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Endo-β-mannanase and superoxide dismutase as enzymatic markers for lettuce seeds thermotolerance

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ABSTRACT: At higher temperatures, lettuce seeds may not germinate, resulting in problems for crop establishment in the field and production. This study aimed to evaluate the maternal effect for thermotolerance on lettuce seeds and find enzymatic markers. We used the thermoinhibition tolerant cultivar 'Everglades', the sensitive 'Verônica', their reciprocal hybrids, F_1 and F_2 generation. Seeds were submitted to germination and enzymatic tests (catalase, superoxide dismutase, ascorbate peroxidase, and endo- β-mannanase). Germination (first and final counting) and speed index were compared through Tukey's test. 'Everglades' and its reciprocal hybrids, have not been influenced by temperature regarding germination compared to the other treatments. Also, the maternal effect was observed for the F_1 generation. Higher temperatures interfere with the activity of all enzymes evaluated, consequently in the physiological parameters. However, superoxide dismutase and endo-β-mannanase have shown direct and more expressive correlation with changes on the physiological parameters compared to the other enzymes. Thus, it is possible to conclude that those enzymes can be used as markers for thermotolerant genotype selection.

Index terms: isoenzymes, higher temperatures, *Lactuca sativa* L., thermoinhibition.

RESUMO: Sob altas temperaturas, sementes de alface não germinam, resultando em problemas para o estabelecimento da cultura em condição de campo. Esse trabalho analisou a possibilidade de presença de efeito maternal na termotolerância em sementes de alface e buscou marcadores enzimáticos para tal. Usaram-se as cultivares Everglades (tolerante) e 'Verônica' (sensível), bem como seus híbridos recíprocos e a geração F_2. As sementes foram analisadas quanto à germinação, bem como atividade enzimática (catalase, superóxido dismutase, ascorbato peroxidase e endo-β-mananase). O percentual de germinação; (primeira contagem e final), bem como a velocidade (IVG) foram analisados por meio de ANOVA e teste de Tukey. A cultivar Everglades e seus híbridos recíprocos não foram influenciados pela germinação em alta temperatura quando comparados com os outros tratamentos. Além disso, o efeito maternal foi observado na geração F_1. Altas temperaturas interferem na atividade de todas as enzimas analisadas nesta pesquisa, consequentemente também nos parâmetros fisiológicos. Contudo, a superóxido dismutase e a endo-β-mananase tiveram suas variações altamente correlacionadas com as mudanças nos parâmetros fisiológicos. O presente trabalho evidenciou o efeito materno para a termotolerância em sementes de alface, bem como o uso da superóxido dismutase e endo-β-mananase como marcadores enzimáticos para tal característica.

Termos para indexação: isoenzimas, altas temperaturas, *Lactuca sativa* L., termoinibição.

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INTRODUCTION

Lettuce (*Lactuca sativa* L.) is a source of considerable amounts of vitamins (A, B1, B2, and B5), calcium, potassium, sodium, phosphorus, iron, silicon, fluorine, magnesium, and antioxidants. It is also a reliable source of insoluble fibbers (Soares and Cantos, 2005). These characteristics makes lettuce a crop known, cultivated, and consumed worldwide. Lettuce has as origin center the Mediterranean region, being mostly adapted to grow in temperatures around 15-25 °C (Zhao et al., 2022). This fact makes lettuce a thermosensitive species, i.e., seeds do not germinate at temperatures over 28 °C (Yoong et al., 2016), creating problems for the crop on the field, especially in tropical regions.

Germination under higher temperature (mentioned as heat stress or HS on some research) have been studied for lettuce (Zhao et al., 2022). Under this stress condition, changes on morphological characteristics may be observed, as thinner blades and longer internodes. Also, decrease on nutritional value is observed (Chen et al., 2011; Song et al., 2015; Zhang et al., 2016). There are reports in the literature for lettuce genotypes in which seeds can germinate at higher temperatures, which is called thermotolerance. As examples there are *Lactuca serriola* UC96US23, germinating around 37 °C (Argyris et al., 2011), *Lactuca sativa* L. PI251246 tolerating germination at up to 33 °C (Yoong et al., 2016), and cultivar 'Everglades', which germination is reported to stands over 70% at 35 °C (Catão et al., 2014; Catão et al., 2016).

