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Effects of different wavelengths of LED light on pollen germination and direction of pollen tube elongation in *Cyrtanthus mackenii*

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Key words: Cyrtanthus mackenii, direction of pollen tube elongation, germination rate, light emitting diode, light wavelength.

Abstract: The effects of different light wavelengths on pollen germination and determination of the direction of pollen tube elongation of *Cyrtanthus mackenii* was examined *in vitro* using light-emitting diodes (LED) with five peak wavelengths: 405 nm (violet), 465 nm (blue), 630 nm (orange), 660 nm (red), and 735 nm (far-red). Pollen grains were cultured on a medium solidified with agar, and maintained at 21°C in an incubator under 3-4 h of continuous lighting or darkness. Neither the pollen germinated under LED lighting and dark conditions nor the rate of pollen germination differed among light conditions, including darkness. However, pollen tubes elongated less in the direction toward light under LED, and elongated with no directional trend in darkness. Moreover, pollen tubes did not elongate toward far-red LEDs. These results suggest that light exerts effects on the factors determining the direction of pollen tube elongation, but not on those controlling pollen germination.

1. Introduction

Light is important for plants, not only as an energy source but also as an environmental signal. Plants respond appropriately to light in their environment, as in seed germination and seedling growth (Godo *et al*., 2011), floral transition (Cerny *et al*., 2003), phototropism (Palmer *et al*., 1993), and so on. Light wavelength is also involved in cell elongation (Braidwood *et al*., 2014).

Pollen tube elongation is of one of the fastest growing plant cell types. The mechanism of pollen tube growth is a multi-stepped one, and it is co-regulated by a variety of essential cellular processes, including exocytosis, actin cytoskeleton organization and activity, calcium and proton physiology, and cellular energetics (Hepler *et al*., 2013). The main factor that attracts the growth tip of the pollen tube is considered to be chemo-attractants from the female gametophyte (Higashiyama and Hamamura, 2008).

In vitro, the effects of light wavelength on pollen germination or pollen tube elongation have been studied in *Pinus roxburghii* (Dhawan and Malik, 1981): pollen tube growth decreased in white light compared to the dark, and increased in red light, although this was counteracted by far red light. In a study of two cultivars of wheat × maize crosses, pollen tube growth was significantly affected by light intensity in one cultivar but not in the other (Campbell *et al*., 2001). Furthermore, the directional growth of pollen tubes of *Nicotiana alata* occurred in cultures incubated in the dark as well as in the light (Lush *et al*., 1998). These results highlight the effects of different wavelengths of light on pollen germination and pollen tube elongation, which vary between species, while few studies have described these effects in detail.

The aim of this study was to verify the effect of different wavelengths of light-emitting diode (LED) light on pollen germination and direction of pollen tube elongation in *Cyrtanthus mackenii* using an *in vitro* experimental system for pollen germination developed by Hirano and Hoshino (2010).

2. Materials and Methods

Plant materials and pollen culture

Mature pollen with anthers that showed dehiscence was collected from *C. mackenii* Hook.f. (Amaryllidaceae) and stored at -20°C. Pollen culture was performed using 1% (w/v) agar, medium that contained 0.01% (w/v) $CaCl_2$, 0.01% (w/v) H_3BO_3, 0.0007% (w/v) KH_2PO_4, 10% (w/v) sucrose, and 0.01% (w/v) yeast extract at pH 5.8 (Hirano and Hoshino, 2009, 2010). The culture medium was sterilized by autoclaving at 121°C for 15 min.

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The effect of light condition on pollen germination rate

Five spots of culture medium with a diameter of 1 cm were put on a glass slide at *ca*. 5 mm intervals. A tiny amount of frozen pollen grains were sown on each coagulated spot of medium using a paintbrush. Two or three prepared slides were horizontally positioned in a petri dish, which was kept at high humidity (Fig. 1A), with toothpicks between the slides to avoid overlapping. Samples were then cultured at 21°C in an incubator (BIOTRON LPH200, NK system, Japan) for 3-4 h under different light conditions. The incubator was divided into five rooms fitted with overhead LED panels (40×10 cm) fabricated using 2,800 indicator-type LEDs of 3 mm diameter (Fujiwara *et al*., 2011; Yano and Fujiwara, 2012). The LEDs were of five types: violet (L405R-36; Epitex Inc., Kyoto, Japan), blue (L460-36; Epitex Inc., Kyoto, Japan), orange-red (L630-36; Epitex Inc., Kyoto, Japan), red (SRK3-3A80-LE; Toricon Co., Shimane, Japan), and far-red (L735-36AU; Epitex Inc., Kyoto, Japan). Each LED emitted a specific peak wavelength (λ_p) of light: 405 nm, 460 nm, 630 nm, 660 nm, and 735 nm, respectively. The petri dishes were set 2.5 cm below the LED panels. In addition to these treatments, lightproof petri dishes wrapped in aluminum foil were set in a room to confirm whether pollen germination was stimulated or suppressed under dark conditions. Five replicated culture slides were prepared per treatment (Fig. 1B).

