Research paper

Orthogonal Test Design for Optimizing Culture Medium for in vitro Pollen Germination of Chinese Chinquapin (*Castanea henryi*)

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【Summary】

Chinese chinquapin (*Castanea henryi* (Skam) Rehder & Wilson) is an ecologically and economically important nut tree species in southern China. Chinquapin suffers from high rates of empty shells, which are directly related to low pollination efficiency. Efficient pollination mainly depends on pollen vigor, which depends on the pollen grain germination rate and the rate of pollen tube growth. We studied the pollen germination rate and pollen tube length of the advanced chinquapin cultivar HCHJA-1; specifically, we compared the effectiveness of different concentrations of 3 key factors by an orthogonal design method: boric acid (H_3BO_3), calcium chloride ($CaCl_2$), and gibberellic acid (GA_3), to determine pollen vigor and optimize the culture medium for in vitro pollen germination. Results indicated that chinquapin pollen germination rates were significantly affected by medium components. The pollen germination percentages varied between 13.02 and 66.99%. Three factors influenced pollen germination in the order of decreasing significance: $GA_3 > H_3BO_3 > CaCl_2$. Pollen tube growth was influenced by these factors as follows: $H_3BO_3 > GA_3 > CaCl_2$. The optimal culture medium for promoting pollen vigor of *C. henryi* HCHJA-1 was 1% agar, 10% sucrose, 20 mg L^{-1} GA_3, 100 mg L^{-1} H_3BO_3, and 50 mg L^{-1} $CaCl_2$.

Key words: *Castanea henryi*, culture medium, orthogonal design, pollen tube growth, pollen vigor.

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研究報告

正交設計優化錐栗花粉離體萌發率的培養基

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摘 要

錐栗是中國南方重要的生態兼經濟型幹果樹種。錐栗常有較高的空苞率，這直接與傳粉效率低有關。有效的傳粉效率主要與花粉活力有關，並取決於花粉萌發率和花粉管生長速度。我們研究了錐栗優良無性系“HCHJA-1”花粉萌發率和花粉管生長長度。具體而言，我們採用正交設計的方法，比較了不同濃度的三個關鍵性因素：硼酸、氯化鈣和激勃酸，測定其花粉活力來優化花粉離體萌發率的培養基。結果表明，不同培養基成分對錐栗花粉萌發率的影響顯著。花粉萌發率在13.02%~66.99%之間變化。三個因素影響花粉萌發率的順序為激勃酸>硼酸>氯化鈣。影響花粉管的生長的順序為硼酸>激勃酸>氯化鈣。最適宜促進錐栗花粉活力的培養基為1%瓊脂、10%蔗糖、20 mg L^{-1}激勃酸、100 mg L^{-1}硼酸和50 mg L^{-1}氯化鈣。

關鍵詞：錐栗、培養基、正交設計、花粉管生長、花粉活力。

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INTRODUCTION

Chestnut trees are among the most important nut crops in China and have been cultivated over a long history (Zou et al. 2013). Chestnuts belong to the genus *Castanea*, including 7 main economically important species: *C. pumila*, *C. crenata*, *C. mollissima*, *C. sativa*, *C. dentata*, *C. seguinii*, and *C. henryi* (Bounous and Marinoni 2005). Chinese chinquapin (*C. henryi*) is a well-known dry fruit that is indigenous to southeastern and southwestern China (Anagnostakis 2010, Fan et al. 2015). It is cultivated as a timber crop in Fujian, Zhejiang, Hunan, Sichuang, and Guizhou Provinces (Xiang et al. 2016). The nut of Chinese chinquapin is rich in nutrients, containing 9.3% protein, 2.7% lipids, 7.4% sugars, and 5.1% amino acids, and has a unique flavor and taste (Xu 2005).

In trees producing nut crops such as the Chinese chinquapin, fertilization is an essential process to enhance fruit yields. Chinquapin is self-incompatible; thus, a tree of a different cultivar is required for effective pollination (Zhang et al. 2016). Successful pollen germination usually leads to good tube growth, which likely promotes fertilization (Sulusoglu and Aysun 2014). Poor pollination always causes empty burs in chinquapin trees, which severely impact yields and economic profits (Zheng et al. 2009). Thus, pollen viability plays a significant role in pollination for high fruit yields. Pollen germination and tube growth are affected by nutrition and plant growth regulators (Maita and Sotomayor 2015, Muengkaew et al. 2016). Nurtrients and plant bioregulators, such as boron (B), calclum (Ca)and gibberellic acid (GA_3) have critical effects on pollination and fruit set in fruit orchards (Nyomora et al. 2000, Okamoto and Miura 2005, Lee et al. 2009). In addition,

it was also found that spring foliar application B, GA_3 and Ca at suitable concentrations, not only can improve pollination, pollen germination and pollen tube growth, but also should be beneficial for nut production (Zhu et al. 1992, Yang et al. 2002, Guo and Xie 2013, Huang et al. 2013, Wang et al. 2014).

