

RELATIONSHIP BETWEEN GENETIC DIVERSITY AND COLD-TOLERANCE OF MAIZE INBRED LINES

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Early maize sowing enables longer growing season with enhanced possibility of achieving higher and more stable yields, and better chances of avoiding summer droughts. For early sowing, cold-tolerant maize genotypes should be used. Breeding maize, tolerant to low temperatures, requires knowledge of genetic diversity and heterotic patterns of breeding material. The objective of this study was to determine genetic diversity of 15 ZP maize inbred lines applying the method of protein markers (UTLIEF method), and to establish correspondence between thus obtained classification with the results of cold test (CT) and field emergence (FE). During two production seasons (2011 and 2014), 15 maize inbred lines were self-pollinated. Pedigree data showed that material belongs to different maturity and heterotic groups. Cold tolerance was assessed in laboratory (2015) by cold test (7.5 °C, 10 days), and field trials on two locations during two successive years (2015, 2016). ZP maize inbred lines showed very good response to stressful conditions of CT and FE. Inbred lines with Lancaster background were more sensitive to low temperatures than inbred lines with BSSS and Iowa Dent background. Based on UTLIEF method two inbred lines with Lancaster background (ZPL 5 and ZPL 7) were grouped by cluster analysis together with Iowa Dent inbred lines, that also expressed better cold tolerance, and thus exceptional consent was achieved with the results of CT and FE. Classification of maize inbred lines based on UTLIEF method, followed by cluster analysis and PCA, showed good agreement with pedigree data, which points out that this method could be successfully applied for genetic classification of breeding material of a wide genetic background.

Keywords: maize, cold-tolerance, genetic diversity, UTLIEF

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INTRODUCTION

Low soil temperature during germination is a major limiting factor in growing plants of tropical origin in regions with moderate climate. Therefore, the prediction of field emergence and early plant growth is of extreme importance for seed production. Sowing maize earlier in the spring provides crops with a longer growing season and avoidance of summer droughts, especially characteristic in regions with cold and wet springs and extremely dry and hot summers (KUCHARIK, 2006). Moreover, this way crops are able to produce more stable and higher yields. There are several reports identifying maize genotypes appropriate for cultivation under cold conditions or early field sowing (ADETIMIRIN *et al.*, 2006; RODRÍGUEZ *et al.*, 2010).

Numerous maize breeding programs were oriented towards improvement of cold tolerance in the past (PEŠEV, 1970). Recently, this issue is raising attention of breeders again (DARKÓ *et al.*, 2011; STRIGENS *et al.*, 2012; REVILLA *et al.*, 2014). For early sowing, cold-tolerant maize genotypes should be used. Breeding of this material requires broadening the gene pool with the material able to germinate and grow vigorously under stressful conditions. Several studies indicated that maize originating from European Flint germplasm performed better cold tolerance compared to the Corn Belt Dent germplasm (STRIGENS *et al.*, 2013; FRASCAROLI and LANDI, 2013; REVILLA *et al.*, 2016). REVILLA *et al.* (2016) explained the significance of creation of synthetic population based on inbreds with favorable alleles of significant QTL for cold tolerance. They proposed that this could be the start material in phenotypic or genomic selection programs with the aim to develop lines with improved cold tolerance.

Maize breeding programs depend on the understanding and knowledge of genetic diversity and relationship among inbred lines and breeding material. This is crucial in terms of the yield, as the major objective in all breeding programs as well as in all other targeted traits. Exploitation of the effect of heterosis in maize more than in any other crop contributed to the significant yield increase (LAUDE and CARENA, 2015). Therefore, the identification of heterotic groups and heterotic patterns in maize is crucial to the success of maize breeding programs (BADU-APRAKU *et al.*, 2016). There are several approaches in assessing genetic diversity between breeding material (i.e. inbred lines, hybrids, populations, landraces and races), which include analysis of pedigree, morphological and heterotic data (BADU-APRAKU *et al.*, 2013), or molecular data, such as protein and DNA markers (BALESTRE *et al.*, 2008; BADU-APRAKU *et al.*, 2016). Although the development of molecular markers and QTL had significant impact on maize breeding, there are just few reports with poor relevance on maize breeding for cold tolerance (LEIPNER *et al.*, 2008).

