

Temperature and GA₃ on ROS and cytogenetic stability during *in vitro* cultivation of strelitzia zygotic embryos

Temperatura e GA₃ na ERO's e estabilidade citogenética durante o cultivo *in vitro* de embriões zigóticos de estrelitzia

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ABSTRACT

Tropical species may require higher temperatures as well as higher growth regulator concentrations for *in vitro* development. Since these conditions may affect plant metabolism, the objective of this study was to identify how different temperatures and gibberellin concentrations may affect the *in vitro* development of strelitzia embryos, analyzing the effect on ROS and cytogenetic stability. Zygotic embryos were cultivated on MS medium supplemented with 5, 10 and 20 μ M GA₃ under temperatures of 25 °C, 30/25 °C and 30 °C. After 60 days, higher embryonic germination rate (72%) and shoot length of plantlets (3.14 cm) were observed on medium containing 20 μ M gibberellic acid (GA₃). At this concentration, there was an increase in nitrate reductase activity with no change in the cytogenetic stability. The temperature influenced only shoot and root lengths, which were highest at 25 °C. At 30 °C, superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities increased compared with those at 25 °C. Thus, the addition of 20 μ M GA₃ to the culture medium and a temperature of 25 °C in the growth room should be used for zygotic embryo culture of strelitzia.

Index terms: Antioxidant; embryo culture; gibberellin; nitrate reductase.

RESUMO

As espécies tropicais podem exigir temperaturas mais altas e maiores concentração de reguladores de crescimento para o desenvolvimento *in vitro*. Em função dessas condições afetarem o metabolismo vegetal, o objetivo foi identificar como diferentes temperaturas na sala de crescimento e concentrações de giberelinas podem afetar os embriões no desenvolvimento *in vitro* de estrelícia, analisando o efeito sobre EROS e a estabilidade citogenética. Os embriões zigóticos foram cultivados em meio MS suplementado com 0, 5, 10 e 20 μ M de GA₃ e mantidos em sala de crescimento sob temperaturas de 25 °C, 30/25 °C e 30 °C. Após 60 dias, observou-se maior germinação dos embriões (72%) e comprimento das brotações (3,14 cm) em meio adicionado com 20 μ M de GA₃. Nessa concentração, houve um aumento na atividade da redutase do nitrato sem alterar a estabilidade citogenética. A temperatura influenciou apenas o comprimento da parte aérea e da raiz, que foram maiores a 25 °C. A 30 °C, a atividade de SOD e APX aumentou em comparação com o tratamento a 25 °C. Assim, a adição de 20 μ M de GA₃ ao meio de cultura e temperatura de 25 °C na sala de crescimento deve ser utilizada para cultura de embriões zigóticos de estrelitzia.

Termos para indexação: Antioxidante; cultura de embriões; giberelina; redutase do nitrato.

INTRODUCTION

Embryo rescue is one of the most feasible techniques for propagation of species with propagation limitations, mainly due to seed dormancy, such as strelitzia (*Strelitzia reginae* Banks ex Aiton). For plants that exhibit chemical and physical dormancy of seeds, which affects germination rates (Gomes et al., 2003; Santos et al., 2003; Paiva et al., 2004; Martins et al., 2020, Santos et al., 2020),

a zygotic embryo culture can be an effective method for plant propagation (Paiva et al., 2004; Paiva; Almeida, 2012). However, although germination was observed in tests conducted *in vitro*, there was no multiplication or formation of somatic embryos (Paiva et al., 2004). Therefore, studies are needed to improve this process, and increase the efficiency of the *in vitro* propagation of the species.

For propagation using embryos, supplementation of the culture medium with gibberellic acid (GA_3) may increase propagation efficiency. For some species, supplementation of the germination medium with GA_3 has been used since this compound regulates the germination of seeds and zygotic embryos. The *in vitro* germination of some species, such as *Maclura tinctoria*, *Cocos nucifera*, and *Syagrus coronata*, has been increased by supplying the medium with GA_3 (Gomes et al., 2010; Medeiros et al., 2015; Montero-Cortés et al., 2011). This growth regulator could be important for cell growth and elongation, leaf expansion and photosynthetic processes, as well as for the activity of important metabolic enzymes, such as nitrate reductase (Gupta; Chakrabarty, 2013; Bezerra et al., 2019). Enhancement of nitrate reductase activity may be beneficial, since this enzyme is responsible for the assimilation of nitrogen, an essential element found in many macromolecules and components of secondary metabolism, including proteins, nucleic acids, cell wall components, hormones, and vitamins (Krapp, 2015).

