Adv. Hort. Sci., 2020 34(3): 349-354 DOI: 10.13128/ahsc-8462

Short note

Improvement of *in vitro* germination of *Cycas revoluta* zygotic embryos using gelrite as gelling agent

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Key words: *Cycas revoluta*, gelrite, germination, *in vitro*, regeneration, zygotic embryos.

OPEN ACCESS

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Citation:
BENJELLOUN J., TAOUFYQ A., EL ABIDINE TRIQUI Z., ALAMI Q.L., LAYACHI R., SMOUNI A., BOUZROUD S., GUEDIRA A., 2020 - *Improvement of in vitro germination of Cycas revoluta zygotic embryos using gelrite as gelling agent.* - Adv. Hort. Sci., 34(3): 349-354

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 15 April 2020
Accepted for publication 18 August 2020

Abstract: An efficient *in vitro* germination protocol for *Cycas revoluta*, a widespread ornamental tree, has been established using zygotic embryos as explants with a focus on mineral composition of the culture media, the gelling agent and cytokinine type. A high percentage of germination, 73% was obtained with SH medium instead of 27% with MS medium. A 100% of germination was obtained with the combination of SH medium and gelrite as gelling agent. The addition of cytokinines prompt shoot formation. An optimum shoot induction occurred using 0.5 mg/l of BAP where an average of 14.1 shoot were produced per explants while 2.2 shoots were formed in the presence of 2iP. A 100% of rooting was observed in the presence of 0.5 mg/l of 2iP whereas only 30% of shoots obtained on the SH medium with 0.5 mg/l of BAP were able to develop roots.

1. Introduction

Cycas revoluta is taxonomically known as the most primitive species among the living cycads (Stevenson, 1990; Jones, 1994). *Cycas revoluta* 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 (Khalighi, 2001; Zarchini *et al*., 2011).

Cycas revoluta is propagated either from seeds, which remain viable for only a short time, or from vegetative offshoots (Demiray *et al*., 2017). As slowly growing plants, they require 3 to 10 years to attain reproductive maturity (Rinaldi, 1999). Germination of *Cycas revoluta* seeds is hard and time consuming (Zarchini *et al*., 2011). Physical dormancy of seed causes delay in seed germination (Frett, 1987). 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 morphogenic potential, which hinder its conservation as well as

favor an effective and rapid mass propagation (Naderi *et al*., 2015). The delay in seed germination along with the slow growth of *Cycas* plants increase the cost of production (Frett, 1987; Litz *et al.*, 2005; Demiray *et al.*, 2017). Thus, conventional methods are not quiet efficient for large-scale propagation of this species. Therefore, other propagation methods are needed (da Silva *et al*., 2014). The use of *in vitro* techniques to accelerate seed germination is a suitable way to conserve many of the endangered species.

Several attempts have been made to establish an efficient protocol for *Cycas revoluta* propagation (Rinaldi and Leva, 1995; Rinaldi, 1999; Naderi *et al.*, 2015; Demiray *et al.*, 2017). However, the results were not satisfying. The present study focuses on developing an efficient *in vitro* germination protocol from mature zygotic embryos (ZEs) with a focus on the gelling agent, the mineral composition of the culture media and the presence of 2-isopetynyl adenine (2iP) or 6-Benzylaminopurine (BAP).

2. Materials and Methods

Plant material

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.

Seed sterilization and zygotic embryos isolation

Seeds were soaked in water for 48 hours in order to soften the sacrotesta, the orange external layer. Once removed, seeds were then flamed with ethanol for 2 minutes in order to eliminate the sclerotesta. Following removal of the sclerotesta, 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 ZE was excised from each megagametophyte.

Zygotic embryos culture

ZEs 1.7 to 2-cm long were placed in culture jars containing 120 ml of culture medium with 2 explants per jar. Two basal mediums Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Schenk and Hildebrandt (SH) (Schenk and Hildebrandt, 1972) were tested for *in vitro* germination. Both media were supplemented with 30 g/L of sucrose and solidified with 0.8% of bacteriological agar type E (BIOKAR Diagnostic) or 0.3% of gelrite (SIGMA-Aldrich). BAP or 2iP (0.5 mg/L) were added to the culture medium. The pH was adjusted to 5.8 with either 1N HCl or KOH prior to autoclaving, at 108 kPa and 120°C for 20 min. Cultures were incubated in a culture room at 25±2°C and in the dark for 21 days, thereafter under a photoperiod of 16h of light/8h of darkness.

