Effects of Temperature on Male Sterility of Two Inbred Lines of Hybrid Rice

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ABSTRACT

The lower critical temperature of temperature-sensitive genic male sterility (TGMS) rice is an environmental factor which has been reported to loosen the sterile pollen of the TGMS line (the A line of the 2 line hybrid system). This study investigated the effect of various temperature conditions on pollen fertility of two TGMS lines (KU-TGMS1 andKU-TGMS3) which were re-grown and transferred to a growth chamber and natural conditions (summer: April to May and winter: December to January at Kasetsart University, Bangkhen campus). Day/night temperature regimes of 26/22 °C, 26/20 °C, 24/18 °C and 22/20 °C were employed under growth chamber conditions of 11.5 hr light/12.5 hr dark and 75% relative humidity. The pollen viability and seed setting were used for male sterility identification. For the pollen viability test, spikelets in each line were collected from the panicle at the R3 to R4 stage. Pollen viability was examined using the I_2-KI staining technique, the fluorescein diacetate staining technique, the 4', 6-diamidino-2-phenylindole (DAPI) staining technique and the germination rate of pollen tubes. The results showed that the maximum percentage of pollen viability and seed set were found at night temperatures of 18–22 °C and 18–20 °C, respectively. For seed setting, 7.67 % was found for KU-TGMS1 at 26/20 °C and the highest seed set rate was 33.63 % for KU-TGMS3 under 24/18 °C. Testing the pollen viability with the DAPI nucleic acid staining technique and the germination rate of pollen tubes in the pistil *(in vivo)* was related with the seed set percentage on each line. These results indicated that the DAPI nucleic acid staining technique and the germination rate of pollen tubes in the pistil *(in vivo)* were suitable for viability testing of pollen in the TGMS lines and these two lines differed in critical temperature

Keywords: pollen viability, temperature, hybrid rice, TGMS line

INTRODUCTION

Rice is one of the most important staple foods for a large part of the world's human population, especially in Asia and the West Indies and rice demand is likely to increase as the population increases (Chen *et al.*, 2011). Hybrid rice is one of the most successful crops utilizing heterosis (Chen *et al.*, 2007; Baranwal *et al.*, 2012). The cultivation of hybrid rice is necessary to meet the rapid increase in the demand for rice on a reducing land base with reduced inputs as hybrid rice has a 15–30% higher yield than that of inbred rice varieties and hybrid rice can perform well under adverse conditions of drought and salinity (Virmani *et al.*, 2003). Because rice is

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self-pollinating, crossing between different parents is quite difficult and requires one of the parents to produce sterile pollen and consequently accept the pollen from another variety. Male sterility is defined as the failure of plants to produce functional anthers, pollen or male gametes. There are two types of hybrid systems to develop hybrid rice. The first system is called a three-line hybrid, which is based on cytoplasmic genic male sterility (CMS). The second system is called two-line hybrid or environment-sensitive genic male sterility, (EGMS); these systems are controlled by nuclear gene expression, which is influenced by environmental factors (Virmani *et al.*, 2003).

The EGMS system using a two-line hybrid has a great number of advantages over the CMS system as the EGMS system is simpler and more effective due to the removal of the maintainer line from the three-line hybrid. EGMS genes are more easily transferred into almost any rice line. Moreover, the ratio of the cultivated area to EGMS line, seed production, and commercial production can be multiplied which reduces the hybrid rice seed cost and furthermore, the EGMS hybrid seed has a 5–10% greater yield than the CMS hybrid (Lopez and Virmani, 2000). In addition, there are no negative effects on the agronomic performance of the EGMS line itself and its resulting hybrids from male sterile cytoplasm and the critical temperature or photoperiod for inducing sterility should be as low as possible for greater stability of the EGMS lines with regard to seed production (Virmani *et al.,* 2003). Male sterility expression in EGMS lines is governed by a single nuclear recessive gene or a pair of nuclear recessive genes that are sensitive to environmental conditions (Borkakati and Virmani, 1996). Many environment-sensitive genic male sterile (EGMS) lines have been developed in China and Vietnam in the past two and a half decades. However, the limitation of this system is hybrid seed production and EGMS multiplication needs appropriate temporal and geographical areas.