However, it is important to balance the concentration of growth regulators, since excesses of these compounds may alter the cytogenetic stability of plants (Samarfard et al., 2014).

In addition, the germination of zygotic embryos and seedling growth may also be affected by environmental conditions with temperature being a limiting factor, as it affects water uptake and the regulation of biochemical and enzymatic reactions (Bewley et al., 2014; Zucareli; Henrique; Ono, 2015; Rodrigues et al., 2020). In studies with *Myrciaria* spp. and *Rehmannia glutinosa*, optimal development was observed when these species were maintained in growth room at 5 °C and 26/18 °C, respectively, which differ from the temperature commonly used (25 °C) (Cui et al., 2000; Picolotto et al., 2007). Since *strelitzia* is a tropical species, it may require temperatures above 25 °C under *in vitro* conditions, which must be investigated. Temperatures for seedling germination and development differ among the species and could induce stress factors, stimulating oxidative stress and increasing reactive oxygen species (ROS) production in some situations (Marutani et al., 2012; Souto et al., 2017).

Thus, the aim of this study was to evaluate how different temperature and GA_3 concentrations may affect the *in vitro* germination of zygotic embryos by analyzing the activity of some antioxidant enzymes and cytogenetic stability.

MATERIAL AND METHODS

Mature seeds collected from *strelitzia* plants cultivated at 21° 45' S latitude, 45° 00' W longitude, and 920 m altitude were sterilized in a laminar flow hood by immersion for 30 seconds in 70% ethanol and then in 2.5% sodium hypochlorite for 15 minutes, washed three times in distilled water and autoclaved (Paiva et al., 2004). After sterilization, the zygotic embryos were excised.

Zygotic embryos were inoculated in MS medium (Murashige; Skoog, 1962) supplemented with 30 g L⁻¹ sucrose and 0.4 g L⁻¹ polyvinylpyrrolidone (PVP) and solidified with 2.5 g L⁻¹ Phytigel® (Paiva et al., 2004). The pH of the medium was adjusted to 5.8, and the medium was autoclaved at 121 °C for 20 minutes.

GA_3 and embryo germination

The effect of different GA_3 concentration [0 (control), 5, 10, and 20 µM] on zygotic embryo germination and seedling development was studied. For each treatment, 32 embryos were placed in the dark at 25 ± 2 °C for seven days after inoculation and then transferred to controlled condition (16-hour photoperiod, temperature of 25 ± 2 °C and photon irradiance of 36 µmol m⁻² s⁻¹). At seven days after inoculation, the zygotic embryo germination (embryo showing radicle protrusion) percentage (%) was evaluated, and at 60 days after inoculation, the germination percentage (%) was evaluated again, along with the root and shoot lengths (cm). Nitrate reductase activity and cytogenetic stability were also determined in seedlings regenerated from zygotic embryos after 60 days of growth on hormone-free medium (control) and medium containing 20 µM GA_3 .

Nitrate reductase activity

The protocol used for this analysis was described by Berger and Harrison (1995), with 9 replicates per treatment in a 2 x 2 factorial arrangement (GA_3 concentration x plant material), for testing shoots and roots.

Cytogenetic stability

To determine the cytogenetic stability of the seedlings regenerated from zygotic embryos, DNA content and ploidy were evaluated by flow cytometric analyses. Nuclear suspensions of 50 mg of leaf were prepared, and the nuclei were released from the cells by cutting samples of this material with a scalpel in 1 mL LB01 nuclear lysis buffer (Doležel; Binarová; Lucretti, 1989). The nuclear suspension was initially filtered through a 50-µm sieve to

remove any fragment and then stained with 25 $\mu\text{L mL}^{-1}$ propidium iodide. Next, the samples were analyzed for 4 minutes with the DNA from at least 6000 nuclei, quantified using fluorescence emission. The reference standard used was a pea leaf (*Pisum sativum*, 9.09 pg). For the analysis, 15 replicates per treatment were performed.