Various methods can be used to estimate pollen viability and germination capacity in Castanea species, such as staining with tetrazolium salts (MTT) (Bounous et al. 1992), fluorescence microscopy (Bounous et al. 1992, Fernando et al. 2006), and in vitro germination (Bryhan and Serdar 2008). In general, in the vitro pollen germination rate is considered to be the best indicator of pollen vigor (Acar et al. 2010). Pollen germination is influenced by several factors including genotype, environment, and medium characteristics (Bal and Abak 2005). Tang et al. (2013a) reported variability in pollen grain germination among 14 Chinese chinquapin cultivars. The pollen germination rate was greatly enhanced as the sucrose concentration increased to 7.5%; however, as the sucrose concentration further increased, the pollen germination rate decreased in some cultivars (Lai et al. 2017). The addition of 0.1~0.5% boric acid to the medium increased pollen germination (Zheng et al. 2004). Zheng et al. (2004) also observed that the highest chinquapin pollen germination (44.3%) occurred in a culture medium component containing 1% agar and 10% sucrose at 30~35℃ over a period of 8 h.

There have been several studies on pollen germination in chinquapin (Zheng et al. 2004, Tang et al. 2013a, b, Yang et al. 2014, Lai et al. 2017); the pollen germination potential and quality were found to differ among genotypes (Bryhan and Serdar 2009). Little information is available on pollen germination of the cultivar *C. henryi* HCHJA-1. Therefore, the objective of this study was to determine the pollen germination and tube growth in *C. henryi* HCHJA-1, using an orthogonal design [$L_9(3)^3$] method. Orthogonal designs have been widely used in agricultural studies, such as fertilization (Xiao et al. 2016), seed germination (Ranil et al. 2015), and pollen germination (Xiong et al. 2016). With an orthogonal design, we could determine optimal levels of various factors with best combinations through a small number of tests, but also save manpower and material resources compared with comprehensive experiments (Shao 2004). Therefore, in this paper, a 10-treatment orthogonal experiment obtained results of a combination of experiments, determined optimal levels of various factors, and developed the best cultural medium combination for chinquapin.

MATERIALS AND METHODS

Plant materials and pollen collection

Castanea henryi HCHJA-1 was collected from a chestnut collection orchard at the Central South University of Forestry & Technology Center of Hongjiang (western Hunan Province, China). Hongjiang is located at 27°9’42’’ -N latitude and 109°49’28’’ -E longitude. The average temperature, annual rainfall, and annual sunshine hours are 17℃, 1246.7 mm, and 1415 h, respectively. The chinquapin genotype HCHJA-1 was selected from individuals growing in mountains and forests in the region. Trees were grown in red soil under ordinary management for commercial fruit production. The average nut weight of HCHJA-1 is 11.0 g, and nuts are rich in nutrients (starch: 37.17%; fat: 0.70%; soluble sugar: 5.52%; protein: 3.72%; vitamin C: 317.3 mg kg^{-1}; N: 0.60%; P: 826 mg kg^{-1}; K: 0.32%; Mg: 612 mg kg^{-1}; Ca: 382 mg kg^{-1}). Mature catkins were randomly collected from 5-year-old *C. henryi* “HCHJA-1” in the full

blooming stage on 18 May 2017. The catkins were brought to the lab and laid on clean black paper sheets at room temperature until the anthers dehisced (Bryhan and Serdar 2009). Pollen grains were harvested in a clean microcentrifuge tube and refrigerated at 4℃ before testing.

Experimental design

We used an orthogonal experiment [$L_9(3)^3$] test design to optimize the culture medium for pollen germination rate of chinquapin (Xiong et al. 2016). The superscript number "3" indicates 3 factors, "3" denotes 3 levels, and subscript number "9" indicates 9 treatments, from T1 to T9. Factors influencing pollen viability are concentrations of [A] H_3BO_3, [B] $CaCl_2$, and [C] GA_3. Each factor had 3 levels of optimization. The control group (CK) denotes pollen germination in medium containing 1% agar and 10% sucrose. Tables 1 and 2 provide information on the experimental parameters.

