Improvement of Pollen Germinability and Storability in Some Japanese Alliums

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Improvement of Pollen Germinability and Storability in Some Japanese *Alliums*

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Introduction

The authors are attempting to breed interspecific *Alliums* from diverse origins for ornamental use. Since asynchronous flowering is a major problem in interspecific hybridization within a large genus such as *Allium,* pollen storage as well as germination protocols for some East Asian *Alliums* were evaluated.

Several authors have studied the long-term cold storage in liquid nitrogen for *Allium* pollen[3,5]. Since the current materials flower every year, only short-term (one-year) pollen storage using ordinary freezer was required.

Using the agar-coated slide method, studies on *Allium* pollen germination had been focused on the composition of the germination medium[2,5,7] and on the effects of relative humidity and temperature in short term (up to one week) storage[1]. Using a modified hanging drop method, the effects of flower age, time of day and variety on the germination of onion pollen were studied by Mann and Woodbury[8]. In this study, we examined the effects of genotype, maturity of the pollen at anther harvest, postharvest drying and/or storage of anthers, and the germination conditions on pollen germinability.

Materials and Methods

Anthers were collected from *Allium tuberosum* 'Tender-Pole' and several *A. chinense*×*A. thunbergii* hybrids and backcross progenies developed by Prof. T. Yoshitake at Fukui University. The *A. chinense*×*A. thunbergii* accessions were tetraploid, except for 87C28 which was hexaploid (Yoshitake, unpublished data). The list of these entries is shown in Table 1.

Evaluation of pollen germination methods

This experiment aimed at identifying a rapid and reasonably precise method for pollen germination. Pollen of freshly dehiscent anthers from 87C23 was kept at 20°C in the dark for 3 h prior to microscopic evaluation of germination. Two trials, at 4 replications each, were performed. In all the experiments, 40 pollen grains were evaluated per replicate.

(a) Agar-coated slide method

About 0.8 ml of the medium (1% agar, 15% sucrose) was dropped in each of the two cavities of depresssion slide glasses. Pollen was brushed onto the medium and then covered with a coverslip. Each slide glass setup was placed in individual petri dishes (Fig. 1a).

(b) Hanging drop method[9]

Table 1. List of materials

Code no.	Parent	Chromosome number*1
87C02	Ra 02×Ya 18	32
87C05	Ra 03×Ya 18	32, 33
87C12	Ra 03×Ya 18	33
87C13	Ra 03×Ya 18	33
87C15	Ra 03×Ya 18	33
87C21	Ra 15×Ya 18	32
87C23	Ra 15×Ya 18	32
87C26	Ra 25×Ya 18	47
87C28	Ra 26×Ya 18	48
87B203	Ra 02× (Ra 26×Ya 17)	32
87B204	Ra 03× (Ra 26×Ya 17)	32
T 01	'Tender-Pole' (*A. tuberosum*)	32*2

*1 Data from T. Yoshitake, Fukui University; genomic n=8; Ra=*A. chinense* and Ya=*A. thunbergii*.

*2 According to Zeven and De Wet[11].

Accession code: C = hybrid cross progeny; B = backcross progeny.