Depending on the environmental factors influencing the expression of the sterility-inducing gene, EGMS is classified into the following categories: 1) PGMS lines (photoperiod-sensitive genic male sterility) are sensitive to the duration of day length for the expression of sterility or fertility. Most PGMS lines remain male sterile under long-day conditions and revert back to fertility under short day conditions (Virmani *et al.,* 2003). For example, most PGMS lines remain male sterile under long day conditions (more than 13.7h daylight) and revert back to fertility under short-day (less than 13h daylight) according to Zhou *et al.* (2001). 2) TGMS (temperature-sensitive genic male sterility) lines are sensitive to the temperature for the expression of male sterility or fertility. For example, most TGMS lines remain male sterile at high temperature (day temperature > 30 °C and night temperature > 24 °C and they revert back to partial fertility at a lower temperature (day < 24 °C and night > 16 °C) according to Virmani *et al* (2003). Most TGMS lines remain male sterile at high temperature and they revert back to partial fertility at a lower temperature. For example, TGMS lines, such as 5460S, IR68945, H89-1 and SA, remain male sterile under conditions of day temperature > 30 °C and night temperature >24 °C. This TGMS line could revert to a fertile one under the conditions of day temperature < 24 °C and night temperature < 16 °C according to Zhou *et al.* (2001).

Critical temperatures of TGMS were reported to be around 23 to 29 °C with variation from line to line (Virmani *et al*, 2003; He *et al.,* 2011). In addition, most TGMS lines remain male sterile at high temperature and they revert back to partial fertility at a lower temperature, so that seed can be produced for propagation. However, some reports showed that different lines have a specific critical temperature for the TGMS line (Chen *et al.*, 1993; Li *et al.*, 2003; Kang *et al.*, 2014). Therefore, the production of seed of TGMS lines or increasing the fertility of pollen for the A line in a two line hybrid rice system are the major problems associated with productivity of

EGMS/TGMS hybrid rice in Thailand because most of the growing area has a high temperature. Thus, to increase the seed production of the A line, information on the critical temperature of TGMS lines grown in Thailand is mandatory. This study examined whether different temperature conditions affect the pollen viability and the seed set of the individual TGMS lines.

MATERIALS AND METHODS

Plant Material

The two lines of the Kasetsart University–Rice Hybrid Breeding Program (KU-TGMS1 and KU-TGMS3) were used to multiply the number of seedlings. Their tillers were separated and re-grown. Before the late vegetative stage or panicle initiation stage, plants were transferred to the growth chamber and subjected to various day/night temperature regimes—26/22 °C, 26/20 °C, 24/18 °C and 22/20 °C with 11.5 hr light/12.5 hr dark and 75% relative humidity. The remaining plants were grown under natural temperature conditions on the Kasetsart University, Bangkhen campus during summer (April to May 2013) when the average temperature was 31.6 to 41.7 °C and winter (December 2012 to January 2013) when the average temperature was 19 to 31.7 °C.

A pollen viability test using staining techniques and pollen germination on the pistil were used to identify male sterility. A sample of 10–15 spikelets from the two main panicles per plant at the R3 to R4 developmental stage of each growing condition was collected to investigate male sterility. The R3 stage was defined by panicle exertion from the boot and the tip of the panicle was above the collar of the flag leaf whereas the R4 stage was defined by more florets on the main stem and the panicle had reached anthesis at about 14–20 d in the growth chamber. In addition, plants at 28–35 d growth under various temperature conditions in the growth chamber were transferred to grow on under natural conditions. The final percentage of seed setting was collected for each line under the various temperature regimes.