Protein polymorphism among different plant genotypes is frequently used as biochemical markers. Protein complexes from different tissues, as specific gene products could indicate the genetic specificity of tested plant material, and therefore could be used as markers for characterization, identification and comparison of hybrids and inbred lines, as well as for seed genetic purity testing (MLADENović DRINIĆ *et al.*, 2002; IQUBAL *et al.*, 2014; STEVANOVIĆ *et al.*, 2016). Seed storage proteins were used as biochemical markers for identification of many plant species (NOLI, 2004; ANJALI and SANJAY, 2012; AKBAR *et al.*, 2012). Ultrathin-layer isoelectric focusing (UTLIEF), applied in this research, is known as fast, cheap and reliable technique for seed protein separation. UTLIEF method for variety identification and genetic purity testing has been standardized by ISTA for species such as maize and sunflower (ISTA, 2015).

The objective of this study was to evaluate cold tolerance of 15 ZP maize inbred lines, to assess genetic diversity of selected material based on UTLIEF method, and to obtain classification

of maize inbred lines to heterotic groups. Additional objective was to establish correspondence between thus obtained classification with the results of cold test (CT) and field emergence (FE).

MATERIALS AND METHODS

Seed material

For this study 15 maize inbred lines developed in Maize Research Institute Zemun Polje were selected. Their pedigree data indicated that material belongs to different maturity and heterotic groups, from FAO 300 to FAO 700, and possess different kernel types (Table 1). All the selected inbred lines are components of commercial ZP hybrids. Seed was obtained by self-pollination in two production years (2011 and 2014), at the nursery field in Zemun Polje. The harvest was performed manually and seed was hand-husked, sieved through 5 mm sieve, and dried until optimal seed moisture between 11 and 13%. Until testing seed produced in 2011 was kept in laboratory storage room where storage conditions were 18°C and 60% RH. Seed produced in 2014 was kept in cold chamber at 5°C and 60% RH. Cold test was performed in November/December 2015.

Table 1. List of maize inbred lines, their pedigree, maturity group and kernel type

Inbred line	Pedigree	Maturity group	Kernel type
ZPL 1	Iowa Dent	350	Dent
ZPL 2	Iowa Dent	400	Dent
ZPL 3	Lancaster	350-400	Dent
ZPL 4	Iowa Dent x unrelated germplasm	400	Dent
ZPL 5	Lancaster	450	Dent
ZPL 6	Lancaster	450	Dent
ZPL 7	Lancaster	550	Semident
ZPL 8	BSSS x Iowa Dent	350	Semident
ZPL 9	BSSS x unrelated germplasm	700	Dent
ZPL 10	BSSS x exotic germplasm	600	Semident
ZPL 11	BSSS x Iowa Dent	400	Dent
ZPL 12	BSSS	600	Dent
ZPL 13	BSSS	700	Dent
ZPL 14	BSSS	700	Dent
ZPL 15	BSSS	700	Dent

Cold test (CT)

The cold test method in rolled paper towels described in handbooks for seed vigour testing (HAMPTON and TEKRONY, 1995; AOSA, 2009) was used. Cold tolerance was analysed by CT with sand (NIJENSTEIN and KRUSE, 2000). Untreated seeds of 15 maize inbred lines were exposed to cold temperatures, 7.5°C for 10 days in rolled paper towels with moist sand and then exposed to optimal conditions 20/30°C temperature, with day/night period lasting 8h/16h. Evaluation of normal seedlings was done after 7 days of germination.

Field emergence (FE)

Cold tolerance was also tested in the field by early sowing at two sites Zemun Polje (chernozem) and Umka (brown forest soil), during two successive years (2015 and 2016). Preparation of the soil was carried out by conventional methods. Sowing was performed manually on both locations, at the beginning of April – 4th April 2015 and 7th April 2016 at Zemun Polje; 2nd April 2015 and 4th April 2016 at Umka. Each genotype was sown in two replications/rows with 100 seeds per replication. Field emergence was recorded every 3-4 days until complete emergence was reached. During germination and seedling emergence soil min and max temperatures were recorded at the depth of 5 cm, as well as the amount of precipitation.

Statistical analysis

Results of CT and FE were observed in percentages, so they were transformed by angular transformation $\arcsin \sqrt{\%}$. Data were then processed by the two-way analysis of variance (ANOVA) for CT and four-way ANOVA for FE. The significance of differences between mean values was tested by LSD test.

