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USE OF MORPHOPHYSIOLOGICAL MARKERS IN INTRASPECIFIC POLYMORPHISM ANALYSIS OF FLAX *Linum usitatissimum* L.

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Abstract

In worsen environmental conditions sustainable agriculture and high quality food production rely on crop diversity and adaptiveness that requires the improved estimates of plant parameters. Flax which is among the recognized promising crops is presently rare in the Tyumen region. Our paper shows the most informative morphometric criteria of adaptiveness for several domestic and foreign flax varieties studied under local conditions. The criteria are for the first time improved by a rapid noninvasive method of chlorophyll measurement in leaves with a SPAD 502 optical counter. The purpose of this work was to estimate response of *Linum usitatissimum* L. genotypes to the environmental factors and to identify signs for use as indicators of adaptability. A total of 20 collection samples of flax of different ecological and geographical origin from Russia (6 samples), Belarus (7 samples), Czechia (2 samples), Ukraine (1 sample), France (1 sample), Canada (1 sample), Australia (1 sample), and Germany (1 sample) were used. Laboratory and field tests were conducted in 2016-2017. Seeds were germinated in Petri dishes on a filter paper moistened with distilled water at 20 °C, germination energy, laboratory germination rate, morphometric parameters and biomass of seedlings were determined. To test the plants at the initial stages of ontogenesis, 20 seeds were sown in 4-fold repeat in the vegetation pots. For field study (Biological station of the Tyumen state university «Biostation Kuchak», Tyumen Province, Nizhnetavdinsky District), 200 seeds of each sample were sown on 1 m^2 plots in 3-fold repetition. Chlorophyll content in leaves was determined using an optical counter SPAD 502 («Minolta Camera Co., Ltd», Japan), three times with a 5 day interval in the laboratory test and seven times (according to the phenological phases — full shoots, herringbone, rapid growth, budding, flowering, green ripeness, and early yellow ripeness) in the field test. As per results obtained the seeds of the studied flax samples had two types of color (brown and yellow), differed in length (3.0-5.4 mm), width (2.1-8.2 mm), weight of 1000 seeds (6.57-4.36 g), shape (elongated-elliptic, ovoid flattened), which indicates sample heterogeneity. Features of plant growth and development at the initial stages of ontogenesis in some cases were confirmed in the field. For example, the length of the shoot in plants of varieties Mayak, Rybin, and Iva in the laboratory test is the largest of the entire experimental group (13.1; 12.5; 12.1 cm, respectively), and in the field, these varieties are in the ‘tall’ group according to plant height (96.1-100.2 cm). The yield of the flax straw was more (166.6-171.7 g/m^2) in the varieties having shoots substantially predominating in the plant biomass (Grant, Mayak, Bertelsdorfer). Field germination rate (62.3-77.3 %) and plant survival during the growing season (70.6-85.8 %) we refer to the informative criteria that characterize a number of ontogenetic interrelated processes. Also, we propose SPAD 502 readings (SPAD index) to evaluate the genotype × environment interactions. Our results show positive correlations between SPAD index and parameters of plant assimilation surface (linear size, area and number of leaves, and plant height), as well as plant survival during the growing season. Significant differences are revealed between the studied samples in the average daily chlorophyll accumulation in the leaves before flowering and chlorophyll degradation at green and early yellow ripeness phases. Significant advantage in flax straw and seed yields have samples with a relatively rapid increase in SPAD index during the first half of the growing season with further uniform decrease towards maturity phase. Thus, Grant, Mayak (Belarus), Bertelsdorfer (Germany), Svalof (Czech Republic), Ottava 770 B See (Canada) are the varieties that combine high adaptive and productive properties.

