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In vitro Maturation and Germination of *Jatropha curcas* Microspores

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ABSTRACT

In the present study, we established an experimental system of *in vitro* maturation and germination of *Jatropha curcas* L. microspore. Our results suggested that the most appropriate medium for *in vitro* culture of *J. curcas* microspores was MS salts+White' vitamins+22% maltose, pH6.5 and the maturation and germination rate were 39.5% and 2.7%, respectively. Liquid medium with 20% sucrose+10% PEG-4000+BK [$Ca(NO_3)_2$ 400 mg/L, $MgSO_4$ 200 mg/L, H_2BO_3 100 mg/L, KNO_3 100 mg/L] +2.5% coconut water, pH 6.5 was optimal for germination of pollen matured *in vivo*. The rate of germination was 71.6%. Pollen matured *in vitro* exhibited a rate of germination of 13.7%, when it was transferred into the optimal medium for pollen germination. © 2010 Friends Science Publishers

Key Words: Germination; *In vitro* maturation; *Jatropha curcas*; Microspore; Pollen

INTRODUCTION

Jatropha curcas L. is deciduous shrubs and is likely to become one of the world's key energy crops. *J. curcas* seed has high oil content, which can be refined into high quality biodiesel, partially or fully replacing petroleum (Berchmans & Hirata, 2008). *J. curcas* with a strong drought resistance can grow on barren semi-arid, thus it will not occupy cropland. Currently, governments in many countries are very concerned about the development and utilization of *J. curcas* because it is a renewable clean energy crop. International and national investors are rushing to establish bases for *J. curcas* cultivation in developing countries. (http://www.greenfacts.org/en/biofuels/figableboxes/jatropha-crop.htm).

J. curcas that derived from the long term of wild or semi-wild state has few female flowers, limited branch, inadequate pollination and very low productivity. Hence it is very important to strengthen basic and applied researches on biology including physiology, biochemistry, breeding and cultivation to improve seed and oil yield of *J. curcas* (Becker & Makkar, 2008). Pollen, as a vector of genetic material, is critical for successful pollination and fertilization, seed set and output of *J. curcas*. However a little knowledge is available on *J. curcas* microsporogenesis (Liu *et al*., 2007) and pollination ecology, the physiological and biochemical basis, molecular mechanism and influencing factors in pollen developmental process. There are many reports that *in vitro* maturation of microspores are all from herbaceous species: lily (Tanaka *et al*., 1980), wheat (Stauffer *et al.*, 1991), maize (Pareddy & Petolino, 1992), snapdragon (Barinova *et al.*, 2002), tobacco (Aziz & Machray, 2003) and *Orychophragmus violaceus* (Zhao *et al.*, 2007), However, so far, no report showed in woody plants. The main objective of this paper was to establish an experimental system for *in vitro* maturation and germination of *J. curcas* microspore, in order to provide operation platform for research in the field of cytology, physiology and biochemistry, genetics and breeding as well as transformation of pollen.

MATERIALS AND METHODS

Materials: *J. curcas* was from the Dry-hot Valley in Liangshan Prefecture, Sichuan, China and the cutting were cultivated in the nursery of the Laboratory of Bio-resources and Eco-environment, College of Life Science, Sichuan University in 2009 early spring. Plants grew under natural conditions, conventional cultivation management in Chengdu, China (30°6'N, 104°1'E). FDA and DAPI were purchased from SIGMA.

Viability and nuclear phase test: The anthers of staminate flowers were removed from flower buds and placed in a drop of 15% sucrose solution on a microscope slide. Pollen grains were released by crushing with a glass rod and cytological observations were made under a microscope (Olympus, Japan). To observe the nuclear phase and the pollen viability, the pollen was stained by DAPI (Kapuscinski, 1995) and FDA (Heslop-Harrison *et al.*, 1984) based on the standard method: For viability detection, FDA

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(1 μg) was dissolved in 1 mL of acetone (stock solution) and diluted to 0.05 μg/mL with 20% sucrose as working solution (Kapuscinski, 1995). Working solution (5 μL) was mixed with 50 μL of pollen suspension to observe and recorded the results under fluorescence microscope. For nuclear phase detection, DAPI (Heslop-Harrison *et al*., 1984) was dissolved in sterile distilled water as stock solution (20 μg/mL). The 5 μL stock solution was mixed with 50 μL of pollen suspension and placed at -20°C for 30 min. Observation was taken under ultraviolet excitation after the material got unfreezen.

