# Assessing Genetic Variability For NUE in Maize Lines from Agricultural Institute Osijek

## Procjena genetičke varijabilnosti za učinkovitost korištenja dušika u inbred linijama kukuruza Poljoprivrednog instituta Osijek

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# ASSESSING GENETIC VARIABILITY FOR NUE IN THE MAIZE LINES FROM THE AGRICULTURAL INSTITUTE OSIJEK

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## SUMMARY

***Maize (Zea mays) is one of the most important crops in the world, with nitrogen (N) fertilization being the most expensive input in its production. Based on a favorable variability, production costs can be reduced by the means of selection for nitrogen use efficiency (NUE). This study aims to analyze the variability in the collection of maize lines in terms of nitrogen use efficiency (NUE) and to identify the loci associated with the yield and grain moisture. The Agricultural Institute Osijek diversity panel comprises of 1,346 maize inbred lines genes with 56,000 SNP (Single Nucleotide Polymorphism) markers. The experiment was carried out with 109 selected inbreds, based on their performance in the long–term trials. The selected inbreds were testcrossed to a common tester from the Iodent heterotic group. The experiments in three nitrogen environments were carried out in 2023: N0 (no nitrogen fertilization), N69 (low nitrogen supply), and Nfull (sufficient nitrogen supply). Association mapping revealed varying associations between the SNPs and the yield/moisture under different nitrogen treatments, highlighting the influence of nitrogen stress on genetic mechanisms governing these traits. The research contributes valuable insights into maize adaptability to nitrogen levels, aiding in the development of strategies for stable and high–yield maize production.***

***Keywords: maize, NUE, SNPs, nitrogen levels, genetic variability***

## INTRODUCTION

Agricultural production environments of Southeastern Europe are often the low–input ones, with a limited N supply (Zorić et al., 2016; Zorić et al., 2022). From the perspective of commercial maize breeding, favorable genetic responses of hybrids are needed in various environmental conditions (Bustos–Korts et al., 2019), with nitrogen availability often being a major constraint. The breeders aim to enrich the germplasm with favorable alleles, conferring tolerance to the low–N conditions by using a selection of progenies manifesting a higher Nitrogen Use Efficiency (NUE; DoVale et al., 2012). In addition to the reduction of production costs, increasing nitrogen utilization in maize also has a positive impact on the environment (Asibi et al., 2019). Nitrogen use efficiency represents the ratio of grain yield to the amount of applied nitrogen fertilizer (Moose and Below, 2009). The cultivars with a higher NUE should be a priority for breeders developing the new cultivars (Balyan et al., 2016; Mălinaş et al., 2022). Furthermore, considerable genetic variability (Agrama et al., 1999) facilitated practicing genomic selection for the NUE in maize (Tadesse Ertiro et al., 2020a).

Different marker densities and population types have been used in the reported studies. Dense genotyping implies a marker density that exceeds the expected number of recombinations in the population (Stange et al., 2013). However, the number of markers used for genomic predictions can improve prediction accuracy only up to the point of saturation of a genetic map, when all recombination events are captured by the markers (Combs and Bernardo, 2013). Genetic variability for many quantitative traits includes numerous loci with moderate effects and a large number of loci with small effects (Kearsey and Farquhar, 1998). The associated

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loci can be categorized as ‘constitutive’ (Collins et al., 2008; that is, the same QTLs are detected in different environments) or ‘adaptive’ (that is, QTLs are detected only in specific environments; Almeida et al., 2013). The use of genomic data in the predictive modeling of maize yield has already found widespread application in the industry (Bernardo, 2020) becoming a state–of–the–art methodology.

The aim of this research was to assess the variability for NUE (Nitrogen Use Efficiency) in the breeding germplasm collection of maize inbred lines from the Agricultural Institute Osijek at different levels of nitrogen application. Association mapping analysis was carried out to investigate the link between loci and the detected genetic variability.