Keywords: *Linum usitatissimum* L., flax, collection sample, chlorophyll counter SPAD 502, simulated and natural conditions

Stresses from environmental factors, known as stochastic events, represent significant deviations from normal conditions and can cause significant damage to the plant population [1]. Sustainable crop production in the south of the Tyumen Province is possible with a combination of the following conditions: selection of plant species tolerant of limiting environmental factors, the creation of new highly adaptable varieties, their accelerated reproduction, and proper environmental placement [2]. Climate changes, manifested in an increase in average annual surface air temperature by 0.7 °C on all continents over the last quarter of the 20th century [3], indicate the need for making adjustments to the strategy for selecting species and varieties of cultivated plants.

The provision of high-quality, environmentally friendly food products to a certain extent is achieved by increasing the number of crops, developing fundamentally new ways to evaluate plant forms and quickly processing information about the biology of their development. Genetic diversity of plants increases the choice and provides protection against adverse conditions.

Flax is one of the crops that deserve attention and introduction into agricultural production. Currently, in the Tyumen Province, it is practically not grown; there is no testing of varieties in state strain-trial stations (SSTS). At the same time, flax growing was developed in Siberia in the 1970s-1980s, and the area under flax in the south of the Tyumen Province was more than 10000 hectares [4]. The introduction and selection of flax varieties, ecologically adapted to local soil and climatic conditions, is important for agricultural practice.

When phenotyping characteristics on seedlings in controlled conditions (modeled in the laboratory) and on adult plants in natural field conditions, it is possible to accelerate the identification of selectively valuable plant forms. Morphological characteristics of seedlings are considered as biometric indicators of the reaction of the forming plant organism to environmental factors. Thus, the ratio of the root and the shoot lengths in standard and stressful conditions can serve as a convenient indicator characterizing ontogenetic development. This indicator is identified among the informative ones during the assessment of the degree of adverse effects on triticale [5], maize [6], safflower [7], and pine [8].

The physiological features characterizing the genotype × environment interaction include the chlorophyll content in the leaves. Stationary methods for determining this indicator using spectrophotometers are laborious, time-consuming, and require removal of plants from agrocenoses. The ability to quickly and accurately determine the amount of chlorophyll using the SPAD-502 optical counter (Minolta Camera Co., Ltd., Japan) in the leaves of wheat [9, 10], barley [11, 12], oats [13], rice [14], potatoes [15], tomatoes [16], celery [17], sugar cane [18], papaya [19], bay [20] and woody [21] plants, eucalyptus [22], peanuts [23], soy [24, 25], arabidopsis [26], and ornamental crops [27] was shown. Differences between species and varieties were revealed according to the amount of chlorophyll when exposed to salinity [28], contrast temperature [29, 30], lack of moisture [31] and in vitro culture [32]. In Russia, studies made using this instrument are fragmented [33].

In this paper, for the first time in the conditions of the south of the Tyumen Province, the variability of morpho-physiological traits has been studied using the example of 20 samples of ordinary flax of different ecological and geographical origins and the most informative criteria determining the adaptive potential of the crop have been established. Rapid diagnosis of the accumulation and degradation of chlorophyll in the leaves at different stages of plant ontogenesis was carried out using a SPAD 502 optical counter.

The purpose of the research was to study the response of ordinary flax genotypes (*Linum usitatissimum* L.) to the effects of environmental factors and to identify population and individual characteristics for use as indicators of adaptive properties.

Techniques. The authors studied 20 collection samples of flax of various eco-geographical origins from Russia (6 samples), Belarus (7 samples), Czech Republic (2 samples), one sample per Ukraine, France, Canada, Australia, and Germany. Laboratory and field experiments were carried out in 2016-2017.

Seeds were germinated in Petri dishes on filter paper moistened with distilled water at 20 °C in a thermostat TS-1/80 SPU (Russia) in accordance with RF State Standard GOST R 52325-2005 [34]. The germinative energy, laboratory germination rate of seeds, morphometric parameters, and seedling biomass were determined.

To test the plants at the initial stages of ontogenesis, 20 seeds were sown in 4-fold repetition in the vegetation vessels. 280 g of soil with a humidity of 60% of the total water capacity was placed in the vessel. Plants were grown on specialized racks (illumination of 5000 lux, 16 h photoperiod). Seed germination, plant height, chlorophyll content in leaves, the weight of the aboveground and underground parts were assessed. To characterize the structure of biomass, the ratio for shoots and roots was calculated.