Statistical analysis

Percentages of germination and plant development were compared using Z test or a fixed model of analysis of variance (ANOVA) depending on the condition. Thirty biological replicates were performed for each condition. In case of significant difference between groups, a Least Significant Difference LSD test was used for means separation. Shoot number, shoot length and number of leaves per shoot obtained from each embryo was analyzed by Z test, at risk of 0.05.

3. Results and Discussion

Effects of the culture medium on germination and regeneration

Nitrogen formulation influences seed germination and callus induction in cycads (Rinaldi, 1999; Demiray *et al.*, 2017). Investigating seed responsiveness to SH and MS medium revealed significant differences in germination (GP) and development percentages (PDP) between the two tested mediums. The results revealed that the highest GP (73%) was obtained with SH medium while the GP was around 57% with MS medium (Table 1). A higher regeneration percentage (around 27%) was also observed with SH medium while only 13% of seeds were able to regenerate (Table 1). Rinaldi and Leva (1995) have found that SH or MS medium, both containing ammonium, promoted shoot formation. Rinaldi (1999) also reported that the percentage of responding explants and the number of regenerated shoots were significantly higher on SH medium than on MS medium. However, the presence of nitrate as a sole source of nitrogen, as in the Klimaszewska and Keller medium (Klimaszewska and Keller, 1985), did not promote shoot regeneration (Rinaldi and Leva, 1995;

Table 1 - Effect of medium culture on germination and plant development of *Cycas revoluta* ZEs.

Medium	Germination (%)	Plant development (%)
SH	73 (*)	27 (*)
MS	57 (**)	13 (**)

* indicate the statistical significance ($p<0.05$) using Z test.

Rinaldi, 1999). This difference might be due to the differences in ammonium amounts found in SH and MS media.

Effect of gelling agents on zygotic embryos development

The gelling agent influences the germination of *Cycas revoluta* ZEs. We found that the germination percentage reached 100% with gelrite used as gelling agent in SH medium while only 73% of ZEs were able to germinate in SH medium supplemented with Agar. Moreover, the highest PDP was obtained with SH medium solidified by gelrite (Fig. 1).

The use of gelrite as gelling agent was previously used to stimulate the growth and the development of *in vitro* cultured plants. In *Sequoia sempervirens*, Fira and Clapa (2008) have reported a higher shoot multiplication with gelrite. Similar finding was also stated in oil palm in which gelrite was proven to be better than Agar giving the highest conversion rate of polyembryoids into plantlets (Palanyandy *et al*., 2020). The micropropagation of Cowpea cultivars (*Vigna unguiculata* L. Walp) was highly assessed using gelrite instead of agar (Aasim *et al.*, 2009). Veramendi *et al*. (1997) have proposed gelrite as a great alternative to agar for micropropagation and microtuberization of *Solanum tuberosum*. Scholten and Pierik (1998) explained this beneficial effect by the inorganic composition and the dynamical interaction between gelling agent-medium-tissue. Later, Puchooa *et al.* (1999) linked the positive effect of gelrite on *in vitro* plant culture to the chemical composition of this gelling agent; with a high content of copper, iron, magnesium, zinc and calcium compared to Agar. In addition to that, Buah (1999) explained the beneficial effect of gelrite on growth of banana by the fact that gelrite provide a better availability of water. Moreover, the diffusion of phenols and other inhibitive molecules in the media culture is facilitated by the use of gelrite (Huang and Chi, 1988; El Abidine Triqui *et al.*, 2008). Thus, it is assumed that the low regeneration rate of seedlings obtained with Agar is probably due to the accumulation of inhibitive compounds in this gelling agent; following this, explants can no longer absorb the mineral salts that are essential for their development. Previous works have reported the positive effect of gelrite on seed germination and development in several plant species (Asif *et al*., 2001; Yamazaki and Miyoshi, 2006; Pech y Aké *et al.*, 2007), however none of them was related to *Cycas revoluta*. This work can be qualified as one of the pioneers highlighting the positive effect of gelrite on *Cycas revoluta* ZEs development.

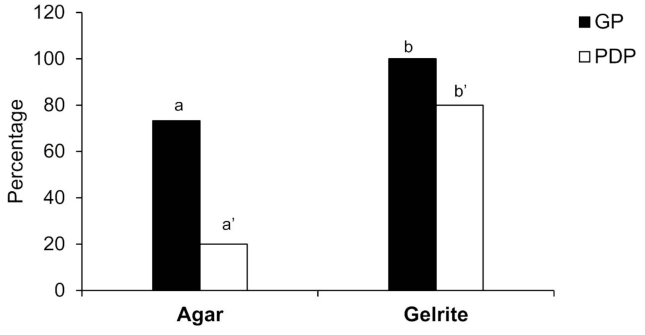

Fig. 1 - Effect of gelling agent (agar or gelrite) on germination (PG) and plant development (PDP) percentages. Bars with different letters indicate the statistical significance ($p<0.05$) according to LSD test.

