UDC 575. 633.15
https://doi.org/10.2298/GENSR2103147B
Original scientific article

PHYSIOLOGICAL AND GENE EXPRESSION CHANGES DURING IMBIBITION IN MAIZE SEEDS UNDER LOW TEMPERATURE CONDITIONS

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Božić M., A. Stanojević, K.Marković, D. Ignjatović Micić, D.Nikolić, M. Milivojević, A. Nikolić (2021). *Physiological and gene expression changes during imbibition in maize seeds under low temperature conditions*. - Genetika, Vol 53, No.3, 1147-1165.

Maize is one of the most important crop species worldwide, but also extremely susceptible to the effects of increasingly higher temperatures and drought during the summer and its flowering and grain filling stage. Different strategies are being utilized to ensure a satisfying yield potential and quality even in the extremely unfavourable environmental conditions, which are the result of climate change. Some of them are cropping pattern changes and sowing alterations, including earlier sowing. Since this implies the exposure to suboptimal temperatures during early developmental stages, it leads to a demand for developing maize lines tolerant to low temperatures during these stages. This research focuses on the first phase of germination, imbibition.

Maize tolerance to low temperatures is a complex trait that includes different mechanisms and strategies that all work together to ensure adaptation and survival, such as cell membrane changes, antioxidative system activation, etc. Additionally, the low temperature response of different maize inbreds varies substantially, so recognizing the different ways in which they respond to low temperatures during imbibition and other earlier stages of development is crucial. To accomplish this, seeds of two maize inbred lines of contrasting susceptibility to low temperatures were selected and exposed to control (20° C) and low temperature (8°C) conditions during the first 24h of imbition and

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then further analyzed to assess their response. This included germination and tetrazolium assays; ascertaining membrane integrity by evaluating cell leakage and lipid peroxidation; determining the antioxidative capacity by assessing superoxide dismutase (SOD) and catalase (CAT) activity; and expression analysis of four genes included in the low temperature response (gibberellin insensitive dwarf 1 gibberellin receptor, *gid1*; fatty acid desaturases 2 and 6, *fad2* and *fad6*; plastid-lipid-associated 2 protein, *pap2*).

The results showed that, while there is not a significant difference in their germination rate, they differ in their survival rate, with more seeds of the tolerant genotype surviving the low temperature period. Significant differences between them were found in cell leakage ($p<0,01$), as well as *gid1*($p<0,05$) and *fad6* ($p<0,05$) gene expression assays. The present research brings light to our understanding of the effect of low temperatures on the first germination stage, - imbibition. It highlights the importance of choosing the right inbreds for earlier sowing and points to certain routes that could be taken for improving and accelerating the breeding process for low temperature tolerance.

Key words: maize, imbibition, low temperature tolerance, abiotic stress response

INTRODUCTION

Maize (*Zea mays*, L.) is the leading and most valuable crop in the world according to data released by the Food and Agriculture Organization, FAO (http://www.fao.org/faostat/en) in 2019, ahead of rice and wheat, with more than one billion metric tons produced annually. Maize is used for many different purposes, like animal feed, human consumption, biofuel, and bioplastic production. Its significance dictates the constant need for production improvement, including yield potential and quality, pest resistance, and abiotic stress tolerance. Climate change and its consequences, such as the considerably higher temperatures and periods of drought during the summer, lead to the development of unfavourable environmental conditions. These conditions have become a significant cause of productivity and yield loss, and a lot of effort is being put into alleviating their negative effects. Maize is especially sensitive to extreme heat occurring during the flowering and grain filling stages in summer, so a reduction in maize yield is expected (LOBELL and ASNER, 2003; HATFIELD and PRUEGER, 2015). It is predicted that for every increased mean global temperature degree, maize crop yields will lower by 7.4% (ZHAO *et al.*, 2017). Some of the most important strategies for minimizing yield losses in maize are cropping pattern changes and sowing alterations (ROSE *et al.*, 2016; TIAN *et al*., 2019), or more specifically earlier sowing (early spring). However, this entails exposure to suboptimal temperatures during earlier developmental stages, such as germination, emergence, V1, etc. – leading to a demand for the development of maize lines tolerant to low temperatures during these stages.

Seed germination is a complex process that begins with seed hydration and culminates in the emergence of the embryonic axis from the seed coat (BEWLEY, 1977). It is affected by several external and internal factors. Essential external factors are temperature, oxygen availability, soil pH, and moisture; while internal include dormancy and plant hormone interaction, most essentially gibberellins (GA) and abscisic acid (ABA) (TAYLORSON, 1987; SRIVASTAVA *et al*., 2002). Maize is a tropical plant and a lot more susceptible to the effects of low temperatures (HOPKINS, 1999). It thrives in temperatures ranging from 28° C to 35°C,

especially when it comes to seed germination (ARNOLD, 1974; YIN *et al.*, 1995). Temperatures lower than 15°C seriously affect germination and seedling development, as well as growth and biomass, and consequently the final yield (FAROOQ *et al*., 2009; SILVA-NETA *et al.*, 2015; NIKOLIĆ *et al*., 2020).

