BIOSCIENCE JOURNAL

ENZYMATIC ACTIVITY AND GENE EXPRESSION RELATED TO DROUGHT STRESS TOLERANCE IN MAIZE SEEDS AND SEEDLINGS

Milena Christy SANTOS[1], Édila Vilela de Resende Von PINHO[2], Heloisa Oliveira dos SANTOS[2], Danielle Rezende VILELA[3], Izabel Costa SILVA NETA[1], Viviane Maria de ABREU[4], Renato Coelho de Castro VASCONCELLOS[5]

[1] Helix Sementes e Mudas, Patos de Minas, Minas Gerais, Brazil.
[2] Department of Agriculture, Federal University of Lavras, Lavras, Minas Gerais, Brazil.
[3] Postgraduate Program in Agronomy and Phytotechnics, Federal University of Lavras, Lavras, Minas Gerais, Brazil.
[4] Bayer Crop Science, São Paulo, São Paulo, Brazil.
[5] Embrapa Cassava and Fruits, Cruz das Almas, Bahia, Brazil.

Corresponding author:
Renato Coelho de Castro Vasconcellos
Email: renatoccv@hotmail.com

How to cite: SANTOS, M.C., et al. Enzymatic activity and gene expression related to drought stress tolerance in maize seeds and seedlings. *Bioscience Journal*. 2021, **37**, e37079. https://doi.org/10.14393/BJ-v37n0a2021-53953

Abstract

Drought stress is a major limiting factor for the development of maize, and the identification of the expression of genes related to this stress in seeds and seedlings can be an important tool to accelerate the selection process. The expression of genes related to tolerance to water deficit in seeds and in different tissues of maize seedlings were evaluated. Four tolerant genotypes (91-T, 32-T, 91x75-T, 32x75-T) and four non-tolerant genotypes (37-NT, 57-NT, 37x57-NT and 31x37-NT) were seeded in a substrate with 10% (stress) and 70% (control) water retention capacity. The expression of 4 enzymes were evaluated: catalase (CAT), peroxidase (PO), esterase (EST), and heat-resistant protein (HRP), as well as the relative expression of 6 genes: *ZmLEA3, ZmPP2C, ZmCPK11, ZmDREB2A/2.1s, ZmDBP3 and ZmAN13* were evaluated in seed, shoots and roots of seedlings submitted or not to stress. There was variation in the expression of CAT, PO, SOD, EST and HRP enzymes among the evaluated genotypes and also in the different tissues evaluated. Higher expression of the CAT and PO was observed in the shoots. There was a greater expression of the EST in the genotypes non-tolerant to water deficit. HRP was expressed only in seeds. In the aerial part of maize seedlings, classified as tolerant, higher expression of genes ZmLEA3 and ZmCPK11 was observed. There was a higher expression of the *ZmAN13* and *ZmDREB2A/2.1S* genes in roots developed under stress conditions and a higher expression of the *ZmPP2C* gene in seeds of line 91-T, which is classified as tolerant to drought stress.

Keywords: Abiotic stress. Proteomic. RT-qPCR. *Zea mays*.

1. Introduction

Drought is a major environmental stressor that negatively affects plant growth and productivity (Ribaut et al. 2009). Plant responses to drought vary according to species, intensity, and duration of stress, genotype, and stage of development, and may manifest at morphological, physiological and molecular levels (Kramer 1983; Wang et al. 2003).

The delay of leaf expansion under water deficit conditions is motivated by the reduction of the cellular expansion and with this, an increase of the stomatal closure is observed, which causes lower transpiration so that the plant can withstand this condition for a longer time (Taiz and Zeiger 2013). Water

restriction in the soil at the time of sowing reduces the emergence and development of seedlings due to interference in the water uptake and cell elongation processes. In this conditions, field emergence and initial seedling development are highly depended on the level of seed vigor (Marcos-Filho 2015; Finch-Savage and Bassel 2016).

Drought stress affects photosynthesis and increases photorespiration, causing an increase in the production of reactive oxygen species (ROS) (Miller et al. 2010). Plants under drought stress can alter their metabolism and invoke various defense mechanisms such as the increased activity of antioxidant enzymes (Chai et al. 2016). Plants protect their cells from the toxic effects caused by ROS using manly antioxidant enzymes, such as catalase (CAT), peroxidase (PO), esterase (EST) and also heat-resistant protein (HRP) (Choudhury et al. 2016). In plants under water stress, there is an increase in the activity of these enzymes related to the increase in drought tolerance (Xiong et al. 2002).

It is known that in response to water stress occurs the increase in the expression of some genes that give the plants a better adaptation to this condition. Among those already studied in maize, we can highlight the genes *ZmLEA3*, *ZmPP2C, ZmCPK11, ZmDREB2A/2.1S, ZmDBP3* and *ZmAN13* for this trait (Koussevitzky et al. 2008; Wang et al. 2009; Hu et al. 2010; Xuan et al. 2011; Caverzan et al. 2012; Zhou et al. 2012; Liu et al. 2013; Marques et al. 2019).

Thus, the identification and understanding of maize drought tolerance mechanisms are fundamental for the development of new commercial cultivars that are more tolerant to water deficit. Little is known about expression in the early stages of development, such as in seeds and seedlings. Therefore, the aim was to study gene expression related to stress caused by water deficit in seeds and tissues of maize seedlings by proteomic and transcriptomic analyses.

2. Material and Methods

Plant material and growth condition

The study was conducted at the Central Seed Laboratory at the Lavras Federal University (UFLA). The selection of genetic materials was done after analyzing the work developed by Abreu et al. (2018). Four contrasting lines were selected: two tolerant lines (T), 91-T and 32-T, and two non-tolerant lines (NT), 57-NT and 37-NT, to drought stress. Four hybrids were also selected, being two tolerant hybrids, 91x75-T and 32x75-T, and two non-tolerant, 37x57-NT and 31x37-NT. The experimental design used was completely randomized with four replicates with a factorial scheme 8x2, being eight genotypes and two conditions of water availability.

For the proteins and transcripts analysis, the seeds of the eight genotypes were seeded in a substrate containing 70% and 10% of water retention capacity in the soil, constituting the conditions of control and stress, respectively. After seven days of sowing, the seedlings were removed from the substrate, washed and separated in aerial part and root. The treatments used in the experiment were dried seeds of the eight selected genotypes, shoot and root under the two contrasting conditions of the eight genotypes.