## MATERIAL AND METHODS

### Genotyping and field trials

The Agricultural Institute Osijek maize diversity collection is comprised of 1,346 inbred lines genotyped over 10 years (2013 – 2023) with Illumina Infinium Maize SNP50 genotyping array (Ganal et al., 2011), yielding 56.109 single nucleotide polymorphisms (SNP). The average call rate for all SNPs was $> 98\%$. Genotyping was carried out at SGS Trait Genetics facilities (Gatersleben, Germany) from total DNA from inbred seeds according to the CTAB methodology (Porebski et al., 1997).

The 150 inbred lines were testcrossed to two testers of Iodent and B37 origin, resulting in 238 testcrossed hybrids in sufficient quantities. Experiments were carried out using the full set of hybrids (238) along with checks. For this research, the 109 B37/Ohio testcrosses were selected from the diversity collection based on their commercial performance and breeding relevance. For this research, the analysis was based on the hybrid seeds obtained by testcrossing the selected inbred lines to a common tester, Os 7215 line from the Iodent heterotic group. The field experiment was set up using an augmented design (Hallauer et al., 2010), where the field was divided into blocks (field rows), each containing at least two hybrids with replications. Field rows contained 54 plots, 50 of which were experimental plots, along with two border plots at each side of the block. The blocking factor was field–row, while the experimental blocks of size 400 were the separate nitrogen treatments. The average number of replications per environment was 1.39. The choice of hybrids for replication was based–on seed availability.

The experiment was set up with three nitrogen treatments: N0 – no nitrogen fertilization in the current season, 0 kg N/ha; N69 – low nitrogen supply, adding nitrogen pre–sowing, without topdressing, 69 kg N/ha added; Nfull – a sufficient supply of 136 kg N/ha added. Adequate supply follows the Regulation on Good Agricultural Practice in the Use of Fertilizers, known as the Nitrogen Directive. Each treatment was assigned a block of 400 plots, each sized 7 $m^2$, with a protective strip of 6 m at the end to prevent treatment overlap between treatments, and a protective strip of two plots on each side. Each nitrogen treatment was considered a separate environment. Planting was done by machine and the planting density was 75 000 plants $ha^{-1}$ designed for experimental planting at the Agricultural Institute Osijek at a fixed depth of 5 cm on April 22, 2023. Cultivation and topdressing (in relevant N environments) were performed at the 5–8 leaf stage, and weed protection was carried out with a tractor sprayer. Harvesting was done mechanically with a Wintersteiger experimental plot harvester at the Agricultural Institute Osijek on 7 October 2023, measuring the grain weight and moisture from each plot. Grain yields were calculated on 14% moisture basis. The weather during conditions from May to August were mostly normal ([25,75] percentiles), according to the Croatian Meteorological and Hydrological Service, except of May (rainy, [75,91] percentile) and August (warm, [75,91] percentile).

### Data preparation and analysis

Genotypic data were filtered for missingness (2.5%) and heterozygosity (2.5%) and imputed using a LinkImpute method (Money et al., 2015) with 30 nearest neighbors. Positions that were not mapped to any of the 10 chromosomes were also filtered. There was a total of 48.734 markers used for further analyses.

Field trial data was analyzed using a restricted maximum likelihood (REML) based mixed–modeling approach (Bates et al., 2014) with the assumption of homogenous variance. The model used to extract variance components was where was genotypic random effect, was nitrogen environment fixed effect, was random interaction term, was a fixed replicate term, and was overall model error. Heritability was estimated on a progeny mean basis following Hallauer et al. (2010). All analyses were carried out in R.

Association mapping was carried out using phenotypic mean values. Inputs for mixed linear model were phenotypic values, Q–matrix, from principal coordinate analysis (PCoA) with six assumed axes, identity–by–descent kinship matrix (K) and marker data, named MLM+Q+K. (Yu et al., 2006). Calculation of Q and K matrices and association mapping were carried out in Tassel software (Buckler et al., 2007) version 5. An arbitrary threshold of $p < 0.0001$ ($-\log(p)>4$) was considered used to assess the significance of loci effects. Genes within 100 Kbp from the associated loci were counted in the *MaizeGDB GBrowser* tool (Zeng et. al., 2022).

