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Effects of Ca^{2+} on *in vitro* pollen germination of three *Acacia* species

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Abstract

We investigated the effects of Ca^{2+} on in vitro pollen germination of *Acacia auriculiformis, Acacia mangium*, and *Acacia crassicarpa* under different concentrations of Ca^{2+} (50 mg.L^{-}1, 100 mg.L^{-}1, 150 mg.L^{-}1, 200 mg.L^{-}1, 250 mg.L^{-}1, 300 mg.L^{-}1, 350 mg.L^{-}1 and 400 mg.L^{-}1) in the present study. Our results revealed that Ca^{2+} could stimulate the pollen germination percentage and the pollen tube growth of *A. auficuliformis* and *A. crassicarpa* under a certain concentration. In vitro pollen germination of *A. mangium* did not require exogenous Ca^{2+}. With high Ca^{2+} concentration, the pollen germination of *A. auriculiformis*, A. *mangium*, and *A. crassicarpa* were obviously inhibited. With the rise of Ca^{2+} concentration, the percentage of pollen germination, the pollen tube length, the pollen tube growth, and the number of pollen tube were increased. However, the pollen germination percentage decreased with high Ca^{2+} concentration.

Keywords: *Acacia, calcium, pollen germination percentage, pollen tube*

Introduction

A. auriculiformis, A. *mangium*, and *A. crassicarpa*, are all fast-growing tropical *Acacia* Mill. species of Mimosaceae, having the characteristics of large biomass, excellent wood properties. They can be used as materials for paper pulp, building, and furniture. In addition, they are useful for soil improvement and greening of barren mountains with nitrogen fixation (Urnbull et al., 1986; Doran and Skelton, 1982; Pan, 1999, He et al., 2006). In China, since the introduction of *Acacia* in Guangdong, Guangxi, Hainan, Fujian and other provinces (regions), the total plantation of *A. auficuliformis* has been over 71 thousand hm^2 (Lin, 2002), while that of *A. mangium* has been more than 250 thousand hm^2 (Li and Deng, 2002). The *Acacia* species play a key role in forestry construction projects of China.

Most *Acacia* species reveal high outcrossing (Moran et al., 1989a, 1989b; Muona et al., 1991). *A. auficuliformis* may be facultative outcrossing (Li et al., 2010), or self-incompatibile species (Bernhardt et al., 1984). The self-incompatibility of *Acacia* species often seriously affects the seed yield in orchard, and the requirements of seedling production cannot be met.
Collecting *Acacia* pollen and artificial pollination improved the production of seed orchard greatly. Collecting the dynamic pollen is the key to success for pollination. Therefore, it is necessary to detect the pollen vitality. The in vitro detection of *Acacia* pollen vitality system has been successfully established (Zhan and Huang, 2015, 2016a, 2016b)

However, testing the pollen vitality is not the only factor in successful pollination. The pollen germination and the normal growth of pollen tube are directly related to fertilization and seed formation. In the presence of Ca^{2+}, pollen germination and pollen tube growth occur, by which pollen tube reaches ovary and ovule, and seeds form.

Ca^{2+} exerts an important influence on starting pollen germination and regulating pollen tube elongation (Pierson and

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Cresti, 1992; Heler, 1997). Ca^{2+} with a given concentration is essential for pollen germination and growth (Daniela and Thomas, 2003). In the presence of Ca^{2+}, the pollen tube elongation of *Hibiscus syriacus* is significantly quicker than that in the absence of Ca^{2+}, showing an obvious increase (Jia et al., 2007). In a certain range of concentration, Ca^{2+} has almost no effect on the frequency of pollen germination, but it mainly affects the percentage of pollen germination and pollen tubes growth.

Without exogenous Ca^{2+}, the germination percentage of pollen is slightly lower. However, pollen still continues to germinate and grow, indicating that exogenous Ca^{2+} is not a necessity for pollen germination (He et al., 2006). Without the addition of exogenous Ca^{2+}, *Fragaria ananassa* pollen can still germinate, indicating that exogenous Ca^{2+} is not a necessity for the pollen germination of *F. ananassa* (Jiang et al., 2007).
Suitable concentration of Ca^{2+} can improve pollen germination and pollen tube growth. On the contrary, high concentration of Ca^{2+} will inhibit or prevent pollen germination and pollen tube growth (Gong et al., 1995; Cheng et al., 2004). In a certain ranges of concentration, Ca^{2+} has different levels of effects on pollen germination percentage and pollen tube elongation of *Aloe vera*. Low concentration of Ca^{2+} is not conducive to pollen tube growth, while high concentration of Ca^{2+} inhibits it (Yang et al., 2010).

