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Physiological quality and protein patterns of corn seeds produced under water and salt stresses

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This study evaluated the effect of water and salt stresses on the physiological quality and on the electrophoresis patterns of heat resistant proteins in corn seeds. The experiments were carried out in the laboratories of seed analysis and biotechnology at the Universidade Federal de Lavras. Corn seeds of the hybrid GNZ 2004 and the inbred line LE 57 were used. The seeds were produced in soils with an electric conductivity of 3 dS m^{-1} (under stress) and 0.4 dS m^{-1} (without stress) and in pots containing substrate with a water holding capacity of 40% (with stress) and 70% (without stress). The randomized complete block design with treatments arranged in a split-plot scheme was used with four replications. Seeds were harvested at different stages of development. Seed physiological quality was evaluated using the tests of germination, artificial aging and cold test. The patterns of heat resistant proteins were evaluated by electrophoresis. The results showed that depending on seed developmental stage there was an effect of water and salt stresses on the seed vigor. The electrophoretical patterns of heat resistant proteins were stable in seeds produced under different stress conditions.

Key words: *Zea mays,* developmental stages, late embryogenesis abundant proteins (LEA proteins).

INTRODUCTION

Seed physiological quality can vary with genotype, seed development stage and also with stress conditions imposed to the plants during the production process, such as water and salt stresses. According to Ferreira et al. (2011), moderate water stress reduces seed germination rate, but it does not affect their vigor. Faria et al. (2004) observed that under natural drying conditions, corn seed germination and vigor are acquired with their development and greater values are observed in the development stage known as ML5 (milk line 5).

The effect of irrigation on different plant development stages was evaluated by Schlichting et al. (2015), who observed that the water deficit during the vegetative stage, corresponding to 12 leaves, reduced corn seed production; however, no effects on seed physiological quality was observed. Irrigation was done by sprinklers based on the evaporation of the Class A tank, applying the coefficient of consumption (Kc) for corn. Another factor that can affect plant and seed development is salinity.

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Plant response to salinity is a complex phenomenon, involving changes at the morphological, physiological and biochemical levels (Fougère et al., 1991). Ferreira et al. (2005) observed that decreasing levels of calcium, magnesium, potassium, and increasing levels of sodium cause an unbalance and nutritional stress on maize.

Programs of seed quality control must guarantee seed genetic purity besides its physiologic quality. This process demands precise methods that can be routinely used by seed producing companies. Genetic purity certification, in Brazil, is generally does by seed, seedling and plant morphological markers at the flowering and maturation phases (Silva et al., 2000). However, for Gomes-Filho et al. (2010), Menezes et al. (2008) and Moreira et al. (2013), many morphological markers do not attend the criteria of discrimination, homogeneity and stability required for cultivar description. These markers can be affected by many environmental factors and especially by stress. In contrast, heat resistant proteins are potential markers that do not present any known catalytic activity (Silva-Mann et al., 2002) presenting polymorphism and stability in seeds, such as in maize (Roveri José et al., 2004).

Therefore, this study aimed to evaluate the effect of water and salt stresses on the physiological quality and on heat resistant proteins patterns of corn seeds harvested at different development stages.

MATERIALS AND METHODS

The experiments were done on November 2013 at the experimental area of the Department of Agriculture and at the Seed Sector of the Universidade Federal de Lavras (UFLA). Seeds of two genotypes, the single cross hybrid GNZ 2004 and its parent line LE 57 were used. Two experiments were carried out aiming to produce seeds under both, stress and no-stress conditions. The first one was conducted in the field, where the salt stress was applied. The second one was conducted in pots, where the water stress was applied.

The first experiment, carried out in the field under salt stress, was set up in a randomized complete block design with treatments arranged in a split-plot scheme, considering the stress factor (with or without salt stress) in the plot, and the stages of development in the subplots, with four replications. Each subplot was composed by 4 five-meter length rows, with a spacing of 0.8 m between rows and 0.2 m between plants, obtaining a density of approximately 62,500 plants ha^{-1}. In each plot subjected to salt stress was applied, directly on the soil 8.650 Kg NaCl, distributed in two applications, the first one immediately after sowing, with 3.460 Kg NaCl (40% of the total applied) and the second one at plant flowering, with 5.190 Kg NaCl. The amount of NaCl applied raised soil conductivity to 3 dS m^{-1}, which is considered stressing for plants. Plots not subjected to salt stress had soil electric conductivity about 0.4 dS m^{-1}.