After culturing, all slides were observed using a microscope (Axiovert 40 CFL, Carl Zeiss, Germany), and germinated pollen grains were counted. Germinated pollen grains with pollen tubes that were shorter than the pollen grain diameter were not counted. We repeated the experiment three times under the same conditions, and are referred to as G1, G2, and G3.

The effects of light condition on the direction of pollen tube elongation

Cover glasses placed over spots of medium with a diameter of 1 cm were prepared. Frozen pollen grains were sown on the spots of medium in the same manner as for the pollen germination experiment. Five cover glasses were vertically set in a humid petri dish using a paper stand to prevent the glasses from sticking. The petri dishes were wrapped in aluminum foil with a slit (8 cm × 0.5 cm) on the upper side for LED light irradiation, and were then cultured at 21°C in the previously described incubator for 3-4 h. To verify the effects of light intensity on pollen tube elongation, two petri dishes were prepared for each light condition: one was placed 3 cm below the LED panel (strong light) and the other was placed 22 cm below (weak light).

After culturing, the direction of pollen elongation was observed using the previously described microscope. The direction of pollen tube elongation was determined as follows:

1. Elongation of the pollen tube tip within a 60° arc centered on the line connecting the LED panel, pollen grain, and floor, and widening in a direction toward the LED light, was defined as "Light" (under LED lighting conditions) or "Upward" (under dark conditions) (Fig. 1C);
2. "Dark" or "Downward" defined the occurrence of pollen tube elongation in the opposite direction of the Light or Upward response;
3. Pollen tube elongation toward a direction that was neither Light/Upward nor Dark/Downward was defined as "Vertical."
4. This experiment was also repeated three times under the same experimental settings and is referred to as D1, D2, and D3.

Statistical analysis

All statistical analyses were conducted with the statistical package R (v.3.0.2) (R Foundation for Statistical Computing, 2014). A generalized linear mixed-effects model (GLMM) with an offset term was used to investigate determinants on the number of germinated pollen grains, assuming Poisson distribution. The light condition, triple experiment design, and subsequent interactions were explanatory variables in the model, and an individual spot of medium was a random factor that considered effects seen on an individual spot compared to other spots on the same slide. The total number of pollen grains per medium was

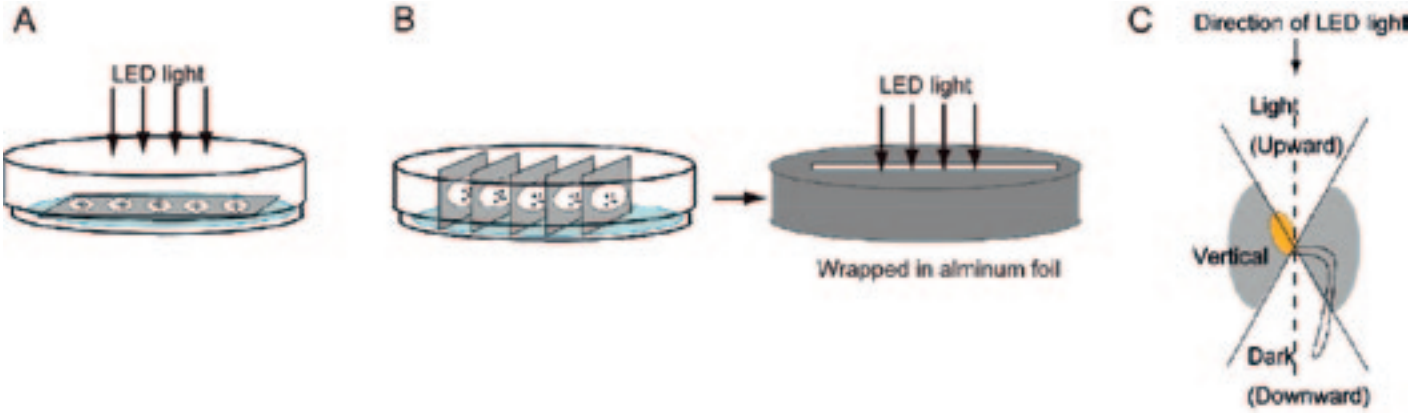


Fig. 1 - Diagrammatic representation of the experimental systems and germinated pollen. A, Petri dish set in prepared slides for pollen germination experiment. B, Petri dishes set in cover glasses and then wrapped in aluminum foil with a slit on the upper side for pollen tube elongation experiment. C, Criterion for determining of the direction of pollen tube elongation.

an offset term. The Akaike information criterion (AIC) was used to select the best models of the GLMMs.