Histograms were obtained using a FACSCalibur® (Becton Dickinson, Franklin Lakes, New Jersey, USA) flow cytometer and analyzed with Cell-Quest software (Dickinson, 1998). The amount of DNA (pg) obtained from the seedlings was calculated using the following equation: amount of DNA (pg) = (position of the G1 peak of the sample/position of the G1 peak of the pea standard) x 9.09.

Temperature and seedling development

After sterilization of the *strelitzia* seeds, the zygotic embryos were cultured on MS medium supplemented with 20 $\mu\text{M GA}_3$. The embryo cultures (20 embryos per treatment) were placed in biochemical oxygen demand (BOD) incubators at different temperature: 25 °C, 30 °C day/25 °C night, and 30 °C. Embryos were maintained in the dark for 7 days (Ulisses et al., 2010) and then under a 16-hour photoperiod. At 7 days after inoculation, the zygotic embryo germination percentage (%) was evaluated, and at 60 days after inoculation, the germination percentage was again evaluated, along with root and shoot lengths (cm).

Temperature and antioxidant metabolism

After 60 days of cultivation, the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) were quantified in seedlings to evaluate the effects of temperature on antioxidant metabolism. Enzymatic extracts were obtained using the method described by Biemelt, Keetman and Albrecht (1998). SOD, APX, and CAT activities were determined following the protocols of Giannopolitis and Ries (1977), Nakano and Asada (1981) and Havir and McHale (1987), respectively.

Experimental design and statistical analysis

The experimental design was a completely randomized design (CRD). The data obtained were analyzed by analysis of variance, and when significant ($P < 0.05$) by the “F” test, the means of the qualitative treatments were compared with the Scott-Knott test ($P > 0.05$). The means obtained in quantitative treatments were analyzed by regression analysis, selecting the equation with the highest coefficient of determination (R^2). The analyses were performed using SISVAR statistical software (Ferreira, 2014).

RESULTS AND DISCUSSION

Germination and radicle protrusion were observed 7 days after inoculation of the zygotic embryos in all treatments. No difference was observed in the average germination percentage among the treatments at that time. After 60 days, for the embryos cultivated in medium with 20 $\mu\text{M GA}_3$, a germination percentage of 72% was observed, whereas in the absence of this growth regulator, the germination percentage was only 41% (Figure 1). Similar results were found for *Cocos nucifera* (L.), where the use of GA_3 increased the germination of zygotic embryos by 50% (Montero-Cortés et al., 2011).

In addition, higher GA_3 concentration promoted shoot development, with a mean length of 3.14 cm for the maximum calculated concentration of 16.62 $\mu\text{M GA}_3$ (Figure 1B). This result is probably associated with the ability of this growth regulator to improve the expression of genes related to the biosynthesis of endogenous gibberellin and cytokinins in the shoot, leading to increased cell size and cell division (Liu et al., 2018; Cruz et al., 2019).

However, GA_3 did not have a significant effect on the growth of the *strelitzia* root system, contrary to some studies indicating that exogenous application of this growth regulator can affect root development, owing to increased expression of genes related to auxin synthesis and transport (Liu et al., 2018).

Nitrate reductase activity

The use of GA_3 affected the activity of nitrate reductase. Higher activity of this enzyme was observed in seedlings from embryos germinated on medium with 20 $\mu\text{M GA}_3$ (682.27 $\mu\text{M NO}_2^- \text{min}^{-1} \text{mg}^{-1} \text{protein}$) compared to seedlings regenerated in the absence of this growth regulator (465.54 $\mu\text{M NO}_2^- \text{min}^{-1} \text{mg}^{-1} \text{protein}$) (Figure 2A).

GA_3 increased the nitrate reductase activity in roots (719.95 $\mu\text{M NO}_2^- \text{min}^{-1} \text{mg}^{-1} \text{protein}$) and shoots (427.86 $\mu\text{M NO}_2^- \text{min}^{-1} \text{mg}^{-1} \text{protein}$) (Figure 2B and 3). Higher concentrations of GA_3 induced an increase in the activity of nitrate reductase, an enzyme essential for nitrogen assimilation, in *Trigonella foenum-graecum* L. (Dar et al., 2015). This higher activity may have contributed to the improved development of the seedlings, since this enzyme is responsible for the reduction of nitrate to nitrite, which is the first reaction in the assimilation of nitrogen, an element present in many essential macromolecules (Krapp, 2015).