Expression analysis of enzymatic activity

For the expression analysis of the enzymes catalase (CAT), peroxidase (PO), esterase (EST) and heat-resistant proteins (HRP), seedlings, root, and shoots were grinded in the presence of liquid nitrogen and PVP (Polyvinylpyrrolidone) on porcelain mortar on ice and subsequently the samples were stored at -80 °C.

For the extraction of the enzymes, 0.2 M Tris HCl pH 8.0 + (0.1% mercaptoethanol) buffer was used, in the proportion of 250 µL per 100 mg of seeds. The material was vortexed and kept overnight in the refrigerator, followed by centrifugation at 14,000 rpm for 30 minutes at 4 °C.

The electrophoretic run was performed in a polyacrylamide gel system with 7.5% (separation gel) and 4.5% (concentration gel). The gel/electrode system used was Tris-glycine pH of 8.9. 60 µl of the supernatant from the samples were applied to the gel and the electrophoretic run was performed at 120 V for 5 hours. After the race, the gels were revealed according to Alfenas (2006), with modifications. The enzyme quantification was performed by ImageJ® software, in pixel^2 (2016).

For the extraction of the heat-resistant protein, the buffer solution (50 mM Tris-HCl-7.5, 500 mM NaCl, 5 mM MgCl2, 1 mM PMSF) was added to the already macerated samples in a proportion of 1:10 (weight of material: volume of extraction buffer) and transferred to 1500 µL microtubes. The homogenates were centrifuged at 14000 rpm for 30 minutes at 4 °C, and the supernatant was incubated in a water bath at 85 °C for 15 minutes and centrifuged again. The supernatant was poured into micro tubes and the pellet discarded. Prior to the gel application, the sample tubes containing 70 µL extract + 40 µL sample buffer (2.5 ml glycerol, 0.46 g SDS, 20 mg Bromophenol blue and the volume completed to 20 ml of Tris extraction buffer pH 7.5) were placed in a water bath with boiling water for 5 minutes. 50 µL of the extract with heat-resistant proteins + sample buffer was applied on a 12.5% SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) (separation gel) and 6% (concentration gel). The electrophoretic run was performed at 150 V and the gel stained in Coomassie Blue at 0.05% for 12 hours and bleached in 10% acetic acid solution (Alfenas 2006). Protein quantification was also performed by ImageJ® software, in pixel^2 (2016).

Expression analysis of transcripts

The transcript expression involved in the drought stress tolerance was done using the RT-qPCR technique and was divided into four steps: RNA extraction and purification, reverse transcription for cDNA synthesis, real-time PCR, and result analysis.

For the RNA extraction, the seeds and the parts of maize seedlings (aerial part and roots) were grinded with liquid nitrogen and the addition of Pure Link RNA Plant® reagent (Invitrogen), following the manufacturer's manual specifications. The RNA integrity and purity were evaluated at all stages using 1.5% agarose gel electrophoresis (stained with GelRed® Nucleic Acid Stain, 10,000 X in Water) and in a spectrophotometer (BioTek® Eon® Microplate Spectrophotometer). The samples were treated with DNAseFree® (Ambiom) to avoid any DNA contamination. In order to prove the efficiency of the DNAse treatment, a conventional PCR reaction was performed and as a positive control, a maize genomic DNA sample was used. The primer used was the one corresponding to the constitutive gene Ubiquitin. A 1.5% agarose gel stained with GelRed® Nucleic Acid Stain was prepared for visualization of possible amplification.

After the extraction and purification process, the mRNAs that were extracted were used as templates for cDNA synthesis, using the cDNA Reverse Transcription cDNA® kit (Applied Biosystems) according to the protocol recommended by the manufacturer. The efficiency of cDNA synthesis was later confirmed by standard PCR.

The target genes were chosen because of their known importance in drought stress tolerance in maize, after a literature review. Sequences of the target genes chosen were found by searching the genomic database of maize sequence in GenBank. Based on these target sequences the primers were designed using Primer Express 3.0 software (Applied Biosystems). The primer sequences used are shown in Table 1. Ubiquitin and Alcoholic Dehydrogenase (ADH) were used as reference genes (Livak and Scmittgen 2001; Scholdberg et al. 2009).

Table 1. Primers used in the RT-qPCR analysis.

Gene	Function		Sequence 5'------3'
ZmLea 3	Osmoprotectant biosynthesis	F	CCACGAGACCACCTACAACT
		R	CCTTTCTGGAGG AGCAAC
ZmPP2C	Positive regulator in abiotic stress resistance	F	GGAAGCTCCGATAACATCACAGT
		R	TCTTTGTCGTCGCCTGATTTC
ZmCPK11	Expression and activity of antioxidant enzymes	F	CCTCCACGACCCCGACAATG
		R	ACCTCTCCGAG CACCCCAAC
ZmDREB2A/2.1S	Binding proteins responding to dehydration	F	GCAGCCCGGAAGGAAGAA
		R	GATGACAGCTGCCACTGACGTA
ZmDBP3	Binding proteins responding to dehydration	F	CATGAGCTGGGATCTATACTAC
		R	CAAGGTATCAACGTCCTCA
ZmAN13	Regulatory function in response to abiotic stress	F	AGCTGTTGCCCAAGTCGAGTT
		R	GCTGGGTCCGGCAACAT

UBI	Reference gene	F	AAGGCCAAGATCCAGGACAA
		R	TTGCTTTCCAGCGAAGATGA
ADH	Reference gene	F	AGGACGCTGAGTTAAGACC
		R	CACATTTGGCAGATCAGTGC

F – forward sequence; R – reverse sequence.

For the expression analysis of the selected genes, the ABI PRISM 7500 Real-Time PCR (Applied Biosystems) was used, with SYBR Green stain as the detection method. cDNA samples obtained from seed, shoot, and root of eight maize genotypes were used in biological duplicates. The efficiency of the drawn primers was determined by the absolute quantification dilution curve.

In the expression assay, 1 µL of cDNA (diluted 1: 5), 0.4 µL of primer forward/reverse (10 µM) and 5 µL of Master MixSYBR green (Applied Biosystems) were used in each reaction, totaling a final volume of 10 µL. Samples were pipetted in technical triplicates, and a control without cDNA (NTC) was included for each pair of primers. The results were normalized using Ct's (threshold cycle) obtained by the expression of the reference genes Ubiquitin (UBI) and Alcoholic Dehydrogenase (ADH). The Ct was determined by the number of cycles in which the fluorescence generated within a reaction crosses the baseline (Threshold cycle, Ct). The relative expression was analyzed by the Pfaffl method (2001).