Soil conductivity was measured four times: the first one was done before the test started to determine the amount of NaCl to be applied on each plot; the second one was done 16 days after the experiment started; the third one was done 98 days after the test had started, just before the second application of salt; and the last one was done when the seeds reached the stage of milk line 5 (ML5). Soil electric conductivity was determined at the Department of Soil Sciences of Universidade Federal de Lavras, according to the method proposed by Raij et al. (2001) for a 1:5 extract (10 cm^3 fine air dried soil for 50 mL water).

The amount of NaCl to be applied on each plot was determined using the following equation:

$$[\%Na] = \frac{[Na^+]\ x\ 100}{T}$$

where, T= soil potential CEC; $[Na^+]$ = sodium concentration [cmolc/dm^3], and [%Na] = sodium percent saturation

A sodium concentration of 60% was required to reach soil conductivity of 3 dS m^{-1}, resulting on the application of 2702.0 mg NaCl Kg^{-1} soil, representing 8.650 Kg NaCl on each plot. Agricultural soil depth was considered as the 0.20 m.

In the second experiment, water stress induction was applied in 30 L pots, containing a sand and soil substrate in the proportion 1:1, in a total of 160 pots: 80 cultivated under water stress (40 with the hybrid and 40 with the parental line) and the other 80 cultivated with no water stress. Soil water capacity was maintained at 40% after pollination in the pots subjected to water stress, or at 70% during seed development in those not subjected to water stress. The experiment was carried out in a randomized complete block design with treatments arranged in a split-plot scheme, considering the stress factor (with or without water stress) in the plot, and the stages of development in the subplots, with four replications.

The seeds started to be harvested at the stage 2 of the milk line (ML2), when the seeds presented 25% of the endosperm solidified, according to the methodology proposed by Hunter et al. (1991). The seeds in the first experiment for both, the hybrid GNZ 2004 and the inbred line LE 57 were harvested at the stages of development ML2, ML3, ML4 and ML5. To the second experiment the seeds of both, hybrid and inbred line were harvested in the stages of development ML3 and ML5.

The stages of milk line were identified by visual inspection, based on a sample of six seeds removed from the middle of five ears. Each seed was cut longitudinally and the embryo and milky contents were removed from one of the halves. The percentage of solidified endosperm was estimated by comparison with the intact half.

Seed physiological quality was evaluated, for each cultivar and each treatment, by the tests of germination, accelerated aging and cold, in four replications. The moisture content of the seed was determined by the oven method at 105ºC for 24 h, using two replicates of each treatment.

The germination test was conducted with four replicates of 50 seeds, sowed between germitest paper towels moistened with distilled water in the ratio of 2.5 mL/g paper. The germination chamber was set at 25ºC and the evaluations of normal seedlings were performed on two counts, 4 and 7 days after sowing. This test was conducted according to the Seed Analysis Ruler [Regras para Análises de Sementes (RAS)] (Brasil, 2009).

The cold test was done as described by Loeffler et al. (1985). Twenty five seeds were distributed on a germitest paper moistened with water at 2.5 times its dry weight. After sowing, the rolls were placed in plastic bags, closed with tape, and maintained in a 10°C chamber for 7 days. Subsequently, the rolls were transferred to the germinator at 30°C. Normal seedling counts were done on the fourth and seventh day after the transfer.

The artificial aging test was performed in “gerbox” where seeds were suspended on a screen inside the box, containing 40 mL water. Seeds remained were incubated for 72 h at a temperature of 42°C, then was performed germination test as described previously.

Heat resistant proteins were extracted from 100 mg of embryo axes of seeds from each treatment, ground in ice cold mortar with 1:10 (embryo weight: extraction buffer volume) buffer (50 mM Tris-HCL-7.5; 500 mM NaCl; 5 mM $MgCl_2$; 1 mM PMSF) and transferred to 1500-μL microtubes. The mixture was centrifuged at 16000 x g for 30 min at 4°C, and the supernatant incubated in a water bath at

Table 1. Mean values (%) of seedlings vigor evaluated by the artificial aging (EA) and cold (TF) tests, of seeds of the hybrid GNZ 2004 and the inbred line LE 57 produced with or without salt stress, and harvested at different stages of milk line (ML).