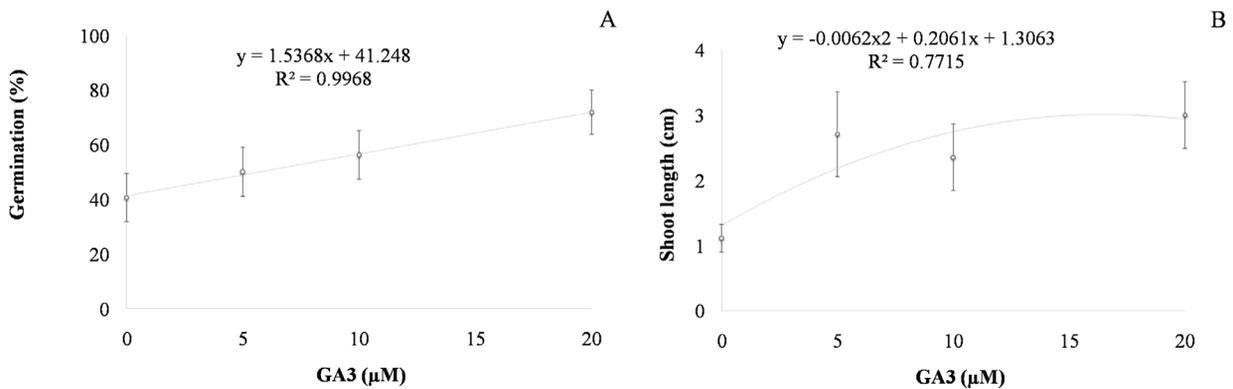


Figure 1: Germination percentage of zygotic embryos (A) and shoot length (B) of strelitzia at different GA₃ concentrations after 60 days of culture. The data are expressed as the mean ± standard error (bars).

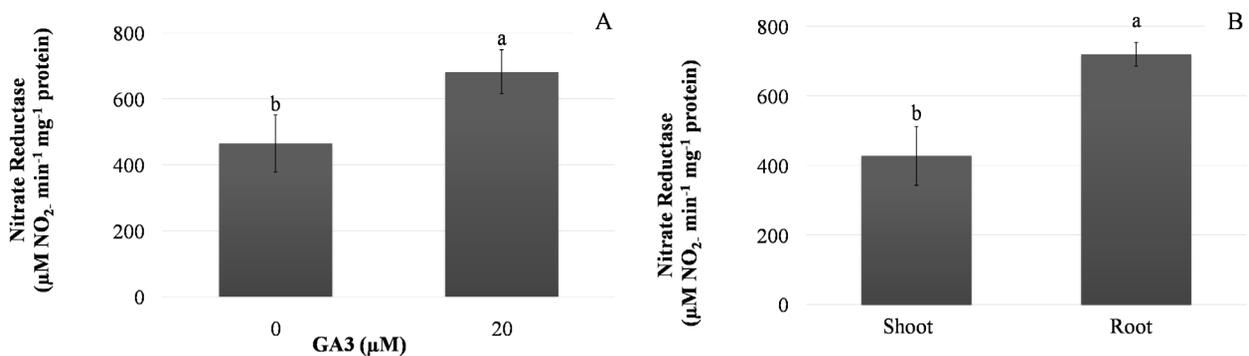


Figure 2: Activity of the nitrate reductase enzyme in strelitzia seedlings cultured *in vitro* in the presence of GA₃ (A) and enzyme activity in the shoot and root (B). The data are expressed as the means ± standard errors (bars). Means followed by the same letter do not differ from each other according to the Scott-Knott test ($P < 0.05$).

This increase in nitrate reductase activity may be a result of the exogenous application of GA₃ (Qin et al., 2019). Regardless of the GA₃ concentration used, there was an increase in nitrate reductase activity in the roots (719.95 μM NO₂⁻ min⁻¹ mg⁻¹ protein), and this value was 41% higher than that in the shoots (427.86 μM NO₂⁻ min⁻¹ mg⁻¹ protein) (Figure 2B and 3). This result can be explained by the fact that species that have high nitrate reductase activity in the leaves use reducing power from the photochemical stage of photosynthesis, and plants grown *in vitro* have a low photosynthetic rate (Robredo et al., 2012). Thus, higher assimilation of nitrogen is necessary in the root system using reducing power derived from respiration and pentose pathways (Esposito et al., 2005). Therefore, translocation of nitrate reduction in the leaf occurs only when there is saturation of the enzymes in the root system.