The thermal conditions of the RT-qPCR reaction were: 2 minutes at 50 °C and 10 minutes at 95 °C for the initiation, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C, and ending with 15 minutes at 95 °C. At the end of the cycling, a denaturation curve of 60-95 °C showed the specificity of the PCR reaction. The data were collected, exported by the program 7500 Fast Software (Version 2.1) and analyzed in an Excel spreadsheet (Microsoft).

3. Results and Discussion

Enzyme activity

Higher expression of catalase (CAT) was observed in the roots under stress conditions, in the four lines evaluated (Figure 1). In general, the hybrid seeds presented lower expression of this enzyme when compared to the lines independent of the evaluated tissue. Higher expression of CAT was also observed in the aerial part of seedlings under drought stress in six of the eight genotypes evaluated. In the hybrids, higher expression was found in the aerial part in both conditions. In the 32-T, 91-T and 57-NT lines, higher expression of the CAT enzyme was observed in seeds.

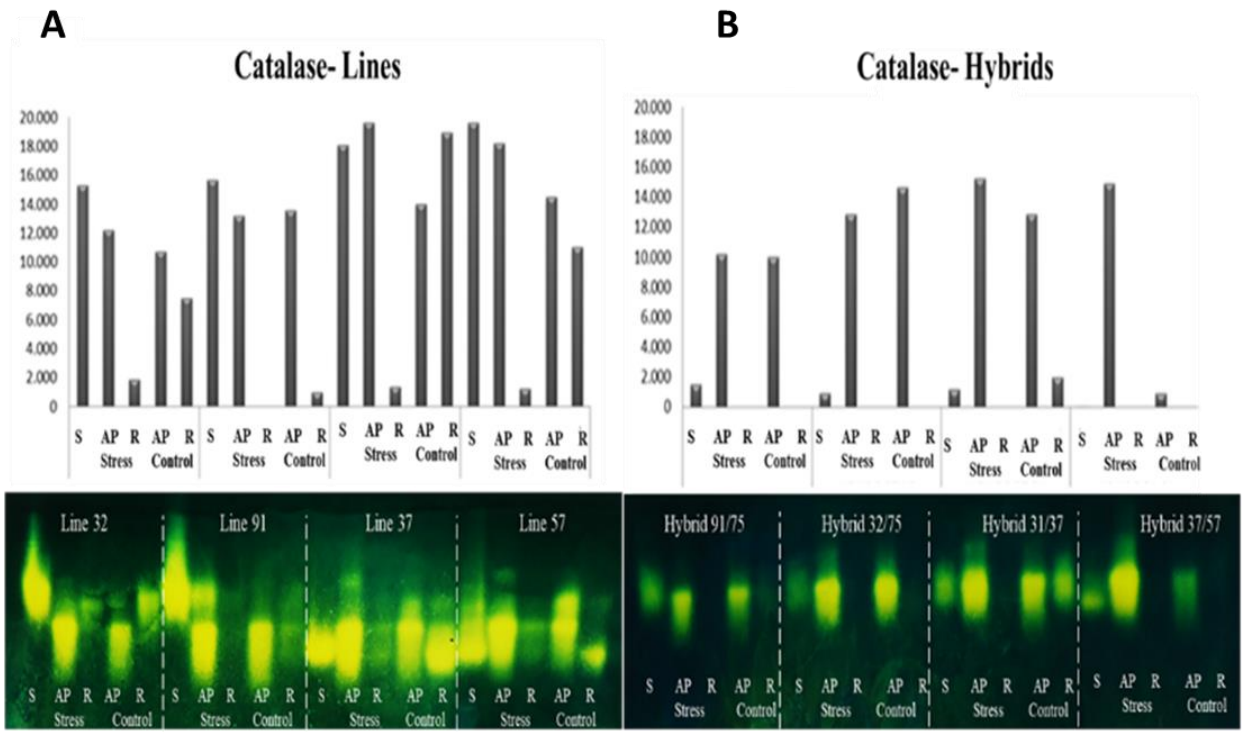

Figure 1. A – expression of the Catalase (CAT) enzyme under two conditions (Stress and Control) of two tolerant lines (T), 91-T and 32-T, and two non-tolerant lines (NT), 57-NT and 37-NT, to drought stress; B – expression of the Catalase (CAT) enzyme under two conditions (Stress and Control) of four hybrids, being two tolerant hybrids, 91x75-T and 32x75-T, and two non-tolerant, 37x57-NT and 31x37-NT. Protein quantification was also performed by ImageJ® software, in pixel^2 (2016).

Catalase is an antioxidant enzyme that catalyzes the conversion of hydrogen peroxide (H_2O_2) to water (H_2O), and its lower activity may be associated with a decrease in oxidative damage prevention mechanisms (Dutra et al. 2015). On the other hand, it has been observed in other research, in different species, the increase of the expression of this enzyme under stress conditions (Deuner et al. 2011).

Like CAT, peroxidase (PO) acts to convert H_2O_2 to O_2. In all genotypes, greater expression of PO was observed in seedling roots under stress condition when compared to that observed in seedlings in the control condition. For all genotypes, an isoform of the enzyme, identified by the arrows in figure 2, can also be observed which was expressed more in roots under the stress condition.