A field study of flax collection samples was performed at the experimental field of the biological station of the Tyumen State University Lake Kuchak (Nizhnetavdinsky District, Tyumen Province, 57°21′N, 66°04°′E) on cultivated sod-podzolic soil of sandy loam granulometric composition. Field trials, observations and records of traits were carried out in accordance with the guidelines [35]. Sowing was carried out in the first decade of May in an ordinary way with 200 seeds per plot with an accounting area of 1 m^2, repeated 3 times. Placement of the plots was randomized. The predecessor is spring barley.

The chlorophyll content in the leaves was determined using an optical counter SPAD 502 (Minolta Camera Co., Ltd., Japan) at λ = 650 nm (maximum absorption of chlorophylls a and b) and λ = 940 nm (taking into account the thickness of the leaf). The instrument was calibrated before starting the measurement. The amount of chlorophyll in 20 leaves from the top of typical plants of each sample was estimated. The middle part of the leaf blade was placed on the bottom of the device, clamped for a few seconds until a numerical value appears. The device automatically remembers all readings and calculates average values (SPAD units). The authors conducted 3 measurements with an interval of 5 days in the laboratory experiment and 7 measurements in the field at different stages of phenological development: sprouting, herringbone, rapid growth, budding, flowering, green ripeness, early yellow ripeness.

The plant height was measured at each phenological phase. During the harvesting period, the number of surviving flax plants in each plot was calculated, and survival was calculated in relation to the number of shoots.

Statistical data processing was performed according to B.A. Dospekhov [36], G.F. Lakin [37], A. Field et al. [38] using the Microsoft Excel spreadsheet processor and the STATISTICA 6.0 software (StatSoft, Inc., USA). Mean values (M) and standard errors of the mean (±SEM) are presented. Differences between mean values of the variants were evaluated using Student's t-test and were considered to be statistically significant at $p < 0.05$ and $p < 0.01$.

Results. The heterogeneous flax material differed in linear dimensions (length, seed width) and 1000-seed weight. In accordance with the International Classification of the species *Linum usitatissimum* L. [39], the samples were divided into two groups: with medium (3 samples) and small (17 samples) seeds (see Table).

According to the color of flax seeds, there are two types, dark (brown) and light (yellow). M.N. Yaglo et al. [40] regarded this trait as an important marker for variety identification. The yellow color is determined by the dominant *YSED1* gene, the dark yellow color by the recessive *ysed2* gene, and the light yellow-brown color by the *rs1* gene [41]. The seed color of the studied samples was brown (of varying intensity) in 19 samples and yellow in one (Ottava 770 B See).

Characterization of seeds of the studied ordinary flax (*Linum usitatissimum* L.) samples (*M*±SEM, laboratory test)