Plant tolerance to different abiotic stress factors, including low temperatures, is a complex trait that includes a number of mechanisms and strategies involved in the stress response, that all work together to ensure adaptation and survival in unfavourable conditions. This is accomplished through a series of molecular, biochemical, and physiological changes (COOK *et al.*, 2004; HANNAH *et al.*, 2005). Low temperature response can mainly be associated with cell membrane changes, as they are the primary injury site of low temperatures (KRATSCH and WISE, 2000). Plants sensitive to low temperatures display this effect more drastically, with cell leakage, delay of seed germination, and even death (ZHUO *et al.*, 2009; RAHOUI *et al*., 2010), while the more tolerant ones are able to delay this outcome. One of the ways they succeed in this is through increasing the membrane unsaturated fatty acid ratio, by activating different fatty acid desaturases (ROUTABOUL and FISCHER 2000). In addition to membrane integrity loss, an important consequence of low temperature stress is the disruption of oxidative metabolism and reactive oxygen species (ROS) accumulation (ALSCHER *et al.*, 1997; MITTLER, 2002). ROS can further harm the cell membranes by damaging membrane lipids, leading to their peroxidation. Plant cells combat this with several different antioxidative enzymes and small molecules, including superoxide dismutase (SOD) and catalase (CAT) (ALSCHER *et al.*, 1997; YOU and CHAN, 2015). These and other processes included in the response to any changes in the environment are of course regulated by a number of different important genes and changes in their expression (NGUYEN *et al.*, 2009; CHEN *et al.*, 2014).

The focus of this research is the first phase of germination – imbibition. Imbibition is the uptake of water by the dry seed, a physical process dependent on differences in water potential (BEWLEY, 1977; HADAS, 2005), and not much is known about the effect of low temperatures on this phase. Since maize varieties respond differently to low temperatures, there is substantial variability in regards to low temperature tolerance in different maize genotypes (FAROOQI and LEE, 2016; MILIVOJEVIĆ *et al.*, 2017). Understanding the different ways in which they respond to this abiotic factor during the earlier stages of development, including imbibition, is crucial. To confront this issue, we have attempted to discover significant differences in the low temperature response that exist between tolerant and susceptible genotypes and are related to some of its pivotal processes. This includes determining the effects of low temperatures on survival and germination completion, membrane integrity, and antioxidative capacities. Additionally, the expression of genes important for germination, lipid accumulation, and membrane lipid desaturation was examined under the low temperature conditions.

MATERIAL AND METHODS

Plant material and experimental design

Seeds of two elite maize inbred lines of contrasting susceptibility to low temperatures were selected for this research. The chosen inbred lines were marked as L_1, considered tolerant, and L_2, considered sensitive, based on previously conducted tests (BANOVIĆ ĐERI *et al.*, 2021). The experiment was carried out in three replicates of 60 seeds for each inbred line. Seeds were

sterilized in 1% sodium hypochlorite (commercial bleach) and then placed on and covered with moist filter paper, before being set in germination boxes. The germination boxes were placed in a growth chamber for 24 hours in the dark at 20° C (control) and 8°C (treatment). A set of 40 seeds from each replicate was sampled immediately after the treatment has finished and used for the tetrazolium, membrane integrity, antioxidative enzyme assays, and gene expression analysis, while the rest was used for the germination rate assay.

Germination assay

For the germination assay, three replicates of 20 seeds from each line were left for seven days in the dark at 20°C, after the initial 24h imbibition period, and germination rates were scored daily until the 7th day. Germination rate was calculated as the percentage of seeds that successfully germinated in this time frame.

Tetrazolium assay

After the 24h imbibition period, three replicates of 10 treated and 10 control seeds were cut longitudinally through the midsection of the embryonic axis and soaked in 0.1% 2, 3, 5 triphenyl tetrazolium chloride solution for 2 hours at 30° C. After the dyeing was completed the seeds were removed from the tetrazolium solution and rinsed in distilled water and then evaluated. They were categorized as viable or non-viable according to the ISTA (International Seed Testing Association) protocol published in 2019.

Membrane integrity assays

Membrane integrity after the cold treatment was examined by electrolyte leakage and lipid peroxidation determination, after the finished imbibition period, and included three replicates of 5 seeds each.

For the electrolyte leakage assays, seeds were submerged in 20 mL of distilled water and left for 1 hour at both treatment and control temperatures, after which the conductivity of the water solution was measured. The water solution with maize seeds was then incubated at 100° C for 10 minutes and the conductivity was measured again. The results were shown as ratios of the first conductivity measurement (before incubation) and the total conductivity (after incubation) (NOBLET *et al.*, 2017). All the measurements were done using a conductivity meter (Consort C6010).

