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RESEARCH ARTICLE

ENZYMATIC AND PHYSIOLOGICAL ALTERATIONS OF LINES MAIZE SEEDS SUBMITTED TO DIFFERENT TEMPERATURES

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ABSTRACT

During seeds germination period, the temperature is an important factor, which influences the seedling establishment in the field. Thus, tolerant cultivars at both low and / or high temperatures in these phases are fundamental in plant breeding programs. The objective in this work was to verify the enzymatic and physiological alterations of lines maize seeds submitted to different temperatures. We used maize lines (L30, L64, L63, L91) from the maize breeding programs of the company *Geneseeds Genetic Resources* Ltda. Seeds from this lines were submitted to germination test, first count of germination, root protrusion and emergence test at different temperatures of 15, 20, 25, 30 and 35°C. We also calculated the emergence speed index (ESI) and the expression of catalase, esterase, malate dehydrogenase, alcohol dehydrogenase and α-amylase enzymes, for all treatments. The experimental design was completely randomized, in factorial scheme of 4x5, with four lines and five temperatures. The maize lines 30 and 64 are susceptible to high temperatures of germination. The temperature variations compromised the physiological quality of lines maize seeds. There is variation in the expression of ADH, MDH, catalase, esterase and alpha amylase enzymes in maize lines of the evaluated lines.

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INTRODUCTION

The climatic conditions in different regions of Brazil are diversified and responsible for the most part of the environmental stresses. The temperature variations can cause alterations in the biochemical and physiological processes, what can directly influence on quality seeds affecting thus, the final stand of plants in the production field, consequently resulting in loss of productivity (Marini *et al*., 2012). With this, the temperature becomes one of the most important factors in seeds germination due to their influence in the absorption of water and others solutions required to the seedlings growth and development (Nerson, 2007). Each specie has a spectrum of limiting and optimum temperature, not only for the final percentage of germination, like also for the speed of germination in which this will occur. The maize seeds to germinate need an adequate moisture and temperature of soil superior to10ºC, and in favorable conditions takes 4 to 7 days to emerge.

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To be a plant from tropical climate, which demand moisture and heat, adverse temperatures of soil promotes low percentage of germination and emergence of their seedlings. Thus, the stress caused by temperature, when occurs for a sufficient period of time, causes irreversible damages to the development of seedlings (Hall, 2001). In low temperatures, the imbibitions can occur, but could be not followed by the embryo growth, or yet, could induce damages to the embryo or to the seedlings, preventing the conclusion of development process. Guan *et al*. (2009) reported that low temperatures induces damages on cellular membranes and also, affect the physiological functions, beyond delay or prevent the germination process, leaving the seeds more susceptible to adverse factors. In turn, the high temperatures can allow the imbibition, but do not allow the embryo growth and the establishment of seedling (Matheus and Lopes, 2009).

Delouche (2004) reported that with the increase of temperature above of the optimal value, the germination becomes more slow and the seeds less vigorous, not resisting to the stress imposed by the environment. Under these stress conditions, the

growth of plants is reduced and the reserve compounds are directed to keep the metabolism active in organs with preferential growth, beyond the enzymatic activation of the antioxidant system. With this, there is an increase in the activity of enzymes like, superoxide dismutase (SOD) that catalyses the dismutation of the superoxide radical into H_2O_2 and O_2, constituting so, an important primary defense against the free radical generated under stress conditions (Carneiro *et al*. 2011). However, if the stress of high and low temperatures be more several a lot of damages can occurs, beginning with lipid peroxidation and advancing for the membranes degradation, and eventually, cellular death (Marini *et al*., 2013).

Inside this context and having the knowledge that the temperature influences on seeds viability and vigor interfering in their metabolic and physiologic process, especial attention are given to this factor, once the temperature directly influence in the final quality of seeds (Mendes *et al*., 2009). With this, the previous characterization of genotypes which presents tolerance to thermal stress, through biochemical and physiological evaluations, can provide parameters that aim in the selection of new cultivars, being essentials in breeding programs, what justify the study related to this stress during the seeds germination process. Based on the above, the aim in this research was to verify the enzymatic and physiological alterations in maize seeds lines submitted to different temperatures.