Pollen viability test

Staining techniques

The florets were collected when the two TGMS lines were at the mature pollen stage and some florets were placed in soluble fixations (25 % acetic acid in 75% ethanol) in glass vials for study using the staining technique. Three techniques were used. In the first technique (I_2–KI staining technique), the anthers were washed and squashed to disperse the pollen. The I_2–KI solutions covered the pollen for 3–5 min before pollen viability was determined under a compound microscope (Pedersen *et al.,* 2004). The second technique (fluorescein diacetate; FDA) staining technique involved preparing FDA stocks in acetone and mixing 0.1% in 99.9% phosphate buffered saline (PBS) buffer. The pollen was stained with FDA for 30–60 min and then the pollen viability was determined under a fluorescent microscope with a filter setting at 388–530 nm (ultraviolet, UV) (Norton, 1966; Boyd *et al*., 2008). The third technique for stained pollen was the 4', 6-diamidino-2-phenylindole (DAPI) staining technique. Pollen staining commenced with the PBS buffer and then was stained with stock of DAPI mixed with McIlvaine's buffer at pH 7.0.The pollen stained with DAPI was observed under a fluorescent microscope and the filter was set at 360–460 nm (UV) (Krasnow *et al*., 2008).

Germination of pollen tube on pistil

To investigate the germination of pollen, TGMS pistils were harvested and *in vivo* pollen germination was observed to determine pollen tube growth inside the pistil. The ovaries at 4 hr after pollination were fixed immediately in fixing solution (25% formaldehyde in 75% ethanol). Then, they were placed in a series of alcohol solutions for rehydration and subsequently the ovaries were transferred to NaOH solution for softening, followed by washing and staining with aniline blue solution for 12 hr. The pollen tube growth in the pistil was observed under a fluorescent microscope with a filter setting at 360–460 nm (UW) according to Shi-qiang *et al* (2008).

The pollen viability percentage was counted and calculated using Equation 1:

$$\text{Pollen viability percentage} = \frac{\text{Number of viable pollen grains}}{\text{Total number of pollen grains observed}} \times 100 \quad (1)$$

Seed setting

The percentage of seed set was counted at the maturity stage after plants had been moved to natural conditions. Total seed production was compared using all florets on the panicle at that time to determine the percentage of seed setting using Equation 2:

$$\text{Seed set percentage} = \frac{\text{Number of speeds}}{\text{Total number of florets observed}} \times 100 \quad (2)$$

The pollen viability and seed set percentage were compared between treatments for the different temperatures and lines using two way analysis of variance and means were compared using Duncan's multiple range test with the level of significance tested at $P < 0.05$. Statistical analysis was conducted using the SPSS software package (version 12; SPSS; Chicago, IL, USA).

RESULTS AND DISCUSSION

Various day/night temperature regimes resulted in increased pollen viability of both TGMS lines, while under natural conditions neither line was able to produce pollen (Figure 1q). After viability testing, the results showed that different temperature conditions were specific for the pollen viability of each KU-TGMS line. Pollen viability testing using the different staining technique produced different percentages from the I_2–KI staining technique, the FDA staining technique and the DAPI staining technique. I_2–KI was used to determine starch organs in the cytosol of pollen. Starch grains were presented only in viable pollen and partially viable pollen with a dark blue color while unviable pollen (non starch grain conditions) lacked binding (Figure 1r). FDA staining with esterase enzyme in the cytoplasm of pollen grains showed light green under a fluorescent microscope (Figure 1a-j). Green fluorescent colors around the pollen grain resulting from staining indicated viable pollen. DAPI staining identified the viability by staining with DNA or the nucleus of the pollen namely, the tube cell and generative cell. This produced two points of blue fluorescence on viable pollen under a fluorescent microscope. Pollen without any nuclei could indicate the pollen was unviable (Figures 1k–1p).