Lipid peroxidation was determined by measuring the content of malondialdehyde (MDA), through the thiobarbituric acid (TBA) reaction, as described by HEATH and PACKER (1968). Maize embryos including the scutellum, separated from the endosperm and the rest of the seed, were frozen and homogenized in liquid nitrogen, and 100 mg of the ground material was diluted in 800μL of trichloroacetic acid (TCA). The solution was chilled to 4°C and then centrifuged for 20 minutes at 13.000 RPM. After separating 1 mL of supernatant, 1 mL of 20% TCA (containing 0.5% TBA) was added. The solution was heated at 95° C for 30 minutes, quickly cooled on ice and centrifuged at 13.000 RPM for 15 minutes. The absorbance of the supernatant was measured at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm), the MDA concentration was determined by its molar extinction coefficient (155 mM−1 cm−1) and shown as nmol of MDA per g of fresh maize seeds (nmol gFW-1).

Antioxidative enzyme assays

Antioxidative enzyme assays included the SOD and CAT activity determination, using three replicates of five seeds each.

SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT), as described by BEAUCHAMP and FRIDOVICH (1971) and GIANNOPOLITIS *et al.* (1977). Following the 24h imbibition period, the embryos were separated, frozen and homogenized in liquid nitrogen, after which 100 mg of the ground tissue was resuspended in 1 mL of extraction buffer (50 mM potassium phosphate buffer, 0,1mM EDTA). The samples were centrifuged for 30 minutes at 13.000 RPM before the supernatant was separated and centrifuged again. The supernatant was separated again and used for protein concentration and SOD activity determination. Protein concentrations were measured according to BRADFORD (1976), using RC DC™ Protein Assay Kit II (Bio-Rad™). The reaction mixture for SOD activity determination (200 µL) contained 50mM potassium phosphate buffer (pH 7,0), 0,1mM EDTA, 13 mM methionine, 75µM NBT, 2µM riboflavin and 5 µg of the enzyme extract. Riboflavin was added last and the reaction mixtures were shaken and illuminated with fluorescent light. The reaction was allowed to proceed for 15 min after which the lights were removed and samples left in the dark. Absorbance was measured at 560 nm, before the beginning and after the end of the reaction (TECAN Infinite 200 Pro Plate Reader). One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate and the results were expressed as U mg−1 of protein extracts added to the reaction mixture (m_{pe}=0,005mg).

CAT activity was measured according to BEERS and SIZER (1952) and AEBI (1984), using the same plant extract that was used for SOD activity determination. The reaction mixture (500 µL) consisted of 50mM potassium phosphate buffer (pH 7,0), 0,1mM EDTA, 16,3mM H_2O_2 and 40 µg of the enzyme extract. The reaction was initiated by adding the extract. The decrease of H_2O_2 was monitored at 240 nm (Ultrospec 3300 Pro, Amersham Biosciences™) and quantified by its molar extinction coefficient (36 M−1 cm−1). The results were expressed as absorbance change in time per mass of added protein, ΔA_{240} min−1 mg−1.

Total RNA purification, cDNA synthesis and gene expression assays

Total RNA was isolated from maize embryos including the scutellum, separated from the endosperm and the rest of the seed after the 24h imbition period. Each of the two inbred lines was represented with ten maize embryos pooled into one sample and three replicates were used for each sample. The tissue was ground in liquid nitrogen using mortar and pestle and stored at -80° C until further use. For the total RNA extraction, 100 mg of the frozen tissue was utilized and it was performed using Gene Jet RNA Purification kit (Thermo Scientific™). Total RNA was further treated with DNase I (Ambion® DNA-free™ kit, Invitrogen™), and RNA concentrations were determined with the NanoDrop™ spectrophotometer (Thermo Scientific™). cDNA was synthesised from 1 µg of the purified, total RNA, using Revert Aid First Strand cDNA synthesis kit with RNase inhibitor (Thermo Scientific™). Real-time PCR analysis was carried out using cyclophilin (*cyp*) as the internal reference gene (recommended by LIN *et al.*, 2014). PCR reactions were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems™). The reaction mixture (10 µl) contained 1x HOT FIREPol® EvaGreen® qPCR

Mix Plus (ROX) (Solis BioDyne™), 0.2 μM of each primer (forward and reverse) and 1 μl of template cDNA diluted 2 times. The thermal cycling conditions included the initial denaturation (95° C for 10 min), followed by 40 cycles of denaturation (95° C for 15 s), primer annealing and extension (appropriate temperature for 60 s).

Genes chosen for this research were the gibberellin insensitive dwarf 1 GA receptor gene (*gid1*), fatty acid desaturase 2 and 6 genes (*fad2*, *fad6*), and the plastid-lipid-associated 2 protein gene (*pap2*). The primers used are listed in Table 1. Primers for the *pap2* gene were designed using Primer 3 (v 0.4.0) online software (http://bioinfo.ut.ee/primer3-0.4.0) and checked in NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast). Sequences for the other primers were taken from LIN *et al.* (2014) for *cyp*, NOBLET *et al.* (2017) for *fad2* and *fad6*, LI *et al.* (2017) for *gid1*. Primer pairs were tested by standard PCR beforehand and expected amplicon sizes of each gene were confirmed by gel electrophoresis. The appropriate amplification efficiencies were calculated according to E=10(-1/slope) method. Relative gene expression was calculated according to LIVAK and SCHMITTGEN (2001) using efficiency correction as in PFAFFL (2004).

