influence on further performance even in hybrids.

Conclusions. At non-freezing low temperatures, the yield of the inbred sunflower lines was positively correlated with the field tolerance score at the early stages of plant growth and development. There was no significant difference in responses of early- and medium-ripening sunflower genotypes to cold exposure. We think that Lakhanov's cold germination test is not appropriate for evaluation of field tolerance in sunflower lines and hybrids. It is assumable that it could be improved with frost, temperature fluctuations and cold acclimation simulations. In addition, advanced, state-of-art, molecular genetic-based approaches may be required to combine numerous genes, which are responsible for cold tolerance, in F_1 hybrids.

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Purpose and Objectives. To evaluate cold tolerance of sunflower in the laboratory and its tolerance in the field, to identify promising combinations, to assess the capacity of a laboratory test to predict tolerance of sunflower in the field and to verify relationships of cold tolerance with ripeness group and yield.

Materials and Methods. Sunflower was grown in compliance with standard farming techniques. In total, 192 F_1 hybrids were obtained. The lines and hybrids were screened for ability to germinate at above-zero low temperature by laboratory test. The field tolerance at the initial stages of plant growth was determined with a 9-point scale. Relationships between the investigated parameters were analyzed by linear regression.

Results and Discussion. The highest field tolerance at the initial stages of plant growth was recorded for Kh4713V, Skh777A and Skh808A lines. However, they only conferred tolerance to some of their hybrids. On the other hand, several hybrids were fairly tolerant in the field though the tolerance scores of their parental lines ranged 1 to 5 points. There was no relationship between the 'emergence – anthesis' period and cold tolerance. A great degree of uncertainty is associated with predicting field tolerance of sunflower from its cold tolerance assessed by laboratory test. This laboratory test cannot be used to predict field tolerance of either lines or hybrids. There was a strong positive correlation between the field tolerance and seed yield in the lines, but the correlation coefficient for the hybrids indicated no significant correlation between these parameters.

Conclusions. At non-freezing low temperatures, the yield of the inbred sunflower lines was positively correlated with the field tolerance score at the early stages of plant growth and development. There was no significant difference in responses of early- and medium-ripening sunflower genotypes to cold exposure. Lakhanov's cold germination test is not appropriate for evaluation of field tolerance in sunflower lines and hybrids.

***Key words**: sunflower, cold tolerance, line, hybrid, linear regression*

ПРОБЛЕМИ В СЕЛЕКЦІЇ СОНЯШНИКУ НА ХОЛОДОСТІЙКІСТЬ

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Вступ. Соняшник вирощують як олійну культуру у всьому світі. Для виробництва соняшнику життєво необхідно мати холодостійкі гібриди. Відповідні методи оцінки холодостійкості є необхідною умовою успішного виведення холодостійкого соняшнику.

Мета та завдання. Оцінити холодостійкість соняшнику в лабораторних умовах та його стійкість у польових умовах, визначити перспективні комбінації, оцінити придатність лабораторного тесту для прогнозування стійкості соняшнику в польових умовах та перевірити зв'язок холодостійкості з групою стиглості та врожайністю.

Матеріали та методи. Соняшник вирощували з дотриманням стандартної агротехніки. Всього отримано 192 гібриди F_1. Скрінінг ліній та гібридів на схожість при плюсовій низькій температурі проводили за допомогою лабораторного тесту. Оцінку польової стійкості на ранніх етапах розвитку рослин визначали за 9-бальною шкалою. Зв'язки між досліджуваними параметрами аналізували методом лінійної регресії.

Результати і обговорення. Найбільша стійкість на ранніх етапах розвитку рослин в польових умовах зареєстрована для ліній Х4713В, Сх777А та Сх808А. Однак вони передали стійкість лише деяким зі своїх гібридів. З іншого боку, кілька гібридів були досить стійкими в польових умовах, хоча показники стійкості їхніх батьківських ліній варіювали від 1 до 5 балів. Не виявлено зв'язку між періодом «сходи – цвітіння» та холодостійкістю. Значний ступінь невизначеності пов'язаний з прогнозуванням польової стійкості соняшнику за його холодостійкістю, оціненою за допомогою лабораторного тесту. Цей лабораторний тест не можна використовувати для прогнозування польової стійкості ліній чи гібридів. Спостерігалася сильна позитивна кореляція між польовою стійкістю та врожайністю насіння у ліній, але коефіцієнт кореляції для гібридів вказував на відсутність суттєвої кореляції між цими параметрами.

Висновки. При позитивних низьких температурах врожайність інбредних ліній соняшнику позитивно корелювала з показником польової стійкості на ранніх стадіях росту та розвитку рослин. Вірогідної різниці у реакції ранньо- та середньостиглих генотипів соняшнику на вплив холоду не виявлено. Результати тесту Лаханова на схожість в умовах холоду не узгоджуються з оцінкою польової стійкості ліній і гібридів соняшнику.

***Ключові слова**: соняшник, холодостійкість, лінія, гібрид, лінійна регресія*