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Warm stratification combined with organic manure application enhances seed germination and improves *Cycas revoluta* growth and development

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Abstract: *Cycas revoluta* (Sago palm) is one of the widespread ornamental plant, used as an indoor and outdoor plant. Seed propagation is extremely hard and time consuming, given the physical dormancy imposed by hard coat. The use of warm stratification improves seed germination by prompting embryos development. As mean to gain more insight on the beneficial effect of warm treatment on seed germination, histological analysis of warm stratified and untreated embryos was conducted. Our results revealed that warm treatment accelerated embryos development, resulting in a rapid differentiation of embryos' tissues. α-amylase, GA_3 and ABA quantification showed that warm stratified embryos accumulated higher and lower amounts of α-amylase and ABA respectively compared to untreated embryos. Regarding plant development, our results showed that organic manures significantly improved *Cycas revoluta* growth and development. The best response was recorded with the application of sheep manure. Indeed, sheep manure addition increased plant height, the number of leaves per plant, stip length and width by nearby 188% and 61%, 36% and 17% respectively. In roots, the presence of nodules had been recorded in the three applied treatments and more importantly in the presence of sheep manure. At the physiological level, sheep manure supplementation improved photosynthetic apparatus and nitrogen content in leaves (by 75%), thereby explaining the growth promotion. Taken together, these results underlined the beneficial effect of organic manure on *Cycas revoluta* growth and development and proposed a new strategy to improve plant growth and development with the use of sheep manure as organic amendment.

1. Introduction

Cycas revoluta is one of the common ornamental trees, grown in temperate, subtropical and tropical areas of the world, more precisely in Miyazaki and Kagoshima Prefectures in Kyushu District down to the Ryukyu Islands, Okinawa Prefecture in Japan (Dehgan *et al.,* 1994; Zarchini *et al.,* 2011). Taxonomically known as the foremost primitive species among the living cycads, this species has been used as an indoor and outdoor landscape plant over decades (Jones, 1994).

Cycas revoluta can either be propagated from seeds or from vegetative offshoots (Demıray *et al.,* 2017). *Cycas* propagation through seeds is extremely hard due to the physical dormancy imposed by the presence of hard coat (Frett, 1987; Zarchini *et al.,* 2011; Ullah *et al.,* 2020). Moreover, seeds also showed a rapid loss of viability along with a low morphogenic potential, which delays their germination and thus limits their use for rapid and effective propagation (Naderi *et al.,* 2015; Demıray *et al.,* 2017). To overcome these limitations, different pre-germination treatments have been applied to accelerate the germination process where several studies have suggested that germination can be improved by mechanical or chemical scarification (Frett, 1987; Zarchini *et al.,* 2011; Fallahabadi *et al.,* 2012; Ullah *et al.,* 2020). Warm stratification has also been proposed as an efficient strategy to enhance seed germination (Benjelloun *et al.,* 2021).

Besides the problem encountered with seeds' propagation, *Cycas revoluta* is a slow growing species that requires up to 10 years to reach the reproductive maturity (Frett, 1987; Rinaldi, 1999). Nutritional management through organic manures is useful to enhance plant growth, yield and quality (El-Sherbeny *et al.,* 2012; Marak *et al.,* 2020). Organic manures are, by definition, derived from animals, plants and microorganisms. A large panel of manures are nowadays available in the local markets at affordable prices (Khairnar and Kaur, 2022). Organic fertilizers act as slow-release fertilizers, providing nutrients in lower amounts over an extensive time period (Shaji *et al.,* 2021). They are considered as natural source of nutrient supply in the soil and ensure the return of essential macronutrients such as nitrogen (2.42%), phosphorus (1.51%) and potassium (0.41%) as well as micronutrients including calcium, magnesium, manganese and sulphur (Parham *et al.,* 2002; Wang *et al.,* 2010; Khaitov *et al.,* 2019). Organic amendments such as animal, green or composted farmyard manures enhance soil's physical properties by reducing bulk density, improving soil water-holding capacity and increasing infiltration rates (Tester, 1990; Werner, 1997; Gopinath *et al.,* 2008). They also heighten the existing soil nutrients, resulting in the improvement of plant growth by increasing nutrient availability (Shaji *et al.,* 2021).

Organic manures have broadly been used to enhance plant growth in many crop species, including wheat (*Triticum aestivum*), sugarcane (*Saccharum officinarum*), rice (*Oryza sativa*) and maize (*Zea mays*) and in ornamental plants such as marigolds, gladiolus (*Gladiolys grandiflorus*) and roses (Shanmugam and Veeraputhran, 2000; Attiyeh *et al*., 2002; Singh *et al*., 2006; Aziz *et al.,* 2010; Abbas *et al.,* 2012; Soomro *et al.,* 2013; Idan *et al.,* 2014; Baruati *et al.,* 2018). We thought to investigate the effect of organic, sheep and horse manure, on *Cycas revoluta* growth and development by assessing morphological, biochemical and physiological analyses. Besides, as mentioned above, warm stratification enhances *Cycas revoluta* seed germination by accelerating embryos development. However, the beneficial effect of warm temperature on seeds' germination is still not fully understood. Thus, we focused on the histological and the biochemical changes occurring in the warm stratified seeds in comparison with the control, to explain the positive effect of warm stratification on seed's germination.