Table 1. Primer sequences used for gene expression analyses

Gene	Orientation	Sequence
gid1	F	5'-TGGTGGACTGATGGACCAA-3'
	R	5'-CCCATCCGATCCATAAAAGA-3'
fad2	F	5'-ATGGTGCCCTACTTCTCGTG-3'
	R	5'-GCCGACCGGGTTGTTGTA-3'
fad6	F	5'-GTATTGTCGGCACAGGTGGA-3'
	R	5'-ACGCTACGTGCTTTCCTACT-3'
pap2	F	5'-CGTTGTGCTACCGGAGAAGT-3'
	R	5'-GGTCGTAAGCAACCACGATT-3'
cyp	F	5'-CTGAGTGGTGGTCTTAGT-3'
	R	5'-AACACGAATCAAGCAGAG-3'

Statistical analysis

All of the results were statistically processed in MSTAT C (MSTAT Development Team, 1989) using the t-test, while graphs were designed in Microsoft Excel (Windows 10).

RESULTS AND DISCUSSION

Germination rate and tetrazolium assay

Optimal temperatures for maize seed germination range from 28° C to 35°C (YIN *et al.*, 1995). Lowering the temperature below the optimum negatively affects germination and seedling development. Maize germination is severely affected by temperatures lower than 15°C and most maize hybrids do not germinate at temperatures lower than 10°C (FRASCAROLI and LANDI, 2013). Additionally, lowering the temperature slows the germination down. Under optimal conditions, maize seeds will begin to germinate after four to five days, while in the suboptimal temperature conditions the process could take up to two weeks (SILVA-NETA *et al.*, 2016).

Germination assays showed no statistically significant difference in the germination rate neither between the genotypes (although the L1 genotype had a better germination rate than L2) nor between the control and treated samples of either genotype (Figure 1). However control seeds started germinating sooner – radicles from the control seeds were emerging by the third day, while the treated seeds entered this stage of germination on the sixth. Low temperatures have a severe negative effect on germination and seedling growth that has been well documented. Nevertheless, the plant's response to low temperatures as well as the factors affecting the response are complex, and the mechanisms included in the whole process are not very well-understood (YANG *et al.*, 2011). Moreover, the genetic background of different maize hybrids influences the ability to germinate under low temperature conditions (ZHANG *et al.*, 2012; HU *et al.*, 2016). There is evidence that not every seed germination phase is equally susceptible to low temperature. For example, BLACKLOW (1972) showed that the maize seed imbition was unaffected by low temperatures and that the rate of water uptake was high regardless of the temperature. This could explain the lack of significant difference between the control and treated samples of both the L1 and L2 genotype, but research including a higher number of maize hybrids and different germination stages is necessary to confirm this assumption.

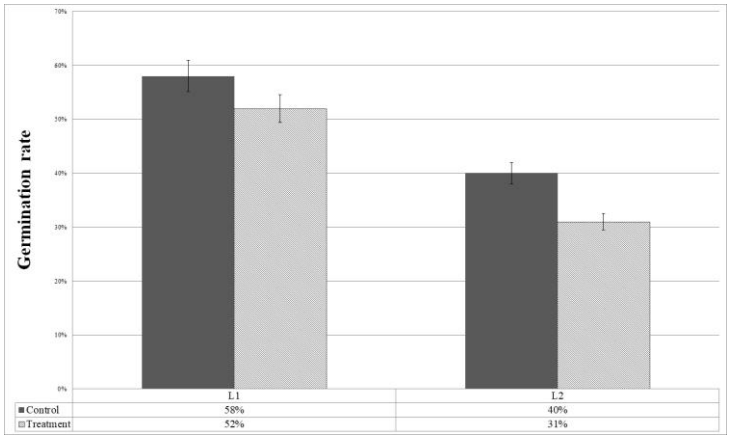

Figure 1. Germination rates of genotypes L1 and L2, seven days after the initial 24h imbibition period under control (20° C) and treatment temperatures (8° C). Values are means ±SE obtained from three replicates and these results were not statistically significant.

The tetrazolium test is one of the most accurate tests for determining seed viability, widely used for maize seeds and seeds of other plant species (DEVRIES and GOGGI, 2006). Parameters used for seed viability assessment are staining intensity, tissue texture, as well as consistency, and the layout of unstained tissue. Seeds that showed no staining were considered unviable, while those with stained radicles and pulmules were considered viable. The staining was more intense in control seeds than in the treated seeds in both genotypes and in the treated seeds, the staining was stronger in the more tolerant genotype, L1 (Figure 2). This was expected and confirms that the genotype L1 is equipped with certain mechanisms which enable survival continued germination even under low temperature conditions. However, based on the genotype scores these results were not statistically significant.