Besides germination-related tests, other techniques can be used to characterize thermotolerance on lettuce. Wei et al. (2021), compared metabolomic changes on lettuce under heat stress being found, among the results, changes on 25 transcription factors linked to heat stress. Oliveira et al. (2021), analyzed how thermotolerance is genetically controlled on lettuce. Among these authors conclusions, the viability of conventional breeding on lettuce aiming for thermotolerance on hybrids is possible. Enzyme endo-β-mannanase activity also was studied on lettuce thermotolerance. This enzyme is related to surrounding-embryo tissue cell wall weakening, allowing its development and growth (Catão et al., 2014), being a temperature-dependent process.

Thermoinhibition is related to endosperm softening (Wang et al., 2019). For this softening to happen, the activity of enzymes such as alcohol dehydrogenase (ADH), malate dehydrogenase (MDH), catalase (CAT), esterase (EST), pyruvate decarboxylase (PDC), glutamate oxaloacetate transferase (GOT), and endo-β-mannanase is essential. This last enzyme is responsible for endosperm mannans hydrolysis during germination (Almeida et al., 2019). Thermosensitive cultivars have more mannose in the cell wall than thermotolerant genotypes; consequently, it is necessary for more time for endo-β-mannanase to complete the hydrolysis and, consequently, germination time is prolonged (Albuquerque et al., 2010; Ferreira et al., 2018).

As mentioned before, as temperature raises, thermosensitive lettuce seed germination is prejudiced by induction of thermodormancy. Although studies have shown treatments which can result on thermodormancy-breaking (Kaya, 2022), production of thermotolerant seeds can be more efficient, by eliminating costs and steps on lettuce production (Oliveira et al., 2021). Study thermotolerance on each cultivar is important so breeding can be efficient and is important to understand mechanisms linked to this process, which may generate markers, as genes or enzymes which can be used as tolls on this process. Considering the potential use of enzyme and protein expression as markers for cultivar selection (Roveri et al., 2004), this research aimed to evaluate the maternal effect on thermotolerance and find possible enzymatic markers for lettuce seeds.

MATERIAL AND METHODS

We conducted this research using two lettuce cultivars: a thermotolerant, 'Everglades', and a thermosensitive, 'Verônica'. Leaf characteristics can differentiate both cultivars. 'Everglades' has wrinkled leaves while 'Verônica' is plain. Besides these cultivars, we used hybrids (F_1 and F_2) from crossings on these cultivars.

For seed production, sowing was executed in 128 cell polystyrene trays, which three seeds per cell. After emergence, only one plant (the most vigorous) was left in each cell, and the others were discharged. ‘Everglades’ are reported as precocious; and thus, to assure that it would be planted from both cultivars flowering at the same time. This cultivar has the sowing staggered at 32 cells per week; to allow crossings on both cultivars to happen.

Seed production was carried at the rural zone of Ijaci city, Minas Gerais – Brazil. Design for seed production was established in completely random. Climatic data during seed production period are presented on Figure 1. The experimental station is located at the coordinates 21º09’24’S e 44º55’34’W at 831 meters altitude. Trays where seedlings were produced, were placed over concrete benches covered with 30% shading screen Sombrite® to reduce solar incidence. Watering was carried according to necessity; and, after 25 days from germination, seedlings were transferred to 10-liter vases filled with soil, sand, and organic compost (2:1:1). From seedling production to harvest, plants received adubation, irrigations, and pulverizations according to crop’s recommendations.

At the flowering time, crossings were carried. Around 4 am, flowers from feminine genitors were emasculated to avoid stigma cutting. Around 8 am, flowers from masculine genitors were collected before opening, avoiding self-pollination. Each flower was emasculated and identified with colored wool at the base of peduncle. When flowers have open and stigma developed, becoming bifid, pollination was conducted by rubbing an opened flower from masculine genitor directly on the stigma from feminine genitor emasculated flowers.

