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CELLULAR AND MOLECULAR BIOLOGY

Orthogonal test design for optimizing culture medium for *in vitro* pollen germination of interspecific oil tea hybrids

RUI ZHAO, XIAO HU, DEYI YUAN, JOSEPH MASABNI, HUAN XIONG & FENG ZOU

Abstract: Oil Tea (*Camellia oleifera*) is an important woody edible oil plant in China. Oil Tea suffers from low rate of fruit set during production, which is related to poor pollination and fertilization. Pollen vigor is directly related to pollination and fertilization. Using the interspecific hybrid Y3 (*C. grijsii* × *C. oleifera*) as plant material, we studied the effects of sucrose, H_3BO_3, $MgSO_4$, and IAA on pollen germination using an orthogonal design to determine the best culture medium. Results indicated that pollen germination rates were significantly affected by medium components and ranged from 29.13% to 56.84%. Pollen tube length was the longest in the T5 medium surpassing the control group by 489.36 µm. $MgSO_4$ turned out to be the most important germination medium component having great effect on the pollen germination rate. The optimal culture medium to promote pollen tube growth of Oil Tea Y3 was: 1% agar, 150 $g{\cdot}L^{-1}$ sucrose, 0.15 $g{\cdot}L^{-1}$ H_3BO_3, 0.07 $g{\cdot}L^{-1}$ $MgSO_4$, and 0.01 $g{\cdot}L^{-1}$ IAA. The results of this paper may provide information for foliar application of Mg and IAA, which can improve pollen tube growth of Oil Tea in practice.

Key words: Oil Tea, pollen germination rate, pollen tube growth, Mg, IAA.

INTRODUCTION

Camellia oleifera is a small evergreen tree species that belongs to the genus *Camellia* in the Theaceae family and produces edible oil from its seed (Xiong et al. 2019a). Tea oil is rich in unsaturated fatty acids and vitamin E and is known throughout the world as the "Oriental Olive Oil" (Gao et al. 2018). At present, the cultivated area of *C. oleifera* in China has exceeded 4.26 million hectares in 2016, but the production of tea oil is only 0.50 million tons (Qin et al. 2018). *C. oleifera* has self-incompatibility, so two or more are required for good pollination (Liao et al. 2014, Gao et al. 2015). Effective pollination mainly depends on good pollen vigor, which depends on the rate of pollen germination and the growth rate of pollen tubes (Ottaviano & Mulcahy 1989). Weak pollen vigor usually leads to lack of pollen germination or poor growth of pollen tubes, which are ineffective for fertilization (Xiong et al. 2016). Poor fertilization is responsible for fruit abortion, which severely impact yields of *C. oleifera* (Gao et al. 2017). Many reports have indicated that pollen vigor is one of the most important factors for the limitation of sexual reproduction of *C. oleifera* (Souza et al. 2017) resulting in low yields (Yuan et al. 2010). Therefore, an *in vitro* germination medium is helpful to study the reproduction process of *C. oleifera* in detail, which can also contribute to better understand and maybe improve the cultivation of Camellia.

Pollen performance has an important role in pollination, and pollen vigor and germination rate are vital factors for pollination and fertilization success (Melekber & Aysun 2014, Sunilkumar et al. 2013). Pollen vigor is influenced by many factors, including species, temperature and storage time, especially culture medium (Stanley & Linskens 1974, Franzon et al. 2005). Therefore, understanding the factors affecting pollen vigor could help with implementing management measures to improve fertilization and fruit yield (De-oliveira et al. 2016). *In vitro* pollen germination and tube growth are affected by nutrients and plant growth regulators (Maita et al. 2015, Muengkaew et al. 2016). Proper application of nutrients and regulators is an effective method to promote pollen germination and tube growth (Lin et al. 2017, Naik et al. 2016, Radovic et al. 2016), and is also beneficial for fruit production (Gao et al. 2012). Nevertheless, only a few studies have examined the effects of nutrients and regulators application on the promotion of pollen germination and on the increase of the rate of fruit set of *C. oleifera* (Gao et al. 2012, Tan et al. 2010, Yuan et al. 2010).

Therefore, in this study, a 10-treatment orthogonal experiment was designed to determine the optimal levels of various nutrients, and consequently develop the best culture medium for pollen germination and tube growth of hybrid clone Y3 (*C. grijsii* × *C. oleifera*).

