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# Establishment of the in vitro evaluation system for pollen viability in Japanese rice cultivars
# （日本のイネ品種における花粉稔性のin vitro 評価系の確立）

**Background**

Rice (*Oryza sativa* L.) is one of the world's most important food crops and ranks second globally after wheat production (Shahbandeh, 2021). Rice provides dietary calories of about 25 % and provides minerals and essential vitamins, including potassium, iron, magnesium, selenium, zinc, folic acid, and vitamin B (Fukagawa and Ziska, 2019). The dramatic world population increase drives researchers and breeders to develop different innovative techniques for improving rice crop production to serve the world.

Rice is a self-pollinated crop; its pollen pollinates the female flower of the same flower for fertilization and seed production. This narrows down its diversity and limits improvement. Several breeding techniques, including artificial cross-pollination, have been used in rice breeding programs to increase genetic diversity by producing new cultivars (hybrids) with improved traits, including grain quality and yield (Sha, 2013). Hybrids produced via artificial cross-pollination are characterized by high performance, such as high yields and high disease, pest, temperature, and drought resistance compared to that of regular rice cultivars (Virmani, 1997). Due to the advantages of hybrid rice, hybrid rice technology is not only a novelty in agriculture but also an innovative strategy for reducing hunger and food insecurity (Olson, 2014).

The success of rice hybrid production via artificial cross-pollination depends on many factors. This includes environmental conditions such as temperature as well as the quality of pollen from the selected male parents (Broussard et al., 2023). Selecting male parents with high pollen quality is challenging without specialized visual assessment tools. Plants may have good vigor and many florets (flowers) but poor pollen quality. Therefore, in vitro pollen quality analysis is potentially the best way of assessing the quality of the pollen for successful fertilization and seed production. Pollen germination rates, pollen tube growth, and pollen tube burst rates can be determined through in vitro pollen quality assessments. These three quality factors are directly associated with fertilization and seed formation in seed plants, including rice (Tran et al., 2023). Therefore, in vitro pollen quality assessment information may serve as a valuable tool for breeders to accelerate the development of new and improved varieties and increase crop productivity.

Pollen grains are the non-motile male gametophytes that affect fertilization in flowering plants (Dresselhaus et al., 2016). Pollen grains are produced within the anther and released after maturation. Mature pollen grains are transferred to the female reproductive region (stigma), where they navigate toward the ovary to reach the female gametophytes for fertilization

(Selinski and Scheibe, 2014). When the male gametes land on a compatible stigma, they hydrate, germinate, and produce generative and vegetative cells (Heslop-Harrison and Heslop-Harrison, 1996; Gao et al., 2016; Zheng et al., 2019). A generative cell typically forms two sperm cells; one fertilizes an egg cell to form a zygote, and another fuses with the polar nuclei to form the endosperm. The vegetative cells form a pollen tube that transfers and delivers the male gametes (sperm cells) from the stigma to the ovule (Adhikari et al., 2020). The pollen tube enters the embryo sac and releases the male gametes when the pollen tube tips burst (Dresselhaus et al., 2016: Zheng et al., 2019).

High-quality pollen grains are required for artificial cross-pollination and breeding to improve the genetics and productivity of crops, including rice. In vitro pollen germination, pollen tube growth, and pollen tube bursting are indicators of pollen quality in many flowering plants, including rice, wheat, maize, sorghum, and rye, and are associated with fertilization, pollen culture success, seed development, and yield (Lange and Wojciechowska, 1976).

Pollen quality analysis is important in plant breeding to ensure successful fertilization and seed production. High-quality pollen must be viable, capable of germinating, and have the ability to fertilize the ovule, contributing to successful seed production. The pollen viability can be assessed in many ways, including (i) in vivo by counting seed set, (ii) using pollen staining dyes, and (iii) using artificial germination media. Assessing the pollen quality using artificial germination media in vitro is simple, time-efficient, and provides accurate information on pollen germination capacity, pollen tube elongation, and pollen tube bursting rate, which are key indicators of pollen quality.