When there is less free Ca^{2+} in pollen intracellular of *Cunninghamia lanceolata*, which cannot meet the needs of pollen germination and pollen tube growth, the supplement of exogenous Ca^{2+} can promote pollen germination and pollen tube growth, but too high concentration will inhibit the germination and growth of pollen (Shen et al., 2010). Similar results were obtained in other plant species (Dong and Zheng, 2007; Yang et al., 2010).

However, there is little known about the effects of Ca^{2+} on pollen germination and pollen tube growth of *Acacia*. Therefore, it has great significance to carry this type of research in *Acacia*. This study deals with the effect of Ca^{2+} on in vitro pollen germination percentage, pollen tube length, and pollen tube growth of *A. auriculiformis, A. mangium* and *A. crassicarpa*, to provide a theoretical basis for increasing seed yield in orchard and for further artificial control pollination in *Acacia* hybrid breeding.

Materials and methods

Study sites and sample trees

The materials were from the Key National Breeding Base of *Acacia* in Xinhui District, Jiangmen City, Guangdong Province (the Longitude of 113°08 'E, and the latitude of 22°18' N), with a subtropical marine climate. Elite individual plants of *A. auriculiformis, A. mangium* and *A. crassicarpa* were sellected from the seed orchard, and the study was conducted between July and December in 2014.

Pollen collection

Before 9:00 am, spikes of *A. auriculiformis, A. mangium* and *A. crassicarpa* on the flowering day were collected into sulfuric acid paper bags, after which they were naturally dried indoors between 25 ° C and 30 ° C. At 10:00 am in the next day, pollen was collected by repeated gentle brushing of the spikes for 3 to 5 times and then the screening of 200 mesh pollen sieve. The pollen was put into a glass bottle with dry silica-gel, and then was vacuumed after covering. A large amount of pure pollen can be collected for testing and storing by this method.

Pollen germination in vitro

Based on the best treatment of in vitro pollen germination (20 % Sucrose+300 mg.L^{-1} boric acid, cultured 24 h at 30 ° C for *A. auriculiformis*, 20% Sucrose+100 mg.L^{-1} Boric acid, cultured 24 h at 28 ° C for *A. mangium*, 15 % Sucrose+100 mg.L^{-1} Boric acid, cultured 24 h at 24 ° C for *A. crassicarpa* (Zhan and Huang, 2015, 2016a, 2016b), different concentrations of $Ca2^{+}$ (Calcium nitrate) (0 mg.L^{-1}, 50 mg.L^{-1}, 100 mg.L^{-1}, 150 mg.L^{-1}, 200 mg.L^{-1}, 250 mg.L^{-1}, 300 mg.L^{-1}, 350 mg.L^{-1}, 400 mg.L^{-1}) were added to screen out the optimum Ca^{2+} concentration for in vitro pollen germination. Two perspectives were selected on each slide, repeated for three times, and then the average was calculated. The germinated pollen number, the pollen tube length and the number of the pollen tube were observed under a microscope for 3 h, 6 h, 9 h, 12 h, and 24 h respectively.

Statistical analysis

One-way ANOVA was performed using SPSS19.0 on the pollen germination percentage and the longest pollen tube length. Duncan's Multiple Range Test at 5 % level was used to compare the differences among treatment means.

A total of 150 *A. mangium* flowers were randomly selected to investigate the number of ovules per flower. A total of 255 seed pods were randomly selected to investigate the seeds number per pod.

Pollen germination percentage (%) = the number of germinated pollen per perspective/the number of pollen per perspective×100.

Pollen viability (%) = the number of germinated pollen per perspective/the number of pollen per perspective×100.

Results

Effects of Ca^{2+} on in vitro pollen germination and pollen tube length

With different concentrations of Ca^{2+}, various effects could be seen on the in vitro pollen germination of *A. auriculiformis, A. mangium* and *A. crassicarpa* (Table1).