Figure 2. Differences in seed viability between the control and treatment seeds, determined by the tetrazolium test in the tolerant genotype, L1 and the sensitive, L2 and shown as different levels of tissue staining.

Membrane integrity assays

The effect of low temperature on membrane integrity in maize seeds during imbibition was examined through electrolyte leakage and lipid peroxidation determination. Cytoplasmic membrane damage and cell leakage are determined by measuring the electrical conductivity of the solutions containing the seeds. The results were shown as ratios of the first conductivity measurement (before incubation at 100° C) and the total conductivity (after incubation). Control seeds of both genotypes showed less electrolyte leakage than the treated seeds ($p<0,01$). A statistically significant difference in electrolyte leakage was detected between the two genotypes ($p<0,01$). Tolerant L1 showed a less notable difference in electrolyte leakage between the control and treated seeds than the sensitive L2 (Figure 3). Higher rates of lipid peroxidation, determined

by measuring the MDA content, were observed in maize embryos exposed to low temperatures during imbibition than the ones in control conditions (L1, $p<0,05$; L2, $p<0,001$). Surprisingly, the sensitive genotype L2 demonstrated lower rates of lipid peroxidation in the treated embryos, than the tolerant, L1, though these results were not statistically significant. Additionally, the difference in MDA content between the control and treated seeds was smaller in the tolerant genotype L1(Figure 3).

It is well known that cell membranes are a primary site of injuries induced by low temperatures and that lowering the temperature under the optimal values causes a series of qualitative and quantitative changes to their composition and structure, known as the thermal phase transition. Phase transition leads to further disruptions to normal cell metabolism and membrane integrity and functioning, causing electrolyte or cell leakage, amongst other consequences (MA *et al.*, 2015; ZHU, 2016). This process is additionally accelerated during imbibition because during this phase cell membrane reconstruction occurs. Dehydrated membrane lipids form the hexagonal II phase, a type of lipid structure or phase also characteristic for injuries induced by low temperatures. During imbition and rehydration, membrane lipids are reorganized and converted into the lamellar phase, typical for the normal, fluid membrane state (MURPHY and NOLAND, 1982). Membrane reorganization can be hindered by abiotic stress factors, such as low temperature, leading to cell leakage, delay of seed germination, and even death (ZHUO *et al.*, 2009; RAHOUI *et al.*, 2010). It is confirmed that low temperatures affect the membrane integrity and lead to an increase in relative conductivity in maize and other plant species (WANG *et al.*, 2008; LI *et al.*, 2012). In addition to membrane integrity loss, low temperature stress, like all abiotic stress factors increases the rate of ROS formation, causing oxidative stress (MITTLER, 2002; NEILL *et al.*, 2002). ROS can further harm the cell membranes by damaging membrane lipids and inducing their peroxidation (ALSCHER *et al.*, 1997; IMLAY, 2003). Lipid peroxidation is often associated with loss of viability since it also affects protein modification and degradation. This, in addition to membrane damage, again leads to cell leakage and death (FAROOQ *et al.*, 2009).

Results of both the assays, electrolyte leakage and lipid peroxidation determination, confirmed this. Significantly higher damages were observed in the treated maize seeds and embryos. Additionally, while there was no significant difference in lipid peroxidation between the genotypes, the tolerant one, L1 showed significantly lower rates of cell leakage than the more sensitive genotype L2. This suggests the possibility that in the tolerant genotypes, such as L1, one of the key mechanisms of low temperature responses and adaptation is membrane integrity preservation, but not with significant help of the antioxidative mechanisms, though research including a higher number of genotypes and different analyses is needed to confirm this.