MATERIALS AND METHODS

Plant materials

Camellia grijsii is one of the most important oil plants in the Theaceae family (Weng 1997), and is distributed in Hunan, Jiangsu, Fujian, Guizhou, Shanxi, Hubei, and Guangxi provinces (Weng 1997, Zou et al. 2013). It is a high-quality cultivated species of *Camellia* with a thick crown, thin peel, strong drought resistance, and high economic value (Lin & Hu 1981). *C. grijsii* flowers from February to April. Its fruit capsules are highly resistant to anthracnose disease (*Colletotrichum camelliae* Massee), which causes fruit capsules of *C. oleifera* to fall off prematurely (Xiong et al. 2019b). The fruit contains edible oil which is conducive to medicinal and nutritional use (Weng 1997).

Camellia oleifera 'Huashuo' is a new large-fruit and high-yield cultivar bred from a *C. oleifera* seedling in 2009, with an average fruit weight of 68.75 g, and a maximum fruit weight of 99.20 g. *C. oleifera* 'Huashuo' blooms from October to December. This new variety has the characteristics of a large fruit, high yield, and high photosynthetic efficiency (Tan et al. 2011).

In early March 2011, we hybridized pollen of *C. oleifera* 'Huashuo' (male parent) with *C. grijsii* (female parent) and harvested several hundred hybrid seeds in autumn. March 2012, we planted the hybrid seeds at the *Camellia* nursery of Central South University of Forestry and Technology. In 2016, the young trees started blooming. We screened for superior Y3 individuals from the F1 generation. We collected uncracked anthers of Y3 in mid-November, 2018 and stored them for two days in a refrigerator at 4 °C before testing.

Experimental design

We used an orthogonal design to optimize the culture medium for pollen germination of *C. oleifera* Y3. The number "4" in the design table indicates the four factors used, namely [A] sucrose, [B] H_3BO_3, [C] $MgSO_4$, and [D] IAA. The number "3" represents the three optimization levels or rates, and the number "9" represents nine pollen treatments from T1 to T9. The control group (CK) consisted of culturing pollen on a medium containing 1% agar. Tables I and II provide additional information of the test parameters.

Table I. Orthogonal experimental design, with 4 factors and 3 levels.

Level	Factor ($g \cdot L^{-1}$)			
	(A) Sucrose	(B) H_3BO_3	(C) $MgSO_4$	(D) IAA
1	100	0.10	0.03	0.01
2	150	0.15	0.05	0.02
3	200	0.20	0.07	0.03

Note: The numbers "1 ~ 3" indicate three levels. The factors affecting pollen viability are the concentrations of [A] Sucrose, [B] H_3BO_3, [C] $MgSO_4$ and [D] IAA.

Pollen culture

We weighed the reagents and labelled them in sequence and dissolved them by adding quantified distilled water. In order to sufficiently dissolve the agar and its content, we heated the mixture in a microwave oven, then cooled it at room temperature. After cooling the medium, we dropped the medium onto a microscope slide using a dropper. When the medium became semi-solid, we used brushes to scatter pollen on the surface of the medium (Xiong et al. 2016). We placed the microscope slides in a culture dish with wet filter paper and incubated them in a constant temperature incubator at 25 °C for 2 h in the dark. Each treatment consisted of 3 replicates.

Measurements

Pollen germination was monitored using a BX-53 microscope (Olympus, Tokyo, Japan). For each sample (slide), we randomly selected 5 optical fields containing no less than 50 pollen grains each (Tan et al. 2010, Yuan et al. 2010). We then counted the number of germinated pollen grains to determine the germination rate. The standard for pollen germination is that the length of pollen tube must exceed its diameter (Bryhan & Serdar 2008). Pollen germination rate (%) is equal to the number of germinated pollen grains divided by the total number of pollen grains (Tan et al. 2010). Pollen tube length was measured using the image processing software Image J (National Institutes of Health, Bethesda, USA). For each treatment, the length of 60 germinated pollen tubes were measured and used to calculate the average length of pollen tube.

Statistical analysis

We used SPSS 19.0 software (IBM company, New York, USA) to analyze the data, and to test the effects of four media on pollen germination rate and pollen tube length using a one-way analysis of variance (ANOVA). Significant differences among means were assessed using Duncan's multiple comparison at $p \leq 0.05$. Figures were drawn using Origin Pro8.5 software (Origin Lab company, Northampton, USA).

