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Studies on the Development of New Ornamental *Allium* through Interspecific Hybridization III. Hybridization of Autumn-Flowering Species through Pull-Style Pollination, Cutflower Culture and Embryo Rescue

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Introduction

Our experience with subgeneric crosses involving spring- and autumn-flowering *Allium* species coincides with those of van der Valk et al.[11] who concluded that strong pre-fertilization barriers, e.g., stylar incongruity, drastically limit the success of wide crosses. Using conventional pollination procedures, such crosses usually result in very low fruit set. Flower stalks usually wilted within two weeks after pollination in wide crosses while selfed inflorescences produced mature seeds, indicating selective embryo abortion, rather than nutrient competition. In some cases, wilting proceeded from the base of the flower stalk, implying that the bulb was somehow affecting the reproductive process. In some ornamental *Alliums*, bulb proliferation occurred at the expense of sexual reproduction[1].

In *Lycoris* Tokugawa and Emoto[10] proposed that the bulb may be competing against the developing seeds for nutrients because the only way they could produce seed from this genus was to cut off the flower stalk around the time of blooming. We have observed that *A. giganteum* cutflowers maintained in tap water produced viable open-pollinated seed. This observation is supported by Rabinowitch's suggestion that, in species with thick flower stalks like leek and onion, the scape seems to be the main source of photosynthate for the developing seeds[9].

We have reported the production of only one trispecific [(*A. chinense*×*A. thunbergii*)×*A. tuberosum* 'Tenderpole'] hybrid using conventional pollination techniques[2]. In this paper we report our attempts to improve this record through modified pollination, cutflower culture and embryo rescue techniques.

Materials and Methods

Materials

The maternal parents consisted of several autumn-flowering clones derived from the basic *A. chinense*×*A. thunbergii* crosses developed by Dr. Yoshitake of Fukui University. In addition to the materials already described in the previous paper[2], we also used the following new clones provided by Dr. Yoshitake: 87C20, 87B207 and 88$F_2$111.

As paternal parents, we used fresh pollen from three 'everflowering' strains of *A. tuberosum* 'Tenderpole' (T01, T02, T03). Through a pollen storage procedure described in an earlier

report[3], we used stored pollen from two spring-flowering *Allium* species (*A. cowanii* and *A. giganteum*) and the summer-flowering cultivar 'Murasame'.

Modified pollination techniques

Around 10 to 15 florets (out of 40-60) were selected and emasculated from each flower stalk. Parchment paper bags were used to exclude unwanted pollen. When the styles became receptive[8], pollination was performed using the standard method of brushing the pollen on receptive stigma, or through the pull-style or cut-style pollination techniques.

The pull-style technique basically involved pulling out the receptive style, brushing its base with the desired pollen, and reinserting it into the ovary. The cut-style technique was done by simply cutting off the receptive style about a millimeter above the ovary and brushing the remaining stump with pollen.

In the crosses involving the Yoshitake hybrids as maternal parents, we opted to use the pull-style pollination technique.

Cutflower culture

Using 87C28 as the maternal parent, we compared the performance of flower stalks cut 0, 7 and 21 days after pollination with uncut flower stalks. The freshly-cut flower stalks were placed in a box provided with drainage holes; fresh tap water was provided through a dripping faucet.

Since the flower stalks which were not included in this experiment began to wilt two weeks after pollination, we decided to randomly cut half of the surviving flower stalks and maintained the cutflowers under the conditions described above.

Embryo culture

Embryo rescue was initiated 4-5 weeks after pollination. After removing extraneous organs, the ovaries were washed with detergent and flowing water for 15 minutes. In the culture chamber, the ovaries were surface-sterilized twice, using the following procedure: the ovaries were soaked in 1% NaOCl solution with a drop of Tween 20[R] (as surfactant) and then rinsed three times with sterile distilled water. Discernible embryos were extracted from ovules; in addition, empty seeds with black seed coat were also cultured in MS media containing 3% saccharose and 0.8% agar adjusted to pH 5.7. The number of germinating embryos was counted about 3-4 weeks after culture.

Seedling transfer

After two months in culture, seedlings with 2-3 leaves and roots with satisfactory growth were transferred from the test tubes to vermiculite media and sprinkled twice a week with a 1% solution of Hyponex[R] and Benlate[R]. Since satisfactory seedling survival was obtained after a month under 16 hr fluorescent lighting, additional incandescent lighting (through a 30 W bulb) was provided to induce bulbing. After a month of the combined illumination, the surviving seedlings were transferred into a partially shaded glasshouse.