Sample, phenotype (origin)	Seed size, mm		Length/width ratio	1000-seed weight, g
	length	width		
Medium seeds ($n = 3$)				
Rucheek, o (Russia)	5.21±0.83	3.54±0.11	1.47	6.12±0.17
Fleez, o (Russia)	5.21±0.12	3.30±0.77	1.58	6.00±0.91
Turquoise, o (Russia)	5.05±0.77	4.31±1.22	1.17	6.57±0.25
Small seeds ($n = 17$)				
36.3.-4, l (Russia)	4.42±0.81	3.25±0.50	1.36	4.51±0.66
Velizhsky Ridge, l (Russia)	3.02±0.12	3.12±0.71	0.97	4.48±0.59
Pechersky Ridge, l (Russia)	4.13±0.99	3.54±0.66	1.17	4.36±0.33
Grant, l (Belarus)	5.01±0.89	3.10±0.63	1.61	5.25±0.34
Mayak, l (Belarus)	3.06±0.12*	5.23±0.32*	0.59	4.50±0.65
Mara, l (Belarus)	4.21±0.54	3.42±0.65	1.23	4.83±1.03
Rubin, l (Belarus)	3.15±0.78	2.11±1.23	1.49	4.66±0.34
Iva, l (Belarus)	4.25±0.75	3.32±0.90	1.28	4.74±0.43
Yarok, l (Belarus)	4.21±0.57	3.15±0.80	1.34	5.00±0.72
Vesta, l (Belarus)	3.35±0.28	3.42±0.64	0.98	5.39±0.94
Glinum, l (Ukraine)	4.45±0.45	2.25±1.12	1.98	4.89±0.77
Bertelsdorfer, l (Germany)	4.06±0.10	8.21±0.21**	0.49	5.25±0.95*
Currong, l (Australia)	4.05±0.45	3.05±0.32	1.33	4.37±0.76
Svalof, l (Czech Republic)	5.34±0.90*	3.15±0.32	1.70	4.59±0.91
Hermes, l (Czech Republic)	4.21±0.91	3.11±0.36	1.35	5.03±0.49
Ottava 770 B See, l (Canada)	5.36±0.21	3.48±0.79	1.54	5.50±0.20
Alizee, l (France)	4.54±0.54	3.27±0.25	1.38	5.61±0.63
Mean of all specimens	4.31±0.55	3.58±0.62	1.30	5.08±0.60

Note. The samples are grouped according to 1000-seed weight [39]; l — long-stalked flax (spinning flax), o — linseed flax (oil flax).

*, ** Differences with the average value of the samples are statistically significant, respectively, at $p < 0.05$ and $p < 0.01$.

The average seed length was 4.31±0.55 mm and varied in samples from 3.02±0.12 (Velizhsky Ridge) to 5.36±0.21 mm (Ottava 770 B See). In seed width, the minimum value (2.11±1.23 mm) was observed in the Rubin variety, the maximum value (8.21±0.21 mm) in the Bertelsdorfer variety, with an average value of 3.58±0.62 mm in samples (see Table). In the majority of the samples studied, the seed linear dimensions did not differ significantly from the average population values. Significant differences were found in the Mayak and Svalof samples in the seed length, in the Mayak and Bertelsdorfer samples in the seed width. The calculation of the index of the ratio of the seed length and width allowed estimating their differences in shape. The seeds of 16 samples, in which the length and width ratio was 1.17-1.98, had an elongated-elliptical shape. In four samples, the seed shape was ovate oblate (length and width ratio 0.49-0.98). By 1000-seed weight, a significant difference with the average population value was noted only in the Bertelsdorfer sample.

Testing of the physiological quality of seeds is carried out by germinative energy and laboratory germination rate in strictly regulated conditions. However, it is believed [42] that these tests are not designed to predict the exact number of seedlings in field conditions, since the impact of stress factors (low soil temperatures, lack of moisture, pathogens, etc.) reduces the rate of germination. In this regard, informative addition to the assessment of the biological properties of seeds was the study of the initial ontogenetic development of plants on the basis of the variability of their morphological features. The germinative energy and laboratory germination rates confirmed the high sowing qualities of the seeds studied. By germinative energy, none of the samples was significantly different from the average value (95.6±0.74%) throughout the experimental group. Laboratory germinative energy of seeds of the Grant (99.8±0.66%) and Glynum (97.1±0.21%) varieties was significantly higher ($p < 0.05$), the Yarok (93.7±0.90%) and Fleez (94.6±0.99%) varieties were below average.

The variation of the characteristic values as influenced by environmental factors is called phenotypic adaptation and is determined by the reaction norm [43]. The ability of seeds to germinate and to form full-fledged seedlings reflects the adaptive properties of the crop in new environmental conditions. The average field germination of seeds in the studied flax samples was 25% lower than in the laboratory, which is typical of the soil and climatic conditions of the Tyumen Province and is consistent with data from other crops [44]. The samples response to cultivation in the field was ambiguous. Indices for 6 samples differed significantly ($p < 0.05$) from the average for the collection, of which Grant, Mayak, Velizhsky Ridge were characterized by high (77.3-76.5%), Hermes, Bertelsdorfer, Rucheek – by low (66.3-67.9%) seed germinative energy. The variability of samples in field germination was higher than in laboratory tests (*Cv* of 25.18 and 9.13%, respectively).