Pollen germination capacity and pollen tube growth can be assessed using artificial culture media, while pollen viability is determined through staining techniques, both providing information quickly (Heslop-Harrison and Heslop-Harrison, 1996). Assessing the pollen viability using stains lacks reliability because it only indicates viability and not pollen germination capacity (Weng et al., 2023), pollen tube growth, or pollen tube bursting. The challenge in assessing germinability for trinucleated rice pollen in artificial media lies in the pollen being very sensitive to the composition of the media, especially sucrose concentration, and incubation conditions (temperature and period). Studies on in vitro pollen germination and tube elongation in rice have been reported (Kariya, 1989; Khatun and Flowers, 1995; Wang et al., 2000); however, information on a standardized pollen germination system in rice is still limited. This hinders efforts to assess pollen quality in vitro, which could otherwise facilitate hybrid production.

Optimal in vitro pollen germination media composition and conditions are essential for basic and applied pollen research (Tushabe and Rosbakh, 2021). Such studies greatly enhance our understanding of pollen and its importance in agriculture. Studying in vitro pollen germination will help us understand pollen's capacity to germinate, elongate the pollen tube, and burst the pollen tube in artificial media, providing useful information that reflects fertilization in typical plants. Assessing pollen germination capacity and observing features of pollen tubes in vitro is achievable when the appropriate medium with optimal components and composition is used. The core components of culture media for in vitro pollen germination are sugar, boron, and calcium nitrate (Tushabe and Rosbakh, 2021). However, other components, such as minerals, vitamins, and growth regulators, can be added to the media to facilitate pollen germination and pollen tube growth (Patel and Mankad, 2014; Jayaprakash, 2018). Sucrose provides energy and maintains the osmotic pressure of pollen grains (Patel and Mankad, 2

014), boric acid facilitates pollen tube growth and maintains cell membrane permeability and cell membrane structure (Jayaprakash, 2018), and calcium is involved in ion balance as well as controlling the permeability of the pollen tube membrane (Fragallah et al., 2019). Among all components, the demand for sucrose is the highest as it provides energy and maintains o smotic pressure during pollen germination and tube growth.

Plant pollen is extremely vulnerable to heat stress, and even short-term fluctuations in temperature during pollen development can drastically reduce pollen quality. Long durations o f high temperatures due to global warming affect crop production, including that of rice (Shi et al., 2016). The effects of heat stress on rice production depend on the growth stage of t he crop. The effects of temperature on the reproductive stage of rice may interfere with poll en production, fertilization processes, and seed production (Shrestha et al., 2022). High or lo w temperatures in rice-growing areas affect rice growth and production. High temperatures m ay affect pollen quality by causing poor pollen germination, while reduced pollen viability m ay also cause poor pollen structure, affecting plant fertility (Khan et al., 2022). Extremely lo w temperatures can drastically impair pollen quality by severely disrupting anther dehiscence (Zeng et al., 2017). When anthers fail to open correctly, pollen release is compromised, creat ing a critical barrier to adequate pollination and fertilization. This chain reaction ultimately r esults in a sharp decline in seed production, posing a serious threat to plant reproductive suc cess. We evaluated the in vitro incubation temperature to identify its effects on pollen germi nation and pollen tube growth rates, providing information relating to plant infertility. High t emperature may cause low to no pollen germination and pollen tube growth because it disru pts the metabolic activity of pollen cells, including ATP and cell membrane syntheses (Loha ni et al., 2024: Zhang et al., 2024).

The aim of this study was to develop the system for the in vitro pollen quality assessments in rice (*Oryza sativa* L.). The first objective, outlined in Chapter 2, examined the effects of sucrose concentrations, incubation periods, and incubation temperatures on in vitro pollen ger mination capacity, pollen tube growth rate, and pollen tube bursting rate in three rice cultiva rs. These factors were found to primarily influence pollen viability and tube development on artificial media. Therefore, they were optimized to help obtain accurate results during in vitr o pollen quality (pollen germination, tube growth, and pollen tube bursting) evaluations. The second objective, described in Chapter 3, was to investigate the in vitro pollen tube growth behaviors and bursting phenomena in rice (*Oryza sativa* L.) to identify the external factors t hat can inhibit fertilization in rice plants. Furthermore, we investigated whether the bursting r ate of the pollen tube is influenced by pollen tube length or the length of time from germin ation to bursting. This study provides insight into specific pollen tube growth and bursting b ehaviors that may be associated with plant infertility in rice plants.