## RESULTS AND DISCUSSION

Yields were the lowest in the N0 treatment at 5.74 t/ha (Table 1), and the highest in the Nfull treatment at 6.25 t/ha. Grain moisture ranged from 16.21% in the N0 treatment to 15.65% in the Nfull treatment, with heritability $H^2=0.544$ for yield and 0.708 for moisture.

**Table 1. The means of treatments ± standard deviations for the grain yield and moisture. The differences were significant in a two–way ANOVA at $p< 0.05$ threshold.**

*Tablica 1. Prosječne vrijednosti tretmana ± standardne devijacije za prinos zrna i vlažnost. Razlike su bile značajne u dvosmjernoj ANOVI na pragu $p<0,05$.*

| **Treatment** / *Tretman* | **Yield (t ha$^{-1}$)** / *Prinos (t ha$^{-1}$)* | **Moisture (%)** / *Vlaga(%)* |
|---|---|---|
| N | $5.74\pm1.59^{b}$ | $16.21\pm2.18^{a}$ |
| N69 | $6.01\pm2.16^{ab}$ | $15.69\pm2.72^{b}$ |
| NFull | $6.25\pm2.12^{a}$ | $15.65\pm3.16^{b}$ |
| ***Variance components$^{2}$*** / *Komponente varijance* | | |
| $\sigma_G$ | 0.845 | 2.7737 |
| $\sigma_E$ | 0.046 | 0.06962 |
| $\sigma_{GEI}$ | <0.01 | <0.01 |
| $\sigma_\varepsilon$ | 2.95 | 4.76773 |
| $H^2$ | 0.544 | 0.708 |

[1] Different letters denote significant differences at $\alpha=0.05$ level according to the Fisher's LSD test / Različita slova označavaju značajne razlike na razini $\alpha=0,05$ prema Fisherovu LSD testu.

[2] Variance components were calculated in a mixed linear model / Komponente varijance su izračunane u mješovitome linearnom modelu

The correlations between the treatments for the yield were significant at the $p < 0.05$ level, with a correlation of 0.293 between N0 and Nfull, 0.491 between N0 and N69, and 0.445 between N69 and Nfull. Similarly, the correlations between treatments for grain moisture were significant at $p < 0.05$, with values of 0.585 between N0 and Nfull, 0.457 between N0 and N69, and 0.467 between N69 and Nfull. The genotype × environment interaction (GEI) was very low.

The diagram (Figure 1) shows the association mapping results between SNPs and yield under different nitrogen treatments. In the N0 treatment (Figure 1a), no loci exceed $-\log(p) > 4$. In the N69 treatment (Figure 1b), there were two loci on chromosome 4 at positions 17,437,105 with 4.08 –log (p) values and 125,521,442 with 4.03 –log (p) values. While in the Nfull treatment (Figure 1c), there were four loci associated with yield. Two loci were detected on chromosome 4 at positions 181,258,688 with 4.08 –log (p) values and 181,263,249 with 4.05 –log (p) values (Table 2). Two loci on chromosome 5 at positions 204,572,992 and 204,573,951 with 4.12 –log (p) values were detected. In the nitrogen–stressed environment, a smaller number of loci influencing yield were included because the genetic mechanisms affecting yield are different under optimal nitrogen levels and stressful conditions with low nitrogen levels (Bänziger et al., 1997; Tadesse et al., 2020b).

