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Enzymes activity as potential molecular markers for drying tolerance and quality in *Coffea arabica* seeds

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ABSTRACT

Antioxidant enzyme expression plays a vital role in the stress response in plants. This study was carried out to evaluate the physiological quality and antioxidant enzyme expression of coffee seeds and plants subjected to drying. Coffee seeds were conventionally dried to 40% and 10% water content, representing nonstressed and stressed conditions, respectively. The tests included germination, accelerated aging, and emergence. The expression of enzymes (SOD, CAT, POX, APX, and 1-cys-prx), together with lipid peroxidation, was analysed. The results showed no significant differences in the physiological test results, except for germination in stressed seeds. We detected differences in antioxidant system enzymes according to the condition applied to the seeds; however, peroxidase, lipid peroxidation, and 1-cys-prx were the only patterns most strongly correlated with seed germination. Nevertheless, only lipid peroxidation and 1-cys-prx were more strongly correlated with germination, indicating its potential as a marker for coffee seed quality.

Key words: Coffee; drought; oxidative stress; 1-cys-prx.

1 INTRODUCTION

Coffea sp., which originated in Ethiopia, is currently a highly important crop worldwide (Tesfa, 2019). However, although the main method for coffee planting is through the use of seedlings, this method depends on high-quality seed production (Carvalho; Almeida; Guimarães, 2014). Additionally, coffee seeds are classified as intermediate (Ellis; Hong; Roberts, 1991; Hong; Ellis, 1990), which means that they have moderate tolerance to drying (10 to 15% water content) and storage. In this case, postharvest drying must be carried out on coffee for proper storage until sowing. Drying is an important step during the seed production process, through which the objective is to reduce the water content of seeds suitable for storage without damaging their quality. In addition, it preserves the seed from physical and chemical alterations that may occur with a high water content to maintain the initial quality of the seeds (Silva et al., 2020).

Despite these advantages, drying can be risky if performed improperly and can lead to irreversible damage and loss of seed quality (Coelho et al., 2015; Taveira et al., 2012). In addition, it can cause damage, such as disruption of membranes; protein denaturation; production of reactive oxygen species; reduced permeability of membranes; increased breathing; fissures; and increased predisposition to pathogenic agents (França-Neto; Krzyzanowsaki; Henning, 2010). In the case of *Coffea arabica* L., which partially tolerates water loss and is sensitive to storage at temperatures below 0°C (Hong; Ellis, 1990), drying can be harmful, as it can reduce physiological quality (Ferreira et al., 2018). This loss of quality can impair the production of vigorous seedlings, which interferes with implantation in coffee plantations (Kurek; Plitta-Michalak; Ratajczak, 2019; Liu et al., 2019).

During the drying process under unfavourable conditions, the production of reactive oxygen species (ROS) may occur. ROS are products of cellular metabolism that regulate plant growth and development, but when in excess, they can be toxic to cells and require their removal (Kurek; Plitta-Michalak; Ratajczak, 2019). The enzymatic antioxidant system present in plant species plays an important role in combating ROS and is strictly related to cell signalling and adaptation to adverse environmental conditions. SOD, POX, CAT, APX and 1-cys-Prx are the main enzymes in this complex system. These proteins mainly remove superoxide and hydrogen peroxide that are produced in different cell compartments (mitochondria, peroxisomes, cytoplasm, and plasma membrane).

Among the antioxidant enzymes, the activity of 1-cys-Prx deserves important attention when the study material is the seed. This enzyme has been reported in several species, including rice, barley, wheat, Arabidopsis, tobacco, and soybean, as it is specific for seeds, is expressed in the aleurone layer, has a monogenic character and is related to the control of damage caused by environmental stress (Ratajczak; Dietz; Kalemba, 2019).

Due to the sensitivity of coffee seeds to drying and storage, ensuring seed quality before sowing for seedling

production is essential. These procedures may take 30 to 60 days (Brasil, 2009), which, combined with the necessity for space, may slow the seedling production process. However, the use of markers for seed quality and tolerance to drying has been studied, and various enzymes have been reported as markers for seed quality and/or tolerance to adverse conditions (Aalenf et al., 1994; Boniecka et al., 2019; Cavasin et al., 2021). These markers may result in faster assays for batch, parental, or seed lot selection regarding quality; however, it is highly important to analyse the potential enzymes for these ends.

In this context, the objective of this research was to verify the correlation between the physiological quality and activity of the antioxidant enzymes superoxide dismutase, peroxidase, catalase, ascorbate peroxidase and 1-cys-peroxidase in coffee seeds and seedlings subjected to different drying conditions.

2 MATERIAL AND METHODS

2.1 Seed harvesting and processing

Coffea arabica L. seeds of the Catuai Amarelo IAC 62 cultivar, produced at the Bom Jardim farm, located in the city of Bom Sucesso, were used in this research, which was carried out at the Central Seed Laboratory of the Federal University of Lavras in the city of Lavras. Coffee fruits were harvested at the cherry maturation stage, and after harvesting, they were mechanically peeled. The seeds were naturally degummed, and in this process, they remained in a tank without water for approximately 20 hours. After degumming, the seeds were allowed to dry in the shade for 24 hours to remove surface water.

The seed samples had an initial water content of 46%. For the drying process, the seeds were evenly spread on the cement terrace and moved every hour. During the afternoon, the seeds, which were still warm, were lined up and covered with canvas to protect them from night dew. The loss of water during drying was monitored by continuous weighing on a precision scale of 0.001 g, and the determination of the water content was carried out by the oven method until the seeds reached the water levels of interest (40 and 10%). To determine the water content under stress (10%) and nonstress (40%) conditions, the seeds were allowed to rest on the terrace for 16 and 2 days, respectively. After the drying periods, the seeds were stored in raffia bags, placed in plastic bags, and kept in a cold chamber with an average temperature of 10-12 °C and a relative humidity of 60 °C until analysis, which were carried out less than 5 days after drying.

The water content of the samples was verified by the oven method at 105 °C for 24 hours. Two subsamples of each material were used according to the Rules for Seed Analysis (RAS) (Brasil, 2009). The results are expressed as the mean percentage (wet basis).