Pollen culture

We dissolved H_3BO_3 and $CaCl_2$ in distilled water at different concentrations, of 20, 50, and 100 mg L^{-1}. We then added 1% agar and 10% sucrose (w/w) to the medium, and warmed it in a microwave to dissolve the sucrose and agar. We dissolved GA_3 in a small volume of alcohol at different concentrations (20, 40, and 60 mg L^{-1}), and added it to the media together with 1% agar and 10% sucrose. When the medium became cold and semi-solid, pollen grains were scattered onto the solidified medium surface using a brush (Xiong et al. 2016). The plates were then

Table 1. Orthogonal experimental design, with 3 factors and 3 levels

Level	Factor		
	(A) H_3BO_3 (mg L^{-1})	(B) $CaCl_2$ (mg L^{-1})	(C) GA_3 (mg L^{-1})
1	20	20	20
2	50	50	40
3	100	100	60

Table 2. Orthogonal array of experiments on the pollen germination rate and pollen tube length in *Castanea henryi* HCHJA-1

Treatment	Factor (mg L^{-1})			Pollen germination	Pollen tube
	(A) H_3BO_3	(B) $CaCl_2$	(C) GA_3	rate (%)	length (μm)
CK	0.0	0.0	0.0	7.48^{az}	36.28^{az}
T1	20.0	20.0	20.0	22.78^{bc}	148.60^{bc}
T2	20.0	50.0	40.0	23.69^{c}	192.10^{ce}
T3	20.0	100.0	60.0	19.15^{bc}	89.03^{ab}
T4	50.0	20.0	60.0	13.02^{ab}	165.08^{cd}
T5	50.0	50.0	20.0	66.99^{e}	414.00^{g}
T6	50.0	100.0	40.0	20.36^{bc}	186.52^{cd}
T7	100.0	20.0	40.0	41.56^{d}	266.38^{ef}
T8	100.0	50.0	60.0	26.91^{c}	239.67^{def}
T9	100.0	100.0	20.0	45.47^{d}	273.65^{f}

Note: Pollen germination rate and pollen tube length were measured after 48 h of incubation.
[z] Different letters in a column indicate a significant difference.

covered and incubated at 35℃ for 48 h in the dark (Tang et al. 2013a, b). Each treatment of 3 replicates.

Measurements

After 48 h, grains of the germinated pollen were counted. Pollen grains were considered germinated when the length of the pollen tube exceeded the grain diameter (Bryhan and Serdar 2009). For each replicate, we randomly selected at least 3 optical fields that contained approximately 50 pollen grains. The germination rate was calculated according to Xiong et al. (2016). For each treatment, the lengths of at least 30 germinated pollen tubes were measured by ImageJ software (National Institutes of Health, USA).

Statistical analysis

We performed a one-way analysis of variance (ANOVA) to test the significance of the 3 factors on the pollen germination rate and tube length. Significant differences among means were assessed using Duncan's multiple comparison at $p \leq 0.05$. Figures were drawn with Origin Pro8.5 software (Origin Laboratory, USA).

RESULTS

Pollen germination rate

The different media components affected pollen germination in *C. henryi*. Significant differences are shown in Tables 2 and 4. The highest percentage of pollen grain germination was found in T5 (66.99%), and the lowest in T4 (13.02%). Germination rates depended on the treatment in the following order: T5 (66.99%) > T9 (45.47%) > T7 (41.56%) > T8 (26.91%) > T2 (23.69%) > T1 (22.78%) > T6 (20.31%) > T3 (19.15%) > T4 (13.02%) (Table 2, Fig. 3). H_3BO_3, $CaCl_2$, and GA_3 were the main factors affecting chinquapin pollen germination rates (Table 3). These results can be compared to the relative order of the R-values: $R_C > R_A > R_B$ (Table 3). Thus, the influence of the 3 factors on chinquapin pollen germination rates occurred in the following order of significance: GA_3 concentration > H_3BO_3 concentration > $CaCl_2$ concentration. The optimal points were A_3, B_2, and C_1, which correspond to H_3BO_3 at 100 mg L^{-1}, $CaCl_2$ at 50 mg L^{-1}, and GA_3 at 20 mg L^{-1}, respectively.