After seed development and maturation, they were harvested and identified as F_1(‘Everglades’ ♀ x ‘Verônica’ ♂) or F_1(ExV), for those from emasculated flowers of ‘Everglades’ cultivar pollinated by ‘Verônica’; F_1 (‘Verônica’♀ x ‘Everglades’♂) or F_1(VxE), for those from ‘Verônica’ emasculated flowers pollinated by ‘Everglades’; ‘Verônica’ and ‘Everglades’ for seeds from auto pollination from each cultivar. Seeds were cleaned, dried, packed, and stored in the cold chamber at 15 °C and 50% RH until experiments were conducted.

For F_2 seed production, at the same conditions mentioned before, were sown seeds from ‘Everglades’, ‘Verônica’, F_1(ExV) and F_1(VxE). A total of six treatments were obtained: ‘Everglades’, ‘Verônica’, F_1(ExV), F_1(VxE), F_2(ExV), and F_2(VxE).

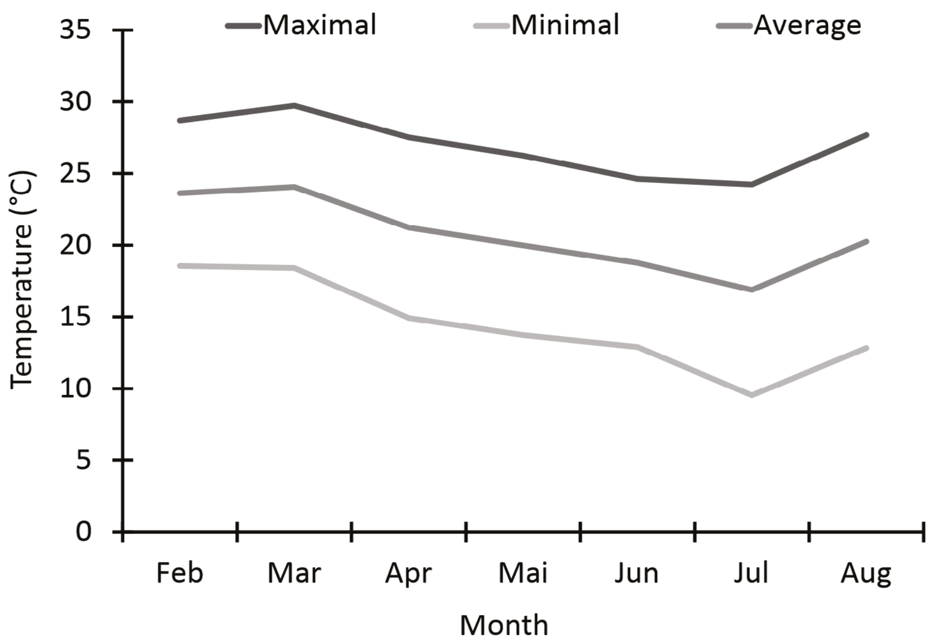

Figure 1. Maximal, minimal, and average temperature on seed production months for the present study for the study region. Source: *Universidade Federal de Lavras* - Meteorological Station (https://portal.inmet.gov.br/).

Germination percentage and vigor (germination speed index) were carried by using seeds from each treatment, placed in a completely random design with four replications (gerbox boxes) of 50 seeds each, a total of 24 samples. Seeds were placed over germination paper in gerbox boxes, with distilled water at 2.5 times the paper mass. Boxes were placed inside BOD germination chamber at 32 °C and 12 hours of light. Daily counting was conducted to verify the germination percentage (G) and speed index (GSI) until the 7^{th} day, when the temperature was lowered from 32 to 20 °C to verify the viability of the remaining seeds, continuing the germination counting for seven more days (total of 14 days, when the second count was carried). The same treatments were also placed in the BOD at 20 °C to verify germination and vigor at the ideal temperature (Brasil, 2009).

Enzymatic analysis was conducted using three samples of 350 fresh seeds from each treatment, which were stored at -86 °C right after harvesting and processing. Seeds were ground at the presence of liquid nitrogen and, polyvinylpolypyrrolidone (PVP) inside a porcelain crucible placed over ice and stored at -86 °C; the whole experiment was conducted at triplicate.

