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In vitro Germination and Viability of *Dendranthema* pollen

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Abstract: Five wild *Dendranthema* species and three cultivars were used to investigate a reliable method to evaluate pollen viability. Pollen grains were cultured in a modified liquid Monnior medium (ME_3) supplemented with PEG 4000 (0-32%) and sucrose (0-30%). Sucrose was not essential for pollen germination and PEG 4000 is an effective inducer of pollen germination in *Dendranthema*. Germination at pH 6.0 was stimulatory. Sixteen percent PEG 4000 combined with 12% sucrose gave the best results and germination percentage was from 69.4 to 76.4% for the above wild species and cultivars, respectively. Percentage of pollen stained with a 2% acetocarmine solution was from 85.3 to 96.3% and no correlation between pollen stainability and germination was found. *D.* × *grandiflorum* pollen could be stored for a short period of 10 days in wet condition at 4°C. However, viability of *D. indicum* and *D. japonicum* pollen was lost after 10 days of storage in the same condition.

Key words: *Dendranthema, in vitro* pollen germination, pollen stainability, pollen storage, polyethylene glycol 4000

INTRODUCTION

Chrysanthemum (*Dendranthema* × *grandiflorum* (Ramat.) Kitamura) is one of the most important global cut flower and pot plants. Breeding of the new pedigrees depends mainly on the conventional crossing method. In the genus *Dendranthema*, there are many wild species which are not yet as materials in breeding programs, although these wild species have useful characters not available among the cultivars. However, wild species and cultivars show extensive variation in flowering response and this may be an obstruction in cross pollination.

In a compatible cross, pollen viability affects seed setting, hence, a quick and reliable method of testing pollen viability is essential to determine the optimum time for pollination. Pollen viability can be assessed by staining with nuclear and by *in vitro* germination tests[1]. In *Dendranthema*, the viability of pollen among species is variable. Endo and Inada[2] reported that *D.* × *grandiflorum* cultivars showed conspicuous variation in pollen stainability. Tsukamoto and Matsubara[3] found that pollen of *D.* × *grandiflorum* cultivars was low germination percentage in sucrose solutions with boric acid. However, pollens of *D. indicum* and its hybrids with *D.* × *grandiflorum* cultured on a modified liquid Monnior medium[4] with polyethylene glycol 4000 showed very high germination percentage[5], respectively.

In addition, information on *Dendranthema* pollen storage is relatively limited. Tanaka[6] mentioned that *D.* × *grandiflorum* pollen had fertilization ability after one month of storage at low temperature, however, no detailed data was showed. The purpose of this study was to establish a method for *in vitro* pollen germination and pollen storage of genus *Dendranthema*.

MATERIALS AND METHODS

Plant materials: Middle October or early November flowering-type of five wild *Dendranthema* species and three cultivars (*Dendranthema* × *grandiflorum*: Mibu-wase, Aomori-kii, Ban-giku and Kishi-no-hakujuji) cultured in non-heated plastic film greenhouse at Iwate University, Japan, were used. Pollen grains were collected from the tubular florets just after flowered between 9.00 and 10.00 am from October to November in 2002.

Pollen germinating media and germination tests: The basal medium for pollen germination was a modified liquid Monnior medium (excepted sucrose) (ME_3 medium[4]). Three wild *Dendranthema* species and one cultivar were used to study the effect of the different levels of polyethylene glycol (molecular weight approximately 4000, PEG 4000) (0 to 32%), sucrose (0 to 30%) and pH (4 to 7) on pollen germination. The pH was adjusted

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with HCl or KOH. Approximately 20 μL of the liquid medium were placed on the slide glass and pollen grains were gently dipped into the medium. The slide glass was placed on a piece of filter paper soaked with the same medium in a plastic dish (90×15 mm), then the dish was incubated for 2 h at 20°C under natural light in room. The pollen grains were then observed under a light microscope. More than 500 pollen grains were observed to judge the percentage germination and 3 repetitions were performed. Pollen grains which the pollen tube had elongated longer than the diameter of the pollen grain were scored as germinated.

Pollen stainability: The pollen was stained with a 2% acetocarmine solution and then observed under a light microscope to evaluate their viability. Darkly stained pollens were counted as viable and pollens with no or very light stain were counted as nonviable ones. Pollen stainability was evaluated about 1,000 grains and 3 repetitions were performed.

Pollen storage: Pollen grains were wrapped in pieces of paraffin paper and then placed in a tight shut plastic bottle with a moist filter paper (wet condition) or a certain amount of silica gel (dry condition) and stored at 4°C. Pollen viability before and after storage was determined by *in vitro* germination tests in which at least 500 pollen were counted and 3 repetitions were performed.