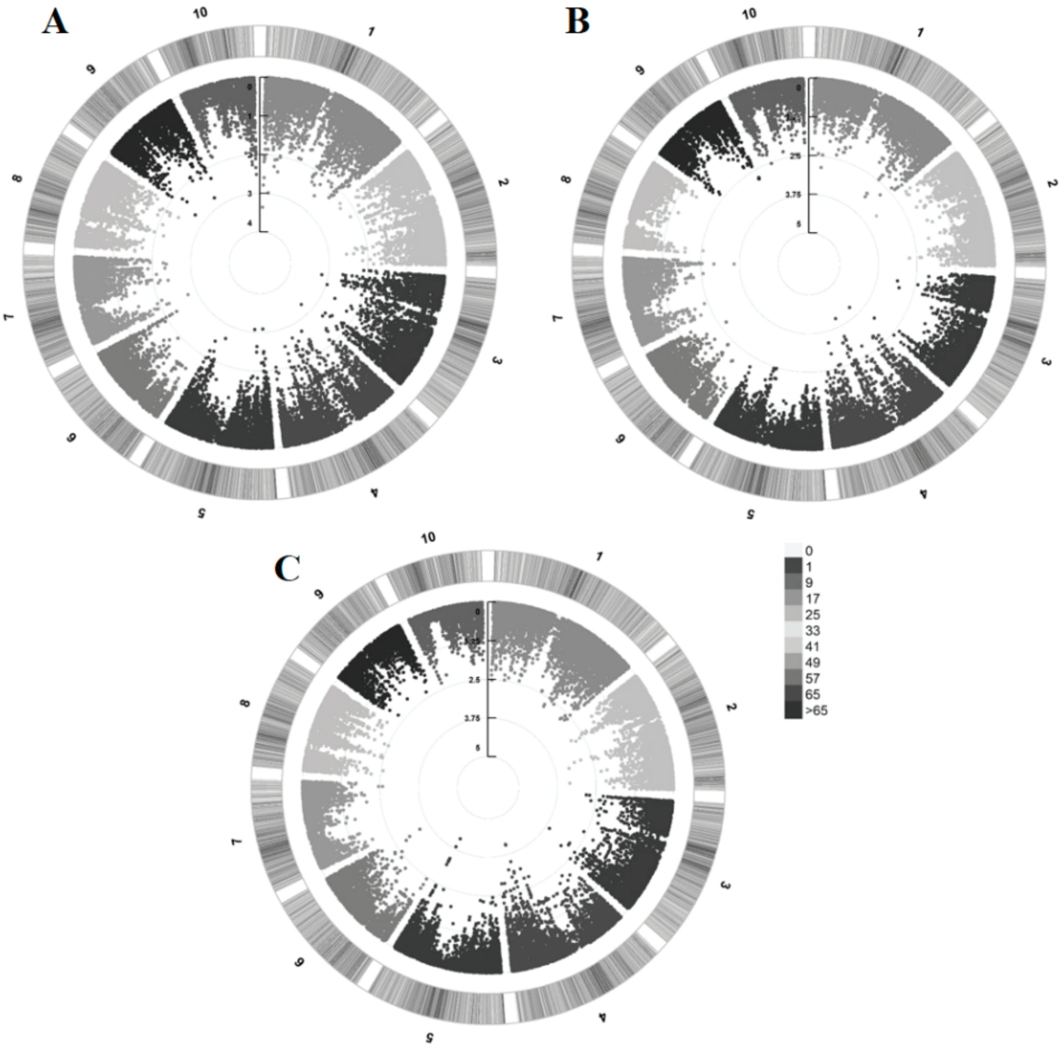

**Figure 1. The Circular Manhattan diagram depicts the association mapping between SNPs and yield using a circular arrangement of data on the diagram. Chromosomes 1–10 are depicted along the circular edge, and genetic markers and their associations influencing yield are represented as –log (p) values in: (a) N0 kg, (b) N69 kg, (c) NFull**

*Grafikon 1. Kružni manhattanski dijagram prikazuje asocijativno mapiranje između SNP–ova i prinosa koristeći kružno raspoređene podatke na dijagramu. Kromosomi 1–10 prikazani su duž kružnog ruba, a genetski markeri i njihove asocijacije koje utječu na prinos predstavljeni su kao –log (p) vrijednosti u: (a) N0 kg, (b) N69 kg, (c) NFull.*