Isolation and purification of microspores: Flower buds of *J. curcas* were surface sterilized with 70% ethanol for 3 min followed washing five times with sterilized water. The anthers were collected from flower buds into a sterilized glass vial (3 mL) and microspores were released by gently pressing with a glass rod in 17% sucrose solution (Zhao *et al.*, 2007). The suspension was filtered though nylon mesh (100 μm pore size), the filtrate collected in a sterile tube and centrifuged at 700 rpm for 5 min. The pellet was washed with 17% sucrose and centrifuged at 700 rpm three times.

***In vitro* culture of microspores:** The purified microspores were mixed with sterilized maturation culture medium and cultured in petri dish (3 cm diameter) with hanging cell culture inserts (30 μm pore size) at 25±1°C in a 14 h light and 10 h dark periods. Fresh maturation culture medium was replaced every two days. The developmental stages and viability of *in vitro* cultured pollen were observed under a fluorescent microscope at the same time.

***In vitro* germination of mature pollen:** Mature pollens were collected before anthesis by earlier explained method. About 20 μL germination culture medium was dropped onto the glass slide and then the collected mature pollen grains were released into it, the glass slide was placed into a moist dish to reduce evaporation and incubated at a constant temperature. Pollen grains were considered germinated when the pollen tube length was greater than the diameter of the pollen grain. The number of germinated pollen grains was counted under microscope after 14 h. The experiments were repeated three times and 500 pollen grains were counted each time.

Statistical analysis: Values reported in this paper were the mean of three replicated treatments. Data were tested at significant levels of $P < 0.05$ using one way analysis of variance (Kleinbaum *et al.*, 1998).

RESULTS

Viability of microspores in various experimental setups: Four combinations of sucrose and maltose plus White vitamins (White, 1963) and the salt constituents from Brewbaker and Kwack medium (BK salts; Brewbaker & Kwack, 1963) were designed to evaluate the effect of carbohydrates on the viability of microspore culture. The results showed that the viability of microspores in the culture medium containing the sole carbon resources maltose was significantly higher than with the sucrose or sucrose plus maltose on third day of culture (Table I). Hence maltose was chosen as the optimum carbon resources for subsequent experiments.

Table I: Effect of sucrose and maltose on viability of microspores in *Jatropha curcas*

Carbohydrates (M)	Other compositions	Viability (%)
Sucrose (0.4)	BK* salts+White's vitamins, pH6.5	4.2**
Maltose (0.4)	BK salts + White's vitamins, pH6.5	52.3**
Sucrose (0.4)+maltose (0.1)	BK salts + White's vitamins, pH6.5	5.8**
Maltose (0.4)+sucrose (0.1)	BK salts + White's vitamins, pH6.5	42.7**

*: The salt constituents in BK medium (Brewbaker & Kwack, 1963) includes: Ca $(NO_3)_2$ 300 mg/L, $MgSO_4$ 200 mg/L, H_2BO_3 100 mg/L, KNO_3 100 mg/L
**P<0.05

Table II: Germination of *Jatropha curcas* pollen in germination medium with different combinations of maltose and PEG-4000

Medium component	Germination frequency (%)
15% maltose +5% PEG-4000 + BK*salt	14.6%**
15% maltose +10% PEG-4000 + BK salt	18.8%**
15% maltose +15% PEG-4000 + BK salt	26.6%**
20% maltose +5% PEG-4000 + BK salt	21.3%**
20% maltose +10% PEG-4000 + BK salt	35.5%**
20% maltose +15% PEG-4000 + BK salt	14.5%**
25% maltose +5% PEG-4000 + BK salt	4.5%**
25% maltose+10% PEG-4000 + BK salt	24.2%**
25% maltose+15% PEG-4000 + BK salt	5.31%**
20% sucrose +10% PEG-4000 + BK salt	49.6%**