For antioxidant lettuce seed enzymes extraction: catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase were used 100 mg seed from each treatment, homogenized in buffer of 400 mM potassium pH 7.8 (375 µL), 10 mM EDTA (15µL), 200 mM ascorbic acid (15 µL) and water (1035 µL). After that, the samples were centrifuged at 1400 rpm at 4 °C for 10 minutes. Enzyme extraction was carried according to protocol proposed by Biemelt et al. (1998).

The activity of SOD, CAT and APX was determined using the Enzyme Linked Immuno Sorbent Assay (ELISA) at 96 well polystyrene microplate. SOD evaluation was carried by using 10 µL extract and 190 µL reaction media [100 mM sodium phosphate pH 7.8 (100 µL), 70 mM methionine (40 µL), 10 mM EDTA (3 µL), water 30 µL), 1 mM blue p-nitro tetrazolium (NBT) (15 µL), and 2 µM riboflavin (2 µL)]. The reaction was conducted at 25 °C, in a chamber equipped with fluorescent light (15 W). After 7 minutes reaction, absorbance at 5600 nm was measured. SOD activity was expressed in enzyme unity consumed by minute per gram of fresh mas (U SOD.min^{-1}.g^{-1} FM) (Giannopolits and Ries, 1977).

For CAT, 10 µL of extract was mixed with 190 µL reaction media [200 mM phosphate buffer pH 7.0 (100 µL), water (80 µL), and 250 mM hydrogen peroxide (10 µL)]. A decrease on absorbance at 28 °C was measured after 3 minutes of reaction. CAT result was expressed by µmol of hydrogen peroxide consumed by minute by gram of protein (mmol H_2O_2.min^{-1}·g^{-1} FM) (Havir and McHale, 1987).

For APX, 10 µL extract was mixed with 190 µL reaction media [200 mM phosphate buffer, pH 7.8 (100 µL), 10 mM ascorbic acid (AsA) (10 µL), water (70 µL), 2 mM hydrogen peroxide (10 µL)]. APX activity is analyzed through a decrease in AsA and absorbance. Absorbance decrease was observed for 3 minutes reaction at 28 °C being APX results expressed by nmol AsA consumed gram of fresh mass per minute (mmol ASA.g NF.min^{-1}) (Nakano and Asada, 1981).

Endo-β-mannanase extraction was carried by 100 mg seeds from each treatment mixed with 300 µL pH 8.0 extraction buffer (0.1 M HEPES, 0.5 M NaCl, 5 mg acid ascorbic per mL buffer). Samples were centrifuged for 30 minutes at 14000 rpm, and 10 µL were collected and applied in agarose gel with 6 mL of locust bean gum (LBG; 24 g agarose, 24 mL citric acid 1M / 0.4 $NaHPO_4 \bullet 2H_2O_2$ pH 5,0). The gel was incubated for 21 hours according to Silva et al. (2004). Endo-β-mannanase activity was calculated according to the method of Downie et al. (1994).

All results collected were analyzed by analysis of variance and Tukey's test at 5% probability, carried through R for Windows software.

RESULTS AND DISCUSSION

Through analysis of variance, significant differences were found for all evaluated characteristics. The germination results for all treatments at 20 °C can be observed in Figures 2 and 3. All treatments but F_1(VxE) have the same values for GSI. Besides, the germination percentage observed was also according to the commercial standard (over 80%) (Kikuti and Marcos Filho, 2012).

