Breeding of Red-flowering Delphiniums using Interspecific Hybrids Produced by in Vitro Germination

Norio Katoh*1, Koji Tokuhiro, Toshihiko Nakabayashi, Hiroyuki Yoshida and Manabu Hagimori

Japan Tobacco Inc., Applied Plant Research Center, 1900 Idei, Oyama, Tochigi 323-0808, Japan
1 Present address: Japan Tobacco Inc., Plant Innovation Center, 700 Higashibara, Toyoda, Iwata, Shizuoka 438-0802, Japan

Interspecific crosses between Delphinium elatum and D. nudicaule or D. cardinale were carried out to produce red-flowering Delphinium varieties. An in vitro germination technique was effective to produce interspecific hybrids. From 800 seeds, 379 interspecific hybrid plants were produced. The form of these hybrid plants was intermediate between that of the parents and their flower color was purplish due to the presence of delphinidin derivatives. Most of these hybrid plants were fertile. The F2 generation was self- and sib-crossed. In the second generation, six plants with pink or red flowers were selected from 224 plants.

Key Words: Delphinium, interspecific hybrid, in vitro germination, pelargonidin, D. nudicaule, D. cardinale.

Introduction

The genus Delphinium in the family Ranunculaceae comprises about 300 species (Wilde 1931). Wild species of Delphinium are mostly distributed in the northern hemisphere. Delphinium includes horticulturally important species, such as D. elatum, D. belladonna and D. grandiflorum, with many cultivars showing mostly blue, white or violet flowers. Legro (1961) succeeded in producing red-flowering delphiniums by interspecific hybridization of red-flowering D. nudicaule and D. cardinale with D. elatum. The red-flowering delphiniums were further improved as garden plants for such important traits as plant height and density of flower spikes, and gave rise to commercial varieties designated as “University Hybrids” in the 1980’s. However, these cultivars were sterile and it became necessary to propagate them vegetatively. Moreover, they have not become popular and they can not be used as breeding materials. It remains to be determined whether the new red-flowering delphinium varieties can be produced by a similar breeding program.

In Delphinium, the pigments of the flowers which comprise anthocyanins (pigmented) and flavonols (colorless), are synthesized through the flavonoid pathway. The blue and white color of the flower of D. elatum is associated with delphinidin derivatives and precursors, respectively, but red or orange flowers of the two wild Delphinium species, D. nudicaule and D. cardinale contain pelargonidin derivatives. Generally, delphinidin is dominant to pelargonidin in various species (Beale 1941). Legro (1961) and Honda et al. (1999) suggested that delphinidin is dominant to pelargonidin in Delphinium based on their crossbreeding results. Thus the flower color of the hybrids between red-flowering wild species and D. elatum was assumed to be blue or purple due to the presence of delphinidin derivatives. When these wild species (2n = 2X = 16) are used as breeding materials crossed with D. elatum (2n = 4X = 32), doubling their chromosomes is necessary to restore the fertility of the hybrids. In this case, one-thirty sixth of the progeny produced by self-crossing or sib-crossing of the hybrids is expected to contain pelargonidin. It is necessary to have more than 105 plants in the second generation to select at least one red-flowering individual at 95% level of probability (n >= log(1 – 0.95)/log(1 – 1/36)).

In the present study, we produced red-, orange- or pink-flowering Delphinium varieties as breeding materials. We used interspecific hybridization and in vitro germination to raise many hybrid plants efficiently. These hybrids were selfed or crossed with each other. Red-flowering fertile plants were obtained from the offspring. Some promising plants as horticultural varieties were selected in later generations. An efficient method to produce red-flowering delphiniums using in vitro germination is described here.

Materials and Methods

Plant materials and cross-pollination

Two wild Delphinium species, D. nudicaule (2n = 2X = 16, Sahin Alphen aan den Rijn, Holland) and D. cardinale (2n = 2X = 16, Fukukaen Nursery & Bulb Co., Ltd., Nagoya, Japan) were used as genetic source of the red color of the flower. D. nudicaule has orange to orange-red flowers and D. cardinale has scarlet flowers. However, horticulturally important traits, such as spike density, flower size, flower number, flower shape are limited. D. elatum cv. Galahad (2n=4X=32, Fukukaen Nursery & Bulb Co., Ltd., Nagoya, Japan) was used as another parent with superior horticulturally important traits.