With 50 mg.L^{-1} and 250 mg.L^{-1} of Ca^{2+} or without Ca^{2+}, the germination percentage of *A. auriculiformis* pollen were higher than 80 %, which was significantly ($P<0.05$) better than that in other treatments. The higher of Ca^{2+} concentration (>250 mg.L^{-1}), the lower of the pollen germination percentage. However, Ca^{2+} had slight effects on pollen tube length of *A.*

Table1
Effects of different concentrations of Ca^{2+} on pollen germination and pollen tube length (PTL)

Ca^{2+} ($mg.L^{-1}$)	*A. auriculiformis*		*A. mangium*		*A. crassicarpa*	
	Germination percentage (%)±s.e.	PTL (μm)	Germination percentage (%)±s.e.	PTL (μm)	Germination percentage (%)±s.e.	PTL (μm)
0	88.73±2.02a	175a	94.27±1.17a	128a	93.97±2.44[a]	232b
50	85.27±6.26a	175a	23.33±5.22f	64b	33.73±2.61[c]	58d
100	62.88±7.78c	175a	26.96e±2.40ef	128a	36.88±1.98[c]	116c
150	46.82±3.50d	140ab	73.59±6.91b	128a	37.97±1.66[c]	116c
200	64.04±5.55c	175a	61.40±9.50c	160a	51.79±3.34[b]	145c
250	87.89±3.01a	175a	33.10±1.79d	160a	96.23±2.32[a]	290a
300	70.38±3.59b	140ab	29.45d±2.09de	128a	19.13d±10.49[de]	58d
350	48.67±2.06d	175a	24.04e±2.78ef	64b	16.04±2.57[e]	58d
400	35.33±2.10e	105b	21.81±2.50f	64b	23.55±4.99[d]	58d

Table 2
The percentage of ovule number per ovary and seed number per pod

Number	4	5	6	7	8	9	10	11	12	13	14	15	16
The percentage of ovule number per ovary (%)	-	-	-	-	-	-	1.4	1.4	18.4	13.5	34.8	24.8	5.7
The percentage of seed number per pod (%)	0.4	0.8	3.1	4.3	7.4	14.1	17.6	20.0	17.2	11.8	1.6	1.6	-

auriculiformis. The pollen tube length of *A. auriculiformis* was the shortest (105 μm) with 400 $mg.L^{-1}$ of Ca^{2+}.

Without Ca^{2+}, the pollen germination percentage of *A. mangium* was as high as 94.27 %, which was significantly ($P<0.05$) larger than that in other treatments. With 200 and 250 $mg.L^{-1}$ of Ca^{2+}, pollen tube length was the longest, almost up to 160 μm. When Ca^{2+} concentration is <200 $mg.L^{-1}$, the pollen germination percentage and pollen tube length increased with the rise of Ca^{2+} concentration, while at >200 $mg.L^{-1}$ Ca^{2+}, the pollen germination rate and pollen tube length was inhibited with the rise of Ca^{2+} concentration. The more the Ca^{2+} concentration, the more severe the inhibition was.

In the treatment without Ca^{2+} and with 250 $mg.L^{-1}$ of Ca^{2+}, the pollen germination percentage of *A. crassicarpa* was up to 93.97 % and 96.23 % respectively, and the pollen tube length of *A. crassicarpa* was up to 232 μm and 290 μm, which was significantly ($P<0.05$) higher than that in other conditions. With low Ca^{2+} concentration, pollen germination percentage and pollen tube length increased with the rise of Ca^{2+} concentration. However, with the Ca^{2+} concentration of higher than 250 mg. L^{-1}, the percentage of pollen germination and pollen tube length decreased with the rise of Ca^{2+} concentration.

At the early stage of culturing *A. auriculiformis* pollen, the pollen germination percentage under Ca^{2+} treatment was higher than that of without Ca^{2+} after 3 h of culturing. In comparison, the pollen germination percentage showed non-significant ($P>0.05$) difference between with and without Ca^{2+} (Figure

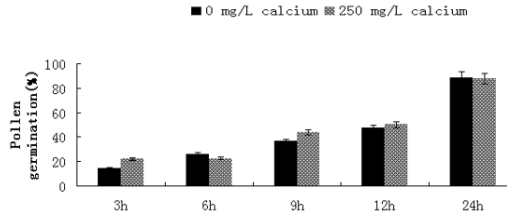

Figure1
In vitro pollen germination of *A. auriculiformis* at 0 $mg.L^{-1}$ and 250 $mg.L^{-1}$ Ca^{2+} concentration