To estimate the effects of light intensity and light wavelengths on the direction of pollen tube elongation, multinomial logistic regression (Venables and Ripley, 2002) was performed using the R package's vector generalized linear and additive model (VGAM). The direction of pollen tube elongation was considered a categorical response variable (Light/Upward, Vertical, Dark/Downward). The explanatory variables were distance from LED panel (long or short); wavelength (405, 460, 630, 660, or 735 nm); and triple experiment design in the case of LED lighting conditions. In the case of analysis for dark conditions, the experiment was the explanatory variable.

3. Results

The effect of light condition on pollen germination rate

The total number of *C. mackenii* pollen grains sown on spots of medium were 9 017 (G1), 6 769 (G2), and 4 257 (G3). The pollen germinated under both LED lightning and dark conditions (Fig. 2). The mean and standard deviation of germination rate under each light condition were 0.30±0.015 at 405 nm, 0.38±0.019 at 465 nm, 0.28±0.018 at 630 nm, 0.34±0.020 at 660 nm, and 0.31±0.023 at 735 nm peak wavelength LEDs, and 0.37±0.018 in darkness. The germination rate varied among the three experiments, but did not differ among the five types of LED and darkness (Table 1).

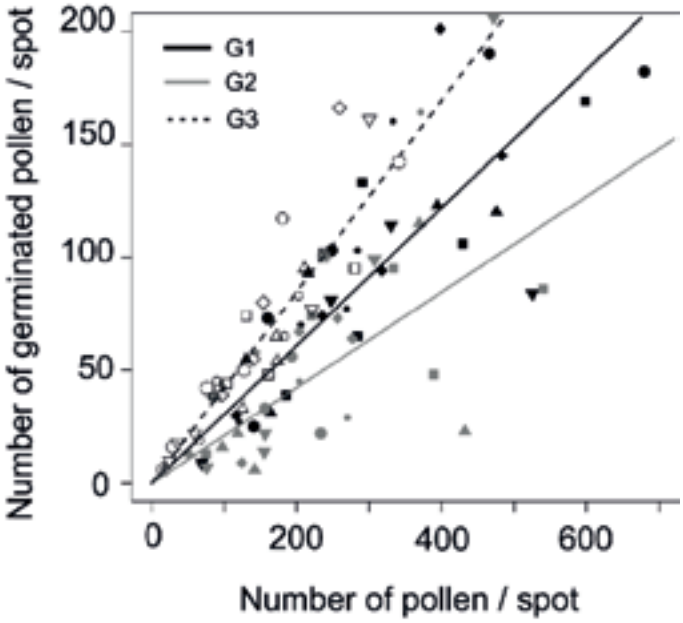


Fig. 2 - Relationships between light condition and each germination experiment and germination rate of pollen. Germinated pollen per total sown pollen on each medium spot under each irradiated LEDs and darkness: 405 nm (squares), 465 nm (circles), 630 nm (triangles), 660 nm (diamonds), 735 nm (upside-down triangles) peak wavelength of the LED, and darkness (small circles). Three lines show the estimated mean number of germinated pollen for three experiments by GLMM. Results of GLMM are shown in Table 1.

Table 1 - Parameters estimated by GLMM to predict number of geminated pollen in different light conditions for three times of germination experiment. The light condition was discarded explanatory variable by AIC

	Coefficient	Z score
Intercept	-1.19	-15.98 ***
Experiment G2	-0.37	-3.40 ***
Experiment G3	0.33	3.06 **

Significance is determined by *Z* scores.
***: significantly different from G1 at $P < 0.001$ and ** $P < 0.01$.

The effect of light condition on the direction of pollen tube elongation

The total number of pollen tubes elongated in the three defined directions as detailed in Fig. 1C for the three experiments was 1 410 for Light, 2 994 for Vertical, and 1 223 for Dark under LED lightning, and 187 for Upward, 421 for Vertical, and 161 for Downward in darkness (Fig. 3). Under LED lightning, the probability of pollen tube elongation classified as Light or Vertical was higher than that classified as Dark (Table 2). However, under darkness, the probability of pollen tube elongation classified as Upward was equivalent to that of Downward, although the Vertical classification was higher. Variability among the three experiments was not found in Light and Upward pollen tube elongation, but was found in Vertical elongation. There was no effect of the distance from LED panel on pollen tube elongation. Under lighting from 735 nm peak wavelength LEDs, the number of Light pollen tube elongations was lower than for Dark pollen tube elongations.