Milk line	GNZ 2004		LE 57	
	TF		EA	
	With	Without	With	Without
ML5	99^{aA}	99^{aA}	97^{aA}	95^{aA}
ML4	90^{aA}	86^{aB}	91^{aA}	87^{aA}
ML3	66^{aB}	36^{bC}	93^{aA}	90^{aA}
ML2	39^{aC}	44^{aC}	74^{bB}	90^{aA}

*Averages followed by the same letter, small cap in the rows and capital in the columns, belong to the same grouping by the Scott-Knott test at 5% probability.

85°C for 15 min and centrifuged again as previously described. The supernatant was poured into microtubes while the pellet was discarded. Before applying the samples into the gel, the tubes containing 70 μL extract + 40 μL sample buffer (2.5 mL glycerol; 0.46 g SDS; 20 mg bromophenol blue, and completed to 20 mL with extraction buffer Tris pH 7.5) were placed in a water bath with boiling water for 5 min. A polyacrylamide SDS-PAGE gel was prepared at 12.5% (separating gel) and 6% (concentrator gel) and each well received 50 μL of the extract + sample buffer. Electrophoresis was done at 150 V, and stained with Coomassie Blue at 0.05%, as described by Alfenas (2006), for 12 h, and de-stained in 10% acetic acid.

Before undertaking statistical analysis of data, the normality as well as the homogeneity of the residuals variances was checked. After that, the analysis of variance was carried out for germination test, artificial aging and cold test. To the salt stress experiment the analyzes were carried out in a randomized complete block design with treatments arranged in a 2×4 split-plot scheme with two stress conditions (with or without) and four stages of seed development (ML2, ML3, ML4 and ML5). To the water stress experiment the analyzes were carried out in a randomized complete block design with treatments arranged in a 2×2 split-plot scheme with two stress conditions (with or without) and two stages of seed development (ML3 and ML5). Means values were grouped by the Scott-Knott test at 5% probability. The statistics analyzes and the Scott-Knott test was performed using the software SISVAR (Ferreira, 2011). The analysis of the heat resistant proteins was qualitative, observing the presence or absence of bands in the gels for each treatment.

RESULTS AND DISCUSSION

There was significant interaction between salt stress and the stages of seed development. A significant double interaction was observed between the stages of seed development and salt stress in the cold test for the hybrid and in the artificial aging test for the inbred line. Also, significant differences were found in the germination and artificial aging test for the hybrid and in the cold test for the inbred line when seeds were harvested at different stages of development.

A significant interaction was observed between stages of development and water stress in the germination test and artificial aging for both, the hybrid and the inbred line. Also, significant differences were found between the two genotypes when the seeds were harvested in different stages of seed development.

The cold test demonstrated that seeds of the hybrid harvested at ML3 under no salt stress had lower vigor than those subjected to stress (Table 1). No significant differences were observed in the cold test for the seeds harvested on any of the other milk lines subjected to stress or not. The artificial aging test of the parent line demonstrated that the stress at ML2 caused lower germination values, which was not observed in any other stage of development.

The germination test of seeds of the hybrid GNZ 2004 and its parental line LE 57 harvested in the stages of development ML2, ML3 and ML4, subjected or not to salt stress, presented lower values than those observed for seeds harvested at ML5 (Table 2). Probably, the seeds harvested at these stages are not physiologically mature and, thus, had lower germination. The artificial aging test demonstrated that seeds of the hybrid harvested in any stage of development had greater germination than those observed in the germination test. It is possible that, under the test conditions, the incubation dried the seeds, inducing their germination. Seeds of the parent line harvested in the stages ML2 and ML3 had lower germination in the cold test than those of the other stages.

In the germination test, both seed sources produced with stress or without it, had greater germination when harvested at ML5 (Table 3). For hybrid seeds, harvested at ML3, it was observed higher germination values on seeds produced under water stress conditions. Lower vigor values were found in the artificial aging test for seeds not subjected to water stress and harvested at ML3 for both the hybrid and the line. In contrast, at ML5 no effect of the water stress was observed on seed vigor for this test.

Greater vigor values were found in the cold test for seeds of the hybrid GNZ 2004 and its parent line LE 57 subjected to water stress, when harvested at the stage of development ML5 (Table 4).

It is possible to state that the seeds of the inbred line

Table 2. Mean values (%) of normal seedlings evaluated by germination test (TG), artificial aging test (EA) and cold test (TF) of seeds of the hybrid GNZ 2004 and the inbred line LE 57 harvested at different stages of milk line (ML).