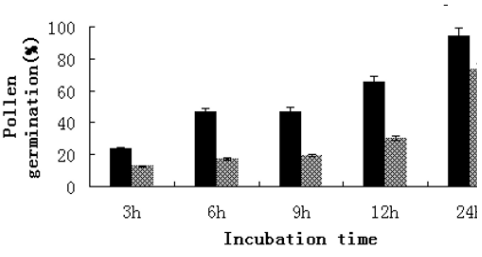

Figure 2
In vitro pollen germination of *A. mangium* at 0 $mg.L^{-1}$ and 150 $mg.L^{-1}$ Ca^{2+} concentration

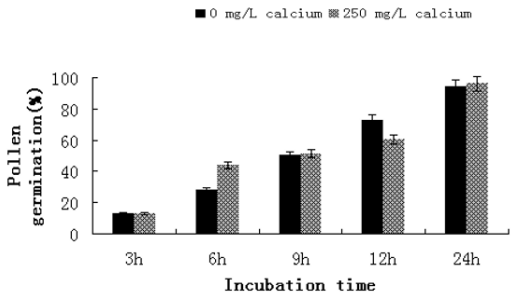

Figure 3
In vitro pollen germination of *A. crassicarpa* at 0 mg.L^{-1} and 250 mg.L^{-1} Ca^{2+} concentration

1) after 24 h of culturing. With Ca^{2+} treatment, the germination percentage of *A. mangium* pollen was lower than that without Ca^{2+} treatment (Figure 2). The pollen germination percentage of *A. crassicarpa* with Ca^{2+} treatment was higher than that of without Ca^{2+} treatment after 3 and 6 h of culturing. However, there was non-significant (P>0.05) difference in the pollen germination percentage between with and without Ca^{2+} treatment after 24 h of culturing (Figure 3).

The percentage of ovule number per ovary and seed number per pod.

The result showed that the number of ovules per flower was between 10 and 16, and 14 ovules was the most common, accounting for 34.8 % of the total ovules per flower, and the seeds number per pod were between 4 and 15, and 11 seeds were the most common, accounting for 20 % of the total seeds number per pod (Table 2).

Discussion

Under natural conditions, the nutrients and mineral elements needed by pollen growing are from stigma and pollen. In addition, Ca^{2+} is required for pollen germination. It is found that the stigma surface of tomato has rich Ca^{2+} (Tirlapur and Shiggaon, 1988). This phenomenon has been confirmed in the research on a variety of plants (Tian and Yuan, 2000). It has been shown from the results of previous study that the culture medium of in vitro pollen germination needs a certain amount of Ca^{2+} (Brewbaker and Kwack, 1963). In this study, with the treatment of Ca^{2+}, the pollen germination percentage increased after the culturing of in vitro *A. auriculiformis* pollen germination. During the early stage of culturing (before 12 h of culturing), under the treatment of 250 mg. L^{-}1 of Ca^{2+}, the pollen germination percentage was higher than that of the treatment without Ca^{2+}. Ca^{2+} with a certain concentration could accelerate the pollen germination percentage of *A. auriculiformis.* However, there was no difference in the pollen germination percentage between treatments with and without Ca^{2+} (88.73 % and 87.98 %) after 24 h of culturing.

Ca^{2+} has a significant role in stimulating pollen tube elongation and pollen tube direction-growth in the process of pollen germination (Yao and Zhao, 2004). The in vitro growth of pollen tube needs to absorb Ca^{2+} from outside, to maintain the Ca^{2+} gradient distribution of pollen tube tip, making it suitable for the polar growth of pollen (Tian and Yuan, 2000). In this study, with the treatment of 250 mg.L^{-}1 Ca^{2+} for in vitro pollen germination of *A. crassicarpa*, the pollen tube length (290 μm) was longer than that in without Ca^{2+} treatment (232 μm), indicating that a certain concentration of Ca^{2+} could improve the pollen tube growth of *A. crassicarpa*.

It is believed that there is not require exogenous Ca^{2+} in pollen germination of *Robinia pseudoacacia* var. *decaisneana,* and the mass concentration of external Ca^{2+} required for *R. pseudoacacia* and tetraploid *R. pseudoacacia* pollen germination is not high (Dai et al. 2012). The results showed that there is enough Ca^{2+} at the internal of pollen to support germination. When the pollen absorbs water, the Ca^{2+} at surface of pollen is released into the culture medium to stimulate pollen germination (Tian et al., 1998, Heslop-Harrison, 1987). In this study, in the process of in vitro pollen germination of *A. auriculiformis* and *A. mangium,* the pollen germination were the best without the addition of Ca^{2+}, showing that the Ca^{2+} inside pollen was sufficient to ensure the pollen germination of *A. auriculiformis* and *A. mangium.*