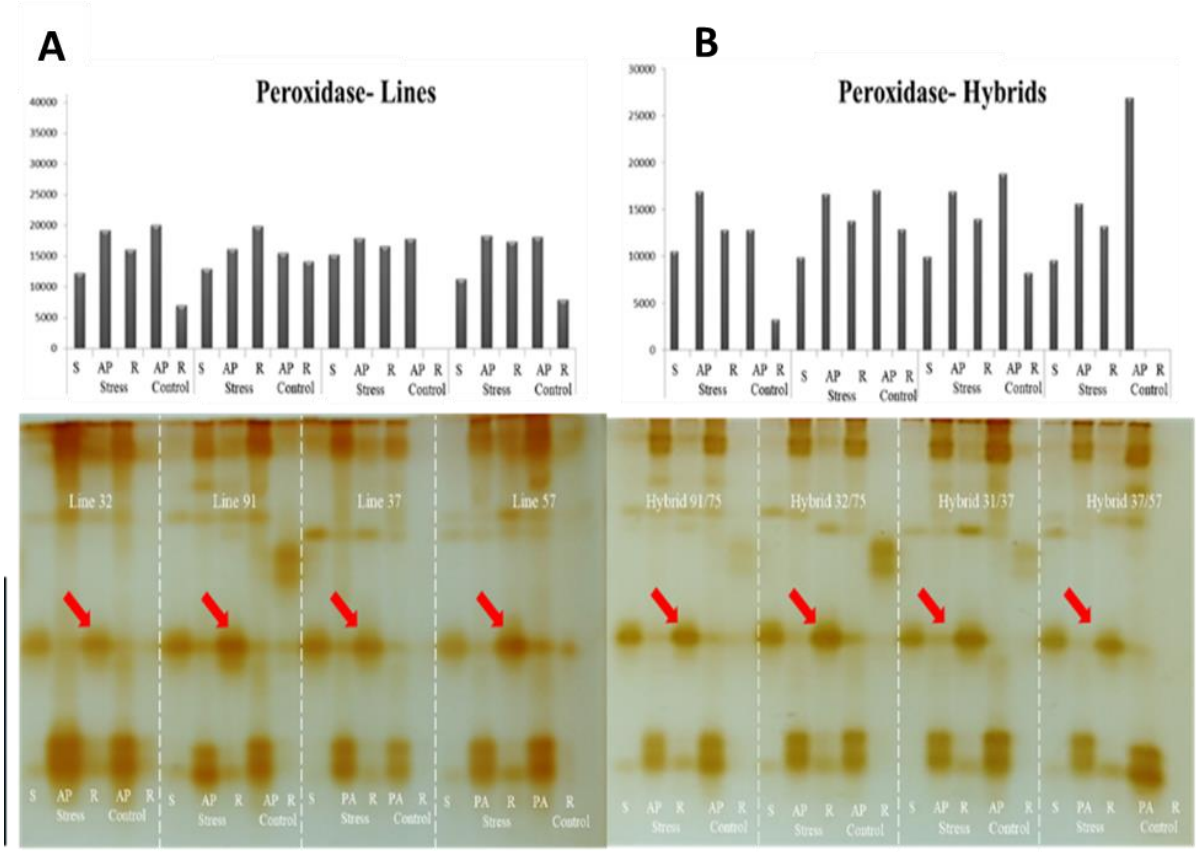

Figure 2. A – expression of the Peroxidase (PO) enzyme under two conditions (Stress and Control) of two tolerant lines (T), 91-T and 32-T, and two non-tolerant lines (NT), 57-NT and 37-NT, to drought stress; B – expression of the Peroxidase (PO) enzyme under two conditions (Stress and Control) of four hybrids, being two tolerant hybrids, 91x75-T and 32x75-T, and two non-tolerant, 37x57-NT and 31x37-NT. Protein quantification was also performed by ImageJ® software, in pixel^2 (2016). The isoform of the enzyme peroxidase (PO) is identified by the arrows.

Except for the PO expression observed in roots under stress condition, higher expression of this enzyme was observed in the aerial part independently of the water retention capacity used. This fact can be explained due to greater respiration, which implies a greater production of free radicals and consequently greater activation of antioxidant systems, such as the peroxidase enzymes. (Silva-Neta et al. 2015).

The esterase enzyme (EST) is one of the most polymorphic isoenzyme systems in plants. EST is an enzyme that is involved in hydrolysis reactions of sterols, which is directly linked to lipid metabolism, such as membrane phospholipids (Santos et al. 2005). Higher expression was observed in seeds for the 91x75-T, 32x75-T, 31x37-NT, 32-T and 91-T genotypes (Figure 3).

There is a greater expression of EST in the seedlings for the 37-NT and 57-NT lines and in the hybrids that have these lines as parental (31x37-NT and 37x57-NT). Lower expression of this enzyme was observed in the 32-T and 91-T lines, regardless of the condition of water retention capacity in the substrate. In the presence of drought stress, there was a greater expression of EST in the seedlings, both for the lines and for the hybrids, except for the 31x37-NT hybrid, in which there was a greater expression in roots under these conditions.

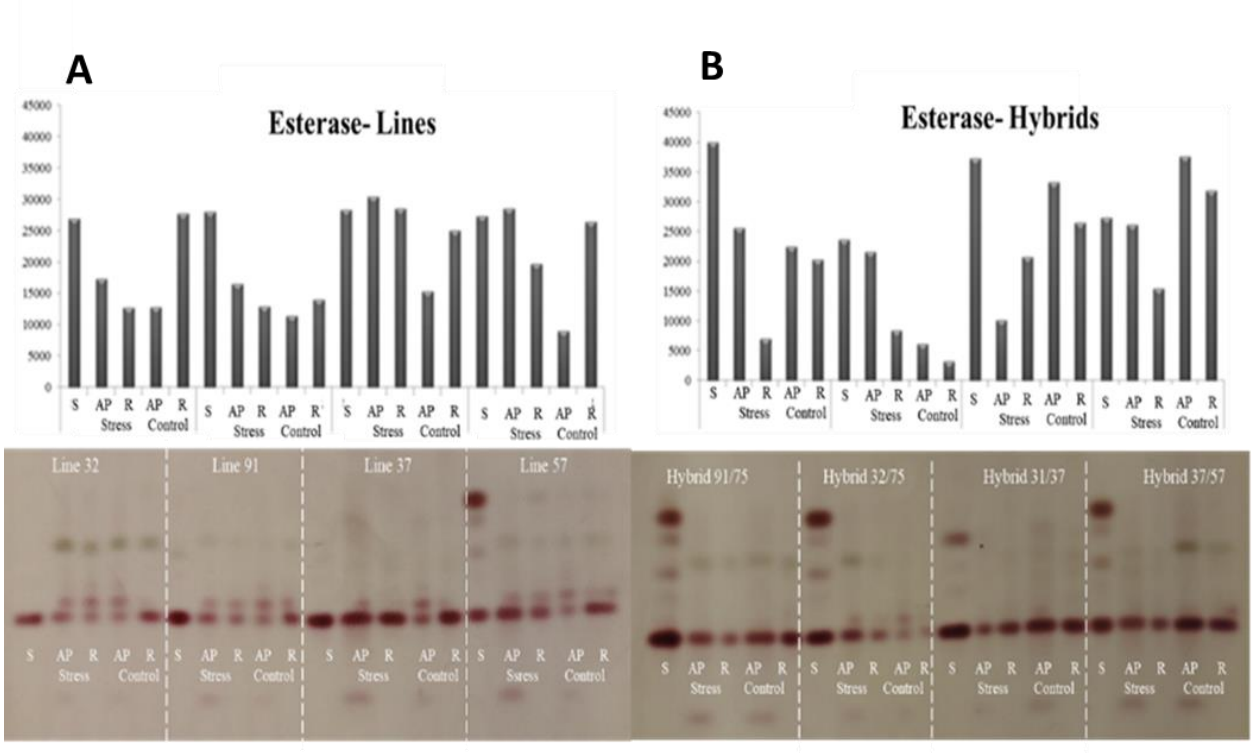

Figure 3. A – expression of the Esterase (EST) enzyme under two conditions (Stress and Control) of two tolerant lines (T), 91-T and 32-T, and two non-tolerant lines (NT), 57-NT and 37-NT, to drought stress; B – expression of the Esterase (EST) enzyme under two conditions (Stress and Control) of four hybrids, being two tolerant hybrids, 91x75-T and 32x75-T, and two non-tolerant, 37x57-NT and 31x37-NT. Protein quantification was also performed by ImageJ® software, in pixel^2 (2016).