Genetic similarity

Genetic diversity of inbred lines was analyzed by Ultrathin Layer Isoelectric Focusing method (UTLIEF), according to the section 8.8.5 of the International Rules for seed testing (ISTA, 2015). Water soluble proteins isolated from 100 individually ground seeds of each line, were separated in the pH gradient of ultrathin polyacrylamide gels, according to their isoelectric point (pI). Presence and absence of bands was recorded and presented as binary data (1, 0). This data were used as matrix for subsequent statistical analyses. Genetic similarity between inbred lines was calculated using Simple matching coefficient (SM), (SOKAL and MICHENER, 1958). Similarity matrices were subjected for cluster analysis applying UPGMA method in SAHN program. Genotype grouping was also done employing Principal Component Analysis (PCA) in EIGEN program. Multivariate analyses (cluster and PCA) were done in NTSYS-pc software.

RESULTS AND DISCUSSION

The application of cold test provided screening of ZP maize inbred lines in terms of low temperature tolerance in early growth stages. Analyzed factors i.e. genotype (G) and the year of seed production (YSP) had significant impact on percentage of seed germination in both CT and FE (Table 2). The two-way ANOVA showed significant influence of the interaction of G x YSP on the results of CT. Variations in meteorological factors during field germination and emergence of seedling in two observed years, contributed to the significant variations in the total number and the percentage of emerged plants. On the other hand, no variation was observed considering locations involved in this trial. Two interactions also had significant influence on the FE, i.e. L x Y and YSP x G (Table 2). Results of this research are in agreement with PEŠEV (1970), who also stated that the tolerance to low temperatures during germination and emergence are influenced by genetic constitution, the amount and type of seed coat injury, seed age and seed maturity as well as, the occurrence of frost before the seed is harvested.

Seed produced in 2014 had average estimate in CT 90.67%, while the older seed, produced in 2011, had lower average estimate of 82.07% (Table 3). The four year storage of seed at 18°C, contributed to the significant decline in seed vigour, comparing to the vigour of the seed

kept one year at 5 °C. Observed results point to the very good response of ZP maize inbred lines to the extremely stressful conditions in CT (7.5°C, 10 days).

Table 2. Analysis of variance of investigated factors for cold test (CT) and field emergence (FE)

Source of variation	df		Mean squares	
	CT	FE	CT	FE
Replicates	1	1	0.01 ^{ns}	28.11 ^{ns}
Year of seed production (YSP)	1	1	657.76**	4154.75**
Genotype (G)	14	14	353.38**	418.31**
YSP x G	14	14	396.91**	452.89**
Location (L)		1		67.40 ^{ns}
Year of sowing (Y)		1		523.69**
L x Y		1		202.99*
L x YSP		1		12.85 ^{ns}
Y x YSP		1		38.32 ^{ns}
L x Y x YSP		1		130.83 ^{ns}
L x G		14		22.78 ^{ns}
Y x G		14		40.70 ^{ns}
L x Y x G		14		30.74 ^{ns}
L x YSP x G		14		84.69 ^{ns}
Y x YSP x G		14		36.14 ^{ns}
L x Y x YSP x G		14		33.32 ^{ns}
Error	29	119	38.73	48.26
Coefficient of variation			8.71%	9.78%

ns, *, ** – non significant, significant at 0.05 and 0.01 probability level, respectively

Maize inbred lines differed significantly in terms of tolerance to low temperatures during germination and early growth. Inbred line ZPL 4 achieved highest estimates in CT (98% and 100%), for seed produced in both years (Table 3). Inbreds ZPL 3 and ZPL 6 showed susceptibility to cold stress in early growth stages, which was expressed only after prolonged storage. Seed of those two inbred lines produced in 2011 and stored four years had the lowest percentage of normal seedlings in CT (27% and 17%, respectively). Interestingly, when the seed produced in 2014 was engaged in CT, percentage of germination and emergence was very high (96%) for both inbreds. This leads to conclusion that better estimate of tolerance to cold can be obtained by testing both newly produced and aged seed of maize inbred lines.

In this research applied temperatures in cold test were even lower than the soil temperatures in field trials, which led to the better discrimination of involved inbred lines in CT. On the contrary, some researchers found that results of CT overestimated FE in most seed lots (NOLI *et al.*, 2008).