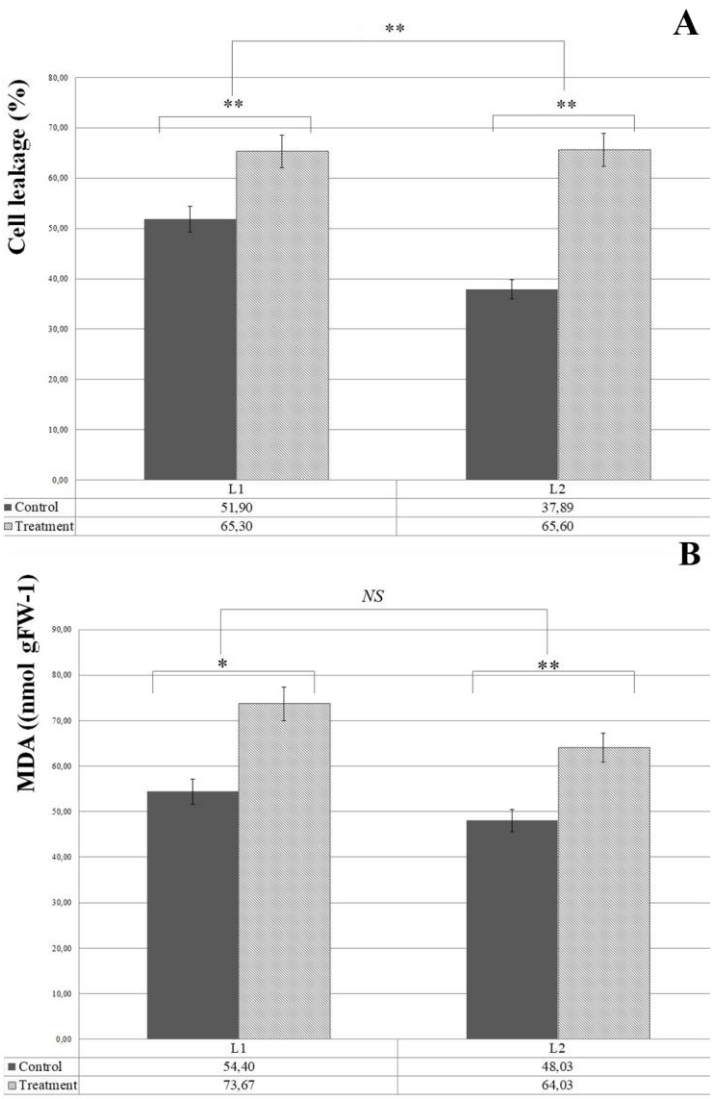

Figure 3. Cell leakage (**A**) and lipid peroxidation level (**B**) in both genotypes (L1 and L2) after the 24h imbibition period under control (20º C) and treatment (8º C). Cell leakage results are shown as ratios of the first conductivity measurement (before incubation at 100° C) and the total conductivity (after incubation); and the lipid peroxidation level was determined by measuring the MDA content of malondialdehyde and shown as nmol of MDA per g of fresh maize seeds (nmol gFW-1). Values are means ±SE obtained from three replicates. The results of the t-test comparisons between the genotypes were shown above the bars. Statistically significant differences were marked with asterisks (* - $p<0.05$; **- $p<0.01$), while those that aren't, are marked with NS (not significant, NS).

Antioxidative enzyme assays

ROS are present in the seeds during imbibition regardless of the environmental conditions. ROS are necessary for normal germination and seedling development, however if their amount in the cells increases they can cause oxidative stress (LEYMARIE *et al.*, 2011). Low temperatures can cause this increase by affecting the activity of systems responsible for their removal and the ROS accumulation leads to severe damages to cell structure and metabolism (SUZUKI and MITTLER 2006; OTT *et al.*, 2007). Plant cells are equipped with antioxidative systems, consisting of several enzymes and small molecules necessary for neutralizing the negative effects of ROS (ZHANG *et al.*, 2010; ZHU *et al.* 2011). Many studies of a large number of plant species have shown the importance of CAT and SOD activity during periods of low temperature stress for ensuring a decrease in cell membrane damage, obtaining normal cell metabolism and ensuring plant survival (LUO *et al.*, 2001; LIU *et al.*, 2014). However, certain abiotic stress intensities can lead to severe ROS accumulation that the antioxidative systems cannot easily neutralize (YOU and CHAN, 2015). JAHNKE *et al.* (1991) demonstrated that suboptimal temperatures negatively affect antioxidative enzymes in maize, particularly SOD, while PRASAD *et al.* (1994) have not observed any difference in CAT activity in low temperature and control conditions. In both genotypes, SOD activity was higher in control embryos, but a smaller decrease in activity was observed in the seeds of the tolerant genotype L1 (Figure 4). CAT activity showed no significant differences between the control and treated seeds of both genotypes (Figure 4).

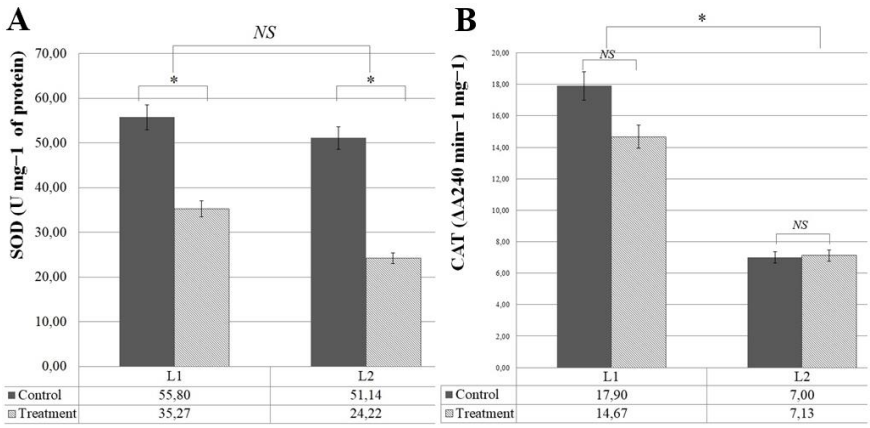

Figure 4. SOD (**A**) and CAT (**B**) activity in both genotypes (L1 and L2) after the 24h imbibition period under control (20º C) and treatment temperatures (8º C). SOD activity is shown as U mg−1 of protein extracts added to the reaction mixture, where U is defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate. CAT activity results were expressed as absorbance change in time per mass of added protein, ΔA240 min−1 mg−1. Values are means ±SE obtained from three replicates. The results of the t-test comparisons between the genotypes were shown above the bars. Statistically significant differences were marked with asterisks (* - $p<0.05$; **- $p<0.01$), while those that aren't, are marked with NS.