**Main results**

Various methods have been used to assess pollen viability, including in vivo seed set counting, in vitro pollen germination assays, and staining techniques using specific dyes. In vitro pollen germination test involves assessing the germination capacity of the pollen grain(s), elongation, and bursting of the pollen tube, while the staining method is used to indicate if the pollen is alive or dead based on the uptake of the dye and the resultant color changes. The quality of pollen grains is strongly correlated with fertilization and seed formation in seed plants. Therefore, pollen quality assessment is

crucial for artificial cross-pollination to ensure fertilization and seed formation. Culture medium composition and conditions for in vitro pollen quality evaluation may vary among species or cultivars, and it may interfere with the in vitro evaluation of pollen germination and tube growth. Incubation time and incubation temperature may interfere with pollen germination and tube growth and, therefore, with the in vitro quality evaluation. Culture media for in vitro rice pollen germination have been developed; however, the information on the system for in vitro pollen viability evaluation in Japanese rice cultivars is still limited. In this study, I conducted experiments to (i) determine the optimal sucrose concentrations, incubation temperature, and incubation period for high pollen germination capacity, pollen tube growth, and pollen tube bursting rates in three Japanese rice cultivars as the parameters for in vitro pollen quality analysis; (ii) identify the most suitable method for pollen quality assessment by comparing pollen staining using fluorescein diacetate (FDA) and in vitro pollen germination; and (iii) analyze the pollen tube growth behaviors and bursting phenomena in vitro.

Fresh mature pollen samples from three Japanese rice cultivars, 'Kitaake', 'Nanatsuboshi' and 'Nipponbare' were used. Pollen samples were cultured on artificial liquid culture media with different sucrose concentrations (0, 10, 20, and 25%) to investigate the suitable sucrose concentration for high rice pollen germination rate, pollen tube growth, and pollen tube bursting rate in vitro. The optimized medium was used to investigate the effects of incubation temperature and incubation time on in vitro pollen germination, tube growth, and pollen tube bursting. The pollen germination, pollen tube growth, and pollen tube burst rates were evaluated for a maximum of 1 h in intervals of 10 min. A pollen viability test using FDA staining was also conducted to test if the pollen grains were viable (alive) or non-viable (dead). The FDA staining test validates the presence or absence of esterase enzyme in pollen grain. Viable pollen is stained green, indicating the presence of esterase enzyme (metabolic active), meaning pollen can undergo the metabolic activity required to grow and perform its function. In contrast, unstained pollen grains indicate non-viable pollen grains.

A 20% sucrose concentration supported high pallyollen germination, pollen tube growth, and pollen tube bursting rates in rice pollen. Optimal in vitro germination conditions were achieved at 20% sucrose concentration and 25 °C for 60 min, enabling easy observation of the germination percentage and length of the pollen tubes. A maximum pollen germination rate of about 80% and a pollen tube length of 285 μm were observed within 1 h of initial incubation in 'Nanatsuboshi'. Pollen tube bursting rates higher than 90% were obtained within 1 h in all three tested rice cultivars. No significant differences were detected between using incubation temperatures of 25°C and 27°C, and both were optimal for pollen germination and pollen tube bursting in 'Nanatsuboshi' pollen. The results from the in vitro study determining if incubation time or pollen tube length influences pollen tube bursting indicated that pollen tube length is a determinant of in vitro pollen tube bursting. The highest pollen tube length frequency of 80.17% was observed in the first 60 min of incubation, while it was 19.83% for incubation periods longer than 130 min.

I developed a system for evaluating in vitro pollen viability whereby high pollen germination rates, pollen tube growth, and pollen tube bursting frequency will be determined. The optimal sucrose concentration, incubation time, and incubation temperature were determined for Japanese rice cultivars. I identified both pollen tube length and incubation time are the key factors influencing in vitro pollen tube bursting. Observations revealed that pollen tubes tended to burst upon reaching their length growth limit (maximum length), regardless of the incubation period. Further studies on the biological processes from pollen germination to bursting may offer an understanding of pollen tube elongation and bursting mechanisms.