The chromosome number of the two wild species is 2n = 16, whereas that of D. elatum is 2n = 32. Therefore, polyploidization of D. nudicaule and D. cardinale was performed
before crossing. Shoots of young seedlings with 3–5 leaves were dipped into a 0.1% colchicine solution at 20°C overnight. Then, the plantlets were cultivated in a greenhouse. In a preliminary experiment, the pollen size showed a positive correlation with the ploidy levels determined by flow cytometry (Partec PA-1, Munster, Germany). Thereafter, the ploidy levels were checked by comparing the pollen size with that of diploid plants as controls (Philippi 1961).

_D. elatum_ (2n=32, 4X) was crossed with somatically doubled _D. nudicaule_ (2n=32, 4X) or _D. cardinale_ (2n=32, 4X), with the two wild species as mother plants and _D. elatum_ as the pollen donor. Flowers of the mother plants were emasculated before anthesis and were hand-pollinated with fresh pollen collected from pollen donors in a greenhouse. At about three weeks after pollination, the fruit capsules whose color changed to brown were harvested and seeds were collected and kept in desiccators.

**Germination of hybrid plants and their cultivation**

A few months after harvest, the seeds were surface-sterilized with a sodium hypochlorite solution containing 1% active chlorine for 10 min and were rinsed twice with sterilized distilled water. Then six seeds were placed in each glass tube containing 1/2 MS medium (Murashige and Skoog 1962, Honda and Tsutsui 1997, Honda et al. 2003) supplemented with 2% sucrose and 0.8% agar in the absence of plant growth substances. The pH of the medium was adjusted to 5.8 with 0.1 N KOH before autoclaving. The seeds were incubated at 24°C under a 12 h photoperiod by using white fluorescent lamps (6 W·m⁻²). After three weeks, only about 10% of the seeds germinated. The seed coat of the seeds that had not germinated was removed partly or wholly using a scalpel. Then the seeds were cultured again on the same medium. They germinated in a few weeks. The germinated plants were potted in sterilized soil and were cultivated in a greenhouse.

**Production of hybrid’s progeny**

To obtain the next generation, seeds were produced by selfing and sib-crossing. In the second generation, plants containing pelargonidin in the flowers were self-crossed and they were sib-crossed with plants having a form similar to that of _D. elatum_. In vitro germination method was used for the seeds of each generation.

**Evaluation of flower color**

In the first and second generations, the plants were classified into flowers with two color types, the “delphinidin type” including pale purple, purple and dark violet flowers, and the “pelargonidin type” including pink, red and orange flowers.

**Anthocyanidin analysis**

For pigment analyses, petal (petal and sepal) samples of 10 individuals randomly selected from the F₁ generation and 6 individuals with flowers of the pelargonidin color type in the F₂ generation were used. Fresh petals were cut into strips and anthocyanins were extracted with methanolic 0.1% HCl overnight in the dark. Extracts were filtered through a 0.45 μm syringe-driven filter unit (Millex-HV, Millipore Co., Bedford, MA, USA) and were concentrated by using SEP-PAK Cartridges (Millipore Co., Milford, MA, USA). Each extract was hydrolyzed with 2N-HCl for 1 h at 95°C to liberate anthocyanidins. After hydrolysis, the concentrates and authentic standards were streaked on Avicel cellulose plates (Funakoshi Co., Ltd.) and were subjected to thin-layer chromatography (TLC) in HO-Ac·HCl·H₂O at 30 : 3 : 10.