Results and Discussion

Preliminary data comparing the efficiencies of hybrid seed production using the standard, pull-style and cut-style pollination techniques are shown in Table 1. The data from selfing show that, in the absence of stylar incongruity, the standard method of pollination is superior to the

modified methods. The reduction in seed yield is obviously due to the damage incurred in the latter two techniques. However, in the case of inter-subgeneric crosses where stylar incongruity is present, the pull-style and the cut-style techniques produced 31% and 8% more hybrid seed per inflorescence, respectively, as compared to the standard pollination method. Seeds from these crosses, however, died out during germination.

Table 1. Seed production using various pollination techniques for subgeneric hybrid production in *A. cowanii*. Data expressed as the number of seeds per pollinated inflorescence (April-May, 1992).

Parents		Pollination method		
Female	Male	Standard	Pull-style	Cut-style
A. cowanii	*A. cowanii*	24.50	13.25	2.25
	A. giganteum	0.00	2.00	1.00
	87B203	1.00	0.00	0.00
	87B204	1.00	1.50	2.50
	T02	1.25	0.75	0.00
Average hybrid seed production		0.81	1.06	0.88
Percent improvement			130.77	107.69

Based on the recent classification proposed by Hanelt[5], most of the reports on interspecific hybridization in *Allium* only involved members of the subgenus *Rhizirideum* (G. Don ex Koch) Wendelbo. We used pollen from *A. cowanii* (subgenus *Amerallium* Traub), *A. giganteum* [subgenus *Melanocrommyum* (Webb et Berth.) Rouy], and a commercial cultivar 'Murasame' along with *A. tuberosum* 'Tenderpole' (subgenus *Rhizirideum*) to fertilize several progenies of the *A. chinense*×*A. thunbergii* (subgenus *Rhizirideum*) hybrid population.

To facilitate the comparison between uncut flower stalks and the cutflower treatment, the reproductive performance data were divided into paired and unpaired treatments (Table 2). Since unpollinated flowers in the *A. chinense*×*A. thunbergii* population usually wilt within 15 days after anthesis, the number of flowers pollinated (NoF) and the fruiting percentage (FP=100×number of fruits harvested (NFH)/NoF) can be used to measure the success of initial pollination. In group IA (uncut flower stalks), the pull-style pollination technique induced fruit development towards maturity in 60% of the 87C12 florets fertilized with T01 pollen. Considering the mean fruiting percentages (MFP=100×NFH/NoF) of the maternal parents, 87C05 (group IA, B), 87C12 (group IA), 87C28 (group IB, 0-14 days) and 88F$_2$111 (group IB) had MFPs greater than 10%. Only 87B203 (group IIA) failed to set fruit. Comparing the gross MFP of cutflowers and uncut flower stalks, cutflower culture increased the fraction of fruits which developed to maturity by 204% (Table 2).

Relative to maternal performances, the MFPs of the pollen parents showed less variation despite the disparity of their genetic background. By avoiding stylar incongruity through the pull-style pollination technique, the pollen were able to germinate near the micropyle and probably fertilize the embryo. In contrast, although the maternal parents shared the same genetic constitution, the marked differences in their fruiting responses (i.e. highly variable MFPs) indicate that other morpho-physiological factors were also important in fruit development.

The viable embryo percentage (VEP=100×number of germinating embryos [NGE]/[6×NoF]; number of ovules/ovary = 6) can also measure the effects of the cutflower treatment. The

Table 2. Reproductive performance of autumn-flowering *Allium* species in subgeneric crosses using pull-style pollination technique, cutflower culture and embryo rescue (Nov. -Dec. 1992).