Another technique for measuring pollen viability involved observing the pollen tube growth in the pistil under a fluorescent microscope (Figures 1s–1x). All viable pollen fluoresced yellow-green in the callose wall of the tube cell when stained with aniline blue at 4 hr after pollination. Germination of the tube occurred in viable pollen. Pollen viability was evaluated by staining and the *in vivo* germination technique. The results indicated that different techniques gave different results for pollen viability. Interestingly, the results in Figure 2 showed that the DAPI nucleic acid staining technique and the germination rate of pollen tube in the pistil *(in vivo)* were more suitable for viability testing of pollen in the inbred rice line when compared with other techniques. The results of seed production (Figure 2e) indicated that the I_2–KI staining could not determine pollen viability because some organelles, starch or membrane occurred on both fertile and sterile pollen (Kakani *et al.*, 2005). The relevant results from the DAPI technique (Figure 2c) were pollen tube germination (Figure 2d) and seed production (Figure2e).

KU-TGMS 3 under 24/18 °C day/night temperature conditions produced the highest percentage of pollen viability with DAPI of 70.58% (Figure 2c). Moreover, the percentages of pollen viability of the two rice lines were very similar under the 26/22 °C, 26/20 °C and 22/20 °C

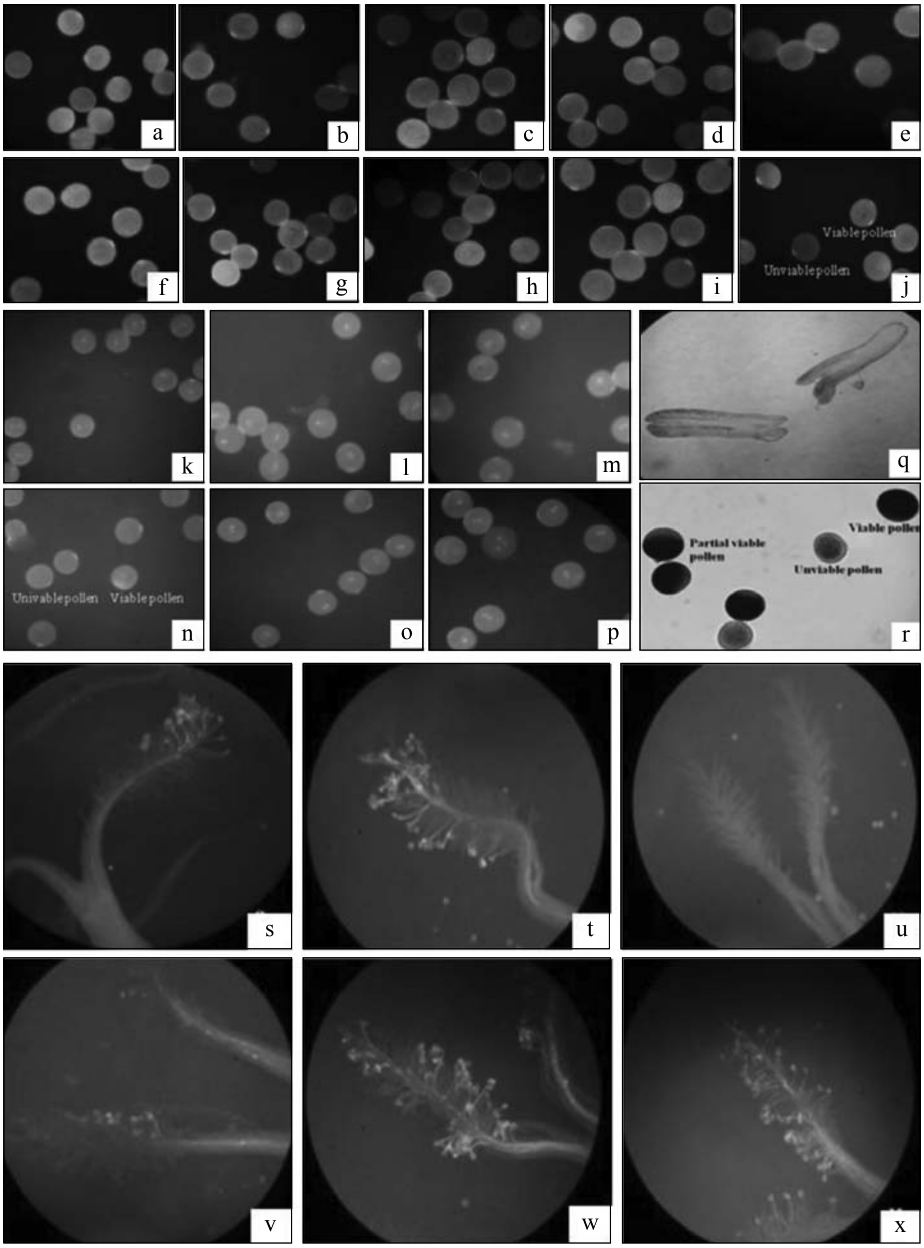

Figure 1 Comparisons between KU-TGMS1 and KU-TGMS3 under various day/night temperature regimes with four techniques: pollen of KU-TGMS1 under (a) Winter; (b) 26/22 °C; (c) 26/20 °C; (d) 24/18 °C; (e) 22/20 °C and KU-TGMS3 under (f) Winter; (g) 26/22 °C; (h) 26/20 °C; (i) 24/18 °C; and(j) 22/20 °C. Nucleus of pollen grains of KU-TGMS1 under: (k) Winter; (l) 26/20 °C; (m) 24/18 °C; and KU-TGMS3 under (n) Winter; (o) 26/20 °C; (p) 24/18 °C (40X); (q) Fertile anther of TGMS lines grown under summer temperature conditions; (r) Types of TGMS pollen staining with I-KI_2 for unviable, viable and partially viable pollen. Comparisons of pollen tube growth in pistil of KU-TGMS1 under (s) Winter; (t) 26/20 °C; (u) 24/18 °C; and KU-TGMS3 under (v) winter; (w) 26/20 °C; and (x) 24/18 °C conditions at 4 hr after pollination (40X).

conditions. KU-TGMS 1 under winter conditions had a higher percentage of pollen viability than KU-TGMS 3. It was found that the germination of pollen tubes in the pistil of KU-TGMS 3 under the 24/18 °C conditions was the highest (53.2%) while KU-TGMS 1 under 26/20 °C conditions and winter conditions had germination rates of 28.5 and 15.18%, respectively (Figure 2d). However, not all temperature conditions were suitable, as only winter, 26/20 °C and 24/18 °C conditions could produce seeds. Interestingly, the percentage of seed production was the highest with 24/18 °C