The identification of growth features in regulated conditions and the possibility of interpreting these data in relation to the field conditions are of particular interest. So, when grown in pots, the longest shoot length was observed in the Mayak (13.1±0.77 cm), Rubin (12.5±0.94 cm), and Iva (12.1±0.31 cm) varieties. According to the results of the field assessment, these varieties were classified as tall (100.0, 96.1, 100.2 cm, respectively). That is, their high growth potential was maintained throughout the entire period of plant development.

Analysis of the structure of raw biomass revealed the predominance of shoots, making in samples 66.7% on average. The high share of shoots (87.5-90.0%) in the formation of plant biomass at the initial stages of ontogenesis was found in samples Velizhsky Ridge, Grant, Iva, Vesta, Glinum, Bertelsdorfer, Ottava 770 B See. In the field trials, stock yield was relatively high for Grant, Glynum, Lighthouse plants (166.5-170.0 g/m^2), seed yield for Grant, Mayak, Bertelsdorfer plants (66.5-77.7 g/m^2).

The environmental plasticity of the tested flax samples was characterized by 80% plant survival during the growing season. The variation of the trait was 30.33% and was higher than for seed germinating energy, laboratory and field germination. Consequently, the survival of plants was largely determined by the indices of individual viability of the samples. Grant, Mayak, Rubin, Currong, Velizhsky Ridge showed the best viability, and Fleez, Mara, Hermes, and Rucheek had the least plasticity. Samples with high resistance to environmental factors on the biological properties of seeds, their ability to germinate, as well as on indicators of linear growth of roots and shoots in early ontogenesis in most cases, minimally deviated from the average values for the collection. Samples with reduced survival often deviated from the mean value in opposite directions, i.e. towards increased shoot length (Mara) and field seed germination (Hermes), and towards reduced laboratory and field germination rates, as well as shoot length (Fleez, Rucheek).

Thus, we used the widely-used tests which are based on the study of the characteristics of plants in laboratory and field tests allow one to reveal the peculiarities of intrapopulation variation at the phenotypic level. However, there are opportunities to improve this traditional methodology. For example, the tests using physiological markers, such as the chlorophyll content, are seemed important.

When analyzing the dynamics of chlorophyll accumulation in leaves in a laboratory test, we did not reveal significant differences between the samples in the first and second measurements with the SPAD 502, which is probably due to the low intensity of linear growth at the initial stages of ontogenesis. The third measurement showed an increase in intrapopulation differences. In six varieties (Mayak, Glynum, Iva, Velizhsky Ridge, Currong, Hermes), the indicators were significantly ($p < 0.05$) lower than the average value, the others did not significantly differ. From the beginning to the end of the experiment, the indicator varied from 1.29 to 9.14 SPAD units. To assess the variability of the chlorophyll content in the leaves, to give a complete statistical description of the analyzed population in laboratory and field conditions, as well as to compare one distribution with another, span diagrams (box and whisker pots) developed by J. Tukey [38] were used (Fig. 1).