*: The salt constituents in BK medium (Brewbaker and Kwack 1963) includes: Ca $(NO_3)_2$ 300 mg/L, $MgSO_4$ 200 mg/L, H_2BO_3 100 mg/L, KNO_3 100 mg/L
**P<0.05

The following experiment was set to evaluate the effect of BK salts and MS salts. The results showed that the viability of microspores culture in the medium BK salts+0.4 M maltose+White vitamins dropped sharply from 81.7% to 17.5%, while that in the medium containing MS salts still maintained 69.5% on fourth day of culture.

Besides under the different concentrations of maltose and pH values, the combination of 22% maltose and pH 6.5 gave the best viability of microspores (Fig. 1). Hence the medium MS salts+White's vitamins+22% maltose, pH 6.5, was the optimal medium for *in vitro* maturation culture of microspores of *J. curcas*. The change of microspores viability was shown in Fig. 2 and Fig. 3 (A-C).

The cytology of microspores during *in vitro* culture: The diameter of microspore rapidly increased in cultural duration *in vitro*, from 71.2 μm at the beginning culture to 82.3 μm after 4 d of culture, then another 2–3 d later, reached 90.1 μm. Similarly, microspores developed into bicellular pollen after 4 d culture and some further became tricellular pollen, formed pollen tubes for another 2–3 d. Most of the pollen with viability developed into tricellular pollen, yielding 39.5% maturation rate and 2.7% germination rate in the medium for maturation (Fig. 3).

Fig. 1: Viability of *Jatropha curcas* microspores cultured in medium with different maltose concentrations and pH values

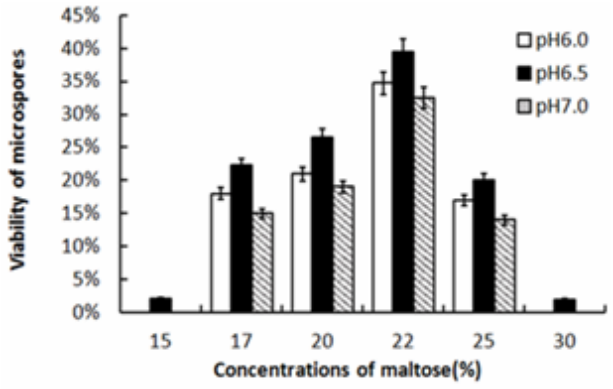
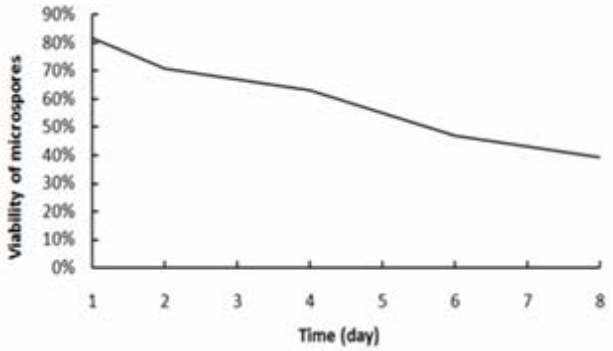


Fig. 2: Viability of *Jatropha curcas* microspores matured *in vitro* over 8 days