Heat-resistant proteins (HRP) are accumulated at the end of the physiological maturity of the seeds and are involved in desiccation tolerance. According to Boucher et al. (2010), an important feature of dry state survival is the ability to protect transient membranes, preventing loss of integrity. Heat-resistant proteins are responsible for the protection and stabilization of the cell membrane. A significant relation between the heat-resistant proteins expression and the physiological quality of the seeds has been described in Abreu et al. (2018) and Silva-Neta et al. (2015).

In Figure 4, the expression of these proteins was observed only in seeds. Andrade et al. (2013) concluded that HRPs differentially express in different genotypes and at different stages of seed development. It also observed that the highest expression is observed in embryos of maize seeds. Lower expression of these proteins was observed in seeds of the 37-NT line and the 32x75-T hybrid.

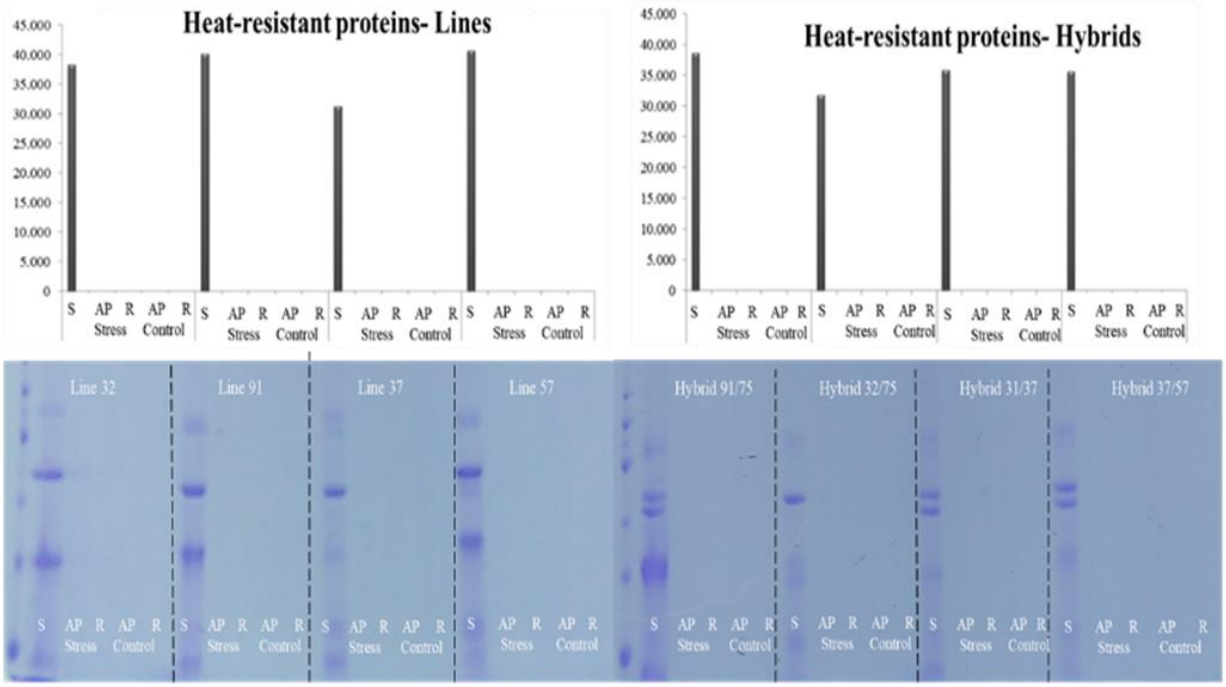

Figure 4. A – expression of the Heat-resistant protein (HRP) enzyme under two conditions (Stress and Control) of two tolerant lines (T), 91-T and 32-T, and two non-tolerant lines (NT), 57-NT and 37-NT, to drought stress; B – expression of the Heat-resistant protein (HRP) enzyme under two conditions (Stress and Control) of four hybrids, being two tolerant hybrids, 91x75-T and 32x75-T, and two non-tolerant, 37x57-NT and 31x37-NT. Protein quantification was also performed by ImageJ® software, in pixel^2 (2016).

The expression of CAT, PO and HRP proteins varies between genotypes and also in the different tissues evaluated. CAT was expressed more in lines than in hybrids. CAT higher expression was also observed in the aerial part of seedlings, mainly in the stress condition. In roots, in general, the expression of this enzyme was lower in the stress condition.

The PO was also expressed more in the aerial part of seedlings. When comparing the two environments, with and without stress, there was a greater expression of this enzyme in roots when the seedlings were developed under drought stress.

All plant tissues are affected by drought stress, however, the root is the first organ to detect variations in the water content available in the soil. It is observed in drought conditions a stimulus in root growth (Pinheiro et al. 2005). The investment in root growth, oppose to the aerial part, favors the exploration of a larger area of soil in search of water, whose availability is greater in deeper regions of dry soils (Rodríguez-Gamir et al. 2010).

In relation to EST, there was a significant variation of the expression between genotypes and tissues. Under the stress condition, the expression was higher in the aerial part of seedlings. In the control environment, the lines had higher expression in the roots, and the hybrids a higher expression in the aerial part of seedlings.

Transcript analysis

In general, the *ZmLEA3*gene had a greater expression in the aerial part in the control condition, except in the 91-T line in which the expression was higher in seeds (Figure 5A).