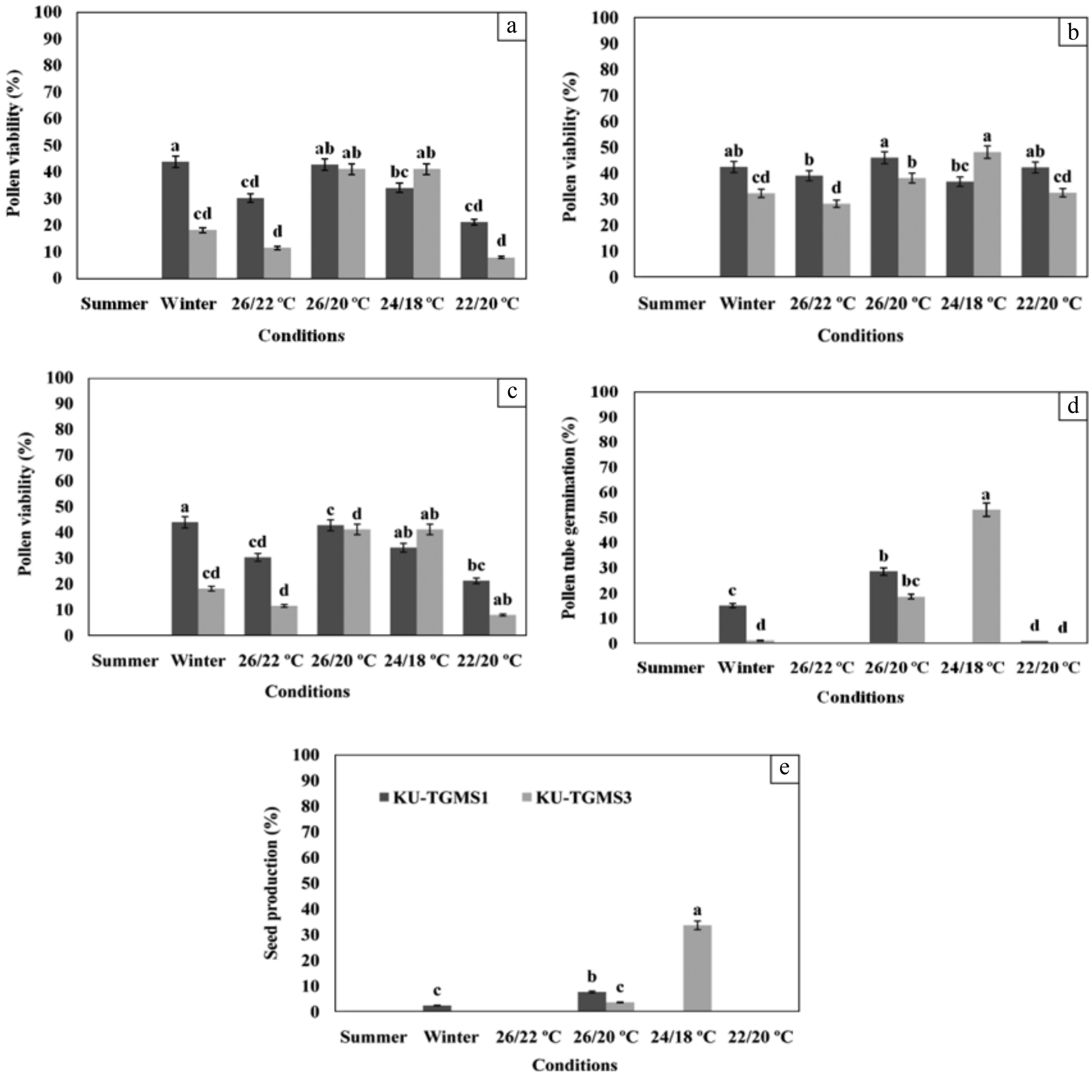

Figure 2 Comparisons of pollen viability percentage and seed setting of KU-TGMS1 and KU-TGMS3 under various temperature conditions: (a) Percentage of pollen viability staining with I_2-KI; (b) Percentage of pollen viability staining with fluorescein diacetate; (c) Percentage of pollen viability staining with 4', 6-diamidino-2-phenylindole; (d) Pollen tube growth in pistil percentage of KU-TGMS1 and KU-TGMS3 at 4 hr after pollination; and (e) Seed percentage of KU-TGMS1 and KU-TGMS3. (Error bars indicate ± SE; Columns with the same lowercase letter are not significantly different by Duncan's multiple range test with the level of significance tested at $P < 0.05$).

(33.63%) as shown in Figure 2e. Furthermore, another set of conditions that was capable of producing seed for the seed set average of KU-TGMS1 and KU-TGMS3 was under 22/20 °C conditions with 7.67% and 3.67%, respectively. On the other hand, the lowest seed production (2.38%) resulted from the winter conditions with KU-TGMS1. DAPI staining resulted in a generative (reproductive) cell containing a two nucleic tube nucleus (that produces the pollen tube) and a generative nucleus (that divides to form the two sperm cells). However, in the fluorescent staining test, both sterile and fertile pollen fluoresced, and the fluorescence faded quickly. Thus, none of the staining methods could effectively distinguish viability and fertile pollen. Consequently, an *in vivo* germination assay was optimized for pollen viability assessment which produced similar results to those reported by Shi-qiang *et al.* (2008).