MATERIALS AND METHODS

The research was conducted at Central Laboratory of Seeds, in the Department of Agricultural of Universidade Federal de Lavras (UFLA), in Lavras-MG. Were used four lines of maize (L30, L64, L63, L91) from the maize breeding program of the Geneseeds Recursos Genéticos Ltda company, produced in 2013 crop. The water content was determined by the oven method at 105° C for 24 hours using two replications of 50 seeds from each treatment (BRASIL, 2009). After this period, seeds were taken to desiccators until the samples cooling and after, was realized the seeds dry weight. The results were expressed in percentage. The germination test was conducted with four replications of 50 seeds, with the sowing in moistened germitest paper in the proportion of 2,5 ml of water per g of paper. Seeds were kept in germinator with 12 hours of light and 12 hours of dark, regulated in the temperatures of 15, 20, 25, 30 e 35ºC.

Conduced together with germination test, was evaluated the percentage of root protrusion and the first count of germination, computing the normal seedlings in the fourth day after sowing (BRASIL, 2009). At seventh day was done the final count of germination and the results were expressed in medium percentage of normal seedlings (BRASIL, 2009). The emergence test was conducted with eight replications of 20 seeds. The sowing was realized in stainless steel containers of 1200 cm^3, containing the mix of soil and sand (2:1), having the field capacity adjusted to 60%. Following, the mixture was placed in bain marie proper for vessels (Alves e Campos, 2001) and installed in acclimatized greenhouse with medium temperature of 25ºC. The temperatures used in bain marie were the same of those used in the germination test (15, 20, 25, 30 and 35 ± 0,5ºC). In bain marie were placed thermometers immersed in substrate which were contained in stainless steel containers to registry the internal temperature of the containers. The monitoring of soil temperature was done twice a day, being the first evaluation in the beginning of morning and the second one in the end of afternoon.

Were realized daily evaluations from the beginning of seedlings emergence, counting the number of emerged seedlings until the stabilization of the stand. Was considered the percentage of normal seedlings at fourteenth day. The emergence speed index was also realized together with germination test, daily and at the same time computing the number of emerged seedlings until the stabilization of stand. The emergence speed index was calculated according Edmond and Drapala (1958). For the enzymatic evaluations were collected two samples of 50 seeds of each treatment kept in the same conditions of the germination test for a period of 72 hours. These seeds were macerated with PVP (polyvinylpyrrolidone) and liquid nitrogen in small container and afterwards stored at -86ºC temperature. For the enzymes extraction was added the extraction buffer (Tris HCl 0,2 M pH 8 + 0,1% of β-mercaptoethanol) in the proportion of 250µL for 100mg of seeds powder. The material was homogenized in vortex and kept in refrigerator during 12 hours followed by the centrifugation at 14000 rpm for 30 minutes at 4ºC and them, applied in polyacrilamide gel.

The electrophoretic run was realized in a discontinuous polyacrylamide gel system at 7,5% (separating gel) and 4,5% (concentrating gel) using Tris-glycine pH 8,9 as standard buffer in the gel electrode system. In each gel channel, was applied 60 µL of the sample supernatant and the running was performed at 150 V for 5 hours. At the end of running, the gels were revealed for the enzymes esterase (EST- EC 3.1.1.1.), catalase (CAT- EC.1.11.1.6.), alpha amylase (α-AMI- EC 3.2.1.1.), alcohol dehydrogenase (EC- 1.1.1.1) and malate dehydrogenase (MDH- EC 1.1.1.37) according the protocols established by Alfenas *et al*. (2006). The evaluation of the gels was realized on transilluminator, being considered the variation of intensity of bands. Was used the completely randomized experimental design in a factorial scheme of (4X5) being four maize lines (L30, L64, L63 and L91) and five different temperatures (15, 20, 25, 30 and 35ºC). The data, previously submitted to the normality tests and homocedasticity of variances, were submitted to analysis of variances and the averages were compared by the Scott-Knott test at 5% of probability. The statistical analyzes were realized with aid of SISVAR® statistical program (FERREIRA, 2011).