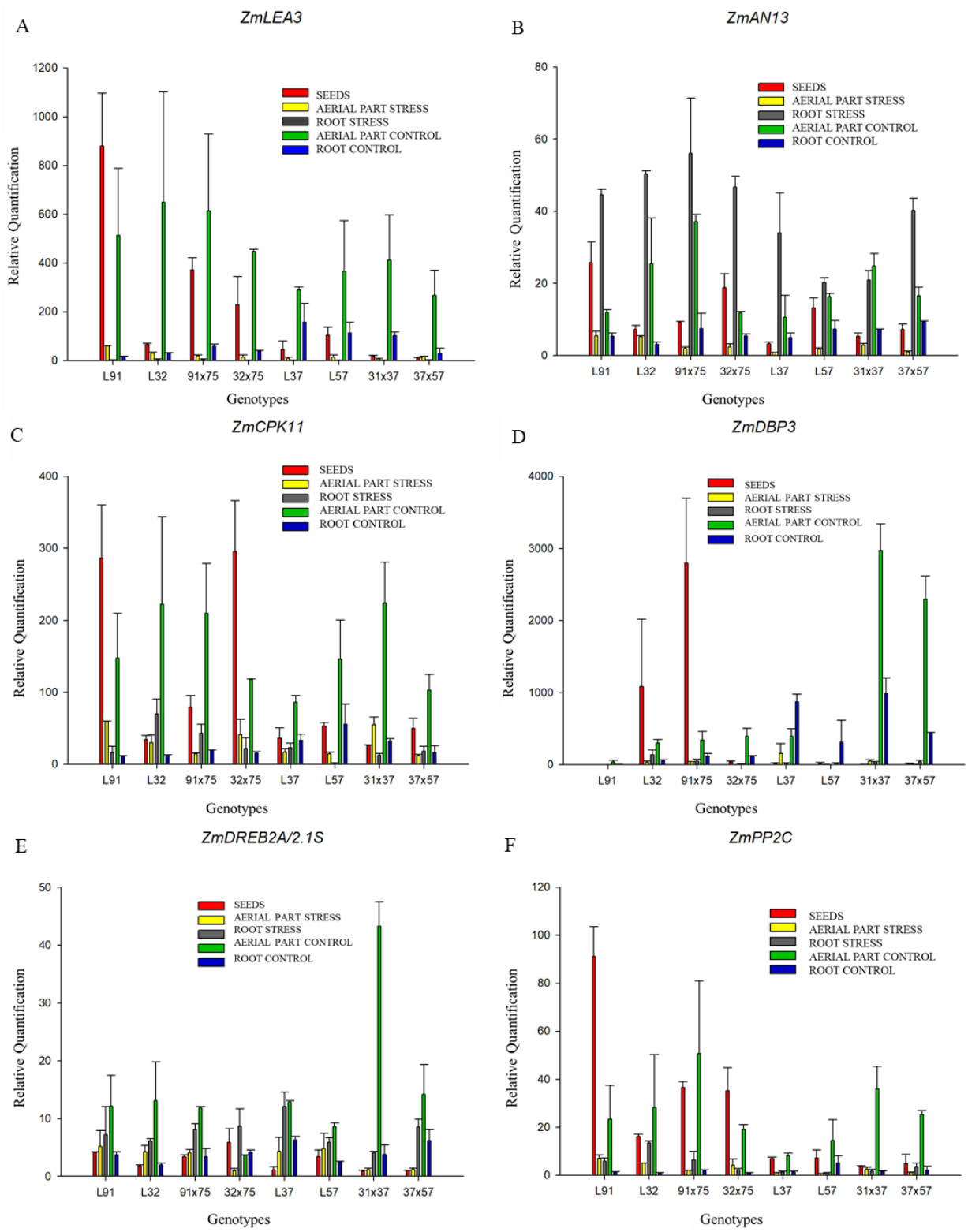

Figure 5. A – relative quantification of the expression of *ZmLEA3* gene; B – relative quantification of the expression of *ZmAN13* gene; C – relative quantification of the expression of *ZmCPK11* gene; D – relative quantification of the expression of *ZmDBP3* gene; E – relative quantification of the expression of *ZmDREB2A/2.1S* gene; F – relative quantification of the expression of *ZmPP2C* gene. The expression was evaluated in seeds, aerial part and root under two conditions (Stress and Control) of eight maize genotypes, two tolerant lines (T), 91-T and 32-T, and two non-tolerant lines (NT), 57-NT and 37-NT, to drought stress and four hybrids, being two tolerant hybrids, 91x75-T and 32x75-T, and two non-tolerant, 37x57-NT and 31x37-NT.

LEA proteins have been widely studied and reported as participants in various developmental processes and accumulated in response to stresses such as drought, salinity, low temperatures or treatment with the ABA phytohormone (Shao et al. 2005). The *ZmLEA3* gene that is expressed under stress conditions

and an overexpression of this gene in tobacco resulted in an increase in tolerance to osmotic and oxidative stress (Liu et al. 2013).

When evaluating the expression of the *ZmLEA3* gene in the aerial part of seedlings in the control condition, lower values were observed in seeds of genotypes 37-NT and 37x57-NT, which are classified as non-tolerant. It is important to note that for all genotypes there was a reduction of ZmLEA3 expression in roots and aerial part, under stress condition. Considering the evaluated genotypes, there was a higher expression in the 91-T line. Lower gene expression was observed in seeds of the 32-T, 37-NT, 57-NT, 31x37-NT and 37x57-NT.

The relative expression of the *ZmAN13* gene is shown in Figure 5B. In the stress condition, it can be observed an increase of expression in the roots of seedlings. It is noteworthy that in the tolerant and responsive genotypes (91-T, 32-T, 91x75-T and 32x75-T) there is a greater expression in roots in the stress condition than in the roots of the other genotypes. In seeds, the highest expression was observed in the 91-T line.

In all genotypes, a lower expression was observed in the aerial part of seedlings submitted to stress, when compared to the expression observed in the control. The functional characterization of the *ZmAN13* gene, a member of the ZNF-AN1 family in maize plants was done by Xuan et al. (2011), whose expression is induced by one or several abiotic stresses.

The *ZmCPK11* is involved in the induction of antioxidant defense. High relative gene expression was observed in seeds of the 91-T line and the 32x75-T hybrid followed by the 91x75-T hybrid that are classified as tolerant and responsive genotypes (Figure 5C). In all genotypes, there was a reduction of ZmCPK11 gene expression in the aerial part of seedlings submitted to stress. This reduction was also observed in roots of the genotypes 37-NT, 57-NT, 31x37-NT, under stress conditions, these being classified as non-tolerant to drought stress.

