

Warm stratification improves embryos development and seed germination of *Cycas revoluta*

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All relevant data are within the paper and its Supporting Information files.

Author Contribution Statement
J.B, A.S and A.G. conceived and designed the experiment. J.B., S.B. performed experiments and collected the data. J.B., S.B., Z.T., Q.L.A., and R.L. contributed reagents/materials/analysis tools. J.B., S.B., A.S. and A.G. analyzed data and wrote the manuscript.

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Abstract: The broad objective of this research is to study the effect of warm stratification on *Cycas revoluta* zygotic embryos length, seed germination and plant development. Four warm treatments were applied to seeds. Our results showed that seeds storage at room temperature or 30°C improved significantly zygotic embryos length. Moreover, time of germination was significantly reduced with the warm stratification. The highest percentage of germination was obtained with seeds warm treated at 30°C for 2 months while only 25% of seeds were able to germinate in the control. Regarding seedlings development, our results demonstrated that warm stratification did not affect plant development. No significant differences have been recorded in all the evaluated parameters except for root length. Taken together, these results underlined the beneficial effect of warm stratification on *Cycas revoluta* seed germination and plant development and proposed a new method to improve seed germination of *Cycas revoluta*.

1. Introduction

The sago palm (*Cycas revoluta* L.) is one of the widespread ornamental trees, grown in temperate, subtropical and tropical regions more precisely in Miyazaki and Kagoshima Prefectures in Kyushu District down to the Ryukyu Islands, Okinawa Prefecture in Japan (Zarchini *et al.*, 2011). Described as one of the most primitive species among the living cycads, *Cycas revoluta* has been used as an indoor and outdoor landscape plant for centuries (Stevenson, 1990; Jones, 1994).

Cycas revoluta is propagated either from seeds, which remain viable for only a short time, or from vegetative offshoots (Demiray *et al.*, 2017). Germination of seed of *Cycas revoluta* is hard and time consuming (Zarchini *et al.*, 2011). Seeds can take 3 to 9 months to initiate germination before they can continue to germinate for periods of a year or more. *C. revoluta* seeds also demonstrates rapid loss of viability and low mor-

phogenic potential, which hinder its conservation (NADERI *et al.*, 2015).

Breaking dormancy is the main problem faced by all *Cycas revoluta* (Frett, 1987). Several attempts have been made to overcome with *Cycas revoluta* seed dormancy problem. Priming treatments of seeds seems to offer a new way to increase seeds germination percentage. Mechanical and chemical scarification has been described widely as an efficient way to improve germination of the hard-seeds species of *Cycas* and some other species known for their hard-coated seeds (Frett, 1987; Rouhi *et al.*, 2010). Indeed, several studies have reported a great responsiveness of Cycads seeds to various pretreatments, namely, scarification, depulping, exposure to chemical substances like potassium nitrate, gibberellic acid or sulfuric acid or soaking in hot water for specific period of time (Zarchini *et al.*, 2011; Millaku *et al.*, 2012). Warm stratification was also used to improve seed germination of many species such as *Sambucus* and *Symphoricarpos* (Baskin *et al.*, 2002). The present study aimed to improve the germination of *Cycas revoluta* seeds through different warm stratification treatments as a way to develop an efficient *in vivo* germination protocol for this ornamental species.

2. Materials and Methods

Plant material

Freshly harvested seeds collected from 50 years old female mature plants grown in Faculty of Sciences garden, University Mohammed V in Rabat (Morocco) were used in this study.

Zygotic embryos length measurement

Seeds were soaked in water for 48 hours in order to soften the sacrotesta; the orange external layer. The sacrotesta was then removed mechanically with a knife. Seeds were then flamed with ethanol for 2 minutes. Sclerotesta layer was mechanically eliminated. The megagametophytes were surface sterilized for 20 minutes by soaking in 30% dilution of NaOCl containing 2-3 drops of Tween-20, followed by 3-4 rinses with sterile distilled water. After surface sterilization, megagametophytes were pooled, longitudinally bisected and the zygotic embryo (ZE) was excised from each megagametophyte. ZEs length was measured and the mean was calculated from at least 20 biological replicates.