Gene expression assays

Expression of *gid1* was increased in both genotypes during the low temperature treatment, and the fold change was higher in the sensitive, L2 (R=8.93) than the tolerant genotype, L1 (R=2.99) ($p<0,05$) (Figure 5). Understanding the role of GA, as one of the most important growth hormones, during seed germination under low temperature conditions is necessary for the complete comprehension of plant growth and development in poor environmental conditions. Evidence suggests that gibberellins are important for growth modulation in these conditions and that their activity depends on the type of stress, intensity, plant genetic background, etc. (COLEBROOK *et al.*, 2014). It is known that GA function through the removal of the DELLA protein repressors, which allows them to stimulate seed germination and other developmental processes (HAUVERMALE, ARIIZUMI, and STEBER, 2012; URBANOVA and LEUBNER- METZGER, 2016), and this is done by binding to the GID1 receptors (UEGUCHI- TANAKA *et al.*, 2005; NAKAJIMA *et al.*, 2006). Rice *gid1* gibberellin insensitive dwarf mutants are more tolerant to low temperature stress conditions (TANAKA *et al*., 2006), while DELLA protein accumulation contributes significantly to low temperature tolerance in *Arabidopsis thaliana* (ACHARD *et al.*, 2008). ACHARD *et al.* (2008) showed that DELLA accumulation is the result of low temperature-induced C-repeat/drought-responsive element binding factor 1, CBF1 expression, that leads to GA-inactivating GA 2-oxidase genes expression. Results of this research suggest that this mechanism could include inactivation of *gid1* as well and that lower *gid1* expression should be expected in the more tolerant maize genotypes.

fad2 expression was downregulated in the treatment conditions, however this difference in expression wasn't statistically significant. Additionally, there was no statistically significant difference in *fad2* expression between the two genotypes neither (L1, R=0.45; L2, R=0.45). On the other hand, *fad6* expression was significantly higher in the sensitive genotype, L2 (R=3.42) than in the tolerant, L1 (R=2.02) ($p<0,05$) (Figure 5). While *fad6* expression was upregulated in both genotypes, this difference between the control and treatment conditions was statistically significant only in L2 ($p<0,05$). It is well known that exposure to low temperatures leads to the increase of the membrane unsaturated fatty acid content and that the unsaturation of membrane lipids is proportional to the cold stress resistance (ROUTABOUL and FISCHER, 2000; UPCHURCH, 2008). The level of membrane lipid desaturation after the exposure to low temperatures depends on the plant genetic background and it differs based on low temperature susceptibility – tolerant genotypes of different plant species, including maize, show a higher increase in the unsaturated fatty acid ratio (PEREIRA DA CRUZ *et al.*, 2010; NOBLET *et al.*, 2017). Enzymes responsible for this change are fatty acid desaturases, and FAD2 and FAD6 belong to this enzyme family. The changes in their expression patterns correspond to the desaturation level in the membrane and these changes point to the direct role of these enzymes when it comes to membrane modifications during low temperature exposure (MATTEUCCI *et al.*, 2011). However, there are instances where this is not the case. NOBLET *et al.*, 2017 showed that *fad2* expression was not induced by low temperature treatments in *Arabidopsis thaliana* and *Glycine max*, and ZHANG *et al*., 2009 showed that while *fad2* expression was increased 1h and 4h after the start of treatment, it started to decrease after that – this coincides with the results of this research. On the other hand, *fad6* expression being higher in the more sensitive type could possibly be explained by the

experimental conditions which were not intense enough to stimulate *fad6* expression in the tolerant L1. This explanation is supported by electrolyte leakage assay results that do not show significant membrane damage.

Expression of *pap2* was higher in the tolerant genotype, L1 (L1, R=1.98; L2, R=0.99), however these differences were not statistically significant (Figure 5). *pap2* gene was chosen based on the leaf transcriptome and differential expression analysis of different maize inbreds from the MRIZP elite core collection.