Milk line	GNZ 2004		LE 57	
	TG	EA	TG	TF
ML5	99[a]	99[a]	98[a]	98[a]
ML4	76[b]	92[b]	51[b]	79[b]
ML3	31[c]	95[b]	17[c]	27[d]
ML2	16[d]	85[c]	8[d]	44[c]

*Averages followed by the same letter, in the columns, belong to the same grouping by the Scott-Knott test at 5% probability.

Table 3. Mean values (%) of normal seedlings in the corn seed germination test (TG) and artificial aging test (EA) of the hybrid GNZ 2004 and the inbred line LE 57 produced with or without water stress, and collected at different stages of milk line (ML).

Milk line	TG				EA			
	Hybrid		Line		Hybrid		Line	
	With	Without	With	Without	With	Without	With	Without
ML5	99[aA]	99[aA]	94[aA]	92[aA]	100[aA]	100[aA]	99[aA]	99[aA]
ML3	77[aB]	65[bB]	35[bB]	48[bB]	97[aA]	66[bB]	93[aA]	76[bB]

*Averages followed by the same letter, small cap in the rows and capital in the columns, belong to the same grouping by the Scott-Knott test at 5% probability.

Table 4. Mean values (%) of normal seedlings after cold test (TF) of corn seeds of the hybrid GNZ 2004 and the inbred line LE 57, collected at different stages of milk line (ML).

Milk line	TF(%)	
	Hybrid	Line
ML5	99[a]	92[a]
ML3	69[b]	65[b]

*Averages followed by the same letter, in the columns, belong to the same grouping by the Scott-Knott test at 5% probability.

and the hybrid presented low germination values when harvested in the stage of development ML2 and ML3 in the cold and germination tests. The greatest germination values were observed on seeds harvested at the stages ML4 and ML5. Greatest vigor values were found in the artificial aging test for seeds harvested at the stages ML2, ML3 and ML4. Seeds harvested at the stage LL5 had high germination and vigor values in all tests, for both the hybrid and the inbred line.

The average values of water contents in the seeds of the hybrid GNZ 2004 harvested at the stages of development ML2, ML3, ML4 and ML5 were 45.9, 43.5, 33.7 and 19.9%, for seeds produced under salt stress, and 45.9, 42.9, 33.2 and 19.3% for the seeds without salt stress. In contrast, the seeds of the inbred line LE 57, harvested at the maturation stages ML2, ML3, ML4 and ML5 presented average water contents of 48.1, 41.4, 36.4 and 17.3%, respectively, for seeds produced under salt stress, and of 47.5, 42.3, 36.7 and 17.6% for seeds produced without salt stress.

The average seed water contents of the line grown under water stress and harvested at the maturation stages ML3 and ML5 were 37.8 and 15.2%, respectively. In contrast, those grown without the water stress had average values of 39.6 and 16.0%. The seeds of the hybrid, harvested at the same stages of development, ML3 and ML5, had average water contents of 38.6 and 17.2% for seeds produced under water stress, and 38.9 and 17.1% for those produced without that stress.

The patterns of heat resistant proteins of the single cross hybrid (GNZ 2004) and its parental line (LE 57) were not affected by either salt or water stresses, as shown by the