RESULTS

Pollen germination rate

Compared to the control media (CK), pollen germination rate was significantly higher in all tested treatment combinations. Germination rate was highest in T7 at 56.84%, which was close to that of T1 (54.59%), T5 (51.85%) and T6 (52.46%). Compared with the germination rate of other three factors, there was relatively low statistical difference between the germination rate of T1 (low sugar concentration) and T4, T5, T6 (medium sugar concentration) or T7 (high sugar concentration), which indicates that the effect

Table II. Orthogonal test on pollen germination rate and pollen tube length of hybrid clone Y3 of *Camellia oleifera.*

Treatment	Factor ($g \cdot L^{-1}$)				Pollen germination rate (%)	Pollen tube length (μm)
	A Sucrose	B H_3BO_3	C $MgSO_4$	D IAA		
CK	0.00	0.00	0.00	0.00	12.10d	95.53d
T1	100	0.10	0.03	0.01	54.59ab	403.84bc
T2	100	0.15	0.05	0.02	41.00b	215.36c
T3	100	0.20	0.07	0.03	31.28bc	172.90cd
T4	150	0.10	0.05	0.03	42.39ab	383.90bc
T5	150	0.15	0.07	0.01	51.85ab	584.89a
T6	150	0.20	0.03	0.02	52.46ab	561.73ab
T7	200	0.15	0.03	0.02	56.84a	560.76ab
T8	200	0.20	0.05	0.03	29.13bc	218.04c
T9	200	0.10	0.07	0.01	32.37bc	420.50b

Note: Pollen germination rate and pollen tube length were measured after 2 hours of culture. Within columns, different letters indicate significant differences at P<0.05.

of sucrose on pollen germination rate is a little similar under these three concentrations (Table IV). The decreasing order of the effect of each treatment on promoting pollen germination of hybrid clone Y3 was: T7 (56.84%) > T1 (54.59%) > T6 (52.46%) > T5 (51.85%) > T4 (42.39%) > T2 (41.00%) > T9 (32.37%) > T3 (31.28%) > T8 (29.13%) > CK (12.10%) (Table II, Figure 3). When compared to the relative order of the R-values (R_C > R_D > R_B > R_A, Table III), we found that the influence of the 4 factors on hybrid clone Y3 of *C. oleifera* pollen germination rates occurred in the following order: $MgSO_4$ > IAA > H_3BO_3 > sucrose concentration. The ideal culture concentration for pollen germination rate was sucrose at $200g \cdot L^{-1}$, H_3BO_3 at $0.15g \cdot L^{-1}$, $MgSO_4$ at $0.03g \cdot L^{-1}$and IAA at $0.02g \cdot L^{-1}$, respectively. Interestingly, in terms of its effect on germination, medium T1 was almost as good as medium T7 (only 2% lower pollen germination), even though its recipe contains half the amount of sucrose and IAA. Thus, T1 is more economically friendly.

Pollen tube length

According to Table II, pollen tube length was longest in T5 (584.89 μm), and shortest in T3 (172.90 μm). The order of effect of each treatment on promoting pollen tube growth was T5 (584.89 μm) > T6 (561.73 μm) > T7 (560.76 μm) > T9 (420.50 μm) > T1 (403.84 μm) > T4 (383.90 μm) > T8 (218.04 μm) > T2 (215.36 μm) > T3 (172.90 μm) > CK (95. 53μm) (Table II, Figure 3). Among these treatments, T5 was the best medium for promoting pollen tube growth of hybrid clone Y3 in this experiment. The nutrient elements and proportion for T5 are sucrose $150g \cdot L^{-1}$, H_3BO_3 $0.15g \cdot L^{-1}$, $MgSO_4$ $0.07g \cdot L^{-1}$, and IAA $0.01g \cdot L^{-1}$. Sucrose, H_3BO_3, $MgSO_4$ and IAA were the main