2. Materials and Methods

Plant material

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

Effect of warm stratification on embryos development

Warm stratification of *Cycas revoluta* seeds was applied as described by Benjelloun *et al.* (2021). Warm treatment was applied after the mechanical removal of the sacrotesta. Two treatments have been applied: the first treatment (T1) consisted on seed storage at 25°C for 2 months. The second treatment (T2) consisted on seed storage at 30°C for 2 months. Meanwhile, control plot (C) was not subjected to any treatment. The embryos from the control plot are 0 month-old.

Zygotic embryos length and width measurements. Zygotic embryos (ZE) were isolated according the protocol described by Benjelloun *et al.* (2021). ZEs length and width were measured in each condition and the mean was calculated from at least 12 biological replicates.

Zygotic embryos germination. Seeds subjected to the three different conditions (C, T1 and T2) were planted in bins containing sterilized soil at 25 cm depth. Cultures were incubated at 25±2°C, with a photoperiod of 16 hours of light and 8 hours of darkness and watered daily depending on soil moisture. Daily observations were performed and seed emergence was recorded. Percentage of germination was then calculated.

Microscopic observation of zygotic embryos. Microscopic observation of zygotic embryos from C and T2 treatments (2 months-old) was conducted using Epson light microscope equipped with an imaging software. For that, zygotic embryos were fixed using a mixture of 95°C ethanol and acetic acid (3:1) for 24 hours as described by Brhadda and Abousalim (2007). ZEs were dehydrated by passing through a series of alcohol baths (70°, 95° and 100°C). After complete dehydration, ZEs were transferred to two successive bath of toluene for 24 hours. Samples inclusion in paraffin was performed in three successive bath of paraffin maintained at 80°C, each bath lasting 60 minutes. The 10-15 µM thick sections, made with a microtome, were spread on perfectly degreased slides. Sections were stained with 1% toluidine blue and viewed with.

Qualitative assay for alpha-amylase activity. The presence of alpha-amylase activity was assessed according to the method of Xie *et al.* (2007). Zygotic embryos and embryoless half-seeds were placed on 2% agar in 9-cm petri dishes. The agar plates included 0.2% of soluble potato starch, 20 mM CaCl2 and 20 mM Sodium succinate pH 5.0. The petri dishes were then incubated at 28°C for 48 hours. After incubation, I_2/KI solution (2.8 mM I_2+ 43.4 mM KI in 0.2 N HCl) was added to the plates. After 5 minutes, the reaction between starch and iodine turned the agar plates to blue-purple. The agar around ZEs or the half-seeds with alpha-amylase activity remained colourless due to starch hydrolysis triggered by alpha-amylase activity.

Quantitative assay for alpha-amylase activity. Alpha-amylase activity was quantitatively determined according to a slightly modified version of the method of Miller (1959) as described by Liu *et al.* (2018). Isolated zygotic embryos were collected, ground and mixed with 100 ml of chilled distilled water. The mixture was soaked in a cooling bath (4°C) for 10 minutes. The mixture was filtered and the extract was then collected and centrifuged at 12000 rpm for 10 minutes at 4°C. The recovered supernatant was heated for 15 min at 70°C. 1 ml of embryos extract was then mixed with 1 ml of 1% soluble starch dissolved in sodium acetate buffer pH 5.6. The mixture was incubated for 15 minutes at 40°C ad then boiled for 5 minutes in the presence of 2 ml of 3,5-dinitrosalicylic acid. The amount of released reducing sugar was measured using a spectrophotometer at 540 nm using maltose as the reducing sugar standard.

Phytohormones quantification in zygotic embryos. Abscisic acid (ABA) and Gibberillic acid (GA_3) quantification in zygotic embryos was performed on a Finnigan LC-MS/MS system (Thermo Electron, San Jose, CA, USA) consisting of a surveyor autosampler, a surveyor MS pump and a Finnigan LTQ linear ion trap mass spectrometer equipped with an ESI source that was operated in negative mode. The data acquisition software used was Xcalibur. The LC separation was carried out by an HiQ Sil C18 column (250 mm × 4.6 mm i.d., 5 m). The two phytohormones were eluted isocratically with methanol/water containing 0.2% formic acid (50:50, v/v) at the flow-rate of 1.0 mL min^{-1}. The injector volume selected was 25 L. LC-MS/MS conditions were as follows: ESI spray voltage, 4 kV; sheath gas flow-rate, 70 arb; auxiliary gas flow rate, 20 arb; capillary voltage, -38 V; capillary temperature, 350°C and tube lens, 95 V. The SRM mode was used for the determination of the phytohormones. GA_3 and ABA were monitored at m/z transitions of 345→239 and 263→153, 219, respectively. The optimized collision energies for GA3 and ABA were 21 and 20 eV, respectively. Selected ion monitoring (SIM) mode was used for the determination of ISTD. ISTD was monitored at m/z 121.

Effect of soil amendments on plant growth and development

Plant material and soil treatments. Cycas revoluta plants, coming from seeds were grown in greenhouse, in 3L pots. After one year of culture, plants were randomized and divided based on them into three similar groups (corresponding to each one of the three soil-substrates-treatments). The three treatments were as follows: (i) plants grown soil substrate only, (ii) plants grown on soil substrate, mixed with sheep manure and (soil: sheep manure = 90:10) (iii) plants grown on soil substrate, mixed with horse manure (soil: horse manure=90:10). Sheep and horse manures were produced by Sardi breed and Arabic breed, respectively. The chemical composition of soil and organic manure are represented in Table 1. In each of the three described treatments, 20 plants-replicates (one plant per pot) were included. During the experiment that lasts six months, all the plants were tri-weekly irrigated with distilled water.