Pollen tube length

When pollen grains were incubated in a germination medium for 48 h in darkness, variations in the pollen tube length were observed among all treatments. There were significant differences in the pollen tube length among the T1 to T9 treatments (Tables 2 and 4). The longest pollen tube was formed in T5, and the shortest in T3; the relative order of lengths among treatments was: T5 (414.00 μm) > T9 (273.65 μm) > T7 (266.38 μm) > T8 (239.67 μm) > T2 (192.10 μm) > T6 (186.52 μm) > T4 (165.08 μm) > T1 (148.60 μm) > T3 (89.03 μm) (Table 2, Fig. 3). H_3BO_3, $CaCl_2$, and GA_3 were the main factors affecting the chinquapin pollen tube length (Table 3), with R-values in the order of $R_A > R_C > R_B$ (Table 3). The relative influence of the 3 factors on the pollen tube length was: H_3BO_3 concentration > GA_3 concentration > $CaCl_2$ concentration (Fig. 2). The optimal points were A_3, B_2, and C_1, similar to those for the pollen germination rate (Fig. 1).

DISCUSSION

Plant growth hormones such as GA_3, indole-3-acetic acid (IAA), and abscisic acid (ABA) influence pollen germination and tube growth (Wu et al. 2008, Maita and Sotomayor 2015). Lower concentrations promote both

Table 3. Range analysis for the pollen germination rate and pollen tube length in *Castanea henryi* HCHJA-1

		(A) H_3BO_3	(B) $CaCl_2$	(C) GA_3
Pollen germination rate (%)	K1	65.62	77.36	135.24
	K2	100.37	117.59	85.61
	K3	113.94	84.98	59.08
	X1	21.87	25.79	45.08
	X2	33.46	39.20	28.54
	X3	37.98	28.33	19.69
	R	16.11	13.41	25.39
Pollen tube length (μm)	K1	429.73	504.01	836.25
	K2	765.60	845.77	645.00
	K3	779.70	549.20	493.78
	X1	143.24	168.00	278.75
	X2	252.20	281.92	215.00
	X3	259.90	183.07	164.59
	R	116.66	113.92	114.16

Note: K_i was obtained by summing the total number of columns corresponding to level i. $\overline{xi}$ is the mean value of Ki (i.e., $K_i/3$), and R is the maximum $\overline{x}_i$ minus the minimum $\overline{x}_j$.

Table 4. Variance analysis for the pollen germination rate and pollen tube length in *Castanea henryi* HCHJA-1

	Factor	SS	DF	*F*	*p*	Significance
Pollen germination rate (%)	H_3BO_3	3,726.034	2	7.654	0.001	*
	$CaCl_2$	2,742.737	2	5.357	0.007	*
	GA_3	8,965.196	2	25.437	< 0.001	*
Pollen tube length (μm)	H_3BO_3	792,594.443	2	28.156	< 0.001	*
	$CaCl_2$	525,482.793	2	17.429	< 0.001	*
	GA_3	589,592.436	2	19.871	< 0.001	*

Note: DF = Degree freedom. *F* value = Mean square of between groups / within groups. The *p*-value indicates a significant difference. SS = Sum of squares between groups. * Significant at the 0.01 level.

pollen germination and pollen tube growth, whereas higher concentrations inhibit these processes (Xiong et al. 2016). Numerous studies have indicated that GA_3 regulates pollen germination rates in many different species (Wu et al. 2008, Chen et al. 2013, Sun et al. 2013). GA_3 plays a significant role in promoting pollen germination in *Armeniaca vulgaris* Shushanggan, Kalayuluke, and Yiliakeyuluke within the range 20~100 mg L^{-1} (Sun et al. 2013). Chen et al. (2013) reported that jujube pollen germination rates increased at GA_3 concentrations of 0~10 mg L^{-1}, and decreased at 10~25 mg L^{-1}. In this study, R-values reflected the degree to which GA_3 influenced germination rates, indicating that the GA_3 concentration was the most important factor for chinquapin. As the concentration increased from 20 mg L^{-1} to 60 mg L^{-1}, germination rates decreased in *C. henryi* HCHJA-1 (Fig. 1). Our results contradict those of a study by Yang et al. (2014), who found that

