Japan. Jour. Crop Sci. 58(1) : 96—102 (1989)

Sterility Caused by Cooling Treatment at the Flowering Stage in Rice Plants

III. Establishment of a method of *in vitro* pollen germination

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Received Jan. 29, 1988

Abstract : Rice pollen grains germinated more than 80% with low variation (standard deviation less than 10%) and their pollen tubes elongated to more than 150 μm within one hour with a simple medium composed of 1% agar, 20% sucrose and 20 $\mu g \cdot ml^{-1}$ (ppm) boric acid, using a Petri dish (4.5 cm in diameter). In addition to the composition of the medium, it is important that the incubation temperature should be around 20°C and that the pollen grains should be shed directly on the medium from the anthers as soon as the flowers begin to open. Under these conditions, their pollen grains began to germinate 2 to 3 min after shedding and attained maximum growth rate within 20 min. After that time, the length of pollen tube increased at a linear rate of ca. 7.5 $\mu m \cdot min^{-1}$ over the next 15 min.

Key words : Boric acid, Incubation temperature, *In vitro* germination, Pollen, Rice.

イネの開花期冷温処理による不稔　第3報　花粉の人工発芽法の確立：刈屋国男（農林水産省北海道農業試験場）

要　旨：イネ花粉の人工発芽は1919年以来多くの報告がある．しかし，発芽率が低い場合や同じ培地でも発芽率の変動が大きい場合もあり，その再現性についても十分検討されていない．本報では下記の方法により高発芽率でしかも再現性の高い人工発芽（発芽率80%以上，標準偏差10% 以下）に成功した．①培地の組成：1% 寒天，20%しょ糖，20 ppm ホウ酸(第1～4表)，②発芽容器：直径4.5 cm シャーレ，③培養温度：20°C前後，なお，18°C以下あるいは24°C以上では発芽率が低下する(第1図)，④花粉の培地への置床：開花後できるだけ早い時期に，穎花から葯をピンセットで採取し，シャーレの縁に軽くたたいて葯内の花粉を直接培地に置床する．

従来の方法に比べ，この方法で安定した高発芽率が得られるようになった主因は，培地にホウ酸(20 ppm)を添加したこと，培養の適温を発見したこと，および確実に新鮮な花粉を供試したことである．

上記の最適条件で花粉発芽の経時変化を観察した結果，置床後2—3分で発芽しはじめ（第2図），およそ20分で発芽率は最大に達した（第2，3図，第5表）．その時の花粉管の伸長速度は7.5 $\mu m \cdot min^{-1}$ であった（第5表）．

キーワード：イネ，花粉，人工発芽，培養温度，ホウ酸．

Satake and Koike reported that the cause of sterility due to cool temperatures at the flowering stage was attributable to the pollen grains being unable to germinate on the stigma[15]. They also found a high correlation between the percentage of sterility and that of pollen grains in which starch granules were abnormally digested[7]. This suggests that the pollen grains with abnormal starch digestion have lost or lowered their germination ability at anthesis. It is an essential step to estimate the germination ability of pollen grains in order to clarify the physiological mechanism of the sterility. Many researchers have tried *in vitro* germination of rice pollen grains using media composed of agar and sucrose or of starch and sucrose[4,9,10,13,14,16,19]. However, a completely successful germination method has not yet been reported. Even when high germination percentages were obtained, the reproducibility was not always high.

This paper presents a method to obtain high germination percentages with high reproducibility. The method uses a simple medium composed of agar, sucrose and boric acid. The time course of germination and tube growth was measured by the method.

Materials and Methods

Rice variety Hayayuki was used throughout the experiments and Norin 19 was added in the experiment on incubation temperature. Twenty seeds were directly sown in a circular pattern in each four liter plastic pot every

week to obtain blooming flowers continuously. Each pot was previously provided with 0.9 g of each N, P and K. Hayayuki was grown in an artificially lit room with a temperature regime of 26°C day-20°C night and 12 hour day-length. Norin 19 was grown in a greenhouse in which the temperature was not controlled. At the booting stage the plants were transferred to a naturally lit room with a temperature regime of 24°C day-19°C night.