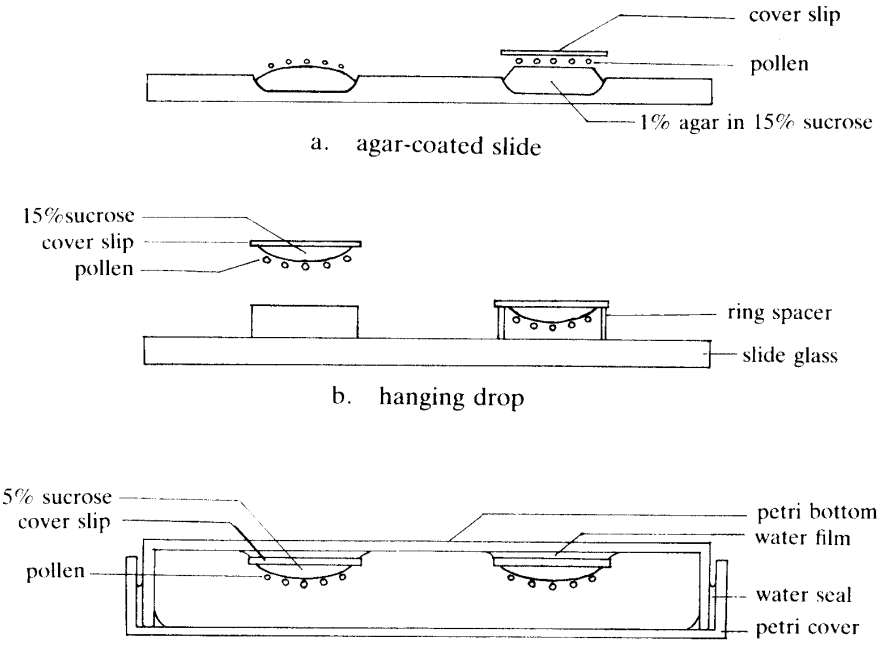


Fig. 1. Methods of pollen germination.

Two ring spacers, 2.5 mm in height and 10 mm in internal diameter, were attached to a slide glass using Vaseline as adhesive. About 0.05 ml of a 15% sucrose solution was dropped on each coverslip and then the pollen sample was stirred in the solution. The coverslips were inverted over the ring spacers and then each slide glass setup was placed in individual petri dishes (Fig. 1b).

(c) Modified hanging drop method

Coverslips were attached to petri dish bottoms moistened with water. About 0.05 ml of 15% sucrose solution was dropped on coverslips and then the pollen sample was stirred in the solution. After covering, the whole petri dish was inverted and 2 ml of water was poured at the rim to make a water seal (Fig. 1c). This method was used in subsequent germination studies.

Studies for germination protocols

A. Effect of pollen drying

Predehiscent mature anthers from 87C26, 87C28, 87B203, 87B204 and 'Tender-Pole' were collected, wrapped in parchment paper and placed in a glass bottle half filled with silica gel for 24, 48 and 72 h at 20°C to dry. Air drying without silica gel was also tested: predehiscent mature anthers from 87C26 and 87B204 were wrapped in parchment paper and placed in a petri dish. Pollen was evaluated for germinability after the prescribed periods.

B. Effects of time, sucrose, light and variety

Mature anthers from 'Tender-Pole' and 87C23 were collected and dried over silica gel for 48 h prior to evaluation. The pollen was allowed to germinate for 1, 2 and 3 h in sucrose solutions ranging from 5 to 25% under dark or light condition.

C. Effects of auxins

The effects of auxins (NAA, IBA and 2,4D) at 10 or 100 ppm in 15% sucrose solutions on the germination of 'Tender-Pole' and 87C23 pollen were evaluated. The pollen was allowed to germinate for 3 h in the dark.

Pollen viability tests by staining and germination

Pollen fertility is often expressed as the percentage of pollen stained with 1% acetocarmine. Pollen was collected from the materials listed in Table 3 and was either stained or germinated under the following protocol: predehiscent mature and newly dehiscent anthers were wrapped in parchment paper and dried on silica gel for 48 h. Using the modified hanging drop procedure, the pollen from those anthers were then germinated in 15% sucrose solution with 10 ppm IBA for 3 h at 20°C in the dark.

Subsequent pollen germination tests followed this protocol.

Low temperature storage

Predehiscent mature and newly dehiscent anthers from 9 entries listed in Table 4 were collected, dried for 48 h on silica gel, then stored, also on silica gel, in 2~3°C, −20°C and −40°C fo 12 months. Pollen germination was observed at two month intervals.

Hypobaric controlled atmosphere storage

To test the effects of controlled atmosphere storage and/or partial vacuum, a vacuum pump [Toyo Jet Sucker JS-75A] was used to draw out air, down to a pressure level of 20 cmHg (0.26 atmosphere), from 10 ml vials containing 'Tender-Pole' pollen (Fig. 2). About 5 ml of either N_2 or CO_2 gas was injected into the designated vials. The stopper rims and the point of injection were sealed with nail polish. No additional gas was injected into the control vials. The vials were stored at 2~3°C, −20°C and −40°C. Pollen was tested at 2 month intervals, for 6 months.