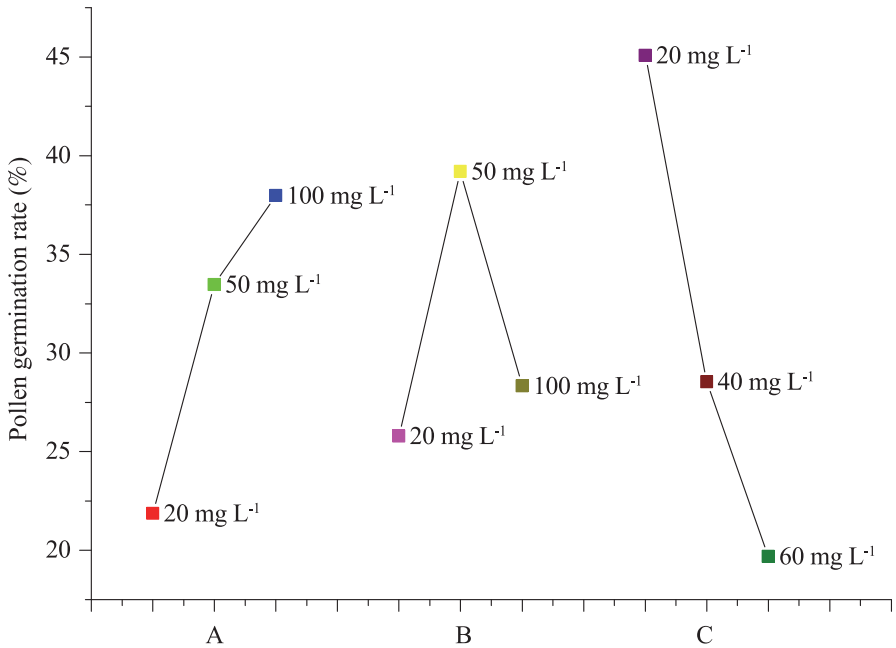

Fig. 1. Influence of 3 factors on the pollen germination rate in *Castanea henryi* HCHJA-1. A: H_3BO_3 concentration; B: $CaCl_2$ concentration; C: GA_3 concentration. Data are based on the values listed in Table 3.

pollen germination rates increased as GA_3 concentrations increased to 60 mg L^{-1}, and then began to decline at higher concentrations in *C. henryi* cv. Bailuzi. Differences in pollen tube length observed in the present study may be a reflection of cultivar variability (Bryhan and Serdar 2008). Thus, the genotype can play an important role in pollen grain germination in chinquapin.

The boron concentration in a suitable range is an essential factor promoting the growth of pollen tubes (Xiong et al. 2016). Previous studies reported that adding boron increases the pollen germination rate. Suitable concentrations of boron promote pollen germination and pollen tube growth in many species (Acar et al. 2010). Chen et al. (2013) found that the addition of 15 mg L^{-1} boric acid to the medium increased pollen tube growth in jujube. Lee et al. (2009) observed that within a range of 0~300 mg L^{-1}, the boric acid concentration was positively correlated with pollen germination and pollen tube growth in pear (*Pyrus pyrifolia*). In our study, R-values indicated that the boron concentration played the second most important role in determining *Castanea* pollen germination rates and was the most significant factor affecting pollen tube growth. From 20 to 100 mg L^{-1}, as the concentration increased, the germination rate increased. This result was similar to that of Yang et al. (2014).

Calcium (Ca^{2+}) is an indispensable element affecting pollen germination and pollen tube growth; it is beneficial to the transportation of assimilates and is directly involved in the growth and regulation of pollen tubes. Higher Ca^{2+} concentrations will cause pollen tube growth to be inhibited or completely stopped (Brewbaker and Kwack 1963). Xiong et al. (2016) found that when Ca^{2+} concentrations ranged from 4×10^{-4} to 1.2×10^{-3} mol L^{-1}, feijoa germination rates and pollen tube length decreased. These values were still