Warm stratification treatments and seeds cultivation

After removing the sacrotesta mechanically, equal samples of seeds were subjected to different treatments. Treatments consisted in seed storage at room temperature (18-20°C) or 30°C for 2 or 4 months depending on the treatment (Table 1). The warm stratification temperatures were chosen based on previous reports that underlined the beneficial effect of seed storage at 18°C and 30°C on seed's germination (Roh *et al.*, 2004; Baldos *et al.*, 2014; Keun *et al.*, 2016). Untreated seeds were cultivated immediately and referred as control treatment (T_0). Seeds of *Cycas revoluta* were then planted in bins containing sterilized soil at 2-5 cm depth. Cultures were incubated at $25\pm 2^\circ\text{C}$, with a photoperiod of 16 hours of light and 8 hours of darkness and watered daily depending on soil moisture.

Table 1 - Different heat treatments used to enhance seed germination of *Cycas revoluta*

Treatment	Type of treatment	Time of application
T_0	Control/Untreated	0 hours
T_1	Room temperature (18-20°C)	2 months
T_2	30°C	2 months
T_3	Room temperature (18-20°C)	4 months
T_4	30°C	4 months

Germination and plant growth parameters recording

Weekly observations were performed and seed emergence was recorded after 10 months of cultivation. The data for the kinetic of germination and time of germination (days) were recorded. Percentage of germination was calculated after ten months of culture. Number of leaves, stip height and width, root length and the length of the most developed leaf were determined at the end of the experiment.

Statistical analysis

Zygotic embryos length, time of germination, number of leaves, stip height and width, root length and the length of the most developed leaf were compared using a fixed model of analysis of variance (ANOVA). For each parameter and condition, means were calculated based on at least thirty biological replicates. In case of significant difference between groups, a Tukey test was used for means separation, at risk of 0.05.

3. Results

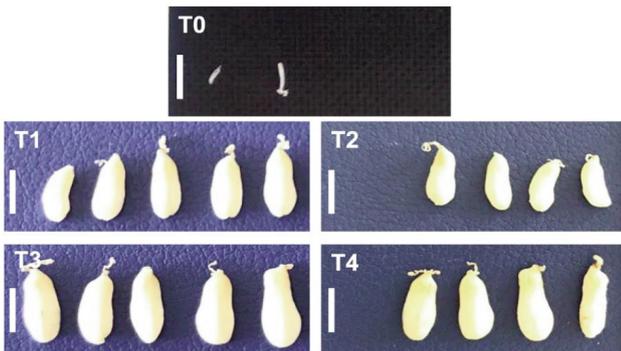
Effect of warm stratification on zygotic embryos length

Zygotic embryos (ZEs) length was investigated in the different warm pre-treatments. Our results showed that all the applied treatments (T_1 , T_2 , T_3 and T_4) influenced ZEs length compared with the untreated seeds (T_0) (Fig. 1). ZEs length of seeds subjected to T_1 , T_2 , T_3 and T_4 was significantly enhanced, but no significant difference was observed between T_3 and T_4 seeds. Indeed, ZEs length increased by 134%, 276%, 300% and 342% in T_1 , T_2 , T_3 and T_4 warm treated seeds respectively when compared to untreated seeds.

Effect of warm stratification on seed's germination

As mean to gain more insight on the effect of warm storage on seed germination, time of germination (calculated starting from the first day of seeds cultivation), percentage of germination and its kinetic were determined for each treatment. The results were summarized in figures 2, 3 and 4.