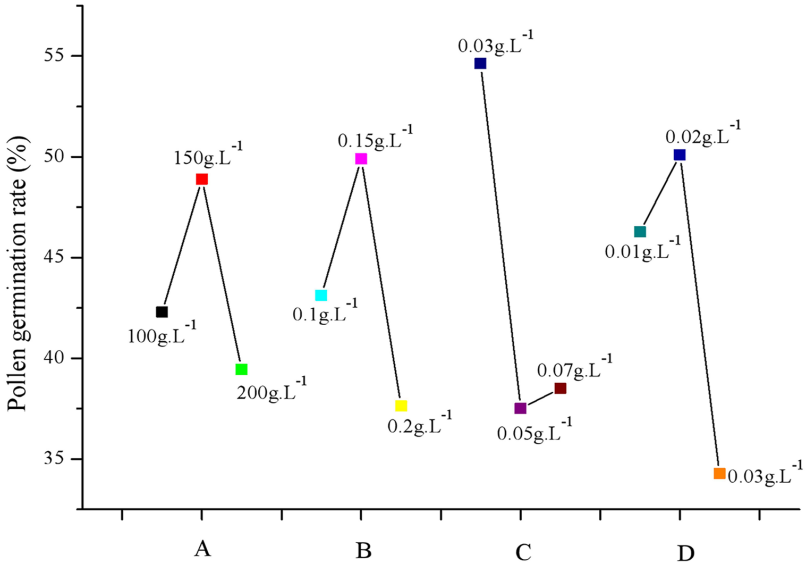

Figure 1. Influence of 4 factors on pollen germination rate in *Camellia oleifera* hybrid clone Y3. A: sucrose concentration; B: H_3BO_3 concentration; C: $MgSO_4$ concentration; D: IAA concentration. Data are based on values listed in Table II.

factors affecting the hybrid clone Y3 pollen tube growth (Table III), with R-values in the order of RA > RC > RD > RB (Table III). The influence of the 4 factors on the pollen tube length was: sucrose > $MgSO_4$ > IAA > H_3BO_3 concentration.

DISCUSSION

Pollen germination is influenced by several factors including nutrition (Imani & Talaie 2006, Bal & Ubak 2005, Maita & Sotomayor 2015, Muengkaew et al. 2016) and plant growth regulators (Kovaleva et al. 2005, Wu et al. 2008). Nutrients, such as sucrose, boron, and magnesium have obvious effects on pollen growth (Chen et al. 2009, Liu et al. 2013, Naik et al. 2016). Sucrose plays an extremely important role and is a significant nutrient for pollen germination (Fei & Nelson 2003, Liu et al. 2013). It helps to protect membranes and preserve protein structure from desiccation, because pollen grains lose water rapidly after release (Kosel et al. 2018). Huang et al. (2011) reported that pollen germination of *C. oleifera* was best when sucrose concentration was 150 g·L^{-1} and began to decrease at 200 g·L^{-1}. The optimum sucrose concentration range for promoting pollen germination is between 100 g·L^{-1} and 150 g·L^{-1} . Similar results were observed in our study, adding 100-150 g·L^{-1} sucrose significantly promoted pollen germination, and was the best at a concentration of 150g·L^{-1}.

Boron plays an important role in stimulating the growth of pollen grains and pollen tubes (Izzet et al. 2010, Lee et al. 2009, Nyomora et al. 2000). Yuan et al. (2010) found that pollen germination of *C. oleifera* was significantly promoted at boric acid concentrations of 0.02-0.10 g·L^{-1} and was best at 0.10 g·L^{-1}. Tanmoy et al. (2018) reported that boric acid concentration of 0.8 g·L^{-1} was a suitable medium for guava pollen germination and pollen tube growth. Lee et al. (2009) stated that the germination rate and tube growth of pear were highest at a boron concentration of 0.2 g·L^{-1}. Our results were similar to those of a study by Jiang et al. (2010), who found that boric acid promoted pollen germination availably within the range of 0.10 g·L^{-1} to 0.15 g·L^{-1}.