In a preliminary experiment, pollen grains germinated when potassium borate ($K_2B_4O_7$) was present in a 1% agar and 20% sucrose medium, but germination did not occur when it was omitted from the medium. In order to decide the optimum concentration, 5, 20, 50, 100, 150, 200 and 500 $\mu g \cdot ml^{-1}$ (ppm) $K_2B_4O_7$ were used. In addition, boric acid (H_3BO_3), which has been often used in experiments on pollen germination, was compared with $K_2B_4O_7$ at the optimum concentration of 20 ppm as boric compound. After selecting the most effective form of boric compound, pollen germination was retested under various concentrations of agar (0.5, 1.0, 1.5%) and sucrose (10, 20, 30%) in order to fix the optimum combination. The effect of incubation temperature on pollen germination was compared in a range of 11-27.5°C.

Agar (Agar powder, Nakarai Co. Ltd.) and sucrose were dissolved in distilled water (no pH ajustment) by heating and the solution was cooled to about 60°C with stirring in a water bath. Other chemicals were added immediately into the agar and sucrose medium with stock solutions 100 times concentrated. The prepared medium was poured into Petri dishes (4.5 cm diameter) and the surplus was removed in order to make a thin layer. Pollen grains were directly shed on the medium from anthers as soon as the flowers began to open. Germinated pollen grains were counted through a microscope about 20 min after shedding of pollen grains on the medium. Any pollen grains with a tube more than half of the grain diameter were regarded as germinated. During the time course of germination, photomicrographs were taken while maintaining the same field of view as far as possible. The percentages of germination and burst pollen grains were shown as the means of 7-10 measurements. In each measurement, 200—300 pollen grains were counted, and pollen tube length of 20 pollen grains out of these germinated ones were measured on the enlarged film.

Results

Table 1 shows the effect of $K_2B_4O_7$ in the range of 0—500 ppm on the germination percentage on a 1% agar and 20% sucrose medium. On the medium without $K_2B_4O_7$ pollen grains immediately burst after shedding. They could germinate, however, on the medium with $K_2B_4O_7$ in the range of 5-100 ppm. The germination percentage reached a maximum of approximately 90% and the standard deviation for the medium was very low at 20 ppm as $K_2B_4O_7$. It decreased as the concentration of $K_2B_4O_7$ increased over 20

Table 1. Effect of $K_2B_4O_7$ concentrations on germination percentage at room temperature (17 ~23°C).

$K_2B_4O_7$ (ppm)	Experiments					Mean
	I	II	III	IV	V	
						%
0	0					0
5					26.3(11.3)	26.3
20				90.1(3.6)	88.5(3.5)	89.3
50			75.6(11.3)	71.8(17.2)	72.1(11.5)	73.2
100	71.2(10.8)	37.1(28.9)	28.7(15.5)			45.7
150		0.8(2.0)				0.8
200		0				0
500	0					0

Basic medium : Agar (1%), Sucrose (20%).
Figures in parenthese show standard deviation.

ppm. There was little or no germination and also no bursting at concentrations greater than 150 ppm $K_2B_4O_7$. Table 2 shows the effect of two kinds of boric compounds at 20 ppm on the pollen germination on 1% agar and 20% sucrose medium. The medium with H_3BO_3 slightly increased the germination percentage and slightly decreased the standard deviation, as compared with $K_2B_4O_7$. Therefore, H_3BO_3 was used in the subsequent experiments instead of $K_2B_4O_7$. As a 1% agar and 20% sucrose medium had been used temporarily as the basic medium the optimum concentration of agar and sucrose was newly examined on the germination at 20 ppm H_3BO_3. There was no difference on germination percentage among three concentrations of agar (89—92%, Table 3) and between 10% and 20% sucrose (87-92%, Table 4), while the germination percentage on a 30% sucrose medium was lower (54-59%, Table 4). When observed under a microscope, the bursting of pollen grains was found to occur earlier and more, and the tube length was shorter on 0.5% agar and 20% sucrose medium or on 1% agar and 10% sucrose one than those on 1% agar and 20% sucrose. From these results, a 1% agar and 20% sucrose medium containing 20 ppm H_3BO_3 was concluded to be the best. These experiments were carried out at room temperature (17-23°C). The variation of germination percentage, however, seemed to increase as the temperature became higher. Therefore, the influence of the incubation temperature on germination percentage was examined in the range of 11-27.5°C using two varieties. As shown in Fig.1, in Hayayuki there were high germination percentages at temperatures in the range of 11-23°C but the germination decreased remarkably when the incubation temperature was higher than 24°C. In Norin 19, germination percentage was lower than that in Hayayuki at all temperatures, and the highest germination percentage was observed in the range of 19-23°C, decreasing when the

Table 2. Effect of boric compounds at 20 ppm on germination percentage at room temperature (17～23°C).