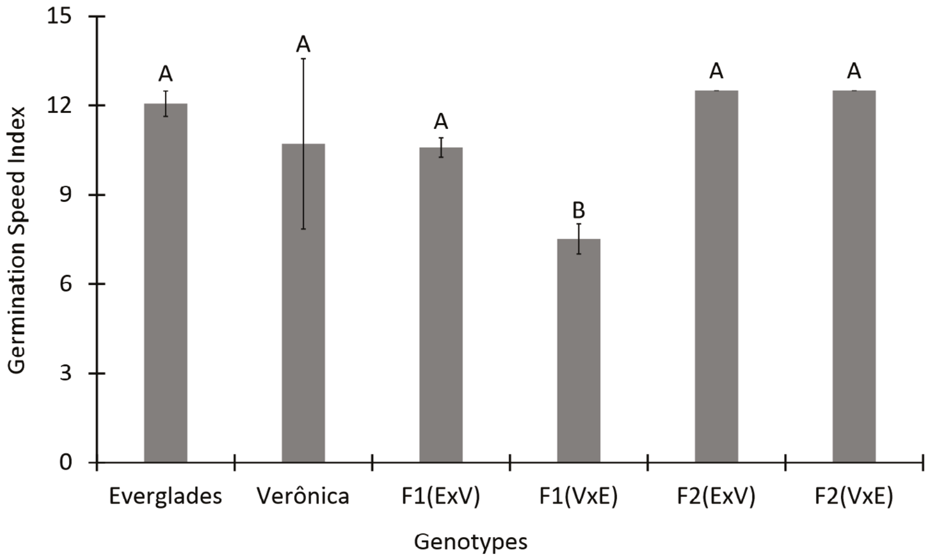

Figure 2. Germination Speed Index for lettuce seeds from different cultivars and hybrids at 20 °C. Same letters indicate no differences through Tukey's test at 5% probability.

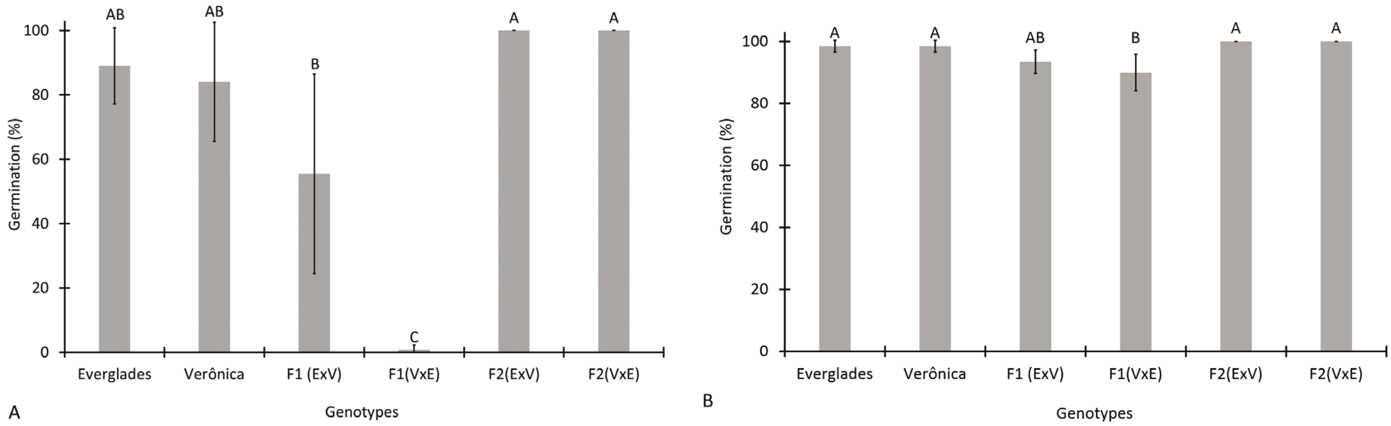

Figure 3. First germination count for lettuce seeds from different cultivars and hybrids at 20 °C. Same letters for each variable (first or final count), indicate no differences through Tukey's test at 5% probability.

Higher values for all parameters were observed for the 'Everglades' when germination conditions were at 32 °C (Figure 4). However, all seeds from all treatments have statistically similar germination after a temperature decrease of 20 °C. For F_1 generation, at 32 °C, F_1(ExV) has higher values when compared to the reciprocal F_1(VxE). However, no statistical differences were observed for the F_2 generation.

For SOD (Figure 5A), higher values were observed for 'Everglades' cultivar and F_2(ExV). Also, higher SOD activity was observed in 'Everglades' when compared to 'Verônica.' The same can be observed by comparing the reciprocal hybrids and F_2 generation; when the 'Everglades' cultivar is the feminine genitor, higher values are observed compared to the reciprocal crossing. For catalase enzyme (Figure 5B), higher activity can be observed only in F1(ExV), with no statistical difference among the other treatments. Regarding APX enzyme (Figure 5C), higher values were observed for treatment F_1(ExV), followed by the reciprocal F_1(VxE) and F_2(VxE).