Plant growth data recording. At the end of the experiment, number of leaves, plant height, stip length and width, root length and density, the number of nodules per plant, their length and width were measured.

Leaf, stip and root nutrient contents. At the end of the experimental period (6 months), *Cycas revoluta* leaves, stips and roots were washed and dried. They were then ground to a fine powder, to pass a 30-mesh screen. 0.5 g of the fine powder of each sample was dry-ashed at 515°C in a muffle furnace, for 5 hours. The ash was dissolved with 3 ml of 6 N HCl and diluted with double distilled water up to 50 ml. The concentrations of P, Na, K, Fe, Cu and Zn were determined using DTPA method as described by Lindsay and Norvell (1978). Nitrogen content was estimated using the Kjeldahl method. Macronutrient (P, Na, K and N) were expressed in % DW, while micronutrients (Fe, Cu and Zn) amounts were expression in mg/Kg DW.

Chlorophyll fluorescence. Chlorophyll fluorescence measurements were performed using a pulse-modulated fluorometer (OS30p, Opti-Sciences, Hudson, NH, USA). Fluorescence measurements were assessed in dark-adapted leaves, using the leaf-clips which were put on the adaxial leaf blades away from the leaf vein. Two measurements were made on each pot. The following chlorophyll fluorescence parameters were determined: maximal photochemical efficiency of PSII (Fv/Fm), the maximum quantum yield of primary photochemistry (Fv/F0) and quantum photosynthetic yield of PSII.

Chlorophyll a, b and total chlorophyll contents. Chlorophyll content was performed as described by Bassa *et al.* (2012) with a slight modification. 0.25 mg of fresh leaves were randomly taken for each treatment. The fresh tissue was fine grounded in a mortar and pestles in the presence of 80% of acetone. The mixture was then centrifuged in 10000 rpm for 1 minute. Samples were analyzed by spectrophotometry at two wavelengths, 645 and 663 nm, using 80% acetone as the blank. The chlorophyll a, b and total chlorophyll contents were calculated according to the following equations: Chl a=0.999A663-0.0989A645; Chl b= 0.328A663+1.77A645 and total chlorophyll content=20.2*Chl a +8.02*Chl b.

Table 1 - Chemical composition of soil and organic amendments before assays launch

	Soil	Horse manure	Sheep manure
pH	7.64±0.05	8.50±0.01	8.3±0.01
CEC (mS/cm)	0.21±0.02	2.13±0.08	2.15±0.04
Potassium (ppm)	120.02±12.50	612.91±5.56	512.38±2.58
Phosphorus (ppm)	58.45±0.71	330.97±0.99	329.16±0.98
Nitrogen (%)	0.038±0.004	1.87±0.01	2.55±0.01
Organic matter (%)	2.88±0.91	44.8±1.2	46.12±1.07
Dry matter (%)	91.13±0.26	97.08±0.03	98.335±0.06
Magnesium (%)	7.25±0.04	0.65±0.01	0.7±0.01
Carbon (%)	1.81±0.005	105.23±0.01	106.12±0.005
Sodium (m eq/100 g)	1.29±0.06	7.35±0.05	7.65±0.15

Statistical analysis

All the analysed parameters have been compared using a fixed model of analysis of variance (ANOVA). For each parameter and condition, means and standard deviation were calculated based on at least twelve biological replicates (except for α-amylase, ABA and GA_3 amounts for which means were calculated based on three independent biological replicates). In case of significant difference between groups, a Tukey test was used for means separation, at risk of 0.05. The relationship between parameters was observed using Pearson coefficient. A principal component analysis was also launched to determine which parameter contribute most to the variation in data.

3. Results and Discussion

Warm stratification affects alpha-amylase activity, GA_3 and ABA contents and enhances zygotic embryos development and seed germination

Seed germination partially relies on the degradation of storage reserves in mature seeds. Sugars from starch hydrolysis are the major source of energy required for seedling emergence (Beck and Ziegler, 1989). Alpha amylase is the major enzyme involved in starch mobilization and its degradation into small organic molecules to provide energy and nutrient indispensable for seed germination and seedling emergence (Ali and Elozeiri, 2017). Quantification in treated (T2) and untreated embryos (C) revealed a huge difference in alpha amylase activity between the two treatments. We observed an increase in alpha amylase activity in 30°C warm stratified embryos (T2), as compared to the untreated plot (C) (Fig. 1b). This finding was also confirmed by the qualitative data (Fig. 1a). The colourless areas around embryoless half-seeds derived from untreated (C) seeds were much smaller than those stored at 30°C for 2 months (T2). Previous work showed that alpha amylase activity substantially increased with the increase of temperature. Indeed, Salisbury and Ross (1995) demonstrated that some enzymes like alpha amylase reactions and thus activities increased with temperature increases from 0°C to 35°C. However, above 40°C, enzyme activities decreased due to their