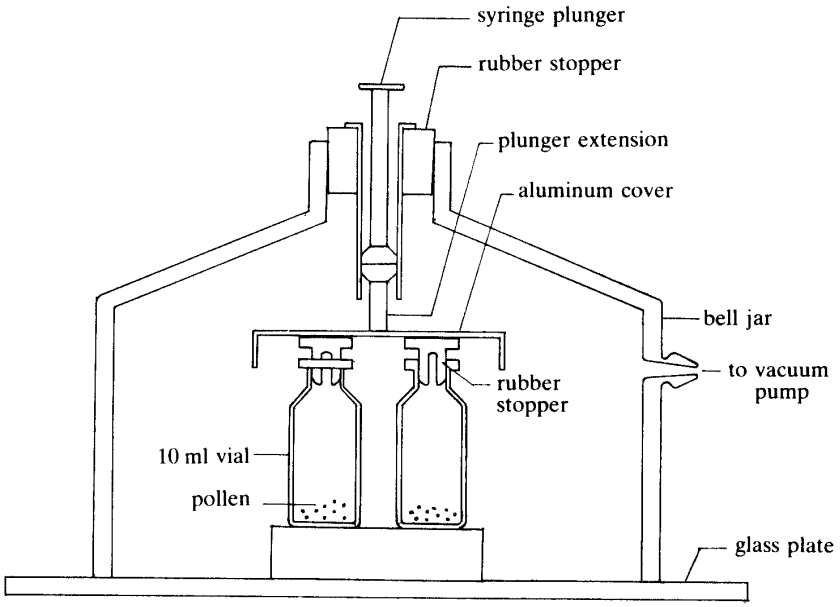


Fig. 2. Vacuum setup for hypobaric pollen storage.

Results and Discussion

Evaluation of pollen germination methods

Using 87C23 pollen, the average pollen germination percentages and standard errors in the two trials for the agar coated slide method, hanging drop method, and the modified hanging drop method were $25.0 \pm 2.4\%$, $27.5 \pm 1.9\%$ and $23.4 \pm 1.8\%$, respectively. The modified hanging drop method had the lowest mean and standard error among the three methods.

Evaluation of pollen germination on the agar medium (Fig. 1a) was complicated by the tendency of the pollen to clump together, obscuring the germination status of some pollen grains. Clumping, which has also been observed in onion[8], may be responsible for the high standard error of this method. This method also requires the preparation of fresh agar media prior to each evaluation.

The hanging drop method (Fig. 1b) allowed the highest pollen germination. However, the germination solution was observed to dry out after the 3 h germination period, increasing the possibility of error if this method is used to germinate pollen for more than 3 h. In addition, care had to be taken to prevent the germination medium from coming into contact with the ring spacer and consequently running down the sides.