2.2 Seed physiological quality

The analyses for evaluating the quality of the coffee seeds were carried out at the Central Laboratory of Seeds at UFLA.

From both conditions (stressed and nonstressed), two hundred seeds were used and divided into four repetitions of 50 seeds each. Sowing was carried out on germitest-type paper moistened with water at an amount equal to 2.5 times the weight of the dry paper. The seeds were subsequently kept in a germinator set at a temperature of 30 °C. The evaluations were carried out at 15 (first germination count) and 30 days after sowing. The number of normal seedlings was computed according to the prescriptions contained in the Rules for Seed Analysis for each culture (Brasil, 2009).

At the end of the germination tests, the seedlings were stored in a -80 °C freezer for subsequent enzymatic analysis. For the accelerated aging test, the Gerbox-type chamber method was used, in which 200 seeds of each were divided into 4 replicates of 50 seeds and distributed on a suspended screen inside a box containing 40 mL of water. Seeds were kept inside a germination chamber at 42 °C for a period of 48 hours. After this period, the seeds were subjected to a germination test according to the methodology described above, during which the percentage of normal plants was determined on the 15th day for the coffee plants after sowing (Filho, 1999).

To carry out the seedling emergence test and emergence speed index (IVE), the seeds were sown in plastic trays (20 cm × 22 cm × 12 cm) containing sand as a substrate for four replications of 25 seeds. The test was conducted in a germination chamber with the temperature regulated to 30 °C and 12 hours of light. The number of emerged plants was counted daily for up to 60 days after sowing. The emergence speed index (IVE) was determined according to Maguire (1962). For the evaluation of the physiological quality of the seeds, a completely randomized experimental design was adopted, with four replications for each species.

After being harvested, the plants were collected at the end of the physiological experiments for biochemical assays. The samples were immediately frozen in liquid nitrogen and stored in a freezer at -80°C until the assays were carried out.

2.3 Proteomic analysis through electrophoresis techniques

Analysis of the enzymes superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC. 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11) were detected via electrophoresis (Alfenas, 2006). For the analysis of these enzymes, the seeds and seedlings from the germination test were macerated in the presence of PVP and liquid nitrogen in a porcelain mortar, and later, the samples were stored at -80 °C.

For the extraction of CAT, SOD and APX enzymes, Tris HCl 0.2 M buffer (pH 8.0) + 0.1% beta-mercaptoethanol was used, and for the POX enzyme, potassium phosphate buffer was used, all in the proportion of 300 µL per 100 mg of seeds and seedlings. The material was homogenized by vortexing and kept for 12 hours in a refrigerator, followed by centrifugation at 14,000 rpm for 30 minutes at 4 °C. The electrophoretic run was performed in a discontinuous system of 7.5% polyacrylamide gels (separating gel) and 4.5% polyacrylamide gels (concentrating gel). The running buffer used was Tris-glycine (pH 8.9). Sixty microliters of the sample supernatant was applied to the gel, and the electrophoretic run was performed at 150 V for 5 hours. For the APX enzyme, 2 mM ascorbic acid was added to the running buffer, and a 30-minute prerun was performed before the protein samples were added to the supernatant.

At the end of the run, the gels were revealed for CAT, SOD and POX enzymes according to Alfenas (2006), with modifications. For CAT, the gel was immersed in 100 mL of H_2O_2 and agitated for 5 minutes, followed by rinsing under running water. Over the gel, 200 mL of solution [2 g of potassium ferrocyanide + iron chloride] was added, and the mixture was incubated at 35 °C until bands appeared. For SOD activity, the gel was immersed in 100 mL of buffer [0.05 M Tris-HCl (pH 8.0)], to which 200 mg of EDTA and 20 mg of NBT were added. The gel was kept at 37 °C until the bands were observed and fixed in 10% (v/v) glycerol solution before registering. The POX gel was revealed by immersing it in 200 ml of buffer [0.1 M sodium acetate (pH 4.5)], to which 64 mg of O-dianisidina bi-HCl and 2 mL of 3% (v/v) H_2O_2 were added, followed by incubation at 37 °C until bands were observed.

For the APX enzyme, the gel was equilibrated in 200 mL of buffer [50 mM sodium phosphate (pH 7.0) + 2 mM ascorbic acid] for 15 minutes, and the solution was subsequently removed. This process was subsequently repeated. incubated for 20 minutes in 200 mL of the second buffer [50 mM sodium phosphate (pH 7.0), 4 mM ascorbic acid, and 2 mM H_2O_2], followed by rinsing for 1 minute in 200 mL of sodium phosphate buffer [50 mM/pH 7.0]. Its development was carried out in 200 mL of solution containing 50 mM sodium phosphate buffer (pH 7.8), 22 mM TEMED and 0.1 mM NBT, and the mixture was maintained under agitation until the appearance of bands.

For each enzyme, three gels were analysed, representing three biological replicates, which were also used as replicates for the quantification of enzymes by ImageJ software (Schneider; Rasband; Eliceiri, 2012). The intensities of the bands were evaluated, and the results are expressed in mm^2.

2.4 Proteomic analysis through Western blotting

The 1-Cys-Prx enzyme was evaluated via Western blotting. Initially, total protein extraction was performed using 150 mg of tissue—seeds and seedlings—from each treatment, which were macerated in the presence of PVP and liquid nitrogen in a porcelain mortar. The extraction buffer was prepared using two solutions, A and B. Solution A was composed of 888 mg of trizma HCl, 530 mg of trizma base and 625 µl of Triton X-100 in a volume of 100 ml of ultrapure water. Solution B contained 32 ml of solution A, 45 g of urea, 15.2 g of thiourea, and 3.6 g of CHAPS in a volume of 100 ml with ultrapure water.

The samples were homogenized in 800 µl of extraction buffer (600 µl of solution B, 1 µl of protease inhibitor, 14 µl of DTT (1 M), 4.6 µl of DNAse, and 20 µl of RNAse (20 mg/ml), completing the final volume with ultrapure water). The samples were incubated on ice for 15 minutes, after which the samples were centrifuged for 10 minutes at 14,000 rpm at 4 °C. After centrifugation, the supernatant was pipetted into a new tube, and the samples were stored at -20 °C. To determine the total protein concentration extracted, Bradford (1976) was used for quantification in a BioTek Eon™ spectrophotometer.