Exp.	$K_2B_4O_7$	H_3BO_3
		%
I	91.7(4.6)	94.1(2.8)
II	84.4(6.8)	92.2(2.8)

Basic medium : Agar (1%), Sucrose (20%). Figures in parentheses show standard deviation.

Table 3. Effect of agar concentrations on germination percentage at room temperature (17～23°C).

Exp.	Agar (%)		
	0.5	1.0	1.5
			%
I	88.8 (7.1)	89.8 (5.4)	90.9 (4.5)
II	89.2 (5.2)	92.4 (1.8)	91.8 (3.7)

Basic medium : Sucrose (20%), Boric acid (20 ppm). Figures in parentheses show standard deviation.

Table 4. Effect of sucrose concentrations on germination percentage at room temperature (17～23°C).

Exp.	Sucrose (%)		
	10	20	30
			%
I	88.4 (3.5)	87.3 (3.5)	59.0 (10.0)
II	90.5 (4.5)	91.6 (5.5)	54.0 (13.0)

Basic medium : Agar (1%), Boric acid (20 ppm). Figures in parentheses show standard deviation.

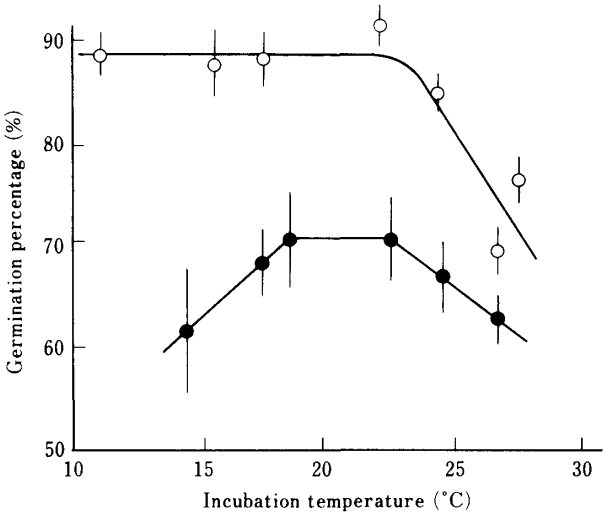

Fig.1. Effect of incubation temperature on pollen germination.
○ : Hayayuki, ● : Norin 19

incubation temperature was less than 18°C or more than 24°C. Although the changes in the germination percentage at incubation temperatures of less than 18°C differed between the two varieties, the best incubation temperature for pollen germination of both was around 20° C.

Fig.2 shows the time course of germination, bursting percentages and pollen tube length on a 1% agar and 20% sucrose medium containing 20 ppm H_3BO_3. Table 5 summarizes comparisons of the increasing rate of pollen tube length and maximum germination values among three concentrations of sucrose. On a 20% sucrose medium, pollen tubes began to elongate within 2-3 min after shedding. They elongated at a linear rate of approximately 7.5 $\mu m \cdot min^{-1}$ until about 15 min and reached the final length of 150-170 μm at around 50 min. Germination percentage began to increase a little later than the beginning of the tube elongation because a pollen grain was regarded as germinated only when the tube length was more than half of the pollen diameter. Germination percentage increased rapidly in a sigmoid curve and reached the maximum value of more than 80% at around 20 min (Fig.3). Bursting percentage began to increase rapidly when germination percentage had reached the maximum value and was 40-50% at 50 min after shedding. On a 10% sucrose medium, the pollen tube length and germination percentage began to increase earlier than those on the 20% sucrose medium, and the tube length elongated at a linear rate of around 11.4 $\mu m \cdot min^{-1}$ until about 10 min. Thus, on a 10% sucrose medium the time required to reach the maximum value of the tube length and germination percentage were reduced. On 30% sucrose medium which is more hypertonic than 10% and 20% sucrose media judging from the decreased size of pollen grains, the pollen tube began to elongate later than that on 10% and 20% sucrose media and the increasing rate was remarkably low, being 2.6 $\mu m \cdot min^{-1}$ linear rate until ca. 45 min. Germination percentage finally reached 38-58% but the bursting scarcely occurred even 50 min after shedding.