Magnesium has been found to play an important role in pollen germination (Chen et al. 2009). Magnesium transport is significantly related to pollen homeostasis and germination

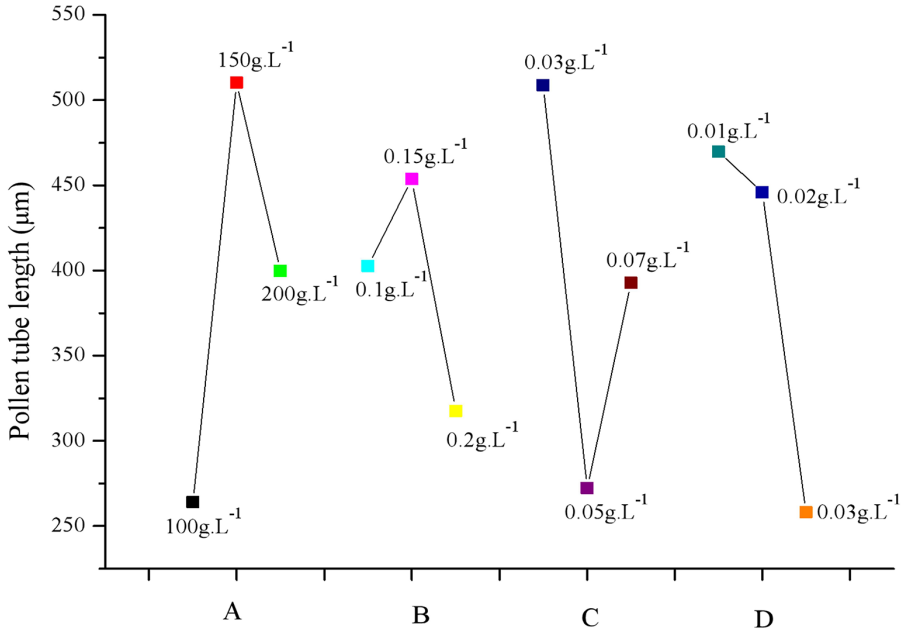

Figure 2. Influence of 4 factors on pollen tube length in *Camellia oleifera* hybrid clone Y3. A: sucrose concentration; B: H_3BO_3 concentration; C: $MgSO_4$ concentration; D: IAA concentration. Data are based on values listed in Table II.

(Chen et al. 2009). Yuan et al. (2010) found that as the concentration of magnesium increased from 0.02 to 0.05 g·L^{-1}, pollen germination was promoted in *C. oleifera*. Han et al. (2014) reported that magnesium inhibited pollen germination and pollen tube growth in *Ziziphus jujuba* to some extent, and the degree of inhibition decreased first and then increased with the increase in concentration. When the magnesium concentration was in the range of 0.01 to 0.05 g·L^{-1}, it promoted both pollen germination and pollen tube growth. According to our results, magnesium played the important role in pollen development of *C. oleifera* Y3 and the best concentration for pollen grain germination was 0.03 g·L^{-1}. Similar results were obtained by Yuan et al. (2010) and Han et al. (2014). However, Jiang et al. (2010) observed that magnesium had no significant effect on pollen germination in *Clivia miniata*. The reason for this difference may be due to variety and genotype differences of plant material.

In general, with the increase in the concentration of growth regulators, the germination rate of pollen *in vitro* will fluctuate (Gokbayraka & Engin 2016). IAA, a plant growth regulator, could promote pollen germination at low concentrations, but can inhibit growth at higher concentrations (Kovaleva et al. 2005). It has also been suggested that IAA at appropriate concentrations promotes pollen tube growth (Abdelgadir et al. 2012, Tian et al. 1996). Tian et al. (1996) reported that 0.005 g·L^{-1} IAA significantly promoted pollen tube growth, while 0.10 g·L^{-1} IAA completely inhibited pollen germination. Tan et al. (2010) observed that pollen germination rate was highest when the concentration of IAA was 0.005 g·L^{-1}. From their research, IAA promoted pollen germination in the range of 0.01 to 0.02 g·L^{-1}. In our study, 0.01 to 0.02 g·L^{-1} of IAA increased the germination rate. This result was agreement with that of Tan et al. (2010). Thus, our results may provide a reference for pollen germination and pollen tube growth in *C. oleifera*.

CONCLUSIONS

Pollen grain performance, including pollen grain germination and tube growth rate, is an important component of successful pollination