A



B

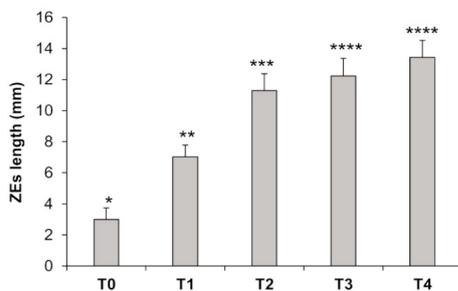


Fig. 1 - Effect of warm stratification treatments on *Cycas revoluta* embryos length. (A) Zygotic embryos isolated from seeds subjected to T_0 , T_1 , T_2 , T_3 and T_4 . Scale bars=1 cm. (B) Zygotic embryos length in the different treatments. Bars are mean \pm SD of at least thirty biological replicates. Asterisks indicate statistical difference according to Tukey test ($p < 0.05$).

Effect of warm stratification on the time of germination

Minimum time of germination (163.33 days) was recorded in seeds stored at 30°C for 4 months followed by those stored at 30°C for 2 months (198.19 days) and those stored at room temperature for 4 months (202 days). Note that the difference between these three treatments was statistically insignificant. Untreated seeds (T_0) and those stored at room temperature for 2 months (T_1) took the maximum time duration for germination with an average of 294.4 days and 256.75 days respectively (Fig. 2).

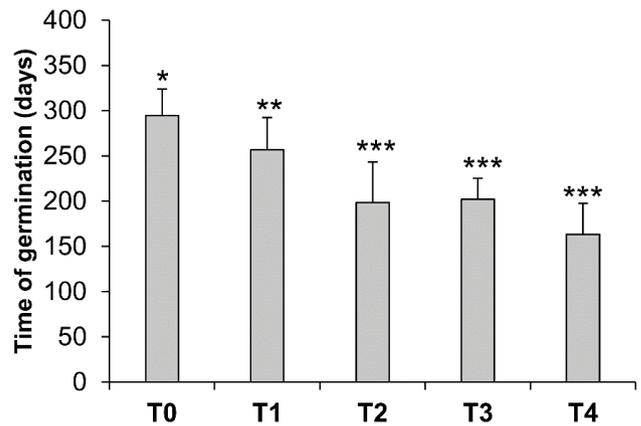


Fig. 2 - Effect of different warm stratification treatments on the time of germination of *Cycas revoluta* embryos. Bars are mean \pm SD of at least thirty biological replicates. Asterisks indicate statistical difference according to Tukey test ($p < 0.05$).

Effect of warm stratification on the percentage of germination

Investigating the percentage of germination of seeds from the four different treatments and the untreated ones revealed that seed germination response varied among the different warm treatments. The highest percentage of germination (49.33%) was recorded in seeds stored for 2 months at 30°C while the lowest value of 8% was observed for seeds stored at room temperature for 4 months (Fig. 3). Untreated seeds showed although a percentage of germination around 25%.

Effect of warm stratification on the kinetic of germination

Investigating the kinetic of germination revealed a high variability between the different treatments applied (Fig. 4). Our data showed that seeds storage at 30°C for two or four months (T_2) allowed seeds to germinate faster. Indeed, seeds started to germinate

after four months of culture whereas, the first germinated seeds appeared after five months and 6 months respectively for T₁ and T₀. Seeds storage at room temperature for four months (T₃) delayed the germination by one month compared to untreated

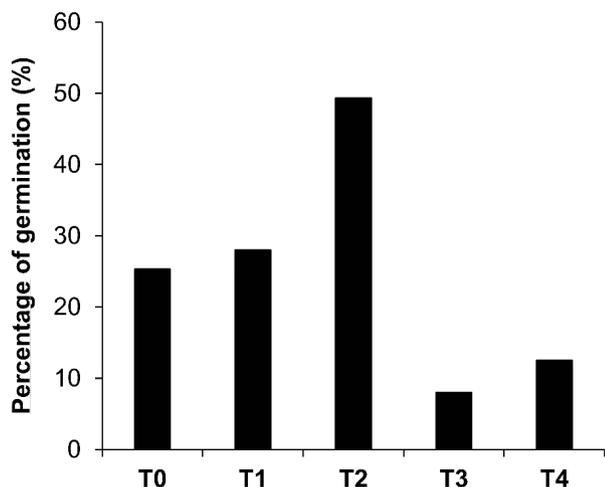


Fig. 3 - Effect of different warm stratification treatments on the percentage of germination of *Cycas revoluta* embryos.