**Results**

**Interspecific hybrid (F₁) generation**

Many tetraploid plants were obtained by the colchicine treatment. For _D. nudicaule_ and _D. cardinale_, 48 out of 90 and 2 out of 5 colchicine-treated plants, respectively, were tetraploid. To obtain interspecific hybrids, _D. nudicaule_ (4X) and _D. cardinale_ (4X) were pollinated with _D. elatum_ pollen. Pods developed normally in most of the plants. Many seeds were obtained from these combinations. When 25 seeds for each of the four strains were sown by the conventional method in a preliminary experiment, only eight seedlings from one strain were observed. These seeds were cultured on 1/2MS medium in glass tubes after sterilization. Three weeks after the start of the culture, about 10% of the seeds had germinated. The seed coat of the seeds that had not germinated was removed partly or wholly and the seeds were cultured again. Thereafter most of the seeds germinated and grew into seedlings (Fig. 1). Finally, a total of 379 interspecific hybrid plants was obtained from 800 seeds. Among these hybrids, only two plants were derived from the cross between _D. cardinale_ and _D. elatum_.

The flower color of the hybrids from _D. nudicaule_ × _D. elatum_ ranged from pale to deep purple. Analysis of the anthocyanidins by TLC showed that these hybrids contained only delphinidin (Fig. 2). Flower shapes ranged from the half-closed _D. nudicaule_ type to the wide open _D. elatum_ type (Fig. 3, Fig. 4A and 4B). The diameter of the flower ranged from 1.3 to 4.5 cm, and the form of the inflorescence of the hybrid varied from the _D. nudicaule_ type to the _D. elatum_ type. Hybrids from _D. cardinale_ and _D. elatum_ had purple flowers that were wide open and 35–45 mm in diameter (Fig. 4C).

The F₁ generation was self-crossed and sib-crossed. Seeds were obtained by both crossing methods but sib-crossing yielded more seeds.

**Second generation (the first generation after F₁)**

In the second generation, 224 flowering plants were produced by the embryo culture method. Among them, six plants showed red or pink flowers (Fig. 4D and 4E). To identify the pigment components of these flowers, the plants were analyzed by TLC, and pelargonidin was detected from all the six plants (Fig. 5). However, a clear trace of delphinidin
was detected in three of them and a faint trace of cyanidin was detected in all the six plants besides pelargonidin.

Analysis of flower color genetics

Segregation of flower color types is shown in Table 1. The flower color of all the plants in the F_1 generation corresponded to the delphinidin type. Among 224 plants in the second generation, six plants with flowers showing the pelargonidin color type were selected. This segregation was reasonably consistent with the expected 35:1 ratio based on the $\chi^2$ test (0.9 < $P$ < 0.95) assuming chromosome segregation. However, the $\chi^2$ test (0.1 < $P$ < 0.2) did not rule out the probability in accordance with the 20.8:1 ratio by chromatid segregation.

Third generation and progeny

In the third generation, 447 flowering plants were obtained by the same method as that used for the second generation. Forty-five plants showed pink, orange or red flowers. Breeding continued and many traits were modified in every generation. In the fifth generation, No. 5-183 (Fig. 4F) and No. 5-570 (Fig. 4G) that were considered to be important materials for the breeding of Delphinium were selected. These plants were 100 cm high with 38 and 40 flowers 4.5 cm in diameter with a carmine red and pink color, respectively.

Discussion

Legro (1961) obtained eight hybrid plants from D. nudicaule (4X) × D. elatum among 55 seeds produced from 277 crosses. He raised seedlings by setting up optimal germination conditions instead of by using the embryo rescue technique. We did not use his germination method. Generally speaking, however, ovule culture and in vitro germination are much more effective to produce interspecific hybrids than conventional sowing. In ovule or embryo culture, the timing of explant of the ovule or embryo affects the efficiency (Collins and Grosser 1984, Hu and Wang 1986). These culture techniques are effective to promote the development of embryos that cannot germinate readily. Honda and Tsutsui (1997) developed interspecific hybrids between D. grandiflorum and D. nudicaule by ovule culture. In this combination, ovule culture was necessary to produce the hybrids, and the period of 20 or 25 days after pollination was more appropriate to explant the ovule and start the culture than 30 days. However, in the present study, in vitro germination was used for mature seeds preserved for more than one month and was effective to promote the germination of hybrid seeds between D. nudicaule or D. cardinalae and D. elatum. To develop plantlets from the excised embryos, addition of plant growth substances to the culture medium was not necessary. The germination rates of the seeds in which the seed coat had been removed wholly and partly were identical. These results suggest that the low germination rate of hybrid seeds might be due to the excessive strength of their seed coat, and not to a defect in their embryo, and that a problem may exist between embryo and seed coat. Hybrid plants cannot be produced easily when D. elatum is