Cytogenetic stability

The use of 20 μM GA₃ to optimize the germination of zygotic embryos and the development of the strelitzia seedlings had no effect on the cytogenetic stability of the seedlings, since there were no differences in stability (Figure 4). In addition, there were no differences in DNA levels, which were 1.65 pg and 1.67 pg for plants grown in the absence and presence of GA₃, respectively. The coefficients of variation for these values were 2.95 and 2.42, respectively.

The use of growth regulators is beneficial to the *in vitro* plant growth and development (Ayub et al. 2019; Mitrofanova et al. 2019; Silva et al., 2019), but it may lead to negative changes in cytogenetic stability (Samarfard et al., 2014, Masouleh; Sassine, 2020). Flow cytometry analyses quantify the DNA content and

estimate the ploidy level, since the size of the genome is highly correlated with the number of chromosomes. This analysis measures the fluorescence emitted by fluorochromes, such as propidium iodide, which binds to DNA and RNA. Thus, when DNA content increases, fluorescence intensity also increases (Doležel et al., 2004). However, the higher concentrations of GA₃, which were associated with higher percentages of germination of the zygotic embryos and better development of *strelitzia* seedlings, did not cause negative effects on the cytogenetic stability of the plants. These results are consistent with the finding that DNA content did not differ when GA₃ was used for *Solanum melongena* shoot elongation (Xing et al., 2010), thus maintaining the cytogenetic stability of the plants grown *in vitro* with GA₃.

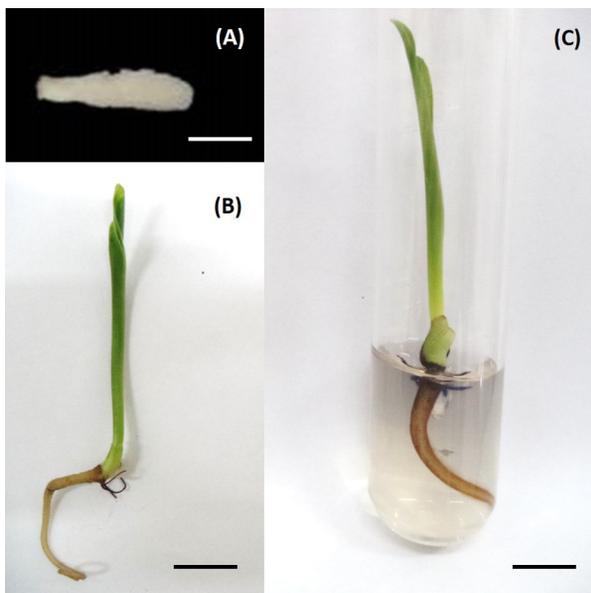


Figure 3: Embryo (bar=25mm) (A), seedlings of *strelitzia* obtained *in vitro* from zygotic embryos grown in the presence of GA₃ after incubation on MS medium supplemented with 20 μM GA₃ for 30 days (bar= 2 cm) (B) and 60 days (bar= 4 cm) (C).

Effect of temperature on zygotic embryo germination and plantlet development

There was no effect of temperature on the germination of the zygotic embryos of *strelitzia* 7 and 60 days after inoculation, with 67% of embryos germinating. However, after 60 days, the shoot length of the seedlings was higher for those cultivated at 25 °C

and those cultivated at alternating temperatures of 30/25 °C (2.72 cm) (Figure 5A). Larger root length values (2.6 cm) were found only at a constant temperature of 25 °C. In contrast, treatment with alternating temperatures of 30/25 °C and a constant temperature of 30 °C produced root lengths of 1.53 cm and 1.17 cm, corresponding to a reduction of 41% and 55%, respectively, relative to of the length observed at a constant temperature of 25 °C (Figure 5B).