After protein extraction and quantification, 60 µg of protein from each sample was applied to a polyacrylamide gel (12% SDS) and run in an electrophoresis tank for approximately 1 hour and 30 minutes at 100 volts. After the run, the proteins were transferred to an Immobilon-P membrane using a Bio-Rad transfer kit. To verify that protein transfer occurred, the membrane was stained with Ponceau and washed with 1x TBST (500 mM Tris + 1.5 M NaCl; pH 7.5).

Protein blocking was performed using 3% BSA for 1 hour, and the samples were incubated with an anti-1-Cys-Prx antibody (Abcam, ab16830) diluted according to the manufacturer's specifications overnight. After this period, the membrane was washed again in TBST (1×) and incubated for 1 hour or 30 minutes with a secondary antibody (HRP-conjugated horseradish peroxidase-conjugated anti-mouse IgG from Vector Laboratories). Antibody detection was performed using photographic films with fixing and revealing solutions. The quantification of the proteins was performed using the ImageJ program, with the analysis of the intensity and measurement of the isoforms obtained from the films (Schneider; Rasband; Eliceiri, 2012). The same incubation protocol was used for immunodetection of the primary anti-GAPDH antibody (Abcam, ab37168), which was used as an endogenous control.

2.5 Lipidic peroxidation

Lipid peroxidation was determined by quantifying the reactive species to thiobarbituric acid, as described by Buege and Aust (1978). Samples of 0.2 g of seeds and seedlings were macerated in liquid nitrogen plus 20% PVP (m/v) and homogenized in 1.5 mL of 0.1% (m/v) trichloroacetic acid (TCA). Two samples were taken from each treatment, and the analyses were performed in triplicate.

The homogenate was centrifuged at 14,000 rpm for 15 minutes at 4 °C. Aliquots (125 µl) of the supernatant were added to the reaction medium (250 µl) containing thiobarbituric acid (TBA; 0.5%) and TCA (10%). The samples were incubated in a water bath at 95 °C for 30 minutes, after which the reaction was stopped by rapid cooling on ice. Readings were determined with a spectrophotometer at 535 nm and 600 nm. It should be noted that three samples were taken from the seeds collected from each treatment, constituting the biological replications, and for the seedlings, each repetition of the germination test was used as a biological replication of each treatment.

2.6 Proteomic assays through spectrophotometry

The extract was obtained by macerating the material in liquid nitrogen, and samples of 0.1 g of seeds or seedlings were obtained according to each treatment. Then, 1.5 mL of extraction buffer containing 375 µl of 100 mM potassium phosphate buffer (pH 7.8), 15 µl of 0.2 mM EDTA (pH 7.0), 75 µl of 10 mM ascorbic acid and 1035 µl of distilled water was added. The extract was centrifuged at 13,000 rpm for 10 minutes at 4 °C, and the supernatant was stored at -20 °C until SOD, CAT and APX enzymatic analyses were performed (Biemelt; Keetman; Albrecht, 1998). All analyses were performed in triplicate.

SOD activity was evaluated by evaluating the ability of nitrotetrazolium chloride (NBT) blue to inhibit photoreduction. Six microliters of enzymatic extract was added to 194 µl of incubation medium containing 100 mM potassium phosphate buffer (pH 7.8), 70 mM methionine, 10 µM EDTA, 1 mM NBT and 0.2 mM riboflavin (Giannopolits; Ries, 1977). The activity of the APX enzyme was determined using an aliquot of 3 µl of the enzymatic extract to which 177 µl of incubation medium containing 90 µl of 200 mM potassium phosphate (pH 7.0), 9 µl of 10 mM ascorbic acid and 9 µl of 2 mM hydrogen peroxide was added (Nakano; Asada, 1981).

For the activity of the CAT enzyme, an aliquot of 3 µl of the enzymatic extract was used, followed by the addition of 177 µl of incubation medium containing 90 µl of 200 mM potassium phosphate (pH 7.0), 78 µl of distilled water and 9 µl of 12.5 mM hydrogen peroxide. Afterwards, the mixture was incubated at 28 °C according to Havir and McHale (1990). The activity of the POX enzyme was measured according to the protocol of Fang and Kao (2000), in which 4 µl of extract was added to 163 µl of reaction buffer containing 50 mM sodium phosphate (pH 6.0) and 0.13% (v/v) guaiacol. After this mixture was added, 33 µl of 0.9% hydrogen peroxide (at a final concentration of 0.15%) was added, and the mixture was immediately placed into a spectrophotometer for analysis.

For the proteomic analysis, three samples of the seeds collected from each treatment were taken, constituting the biological replications, and for the seedlings, each repetition of the germination test was used as a biological repetition of each treatment.

2.7 Statistical analysis

The experimental design used was completely randomized with 2 treatments (under stress or not). All analyses were conducted individually for the seeds and seedlings. Data analysis was carried out using the software R for Windows (R Core Team, 2022) with the packages car (Fox; Weisberg, 2019) and rstatix (Kassambara, 2023). The normality of the data was analysed through the Shapiro–Wilk test, and variance homoscedasticity was analysed through Levene's test. Nonnormal data were transformed into arcsines with a square root of x/100, and normality was tested. Treatments were compared by Student's t test, with the correct adaptations for the formulae according to variance homoscedasticity. Additionally, through the same software, correlation analysis was carried out using the stats (R Core Team, 2022), psych (Revelle, 2024) and Performance Analytics (Peterson; Carl, 2020) packages.

3 RESULTS

3.1 Seed quality

Among the tests used to evaluate the physiological quality of the coffee seeds, the germination test was the only test for statistical significance (Table 1). In seeds that had a reduced water content (to 10%), a lower percentage of germination (78%) was verified in relation to those that were dried to 40% water content (90%). In general, high physiological quality coffee seeds were observed.

Table 1: Average results of the first germination count (FC), germination at final count (G), accelerated aging (AE), emergence (E) and emergence speed index (ESI) of coffee seeds seeds produced or not under water restriction.