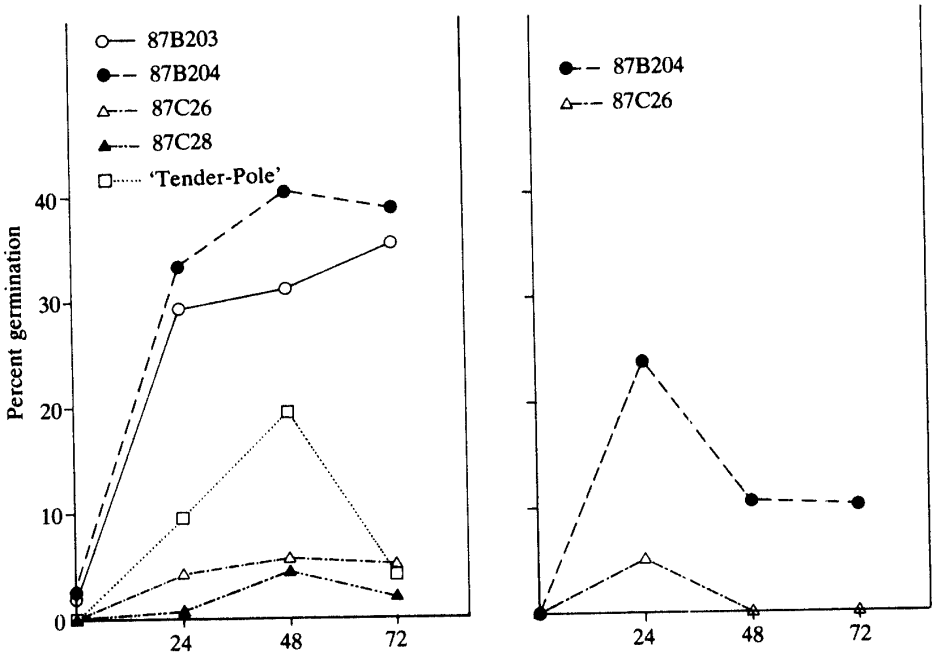
The low pollen germination percentage observed in the modified hanging drop method (Fig. 1c) was offset by the low standard error, ease and simplicity of the operation. Compared to the standard hanging drop method, this modified procedure was easier to implement and less prone to droplet evaporation. Compared to the agar-coated slide method, both hanging drop procedures had no problems with pollen clumping, since the pollen grains were stirred in for even distribution. The hanging drop procedures also allowed the subsequent addition of 1% acetocarmine in order to terminate pollen germination and to clarify pollen images upon microscpic evaluation.

Studies for germination protocols

A. Effect of pollen drying

Preliminary observations had indicated that around 20% of the pollen grains from any freshly dehisced anther of the *Allium* genotypes in this trial were immature, binucleate ones. Such pollen grains are classified as dessication tolerant, and they can survive low temperatures if the pollen is dried to a low moisture content prior to storage[4]. Drying is needed in order to minimize the deleterious effect of ice crystal formation in the cryopreserved cells[6].

Pollen of the freshly harvested anthers showed negligible germination, whereas pollen germinability increased after 24 and 48 h storage on silica gel. These facts indicate that a drying period is needed for pollen maturation and germination (Fig. 3a). The graph shows that 48 h may be the optimum drying time for most of the genotypes; extending the drying period drastically reduced pollen germinability in 'Tender-Pole'. Chang and Struckmeyer[1] also observed that onion pollen germinated best when stored at low (20%) humidities as compared to higher (50% and 80%) relative humidities prior to germination.



a. Drying on silica gel b. Air drying

Fig. 3. Effects of pollen drying time (h) and method.

In the air drying experiment, fewer anthers dehisced as compared to those dried on silica gel. In addition to the lower germinability, the germination percentage of the air dried pollen considerably declined after 24 h of storage (Fig. 3b). The poor performance of the air dried pollen may be due to the higher ambient humidity in the storage area.

B. Effects of time, sucrose, light and variety

The effects of time, sucrose concentration, light and variety on pollen germination percentages are shown in Table 2. The germination rates increased with time, but the degree of response was dependent on the genotype and light condition. In general, pollen germinated best in 10 to 20% sucrose solutions. For a 3 hour germination period under dark condition, 'Tender-Pole' pollen germinated best in 15 to 20% sucrose while 87C23 pollen did comparatively

Table 2. Average pollen germination percentage in 87C23 and 'Tender-Pole' as affected by time, light and sucrose

Allium accession	Light or dark	Sucrose concentration (%)	Time (h) 1	2	3
'Tender-Pole'	Light	5	4.2	8.3	1.7
		10	6.7	6.7	12.5
		15	13.3	27.5	33.3
		20	15.7	25.0	n.d.*
		25	9.2	10.0	31.7
'Tender-Pole'	Dark	5	1.7	2.5	7.5
		10	5.0	9.2	16.7
		15	41.3	40.0	39.2
		20	8.3	11.7	23.3
		25	15.8	16.7	20.8
87C23	Light	5	13.3	20.8	26.7
		10	7.5	36.7	54.2
		15	11.7	30.0	41.7
		20	5.0	21.7	55.8
		25	7.5	16.7	25.8
87C23	Dark	5	19.2	26.3	31.7
		10	12.5	35.8	58.3
		15	9.2	30.0	60.0
		20	3.3	15.0	46.7
		25	5.8	20.0	40.8