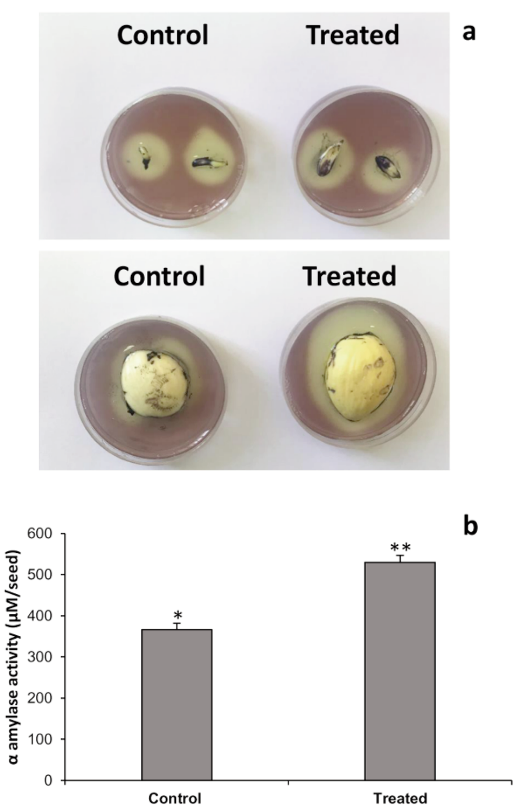

Fig. 1 - Qualitative (a) and quantitative (b) determination of α-amylase activity in untreated (C) embryos and those stored at 30°C for 2 months (T2). The presence of α-amylase was qualitatively determined in embryos and embryoless half-seeds. The quantitative determination of α-amylase activity was performed on untreated and treated embryos. Values are mean ± SD of three biological replicates. Asterisk (*) showed statistical significance according to T-student test (p<0.05).

denaturation (Salisbury and Ross, 1995). Sari (2021) showed that the highest alpha amylase activities were recorded in rice seeds (*Oryza sativa* var Cisokan) at 30-40°C. They noticed the absence or the decrease in alpha-amylase activity in temperature below 28°C and above 40°C, respectively (Sari, 2021). Here, we reported a notable and significant increase in alpha-amylase activity in warm treated seeds (T2), which can be explained by the beneficial effect of moderate temperatures (30°C in our case) on alpha-amylase enzyme.

Seed dormancy and germination are mainly regulated by two major antagonist phytohormones, abscisic acid (ABA) and gibberellin (GA). ABA positively regulates the induction and the maintenance of seed dormancy while GA enhances germination (Tuan *et al.,* 2018). Investigating ABA and GA_3 amounts in pre-treated (T2) and untreated (C) embryos of *Cycas revoluta* revealed a notable difference in ABA and GA_3 amounts. Untreated embryos (C) accumulated more ABA than warm stratified embryos (T2) (Table 2). However, GA_3 amount in warm treated (T2) seeds was higher than in the control (C). This difference was although statistically insignificant. It was previously reported that the balance of seed ABA/GA levels is a pivoting regulatory mechanism underlying the maintenance and release

Table 2 - GA_3 and ABA contents in untreated (C) and warm stratified (T2) embryos

Treatments	GA_3	ABA
Untreated embryos (C)	68.91±1.35 (*)	42.09±0.44 (*)
Warm stratified (T2) embryos	65.15±0.81 (*)	30.11±1.29 (**)

Values are mean ±SD of three independent biological replicates. (*) Showed statistical differences according to t-student test (p<0.05).

of seed dormancy. Seeds ‘dormancy studied in *Arabidopsis thaliana* revealed that the suppression of GA_3 biosynthesis and ABA catabolism inhibited seeds ‘ germination and resulted in dormancy implementation (Chen *et al.,* 2020). The improvement of rice seed germination was linked to the trigger of the glycolytic metabolism and the restoration of GA/ABA balance in seeds (Yang *et al.,* 2022). Warm stratified embryos accumulated more GA_3 than ABA. Besides, as mentioned above, warm treated embryos showed higher alpha-amylase activity, reflecting a strong glycolytic activity. Thus, the lower ABA content along with the increased GA_3 levels and alpha-amylase activity suggest that those embryos can easily germinate and explained the stimulatory effect of warm stratification on embryos germination that has been previously reported by Benjelloun *et al.* (2021).

Cycas revoluta seeds have not the ability to immediately germinate after seed shed. This finding was attributed to embryo immature stage as previously reported in *Cycas rumphii* (De Silva and Tambiah, 1952), *Cycas revoluta* (Dehgan and Schutzman, 1989) and *Eucephalartos natalensis* (Woodenberg *et al.,* 2014). In this study, histological analysis showed that untreated embryos displayed a rudimentary structure subtended by a long suspensor as expected (Fig. 2a). Treated embryos (T2) was although observed to undergo considerable growth and development which confirmed our earlier observation (Fig. 2b) (Benjelloun *et al.,* 2021). Microscopic observation of embryos from control plot (C) or subjected to treatment 2 (T2) thin longitudinal sections stained with toluidine blue revealed that untreated embryos were at early stage of development, which corresponds with early stage of globular embryo (Fig. 2). The