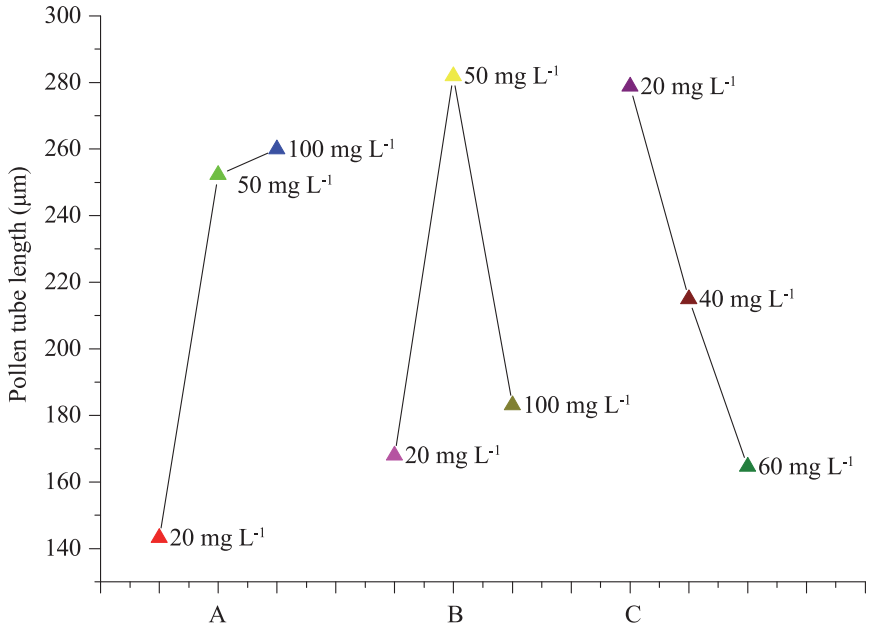

Fig. 2. Influence of 3 factors on the pollen tube length in *Castanea henryi* HCHJA-1. A: H_3BO_3 concentration; B: $CaCl_2$ concentration; C: GA_3 concentration. Data are based on the values listed in Table 3.

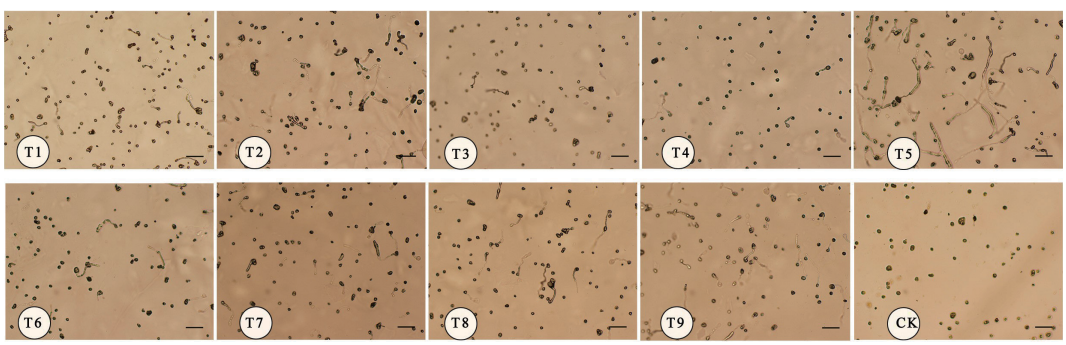

Fig. 3. Pollen germination and pollen tube length in *Castanea henryi* HCHJA-1 after incubation for 48 h. For treatment symbol, T1~CK, refer to Table 2. Scale bars: T1~CK=200 μm.

higher than those of the control group, meaning that Ca^{2+} can promote pollen germination and pollen tube growth. Tang et al. (2013b) also reported that pollen germination and tube growth were enhanced as Ca^{2+} concentrations increased to 50 mg L^{-1}, and then decreased at higher concentrations in *C. henryi* cv. Tiezhen. In our study, from 20 to 100 mg L^{-1}, germination rates increased at first, and then decreased (Fig. 1). The $CaCl_2$ concentration was the third influential factor in the present study. Our results are in general agreement with those of Tang et al. (2013b), who found that the optimal $CaCl_2$ concentration for pollen germination and pollen tube length was 50 mg L^{-1}. Our results may provide useful information to facilitate chinquapin pollen quality assessment and germination capacity.

CONCLUSIONS

Pollen grain performance, including the pollen grain germination and tube growth rates, is an important component of successful chestnut tree fertilization. In this paper, we studied 3 factors that affect *Castanea* pollen vigor and found that each significantly influenced pollen germination and pollen tube length to a different extent. Pollen germination responded as follows, in order of significance: GA_3 concentration > H_3BO_3 concentration > $CaCl_2$ concentration; pollen tube length showed the following: H_3BO_3 concentration > GA_3 concentration > $CaCl_2$ concentration. Optimal conditions for promoting both pollen germination and pollen tube length were 100 mg L^{-1} H_3BO_3, 50 mg L^{-1} $CaCl_2$, and 20 mg L^{-1} GA_3. These results provide important information on pollen grain germination and tube growth in this chinquapin cultivar, which may be useful for foliar application during flowering and may potentially improve nut yields.

