Pollen Morphology of Interspecific Hybrids of *Brassica oleracea* and *B. campestris*

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Abstract. Pollen morphology was examined in amphidiploid and amphihaploid interspecific hybrids between *Brassica oleracea* L. and *B. campestris* L. Pollen of the amphidiploid interspecific hybrids between *B. oleracea* var. *capitata* and *B. campestris* var. *pekinensis*, and between *B. campestris* var. *chinensis* and *B. oleracea* var. *capitata*, were significantly longer and wider than those of their diploid parents, presumably due to the phenotypic expression of the hybrid genomes and ploidy effects. The exine ridges and pores of the amphidiploids were well-developed and significantly larger than those of their diploid parents, but they were poorly developed in the amphihaploids.

Successful production of resynthesized *Brassica napus* from its progenitors, *B. oleracea* and *B. campestris*, raised great interest in the breeding of *Brassica* (U, 1935). Thereafter, interspecific hybridization of these species frequently has been used to develop new types of vegetables, fodder crops, and oil seeds (Hossain et al., 1988). Many artificially synthesized *B. napus* recently have been released as cultivars (Namai et al., 1980). The fundamental problems of the interspecific hybrids, pollen sterility, and low seed production constitute a severe hindrance to their use for any agronomic potential (Hossain et al., 1987).

We studied the pollen morphology of *B. campestris* and *B. oleracea*, and their amphidiploid and amphihaploid interspecific

hybrids, in relation to pollen fertility. To our knowledge, there is no previous report on pollen morphology; i.e., pollen size, shape, and pore characteristics of the interspecific hybrids of these two taxa.

The species used were *B. oleracea* var. *capitata* cv. Yoshin (cabbage), *B. oleracea* var. *alboglabra* cv. Senyo shirobana (Chinese kale), *B. campestris* var. *pekinensis* cvs. Kaga and Kenshin (Chinese cabbage), and *B. campestris* var. *chinensis* cv. Honsaitai (Chinese vegetable). Seven plants from each of the parental species were used in the crosses. Interspecific hybrids in various cross combinations were developed through in vitro embryo and ovary culture (Hossain et al., 1987; Inden et al., 1987). The interspecific hybrids were sterile. Colchicine 0.3% (w/v) was applied at the six-to eight-leaf stage to double the chromosomes and restore fertility.

Mitotic chromosomes were counted in 10 metaphase cells in root tip meristematic tissue from the hybrids and the parental plants. The root tips were pretreated with 2 mM 8-hydroxyquinoline and fixed in 3 absolute ethanol : 1 glacial acetic acid. After fixation, the root tips were washed and treated with

an enzyme solution containing 4% (w/v) cellulase, 1% (w/v) pectolyase, 7.5 mM KCl, and 7.5 mM EDTA, then stained with 2% (w/v) aceto-orcein for 20 min (Hossain et al., 1988). Pollen viability was estimated by counting 1000 pollen grains stained with acetocarmine.

All plants were held for 3 weeks under 16-hr daylength at 5C in a phytotron to induce flowering and then were grown in a glasshouse under natural daylight conditions at 20 ± 5C for flowering.

Flowers were collected from glasshouse-grown plants a few hours after anthesis. Anthers were removed and pollen grains of each plant were dusted on a polished aluminum disk stub covered with double-faced transparent tape. The sample on each stub was sputter-coated with 60:40 gold-palladium alloy ≈ 20 nm thick with an ion coater (Eiko, IB-3) and were examined with a scanning electron microscope (SEM) (Akashi, Alpha-10) at a 35° sample stub inclination. Three groups of 10 pollen grains per plant were examined at random. The data were analyzed by analysis of variance and the means were separated by Duncan's multiple range test at *P* = 0.05.

Pollen length, width, and pore characteristics of the amphidiploids and amphihaploids interspecific hybrids are listed in Table 1. The size and shape of pollen grains varied between and among cultivars of the two species. Amphidiploid hybrids of 'Yoshin' × 'Kaga' and 'Honsaitai' × 'Yoshin' had significantly larger pollen grains than the others (Table 1, Fig. 1B). The pollen lengths and widths of the amphihaploid hybrids between 'Yoshin' × 'Kenshin' and between 'Senyo shirobana' × 'Kenshin' were significantly smaller than those of their diploid parents in the interspecific hybridization (Table 1, Fig. 1E). Maas (1977) reported that the sizes of pollen corresponded with ploidy levels in *Fragaria*. The pollen grains examined in each of the hybrids and the parents were monad and tricolpate, with the pore extending almost the full length of the grain. The pollen shape of the interspecific hybrids resembled that of the female parents.