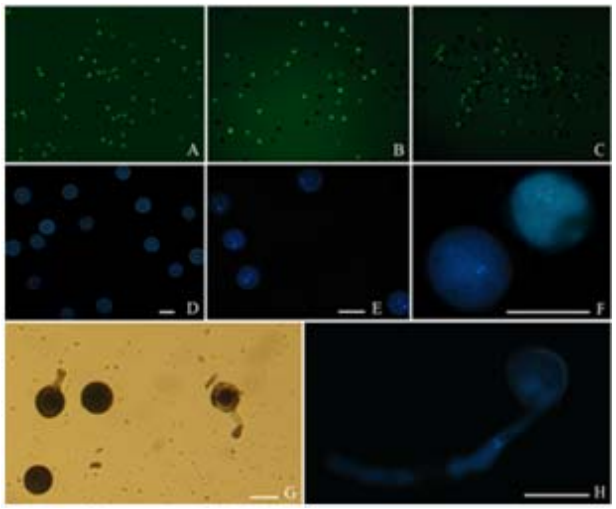


The impact of carbohydrates during *in vitro* pollen germination: In this experiment, we designed nine combinations of different maltose and PEG-4000 concentrations. When maltose was used at low concentrations (bellow 20%), PEG-4000 significantly enhanced the germination rate of pollen. The medium with PEG-4000 concentration greater than 10% decreased germination rate when maltose was over 20% (Table II). From another point of view, more than 20% of maltose suppressed pollen germination even if PEG-4000 was 5%. The combination 20% of maltose+10% PEG-4000 gave the best pollen germination (Table II). However, replacement of maltose with sucrose gave an improved germination (49.6% of pollen germination; Table II). Thus the medium 20% sucrose+10% PEG-4000+BK salts was considered as basic germination medium in subsequent experiments.

Effect of light and temperature on pollen germination: Dark improved germination of *J. curcas* pollen. Compared with the light, the germination frequency of pollen in dark was 30% higher when temperature was 25°C to 28°C (Fig. 4A). If temperature was over 28°C in dark, the germination frequency showed a sharp decline. The germination of *J. curcas* pollen was less sensitive to temperature. The temperature 25°C to 28°C in dark and of 25°C to 30°C in light had no significant effect on pollen germination (Fig. 4A). But low temperature (0-4°C) pretreatment for 20 min increased the rate and synchronism of pollen germination (Fig. 4B).

Fig. 3: *In vitro* maturation of *Jatropha curcas* microspores

(A) Viability of microspore after about 1 days of *in vitro* culture; (B) Viability of microspore after about 4 days of *in vitro* culture; (C) Viability of microspore after about 8 days of *in vitro* culture; (D) Microspores *in vitro* cultured for 1 day; (E) Bicellular pollen derived from microspore after about 4 days of *in vitro* culture; (F) Tricellular pollen derived from microspore *in vitro* culture for about 8 days.(G) Germination of *in vitro* matured pollen under the light and fluorescence microscope. Horizontal bars in panels D = 70 μm, E = 80 μm and F–H = 100 μm