For endo-β-mannanase (Figure 5D), higher expression can be observed for F_1 (ExV) and F_2(ExV), followed by F_2(VxE) and 'Everglades' cultivar. Statistically different values were observed for generation F_1, when 'Everglades' is the female genitor, with higher values than the reciprocal. Results for Endo-β-mannanase and superoxide dismutase follow the same pattern of germination, in which F_1 and F_2, in which 'Everglades' was the feminine genitor had intermediary results. These results indicate that both enzymes can be correlated to thermotolerance.

High temperatures negatively interfere over with lettuce's physiological parameters. 'Everglades' cultivar, as the hybrids using it as feminine genitor have less influence of temperature over seed germination compared to the other treatments. Enzyme endo-β-mannanase and superoxide dismutase have higher activity in thermotolerant cultivars, as could be observed of F_1 and F_2 which have 'Everglades' as female genitor. This shows the potential of this enzyme as thermotolerance marker on lettuce.

The seed germination for all treatments after the temperature decreases to 20 °C (Figure 2) confirms the thermoinhibition effect on lettuce seeds (Catão et al., 2016). This result confirms that the 'Everglades' cultivar is thermotolerant and agrees with various reports which find higher germination compared to thermosensitive cultivars (Nascimento et al., 2013; Catão et al., 2014; Catão et al., 2016; Catão et al., 2018).