Condition	FC	G	AE	E	ESI
Under Stress	94.0 a	78.0 b	94.0 a	96.0 a	0.32 a
No Stress	96.0 a	90.0 a	95.0 a	74.0 a	0.24 b
CV	3.60	0.80	4.65	6.49	11.69

*Averages followed by the same lowercase letter in the column, do not differ by Student's T test.

3.2 Superoxide dismutase activity

The results from SDS–PAGE and spectrophotometry were slightly different. SOD expression was greatest in seedlings under no stress, followed by that in seeds under the same conditions (Figure 1B and E), and increased SOD expression was detected in stressed seeds through spectrophotometry (Figure 1F), followed by that in nonstressed seeds, which was the second highest treatment.

3.3 Catalase and peroxidases

Similar to superoxide dismutase, changes in the pattern of catalase (CAT) were found depending on the technique applied. The same pattern of expression was not observed for the seedlings via electrophoresis, while the same pattern was observed via spectrophotometry for the seeds (Figure 2A, C, and E). Through electrophoresis, higher expression was found for stressed seeds, while higher expression was found for nonstressed seedlings, as we observed by spectrophotometry.

For ascorbate peroxidase (APX) (Figure 2B, D, and F), we detected increased expression in the seeds, independent of the presence/absence of stress. The expression of these genes was lower in the nonstressed seedlings (Figure 2B and D). However, through spectrophotometry, no APX activity was detected.

Peroxidase (POX) activity was analysed only through electrophoresis. In this study, we detected increased expression of this enzyme in plants under stress conditions, with slightly decreased expression in nonstressed plants (Figure 3). Lower expression of this enzyme was detected in nonstressed plants.

3.4 Lipid peroxidation

Lipid peroxidation was measured through spectrophotometry by measuring malondialdehyde (MDA) content. In coffee, the MDA content was greater in seeds under drying stress than in those not under stress. In the seedlings, the activity was lower than that in the seeds and was statistically similar for both treatments (Figure 4).

3.5 1-CYS-PRX

The expression of 1-Cys-PRX was greater in stressed coffee seeds than in stressed coffee seedlings (Figure 5). No expression of this enzyme was detected in nonstressed seedlings.

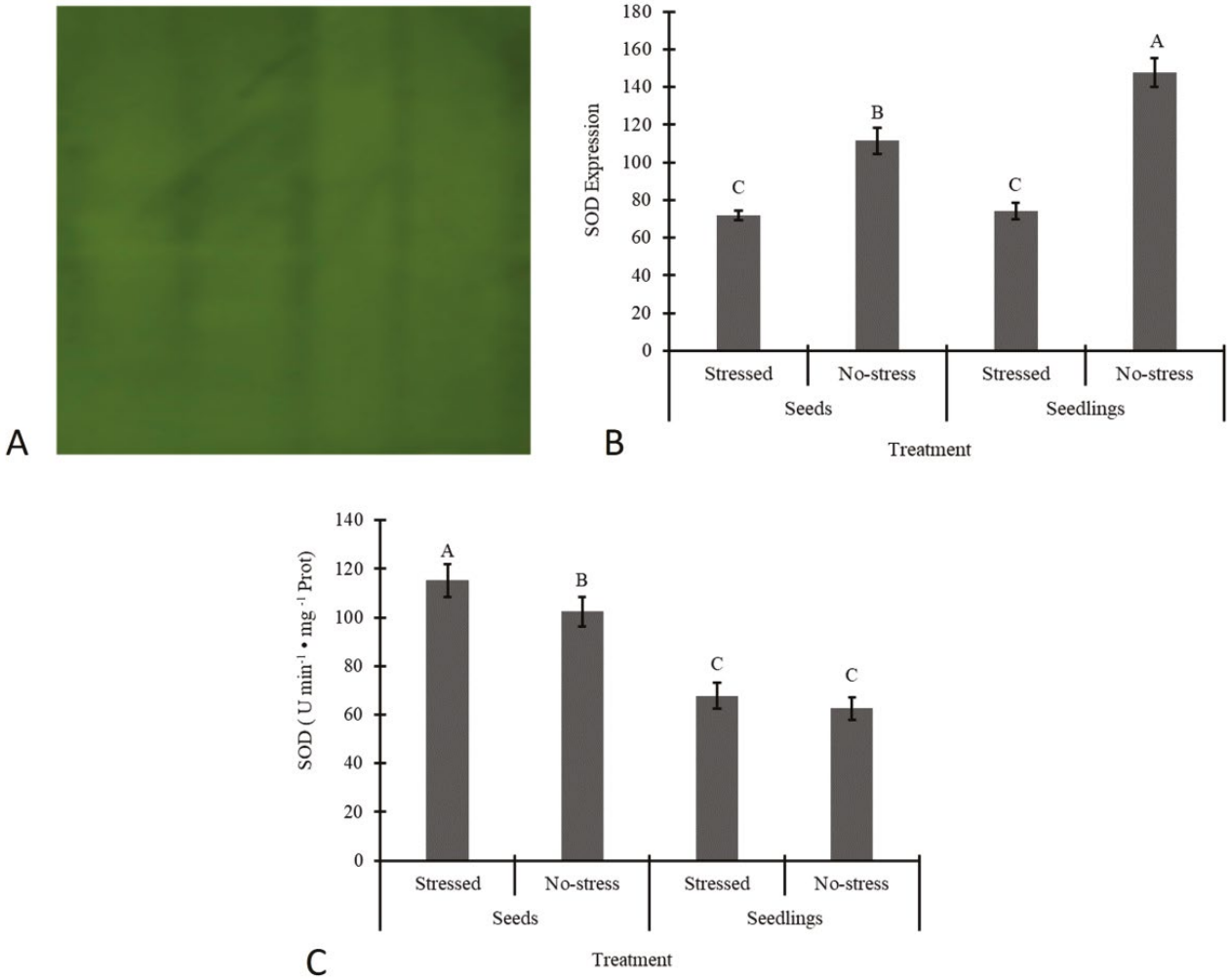

Figure 1: Superoxide dismutase enzyme activity analysis in gel electrophoresis (A), its expression estimation trough ImageJ (B), and activity measured trough spectophotometry (C) in coffee seeds stressed (dried to 10% water content) and nonstressed (dried to 40% water content) and seedlings developed from these seeds. Averages followed by same letters (for each tissue) indicates no differences among stress conditions according to Student's T test.