The effect of exogenous Ca^{2+} on pollen germination and pollen tube growth is determined by the content of intracellular free Ca^{2+}. With low Ca^{2+} concentration, pollen tube stops growing or growing slowly, and deformity appears due to the lack of enough Ca^{2+} for the stimulation. The intracellular free Ca^{2+} in *Amygdalus persica* pollen is less enough to meet the demands for pollen germination and pollen tube growth. When 1.0 mmol.L^{-}1 of Ca^{2+} is added, the pollen germination percentage of *A. persica* increased significantly. When 0.1 mmol.L^{-}1 of Ca^{2+} is added, pollen tube growth improved greatly. A certain concentration of exogenous Ca^{2+} can improve pollen germination and pollen tube growth, while high concentration of exogenous Ca^{2+} inhibited the pollen germination and pollen tube growth (Xue et al., 2007). It is reported by Nian et al. (2005) that with low or no Ca^{2+}, the pollen tube tip easy to break, while high Ca^{2+} concentration will prevent the growth of pollen tube. They also found that at the concentration of up to 350 mg. L^{-}1 Ca^{2+}, the germination percentage and the pollen tube length of *A. auriculiformis* were inhibited significantly. When the Ca^{2+} concentration reached 200 mg. L^{-}1, the germination percentage and the pollen tube length of *A. mangium* were inhibited. The higher Ca^{2+} concentration, the more severe the inhibition was. When the concentration of Ca^{2+} increased to 400 mg. L^{-}1, the pollen germination percentage dropped by 39.59 %, as low as 21.81 %, and the pollen tube length was only 64 μm. The results showed that the Ca^{2+} concentration in *A. mangium* pollen was higher than that in *A. auriculiformis*. Therefore, with low concentration of Ca^{2+}, the pollen germination and the pollen tube growth of *A. mangium* pollen was inhibited.

With the treatment of high concentration Ca^{2+}, the pollen germination percentage and pollen tube growth of *A. auriculiformis, A. mangium* and *A. crassicarpa* were all greatly inhibited. High concentration of Ca^{2+} may affect the physiological activities of cytoskeleton in the pollen tube, resulting in the formation of thick wall in the pollen tube tip. Therefore, the pollen tube growth was affected (Steer and Steer, 1989), and high concentration of Ca^{2+} might be toxic to cells, resulting in excessive cell turgor, thereby limiting the extending of pollen tube (Dong and Zheng, 2007). With high concentration of Ca^{2+}, pollen tube growth is inhibited, which may be due to the hurt of Ca^{2+} with pull-channel (Sun and Sun, 2001).

We observed a total of 8 pollen tubes at the concentration of 250 mg. L^{-1} Ca^{2+} from the composite pollen grains of *A. auriculiformis* in this study, while with treatment of 200 mg. L^{-1} Ca^{2+}, five pollen tubes appeared in the composite pollen grains of *A. mangium*. With the treatment of 250 mg. L^{-1} Ca^{2+}, the composite pollen grains of *A. crassicarpa* produced 13 pollen tubes. Based on the stigma size of *A. mangium*, the stigma generally accepted 1-2 composite pollen grains (Huang et al. 2014), and the ratio of one composite pollen was 59.4 %, and that with two was 27.4 % (Li, 2010). As shown in Table 2, the percentage of seed number within pods did not coincide with that of ovules. The reason for such difference and the relation with the number of pollen tubes were worthwhile of further study. It was speculated that in addition to the viability of pollen and pollen tube number, self-incompatibility of *Acacia* species was an important factor affecting seed number, which still needed further verification.

This study revealed an effective method to improve the the in vitro pollen germination system of *A. auriculiformis, A. mangium* and *A. crassicarpa* and provided theoretical support and guidance for artificial pollination in future breeding program. With the supplement of a certain concentration of Ca^{2+}, the pollen tube growth was stimulated in the in vitro pollen germination system of *Acacia*. The research results lay a solid foundation for further understanding of the pollen germination of *Acacia* species. Meanwhile, it also provides a theoretical basis for improving the success rate of the artificial pollination in *Acacia*, the fruit setting percentage, and seed yield.

However, the mechanism of seed number per pod and self-incompatibility in *Acacia* still needs further studies. Because of the uncertainty in natural environment, whether the germination and growth of *Acacia* pollen in vitro are same as the germination and growth of pollen in the natural state still needs to be confirmed.

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