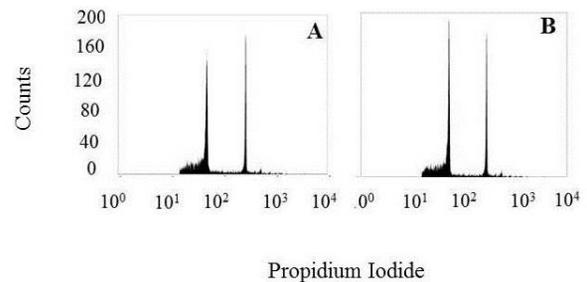


Figure 4: Histograms of the relative intensity of propidium iodide using nuclei from the leaves of *strelitzia* seedlings grown in MS medium in the absence of GA₃ (A) or in the presence of 20 μM GA₃ (B).

After 60 days, the length of the aerial part was greater at a constant temperature of 25 °C and at alternating temperatures of 30 / 25 °C and the root length was greater at 25 °C, showing that higher temperatures are not ideal for the development of this species. In many cases, this reduction in growth with increasing temperature is related to metabolic changes, since protein denaturation, enzyme inactivation and increased production of ROS occur, leading to loss of vigor and abnormal plant development (Hemantaranjan et al., 2014). However, the presence of an antioxidant system can minimize the deleterious effects of ROS (Hasanuzzaman et al., 2012; Soares et al., 2016). During *strelitzia* development at higher temperatures, there was also an increase in the activity of some antioxidant enzymes, such as SOD and APX (Figure 6). This higher SOD activity prevents hydroxyl radical formation via the Haber-Weiss reaction (Gill; Tujeta, 2010; Silva et al., 2017, Del Río et al., 2018). However, since it is the first line of plant defense, it leads to the formation of H₂O₂, which also causes oxidation problems in the system. The increased activity of this enzyme alone is thus not sufficient to ensure the efficiency of the antioxidant system. Therefore, the presence of enzymes such as APX and CAT, which lead to the dismutation of H₂O₂ to H₂O

and O_2 , is important to reduce the effects of oxidative stress (Gill; Tujeta, 2010; Silva et al., 2017, Acosta-Moto et al., 2019; Abreu, et al., 2020).

Temperature and antioxidant metabolism

Strelitzia seedlings that developed at 30 °C showed higher SOD and APX activities compared to seedlings grown at a constant temperature of 25 °C and alternating temperatures of 30/25 °C (Figure 6).

The SOD activity was 0.91 U SOD μg^{-1} protein at 30 °C, corresponding to a 40% and 32% increase in the activity of this enzyme compared to that at 25 °C (0.55 U SOD μg^{-1} protein) and 30/25 °C (0.62 U SOD μg^{-1} protein), respectively (Figure 6A).

The APX activity was 10.28 μmol ascorbate (AsA) min^{-1} μg^{-1} protein at the highest temperature (30 °C), 7.73 μmol AsA min^{-1} μg^{-1} protein at 25 °C and 6.68 μmol AsA

min^{-1} μg^{-1} protein at 30/25 °C, corresponding to a 25% and 35% increase in APX activity at 25 °C and 30/25 °C, respectively, in comparison to the activity at 30 °C (Figure 6B). In contrast, the average CAT activity was 0.30 μmol H_2O_2 min^{-1} mg^{-1} protein and did not differ among the evaluated temperatures.

Analysis of the strelitzia seedlings indicated that APX activity was higher at the highest temperature; however, CAT activity did not exhibit a considerable increase at the highest temperature compared to the lower temperatures, and no difference was observed. This may result from CAT operating in the absence of a reducing agent, becoming energy efficient for the removal of high concentrations of H_2O_2 only, whereas APX is responsible for the removal of H_2O_2 when present in small amounts owing to its higher affinity for this substrate (Gill; Tujeta, 2010, Silva et al., 2017). However, CAT has a high