![Fig. 1. Segregation of flower color types in each generation.](image1)

![Fig. 2. Thin-layer chromatography analysis of anthocyanidin pigments of the hybrid plant between D. nudicaule (4X) and D. elatum. del: delphinidin, cya: cyanidin, per: pelargonidin.](image2)
Katoh, Tokuhiro, Nakabayashi, Yoshida and Hagimori

the maternal plant crossed with \textit{D. nudicaule} or \textit{D. cardinale} (Legro 1961). Therefore, in our study, \textit{D. elatum} was used only as a paternal plant.

It was observed that sib-crossing yielded more seeds than selfing. In some of the \textit{Delphinium} species, Bosch (1999) reported that the seed set after selfing averaged 72% of the seed set after outcrossing, and Darwin (1876) reported a value of 59\% for \textit{Delphinium consolida} (= \textit{Consolda regalis}), suggesting that partial self-compatibility is widespread in the tribe Delphineae. It appeared that our breeding materials also displayed a partial self-compatibility.

In the present study, all the F$_1$ hybrid plants had blue or purple flowers associated with the presence of delphinidin derivatives. Honda et al. (1999) also detected only delphinidin in the F$_1$ hybrids between \textit{D. cardinale} and \textit{D. grandifolium}. These results indicate that delphinidin is dominant to pelargonidin in \textit{Delphinium}. Therefore, the genotype controlling the delphinidin expression in \textit{D. elatum} was represented by “\textit{DDDD}”, whereas the genotype controlling the pelargonidin expression in \textit{D. nudicaule} (4X) and \textit{D. cardinale} (4X) was represented by “\textit{dddd}”. In this case, the genotype of the F$_1$ hybrids was assumed to be “\textit{DDdd}”, controlling the delphinidin expression. In the second generation, six plants that contained pelargonidin as flower pigment were observed in 224 plants. This ratio (6/224) was close to the theoretically expected ratio of 1/36 based on the $\chi^2$ test. The results suggested that “\textit{dddk}” was the genotype of the plants that contained pelargonidin. However, pelargonidin coexisted with delphinidin in three plants, and thus delphinidin was not simply dominant to pelargonidin. Moreover, cyanidin

![Fig. 3. Flower shape of plants used as materials. A: \textit{D. elatum}, B: \textit{D. cardinale}, C: \textit{D. nudicaule}.](image3)

![Fig. 4. Hybrid plants cultured in the F$_1$ (A–C), the 2nd (D, E) and 5th generations (F, G).](image4)

![Fig. 5. Thin-layer chromatography analysis of anthocyanidin pigments in the second generation. Lanes 1--6: selected plants, del: delphinidin, cya: cyanidin, per: pelargonidin.](image5)

<table>
<thead>
<tr>
<th>Table 1. Segregation of flower color types in each generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>1st</td>
</tr>
<tr>
<td>2nd$^{11}$</td>
</tr>
</tbody>
</table>

$^{11}$ reasonably consistent with the theoretical ratio in the case of chromosome segregation. ($\chi^2=0.0082, 0.9<P<0.95$).
was observed in six plants as indicated by TLC. Further studies should be carried out to analyze the biosynthesis of anthocyanidins in *Delphinium*. The flower color of the F₁ hybrid generation ranged from pale to deep purple. The flowers of the hybrids derived from a cross with *D. nudicaule* individuals with reddish flowers tended to be deep purple, while those of the hybrids derived from a cross with *D. nudicaule* individuals with bright orange flowers were pale purple. To elucidate the detailed mechanism of flower color expression, the amount of anthocyanidin pigments should be determined.

In the present study, new red-flowering delphiniums were obtained in the second generation. We conclude that the in vitro germination technique is effective to produce hybrid *Delphinium*. Breeding was pursued by using this technique and many plants were selected in the progeny of the fourth generation. Since these plants can be propagated by using a tissue culture technique, they could be developed as cultivars. We hope that the breeding method described in this study will contribute to future *Delphinium* breeding.

Acknowledgement

We thank Ms. Tokiko Abe for her outstanding technical assistance.

Literature Cited