* No data

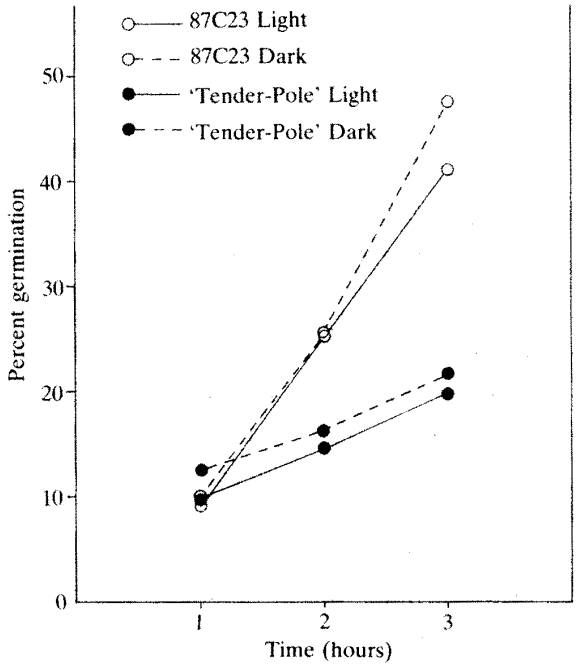


Fig. 4. Effects of light and time on *Allium* pollen germination. Averaged over five sucrose concentrations: 5, 10, 15, 20 and 25%.

better at 10 to 15% sucrose. In contrast with other *Allium* species[2,5], the 25% sucrose did not drastically reduce the germination percentage.

The pollen germination percentages for both genotypes, averaged over five sucrose percentages, from 1 to 3 h are shown in Fig. 4. The effect of light was not pronounced but pollen from both genotypes grown in the dark consistently showed a higher germination percentage. Thus, in subsequent tests, the pollen was germinated in 15% sucrose solution at 20°C in the dark for 3 h.

C. Effects of auxins

At 100 ppm concentration, all the auxins were inhibitory to pollen germination, although IBA had the least negative effect (Fig. 5a, b). At 10 ppm concentration, the three auxins improved the germination of 87C23 pollen and IBA showed the greatest effect, improving germination almost 3 times as compared to the control (Fig. 5a). The same degree of improvement was also observed at 10 ppm IBA in 'Tender-Pole' pollen (Fig. 5b). Kwan *et al.*[7] also observed the same depression in onion pollen germination with increased levels of IAA.