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LITERATURE CITED

Acar I, Ak BE, Sarpkaya K. 2010. Effects of boron and gibberellic acid on in vitro pollen germination of pistachio (*Pistacia vera* L.). Afr J Biotechnol 32(9):5126-30.

Anagnostakis SL. 2010. Chinese Chinquapin *Castanea henryi*. Arnoldia 68(2):61-2.

Bal U, Abak K. 2005. Effects of sucrose, maltose, pH and phloroglucinol on the germination of globe artichoke pollen in vitro. Eur J Hort Sci 70(3):S142-8.

Bounous G, Marinoni DT. 2005. Chestnut: botany, horticulture, and utilization. Hort Rev 31:291-347.

Bounous G, Paglietta R, Peano C. 1992. Methods for observing chestnut pollen viability, germinability and pollen tube growth. Proceedings of the International Chestnut Conference. Morgantown, West Virginia, USA. p 76-9.

Brewbaker JL, Kwack BH. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. Am J Bot 50:859-65.

Bryhan N, Serdar U. 2008. Assessment of pollen viability and germinability in some European chestnut genotypes (*Castanea staiva* L.). Hort Sci (Prague) 35(4):171-8.

Bryhan N, Serdar U. 2009. In vitro pollen germination and tube growth of some European chestnut genotypes (*Castanea sativa* Mill.). Fruits 64(3):157-65.

Chen W, Yuan D, Zhang R, Han Z, Fan X. 2013. Effects of boric acid and plant growth regulators on pollen germination in *Zizyphus jujuba* Mill. Acta Agric Univ. Jiangxiensis 35(3):496-501. [in Chinese with English abstract].

Fan X, Yuan D, Tang J, Tian X, Zhang L, Zou F, Tan X. 2015. Sporogenesis and gametogenesis in Chinese chinquapin (*Castanea henryi* (Skam) Rehder & Wilson) and their systematic implications. Trees 29:1713-23.

Fernando DD, Richards JL, Kikkert JR. 2006. In vitro germination and transient GFP expression of American chestnut (*Castanea dentata*) pollen. Plant Cell Rep 25:450-6.

Guo S and Xie P. 2013. Effects of exogenous substance on starch synthesis and related enzymes activity in Chinese chestnut. Sci Silv Sin 49(4):135-40. [in Chinese with English abstract].

Huang Y, Wang Q, Guo Y, Cheng D, Li S, Duan P. 2013. Effects of five foliar fertilizers on yield and quantity of Chinese chestnut. Nonwood For Res 31(3):143-5. [in Chinese with English abstract].

Lai J, Gong B, Jiang X, Zhang J, Hu Q, Wu

C, Wu Q. 2017. Study on germination rate and viability of pollen for *Castanea henryi*. J. SW For Univ. 37(1):42-8. [in Chinese with English abstract].

Lee SH, Kim WS, Han TH. 2009. Effects of post-harvest foliar boron and calcium applications on subsequent season's pollen germination and pollen tube growth of pear (*Pyrus pyrifolia*). Sci Hort 122(1):77-82.

Li G, Yu S, Yang XS, Chen QF. 2011. Study on extraction technology for chlorogenic acid from sweet potato leaves by orthogonal design. Proc Environ Sci 8:403-7.

Maita S, Sotomayor C. 2015. The effect of three plant bioregulators on pollen germination, pollen tube growth and fruit set in almond [*Prunus dulcis* (Mill.) D.A. Webb] cvs. Non Pareil and Carmel. Electr J Biotechnol 18:381-6.

Muengkaew R, Chaiprasart P, Wongsawad P. 2016. Calcium-boron addition promotes pollen germination and fruit set of mango. Int J Fruit Sci 17(2):147-58.

Nyomora AMS, Brown PH, Pinney K, Polito VS. 2000. Foliar application of boron to almond trees affects pollen quality. J Am Soc Hort Sci 125(2):265-70.

Okamoto G, Miura K. 2005. Effect of prebloom GA application on pollen tube growth in cv. Delaware grape pistils. Vitis 44(4):157-9.