Maternal parents	Cutflower culture	Paternal parents																		Maternal	
		T01			T02			T03			*A. cowanii*			*A. giganteum*			'Murasame'				
	DAP	NoF	FP	VEP	NoF	FP	VEP	NoF	FP	VEP	NoF	FP	VEP	NoF	FP	VEP	NoF	FP	VEP	MFP	MVEP
Group I (paired crosses)																					
A. Uncut flower stalks																					
87C05	-	32	3.1	0.5	52	21.2	0.6	43	14.0	0.8	40	0.0	0.0	55	14.5	0.0	58	13.8	0.0	12.1	0.3
87C12	-	20	60.0	6.7	23	0.0	0.0	22	0.0	0.0	22	0.0	0.0	34	5.9	1.0	26	7.7	1.9	10.9	1.5
87C20	-	43	0.0	0.0	48	0.0	0.0	39	2.6	0.9	22	0.0	0.0	30	0.0	0.0	20	30.0	0.0	3.5	0.2
87C21	-							253	2.4	0.0										2.4	0.0
87C28	-	62	9.7	0.0	80	12.5	0.0	76	7.9	0.0	98	7.1	0.0	60	6.7	0.0	48	10.4	0.0	9.0	0.0
88F$_2$111	-													15	6.7	1.1	15	6.7	1.1	6.7	1.1
Paternal MFP			12.1			10.3			4.4			3.8			7.7			13.2		7.7	
Paternal MVEP				1.0			0.2			0.2			0.0			0.3			0.4		0.3
B. Cut flower stalks																					
87C05	7	56	21.4	2.1	55	25.5	0.0	42	31.0	3.2	22	54.5	0.8	34	20.6	1.0	29	27.6	0.0	27.7	1.3
87C12	10	40	7.5	0.8	19	0.0	0.0	61	18.0	3.8	29	3.4	0.6	41	4.9	0.0	26	3.8	0.0	8.3	1.3
87C20	9	30	6.7	1.1	46	2.2	0.4	38	2.6	0.0	23	52.2	13.8	14	0.0	0.0	13	0.0	0.0	9.8	2.2
87C21	6							174	8.6	0.8										8.6	0.8
87C28	0	41	12.2	0.0	68	10.3	0.2	46	13.0	0.0	75	12.0	0.0	32	15.6	0.0	55	12.7	0.0	12.3	0.1
87C28	7	54	9.3	0.0	37	18.9	1.4	66	13.6	0.0	58	12.1	0.0	60	8.3	0.0	63	6.3	0.0	10.9	0.1
87C28	14	65	9.2	0.0	54	3.7	0.0	44	4.5	0.0	49	6.1	0.0	55	27.3	5.2	47	10.6	0.0	10.5	0.9
87C28	21	45	6.7	0.0	47	2.1	0.0	72	0.0	0.0	31	3.2	0.0	33	0.0	0.0	39	0.0	0.0	1.9	0.0
88F$_2$111	10													36	13.9	0.9	15	46.7	7.8	23.5	2.9
Paternal MFP			10.9			9.8			10.5			15.7			12.8			11.1		11.6	
Paternal MVEP				0.6			0.3			0.9			1.2			1.1			0.4		0.8
Group II (unpaired crosses)																					
A. Uncut flower stalks																					
A. virgunculae		18	0.0	0.0							16	6.3	1.0	10	0.0	0.0	28	0.0	0.0	1.4	0.2
87C23	-	48	2.1	0.0	67	10.4	0.0	37	0.0	0.0	82	1.2	0.0	96	8.3	0.0	52	5.8	0.0	5.2	0.0
87B203	-	42	0.0	0.0	26	0.0	0.0	38	0.0	0.0	50	0.0	0.0	15	0.0	0.0	33	0.0	0.0	0.0	0.0
87B204	-	100	1.0	0.0	91	2.2	0.4	100	1.0	0.0	72	4.2	0.5	75	4.0	0.2	57	8.8	1.5	3.2	0.3
87B207	-	50	0.0	0.0	27	0.0	0.0	36	0.0	0.0	30	0.0	0.0	42	7.1	0.0	5	0.0	0.0	1.6	0.0
Paternal MFP			0.8			4.3			0.5			2.0			5.9			5.1		3.0	
Paternal MVEP				0.0			0.2			0.0			0.2			0.1			0.5		0.1
B. Cut flower stalks																					
87C02	9	52	0.0	0.0	39	10.3	0.0	29	6.9	0.0	40	7.5	0.0	48	8.3	0.0	40	0.0	0.0	5.2	0.0
87C07	1				18	11.1	0.0	23	0.0	0.0	9	0.0	0.0	12	8.3	2.8	21	0.0	0.0	3.6	0.4
87C13	5	48	4.2	0.3	52	1.9	0.3	39	5.1	0.0	24	8.3	0.0	42	4.8	0.8	30	10.0	0.6	5.1	0.4
87C15	9	18	0.0	0.0	44	0.0	0.0	50	2.0	0.0	45	4.4	0.0	48	2.1	0.0	48	6.3	0.3	2.8	0.1
88F$_2$111	10	38	13.2	0.0	53	32.1	3.8	52	40.4	4.2	49	34.7	5.1							31.3	3.5
Paternal MFP			4.5			11.7			13.5			14.4			5.3			4.3		9.4	
Paternal MVEP				0.1			1.1			1.1			1.5			0.4			0.2		0.8
A. Uncut flower stalks																				5.3	0.2
B. Cut flower stalks																				10.9	0.8
C. Percent improvement (100×B/A)																				203.5	366.6