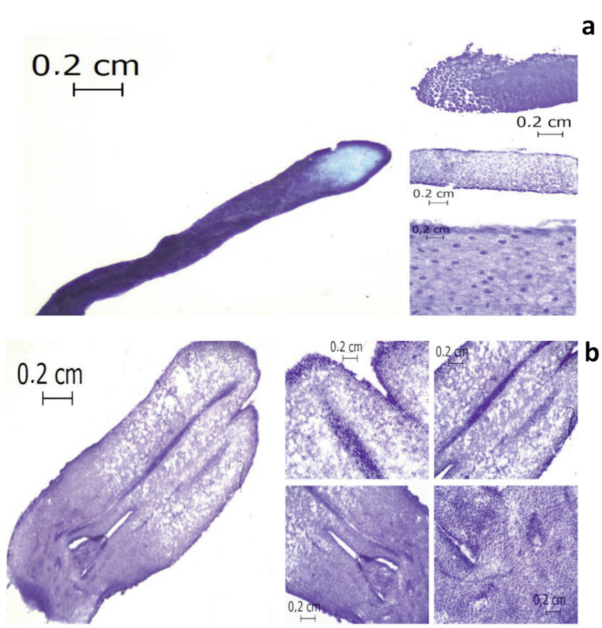

Fig. 2 - Histology of (a) embryos from the control plot (0 month) (C) and (b) embryos stored at 30°C for 2 months (T2). The embryos were stained darkly with toluidine blue. Bar, 200 µm. Observations have been made for the control, using embryos isolated immediately after seed shed. For those subjected to T2 treatment, the observations were performed after 2 months of storage at 30°C. Untreated embryos (C) displayed a rudimentary developmental stage, cells of the embryo are thin, showed small size in comparison with cells of the suspensor (longer cells). Warm stratified embryos (T2) showed a differentiated structure with two apparent cotyledons, shoot and root meristem can be easily identified.

embryo tissue had no intercellular spaces. Cells were bounded by thin walls with prominent nuclei. Warm stratified embryos showed several morphological, histological, and cellular differentiation (Fig. 2). Those embryos were able to reach cotyledonary stage in only 2 months. Shoot and root meristems can be differentiated. Shoot meristem is well developed and flanked by cotyledonary protuberances. Procambium tissue, a meristematic tissue concerned with providing the primary tissues concerned with providing the primary tissues of the vascular system, was well developed. This phenomena has been earlier explained by Devillez in 1976 in *Taxus baccata* by the fact that warm stratification prompted after-ripening in underdeveloped embryos along with the suppression of the morphological dormancy (Devillez, 1976). Chien *et al.* (1998) have found that warm stratification promoted embryos development in *Taxus* species, the embryos reached the double of their size after six months of warm stratification (Chien *et al.,* 1998). This finding is consistent with our previous observation, in which we reported that 2 and 4 months' exposure to warm treatment significantly increases *Cycas revoluta* embryos' length (Benjelloun *et al.,* 2021). Seed germination in dormant seeds generally occurs as a result of the metabolic activation (Bewley and Black, 2013), supplying cells with the energy required for cell differentiation, expansion and development. For instance, Woodenberg *et al.* (2013) showed that *Encephalartos natalensis* developed embryos (at the cotyledonary stage) accumulated high amounts of starch compared to the other stages. They also suggested that the accumulated starch serves more as carbohydrate reserve during germination and seedling establishment than during the embryo growth in the ovule (Woodenberg *et al.,* 2014).

Given the beneficial effect of warm treatment on zygotic embryos germination, we thought to examine growth parameters (length and width) of zygotic embryos deriving from seeds stored at 25°C (T1) or 30°C (T2) for 2 months, in comparison with untreated seeds (C) isolated directly after seed shed (0 month). Our results showed that seed storage for 2 months at 25°C (T1) or 30°C (T2) significantly increased zygotic embryos' length and width, compared to the control. We noticed that ZEs length increased by 126% and 221% with T1 and T2 treatments, respectively (Table 3). Similarly, we were able to record a 5 to 7 times increase in ZEs width, when seeds were stored at 25°C or 30°C respectively. Germination percentage

Table 3 - Effect of warm stratification on zygotic embryos length, width and germination

Parameters	Control (C)	25°C (T1)	30°C (T2)
ZEs length	3.46 ± 1.14 a	7.84 ± 1.29 b	11.14 ± 1.20 c
ZEs width	0.09 ± 0.02 a	0.51 ± 0.04 b	0.68 ± 0.03 c
Germination percentage	25.33 ± 1.63 a	28.00 ± 1.60 a	49.33 ± 1.70 b

Values are mean ± SD of at least 12 biological replicates.
For each analysed parameter, different letters indicate statistical difference according to Tukey test (p<0.05).

was also stimulated with the application of warm treatment. A significant increase in the percentage of germination was reported with the application of T2 treatment. Indeed, the highest germination percentage of 49.33% was recorded with seeds pre-treated at 30°C for 2 months. Meanwhile, no significant differences were detected between the control plot (25.33%) or T1 treatment (28.00%). These data strongly showed the stimulatory effect of warm treatment on ZEs development and germination, thereby confirming our earlier observations (Benjelloun *et al.,* 2021).

Organic amendments stimulate Cycas revoluta growth

Investigating the effect of soil organic amendments on *Cycas revoluta* growth in greenhouse conditions revealed a stimulatory effect of organic manures on plant growth (Fig. 3).

All fertilization treatments increased plant growth attributes except for root length. We found that both horse and sheep manures significantly increased plant height and stip length, but no significant differences were recorded between the different manures (Table 4).

Fig. 3 - Effect of organic amendments on *Cycas revoluta* growth. (Control) one-year-old *Cycas* plant grown in soil substrate only, (HM) one-year-old *Cycas* plant grown in a mixture of soil and horse manure and (SM) one-year-old *Cycas* plant grown in the presence of mixture of soil and sheep manure. Scale bar = 13 cm.

Regarding root development, a significant increase in root length and root density was observed with the use of sheep manure. Nodules' number also increased in a significant way with sheep manure amendment, while comparable values were recorded in the control treatment and in the presence of horse manure (Table 4). Besides nodules 'number, nodules' length and width was notably variable. Significant differences in nodules' number have been recorded between the three culture conditions.