Ranil RHG, Niran HML, Plazas M, Fonseka RM, Fonseka HH, Vilanova S, et al. 2015. Improving seed germination of the eggplant rootstock *Solanum torvum* by testing multiple factors using an orthogonal array design. Sci Horticult 193:174-81.

Shao C. 2004. Probability and mathematical statistics. Beijing: China Forestry Publishing. Chapter 10, Experimental Design; p 305-19.

Sun H, Liao K, An X, Cheng X, Liu J, Qiu C. 2013. Influence of plant growth regulators on pollen germination and growth of Xinjiang apricots. Agric Sci Technol 14(2):262-8.

Sulusoglu M, Aysun C. 2014. In vitro pollen viability and pollen germination in cherry laurel (*Prunus laurocerasus* L.). Sci World J 657123. http://dx.doi.org/10.1155/2014/657123.

Tang J, Yuan D, Fan X, Yang F, Chen W. 2013a. The influence of nutrient elements on pollen germination percentage of *Castanea henryi*. Acta Agric Univ. Jiangxiensis 35(4):782-5. [in Chinese with English abstract].

Tang J, Yuan D, Fan X, Yang F, Gao W, Yang Y. 2013b. Inflorescence traits and pollen germination characteristics of chinquapin. J Fujian Agric Fore Univ. (Natural Science Edition) 42(5):503-7. [in Chinese with English abstract].

Wu J, Yuan Q, Zhao J. 2008. Pollen tube growth is affected by exogenous hormones and correlated with hormone changes in styles in *Torenia fournieri* L. Plant Growth Regulat 55(2):137-48.

Wang L, Yuan D, Shen G, Tian S, Sun X. 2014. Effects of spraying boron fertilizer at flowering stage on photosnthetic characteristics and fruit-setting rate in *Castanea mollissima*. Nonwood For Res 32(2):58-61. [in Chinese with English abstract].

Xia P, Guo H, Zhao H, Jiao J, Deyholos M, Yan X, et al. 2016. Optimal fertilizer application for *Panax notoginseng* and effect of soil water on root rot disease and saponin contents. J Ginseng Res 40:38-46.

Xiang H, Yuan D, Fan X, Zhu Z, Jia B. 2016. The analysis of genetic diversity of *Castanea henryi* by using SRAP. J Plant Gene Resources 17(6):1072-81. [in Chinese with English abstract].

Xiong H, Zou F, Yuan D, Zhang X, Tan X. 2016. Orthogonal test design for optimizing the culture medium for in vitro pollen germination of feijoa (*Acca sellowiana* cv. Unique). New Zealand J Crop Horti Sci 44(3):192-202.

Xu J. 2005. The effect of low-temperature storage on the activity of polyphenol oxidase

in *Castanea henryi* chestnuts. Postharvest Biol Technol 38:91-8.

Yang F, Yuan D, Fan X, Tang J, Chen W, Gao W. 2014. Effects of H_3BO_3 and plant growth regulators on pollen germination rate in *Castanea henryi*. Nonwood For Res 32(1):70-5. [in Chinese with English abstract].

Yang J, Liu X, Wang A, Xie P. 2002. Several plant growth regulating agent effects to young *Castanea mollissima* growth and seed. Econ For Rese 20(4):42-4. [in Chinese with English abstract].

Zhang X, Yuan D, Zou F, Fan X, Tang J, Zhu Z. 2016. A study on the Xenia Effect in *Castanea henryi*. Horti Plant J 2(6):301-8.

Zheng C, Pan D, Zhang D, Wu X, Lv J, Yu X, Wu F, Zheng J. 2009. Studies on regularities of growth and development of chinquapin (*Castanea henryi*) fruits. Acta Hort 844:153-61.

Zheng C, Yu X, Wang X, Shen W. 2004. A study on the viability of the pollen of *Castanea henryi* Rehd et Wils. Acta Agric Univ. Jiangxiensis 26(2):200-2. [in Chinese with English abstract].

Zhu C, Liu Q, Zhao L, Ju G, Wang R.1992. The effects of PGR on the growth, flowering and seed yield of Chinese chestnut. For Res, 5(3):311-6. [in Chinese with English abstract].

Zou F, Guo S, Xie P, Lv W, Xiong H, Li G. 2013. Sporogenesis and gametophyte development in the Chinese chestnut (*Castanea mollissima* Blume). Taiwan J For Sci 28(4):171-84.