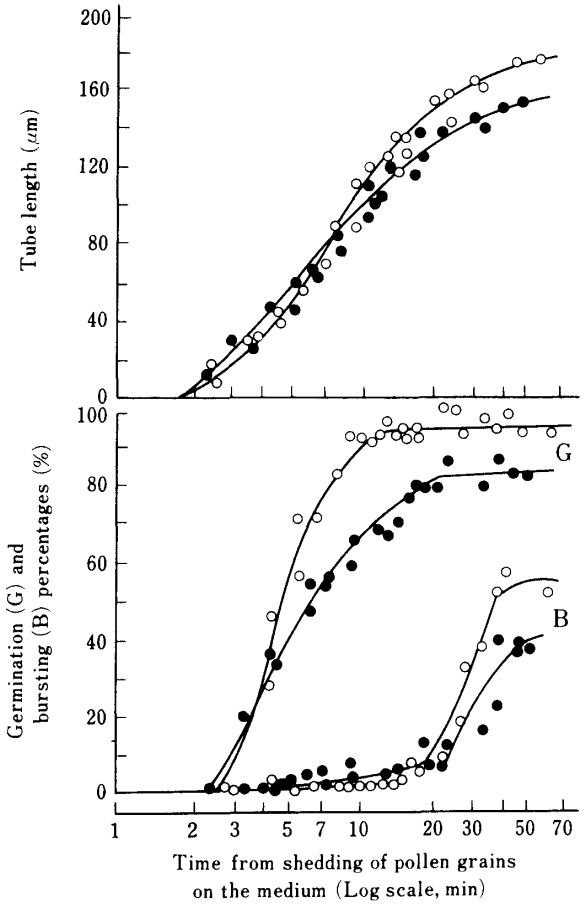

Fig.2. Time course of germination and bursting percentages, and tube length of pollen grains on 1% agar, 20% sucrose and 20 ppm H_3BO_3 medium at an incubation temperature of 20°C.
○, ● : Repetition

Discussion

Using the methods of Sasahara and Katsuo[13] and Yamada and Hozumi[19], the author first examined pollen germination on soluble starch-sucrose and agar-sucrose medium using van Tieghum's cell. Consistent

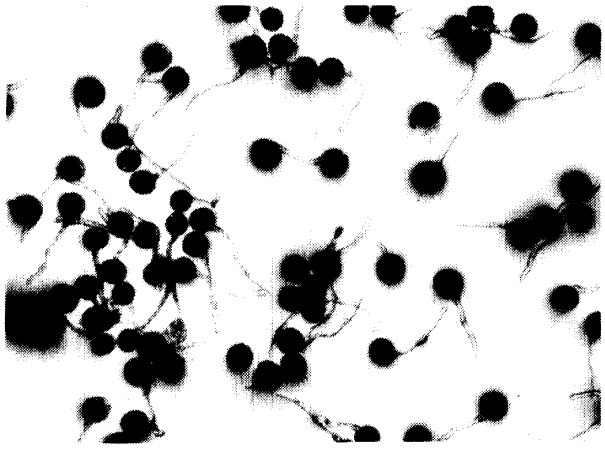

Fig.3. Pollen germination on 1% agar, 20% sucrose and 20 ppm H_3BO_3 medium at an incubation temperature of 20°C (at 20 min after shedding on the medium).

germination to a high percentage was not obtained with these media, even at various concentrations and at various ranges of humidity in the cell. Cosistently high germination was attained by adding boron to the medium of which the effect on pollen germination had been recognized in some plants[1,3,11,12,17]. No attempt to add boron had been reported so far in rice. The author succeeded in getting the most constant germination percentage of more than 80% on 1% agar and 20% sucrose medium containing 20 ppm H_3BO_3 in a Petri dish (4.5 cm in diameter). In this case, the concentration of boron which was contained in the medium was different between two compounds (Table 2). As compared with them at 20 ppm as boric compounds, boron concentration of H_3BO_3 (3.5 ppm) was almost as much as that of $K_2B_4O_7$ (3.7 ppm). Consequently, the active compound for consistently high germination was boron of 3.5 ppm.