It was found that the results from the DAPI technique and the pollen tube germinations were relevant. The KU-TGMS3 line under 24/18 °C had the highest percentage of pollen viability when stained with DAPI and with measurement of pollen tube growth and subsequently it produced the highest seed number, with an average of 70.58% pollen fertility with stained DAPI producing 53.23% pollen tube growth in the pistil and a 33.63% seed setting rate. These results indicated that the two TGMS lines responded to different critical temperatures for pollen viability which was similar to the results of Virmani *et al*, 2003 and He *et al.,* 2011. The TGMS lines were sensitive to temperature for the expression of male sterility or fertility by a single nuclear recessive gene or a pair of nuclear recessive genes that were sensitive to environmental conditions (Borkakati and Virmani, 1996). Thus, TGMS could revert back to being fertile under conditions of day and night temperature that were lower than their critical temperature (Zhou *et al*., 2001). The TGMS lines in this research usually produced sterile pollen or less seeds under the summer conditions (average temperature of 36.6 ± 1.0 °C). In addition, most of the growing season in Thailand involves a high temperature so that the TGMS lines could not produce viable pollen except during the winter season.

The experiment showed all TGMS lines were sensitive to a critical temperature lower than 23 °C. In addition, based on these experiments, the midpoint value of the day/night temperature conditions could be used—namely, 24 °C (26/22 °C), 21 °C (22/20 °C), 23 °C (26/20 °C) and 21 °C (24/18 °C). The results of seed setting indicated that both KU-TGMS1 and KU-TGMS3 could produce seeds (Figure 2e) under various conditions with the temperature ≤ 23 °C (the average temperatures were 23 °C (26/20 °C) and 21 °C (24/18 °C) as shown in Figure 2e). These results were similar to the reports of Virmani *et al.*, 2003 and He *et al.,* 2011 who found many TGMS lines with a critical temperature around 23 to 29 °C, varying from line to line. Among all the TGMS lines with different temperature conditions, only KU-TGMS3 produced a high seed percentage under 24/18 °C conditions. The result indicated that changing the temperature affects the ability to produce pollen and its viability. Interestingly, both KU-TGMS1 and KU-TGMS3 could produce viable pollen with a low night temperature (18–22 °C) even when some day conditions were higher than the critical temperature (24 °C and 26 °C). These results pointed to night temperature having a greater effect on pollen viability than day temperature. However, the effect of low temperature on pollen germination was clearly different with the other techniques. The percentages of pollen germination in the pistil were involved with the seed set percentage. Similar results were reported by Chakrabarti *et al.* (2011) who found maximum pollen germination (85.2%) in the PBW 343 variety of wheat, when the temperature was 18.4 °C. In all the varieties, pollen germination was maximum between mean temperatures of 18–20 °C which occurred when the variety experienced very low temperature during anthesis in January.

Consequently, it could be concluded that the best critical temperature and technique for pollen viability testing of Thai TGMS lines could be used to determine the recommended line that could increase seed production of the A line in a two line hybrid rice system.

CONCLUSION

The pollen formation or pollen viability of two different TGMS lines responded differently to different temperatures, with KU-TGMS1 producing pollen under winter and 26/20 °C conditions while KU-TGMS 3 produced pollen under 24/18 °C conditions. Furthermore, the different TGMS cultivars required a distinct temperature to produce seed; KU-TGMS1 required winter or 26/20 °C conditions and KU-TGMS 3 required 24/18 °C conditions. Thus, both the pollen viability and seed set of each cultivar were promoted by different temperature regimes. Growing TGMS under 26/20 °C provided the optimum conditions to revert back to fertile pollen for KU-TGMS 1, while for KU-TGMS 1 the optimum reversion conditions were 24/18 °C.

In addition, the results indicated that the DAPI nucleic acid staining technique and the germination rate of pollen tube in the pistil *(in vivo)* were suitable for testing the viability of pollen in inbred rice lines when compared with seeds production. However, pollen viability should be tested using various methods.

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