The pollen exine varied greatly among the

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Table 1. The pollen characteristics of the *Brassica* interspecific hybrids (female parent × male parent) and their parents.¹

Cultivar/ hybrid	Species	Ploidy level ²	Chromosome no.	Shape ³	Pollen			Exine		Pore		Pollen fertility (%)
					Length (μm)	Width (μm)	Length : width ratio	Texture ⁴	Shape ⁵	Length (μm)	Width (μm)	
Yoshin	(<i>B. oleracea</i>)	D	18	NP	34.4 f	19.1 de	1.80 c	RRi	I	1.08 d	1.20 c	98.0
Senyo shirobana	(<i>B. oleracea</i>)	D	18	NP	38.5 d	18.8 de	2.05 b	RRi	I	1.37 c	0.97 e	84.0
Kaga	(<i>B. campestris</i>)	D	20	Ob	36.0 e	18.5 e	1.95 bc	PRi	I	1.57 b	1.05 d	98.5
Kenshin	(<i>B. campestris</i>)	D	20	Ob	38.5 d	20.3 c	1.90 c	PRi	I	0.81 e	0.82 f	97.0
Honsaitai	(<i>B. campestris</i>)	D	20	BP	40.4 c	19.7 cd	2.05 b	PRi	I	1.56 b	1.02 de	92.5
Yoshin × Kaga		Ad	38	NP	48.6 a	25.5 a	1.91 bc	PRe	I	1.80 a	1.48 a	75.0
Honsaitai × Yoshin		Ad	38	BP	44.0 b	24.0 b	1.83 c	RRi	I	1.60 b	1.30 b	81.5
Yoshin × Kenshin		Ah	19	NP	35.4 e	15.9 f	2.23 a	PRi	I	1.00 d	0.98 e	3.0
Senyo shirobana × Kenshin		Ah	19	NP	20.6 g	13.5 g	1.53 d	PRe	R	0.34 f	0.29 g	11.5

¹Means separation within columns by Duncan's multiple range test, *P* = 0.05.

²D = Diploid, Ad = amphidiploid, Ah = amphihaploid.

³NP = Narrowly prolate, Ob = oblong, BP = broadly prolate.

⁴RRi = Reticulately ridged, PRi = prominently ridged, PRe = prominently reticulated.

⁵I = Irregular, R = round.

Fig. 1. Pollen morphology of amphidiploid and amphihaploid interspecific hybrids between *Brassica oleracea* and *B. campestris* (A-F). (A) A female parent 'Yoshin'; (B) an amphidiploid of 'Yoshin' $\times$ 'Kaga', arrowheads indicate minute protuberances in pores; (C) a male parent 'Kaga'; (D) a female parent 'Senyo shirobana'; (E) an amphihaploid of 'Senyo shirobana' $\times$ 'Kenshin'; (F) a male parent 'Kenshin'. Scale line equals 20 μ m.

parents and the hybrids. The exine of 'Yoshin' and 'Senyo shirobana' (*B. oleracea*) were reticulately ridged, whereas 'Kenshin', 'Kaga', and 'Honsaitai' (*B. campestris*) had prominently ridged exine (Fig. 1 A, C, D, and F). The interspecific hybrids of 'Yoshin' $\times$ 'Kaga' had prominently ridged exine, while the hybrid between 'Honsaitai' $\times$ 'Yoshin' was reticulately ridged. The exine character of the interspecific hybrids was closer to that of the pollen parents.

Significantly larger pollen pores were found in the two amphidiploid hybrids of 'Yoshin' $\times$ 'Kaga' and 'Honsaitai' $\times$ 'Yoshin' than in the other hybrids. An amphidiploid interspecific hybrid had distinct minute protuberances in pollen pores (Fig. 1B, arrows). When

B. campestris is used as the male parent in crosses to *B. oleracea*, the minute protuberances are distinct. This phenomenon is probably controlled by the pollen parent, or it may be the result of the phenotypic expression of the hybrid genomes (AACC).

Pollen fertility of the amphidiploid hybrids was significantly higher than amphihaploids (Table 1). Pollen of the amphihaploid hybrids was mostly sterile and poorly developed. The sterile and aborted pollen can also be identified through pollen morphology by light microscopy.

The present results reveal that pollen length, width, and pore characteristics can provide evidences for distinguishing the amphidiploid and amphihaploid interspecific hybrids.

These will be useful in practical breeding and backcrossing in interspecific hybridization in *Brassica*.