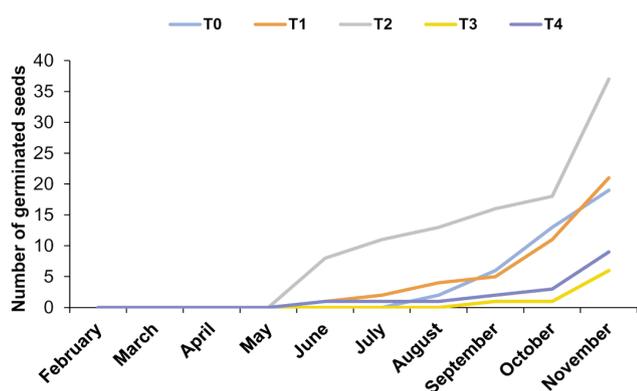


Fig. 4 - Effect of different warm stratification treatments on the kinetic of germination of *Cycas revoluta* embryos.

seeds (T₀). These data joined those related to time of germination.

Effect of warm stratification on plant's growth and development

Seeds pre-treatment with temperature affected zygotic embryos length and their germination. These results prompted us to see whether the pre-treatment can influence seedlings growth and development. Several growth parameters namely, number of leaves per plant, stip height and width, root length and the length of the most developed leaf were evaluated. Table 2 summarized the results.

Data analysis had shown that seeds pre-treatment did not affect the number of leaves per plant. Seeds storage for 2 months at 30°C gave a maximum number of leaves per plant (1.50±0.59) while the lowest value was obtained with T₄ treatment (1.14±0.37). Note that these differences remained insignificant. Leaf length was also not significantly affected by the priming treatments. It was found that maximum leaf length was 57.33±3.05 cm in seedlings subjected to T₃ treatment while a minimum of 48.22±4.23 cm was recorded with T₁ treatment.

Stip height showed no significant difference between the different treatments. Stip width, on the other hand, displayed significant variations between the different treatments. A significant increase in stip width was observed in seedlings subjected to T₁ and T₂ as compared to the control (T₀) while non-significant changes was observed between the remaining treatments(T₃ and T₄).

Regarding root development, root length showed significant variations between the different treatments. Seeds storage for 2 months at 30°C resulted in a significant increase in root length compared to untreated seeds while a decrease in root length was observed with T₃ and T₄ treatments.

Table 2 - Effect of different priming treatments on growth parameters of *Cycas revoluta* seedlings

Treatments	Number of leaves/plant	Stip height (cm)	Stip width (cm)	Leaf length (cm)	Root length (cm)
T ₀	1.31±0.47 a	3±0.47 a	1.81±0.19 b	50.63±6.24 abc	26.62±3.15 bc
T ₁	1.20±0.42 a	3.92±0.32 a	2.10±0.24 a	48.22±4.23 bc	21.60±2.07 cd
T ₂	1.50±0.59 a	3.77±0.45 a	2.15±0.22 a	55.91±4.25 ab	35.42±2.69 a
T ₃	1.33±0.57 a	3.5±0.86 a	1.86±0.30 ab	57.33±3.05 a	31.66±2.31 ab
T ₄	1.14±0.37 a	3.50±0.47 a	1.58±0.15 b	49±5 c	21±3 d

Values are mean ± SD of at least thirty biological replicates. Values with different letters indicate the statistical significance (p<0.05) according to Tukey test.