RESULTS AND DISCUSSION

Based on variance analyses, was possible to observe significant differences between the maize lines and between the temperatures of germination and emergence, as well as for the interaction of the evaluated factors ($p < 0,05$). Similar results were reported by Vaz-de-Melo *et al*. (2012) evaluating the temperature effect in popcorn cultivars. These authors verified that occurred significant interaction in the germination, shoot length, fresh mass and dry mass. The

medium water content of seeds in the moment of the test was of 12,8 % with maximum variation of 1%. For the root protrusion datas was possible to observe that there was no significant difference between maize lines in the temperatures of 15 and 25 ºC. In the temperature of 20 ºC, the maize lines L63 an L91 did not present statistical difference for root protrusion, however these lines were superior in comparison with L30 and L64 (Table 1).

Table 1. Root protrusion (%) of lines maize seeds submitted to different temperatures during the germination

Temperature (°C)	Maize Lines			
	L30	L64	L63	L91
15	100 aA	100 aA	100 aA	100 aA
20	92 cB	96 bB	100 aA	98 aA
25	100 aA	100 aA	100 aA	100 aA
30	93 bB	96 aB	98 aA	99 aA
35	93 bB	96 aB	99 aA	97 aA
CV				2,10

Means followed by the same lower case letter in the line and capital letter in the column do not statistically differ by the Scott-Knott test at 5% significance.

In temperatures higher than 30ºC the line L30 presented results that characterizes this line like most sensible to the temperature. Analyzing the temperature inside each line, was verified that the lines L30 and L64 presented the same behavior, being that some temperatures (20, 30 and 35°C) reduced the root protrusion percentage. The lines L63 and L91 did not present variations in root protrusion when submitted to different temperatures. For the most part of species the optimum temperature of germination, where exists higher germinability achieved in a short period of time, is between 15and 30°C; the maximum varies between 35 and 40°C, and the minimum can reach the freezing point. Above the maximum temperature, seeds generally die in few days and below the minimum temperature, seeds do not germinate within a reasonable period of time (Vaz-de-Melo *et al*., 2012).

Table 2. Normal seedlings (%) in the first count of germination Root protrusion (%) of lines maize seeds submitted to different temperatures during the germination

Temperature (°C)	Maize Lines			
	L30	L64	L63	L91
15	65 aC	61 aC	21 bC	20 bC
20	66 aC	63 aC	32 bC	39 bB
25	90 aA	92 aA	89 aA	95 aA
30	73 cB	87 bB	94 aA	99 aA
35	22 cD	16 cD	77 bB	91 aA
CV				3,25

Means followed by the same lower case letter in the line and capital letter in the column do not statistically differ by the Scott-Knott test at 5% significance.

In data's related to the first count of germination (Table 2), was observed that the lines L30 and L64 tolerates low germination temperature of 15ºC, with values of 65 and 61% of normal seedlings in the first count of germination, respectively, followed by the lines L63 and L91 with medium results of 21 and 20%. However, when was used the temperatures of 30 and 35ºC was observed that the lines L63 and L91 were statically superior that the lines L30 and L64. Between the lines was possible to observe higher sensibility in the materials L30 and L64 in high temperature of germination, as well as the L63 and L91in low temperatures. Thus, the low temperatures are considered one of the limiting factors in the productivity of plants and, frequently cause damages in the germination and in the development of maize seedlings, as well as, the high temperatures can allow the imbibition but do not allow the embryo growth and the seedling establishment (Matheus and Lopes, 2009).

Table 3. Germination (%)Root protrusion (%) of lines maize seeds submitted to different temperatures during the germination

Temperature (°C)	Maize Lines			
	L30	L64	L63	L91
15	73 aC	68 bC	27 cD	29 cD
20	79 aB	70 bC	41 cC	43 cC
25	94 aA	94 aA	97 aA	97 aA
30	75 cC	89 bB	96 aA	99 aA
35	28 cD	23 dD	82 bB	93 aB
CV				4,38

Means followed by the same lower case letter in the line and capital letter in the column do not statistically differ by the Scott-Knott test at 5% significance.