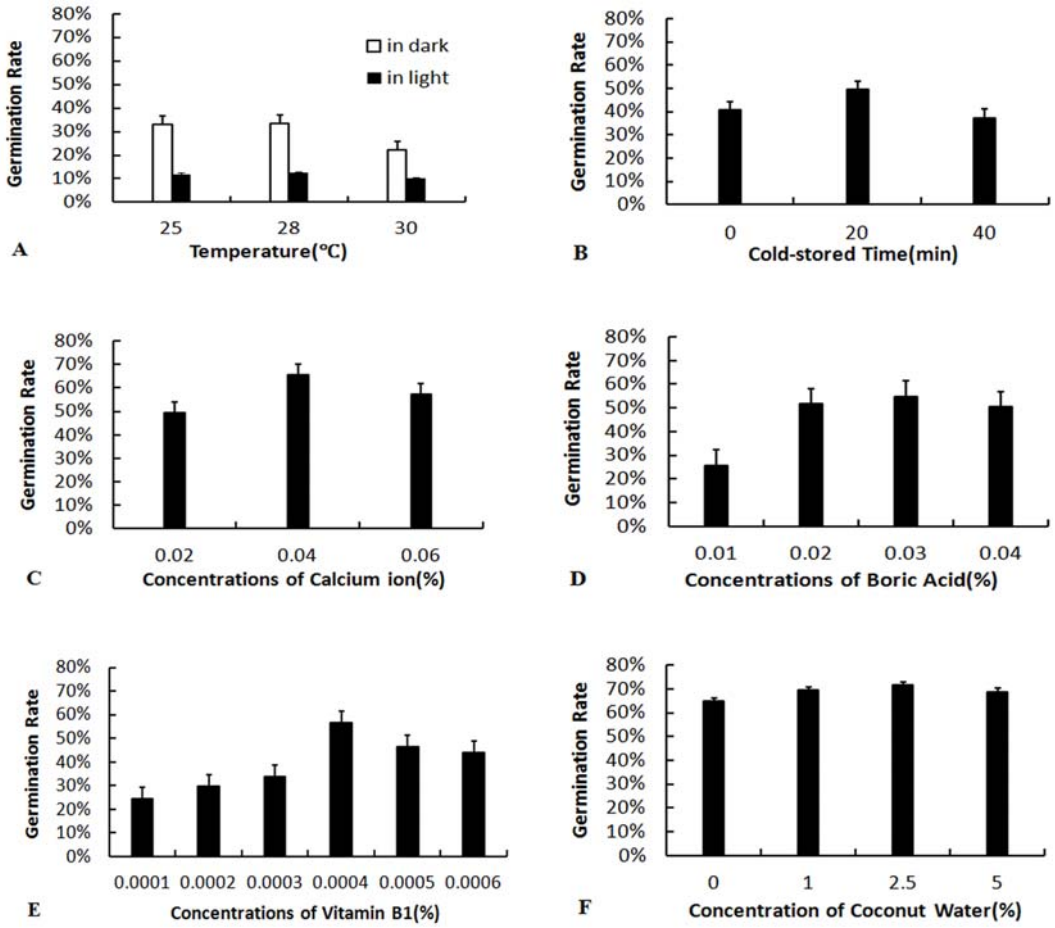
Effects of Ca^{2+} and B concentrations on pollen germination: Increase in calcium ion (Ca^{2+}) concentration in the basic medium had a significant impact on pollen germination rate. The germination rate peaked at 65% with the mean tube length of 503 μm when the Ca^{2+} concentration was 0.04% (Fig. 4C). On the other hand for boric acid, the germination rate peaked when the boric acid concentration was 0.03% (Fig. 4D).

Influence of organic active substances on pollen germination: To explore the impact of physiologically active substances on pollen germination, we set various media containing different concentrations of vitamin B6, B1 and coconut water. The results showed that vitamin B6 had no obvious effect on the germination of *J. curcas* pollen, but vitamin B1 promoted the germination of the pollen and elongation of the pollen tube (Fig. 4E); the mean tube reached 1013 μm. Besides, the addition of coconut water in the basic medium significantly contributed to germination of pollen (Fig. 4F).

Finally, most effective germination medium suitable for *J. curcas* pollen was: 20% sucrose+10%PEG+BK salts (Ca^{2+} supplemented to 0.04%) +2.5% coconut water, pH 6.5. The pollen germination rate of *J. curcas* reached 71.6% cultured in this medium. *In vitro* germinated pollen showed diversity in morphology, some pollen formed into V-shaped tubes and some into Y-shaped (Fig. 5). The

Fig. 4: Germination frequencies of pollen in *Jatropha curcas* under different conditions

(A) Germination of *J. curcas* pollen in dark and light with different temperature. (B) Germination of *J. curcas* pollen under different cold-stored time. (C) Germination of *J. curcas* pollen in the germination medium supplemented with different concentrations of calcium ion. (D) Germination of *J. curcas* pollen in the germination medium supplemented with different concentrations of boric acid. (E) Germination frequency of *J. curcas in vivo* matured pollen in germination medium supplemented with different concentrations of vitamin B1. (F) Germination frequency of *J. curcas in vivo* matured pollen in germination medium supplemented with different coconut water concentration



germination rate of *in vitro* matured pollen grains showed 13.7% when they were transferred to the optimized germination medium (Fig. 3G).