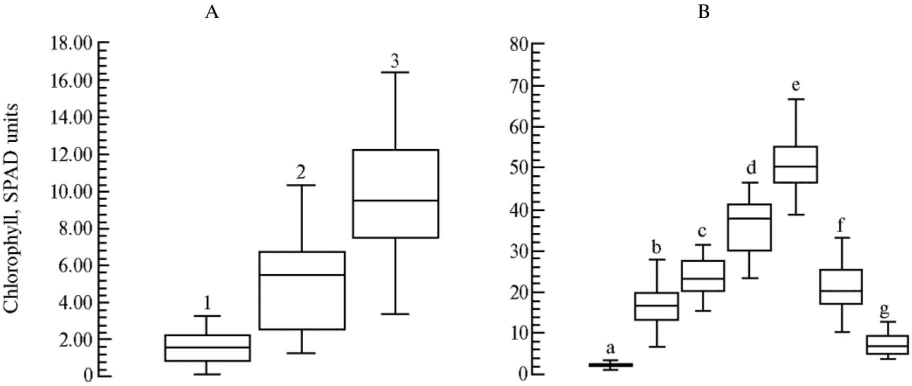

Fig. 1. Chlorophyll content in leaves of flax (*Linum usitatissimum* L.) collection samples with SPAD 502 (Minolta Camera Co., Ltd., Japan) in laboratory (A) **and field** (B) **trials:** 1, 2, 3 — counted with an interval of 5 days; a — full shoots, b — herringbone, c — rapid growth, d — budding, d — flowering, e — green ripeness, g — early yellow ripeness; line — arithmetic average; □ — standard error, ±; ⊥ — the trait minimum; T — the trait maximum (n = 20; field tests were performed at the biological station of Lake Kuchak, Tyumen State University, Nizhnetavdinsky Region, Tyumen Province, 2016-2017).

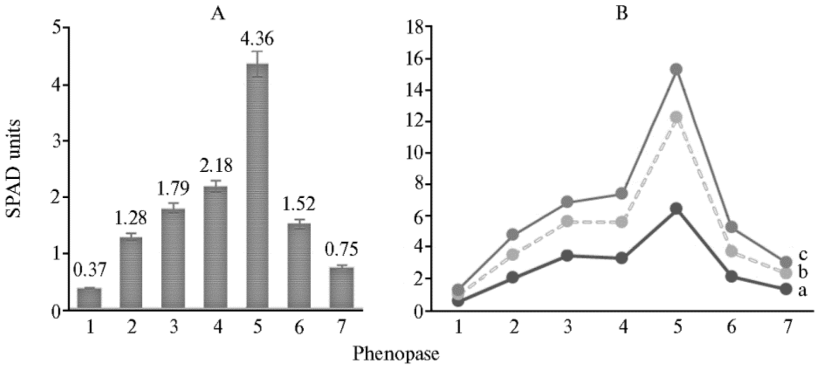

Fig. 2. Average daily accumulation and degradation of chlorophyll in flax (*Linum usitatissimum* L.) leaves during phenological phases for all samples on average (n = 20) (A) **and in the samples with significant differences** (n = 3) (B)**:** 1 — full shoots, 2 — herringbone, 3 — fast growth, 4 — budding, 5 — flowering, 6 — green ripeness, 7 — early yellow ripeness; a — Grant, b — Svalof, c — breeding sample 36.3.4 (field tests were carried out at Lake Kuchak biostation of Tyumen State University, Nizhnetavdinsky Region, Tyumen Province, 2016-2017).

Under natural conditions, the highest values (70.05 SPAD units) for chlorophyll were observed in flax leaves during flowering. During seed formation and ripening, the index dropped to 4.23-13.24 SPAD units. The nature of changes in the amount of chlorophyll can significantly affect seed yield and quality [45]. The minimum daily average accumulation of chlorophyll was recorded in the seedling phase (0.37 SPAD units). The further development of plants was associated with an increase in the relative rate of chlorophyll accumulation during sprouting—herringbone period (up to 1.29 SPAD units), herringbone—rapid growth (up to 1.79 SPAD units), rapid growth—budding (up to 2.18 SPAD units), budding—flowering (up to 4.36 SPAD units). From flowering to green and early yellow ripeness, the indices sharply decreased and amounted to 1.52 and 0.75, respectively (Fig. 2).