The use of seeds with high germinative capacity and vigor is essential for the quick and uniform establishment of the plants stand when this is found under huge diversity of environmental conditions (Del Giúdice *et al*., 1998). So, high values of germination and vigor are essential to determine the quality of lots, as well as the indication of materials that better adapt to different climate conditions found in different maize areas production around the country. In relation to the percentage of germination, was possible to observe that the lines presented variations when germinated at 15 and 20°C, being that the L30, have values superior (73 and 79%) when compared to the others lines (Table 3). When it is observed the temperature of 35°C, the line L91 was superior than the others with medium average of 93%. Li *et al*. (2013), working with induction to cold tolerance in wheat seeds during the germination, verified that the seeds when germinated at 12ºC, presented germination taxes inferior than the germination of the same materials at 22ºC, For lines which tolerate low temperatures of germination (L30 e L64), was possible to observe that the high values of germination were observed in temperatures of 25°C.

For the lines L63 and L91, the high percentages of germination were observed in the temperatures of 25 and 30^{o}C and these results are in agreement with those observed in the first count of germination, as well as with the root protrusion. In this sense, the choice of high quality seeds and adapted to the local conditions can be the reason of a crop production success or failure (Sans and Santana, 2005). The temperatures of 15 and 20°C also contributed for the emergence reduction in L63 and L91 when compared to L30 and L64 lines. In temperature of 25°C was verified higher emergence index of seeds in all lines (Table 4). The low temperature of soil during the sowing contributes for the delay of seedlings germination and emergence, and consequently affects the emergence speed (Tables 4 e 5). However, must be highlight that the maize presents genetic viability for the germination in low temperatures, therefore is necessary to select the genotypes that presents this characteristic and use in this conditions, ensuring higher successes of the field (Cruz *et al.,* 2007). The

low temperatures (15 and 20°C) delayed the emergence speed for the lines L30, L64 and L91, followed by L63 respectively. In temperature of 25°C was observed higher percentage and consequently a higher vigor of seeds. For the percentage of emergence in this temperature, all the lines did not present statistical differences.

Table 4. Emmergence (%) of lines maize seedlings submitted to different temperatures during the germination

Temperature (°C)	Maize Lines			
	L30	L64	L63	L91
15	40aD	36aC	06bE	12bE
20	54aC	58aB	24cD	28bD
25	96aA	94aA	92aA	96aA
30	68bB	64cB	72bB	78aB
35	10cE	12cD	58aC	48bC
CV				9,27

Means followed by the same lower case letter in the line and capital letter in the column do not statistically differ by the Scott-Knott test at 5% significance

Table 5. Emmergence speed (days) of lines maize seedlings submitted to different temperatures of soil

Temperature (°C)	Maize Lines			
	L30	L64	L63	L91
15	8aC	7aC	9bB	8aC
20	7aB	6aB	8bB	7aC
25	4aA	4aA	5bA	5bA
30	3aA	3aA	4bA	4bA
35	6bB	7cC	5aA	6bB
CV				1,34

Means followed by the same lower case letter in the line and capital letter in the column do not statistically differ by the Scott-Knott test at 5% significance.

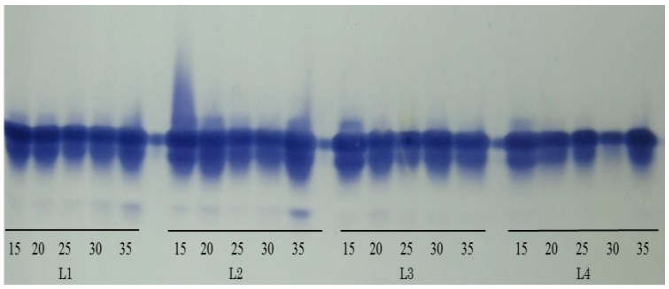

Figure 1. Electrophoretic pattern of alcohol dehydrogenase enzyme (ADH) in maize seeds pre-germinated in different temperatures (L1-30; L2-64; L3-63; L4-91)