Table 3. Average estimates of germination in cold test (CT) and field emergence (FE) of ZP maize inbred lines

Line	CT (%)			FE (%)		
	2011	2014	Average	2011	2014	Average
ZPL 1	93	98	95.50 ^{abc}	80	95	87.38 ^{bc}
ZPL 2	93	94	93.50 ^{bcd}	77	83	79.75 ^{de}
ZPL 3	27	96	61.50 ^{gh}	64	98	81.00 ^c
ZPL 4	98	100	99.00 ^a	84	95	89.38 ^{bc}
ZPL 5	85	88	86.50 ^{ef}	83	88	85.13 ^{cd}
ZPL 6	17	96	56.50 ^h	69	83	76.00 ^e
ZPL 7	94	94	94.00 ^{bcd}	89	96	92.38 ^{ab}
ZPL 8	77	90	83.50 ^f	83	95	88.63 ^{bc}
ZPL 9	89	82	85.50 ^{ef}	94	73	83.38 ^{cd}
ZPL 10	88	70	79.00 ^{fg}	93	93	93.00 ^{ab}
ZPL 11	90	86	88.00 ^{def}	87	93	89.75 ^{bc}
ZPL 12	97	92	94.50 ^{bcd}	96	97	96.50 ^a
ZPL 13	97	92	94.50 ^{bcd}	92	97	94.50 ^a
ZPL 14	94	98	96.00 ^{ab}	75	97	85.88 ^{bc}
ZPL 15	92	84	88.00 ^{def}	81	90	85.13 ^{cd}
Average	82.07	90.67	86.37	82.90	91.47	87.18

Estimates marked with the same letter do not differ significantly at the 0.05 probability level based on LSD test

Seed produced in 2014 had higher average percentage in FE (91.47%), compared to the seed produced in 2011 (82.90%). The difference was even more pronounced in field trials in 2015 due to more stressful meteorological conditions. Namely, minimum soil temperatures in 2015 did not exceed 10 °C, at least for the first two weeks on the location Zemun Polje, while on the other location for 19 days after sowing the lowest soil temperature was below this point. In 2016, although the sowing was performed on 4th and 7th of April, period of germination and emergence was characterized by extremely warm soil and air temperatures. The temperatures below 7 °C, on both locations were recorded only in the last three days of the field trial, when the emergence of plants was almost complete. Lower soil temperatures and higher precipitation in 2015 during the period of germination and emergence contributed to the significant differences in rates of germination and final field stand of observed maize inbred lines.

Inbred line ZPL 12 had the highest average percentage of FE across locations and years (96.50%). This was noticed for the seed produced in both 2011 and 2014 (96% and 97%, respectively). Inbred lines with Lancaster background ZPL 3 and ZPL 6 had the poorest estimates of FE. This shows that CT is a valuable test which can reliably predict the response of maize inbred lines to the stressful field conditions. Such results are compatible with QUEIRO (2012) who found that tests such as cold germination, the thermogradient plate and coleoptile growth at chilling temperatures can be efficiently used to identify superior lines and hybrids with the ability to perform well under chilling stress. PEŠEV (1970) stated that the degree of tolerance to low temperature is strongly dependent on the germination potential of the maternal parent of a hybrid. Therefore, those two inbreds are not desirable as maternal components of hybrids. On the other

hand ZP maize inbred lines with BSSS and Iowa Dent background are potentially good sources of tolerance to stressful conditions during germination and early growth.

The other objective of this research was to establish concurrence in classification of inbred lines by CT/FE with the one obtained by UTLIEF method.

Total number of detected protein fractions was 16, out of which 14 were polymorphic (87.5%). The largest number of polymorphic bands was focused in a pH range from 5.8 to 10. Analysis of protein seeds using UTLIEF method showed uniform protein pattern for inbreds ZPL 3 and ZPL 6, while the other 13 inbred lines had specific protein profiles with unique band distribution.

Calculated genetic similarity was in a range from 0.44 to 1. The lowest similarity was detected between ZPL 2 and four inbreds (ZPL 9, ZPL 12, ZPL 14 and ZPL 15), and the highest between sister lines ZPL 3 and ZPL 6. The differentiation of material so genetically close was not possible by the method of protein markers, so more sophisticated molecular markers should be applied (MLADENOVIC DRINIC *et al.*, 2002; REVILLA *et al.*, 2016).