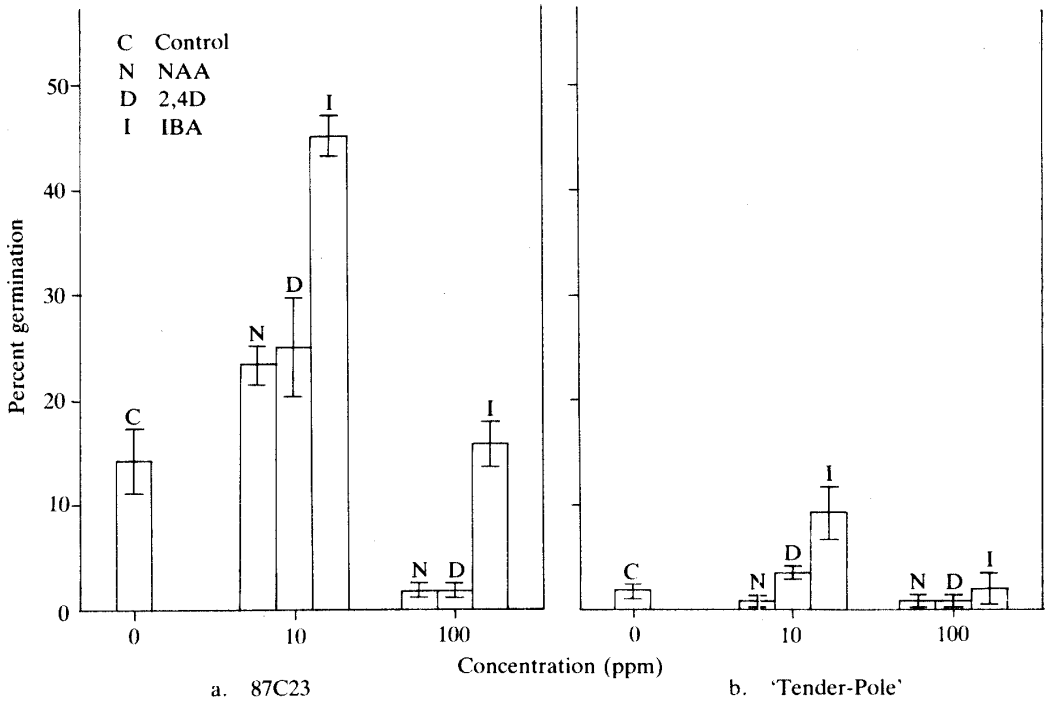


Fig. 5. Effects of auxins on *Allium* pollen germination.

Pollen viability tests by staining and germination

The data in Table 3 clearly show that there is no relationship between the percentages of stained pollen and the corresponding germination percentages. Thus, in subsequent experiments, pollen germination was used to measure the treatment effects on *Allium* pollen.

Differential staining with Giemsa, neotetrazolium chloride, or gentian violet was tried but failed (data not shown). Giemsa and gentian violet completely stained all pollen while neotetrazolium chloride was able to stain only the pollen cell wall. With the simplicity and speed of the modified hanging drop procedure, indirect evaluation of pollen viability in *Alliums* through staining seems superfluous.

Table 3. Comparison of acetocarmine staining and germination percentages in *Allium* pollen

Accession	Stained	Germinated
87C05	60.0	0.0
87C15	60.0	0.0
87C21	76.0	1.0
87C23	93.0	24.0
87C26	66.0	1.0
87C28	76.0	0.0
87B203	93.5	16.0
87B204	90.0	16.5
A. thunbergii	73.0	39.5
'Tender-Pole'	88.1	15.0

Low temperature storage

Allium pollen can be stored in liquid nitrogen[3,5]. However, storage in liquid nitrogen seemed unnecessary since the aim was only to store pollen to allow crosses between species flowering at different times in a year.

Pollen germination data in Table 4 were interpreted to indicate the suitability of the pollen for cold storage. Although pollen germinability decreased with time, the data clearly show the need of storing pollen with high initial germination percentages. Provided that pollen germination after two-month storage is beyond 50%, enough pollen (20–30%) will germinate for use in breeding after a year of storage. Storing pollen with low initial germination ability is futile.

The poor performance of 87C28 pollen may be due to problems with ploidy, because this accession is hexaploid. Pollen from the two triploid *A. chinense* accessions (Ra 38 and Ra 40) also showed very low germination after storage (data not shown).

The average effects of the 3 cold storage temperatures on pollen germination of the genotypes listed in Table 4 are shown in Table 5. Storage at 2~3°C can preserve the germinability of more than 50% of the viable pollen up to 8 months. Thus, when crosses between autumn and spring flowering *Alliums* are planned, pollen storage in 2~3°C will suffice provided that the

Table 4. Percent pollen germination of nine *Allium* accessions averaged over 3 cold storage temperatures (2~3, −20 and −40°C) for 12 months

Allium accession	Storage period (months)					
	2	4	6	8	10	12
87C02	51.1	47.8	37.2	37.9	31.2	19.6
87C05	14.4	1.4	3.3	1.9	0.8	0.8
87C12	13.4	3.6	4.4	1.7	1.1	0.2
87C13	31.7	16.9	18.8	16.5	12.3	9.4
87C15	11.7	13.9	4.6	0.4	1.0	0.8
87C21	65.0	47.5	52.8	48.8	40.4	33.1
87C23	64.2	58.9	60.3	55.4	37.5	26.9
87C28	31.2	0.8	2.9	1.9	0.2	0.6
'Tender-Pole'	19.6	0.8	12.7	3.0	3.9	2.9

Table 5. Percent pollen germination in 3 cold storage temperatures averaged over 9 *Allium* accessions