RESULTS

Establishment of pollen germination medium: Preliminary experiments with agar or liquid medium with 15, 20 and 30% sucrose containing 10 mg L^{-1} boric acid (data not shown) were carried out. No pollen germination was observed.

Effects of PEG 4000 concentration: The effect of PEG 4000 of the medium on *in vitro* germination of pollen was examined using three wild *Dendranthema* species and one cultivar. In this experiment, as an alternative osmoticum PEG 4000 was substituted for sucrose in ME_3 medium. Sucrose was not essential for pollen germination and the effects of four concentrations of PEG 4000 are given in Table 1. For *D. boreale*, approximately 21.1% pollen germinated at 16% PEG 4000 only. Germination percentages were 3.8 to 8.2% at 14 % PEG 4000 and reached maximum of between 26.7 and 30.4% at 16% PEG for other species.

Effects of combining PEG 4000 with sucrose: As preliminary study, *D.* × *grandiflorum* cv. Mibu-wase was used to investigate the effect of combining PEG 4000 (0 to 32%) with sucrose (0 to 25%) of the medium on pollen germination, showing the best result in the medium accompanied with combination of 16% PEG and 12% sucrose, no pollen germination was, however, observed in the medium without PEG (Table 2). Therefore, pollens of three wild *Dendranthema* species and one cultivar were cultured in the medium supplied with 16% PEG and different concentration of sucrose (8 to 30%). The highest germination percentage showed on the medium with combination of 16% PEG and 12% sucrose for all species (Table 3), as *D.* × *grandiflorum* cv. Mibu-wase.

Table 1: Effect of PEG 4000 concentration on in vitro germination of pollen[z]

PEG 4000 (%)	Di	Db	Dw	DgM
14	5.0±0.2a[y]	0a	3.8±0.4a	8.2±0.4ab
16	26.7±0.2b	21.1±0.2b	28.6±0.4b	30.4±0.4a
17	6.1±0.1ab	0a	4.1±0.2a	7.6±0.4b
18	5.4±0.1a	0a	2.1±0.3c	6.3±0.5b

[z]Di, *D. indicum;* Db, *D. boreale;* Dw, *D. weyrichii;* DgM, *D.* × *grandiflorum* cv. Mibu-wase, [y]Means±SE followed by the same letter are not significantly different at a 0.05 probability level by t-Test

Table 2: Effect of combination of PEG 4000 and sucrose on pollen germination of *D.* × *grandiflorum* cv. Mibu-wase

Sucrose (%)	PEG 4000 (%)	Pollen germination (%)
0	0	0a[z]
0	16	4.6±1.2b
8	0	0a
8	8	5.2±0.4bc
8	16	6.6±1.0c
8	32	4.0±0.9b
12	0	0a
12	8	27.8±1.0d
12	16	69.9±1.7e
12	32	47.0±0.6f
25	0	0a
25	8	0.2±0.4bc
25	16	0.6±1.6c
25	32	0a

[z]Means±SE followed by the same letter are not significantly different at a 0.05 probability level by t-Test

Table 3: Effect of sucrose concentration of the medium with 16% PEG 4000 on pollen germination[z]

Sucrose (%)	Pollen germination (%)			
	Di	Db	Dw	DgA
8	10.0±0.5a[y]	5.5±0.4a	19.7±0.4a	12.1±0.4a
12	76.4±0.3b	75.9±0.6b	69.6±0.5b	71.4±0.5b
18	41.5±0.3c	33.1±0.2c	21.4±0.4a	45.3±0.6c
25	1.6±0.3d	1.0±0.6d	3.1±0.6c	23.1±0.6d
30	0d	0d	0d	0e

[z]Di, *D. indicum;* Db, *D. boreale;* Dw, *D. weyrichii;* DgA, *D.* × *grandiflorum* cv. Aomori-kii; [y]Means±SE followed by the same letter are not significantly different at a 0.05 probability level by t-Test

Effects of pH of medium: Percentage germination in pollens of three wild *Dendranthema* species and one cultivar were measured under different pH conditions (4.0-7.0). For *D. boreale*, percentage of germination varied between 19.7 and 72.1% in the pH range of 5.0 and 6.0,

although pollen did not germinate at pH 4.0 and 7.0. For other species, at pH 6.0 pollen germination was significantly higher ($p < 0.05$) than that at pH 4.0, 5.0 and 7.0 (Table 4).

In the following studies, pollen germination was assayed on the ME_3 medium (pH 6.0) containing 16% PEG and 12% sucrose.