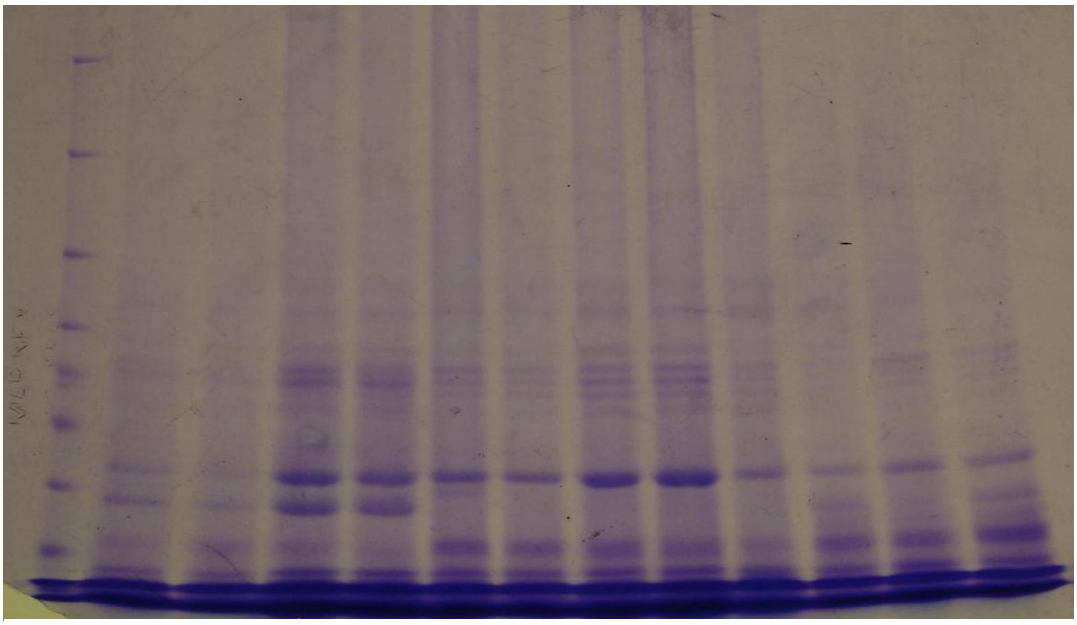

Figure 1. Heat resistant protein electrophoretic patterns of seeds of the single cross hybrid GNZ 2004 (H) and its parent line LE 57 (L) produced under water stress (ch) or not (sh), or under salt stress (cS) or not (sS), and harvested at different development stages, ML2 (2), ML3 (3) and ML5 (5); protein standard (P).

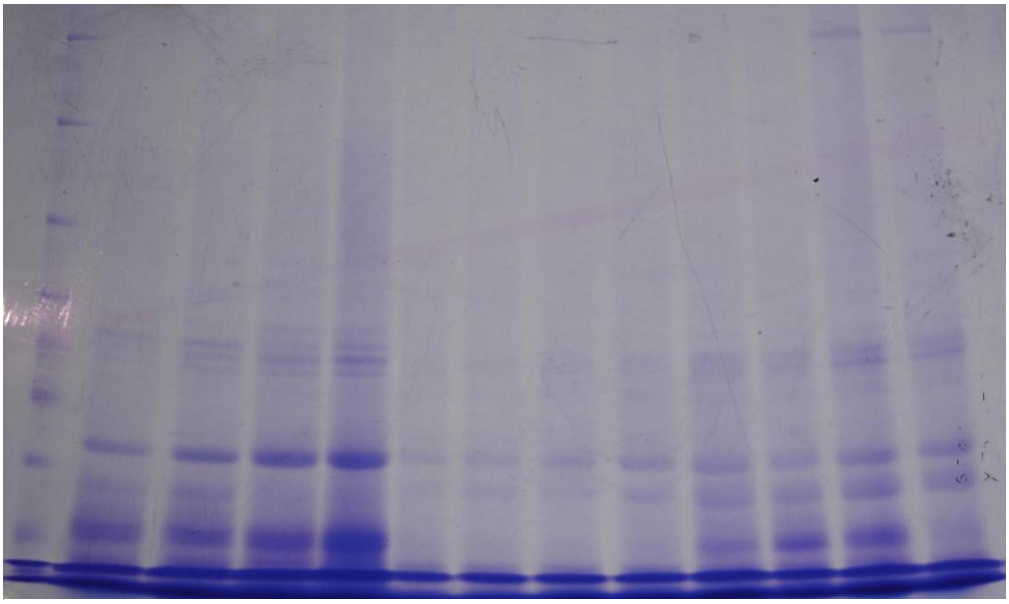

Figure 2. Heat resistant protein electrophoretic patterns of seeds of the single cross hybrid GNZ 2004 (H) and its parent line LE 57 (L) produced under water stress (ch) or not (sh), or under salt stress (cS) or not (sS), and harvested at different development stages, ML2 (2), ML3 (3), ML4 (4) e ML5 (5); protein standard (P).

zymograms (Figures 1 and 2). Also, lower protein expression was observed at the maturation stages LL2 and LL3, reflected by the low band intensity. Greater expression of these proteins was found at the maturation

stage LL5. Similar results were found by Faria et al. (2004) in corn seeds harvested at different development stages.

This study demonstrated that it is possible to identify the cultivar, certifying their genetic purity in early development stages. Moreover, the patterns of these proteins are stable, even when the seeds are produced under different stress conditions.

Seeds of corn lines subjected to artificial or natural drying, even those that showed large variations on germination, had stable patterns of heat resistant proteins (Roveri José et al., 2004).

Conclusions

Salt and water stresses affect seed vigor depending on their development stage. Greater germination levels and vigor were observed on corn seeds harvested at the maturation stage ML4 and afterwards. Heat resistant proteins presented stable patterns, even when the seeds are produced under water or salt stresses.

Conflict of Interest

The authors have not declared any conflict of interest.

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