Temperatures (°C)	Storage period (months) 2	4	6	8	10	12
2~3	32.5	21.0 (35.4)*	19.1 (41.1)	17.2 (47.2)	7.1 (78.0)	4.8 (85.3)
−20	30.9	20.2 (34.8)	21.8 (29.6)	20.0 (35.3)	18.8 (39.3)	14.7 (52.6)
−40	28.8	24.5 (14.8)	24.7 (14.2)	18.6 (35.3)	16.9 (41.2)	12.3 (57.3)

* Percent reduction in pollen germination relative to actual germination after 2 months of cold storage.

initial germination is high. This result is in contrast with those of *A. victorialis* ssp. *platyphyllum*, whose pollen became non viable after only 2 months at 5°C storage[5]. When pollen needs to be stored for one year, as in late autumn flowering by early autumn flowering crosses, storage at −20°C is possible. The advantage of −40°C storage was quite pronounced in the first 6 months of storage but, beyond this period, pollen germinability dropped to levels similar to those stored at −20°C.

Hypobaric controlled atmosphere storage

Storage in partial vacuum, or hypobaric storage, reduces the amounts of gases available to the stored products. It has been shown to be very effective for fruits and cutflowers[10]. Although

Table 6. Percent germination of 'Tender-Pole' pollen after modified atmosphere storage in 3 temperature regimes

Temperature (°C)	Atmosphere*1	Months 0	2	4	6
2~3	PV	26.7	10.0 (37.4)*2	18.8 (70.4)	4.2 (15.7)
	PV+N_2	18.8	14.4 (76.6)	13.1 (70.0)	9.2 (48.9)
	PV+CO_2	27.5	31.3 (113.8)	14.4 (52.4)	6.7 (24.4)
−20	PV	26.7	15.6 (58.4)	15.0 (56.2)	14.2 (53.2)
	PV+N_2	18.8	16.9 (89.9)	7.5 (39.9)	5.8 (30.9)
	PV+CO_2	27.5	16.3 (59.3)	11.3 (41.1)	6.7 (24.4)
−40	PV	26.7	8.8 (33.0)	0.6 (2.2)	7.5 (28.1)
	PV+N_2	18.8	14.4 (76.6)	11.9 (63.3)	5.8 (30.9)
	PV+CO_2	27.5	12.5 (45.4)	7.5 (27.3)	7.5 (27.3)

*1 PV is partial vacuum at 20 cmHg Abs; N_2 is 5 ml of nitrogen gas while CO_2 is 5 ml of carbon dioxide gas per 10 ml vial.

*2 Germination percentage relative to initial (0 month) percent germination.

the treatments did not dramatically preserve pollen germinability, storage of *Allium* pollen for 6 months in partial vacuum was most effective at −20°C, but it was not beneficial at 2~3°C (Table 6). The addition of nitrogen gas helped to maintain pollen germinability at 2~3°C, but it showed no pronounced useful effects at lower temperature conditions. The addition of carbon dioxide was the least effective probably because high levels of carbon dioxide can cause effects similar to anaerobiosis[10]. These results indicate the need for further trials using pollen with higher germinability and different gas concentrations.

Summary

In order to expedite the development of ornamental *Alliums* from *Allium tuberosum* 'Tender-Pole' and several *A. chinense*×*A. thunbergii* progenies of different flowering seasons, studies on pollen storage and germination were implemented.

A modified hanging drop method was developed to evaluate pollen germination. As most of the pollen was immature at anther dehiscence, drying over silica gel for 48 h promoted pollen maturation and greatly improved germinability. Better pollen germination was obtained by keeping pollen in 15% sucrose and 10 ppm IBA solution for 3 h at 20°C in the dark. Cold storage maintained the germinability of about 50% of the viable pollen up to 6 months at 2~3°C and up to 12 months at −20°C. Pollen germination rate varied among entries.

Preliminary examination of hypobaric (20 cmHg) controlled atmosphere (nitrogen or carbon dioxide gas) for pollen storage indicates that carbon dioxide gas is ineffective and that additional nitrogen gas can maintain germinability of 50% of the viable pollen of *A. tuberosum* 'Tender-Pole' up to 6 months at 2~3°C.

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