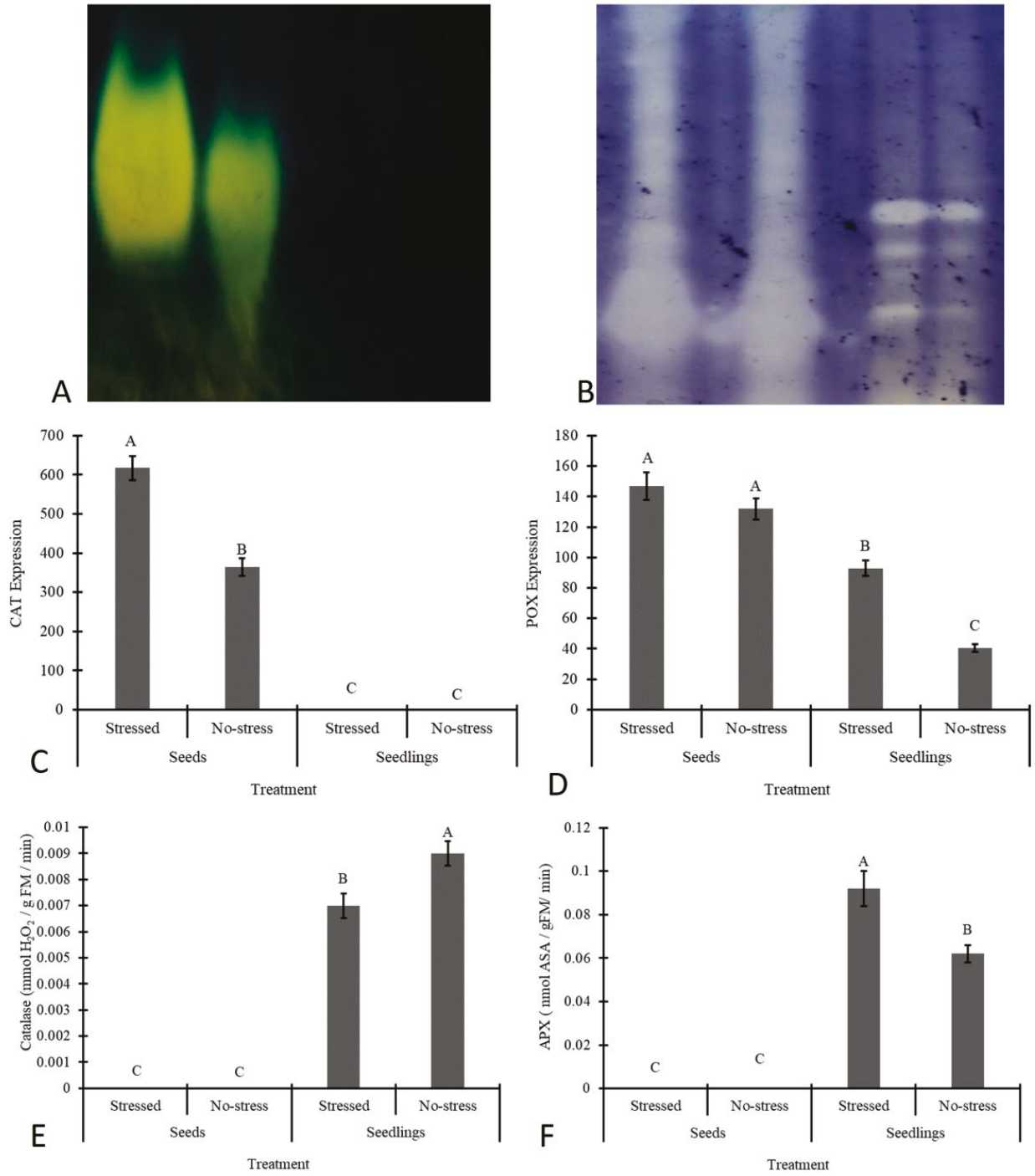

Figure 2: Catalase (A, C, E) and ascorbate peroxidase (B, D, F), enzyme activity analysis in gel electrophoresis (A-B), its expression estimation trough ImageJ (C-D), and activity measured trough spectophotometry (E-F) in coffee seeds stressed (dried to 10% water content) and nonstressed (dried to 40% water content) and seedlings developed from these seeds. Averages followed by same letters (for each tissue) indicates no differences among stress conditions according to Student's T test.

3.6 Correlation tests

We observed correlations between physiological results and those from biochemical assays carried out for seeds (Table 2). A negative correlation was found for germination and lipid peroxidation, 1-CYS-PRX, and peroxidase. Similarly, the emergence speed index was negatively correlated with catalase and superoxide dismutase (both from electrophoresis). An electrophoresis assay revealed a positive correlation between germination and both catalase and superoxide dismutase activity and between the emergence speed index and 1-CYS-PRX and peroxidase activity.