4. Discussion and Conclusions

In this study, light neither inhibited nor promoted *Cyrtanthus* pollen germination, and the different wavelengths

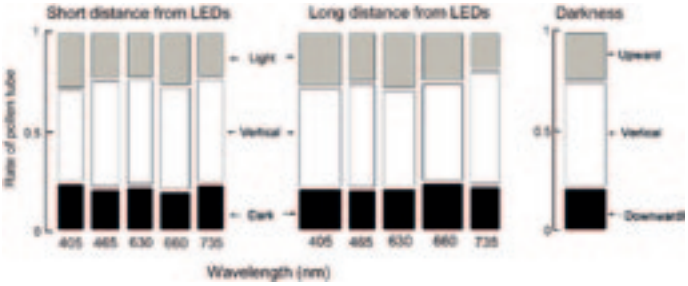


Fig. 3 - Rates of pollen tube elongation in three directions for each irradiated LEDs for the two distances (3 and 22 cm) from LED panels and for shading. The three directions are expressed as follows: toward light or upward (Light or Upward, grey culms), away from light or downward (Dark or Downward, black culms), and in a direction that is neither Light/Upward nor Dark/Downward (Vertical, white culms). The rates are calculated using sum of the number of pollen tubes in five replications of all experiments. The width of culm shows the number of pollen tubes.

Table 2 - Coefficients estimated by multinomial logistic regression models that predict probability of pollen tube elongation toward to the Light/Upward and Vertical directions based on Dark/Downward direction in short distance from 405 nm peak wavelength LED panel in D1

		LEDs irradiation		Darkness	
Direction		Light	Vertical	Upward	Vertical
Intercept		0.23*	0.72***	-0.02 N.S	0.59 **
Experiment	D2	0.05 N.S	0.30***	0.19 N.S	0.42 N.S
	D3	0.03 N.S	0.11 N.S	0.27 N.S	0.54 *
Distance	long	-0.007 N.S	-0.02 N.S		
Wavelength	465 nm	-0.09 N.S	0.14 N.S		
	630 nm	-0.05 N.S	0.08 N.S		
	660 nm	-0.10 N.S	0.03 N.S		
	735 nm	-0.37**	0.10 N.S		

Significant effects of distance and type of LED lights on the direction of pollen tube elongation are determined by *Z* scores.
***: significantly different at $P < 0.001$, **$P < 0.01$, *$P < 0.05$ and NS: non-significant.

of LED light also did not affect the pollen germination rate. However, pollen tubes were less likely to elongate toward the opposite direction of light, although they elongated with no directional trend in darkness. These results suggest that light has some effect on the factors determining the direction of pollen tube elongation, but not on the factors controlling pollen germination.

The growth direction of a pollen tube is continuously reoriented by external signals and physical obstacles (Cheung and Wu, 2008). Without any chemotropic attractants or physical directional control caused by configuration of female tissue or gametophytes, pollen tubes elongated randomly *in vitro* (Horade *et al*., 2013). However, the pollen tubes did not show negative phototropism under LED lighting from only one direction in the present study. Therefore, it can be assumed that there is an integrative mechanism that causes unequal elongation of the pollen tube tip, e.g. perception of the light environment (Casal, 2013), transcription factors such as phytochrome interacting factors (PIFs) (Chen and Chory, 2011), or hormones and growth-related genes such as for hypocotyl cells (Braidwood *et al*., 2014).

In a unique manner, the pollen tubes did not elongate toward light from 735 nm peak wavelength LEDs. This result was consistent with previous studies that examined the effect of different light wavelengths in combination with hormones on pollen tube growth in *Arachis hypogaea* (Chhabra and Malik, 1978) and *Pinus roxburghii* (Dhawan and Malik, 1981). Considering the red:far-red ratio, phytochrome undergoes a conformational change from the active far-red form into the red-light-absorbing, active red form, and derepresses PIF activity, resulting in an increase in cell elongation. If the phytochrome exists in pollen tube tips and causes local cell elongation at the point irradiated with far-red light, the phenomenon of pollen tube elongation away from light may be explained.

In conclusion, our results demonstrate that the different wavelengths of LED light affected the direction of pollen tube elongation, but not pollen germination, in an *in vitro* experimental system. Further studies are necessary to clarify the influence of light wavelength on determination of the direction of pollen tube elongation.

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