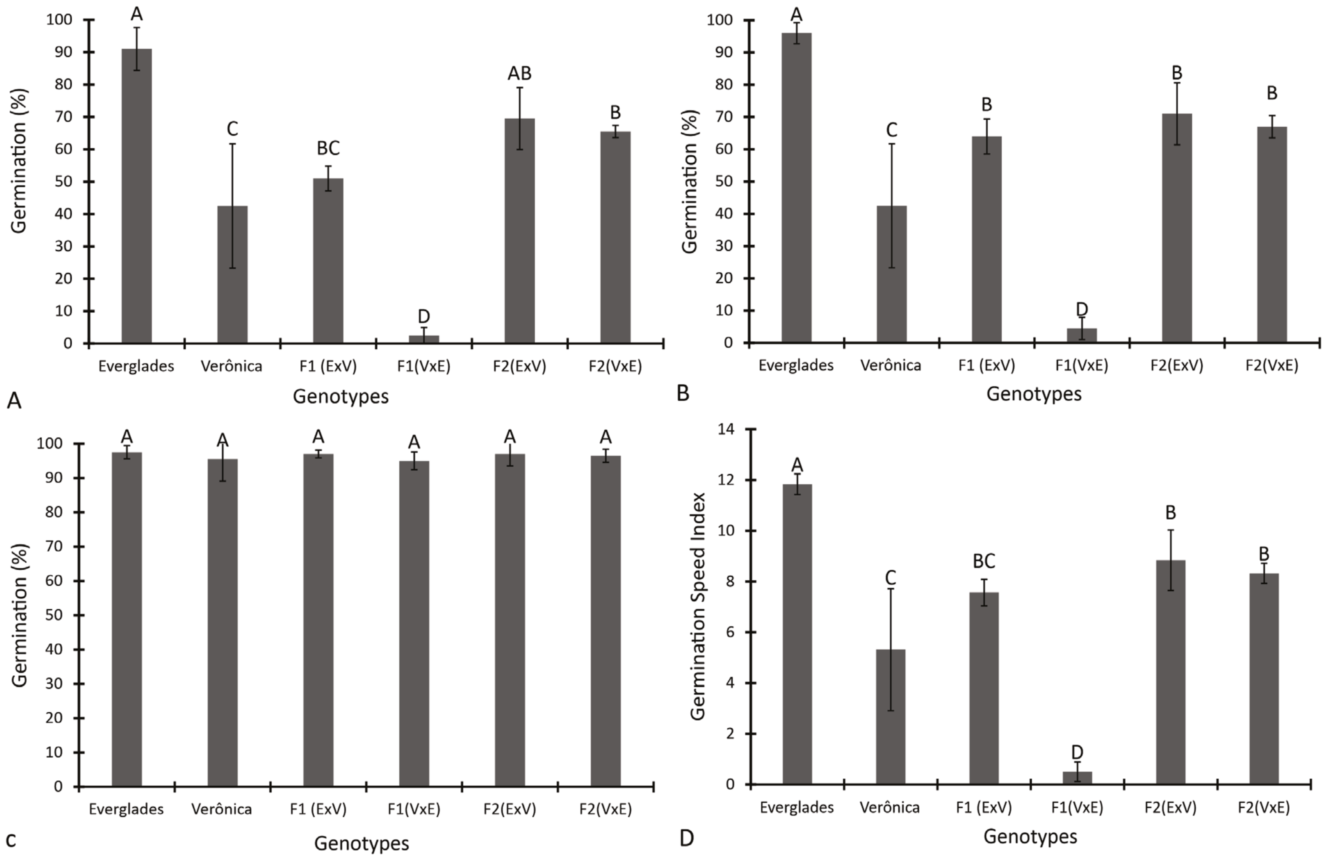

Figure 4. Germination Speed Index, first (7 days at 32 °C), and final (7 more days at 20 °C) germination counting for lettuce seeds from different cultivars and hybrids at 20 °C. Same letters for each parameter indicate no differences through Tukey's test at 5% probability.