Manhattan plot (Figure 2) shows the association mapping between single nucleotide polymorphisms (SNPs) and grain moisture under different nitrogen treatments. In the N0 treatment (Figure 2a), 8 loci associated with soil moisture were identified with significance values of –log (p) less than 4 (Table 2). These include one locus on chromosome 1 at position 287,655,675 and one locus on chromosome 10 at position 9,591,402 with –log (p) values of 7.72. Additionally, two loci on chromosome 7 at positions 3,670,125 and 3,672,327 with –log (p) values of 5.41, and two loci on chromosome 2 at positions 3,191,417 and 3,585,427 with –log (p) values of 4.55 were identified. Furthermore, two additional loci were identified on chromosome 9 at positions 134,975,544 with an –log (p) value of 4.41, and 135,602,225 with an –log (p) value of 4.13.

In the N69 treatment (Figure 2b), 4 loci were observed, with two on chromosome 5 at positions 14,523,812 with an –log (p) value of 5.42 and 14,214,336 with an –log (p) value of 4.62. One locus was found on chromosome 8 at position 11,503,503 with an –log (p) value of 5.42 and on chromosome 1 at position 15,170,790 with an –log (p) value of 4.46.

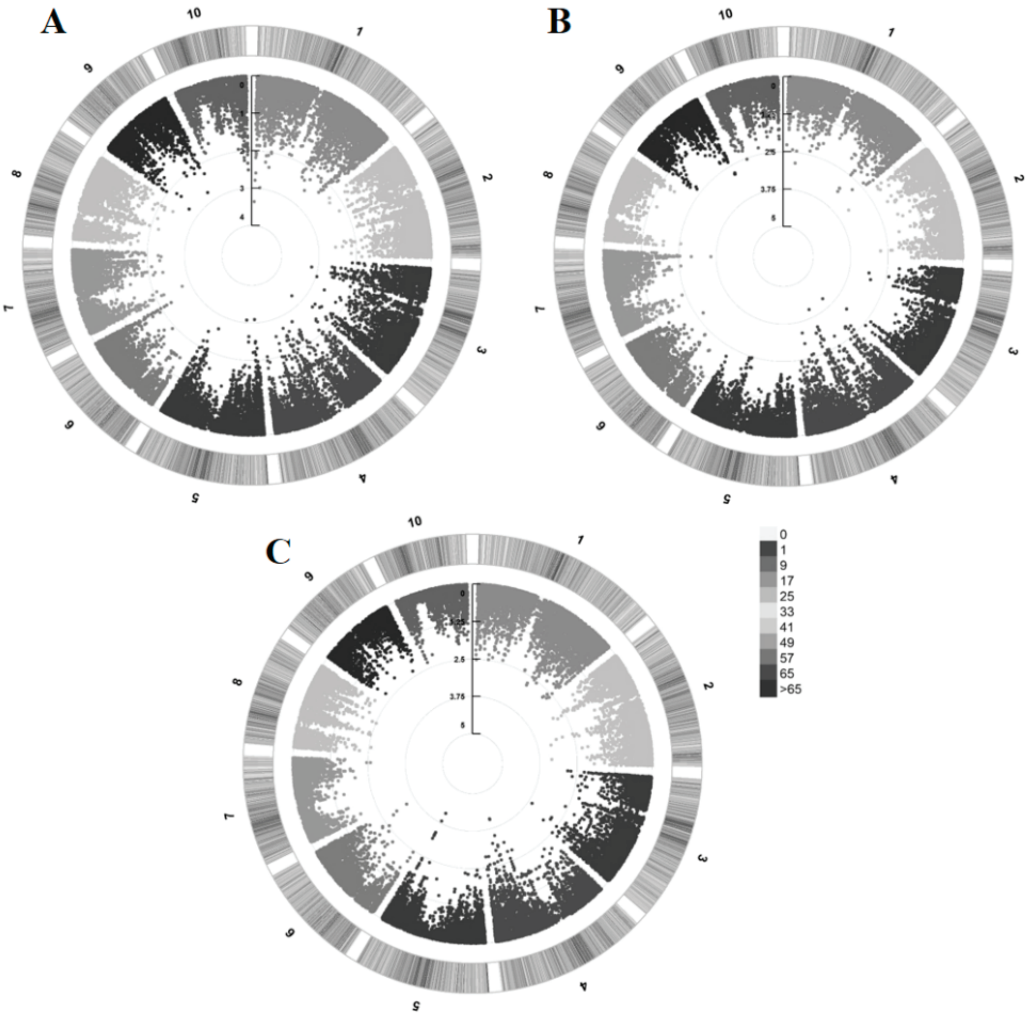

**Figure 2. The Circular Manhattan diagram depicts the association mapping between SNPs and grain moisture using a circular arrangement of data on the diagram. Chromosomes 1–10 are depicted along the circular edge, and genetic markers and their associations influencing moisture are represented as –log (p) values in: (a) N0 kg, (b) N69 kg, (c) NFull**

*Grafikon 2. Kružni manhattanski dijagram prikazuje mapiranje asocijacija između SNP–ova i vlažnosti zrna koristeći kružno raspoređene podatke na dijagramu. Kromosomi 1–10 su prikazani duž kružnog ruba, a genetski markeri i njihove asocijacije koje utječu na vlažnost predstavljene su kao –log (p) vrijednosti u: (a) N0 kg, (b) N69 kg, (c) NFull.*

In the NFull treatment, only one locus on chromosome 5 at position 4,777,817 with an –log (p) value of 4.01 was included. Under low nitrogen stress, a tolerant genotype exhibits a higher expression of genes compared to intolerant genotypes (Singh et al., 2022). The presence of multiple significant loci in the N0 treatment can be explained by a moderate positive correlation between the N0 and Nfull treatments. This correlation suggests that some genes or genetic factors that were more