Table 4 - Effect of organic amendments (horse and sheep manures) on the agro-morphological parameters of *Cycas revoluta* plants

Parameters	Soil	Soil-horse manure	Soil- sheep manure
Number of leaves per plant	1.69 ± 0.48 a	2.94 ± 0.99 b	2.73 ± 0.47 b
Plant height (cm)	20.17 ± 2.40 a	57.43 ± 4.53 b	58.14 ± 2.91 b
Stip length (cm)	3.19 ± 0.32 a	4.35 ± 0.34 b	4.34 ± 0.42 b
Stip width (cm)	3.52 ± 0.34 a	4.15 ±0.38 b	4.17 ± 0.32 b
Root length (cm)	21.14 ± 1.68 a	21.11 ± 3.60 a	32.02 ± 2.51 b
Root density	1.13 ± 0.23 a	1.23 ± 0.20 ab	1.49 ± 0.29 b
Number of nodules per plant	3.18 ± 1.93 a	4.31 ± 1.19 a	12.67 ± 2.05 b
Nodules length (cm)	0.81 ± 0.12 a	0.95 ± 0.19 b	1.22 ± 0.21 c
Nodules width (cm)	0.84 ± 0.12 a	1.39 ± 0.30 b	1.35 ± 0.35 b

Values are mean ± SD of at least twelve independent replicates.
For each parameter, values with different letters indicate statistical differences according to Tukey test (p<0.05).

The highest nodules' length values were recorded in the presence of sheep manure. Regarding nodules' width, the statistical analysis revealed a significant difference between the control and the different manures treatments. The highest width values were interestingly reported with the use of horse manure. The cross-section of these nodules revealed the presence of blue-green halo, which can potentially indicate the presence of cyanobacteria (Fig. 4). Further analysis should be conducted to confirm these observations.

Organic amendments have the ability of binding minerals like, magnesium, potassium and calcium in a colloidal form (humus and clay), which can promote the formation of stable aggregate of soil particles at desired porosity to support plant growth (Azarmi *et al.,* 2009; Chang *et al.,* 2010). Here, we found that organic manures (sheep or horse manures) significantly promoted plant height, number of leaves per plant and stip length. Indeed, manure supplementation increases macro and micronutrient contents as well as soil physico-chemical properties, which can ultimately lead to a better vegetative growth (Adekiya *et al.,* 2020). Root development can be significantly influenced by soil mineral composition. The presence of sufficient nutrients prompted the development of root system. Gregory (1994) compared

Fig. 4 - *Cycas revoluta* plant presenting a globular-like structures or nodules in the underground part of the plant. (a) *Cycas revoluta* root system with several nodular structure, (b) a single nodule and (c) isolated nodule presenting a blue-green halo which indicates the presence of cyanobacteria. black narrow pointing the blue-green halo. Scale bar = 1 cm.

the influence of fertilization on root growth to its beneficial effect on shoot growth. Smith showed that additional nitrogen levels can result in a better leaf growth and number of another cycadales species; *Zamia integrifolia* (Smith, 1978). Here, we found that sheep manure allowed a better development of root system, as evaluated by root length and density (Gregory, 1994). In quinoa (*Chenopodium quinoa*), Kakabouki *et al.* (2019) have linked the better development of root system in plants grown in a fertilized soil with the presence of high amounts of nitrogen (Kakabouki *et al.,* 2019). Sheep manure used in this study displayed the highest nitrogen content (2.55 ± 0.01%) (Table 1), thus explaining the better plant development recorded. Besides promoting shoot and root development, sheep manure supplementation resulted in a significant increase in the number of nodules per plant (Table 4). It has been previously reported that *Cycas revoluta* forms beneficial association with the blue green algae, ensuring nitrogen fixation. This symbiotic nitrogen fixation, occurring in cycads coralloid roots has been reported to arise nitrogen at a significant rate (Halliday and Pate, 1976; Grove *et al.,* 1980). Nitrogen fixation by coralloid roots was estimated to be comprised between 18.8 kg N/ha and 35 kg N/ ha (Smith, 1978). Dehgan (1983) showed that nitrogen fixation in the coralloid roots contributes significantly to plant growth (Dehgan, 1983).

The presence of great number of nodules per plant in *Cycas* plants grown in the presence of sheep manure could indicate a better nitrogen fixation potential, which can increase plant supplying with sufficient amount of nitrogen. Besides cyanobacteria - *Cycas* roots association, previous reports showed also the presence of endophytes in regular and coralloid roots of *Cycas bifida* (Zheng *et al.,* 2018). Endophytic bacteria have been associated with the growth promotion of several crop species such as maize (Alkahtani *et al.,* 2020), wheat (Khan *et al.,* 2017), tomato (*Solanum lycopersicum*) (Amaresan *et al.,* 2012), rice (*Oryza sativa*) (Khan *et al.,* 2020) and chilli (*Capsicum annuum*) (Amaresan *et al.,* 2012). The plant growth is promoted through improved nutrient acquisition, including nitrogen fixation and the production of plant growth promoting substances such as indole acetic acid and cytokinins (Miliute *et al.,* 2015). Therefore, it could be more interesting to investigate the presence of these beneficial microorganisms in the observed nodular structures, reported in this work.