Iwanami[6] summarized his papers on incubation temperatures for pollen germination on artificial culture media and showed the optimum temperature was generally 20-30°C in many plants. In rice, Enomoto et. al.[2] observed that the maximum and minimum incubation temperatures for the pollen germination were 42-45°C and 12-15°C, respectively. Sasahara and Katsuo[13] and Sawada[16] observed pollen germination at 24-29°C and 25-28°C, respectively. The optimum temperature for the constant high germination in the present paper was around 20°C (Fig.1) which was somewhat lower than the temperature used by Sasahara and Katsuo[13] and Sawada[16]. This suggests that the medium should be kept around 20°C before and during the incubation of the pollen grains, because air temperature at the flowering stage is generally higher than this. Kubo[8] studied pollen germination of *Zea mays* from July to December. The experiments with the same procedure did not always produce the same results and the optimum concentration of medium varied with different seasons. If the medium had been kept at the optimum incubation temperature, the constant high germination percentage might have been obtained on the same medium throughout the experimental season.

As for the thickness of the layer, Kubo[8] reported that the highest germination percentage was obtained on 20 μm thick layer of 10-40% gelatin medium with van Tieghum's cell in *Zea mays*. Yamada and Hozumi[19] and Sasahara and Katsuo[13] also observed in their experiments with the same cell in rice that the layer of the medium had to be made thinner. It is difficult to make thin layers uniformly

Table 5. The rate (b) of pollen tube growth and the maximum values of germination at three concentrations of sucrose at an incubation temperature of 20°C.

Conc. of sucrose		Tube growth				Germination		Bursting	
		b	Range	Max.	Range	Max.	Range	Max.	Range
%		μm·min^{-1}	min	μm	min	%	min	%	min
10		11.5**		120		92		62	
		10.8**		125		95		50	
		11.6**	<10	120	<15	95	<10	48	<40
		11.5**		130		79		42	
	Ave.	**11.4**		**124**		**90**		**51**	
20		7.9**		174		94		52	
		7.1**	<15	150	<50	80	<20	40	<50
	Ave.	**7.5**		**162**		**87**		**46**	
30		2.4**		144		58		8	
		2.8**	<45	132	<60	52	<50	2	<100
		2.5**		112		34		2	
	Ave.	**2.6**		**129**		**48**		**4**	

b : Regression coefficient of the linear line, which the limited range was estimated from the Figure by visual observation.

** : Significant at 1% level.

with these methods and as a consequence, germination percentage will be expected to vary. In the method reported here, it was found not necessary to adjust the thickness of the medium in the Petri dish because pollen grains germinated with a high percentage regardless of the thickness of the layer. A thinner layer, however, facilitates a microscopical observation and photography of the germinated pollen grains. In addition to the composition of the medium and the incubation temperature, it is important to shed the pollen grains directly on the medium as soon as the flowers begin to open because of the short life of rice pollen grains[9].

By establishing the incubation method for cosistent germination, it was possible to compare the time course of the germination of rice pollen grains at different concentrations of sucrose. Two types of pollen tube growth have been reported i.e. a sigmoid curve type[18] and a linear rate over a limited time[1,5,12]. The pollen tube growth of rice showed the latter form at all concentrations of sucrose. Brewbacker and Majumder[1] and Raghavan and Baruah[12] reported for *Petunia inflata* and *Areca catechu* that an initial lag phase from shedding of pollen grains till the germination and the time required to reach the maximum length were very long, being 0.5-1.0 hour and more than 6-8 hour, respectively. In contrast, these lag phases were very short in rice, being 2-3 min and approximately 1 hour, respectively on the optimum medium of 20% sucrose, and still shorter on a 10% sucrose medium. Furthermore, the increasing rate of the tube growth in rice was very fast, being approximately 7.5 $\mu m \cdot min^{-1}$ until about 15 min on the optimum medium, as compared with 1.1-2.1 $\mu m \cdot min^{-1}$ in *Petunia inflata*[1] and *Areca catechu*[12]. Thus, faster increasing rate of the pollen tube and the shorter time to maximum value may have a close relation to the short life of rice pollen grains[9].

By using this method, the germination ability will be further investigated in relation to sterility caused by cooling treatment.

Acknowledgement

The author wish to thank Dr. B.S. Vergara, the International Rice Research Institute and Dr. B.D. Patterson, Plant Physiology Group, Division of Food Research, Commonwealth Scientific and Industrial Research Organization, for their critical reading the manuscript and making useful comments.

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* In Japanese.
** In Japanese with an English summary.