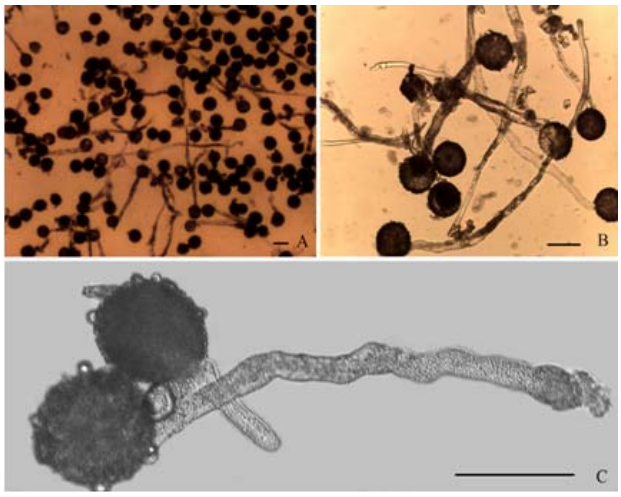
DISCUSSION

Our experiments have documented that microspores of *J. curcas* could develop into mature pollen with biological function (generation of pollen tube) under the artificial conditions. The maturation rate of *J. curcas* microspores was 39.5% in optimal medium, a significant improvement over wheat (1%: Stauffer *et al*., 1991), maize (14%: Pareddy & Petolino, 1992) *and O. violaceus* (19.3%: Zhao *et al.*, 2007).

Developmental process of *J. caucas* microspores *in vitro* was similar to *in vivo* in this study. It required 7–9 d for late microspores to develop into mature pollen in planta and about another 2 d for pollination and germination. Our observation showed that the mature *J. caucas* pollen is was tricellular pollen (Fig. 3) instead of bicellular pollen as reported previously (Liu *et al.*, 2007). In the process of *in vitro* culture, microspores were uninucleate, accompanied by changes in size (the initial mean diameter of the small spores 71.19 μm, after 8 days, the mean diameter increased to 108 μm) developed into trinucleate pollen through binucleate stage. Moreover, results of hanging culture experiment showed that culture in this cell culture inserts were much better than those in the liquid medium. It is likely that in hanging cell culture inserts, cell growth could more closely mimicked as occurs *in vivo*.

Sucrose as an osmotic regulator and carbon resource, has been widely used in tissue culture. However, viability of microspores in medium supplemented with sucrose was much lower than that with maltose in the maturation culture of *J. curcas* microspores. This contribution of maltose to *in vitro* maturation of microspores has been reported in barley (Scott & Lyne, 1994), snapdragon (Barinova *et al.,* 2002) and *O. violaceus* (Zhao *et al.*, 2007). Addition of sucrose to

Fig. 5: *In vitro* germination of *Jatropha curcas* pollen. Horizontal bars in panels A–C = 100µm



the maltose-containing medium decreased the viability of microspores, whereas supplement of maltose to the sucrose-containing medium increased viability of microspores (Table I). This information indicated that sucrose was detrimental to the development of microspores in culture. In contrast to promoting *in vitro* microspores maturation of maltose, sucrose was superior to maltose during pollen germination process of *J. curcas*. Both sucrose and maltose are disaccharides. The blocking of maltose to pollen germination of *J. curcas* may be from maltose itself rather than its decomposition product, glucose. The difference between maltose and sucrose is that sucrose contains fructose and glucose but maltose contains two glucose molecules. This assertion can be supported from the observation of Okusaka and Hiratsuka (2009) on "Fructose inhibits pear pollen germination without loss of viability". The mechanism maintains to be clarified. Both inorganic salts in MS medium and BK medium have been used in maturation culture of microspores *O. violaceus* (Zhao *et al.*, 2007), tobacco (Aziz & Machray, 2003), maize (Dupuis & Pace, 1993). In maturation culture of microspores on *J. curcas*, MS salts producing larger amount of mature pollen than BK salts indicated that inorganic salts had an important impact on male gametophyte of *J. curcas*. Further optimizing the combination of inorganic salts will more remarkably improve mature frequency of *J. curcas* microspores.