Pollen stainability and germination of wild *Dendranthema* species and cultivars: Pollens of five wild *Dendranthema* species and four cultivars were stained with a 2% acetocarmine solution and then observed under a light microscope to evaluate their viability. Pollen stainability was not different (85.3 to 86.0%) between *D.* × *grandiflorum* cvs. Mibu-wase and Aomori-kii, but significantly lower ($p< 0.05$) than the five wild *Dendranthema* species and two cultivars (89.4 - 96.3%) (Table 5). However, pollens of these species were cultured in ME_3 medium supplemented with combination of 16% PEG and 12% sucrose (pH 6.0), percentage of germination was between 63.0 and 78.7%, showing distinct difference ($p < 0.05$) in these species and cultivars respectively (Table 5).

Pollen storage: Pollens of two wild *Dendranthema* species and two cultivars were stored in different conditions. After 10 and 30 days of storage, percentage of pollen stained with 2% aceto carmine solution reduced little less than that of fresh pollen (data not shown), however, germination percentage of pollen in vitro cultured in ME_3 medium supplemented with combination of 16% PEG and 12% sucrose (pH 6) decreased dramatically (Table 6). Germination percentage of fresh pollen was between 56.5 and 78.7%. When pollens were stored in wet condition with a moist filter paper at 4°C, germination percentage decreased to one fourth compared with fresh pollen for 2 cultivars of *D.* × *grandiflorum* and one eighth for *D. indicum* and *D. japonicum* after 10 days. After 30 days of storage, germination percentage of pollens of *D. indicum* and *D.* × *grandiflorum* cv. Aomori-kii decreased to 1.0 and 2.8%, but no pollen germination was observed in *D. japonicum* and *D.* × *grandiflorum* cv. Ban-giku. When pollen was stored in dry condition, however, the viability was lost after 10 days for all species and cultivars (Table 6).

Table 4: Effect of pH on pollen germination[z]

	Pollen germination (%)			
pH	Di	Db	Dw	DgA
4	0.4±0.1a[y]	0a	2.4±0.1a	3.1±0.3a
5	15.2±0.2b	19.7±0.3b	22.6±0.2b	17.7±0.7b
6	78.9±0.8c	72.1±0.8c	63.0±1.7c	53.5±0.4c
7	14.2±0.2b	0a	16.0±0.1d	21.9±0.2b

[z]Di, *D. indicum;* Db, *D. boreale;* Dw, *D. japonicum;* DgA, *D.* × *grandiflorum* cv. Aomori-kii; [y]Means±SE followed by the same letter are not significantly different at a 0.05 probability level by t-Test

Table 5: Pollen stainability and germination in *Dendranthema* species and cultivars[z]

Species and cultivars	Pollen	
	Stainability (%)	Germination (%)
Di	95.9±0.1a[y]	78.7±1.7a
DiA	92.6±0.8b	64.1±0.9b
Db	96.3±0.1a	78.8±0.8a
Dj	89.4±0.6b	56.5±0.8c
Dw	92.0±2.0b	63.0±1.7b
DgM	85.3±1.2c	73.5±0.4e
DgA	86.0±0.5c	78.4±1.0a
DgB	90.8±2.8b	69.2±0.4f
DgK	92.4±3.4b	71.4±2.1ef

[z]Di, *D. indicum;* Db, *D. boreale;* DiA, *D. Indicum* var *aphrodite* Dj, *D. japonicum* DgM, *D.* × *grandiflorum* cv. Mibu-wase; DgA, cv. Aomori-kii DgB, cv. Bann-giku; DgK, cv. Kishi-no-hakujuji

[y]Means±SE followed by the same letter are not significantly different at a 0.05 probability level by t-Test

Table 6: Pollen germination after storage[z]

Species and cultivars	Treatment			
	Wet			Dry
	0 day	10 days	30 days	10 days
Di	78.7±1.7a[y]	19.9±1.7b	1.0±0.1c	0d
Dj	56.5±0.8a	7.1±1.2b	0c	0d
DgA	78.4±1.0a	20.2±1.3b	2.8±0.3c	0d
DgB	69.2±0.4a	17.7±0.7b	3.0±0.3c	0d

[z]Di, *D. indicum;* Dj, *D. japonicum;* DgA, *D.* × *grandiflorum* cv.Aomori-kii; DgB, cv. Bann-giku

[y]Means±SE followed by the same letter are not significantly different at a 0.05 probability level by t-Test

DISCUSSION

Tsukamoto and Matsubara[3] reported that pollen germination of *D.* × *grandiflorum* was 10% in liquid medium with sucrose and boric acid. Ikeda and Numata[7] showed that germination percentage was very low (5%) on an agar medium. However, in our preliminary studies, no pollen of *D.* × *grandiflorum* and wild species germinated in/on liquid or agar medium with sucrose and boric acid (data not shown).