Organic manures have a positive effect on Cycas revoluta seedlings photosynthetic apparatus

Chlorophyll a, b and total chlorophyll contents significantly increased with the application of different organic manures (Fig. 5). An average increase (2.11 and 2.67 mg/g FW) of chlorophyll a content was recorded with the application of horse manure and sheep manure respectively. Similar trend was also observed with chlorophyll b (3.61 and 4.61 mg/g FW) and total chlorophyll (71.06 and 82.98 mg/g FW) contents. Regarding the chlorophyll fluorescence, our results showed an increase in chlorophyll fluorescence attributes mainly the potential activity of PSII, the maximum quantum yield of primary photochemistry and effective quantum yield of PSII with the application of either horse or sheep manure (Fig. 5). Note that no significant difference has been observed in the potential activity of PSII between the control and plants grown in horse manure-soil mixture.

It is well established that organic fertilizers improved plant growth and development, by improving soil physico-chemical properties and nutrient availability to plants (Eneji *et al.,* 2001; Azarmi *et al.,* 2009; Osama *et al.,* 2016). This latter seems to directly affected photosynthesis process (Osama *et al.,* 2016). Several studies conducted on different plant species such as sugarcane (*Saccharum officinarum*), Soybean (*Glycine max*), Potato (*Solanum tuberosum*) and Kiwifruit (*Actinidia deliciosa*) have underlined the positive effect of organic amendments on chlorophyll

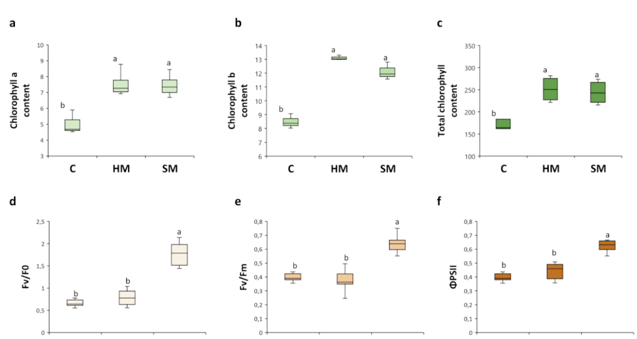

Fig. 5 - Effect of organic amendments - horse manure (HM) and sheep manure (SM) - on the photosynthetic attributes. (a) chlorophyll a content, (b) chlorophyll b content, (c) total chlorophyll content, (d) the maximum quantum yield of primary photochemistry (Fv/Fm), (e) potential activity of PSII (Fv/Fo) and (f) effective quantum yield of PSII (ΦPSII) of *Cycas revoluta* plants. Values are mean ± SD of at least six independent replicates. For each parameter, values with different letters indicate statistical differences according to Tukey test ($p<0.05$).

content (Ghosh *et al.,* 2004; Bokhtiar and Sakurai, 2005; Najm *et al.,* 2012; Sharma *et al.,* 2022). Sharma *et al.* (2022) associated this beneficial effects on photosynthetic properties with the presence of high nitrogen amounts conferred by organic manure supplementation (Sharma *et al.,* 2022). Moriwaki *et al.* (2019) explained the positive effect of nitrogen on photosynthetic attributes (photosynthetic quantum yield) by the increase in thylakoid density, which can enhance green light absorption (Moriwaki *et al.,* 2019). In *Cycas revoluta*, we found that organic manures, more precisely sheep manure, significantly improve plant photosynthetic attributes (Fv/Fm and ΦPSII). This can likely be attributed to the high nitrogen content of sheep manure along with the presence of great number of nodules per plant.

Organic manures modify mineral allocation in Cycas revoluta plants

The mineral contents of *Cycas revoluta* botanical parts (leaf and root) were determined after six months of culture. Our results showed a huge difference in macronutrients and micronutrients amounts between the control plants and those grown in the presence of either horse or sheep manure (Table 5). Regarding sodium content, the highest and lowest sodium amounts was recorded in roots grown in the presence of sheep manure and leaves collected from the control plants. Potassium content was highly variable between the three different treatments. The highest potassium content of 15.02±2.18% was detected in roots isolated from plants grown in the presence of sheep manure. Meanwhile, the lowest potassium content of 3.2±0.64% was this time recorded in leaves under sheep manure treatment. The highest nitrogen content was recorded in roots of the control plants and leaves collected from plants grown in the presence of sheep manure. However, no significant differences have been recorded in phosphorus amount. For iron, the highest and the lowest amounts of 7.02±0.39 mg/Kg and 11.83±0.50 mg/Kg were observed in leaves collected from plants grown in the presence of sheep manure and roots of the control plants, respectively. The highest copper and zinc contents were recorded in leaf under horse manure treatment and root of the control plants respectively while the lowest amounts were found in roots of the control plots and roots under horse manure treatment, respectively.

Organic fertilization is known to affect the concentrations and the uptake of several macro and

Table 5 - Mineral composition of roots and leafs of *Cycas revoluta* plants subjected to the three following treatments (Control "C", Horse manure "HM" and Sheep manure "SM") for six months

Treatments	Nitrogen (%)	Phosphorus (%)	Sodium (%)	Potassium (%)	Iron (mg/Kg)	Cupper (mg/Kg)	Zinc (mg/Kg)
Root							
C	1.75±0.09 a	0.33±0.05 a	1.50±0.36 a	4.55±0.15 cd	11.56±0.21 a	46.33±2.08 d	338.66±9.08 a
HM	1.44±0.09 c	0.36±0.08 a	1.95±0.26 a	11.42±1.11 b	10.99±0.38 ab	49.83±2.83 d	89.90±3.34 d
SM	1.60±0.14 ab	0.43±0.08 a	4.03±0.77 b	15.02±2.18 a	11.15±0.52 ab	47.50±3.44 d	168.0±15.93 c
Leaf							
C	1.19 ±0.07 d	0.43 ±0.05 a	1.12 ±0.13 a	4.55 ±0.05 cd	7.92 ±0.72 c)	67.0 ±2.00 bc	282.50 ±4.5 b
HM	1.01 ±0.11 de	0.38 ±0.09 a	1.40 ±0.20 a	10.50 ± 1.21 b	8.12 ±0.59 c	85.00 ±3.60 a	166.33 ±18.55 c
SM	1.77 ±0.18 a	0.35 ±0.05 a	2.31 ±0.10 a	2.68 ±0.08 d	7.02 ±0.37 d	61.00 ±1.82 c	245.50 ±24.49 b