Matrices of genetic similarity were used for dendrogram construction using UPGMA method (Figure 1). Dendrogram consisted of two main groups (I and II). Group I encompassed five inbred lines. Genotypes with Iowa Dent background (ZPL 1 and ZPL 2) were classified in one cluster, Lancaster lines (ZPL 3 and ZPL 6) formed the other, while inbred ZPL 4 (Iowa dent x unrelated germplasm) clustered separately. Inbred ZPL 2 expressed very good laboratory results, while the results of FE were low and statistically not different from ZPL 3 and ZPL 6, also from this cluster branch.

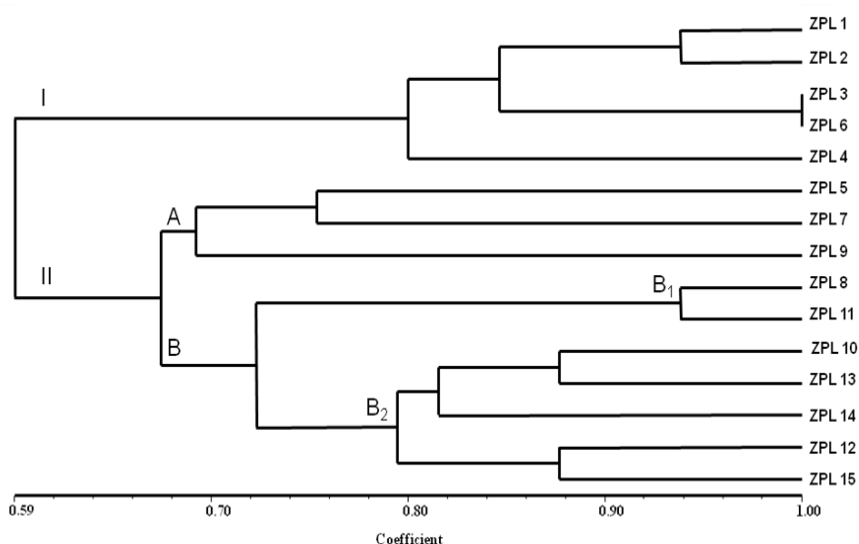


Figure 1. Cluster analysis of genetic similarity according to Simple Matching coefficient for 15 ZP maize inbred lines based on UTLIEF method

Group II comprised of two clusters - A and B. Cluster A consisted of three inbred lines: ZPL 5 and ZPL 7 (Lancaster) were classified together, while ZPL 9 (BSSS x unrelated germplasm) clustered separately. Subclusters B1 and B2 formed cluster B. Inbreds ZPL 8 and ZPL 11 (BSSS x Iowa dent) clustered in B1, while B2 encompassed five BSSS genotypes (ZPL 13, ZPL 14, ZPL 12 and ZPL 15) and ZPL 10 of BSSS x exotic germplasm.

Disagreement between pedigree data and results of cluster analysis was noticed only in the classification of inbreds ZPL 5 and ZPL 7. Those inbreds possess Lancaster background, but were grouped in Group II, together with inbreds with BSSS background. Such classification of those two inbred lines can be explained by the contribution of the other parent germplasm involved in the breeding process. Moreover, those two lines performed well in CT – i.e. presented as tolerant to low temperatures during germination and emergence, and accordingly showed similarity with inbreds of that part of cluster.

PCA results (Figure 2) were in accordance with cluster analysis. Congruence of those two methods was observed also by SRDIĆ *et al.* (2011). The first two components (PC1 and PC2) explained 37.76% and 17.05% of variation, respectively (in total 54.81% of variation). The identical grouping of four lines (ZPL 1, ZPL 2, ZPL 3 and ZPL 6) was presented both by PC and cluster analysis. According to PC1 the closest line to these four lines was ZPL 4, the same as in cluster analysis.