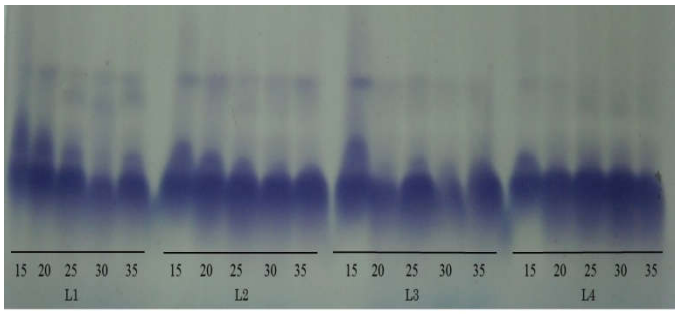

Figura 1. Electrophoretic pattern of malate dehydrogenase enzyme (MDH) in maize seeds pre-germinated in different temperatures (L1-30; L2-64; L3-63; L4-91)

However, with the increase of temperature to 35°C, can be observed a delay on seedlings emergence, which minimizes the emergence speed for the lines L30, L91 and L64, respectively. Statistically superior, the L63 presented better vigor than the others, in the emergence speed (Table 5). According Cruz *et al*. (2007), low soil temperatures delay the germination, decrease the reserve remobilization and consequently the emergence speed. Temperatures above the value considered optimum to the germination, also takes to the delay in the germinative process taking seeds to the low vigor (Delouche, 2004). In general, in the temperatures of 15 and 20°C could be observed better performance from lines L30 and L64. Already the lines L63 and L91, presented better results in the factors evaluated in temperatures of 30 and 35°C.In temperature of 25°C, all the lines presented good physiological performance.

Like verified in this work, the temperature can cause significant difference on germination, as well as on vigor of maize seeds. These differences of the behavior in the germination and emergence related to the temperature, cause also, deterioration in level of membranes and also interference in respiratory process (Coutinho *et al*., 2007; Mendes *et al*., 2009). Thus, the alterations in physiological quality of seeds are directly related to the integrity of enzymes and structural protein. According Devi *et al*. (2007) is fundamental to verify also the answer of thermal stress in seeds, through the activity of enzymes associated to the reserve hydrolyses, to respiration as well as, by the integrity of cellular membranes by being these, associated to the essential process of germination. Can be observed in Figures 1 and 2 that the lines L30 and L64 presented an increase in the activity of enzymes ADH and MSH. These results can be explained by an increase of the germinative metabolism with elevation of germination temperature. So, the activity of these enzymes contributed for the maintenance of physiological quality even in adverse situations.

The lines L63 and L91 presented reduction on enzymatic activity with the increase of temperature, being these lines classified like tolerant materials to high temperature of germination, based on physiological tests also. However, can be noted that, as well as ADH, the MDH increased the enzymatic activity in temperature of 15°C. The ADH enzyme acts in the anaerobic metabolism, where the acetaldehyde is reduced to ethanol, a compound with low toxicity, and reduces the speed of the deterioration process (Veiga *et al*., 2010). When the activity of ADH enzyme decreases, the seeds becomes more susceptible to the deleterious actions of the acetaldehyde (Zhang *et al*., 1994). So, this explains the increase of their activity in temperatures of 15°C. The MDH enzyme is connected to the energy generation for metabolic process, like seeds germination and in the movement of malate through the mitochondrial membrane generating energy and fixing CO_2 in pants (Taiz and Zeiger, 2009).

Thus, the main alterations related to this enzyme is connected to the seeds deterioration process, due to the reduction in the respiratory activity, causing the degradation and the inactivation of other enzymes (Copeland and McDonald, 2001). The high activity of this enzyme at 15°C, contributed also, for the higher germination of L30 and L64 than the others

lines. In the enzymatic evaluation of esterase (Figure 3), was observed that independently of the evaluated lines be tolerant or no to high temperatures of germination, there was lower activity of this enzyme in L63. However, this same line presents specific bands in temperature of 15°C, could being related to non-tolerance to low temperatures of germination, according verified in the physiological tests. Esterase is a degradative enzyme, which is involved in hydrolyses reactions of esters, which is directly connected to the lipid metabolism (phospholipids). Many of these lipids are constituents of the plasmatic membrane and their degradation increases when increase the level of seeds deterioration (Santos *et al*., 2004).