4. Discussion and Conclusions

Cycas revoluta is an ornamental tree that has been widely used as an indoor and outdoor landscape. However, despite its importance in landscape design, it is facing problems regarding its germination mainly due to the hard-coat (Ullah *et al.*, 2020). Different methods have been tested to overcome hardseededness. These include mechanical, chemical, and physical scarification treatments to make the seed coat permeable for water uptake. Several reports have shown that seeds pre-treatment with sulphuric acid (H_2SO_4), potassium nitrate (KNO_3) or GA_3 results in a better germination results. Indeed, *Zamia furfuracea* and *Cycas revoluta* germinations were assessed by Schutzman, using a chemical scarification with concentrated H_2SO_4 and then soaking them in gibberellic acid (GA_3) (Schutzman, 1984, 1989). However, these pretreatments are not always reliable with cycads (Dehgan and Yuen, 1983). For instance, Zarchini *et al.* (2011) have found that the use of sulphuric acid even at lower concentrations seems to affect negatively seed's germination while combined with hot water seems to improve *Cycas revoluta* seed germination.

It is well admitted that seeds storage in a warm environment usually results in relatively rapid embryos development (Baskin *et al.*, 2002; Merritt *et al.*, 2007; Razavi and Hajiboland, 2009). However, no previous work has been conducted to study the effect of seed storage in warm conditions on *Cycas revoluta* germination and seedlings development. Thus, we investigated the effect of warm storage on zygotic embryos development, seeds germination and growth of young seedlings of *Cycas revoluta*. Our results showed that warm storage at 30°C for 2 months or 4 months speeded the germination when compared to the untreated plot (Fig. 2). Seeds storage at 30°C for 2 months improves also the germination percentage. Meanwhile, the prolonged storage at 30°C for 4 months reduced the germination percentage by half compared to the untreated plot which suggest that prolonged storage have an inhibitive effect rather than stimulating the germination (Fig. 3). Chen *et al.* (2007) have found that *Prunus campanulata* seeds required 4-6 weeks of warm followed by 8 weeks of cold stratification for maximum germination percentage. This finding was explained by the accumulation of high amounts of GA as a result of the cold stratification while GAs in warm stratified embryos were significantly low. Thus,

Prunus warm stratified embryos failed to germinate since GAs content was very low. In *H. salicornicum* and *S. imbricate*, seeds germination was significantly improved when seeds were stored at $40\pm 2^\circ C$ for three months (El-Keblawy, 2013). Warm stratification for at least 1 month appeared to be essential for the germination of Japanese snowbell (*Styrax japonicus*) (Baskin, 2009). Indeed, Roh and Bentz (2003) found that without warm stratification, seeds were not able to germinate. Seed's dormancy in some orchids, mainly *Epipactis palustris* and *Goodyera pubescens*, could be overcome by a warm incubation of seeds followed by cold storage. This was explained by the fact that warm and cold stratification increased seeds permeability to water thereby softening the testa (Roh and Bentz, 2003). Thus, the increase observed in germination percentage could be attributed to the increase of seed's hard coat permeability caused by the warm storage which allow the removal of the physical barrier to water absorption. Besides the improvement of seeds germination percentage and the reduction of the germination time, warm storage improved significantly zygotic embryos length which suggest that warm scarification result in a better development of zygotic embryos. Regarding seedlings development, our data showed that warm scarification did not affect plant development (Fig. 5). Indeed, no significant differences have been recorded in all the evaluated parameters except for root length. Taken together, these results suggest that warm scarification improved seeds development and germination by prompting zygotic embryos length, increasing germination percentage and reducing time of germination.



Fig. 5 - *Cycas revoluta* plants obtained from pre-treated seeds with temperature (T1, T2, T3 and T4) and untreated seeds (T0).

This study aims to study the effect of seeds storage at different temperatures on *Cycas revoluta* seeds germination and development in order to develop an efficient *in vivo* germination protocol that can be used for mass production of this ornamental tree. Based on our results, we found that seeds storage at 30°C for 2 months or 4 months reduced time of germination. However, the highest percentage of germination was only assessed when seeds were stored at 30°C for 2 months. Taken together, this protocol represents a useful and potential method to improve commercial mass propagation of *Cycas revoluta*.

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