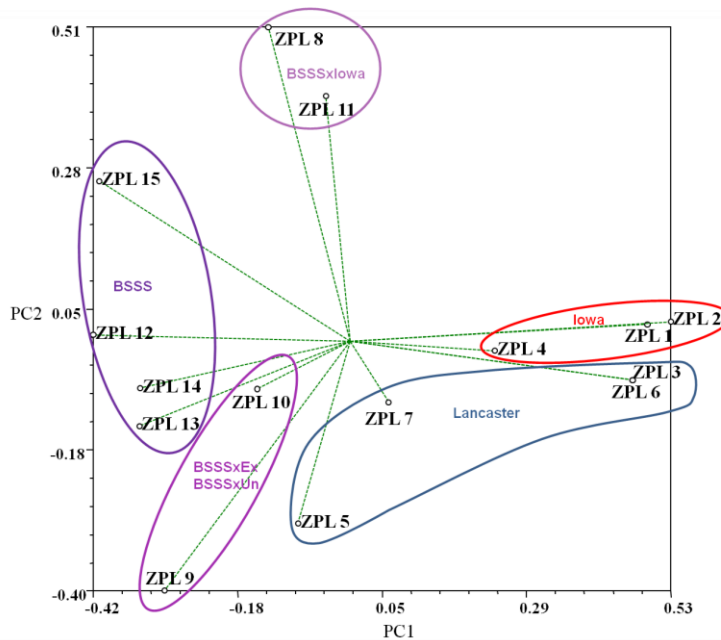


Figure 2. PCA of genetic similarity according to Simple Matching coefficient for 15 ZP maize inbred lines based on UTLIEF method –Ex – exotic, Un - unrelated

Results of PCA also showed very good correspondence with pedigree data. The differentiation of pedigree groups could be observed. Genotypes with BSSS x Iowa dent pedigree were placed between inbreds with BSSS and Iowa Dent background. Also, lines originated from BSSS and unknown or exotic germplasm were positioned close to each other, and next to BSSS.

The classification of maize inbred lines based on protein markers i.e. UTLIEF method, by cluster analysis and PCA showed very good concurrence with their pedigree data, indicating that this method could be successfully applied in characterization of breeding material of wide genetic background. This method proved its efficiency in studies of genetic polymorphism on inter and intra sorghum variety research (LIU *et al.*, 2010). On the other hand these markers should be used only for obtaining preliminary information, while for the more precise results DNA based markers should be used.

Based on good agreement between genetic similarity according to UTLIEF method and the results of vigour test, it can be concluded that both cold test and UTLIEF are valuable tools for screening inbred lines for cold-tolerance. The prediction of potentially perspective hybrid combinations could be successfully obtained by screening the material by UTLIEF method, while on the other hand the information of highly tolerant inbreds could be obtained by CT. Combining those methods provides necessary information in planning and prediction of breeding high yielding and cold tolerant maize hybrids.

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ODNOS IZMEĐU GENETIČKE DIVERGENTNOSTI I TOLERANTNOSTI NA NISKE TEMPERATURE SAMOOPLODNIH LINIJA KUKURUZA

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Izvod

Rana setva kukuruza omogućava duži vegetacioni period čime su obezbeđeni uslovi za veće i stabilnije prinose, kao i veću mogućnost izbegavanja letnje suše. Rana setva podrazumeva gajenje genotipova sa osobinama dobre klijavosti u stresnim uslovima. Oplemenjivanje kukuruza tolerantnog na niske temperature zahteva poznavanje genetičke divergentnosti i pripadnosti selekcionog materijala heterotičnim grupama. Cilj ovog rada bio je ispitivanje genetičke divergentnosti 15 ZP samooplodnih linija kukuruza primenom proteinskih markera i utvrđivanje saglasnosti postignute klasifikacije sa rezultatima vigor testa i nicanja u polju. Seme je dobijeno ručnom samooplodnjom 2011. i 2014. godine. Za ispitivanje je odabrano 15 samooplodnih linija kukuruza iz različitih heterotičnih grupa, stvorenih u Institutu za kukuruz “Zemun Polje”. Tolerantnost na niske temperature je testirana u laboratoriji (2015) hladnim testom (7.5 °C, 10 dana) i ranom setvom u polju na dva lokaliteta Zemun Polje (černoze) i Umka (lesivirana gajnjača) u toku dve godine (2015, 2016). Rezultati ukazuju na veoma dobru reakciju ZP samooplodnih linija na stresne uslove ispitivanja u hladnom testu i ogledima u polju. Samooplodne linije poreklom od Lancaster populacije bile su osetljivije na niske temperature od linija BSSS i Iowa Dent porekla. Primenom UTLIEF metode izvršena je identifikacija dve linije Lancaster porekla (ZPL 5 i ZPL 7) koje poseduju bolju tolerantnost na niske temperature i tako je postignuta izuzetna saglasnost sa rezultatima vigor testova. Klasifikacija samooplodnih linija na osnovu proteinskih markera putem klaster i PC analize pokazala je dobru saglasnost i sa pedigre podacima, što ukazuje da se UTLIEF metoda može uspešno koristiti za karakterizaciju materijala široke genetičke osnove.

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