expressed or activated in the N0 treatment also had some association with phenotypic characteristics in the Nfull treatment. However, it is important to note that the same loci did not have the same level of expression or impact across different treatments. In other words, although some genes may have been more significant or active in the N0 treatment, it does not necessarily mean that they will have the same level of expression or impact in other treatments such as Nfull. This suggests that specific treatment conditions could have different interactions with genes or genetic factors, because genes can also be switched on and off in response to environmental stimuli (Bernardo, 2010). Resulting in variation in the expression or impact of loci on phenotypic characteristics.

**Table 2. A list of locus names and positions on the chromosome under various treatments of N for the yield and moisture traits at –log (p) > 4 values.**

*Tablica 2. Popis imena lokusa i njihovih pozicija na kromosomu pod različitim tretmanima N za osobine prinosa i vlažnosti pri –log (p) vrijednostima >4.*

| Treat. | Trait | Marker | Chr. | Position | -log (p) | Candidate gene |
|---|---|---|---|---|---|---|
| **N0** | Moisture / *vlaga* | PZE-101240610 | 1 | 287655675 | 7.72 | Zm00001d034240 |
| | | PZE-110011078 | 10 | 9591402 | 7.72 | Zm00001d023542 |
| | | PZE-107005432 | 7 | 3670125 | 5.41 | LOC542352 |
| | | PZE-107005445 | 7 | 3672327 | 5.41 | Zm00001d018738 |
| | | PZE-102006073 | 2 | 3191417 | 4.55 | Zm00001d001939 |
| | | SYN2674 | 2 | 3585427 | 4.55 | Zm00001d001962 |
| | | PZE-109086221 | 9 | 134975544 | 4.41 | Zm00001d047549 |
| | | SYN30020 | 9 | 135602225 | 4.13 | Zm00001d047560 |
| **N69** | Moisture / *vlaga* | PZE-105028002 | 5 | 14523812 | 5.42 | Zm00001d013573 |
| | | SYN37797 | 8 | 11503503 | 5.42 | Zm00001d008525 |
| | | PZE-105027793 | 5 | 14214336 | 4.62 | Zm00001d013555 |
| | | SYNGENTA12708 | 1 | 15170790 | 4.46 | Zm00001d027854 |
| | Yield / *prinos* | SYN30708 | 4 | 17437105 | 4.08 | LOC100502003 |
| | | SYN25513 | 4 | 125521442 | 4.03 | Zm00001d050837 |
| **Nfull** | Yield/ *prinos* | SYN1883 | 5 | 204572992 | 4.12 | Zm00001d017699 |
| | | SYN1873 | 5 | 204573951 | 4.12 | Zm00001d017700 |
| | | PZE-104104870 | 4 | 181258688 | 4.08 | Zm00001d052148 |
| | | PZE-104104913 | 4 | 181263249 | 4.05 | Zm00001d052152 |
| | Moisture / *vlaga* | PZE-105010938 | 5 | 4777817 | 4.01 | Zm00001d013109 |