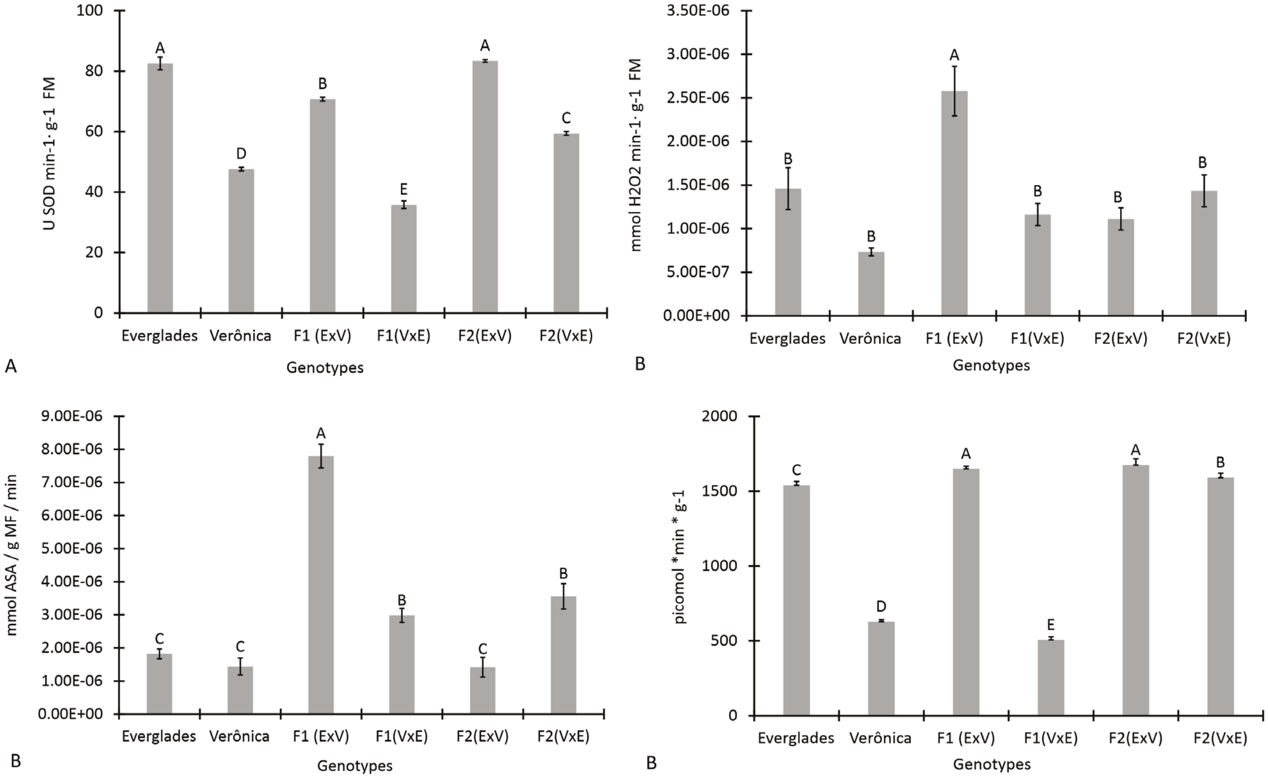

Figure 5. Activity of superoxide dismutase (A), catalase (B), ascorbate peroxidase (C), and quantification of endo-β-mannanase (D) on lettuce seeds according to cultivars and reciprocal hybrids. Same letters indicate no statistical differences according to Tukey's test at 5% probability.

Differences found among F_1 hybrids show the maternal influence over the expression of thermoinhibition tolerance. Similarly, the maternal effect was observed for tomato seeds germination at higher temperatures, and differences were found for their reciprocal hybrids for the cultivar San Vito and Fontana (Nascimento et al., 2016).

Thermoinhibition for lettuce seeds is linked to endosperm is triploid (Orsi and Tanksley, 2009) and major of the maternal origin. Endosperm can delay or prevent germination, acting as a physical barrier for radicle emission, especially in unfavorable conditions. Endosperm weakening (softening) is a prerequisite for radicle emission at elevated temperatures (Nascimento et al., 2001). This information is essential for genotype selection on plant improvement programs once two-thirds of the endosperm are from the feminine genitor.

Mannose is a reserve component from lettuce seeds endosperm, and endo-β-mannanase production is essential for softens the endosperm, consequently allowing radicle protrusion and germination. Endo-β-mannanase activity incudes also cell wall extension, defense, and limited mobilization of hemicellulose with other enzymes, to supply carbohydrates during some phases of seed and plant development (Bewley, 1997).

Higher temperatures cause oxidative stress, forming oxygen-reactive species (Jaleel et al., 2007). Seeds have enzymatic antioxidant systems to act against this species, , the primary defense against free radicals generated under stress conditions. Among these enzymes superoxide dismutase (converts the superoxide radical into H_2O_2 and O_2), catalase, and ascorbate peroxidase (transform H_2O_2 into H_2O and O_2) (Catão et al., 2016).

Thus, the reduction in the expression of these enzymes can result in a decrease in oxidative damage prevention. Higher temperatures during germination reduce the activity of these enzymes during photorespiration, resulting in free radical accumulation (Foyer and Noctor, 2003).

The knowledge of isoenzymes activity allows the identifying abiotic factors-related variations as the species respond to temperature. Thus, the biochemical markers can select elevated temperature tolerant cultivars, contributing to genetic improvement programs in tropical and subtropical regions (Catão et al., 2014). Catão et al. (2016) observed that higher temperatures interfere with seed biochemical and physiological quality, and isoenzymes can be used as markers, for lettuce seeds storage at different temperatures.

As was observed in this study, it is evident that temperature directly influences lettuce seed physiological parameters. The activity of enzymes SOD and endo-β-mannanase followed a similar pattern to the observed physiological parameters compared to the other evaluated enzymes. Also, 'Everglades' cultivar (thermotolerant) and the hybrids F_1 and F_2 having it as feminine genitor, have higher values on both enzymes, showing that SOD and endo-β-mannanase together can be promising indicators for lettuce seed thermotolerance.

As a worldwide-consumed crop, lettuce must be produced on a wide range of conditions, being higher temperatures one of those. Although species is characterized as a thermosensitive (Zhao et al., 2022), many genotypes are reported as thermotolerant, being possible the introduction of thermotolerance on cultivars of interest through breeding (Oliveira et al., 2021). However, for this, to be viable, characterization of which cultivar is or not thermotolerant, studies must be carried. Those can be germination-related tests or even by use of markers, as superoxide dismutase and endo-β-mannanase can be a fast test to identify thermotolerant cultivars and consequently speed breeding programs activities.

CONCLUSIONS

There is a material effect on the tolerance of thermoinhibition in lettuce seeds.

Conventional breeding can be used to develop thermotolerant cultivars.

Endo- β-mannanase and superoxide dismutase quantification can be used as markers for lettuce progeny selection regarding thermoinhibition tolerance.

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