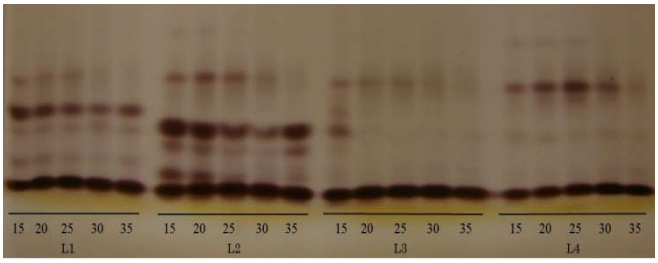

Figure 2. Electrophoretic pattern of esterase enzyme (EST) in maize seeds pre-germinated in different temperatures (L1-30; L2-64; L3-63; L4-91)

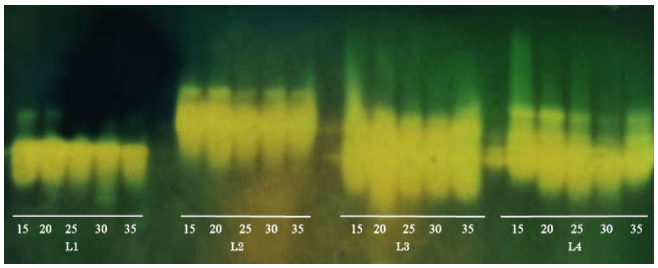

Figure 3. Electrophoretic pattern of catalase enzyme (CAT) in maize seeds pre-germinated in different temperatures (L1-30; L2-64; L3-63; L4-91)

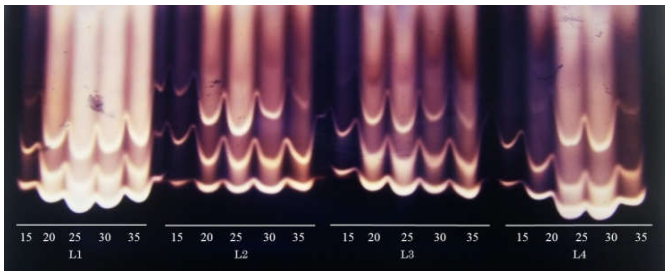

Figure 4. Electrophoretic pattern of alpha amylase enzyme in maize seeds pre-germinated in different temperatures (L1-30; L2-64; L3-63; L4-91)

For the catalase enzyme (Figure 4), was possible to observe that in lines which showed to be tolerant to high temperatures of germination (L63 and L91), there was a lower activity of this enzyme. At temperature of 25°C was also observed lower expression. The catalase enzyme is related to deterioration process in seeds. So, is an important antioxidant enzyme, that catalyzes the conversion of hydrogen peroxide in water, protecting the cells from the oxidation caused by free radicals. Together with other enzymes, catalase is known like the free radical remover, cause performs a paper of detoxification of H_2O_2 (toxic for plants) in cells, transforming the hydrogen peroxide into water and oxygen. The free radicals liberated during the formation of H_2O_2 damage the cellular membranes, what culminates in destructives reactions. Thus, the functioning of mitochondrias, where the chemical reactions of respiration occurs, is compromised, together with the supply of energy and secondary compounds for the synthesis of proteins (Mcdonald, 1999).

About the alpha amylase enzyme expression, the main differences verified were related to lower temperatures of germination (15 and 20°C). All lines presented also, lower germination in this temperature. Beyond the antioxidant enzymes, the expression of enzymes α and β amylases are extremely important for that maize seeds germinates in conditions of low temperatures. The development of the alpha amylase activity constitute an important event, could being detected during the beginning of seeds germination, being their main paper, to provide substrates for the utilization of seedlings until it becomes photosynthetically self-sufficient (Nedel *et al*., 1996). In maize, the alpha amylase enzymes, when promote the hydrolyses of starch, turns available the necessary carbohydrates for the embryo development, enabling thus, the germinative process (Franco *et al*., 2002). However, Oliveira (2013) points out that in addition to the amylase genes, many others genes could be involved in the control of physiological quality character of seeds, for example, the genes directly related with respiration

Conclusions

The 30 and 64 maize lines are susceptible to high temperatures of germination. The variations of temperature commit the physiological quality of maize lines seeds. There is variation in the expression of ADH, MDH, catalase, esterase and alpha amylase enzymes in maize lines of the evaluated lines.

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