## CONCLUSION

Based on the results of our study, we can conclude that there is a significant variability in the maize germplasm collection from the Agricultural Institute Osijek concerning nitrogen use efficiency at different levels of nitrogen application. Furthermore, mapping analysis using a set of maize inbred lines has demonstrated that the number of associated loci for yield and grain moisture varies at different levels of applied nitrogen per hectare. The moderate correlations between treatments were significant, indicating a connection between applied nitrogen levels and yield components, as well as grain moisture (except NFull vs. N0 for yield). Mapping associations between the SNPs and yield, as well as grain moisture, under different nitrogen treatments, have revealed different patterns of associations depending on nitrogen levels. In the treatments with lower nitrogen levels, a smaller number of significant loci were identified, suggesting different genetic mechanisms influencing yield and grain moisture under optimal and stressful conditions with low nitrogen levels. These results provide important insights into the genetic basis of maize adaptability to different nitrogen levels and emphasize the importance of considering genotype-environment interactions in breeding to achieve stable and high maize production under various growing conditions. It is important to note that this paper presents single–year results with GxE embedded in the environmental effect.

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# PROCJENA GENETIČKE VARIJABILNOSTI ZA UČINKOVITOST KORIŠTENJA DUŠIKA U INBRED LINIJAMA KUKURUZA POLJOPRIVREDNOGA INSTITUTA OSIJEK

## *SAŽETAK*

***Kukuruz (Zea mays) jedna je od najvažnijih kultura u svijetu, pri čemu je gnojidba dušikom najskuplji trošak proizvodnje. Na osnovi povoljne varijabilnosti, troškovi proizvodnje mogu se smanjiti selekcijom glede efikasnosti korištenja dušika (NUE). Ovo istraživanje ima za cilj analizirati varijabilnost u kolekciji linija germplazme kukuruza u pogledu efikasnosti korištenja dušika (NUE) i identificirati lokuse povezane s prinosom i vlagom zrna. Poljoprivredni institut Osijek u okviru svoje oplemenjivačke germplazme sadrži 1346*** **inbred** ***linija kukuruza genotipiziranih s 56 000 SNP (oznaka kratice na hrvatskome i na engleskom) markera. Eksperiment je proveden s odabranih 109 linija na temelju njihovih rezultata u dugogodišnjim ispitivanjima. Odabrane linije testirane su zajedničkim testerom heterotične skupine Iodent. Ispitivanja su provedena 2023. godine i uključivala su tri scenarija opskrbljenosti dušikom: N0 (bez gnojidbe dušikom), N69 (niska opskrba dušikom) i Nfull (dovoljna opskrba dušikom). Asocijativno mapiranje otkrilo je različite asocijacije između pojedinačnih polimorfizama nukleotida i prinosa/vlage pod različitim uvjetima dušika, ističući utjecaj dušičnoga stresa na genetičke mehanizme koji upravljaju tim karakteristikama. Istraživanje pruža vrijedne uvide u prilagodljivost kukuruza razinama dušika, što pomaže u razvoju strategija za stabilnu i visokoprinosnu proizvodnju kukuruza.***

***Ključne riječi: kukuruz, NUE, SNP, tretmani dušikom, genetička varijabilnost***

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