Despite a common pattern of chlorophyll accumulation and degradation of, the studied samples differed significantly among themselves (see Fig. 2, B). Thus, the highest intensity of chlorophyll accumulation in the leaves was revealed in Grant, i.e. the pigment level during growing season increased daily by 2.71 SPAD units. Regarding other samples, the variety was characterized by a rapid increase in the chlorophyll content before flowering and pronounced degradation during seed formation. The variety showed a higher yield of stock (170.0 g/m^2) and seeds (68.3 g/m^2), with average population values of 147.1 and 59.85 g/m^2, respectively. During the growing season in the breeding sample 36.3.4, an increase in chlorophyll per day was slow (1.42 SPAD units), the lowest rate (1.53 SPAD units) as compared to other samples was during flowering phase, the stock yield was low (100.2 g/m^2). Destruction of chlorophyll in green ripeness (1.58 SPAD units) and early yellow ripeness (0.70 SPAD units) was relatively slow, and seed yield was 33.3 g/m^2. Svalof variety ranked an intermediate position between the described samples according to average daily chlorophyll accumulation in leaves (2.10 SPAD units during the growing season). It stood out by this indicator during plant flowering (2.43 SPAD units), seed formation and maturation (1.57-1.00 SPAD units), as well as by seed productivity (116.5 g/m^2). In general, for the studied samples, seed yield varied from 33.3 to 116.5 g/m^2, and stock from 100.2 to 171.7 g/m^2.

Correlation coefficients revealed interrelations of chlorophyll accumulation with other laboratory and field indicators. For plant height in the laboratory and field test the values were respectively $r = 0.65$ and $r = 0.89$; for the number of leaves $r = 0.36$ and $r = 0.25$; for leaf area $r = 0.35$ in the laboratory test (in the field correlation was weak). Positive correlations were found between the SPAD 502 measurements and plants survival rate in the budding phase ($r = 0.22$) and early yellow ripeness ($r = 0.24$).

The data we obtained are consistent with the concept that chlorophyll content in leaves is a significant parameter of plant physiological status [46, 47]. The chlorophyll content per unit of leaf area (chlorophyll density) [48] is an informative indicator of photosynthetic activity, growth and development of many crops. SPAD 502 readings are considered convenient criteria to estimate photosynthesis process with regard to changes caused by environmental factor, which allows selection of genotypes that adapt to stress [49-51].

According to the results of variance analysis, differences in factors were found in terms of the proportion of influence on the content of chlorophyll in the leaves in the total phenotypic variability. The accumulation of chlorophyll was primarily determined by the conditions of growing flax plants (46.2%), as well as the interaction of this factor with the genotype (34.4%). Genotypic differences were 16.6%. The simultaneous effect of other factors on the trait was insignificant.

So, by laboratory and field tests, population and individual traits crucial for flax plant adaptation to new agro-ecological conditions are identified. Testing of seeds shows that morphological traits of seedlings and young plants, the indices of root and shoot development, the structure of biomass can be informative criteria for biological state of varieties, along with traditional indicators of germinative energy and laboratory germination rate. The patterns of variability of some traits (shoot length, plant biomass, chlorophyll content) detected in flax samples at the initial stages of ontogenesis are confirmed in field trials, which gives grounds for selecting genotypes with useful traits in simulated laboratory conditions. In the field, the field germination rate and plant survival during the growing season can be used to identify flax varieties resistant to hydrothermal stress, since these indicators sufficiently characterize a number of interrelated ontogenetic processes and reflect the plant response to environmental factors. Assessment of genotypes in the field trial is based on a number of traits: plant height, linear dimensions, leaf area and number. When selecting flax genotypes, it is convenient to use the SPAD 502 optical chlorophyll counter, which allows breeder significantly reduce the time of evaluation without losing objectivity. The relationships between the measured indicators (SPAD units), morphological features and plant survival at different stages of ontogenesis are revealed. By a comprehensive study, Grant, Mayak (Belarus), Bertelsdorfer (Germany), Svalof (Czech Republic), Ottava 770 B See (Canada) varieties combining high adaptive and productive properties are suggested as a starting material in breeding genetic programs.

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