Tang *et al.*[5] reported that pollen of *D. indicum* and its hybrids with *D.* × *grandiflorum* germinated in ME_3 medium supplemented with sucrose alone and germination percentage was more higher in the medium supplemented with PEG 4000. The similar phenomena were generated on pollen of *Capslla bursa-pastoris*[4], *Fagopyrum esculentum*[8]. In these plants, sucrose was essential for pollen germination and PEG 4000 could be used as an alternative osmoticum in pollen germination medium. However, in the present study, pollen of *D.* × *grandiflorum* did not germinate in the medium with sucrose alone, but germinated in the medium with PEG

4000 alone and germination percentage increased with sucrose application. This result was consonant with Wang's study[9], showing that sucrose was not essential in pollen germination of *D.* × *grandiflorum,* but could accelerate its germination.

It has been reported that absorption of PEG by plant cells is inversely related to its molecular size[10]. This indicates that high molecular weight PEG was possibly not absorbed by pollen, but worked as an osmoticum in the medium. Theoretically, when the osmotic pressure of a medium is lower than that of the pollen, water is forced into the pollen grain from the medium. As a result, the best result of pollen germination can be obtained in the medium with optimal molecular weight PEG and the cell wall at the aperture of the pollen grain cannot sustain the additional pressure and bursting occurs at a high molecular weight PEG. Wang[9] investigated the effect of the molecular weight of PEG on *D.* × *grandiflorum* pollen germination using various molecular weight PEGs and showed that only PEG 1500 stimulated pollen germination. However, in our preliminary studies, *Dendranthema* pollen germination was lower in medium supplemented with PEG 1540 and 6000 than that with PEG 4000 and there was a little bursting of pollen grains in these media (data not shown). The similar results were obtained on pollen germination of *C. bursa-pastoris*[4].

In this study, germination percentage of *Dendranthema* pollen increased significantly in the medium with combination of PEG and sucrose compared with PEG only. Likewise, in *C. bursa-pastoris*[4], as well as *D. indicum* and its hybrids with *D.* × *grandiflorum*[5], pollen germination percentage was higher in the medium supplemented with PEG and sucrose than that with PEG alone. Moreover, in the present study, the highest percentage was obtained in ME_3 medium supplemented with combination of 16% PEG and 12% sucrose for *Dendranthema*, in common with *D. indicum* and its hybrids with *D.* × *grandiflorum*[5].

Leduc *et al.*[4] reported that the pollen germination substantially increased at pH 4.0 in *C. bursa-pastoris.* In *Fagopyrum esculentum*, the maximum germination of pollen was observed at a pH 5.0[8]. However, in this study, germination of *Dendranthema* pollen was highest at a pH 6.0 and significantly decreased at pH 7.0, 5.0 and 4.0. Tang *et al.*[5] also showed the similar result in *D. indicum* and its hybrids with *D.* × *grandiflorum.*

In *D.* × *grandiflorum*, pollen stainability was not different (85.3-86.0%) between two cultivars Mibu-wase and Aomori-kii, while germination percentage differed significantly (73.5-78.4%) between these two cultivars. However, both pollen stainability and germination percentage did not differ between cvs. Ban-giku and Gishi-no-hakujuji. It was, therefore, not possible to find any correlation between pollen stainability and germination in *D.* × *grandiflorum* and this was consistent with result of Kawase and Tsukamoto[11]. Likewise, no correlation between pollen stainability and germination of wild species was observed.

Ikeda and Numata[7] reported that the ray floret extract by ethyl ether or ethyl acetate advanced pollen germination of *D.* × *grandiflorum* cv. Salmon. In the present study, however, the effect of the ray floret extract on pollen germination was not investigated. Therefore, further examination in genus *Dendranthema* is needed.

Ikeda and Numata[7] reported that *D.* × *grandiflorum* cv. Salmon pollen could be stored for a short period of 10 days or so at low temperature (1°C) and for 35 days with benzene and toluene. In the present study, however, when *Dendranthem* pollen was stored in wet condition at 4°C, pollen germination decreased to one fourth for *D.* × *grandiflorum* and one eighth for *D. indicum* and *D. japonicum* compared with fresh pollen after 10 days; after 30 days, pollen germination significantly decreased to 0-3.0%. Further study, such as, effect of benzene and toluene on pollen storage is necessary.

It may be concluded from the present study that the modified liquid Monnior medium (ME_3 medium[4]) supplemented with combination of 16% PEG and 12% sucrose and then pH adjusted to 6.0, could be used for pollen germination in genus *Dendranthema*. There was no correlation between pollen stainability and germination in genus *Dendranthema*. *D.* × *grandiflorum* pollen could be stored for a short period of 10 days in wet condition at 4°C. However, viability of *D. indicum* and *D. japonicum* pollen was lost after 10 days of storage in the same condition.

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