Values are mean ±SD of three independent biological replicates. Values with different letters indicate statistical differences according to Tukey test (p<0.05).

micronutrients such as nitrogen, potassium and phosphorus by plants, independently from the irrigation system (Yang *et al.,* 2004). The highest nitrogen amounts were recorded in the aerial parts of *Cycas* plants grown in the presence of sheep manure, thus explaining the positive effect of sheep manure supplementation on vegetative growth and photosynthetic attributes. Potassium concentration was significantly more in the roots isolated from plants grown in the presence of either sheep or horse manure than in plants grown without organic manure addition. Maximum increase of leaf K amount was detected with the addition of horse manure. The increase in K concentration, resulting from organic matter addition might be attributed to K concentration in the organic fertilizers (Table 1), as previously shown for *Zea mays* (Aziz *et al.,* 2010) and *Brassica juncea* (Aziz *et al.,* 2006).

The interrelationship between plant morphological, biochemical, and physiological attribute using the Pearson's correlation matrix revealed the existence of a significant positive correlation of potassium content in roots with leaves number (r=0.89), stip width (0.75), nodules' number (r=0.72), nodules' length (r=0.59), nodules' width (r=0.54), chlorophyll a (r=0.77), chlorophyll b (r=0.81) and total chlorophyll (r=0.78) contents. Similarly, root sodium content was positively correlated with root length (r=0.81), nodules' number (r=0.80), their length (r=0.61) and root potassium amount (r=0.68). Root sodium amounts were also positively correlated with photosynthetic attributes, more precisely with Fv/F0 (r=0.84), Fv/Fm (r=0.76) and PSII (r=0.79) (Fig. 6a). Interestingly, root zinc amounts were negatively correlated with stip length (r=-0.78) and width (r=-0.59), leaf length (r=-0.9), chlorophyll a (r=-0.84), chlorophyll b (r=-0.96) and total chlorophyll contents (r=-0.87). PCA (Principal component analysis) showed that variables explained 66.9% of the variation in the first two axes (Fig. 6b), which is why 42.6% and 24.3% variances were accounted respectively, for the first and second principal components. The first principal component counted more attributes than the second principal component. Results from this study strongly suggest that the application of sheep manure significantly affected the plant root growth (root length), the photosynthetic attributes (Fv/Fo, PSII, Fv/Fm) and also root sodium content. Horse manure seems to strongly affected copper amounts in both leaves and roots.

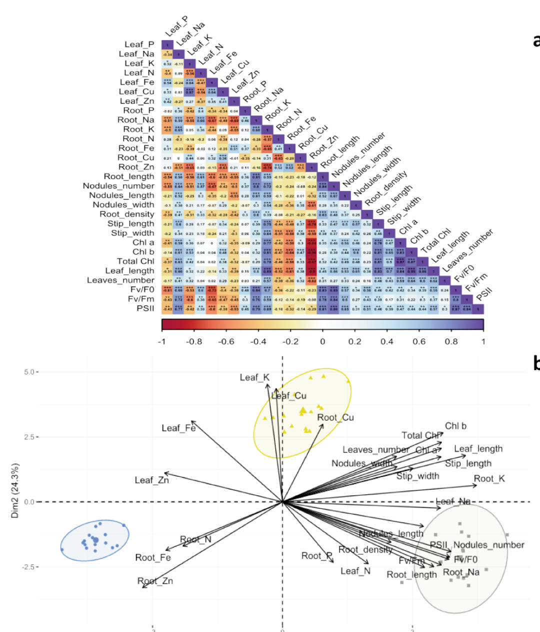

Fig. 6 - Pearson correlation (a) and Principal component analysis (b) gathering all the agro-morphological and physiological parameters that have been used for this study.

4. Conclusions

Here, we demonstrated that warm stratification significantly enhanced *Cycas revoluta* seeds' germination by promoting embryos' length. In the present work, we found that warm stratification increased α-amylase (key enzyme involved in starch mobilization and degradation) activity triggered by the moderate temperature imposed during the warm treatment application. Decreased ABA content was also detected in warm stratified embryos, thus explaining the highest germination percentage recorded in the previous work (Benjelloun *et al.,* 2021). At the histological level, we found that warm stratification triggers cell differentiation and embryos development to reach cotyledonary stage after 2 months of treatment only. Plant growth was highly stimulated by the application of organic manures, more specifically by sheep manure application. Indeed, sheep manure application improves plant growth, namely plant height, root length and density, the number of nodules per plant, nodules 'length and width, nitrogen allocation and photosynthetic attributes. Overall, these data strongly recommend the use of warm stratification to enhance *Cycas revoluta* seed's germination followed by sheep manure application to accelerate *Cycas revoluta* growth, thereby offering new insights on the use of biological agriculture inputs for sustainable production of horticultural plants.

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