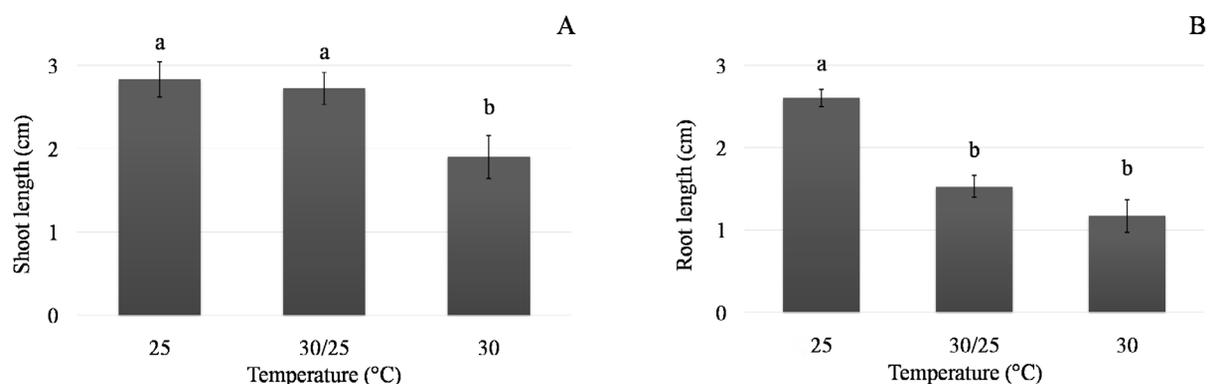


Figure 5: Effect of constant temperatures of 25 °C and 30 °C and alternating temperatures of 30/25 °C (day/night) on shoot length (A) and root length (B) after 60 days. The data are expressed as the means \pm standard errors (bars). The means followed by the same letter do not differ from each other according to the Scott-Knott test ($P < 0.05$).

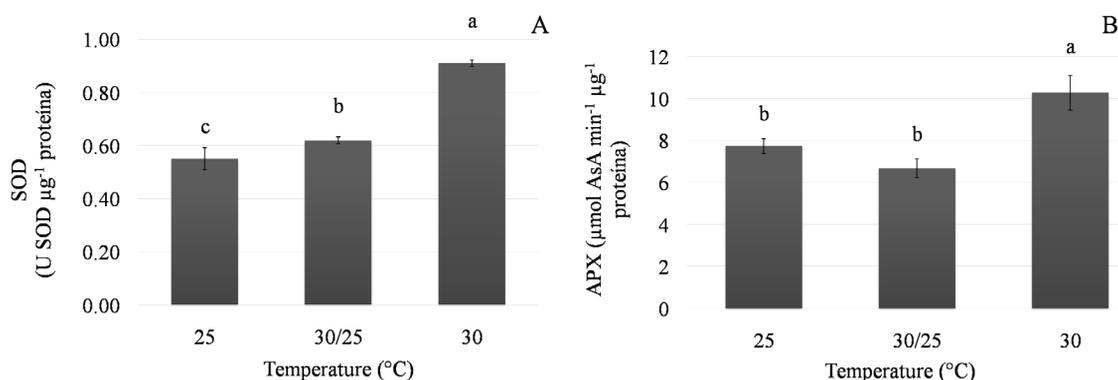


Figure 6: A) Superoxide dismutase (SOD) and B) ascorbate peroxidase (APX) enzyme activities in strelitzia seedlings under different temperature conditions (25 °C, 30/25 °C and 30 °C). The data are expressed as the means \pm standard errors (bars). The means followed by the same letter do not differ from each other according to the Scott-Knott test ($P < 0.05$).

conversion rate and can convert 6 million molecules of H₂O₂ to H₂O and O₂ per minute, and therefore, its activity is needed for the reduction in oxidative stress in many species (Gill; Tujeta, 2010). Thus, the increased SOD and APX activities alone may not have been sufficient to alleviate the stress caused by high temperatures, compromising the development of *strelitzia*.

In addition, for many species under extreme conditions, increased activity of antioxidant enzymes is not sufficient to alleviate oxidative stress, owing to the very high production of ROS, preventing their elimination (Silva et al., 2017) and leading to limitations on *in vitro* growth, development and establishment of plants. Thus, the temperature of 25 °C, commonly used in growth rooms, allows better development of *strelitzia* seedlings.

CONCLUSIONS

Supplementation of the culture medium with 20 µM GA₃ improved zygotic embryo germination and plantlet growth, provided high nitrate reductase activity and did not modify the cytogenetic stability of the seedlings. The recommended temperature for the growth of *strelitzia* seedlings is 25 °C, since the highest activity of the antioxidant enzymes (SOD and APX) occurred at 30 °C. Thus, the addition of 20 µM GA₃ to the culture medium and a temperature of 25 °C in the growth room should be used for *in vitro* cultivation of zygotic embryos of *strelitzia*.

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