Legend: DAP days after pollination; NoF number of pollinated flowers; FP fruiting percentage; VEP viable embryo percentage; MFP mean fruiting percentage; MVEP mean viable embryo percentage

87C20×*A. cowanii* cross of the cut flower treatment in group I had the highest VEP of 13.8% in the whole trial. In terms of mean viable embryo percentage (MVEP) after pollination with the six male parents, 87C20 flower stalks cut 9 days after pollination produced 11 times more viable embryos as compared to the uncut 87C20 flower stalks. Comparing the gross MVEP of cutflowers and uncut flower stalks, cutflower culture increased the fraction of viable embryos produced from these crosses by 367% (Table 2).

With an R^2 value of 0.51, MFP is a poor predictor of MVEP. Most of the harvested fruits contained "empty" seed with black seed coats indicating the presence of post-fertilization barriers which terminated embryogenesis at an early stage.

The cutflower trial using 87C28 was based on the hypothesis that bulbs play a role in terminating "unwanted" embryogenesis. Although the MFP data for 87C28 in Table 2 are inconclusive, the MVEPs underscore the advantage of cutting the flower stalk within 2 weeks from pollination. The positive relationship between the yield of viable embryos and the time of cutting (up to the 14th day) indicates that (a) the 87C28 flower stalks, slender as they were[2], depended greatly on the bulbs to help sustain early embryo growth and that (b) an unknown stimulus was probably released from the bulb between 14 and 21 days after pollination which resulted in the termination of embryogenesis.

Aside from avoiding this putative "selective antiembryogenic" stimulus, cutflower culture can provide a less stressful and drought-free environment to the developing fruits since this can be done indoors. For example, onion ovaries exposed to direct sunlight can reach temperatures of 60℃, resulting in total embryo abortion[9]. Since we conducted the experiment in late autumn, such a temperature effect on fruit-set was not important in this trial. Nutrient competition, which can lead to embryo abortion, can be discounted since many of the open pollinated inflorescences produced a lot of normal seeds.

With such improvement in the number of viable embryos per pollinated ovule, the cutflower culture method deserves more investigation. For example, although pathogenic contamination is minimized because of the continuous flow of chlorine-treated tap water, its occurrence at the late embryo development phase must be inhibited through suitable preventive measures. Since this maternal population produces slender flower stalks, the use of periodic "pulsing" treatments—dipping the cutflowers in sugar and mineral or hormone solutions may improve embryogenesis.

In Table 3 the effects of the combined pull-style, cutflower, and embryo culture techniques are expressed in terms of pollination efficiency (PE =100×number of transplanted seedlings [NTS]/[6×NoF]. In previous reports dealing with *in vitro* culture of intra *Rhizirideum* crosses, the maximum PE ranged from 11.1% for *A. cepa*×*A. fistulosum*[4], 14% for *A. chinense*×*A. fistulosum*[7] and 2.8% for *A. chinense*×*A. thunbergii*[8].

Using cutflowers, maximum pollination efficiencies of 9.4%, 4.1% and 2.8% were observed in inter-subgeneric crosses (87C20×*A. cowanii*, $88F_2111$×*A. cowanii*, and 87C07×*A. giganteum*, respectively). Intra *Rhizirideum* crosses like $88F_2111$×TO3, 87C05×TO3, and $88F_2111$×TO2 also had respectable pollination efficiencies (3.5%, 3.2% and 2.5%, respectively). It should be emphasized, however, that the current results involve trispecific hybrids since the maternal parents are all interspecific hybrids. The overall pollination efficiency, as affected by cutflower culture, was 0.51% (95 transplanted seedlings out of 18540 pollinated ovules) which is 391% more than the 0.13% (22 transplanted seedlings out of 16074 ovules) obtained from uncut flower stalks.