Szczegielniak et al. (2005) observed that *ZmCPK11* transcripts were present in tested organs of the maize plant. The expression was relatively higher in seeds and seedlings and lower in the stems, roots, and leaves.

Among the genotypes classified as tolerant, there was a greater expression of *ZmDBP3* in seeds of the 32-T line and the 91x75-T hybrid and lower in line 91-T. In the other genotypes the expression of this gene in seeds was small or null. In all genotypes, there was expression of this gene in the aerial part of seedlings developed in the control condition, with emphasis on the expression in this condition in the 31x37-NT and 37x57-NT genotypes (Figure 5D). Overexpression of the *ZmDBP3* gene extracted from maize leaves of seedlings increased the tolerance to cold and drought in transgenic plants of Arabidopsis (Wang et al. 2009).

In general, there was a lower expression of this in different tissues for the *ZmDREB2A/2.1S* gene. For most genotypes, the expression was relatively higher in the aerial part of seedlings in the control condition. Here, the expression in roots was higher when grown under the stress condition. Liu et al. (2013) found that *ZmDREB1*-like genes were significantly expressed in roots (Figure 5E).

In all genotypes, there was a lower expression of the ZmDREB2A/2.1S gene in the aerial part of seedlings, and higher in roots under stress conditions. The DREB gene encodes a transcription factor that is involved in the activation of other genes related to tolerance to drought. DREB proteins act at the top of the cascade of molecular events, inducing defense responses against cell dehydration (Maruyama et al. 2009).

The relative expression of the *ZmPP2C* gene is shown in Figure 5F. In tolerant and responsive genotypes, there was a greater relative expression in seeds, with emphasis on the 91-T line, in relation to the expression of this in seeds of genotypes classified as non-tolerant to water stress. Liu et al. (2009) observed overexpression of the *ZmPP2C* gene also in Arabidopsis plants exposed to water and saline stresses.

In all genotypes, there was a reduction of *ZmPP2C* expression in the aerial part of seedlings developed under drought stress. In the genotypes classified as tolerant there was a higher expression of this gene under stress conditions than that observed in roots developed in control condition. The *ZmPP2C* gene may act as a positive regulator in abiotic stress resistance. (Hu et al. 2010).

In relation to the transcript expressions, the 91-T line had greater expression of the *ZmLEA3*, *ZmAN13*, *ZmCPK11* and *ZmPP2C* genes was observed in seeds. Abreu et al. (2017) also evaluated this line and

considered it promising for drought tolerance by evaluations of grain yield, prolificacy, and intervals between female and male flowering. When evaluating genotypes with different levels of tolerance at high temperatures, Dutra et al. (2015) concluded that the 91-T line is more tolerant in high-temperature conditions. Considering these results, it is inferred that the 91-T line is promising for drought and high temperatures tolerance.

4. Conclusions

The expression of all enzymes varied between genotypes and also in the different tissues evaluated. CAT higher expression was also observed in the aerial part of seedlings, mainly in the stress condition. There was a greater expression of PO in roots when the seedlings were developed under drought stress. There was a significant variation of the EST expression between genotypes and tissues and in the control environment, the lines had higher expression in the roots. Through the proteomic analysis, it was not possible to associate a marker with drought stress tolerance, considering the genotypes classification.

Most of the evaluated genes have a higher expression in the aerial part of seedlings when developed in substrate with the capacity of water retention favorable for the development of seedlings (70% of the capacity of water retention). However, it is worth mentioning the higher expression of the *ZmPP2C* gene in seeds of the 91-T line which was classified as tolerant to drought stress.

Authors' Contributions: SANTOS, M.C.: conception and design, acquisition of data, analysis and interpretation of data, drafting the article; VON PINHO, E.V.R.: analysis and interpretation of data, drafting the article; SANTOS, H.O.: analysis and interpretation of data, drafting the article; VILELA, D.R.: acquisition of data, drafting the article; SILVA NETA, I.C..: acquisition of data, analysis and interpretation of data; ABREU, V.M.: acquisition of data, analysis and interpretation of data; VASCONCELLOS, R.C.C.: analysis and interpretation of data, drafting the article. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

Acknowledgments: The authors would like to thank the funding for the realization of this study provided by the Brazilian agencies CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil), Finance Code 001, and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brasil).

References

ABREU, V.M., et al. Combining ability and heterosis of maize genotypes under water stress during seed germination and seedling emergence. *Crop Science*. 2018, **59**(1), 33-43. https://doi.org/10.2135/cropsci2018.03.0161

ABREU, V.M., et al. Indirect selection for drought tolerance in maize through agronomic and seeds traits. *Revista Brasileira de Milho e Sorgo*. 2017, **16**(2), 287-296. https://doi.org/10.18512/1980-6477/rbms.v16n2p287-296

ALFENAS, A.C. *Eletroforese de isoenzimas e proteínas afins: fundamentos e aplicações em plantas e microrganismos*. 2 ª ed. Viçosa, MG: Universidade Federal de Viçosa, 2006.

ANDRADE, T., et al. Physiological quality and gene expression related to heat-resistant proteins at different stages of development of maize seeds. *Genetics and Molecular Research.* 2013**,** **12**(3), 3630-3642. https://doi.org/10.4238/2013.September.13.7

BOUCHER, V., et al. MtPM25 is an atypical hydrophobic late embryogenesis-abundant protein that dissociates cold and desiccation-aggregated proteins. *Plant, cell & environment.* 2010, **33**(3), 418-430. https://doi.org/10.1111/j.1365-3040.2009.02093.x

CAVERZAN, A., et al. Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection**.** *Genetics and molecular biology.* 2012, **35**(4), 1011-1019. https://doi.org/10.1590/S1415-47572012000600016

CHAI, Q., et al. Regulated deficit irrigation for crop production under drought stress. A review. *Agronomy for Sustainable Development.* 2016, **36**(1), 1-21. https://doi.org/10.1007/s13593-015-0338-6

CHOUDHURY, F.K., et al. Reactive oxygen species, abiotic stress and stress combination. *The Plant Journal*. 2016, **90**(5), 856-867. https://doi.org/10.1111/tpj.13299

DEUNER, C., et al. Viabilidade e atividade antioxidante de sementes de genótipos de feijão miúdo submetidos ao estresse salino. *Revista Brasileira de Sementes.* 2011, **33**(4), 711-720. https://doi.org/10.1590/S0101-31222011000400013