Effect of cytokinines on shoot development

Even though germination percentage reached 100% with SH medium solidified with gelrite (Fig. 2), the number of shoots per ZE was significantly lower (Table 2).

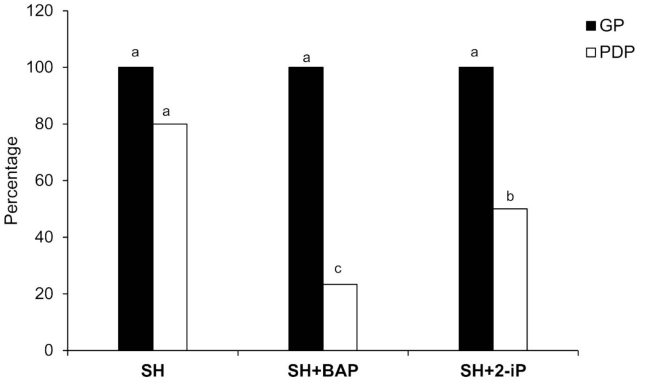

Fig. 2 - Effect of plant regulators (BAP and 2-iP) on germination (GP) and plant development (PDP) percentages of *Cycas revoluta* zygotic embryos cultured on SH medium. ZEs were cultured on SH medium solidified with gelrite and supplemented with 0.5 mg/l of BAP or 0.5 mg/l of 2iP. Bars with different letters indicate the statistical significance ($p<0.05$) according to LSD test.

Table 2 - Effect of phytohormones on the number of shoot/ explant, number of leaves/ shoot and shoot length of *Cycas revoluta* ZEs.

	SH	SH + BAP	SH + 2-iP
Number of shoot/ explant	1.6 (***)	14.1(*)	2.2 (**)
Shoot length	0.7 (**)	0.52 (**)	2.73 (*)
Number of leaves/shoot	1.81 (**)	9.13 (*)	3.6 (**)

* indicate the statistical significance ($p<0.05$) using Z test.

The addition of phytohormones, likely BAP and 2iP, increased shoot number in *Cycas revoluta* ZEs. The average of shoots developed per explant was around 14.1 in the presence of BAP and 2.2 when ZE were cultured in SH medium supplemented with 2iP (Table 2). However, shoot length was significantly higher in the presence of BAP with 9.3 leaves per shoot versus 1.7 and 3.6 when cultured in the absence of phytohormones or with BAP added to the media culture (Fig. 3 d, e, f). Shoot differentiation was previously obtained from zygotic embryos of *Cycas revoluta* in the media with BAP (0.5 mg/l) and 2.4-D (1 mg/l) (Rinaldi and Leva, 1995). Naderi *et al*. (2015) found that BAP stimulated shoot regeneration from *Cycas* ZEs while other phytohormones like 2.4-D or Kinetin alone or in combination with BAP failed.