PEG is relatively inert metabolically in pollen and can not enter the cells (Subbaiah, 1984). The incorporation of PEG to the medium has effectively improved pollen germination of *J. curcas*. The possible mechanism is considered as that PEG can regulate the permeability of plasma membrane and to give stability to the pollen tube membrane (Read *et al*., 1993).

Rapid pollen tube growth requires constant fusion of vesicles forming the plasmalemma and continuous secretion of cell wall material. Boron (B) is considered to abridge the connection of cellular wall materials, such as glycoproteins containing hydroxyl-rich mannose and fucose and thus involved in pollen wall formation (Blevins & Lukaszewski, 1998). Through its effect on H^+-ATPase activity, B also affects pollen germination, tube growth and oscillation behavior (Feijo *et al.*, 1995; Holdaway-Clarke *et al*., 2003; Wang *et al.*, 2003). Supplementing higher concentrations of B in medium is conducive to the germination and tube growth of *J. curcas* pollen. Ca^{2+} is also critical factor for pollen germination and tube growth (Holdaway-Clarke & Hepler, 2003). It participates in these processes by regulation of growing cell wall, vesicle trafficking, actin dynamics (Chebli & Geitmann, 2007) and growth rate oscillations (Messerli & Robinson, 1997; Geitmann & Cresti, 1998; Holdaway-Clarke *et al.*, 2003). More than 300 mg/L of Ca^{2+} can significantly improve germination of *J. curcas* pollen. Apart from boric acid and calcium nitrate being necessary for pollen germination, the addition of coconut water significantly improve germination of *J. curcas* pollen.

Medium pH is a critical condition for *in vitro* microspore mature and germination. For *J. curcas* microspore mature culture and germination *in vitro*, the optimum is pH 6.5, as reported for Snapdragon (Barinova *et al*., 2002). However, for *O. violaceus*, the optimum pH is 7.0 (Zhao *et al*., 2007). Dupuis and Pace (1993) reported that a pH range of 7-8 was conducive to the maintenance of viability of maize microspores, while detrimental for starch accumulation. Hence closer to neutral pH is more appropriate for microspore culture and germination of pollen.

Temperature is also crucial factor for pollen germination. Pollen of many plants including *Arabidopsis thaliana* (Boavida & McCormick, 2007; Daher *et al.,* 2009), *Limonium perezii* (Zhang *et al.*, 1997) and cotton (Kakani *et al.,* 2005) gave bell-shaped curve e.g., the germination rate increase and then decline with increasing temperature and a narrow peak. While in this study, *J. curcas* maintained a wide peak from 25 to 28°C (Fig. 4A). Low temperature pretreatment (0 − 4°C) and darkness improved pollen germination of *J. curcas* (Fig. 4B).

In vitro mature and germination of *J. curcas* microspores established in this paper makes it possible the study of biological process in pollen development and its mechanism under artificial conditions. *In vitro* pollen germination is considered the best model system to detect alterations in germination or tube growth performance and study cellular processes involved in polarity and tip growth, as assays for defects in these parameters are difficult to perform *in vivo* (Boavida & McCormick, 2007). On the other hand, *J. curcas* is introduced instead of native plant in many countries of Asia, Africa and Latin America and improved varieties with desired straits that are not available for particular cultural conditions yet (Divakara *et al.*, 2010). Every pollen grain represents a special genotype. *In vitro* maturation and germination of *J. curcas* microspores can be

used for pollen selection (Touraev *et al*., 1995). Moreover, *in vitro* maturation and germination of *J. curcas* microspores can provide a novel system for transformation and thus have great potential for genetic improvement.

In conclusion, we established an experimental system of *in vitro* maturation and germination of *J. curcas* microspore, the medium: MS salts+White' vitamins+22% maltose, pH 6.5 and hanging culture were optimal for *J. curcas* microspore maturation; medium: 20% sucrose+10% PEG-4000+BK [Ca $(NO_3)_2$ 400 mg/L, $MgSO_4$ 200 mg/L, H_2BO_3 100 mg/L, KNO_3 100 mg/L] +2.5% coconut water, pH 6.5 was the most appropriate for pollen germination and the pre-cold store time was necessary.

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