Kahane et al.[6] reported that they were able to induce bulbing and subsequent dormancy in *A. cepa* by exposing plantlets aseptically growing in test tubes to 16 hr daylengths provided by a

Table 3. Pollination efficiency based on the number of transplanted seedlings at the 2-3 leaf stage (22 Jan., 1993) and percent survival during the glasshouse culture period (30 May, 1993).

Maternal parents	Cutflower culture	Paternal parents												Maternal	
		T01		T02		T03		*A. cowanii*		*A. giganteum*		'Murasame'		MPE	MPS
	DAP	PE	PS	PE	PS	PE	PS	PE	PS	PE	PS	PE	PS		
A. Uncut flower stalks															
A. virgunculae								1.0	0.0					0.23	0.0
87C05		0.5	0.0	0.3	0.0	1.2	33.3							0.30	50.0
87C12		4.2	20.0							0.5	0.0			0.68	50.0
87C20														0.00	0.0
87B204				0.4	0.0			0.5	100.0	0.2	0.0	1.2	75.0	0.30	71.4
88$F_2$111												1.1	0.0	0.56	0.0
Paternal MPE		0.24		0.12		0.07		0.11		0.07		0.24		0.13	
Paternal MPS			16.7		0.0		33.3		66.7		0.0		60.0		33.3
B. Cut flower stalks															
87C02	9									0.4	0.0			0.07	0.0
87C05	7	0.6	100.0			3.2	50.0	0.8	0.0	1.0	100.0			0.91	61.5
87C07	1									2.8	0.0			0.40	0.0
87C12	10	0.8	0.0			2.2	25.0							0.77	50.0
87C13	5	0.4	0.0	0.3	0.0					0.8	0.0	0.6	0.0	0.35	0.0
87C15	9													0.00	0.0
87C20	9	0.6	100.0					9.4	46.2					1.42	50.0
87C28	0			0.2	100.0									0.05	100.0
87C28	7			0.9	0.0									0.10	0.0
87C28	14									2.7	33.3			0.48	33.3
88$F_2$111	10			2.5	12.5	3.5	0.0	4.1	25.0	1.9	0.0	3.3	0.0	2.61	20.0
Paternal MPE		0.20		0.37		0.61		0.95		0.73		0.15		0.51	
Paternal MPS			50.0		16.7		22.2		34.6		25.0		0.0		26.3
Overall MPE														0.34	
PI (100×cut flower stalk MPE/uncut flower stalk MPE)														390.36	

Legend: DAP days after pollination; PE pollination efficiency; PS percent survival; MPE mean pollination efficiency; MPS mean percent survival; PI percent improvement

fluorescent light enriched with incandescent light. We attempted to induce early bulbing among the seedlings growing in vermiculite one month after they were extracted from the test tubes. After another month of exposure to 16 hr daylengths through a 40 W fluorescent bulb and a 30 W incandescent bulb, we noticed that around 74% of the seedlings had died while the rest of the survivors showed glossy green, watery and weak leaves and pronounced basal leaf sheath development. The death of most of the seedlings can be blamed on insufficient light intensity (and photosynthesis) and a deranged physiology due to the strong bulbing stimulus. The survivors were then transferred to a partially shaded glasshouse to restore their vitality. The data on the number of survivors (NoS) is expressed in terms of percent survival (100×NoS/NTS) at the glasshouse. The genetic identity of these hybrids will be described in a future report.

Summary

A pull-style pollination technique designed to avoid stylar incongruity was observed to increase the number of hybrid seeds produced per pollinated inflorescence by 31% relative to the standard pollination procedure. Compared to uncut inflorescences, cutflower culture implemented within 14 days from pollination, which was designed to avoid putatively unfavorable stimuli emanating from the bulb during embryogenesis, improved the fraction of viable embryos per pollinated ovule by 367%. Using the pull-style pollination, cutflower culture and embryo rescue techniques, we obtained 95 transplantable (2-3 leaf stage) putative trispecific hybrid seedlings using the Yoshitake hybrids (*Allium chinense*×*A. thunbergii*) as maternal parents. The successful pollinator parents included *A. tuberosum* 'Tenderpole' (subgenus *Rhizirideum*), *A. cowanii* (subgenus *Amerallium*), *A. giganteum* (subgenus *Melanocrommyum*) and the commercial cultivar 'Murasame'.

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