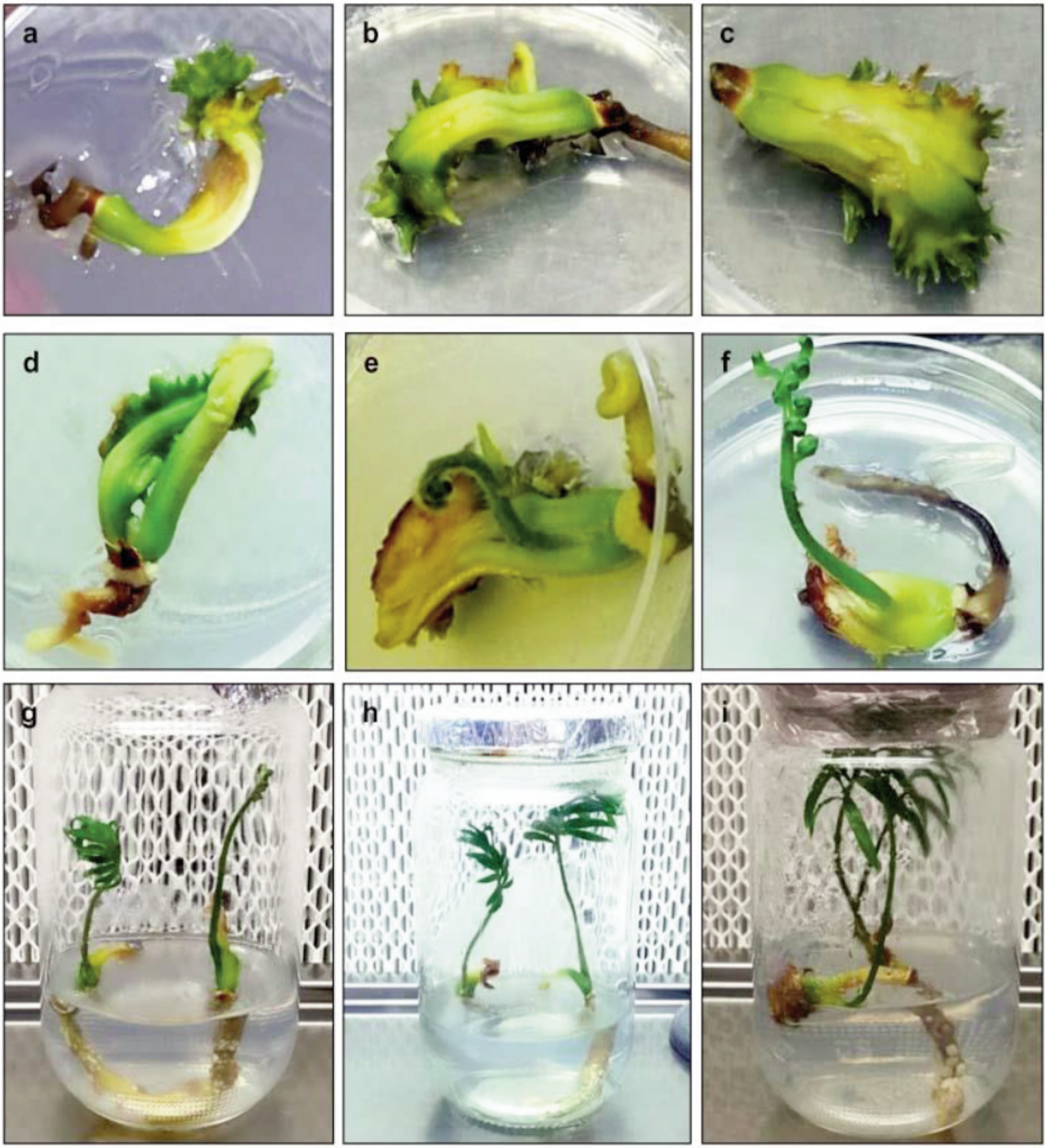

Fig. 3 - Effect of phytohormones on zygotic embryos development. (a) shoot induction in SH basal medium, (b, c) shoot regeneration in SH medium supplemented with 0.5 mg of BAP and 0.5 mg of 2iP respectively, (d, e, f) shoot elongation SH, SH+BAP and SH+2iP Prespectively, (g, h, i) rooted plants after 3 months of culture in SH, SH+BAP and SH+2iP, respectively.

The combination of BAP (0.2 mg/l) and 2,4-D (0.02 mg/l) induced adventitious shoots from mature *C. revoluta* ZEs (Motohashi *et al*., 2008). Shoot elongation was although promoted by the presence of 2iP. Our data showed that shoot length developed in SH medium supplemented with 2iP was significantly higher than those obtained in the presence of BAP. Root development was obtained in all growth conditions. We noticed that the shoots developed on the SH medium supplemented with 0.5 mg/l of 2iP were all rooted (100%) whereas only 30% of shoots obtained on the SH medium with 0.5 mg/l of BAP developed roots instead of 50% on SH medium (free from plant growth regulators) (Data not shown). In addition to the high rooting percentage obtained in the presence of 2iP, we also noticed that the presence of 2iP promoted the development of primary and/or secondary roots with likely meristematic structures identified as nodules (Fig. 3i). This finding was previously reported by Dhiman *et al*. (2000), who suggested that such nodules are meristematic zones with distinct organogenic potential, which can probably be evolved to embryos or seedlings depending on the culture conditions.

4. Conclusions

This study aims to improve an efficient *in vitro* germination protocol from mature zygotic embryos of *Cycas revoluta*. Based on our results, we found that SH medium was beneficial for seeds germination rather than MS medium. Moreover, the use of gelrite, instead of Agar, enable us to obtain a 100% of seed germination. An average of 14.1 shoots per zygotic embryo was thus obtained with the addition of 0.5 mg/L BAP to the culture media. Taken together, this protocol represents a useful and potential commercial method for *Cycas revoluta* mass propagation.

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