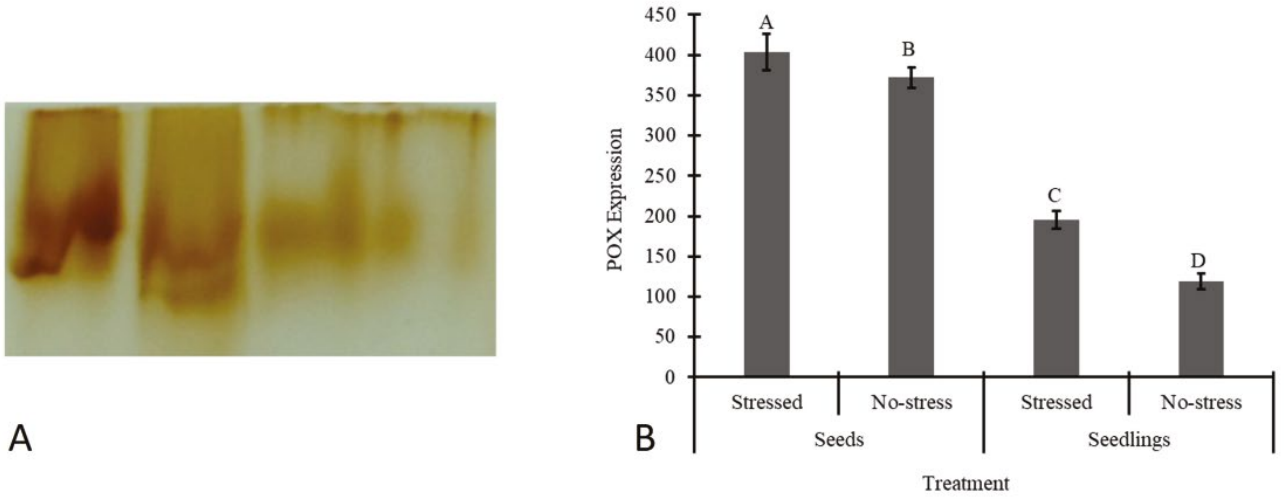

Figure 3: Peroxidase activity analysis in gel electrophoresis (A), its expression estimation trough ImageJ (B), in coffee seeds stressed (dried to 10% water content) and nonstressed (dried to 40% water content) and seedlings developed from these seeds. Averages followed by same letters (for each tissue) indicates no differences among stress conditions according to Student's T test.

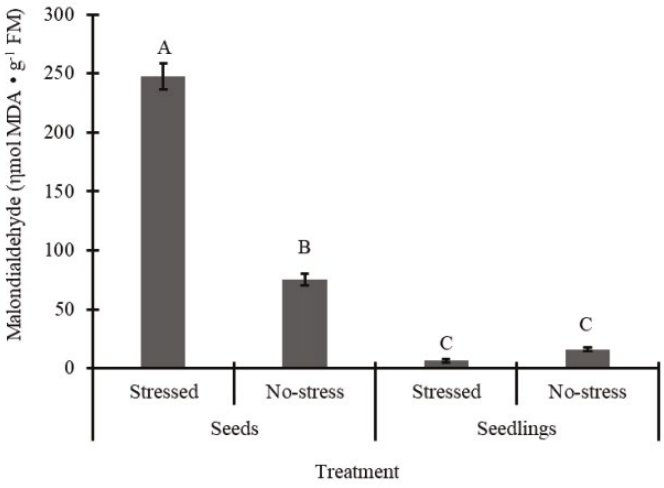

Figure 4: Lipid peroxidation estimated by malondialdehyde content in coffee seeds stressed (dried to 10% water content) and nonstressed (dried to 40% water content) and seedlings developed from these seeds. Averages followed by same letters indicates no differences among treatments according to Tukey's test at 5% probability.

A significant correlation was also found among the biochemical parameters for the seeds. (Table 2). Lipid peroxidation was positively correlated with 1-CYS-PRX and peroxidase, as 1-CYS-PRX was positively correlated with peroxidase and catalase to superoxide dismutase (Table 2). A negative correlation was also found for the seed biochemical parameters, as lipid peroxidation was negatively correlated with SOD and CAT activity (trough electrophoresis), and the same was observed between these two enzymes and 1-CYS-PRX and peroxidase (POX).

In the biochemical tests of the seedlings, we also detected correlations between those parameters and the physiological results (Table 3). A positive correlation was found between germination and lipid peroxidation and between germination and superoxide dismutase (both by electrophoresis and spectrophotometry). For the ESI, a positive correlation was found for SOD (spectrophotometry) and

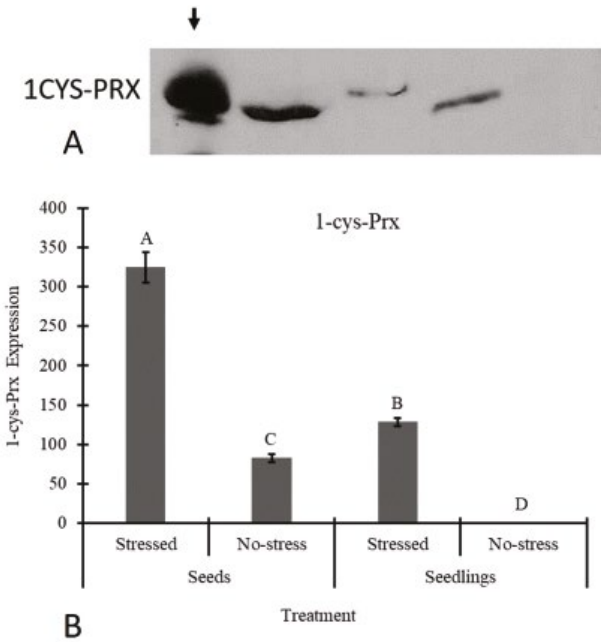

Figure 5: Expression of 1-CYS-PRX enzyme trough Western blotting (A) and measurement of bands trough ImageJ sotware (B) in coffee seeds stressed (dried to 10% water content) and nonstressed (dried to 40% water content) and seedlings developed from these seeds. Averages followed by same letters indicates no differences among treatments according to Tukey's test at 5% probability. Arrow indicates the sample from rice seeds, used as positive witness.

Table 2: Pearson's correlation index and p values between the parameters of first count (FC), germination (G), accelerated ageing (AE), emergence (E), emergence speed index (ESI), lipid peroxidation (LP), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), 1-CYS-PRX, and peroxidase (POX) for coffee seeds submitted or not to stress conditions.