DUTRA, S.M.F., et al. Genes related to high temperature tolerance during maize seed germination. *Genetics and Molecular Research.* 2015, **14**(4), 18047-18058. https://doi.org/10.4238/2015.December.22.31

FINCH-SAVAGE, W.E., BASSEL, G.W. Seed vigour and crop establishment: extending performance beyond adaptation. Journal of Experimental Botany. 2016, **67**(3), 567-591. https://doi.org/10.1093/jxb/erv490

HU, X., et al. Enhanced tolerance to low temperature in tobacco by over-expression of a new maize protein phosphatase 2C, ZmPP2C2. *Journal of plant physiology.* 2010, **167**(15), 1307-1315. https://doi.org/10.1016/j.jplph.2010.04.014

HUSSAIN, H.A., et al. Interactive effects of drought and heat stresses on morpho-physiological attributes, yield, nutrient uptake and oxidative status in maize hybrids. *Scientific reports*. 2019, **9**(1), 1-12. https://doi.org/10.1038/s41598-019-40362-7

KOUSSEVITZKY, S., et al. Ascorbate peroxidase 1 plays a key role in the response of Arabidopsis thaliana to stress combination. *Journal of Biological Chemistry.* 2008, **283**(49), 34197–34203. https://doi.org/10.1074/jbc.M806337200

KRAMER, P.J, 1983. Water deficits and plant growth. In.: KRAMER, P.J. (ed). *Water relations of plants.* New York: Academic Press, pp. 342-389.

LIU, T., et al. Identification of proteins regulated by ABA in response to combined drought and heat stress in maize roots. *Acta physiologiae plantarum.* 2013, **35**(2), 501-513. https://doi.org/10.1007/s11738-012-1092-x

LIU, Y., et al. A maize early responsive to dehydration gene, ZmERD4, provides enhanced drought and salt tolerance in Arabidopsis. *Plant Molecular Biology Reporter.* 2009, **27**(4), 542-548. https://doi.org/10.1007/s11105-009-0119-y

LIVAK, K.J. and SCMITTGEN, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta Ct) Method. *Methods,* 2001, **25**(4), 402-408. https://doi.org/10.1006/meth.2001.1262

MARCOS-FILHO, J. *Fisiologia de sementes de plantas cultivadas*. Londrina: ABRATES, 2015.

MARQUES, T.L., et. al. Expression of ZmLEA3, AOX2 and ZmPP2C genes in maize lines associated with tolerance to water deficit. *Ciência e Agrotecnologia*. 2019, **43**, e022519. https://doi.org/10.1590/1413-7054201943022519

MARUYAMA, K., et al. Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. *Plant physiology*. 2009, **150**(4), 1972-1980. https://doi.org/10.1104/pp.109.135327

MILLER, G., et al. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell & Environment*. 2010, **33**(4), 453-467. https://doi.org/10.1111/j.1365-3040.2009.02041.x

PFAFFL, M.W. A new mathematical model for relative quantification in realtime RT–PCR. *Nucleic Acids Research*. 2001, **29**(9), e45-e45. https://doi.org/10.1093/nar/29.9.e45

PINHEIRO, H.A., et al. Drought tolerance is associated with rooting depth and stomatal control of water use in clones of *Coffea canephora*. *Annals of Botany.* 2005, **96**(1), 101-108. https://doi.org/10.1093/aob/mci154

RIBAUT, J.M., BETRAN, J., MONNEVEUX, P. and SETTER, T., 2009. Drought tolerance in maize. In BENNETZEN, J.L. and HAKE, S.C. (Ed.). *Handbook of maize: its biology*). New York: Springer, pp. 311-344.

RODRÍGUEZ-GAMIR, J., et al. Citrus rootstock responses to water stress. *Scientia horticulturae.* 2010, **126**(2), 95-102. https://doi.org/10.1016/j.scienta.2010.06.015

SANTOS, C.M.R., et al. Modificações fisiológicas e bioquímicas em sementes de feijão no armazenamento. *Revista Brasileira de Sementes*. 2005, 27(1), 104-114. https://doi.org/10.1590/s0101-31222005000100013

SCHOLDBERG, T.A., et al. Evaluating precision and accuracy when quantifying different endogenous control reference genes in maize using real-time PCR. *Journal of agricultural and food chemistry.* 2009, **57**(7), 2903-2911. https://doi.org/10.1021/jf803599t

SHAO, H., LIANG, Z. and SHAO, M. LEA proteins in higher plants: structure, function, gene expression and regulation. *Colloids and surfaces B: Biointerfaces.* 2005, **45**(3-4), 131-135. https://doi.org/10.1016/j.colsurfb.2005.07.017

SILVA NETA, I.C., et al. Expression of genes related to tolerance to low temperature for maize seed germination. *Genetics and Molecular Research.* 2015, **14**(1), 2674-2690. https://doi.org/10.4238/2015.March.30.28

SZCZEGIELNIAK, J., et al. A wound-responsive and phospholipid-regulated maize calcium-dependent protein kinase. *Plant physiology.* 2005, **139**(4), 1970-1983. https://doi.org/10.1104/pp.105.066472

TAIZ, L. and ZEIGER, E. *Fisiologia Vegetal.* 5ª ed. Porto Alegre, RS: Artmed, 2013.

WANG, C. and DONG, Y. Overexpression of maize ZmDBP3 enhances tolerance to drought and cold stress in transgenic Arabidopsis plants. *Biologia*. 2009, **64**(6), 1108-1114. https://doi.org/10.2478/s11756-009-0198-0

WANG, W., VINOCUR, B. and ALTMAN, A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta.* 2003, **218**(1), 1-14. https://doi.org/10.1007/s00425-003-1105-5

XIONG, L., SCHUMAKER, K.S. and ZHU, J.K. Cell Signaling during Cold, Drough, and Salt Stress. *The Plant Cell.*2002, **14**(1), 165-183. https://doi.org/10.1105/tpc.000596

XUAN, N., et al. A putative maize zinc-finger protein gene, ZmAN13, participates in abiotic stress response. *Plant Cell, Tissue and Organ Culture (PCTOC).* 2011, **107**(1), 101-112. https://doi.org/10.1007/s11240-011-9962-2

ZHOU, M.L., et al. Aldehyde dehydrogenase protein superfamily in maize. *Functional & integrative genomics.* 2012, **12**(4), 683-691. https://doi.org/10.1007/s10142-012-0290-3

Received: 15 April 2020 | **Accepted:** 21 July 2020 | **Published:** 29 December 2021