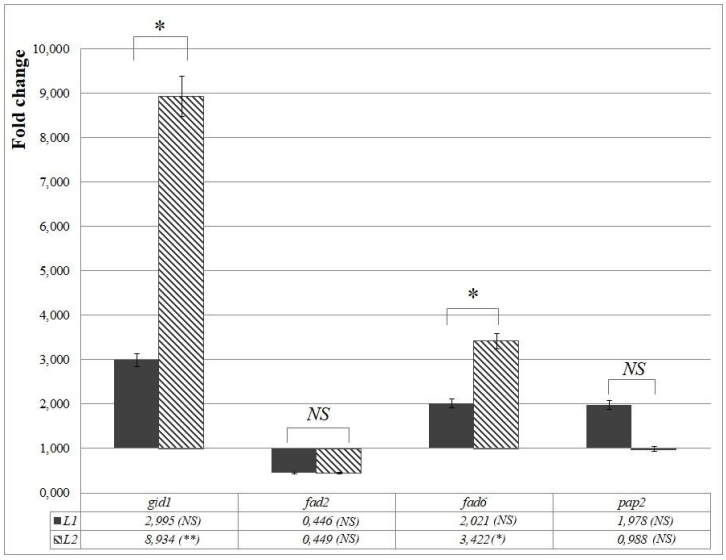

Figure 5. The effect of low temperature on expression patterns of *gid1, fad2, fad6* and *pap2*, in both genotypes (L1 and L2) after the 24h imbibition period, expressed as the fold change between their expression in control (20° C) and treatment conditions (8° C). Values are means ±SE obtained from three replicates. The results of the t-test comparisons between the genotypes were shown above the bars, while the differences between the control and the treatment in the same genotype were shown in the table under the graph. Statistically significant differences were marked with asterisks (* - $p<0.05$; **- $p<0.01$), while those that aren't, are marked with NS.

This research revealed certain genes involved in the abiotic stress response and one of them was *pap2* (BANOVIĆ ĐERI *et al.*, 2021). Although this gene did not show expression regulation dependent on the duration of cold exposure in the V4 stage, the same is not true during imbibition. Plastid-lipid associated proteins (PAPs) are necessary for lipid and carotenoid storage and stabilization during periods of unfavourable conditions. Their accumulation in plastids is accelerated by different stress factors, both abiotic and biotic (KIM *et al.*, 2001; MURPHY, 2004) and their expression is stimulated by the creation of ROS, more precisely H_2O_2 (MANAC'H and KUNTZ, 1999; LANGENKAMPER *et al.*, 2001). The role of PAPs during the stress conditions is suggested to be in the sequestration of hydrophobic molecules (LEITNER-DAGAN *et*

al., 2006), as well as the modulation of photosynthetic efficiency (MONTE *et al.*, 1999) and thylakoid reparation and/or degradation and removal (SMITH *et al.*, 2000). Additionally, it has been shown that their increased expression results in improved plant performance under these conditions (REY *et al.*, 2000). Data on the PAP expression, particularly *pap2*, in early stages of development under low temperature conditions are insufficient, but the results of this research and the increased expression in the tolerant genotype, L1 allude to them having an important role in surviving periods of stress even this early in the development of maize.

CONCLUSIONS

The present research brings some light to the understanding of the effect of low temperatures on the first germination stage, imbibition. It highlights the difference in susceptibility to low temperatures between different maize inbreds during this stage and accentuates the importance of choosing the right inbreds for earlier sowing. Additionally, it points to certain routes that could be taken for breeding maize inbreds tolerant to low temperatures. For example focusing more on the effect low temperatures have on membrane integrity, gibberellin signalling network, and lipid storage and stabilization - and using the acquired knowledge to develop tools to improve and accelerate the breeding process.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia. upisati broj institucijalnog projekta

Received, November 30th, 2020
Accepted April 28th, 2021

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FIZIOLOŠKE I PROMENE U GENSKOJ EKSPRESIJI TOKOM IMBIBICIJE SEMENA KUKURUZA U USLOVIMA NISKE TEMPERATURE

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Izvod

Kukuruz je usev od izuzetne važnosti za poljoprivredu, koji se takođe odlikuje značajnom osetljivošću na visoke temperature i sušu tokom letnjih meseci, odnosno tokom faze cvetanja i nalivanja zrna. Jedna od strategija obezbeđivanja zadovoljavajuće produktivnosti i prinosa kukuruza, uprkos nepovoljnim uslovima životne sredine, jeste ranija setva. Ipak u tom slučaju su biljke kukuruza izložene suboptimalnim temperaturama u ranim fazama razvića, što stvara potrebu za razvojem linija tolerantnih na niske temperature u tim fazama, što je i fokus ovog istraživanja. Odgovor kukuruza na niske temperature je kompleksan i uključuje niz različitih mehanizama i strategija koje obezbeđuju opstanak, a takođe zavisi i od samog genotipa, faze razvića, itd. Iz tog razloga, odgovor kukuruza na niske temperature (8°C) je posmatran tokom faze imbibicije, kod dva genotipa, i to na nivou promena genske ekspresije (*gid1*; *fad2*; *fad6*; *pap2*), aktivnosti antioksidativnih enzima (SOD, CAT), integriteta ćelijskih membrana, stopa germinacije, kao i samog preživljavanja. Rezultati ukazuju da postoje statistički značajne razlike između genotipova kada je reč o stopi preživljavanja, integritetu ćelijskih membrana i ekspresiji gena *gid1*i *fad6* ($p<0,05$). Ovo istraživanje ukazuje na moguće mehanizme aklimatizacije na niske temperature tokom faze imbibicije i ističe značaj odabira odgovarajućih linija za raniju setvu.

Primljeno 30.XI.2020.
Odobreno 28. IV. 2021.