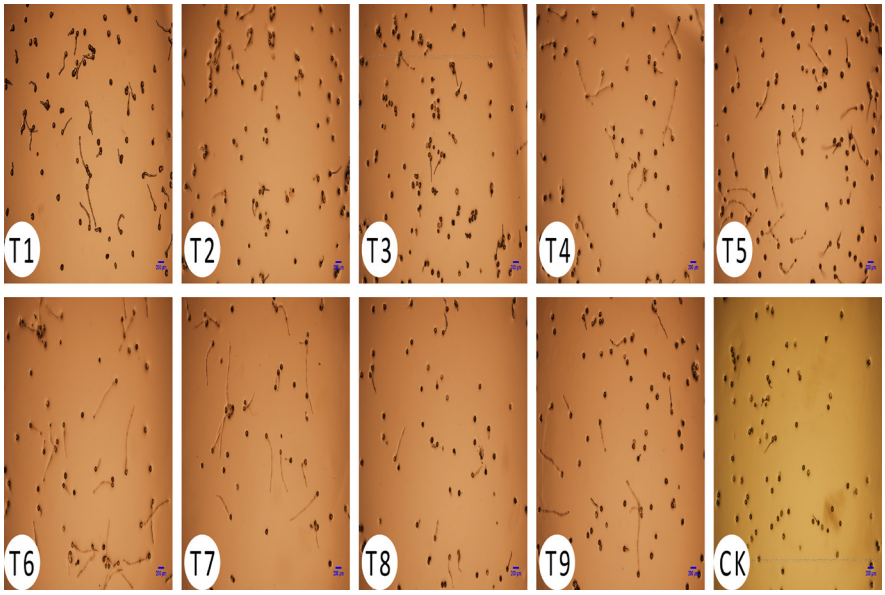

Figure 3. Pollen germination and pollen tube length after incubation for 2 h in *Camellia oleifera* hybrid clone Y3. For symbol T1~CK, represents different treatments of pollen, refer to Table II.

Table III. Range analysis for pollen germination rate and pollen tube length in *Camellia oleifera* hybrid clone Y3.

		(A) sucrose	(B) H_3BO_3	(C) $MgSO_4$	(D) IAA
Pollen germination rate (%)	K1	126.87	129.35	163.89	138.81
	K2	146.70	149.69	112.52	150.30
	K3	118.34	112.87	115.50	102.80
	X1	42.29	43.12	54.63	46.27
	X2	48.90	49.90	37.51	50.10
	X3	39.45	37.62	38.50	34.27
	R	9.45	12.28	17.12	15.83
Pollen tube length (μm)	K1	792.10	1208.24	1526.33	1409.23
	K2	1530.52	1361.01	817.30	1337.85
	K3	1199.30	952.67	1178.29	774.84
	X1	264.03	402.75	508.78	469.74
	X2	510.17	453.67	272.43	445.95
	X3	399.77	317.56	392.76	258.28
	R	246.14	136.11	236.35	211.46

Note: Ki was obtained by summing the total number of columns corresponding to level i, that is, the sum of pollen germination rate or pollen tube length corresponding to three concentration levels of each factor. Xi is the mean value of Ki (i.e., Ki/3), and R is the maximum Xi minus the minimum Xi.

Table IV. Variance analysis for the pollen germination rate and pollen tube length in *Camellia oleifera* hybrid clone Y3.

	Factor	SS	DF	*F*	*p*	Significance
Pollen germination rate(%)	sucrose	3094.022	3	7.941	0.001	*
	H_3BO_3	3350.616	3	9.307	< 0.001	*
	$MgSO_4$	4334.112	3	17.581	< 0.001	*
	IAA	3910.728	3	13.389	< 0.001	*
Pollen tube length (μm)	sucrose	1823941.580	2	18.414	< 0.001	*
	H_3BO_3	567523.617	2	5.011	0.008	*
	$MgSO_4$	1675928.678	2	16.639	< 0.001	*
	IAA	1610034.936	2	15.868	< 0.001	*

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

and fertilization for *C. oleifera*. In this paper, pollen tube length was affected by added components in the following decreasing order of effect: sucrose > $MgSO_4$ > IAA > H_3BO_3. The optimum medium for pollen tube growth was 1% agar, 150 g·L^{-1} sucrose, 0.15g·L^{-1} H_3BO_3, 0.07 g·L^{-1} $MgSO_4$, and 0.01 g·L^{-1} IAA. These results may provide useful information for promoting pollen tube growth of *C. oleifera* in practice.

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Zhao Rui, Xiong Huan and Zou Feng conceived the experiment and developed the experimental design. Hu Xiao provided the oil tea materials. Yuan Deyi secured funding for the research and was responsible for overall project administration. Zhao Rui, Zou Feng and Joseph Masabni contributed to the preparation of the manuscript. All authors have read and agreed to the published version of the manuscript.