		Physiological Tests					Spectophotometry Assays				1-Cys-PRX	Electrophoresis			
		FC	G	AE	E	ESI	LP	CAT	SOD	APX		CAT	POX	SOD	APX
Physiological tests	FC		0.75	0.1	-0.38	-0.69	-0.77		-0.7		-0.72	0.72	-0.67	0.71	-0.8
	G	*0.03*		0.42	-0.5	-0.97*	-0.98*		-0.74		-0.99*	0.97*	-0.98*	0.96*	-0.78
	AE	*0.81*	*0.3*		-0.48	-0.37	-0.5		-0.22		-0.5	0.53	-0.51	0.57	-0.28
	E	*0.35*	*0.21*	*0.23*		0.56	0.59		0.1		0.61	-0.66	0.57	-0.71	0.1
	ESI	*0.06*	*0*	*0.37*	*0.15*		0.97*		0.74		0.98*	-0.97*	0.98*	-0.93*	0.63
Spectrophotometry Assays	LP	*0.02*	*0*	*0.21*	*0.13*	*0*			0.78		0.99*	-0.98*	0.99*	-0.97*	0.73
	CAT														
	SOD	*0.05*	*0.03*	*0.6*	*0.8*	*0.04*	*0.02*				0.71	-0.69	0.73	-0.61	0.65
	APX														
1-CYS-PRX		*0.05*	*0*	*0.21*	*0.11*	*0*	*0*		0.05			-0.99*	1*	-0.98*	0.7
Electrophoresis Tests	CAT	*0.04*	*0*	*0.18*	*0.07*	*0*	*0*		0.06		0		-0.98*	0.98*	-0.65
	POX	*0.07*	*0*	*0.19*	*0.14*	*0*	*0*		0.04		0	0		-0.97*	0.68
	SOD	*0.05*	*0*	*0.14*	*0.05*	*0*	*0*		0.11		0	0	0		-0.7
	APX	*0.02*	*0.02*	*0.51*	*0.82*	*0.09*	*0.04*		0.08		0.05	0.08	0.06	0.05	

Values above the grey diagonal line indicates the Pearson's correlation index , those below the same line (in italic) indicates the significance (p value) for the correlation between the parameters. Cells for CAT and APX for spectrophotometry assays are empty due the no-detection for these enzymes in seeds. Symbol * highlights parameters with significative correlation.

Table 3: Values for Pearson's correlation index and p values between the parameters of first count (FC), germination (G), accelerated ageing (AE), emergence (E), emergence speed index (ESI), lipid peroxidation (LP), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), 1-CYS-PRX, and pwroxidase (POX) in coffee seedlings submitted or not to stress condition.

		Physiological Tests					Spectophotometry Assays				1-Cys-PRX	Electrophoresis			
		FC	G	AE	E	ESI	LP	CAT	SOD	APX		CAT	POX	SOD	APX
Physiological tests	FC						0.67	0.53	-0.76	-0.72	-0.74		-0.72	0.7	-0.23
	G						0.93*	0.89	-0.99*	-0.9	-0.98*		-0.93*	0.97*	-0.11
	AE						0.4	0.71	-0.43	-0.26	-0.47		-0.45	0.42	0.23
	E						-0.6	-0.62	0.57	0.6	0.61		0.73	-0.58	-0.13
	ESI						-0.94*	-0.88	0.97*	0.94*	0.98*		0.95*	-0.98*	0.04
Spectrophotometry Assays	LP	*0.07*	*0*	*0.32*	*0.12*	*0*		0.93*	-0.93	-0.97*	-0.97*		-0.96*	0.96*	0.19
	CAT	*0.17*	*0*	*0.05*	*0.1*	*0*	*0*		-0.89	-0.85	-0.93*		-0.92	0.92	0.25
	SOD	*0.03*	*0*	*0.29*	*0.14*	*0*	*0*	*0*		0.91	0.98*		0.96*	-0.99*	0.08
	APX	*0.04*	*0*	*0.53*	*0.12*	*0*	*0*	*0.01*	*0*		0.94*		0.96*	-0.96*	-0.2
1-CYS-PRX		*0.03*	0	0.24	0.11	0	0	0	0	0			0.97*	-0.99*	-0.01
Electrophoresis Tests	CAT														
	POX	*0.04*	*0*	*0.26*	0.04	0	0	0	0	0	0			-0.97*	-0.13
	SOD	*0.05*	*0*	*0.3*	*0.14*	*0*	*0*	*0*	*0*	0	0		0		0.05
	APX	*0.58*	*0.79*	*0.59*	*0.75*	*0.93*	*0.64*	*0.55*	*0.86*	*0.64*	*0.97*		0.76	0.9	

Values above the grey diagonal line indicates the Pearson's correlation index , those below the same line (in italic) indicates the significance (p value) for the correlation between the parameters. Correlation between physiological tests were removed because are repeating the tests for table 2. Results for Catalase in electrophoresis are empty because this enzyme was not detected for seedlings through this technique. Symbol * highlights parameters with significative correlation.

POX. Additionally, negative correlations were found between germination and SOD (spectrophotometry) and 1-CYS-PRX, while ESI was negatively correlated with lipid peroxidation and superoxide dismutase (electrophoresis).

Among the results for the biochemical parameters of the seedlings, a positive correlation was found between lipid peroxidation and catalase (spetophotometry) and superoxide dismutase (Table 3). The enzyme 1-CYS-PRX was positively correlated with SOD and APX (spectrophotometry), as was POX, which was also positively correlated with SOD and APX.

4 DISCUSSION

Seed quality is essential for crop production. In a low-quality seed batch, germination will be uneven, resulting in seedlings developing faster than others, and consequently, production will be affected. In the present work, the physiological quality observed both by the germination test and by the vigor tests was high for both treatments. Determining the physiological quality of seeds is essential for their launch on the market to ensure the uniform establishment of crops in the field and to obtain high yields (Souza et al., 2021). Drying can negatively affect the quality of coffee seeds if it is carried out incorrectly, given the tolerance of the species to desiccation (Ellis; Hong; Roberts, 1991). Correct drying and storage methods must be used to ensure the production of quality seedlings, as these two processes are the main causes of reduced seed viability and vigor (Vitis et al., 2020).

Rosa et al. (2005) evaluated different drying temperatures on maize seeds and did not detect differences in superoxide dismutase activity at the different temperatures used. In addition, as observed for coffee, it was possible to observe differences between the evaluated treatments, which were not observed in the vigor tests used in this research. Thus, this tool is useful for understanding the mechanisms involved in tolerance to desiccation in seeds and can possibly be used as a marker to measure the response of seeds to desiccation (Cavasin et al., 2023; Wei et al., 2021).

SOD is the first cell defense mechanism against oxidative stress (Liu; Soundararajan; Manivannan, 2019). This enzyme converts the ion superoxide, which is highly reactive, into hydrogen peroxide, which is still harmful but less reactive. Abiotic stress, such as drying, results in changes in seed metabolism, and an increase in respiration is among those changes. As a result, there is an increase in superoxide ion accumulation and, consequently, oxidative stress if this enzyme does not act. Thus, higher expression of this enzyme may indicate that seeds effectively respond to stress, such as drying, and prevent ROS accumulation (Guaraldo et al., 2023).

In the present research, the expression of antioxidant enzymes verified through the development of electrophoresis gels proved to be important for the differentiation of the evaluated treatments. The activity of CAT and POX enzymes, for example, in seeds, was greater under stress conditions. In some studies, it has been observed that increased expression of these enzymes is associated with increased production of ROS, which are products of oxidative stress caused by stress conditions such as drying (Guaraldo et al., 2023; Oliveira et al., 2022).

Coelho et al. (2015), when evaluating the physiological and biochemical changes in coffee seeds subjected to fast and slow drying, also observed an increase in POX expression associated with a reduction in seed water content. The enzymes POX, CAT and APX act on the same substrate, hydrogen peroxide, which is the product of superoxide dismutation carried out by SOD.

Malondialdehyde is the final product of lipid peroxidation (Sadak, 2022; Sadak; Bakhoum, 2022); thus, the higher the malondialdehyde content in the cell is, the greater the damage to cell components that contain lipids (Sadak, 2022). Lipid peroxidation may occur in either reserves or membranes (cells or organelles). This stress may result in damage, which leads to internal cell content lixiviation for the environment (Dhindsa; Plumb-Dhindsa; Thorpe, 1981; Parkhey; Naithani; Keshavkant, 2012). In addition to cellular damage, the final product of lipid peroxidation, MDA, is toxic to the cell, resulting in more than structural damage. As seeds lose vigor, this damage cannot be repaired or prevented, resulting in lower germination for the batch and consequently less efficiency in seedling production (Espindola et al., 1994; Oliver et al., 2020). For coffee, the content of MDA in stressed seeds corroborates the reduction in germination, which may be an indicator of the occurrence of oxidative stress in seeds.

The greater activity of 1-cys-Prx in seeds than in seedlings indicated that this enzyme is synthesized and accumulates in seeds and is degraded during germination and seedling growth. The same pattern was verified in previous research involving different species: Norway maple (Ratajczak; Dietz; Kalemba, 2019), milk thistle (ElSayed et al., 2019), *Canabis sativa* (Cattaneo et al., 2021), and soybean (Nishizawa; Komatsu, 2011).

Although accurate, germination tests can take a long time and require larger structures as the number of samples to be tested increases, as in coffee, which takes up to 30 days for final results (Brasil, 2009). Considering this, many studies have searched for other viable tests that may accurately predict seed physiological quality faster and with less space (Wei et al., 2021). In this case, the search for molecular markers on seeds is a potential method. In some studies, molecular markers have also been used for the selection of soybean cultivars with higher physiological quality and greater tolerance to environmental stress conditions, such as drying, according to the results of gene expression through proteomic and transcriptomic analyses (Moreno et al., 2019).

Not all methods for enzyme assays are available and, in some cases, cannot be accessible. Thus, the use of alternative methods can lead to the use of enzymes as molecular markers. However, there is a need for correlation among the results of both techniques, so they can be used. A comparison of methods is common, as researchers are seeking both more effective and lower-cost methods (Cavasin et al., 2023). However, no studies have compared the spectrophotometric and electrophoretic results for SOD, CAT, and APX in seeds.

The differences between the results can be attributed to the effects of seed and seedling components that interfere with the analysis and may be extracted at higher or lower amounts depending on the method applied (Varnavides et al., 2022). Additionally, it may be highlighted that the protocols for antioxidant-enzyme assays have different focuses. Electrophoresis assays analyse the active enzymes, i.e., those in the correct conformation (Alfenas, 2006). However, the spectrophotometry assays analyse all forms of the enzyme – active or not – present in the cell (Biemelt; Keetman; Albrecht, 1998). As we analysed the enzymatic results together with those from germination tests, we can infer the best technique to analyse each enzyme as well as the best enzymes to be used as markers for coffee seed quality.

The imposition of stress on coffee seeds effectively differentiated the results for the enzymatic patterns, which allowed a better indication of the changes. We found that the expression of these proteins may change depending on the stress and tissue tested, indicating their potential as molecular markers.

Our research revealed correlations between physiological results and biochemical assays. However, the correlation between enzyme activity and physiological results depends on the technique used (electrophoresis or spectrophotometry) and tissue collected. Peroxidase activity, although analysed only through electrophoresis, was correlated with coffee seed germination, as relatively high expression was detected in stressed seeds; however, similar (but significantly different) expression in nonstressed seeds may hinder the use of peroxidase activity as a molecular marker.

For lipid peroxidation and 1-Cys-Prx, the greater expression in stressed seeds and the difference in expression between the other treatments may indicate the potential of both as molecular markers for determining coffee seed quality. While coffee seed quality may range from 30 (germination test) to 60 days (emergence test), enzyme assays can be carried out faster, require lower amounts of material, and may proceed with a larger number of samples at the same time. However, as cited by Cavasin et al. (2023), as we found the potential molecular marker, its use depends on correct adaptations and extra tests until it can be finally used. Nevertheless, according to our research, 1-Cys-Prx and the malondialdehyde content (lipid peroxidation) can potentially be used as molecular markers for coffee seed quality analysis.

5 CONCLUSIONS

There is a correlation between coffee seed quality and the activity of antioxidant system enzymes, which depends on the tissue used. There is potential use for the analysis of antioxidant system enzymes as markers for coffee seed quality. The enzyme used for analysis depends on the tissue (seed or seedling) and technique (electrophoresis or spectrophotometry) available. The enzymes 1-cys-prx and lipid peroxidation are potential markers for detecting drying stress in coffee plants and are more highly expressed in seeds than in seedlings.

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7 AUTHOR CONTRIBUTIONS

Conceptual Idea: von Pinho, E.V.R.; Santos, H.O.; Methodology design: Freitas, A.S.; von Pinho, E.V.R.; Santos, H.O.; Pereira, E.M.; Data collection: Freitas, A.S.; Pereira, E.M.; Data analysis and interpretation: Freitas, A.S.; Pereira, E.M.; Pereira, W.V.S.; and Writing and editing: Freitas, A.S.; Pereira, W.V.S.

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