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Survival of in Vitro-grown Apical Meristems of *Pyrus* Following Cryopreservation

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Abstract. Apical meristems of four pears (*Pyrus communis* L. cv. Beurre Hardy, *P. koehnei* Schneider, *P. cossonii* Coss. and Dur., and a hybrid, *P. dimorphophylla* Makino × *P. fauriei* Schneider) were tested for their ability to survive immersion in liquid nitrogen. Plantlets were grown in vitro at 25C or cold-hardened for 1 week at -1C before cooling at rates of 0.1, 0.3, 0.5, and 0.8 C/rein to -40C, followed by plunging the vials into liquid nitrogen. Vials were thawed for 1 min at 40C. A cryoprotectant mixture of polyethylene glycol, glucose, and dimethylsulfoxide (DMSO) was used. Regrowth of meristems ranged from 0% to 61% for plants grown at 25C and from 5% to 95% for cold-hardened plants. Cold-hardening significantly improved the recovery rates of all species tested. Survival rates increased as cooling rates decreased. Survival rates were not linked to the geographic origin of the species tested.

The development of methods to successfully store apical meristems in liquid N₂ is needed to aid in the conservation of genetic resources. Long-term storage methods for *Pyrus* spp. are needed to provide a backup collection of the 26 species and numerous cultivars present at the National Clonal Germplasm Repository, Corvallis, Ore. Cryopreservation may be a useful method for long-term storage using a minimum of space and maintenance.

In freezing actively growing apical meristems of *Pyrus* species, variable survival over a range of conditions for dehydration could be expected. The rate of cooling and the pre-freezing temperature must be taken into account (15). Relatively slow cooling rates may have the net effect of greater dehydration at a given temperature than faster rates, while the same level of dehydration would be reached at a lower temperature at faster cooling rates.

Enhanced survival has been found in callus of several species that were cold-hardened before cryopreservation (8, 10, 12). One week of cold-hardening was found to in-

crease the survival of cryopreserved apical meristems from tissue-cultured *Rubus* plants (6). Dormant apple buds survived best in liquid N₂ when the tissue had ≤20% liquid water at the precooking temperature and the cell solute concentration had increased from about 2 mmol to 5 mmol with dehydration, leading to higher survival rates (14).

Field-grown pear species and cultivars vary in hardiness to freezing damage of cambial cells from temperatures of -16C to -40C (5). *Pyrus communis* cultivars alone vary in hardiness from -20 to -35C (J.M. Montano, unpublished results). Other species ex-

hibit somewhat greater or lesser hardiness that often is well-correlated with the geographic origin of the plant (7). Sakai (9) found that, in the cryopreservation of woody twigs, the greater the hardiness, the higher the pre-cooking temperature that is required for survival. Cryopreservation of *Pyrus* spp. has been limited to the cooling of dormant buds during midwinter (4, 8) and to protoplasts and suspension-cultured cells (1, 16).

Both cold-hardening and dehydration due to pretreatment and cooling rate play important roles in conditioning meristems to survive immersion in liquid N₂ (13, 14). This study compares the survival of apices of four species of the genus *Pyrus* that were frozen at four rates following growth under cold-hardening or growth room conditions.

Tissue culture conditions. Tissue-cultured plants of *P. communis*, *P. koehnei*, and *P. cossonii* and of a hybrid, *P. dimorphophylla* Makino × *P. fauriei*, were grown on the medium of Cheng (2) with benzyladenine at 2 mg·liter⁻¹ and 0.6% agar at pH 5.2. Growth conditions included a 16-hr photoperiod at 25C. Cold-hardening of in vitro plants involved 22C days (8 hr) and -1C nights (16 hr) for 1 week before excision of meristems (6). Dissected meristems (0.8 mm) of both treatments were grown for 48 hr on medium with 0.8% agar and 5% DMSO added. Meristems were grown under the same conditions as the parent plants.

Addition of cryoprotectant. Following cold-hardening, meristems were transferred to 0.25 ml of liquid medium in 1.2-ml plastic cryotubes on ice. The cryoprotectant PGD (3), a mixture of 10% each of polyethylene glycol (M, 8000), glucose, and DMSO in water, was added dropwise up to 1.2 ml over 30

Table 1. Influence of cold-hardening or growth at 25C (non-hardened) on the survival of meristems of *Pyrus* spp. after cryopreservation in liquid N₂ (all cooling rates combined).

Species	No. meristems tested					
	Cold-hardened			Non-hardened		
	Living	Dead	Total	Living	Dead	Total
<i>P. communis</i>	73	57	130	9	99	108
<i>P. hybrid</i>	35	107	142	6	113	119
<i>P. koehnei</i>	31	110	141	1	114	115
<i>P. cossonii</i>	42	108	150	7	85	92
Total	181	382	563	23	411	434
	df			G ²		
<i>P. communis</i>	1			66.34***		
<i>P. hybrid</i>	1			20.83***		
<i>P. koehnei</i>	1			32.89***		
<i>P. cossonii</i>	1			16.45***		
Species totals	3			40.15***		
Total	7			176.67***		

²G values calculated as in Sokal and Rohlf (11).

***Significant at *P* < 0.001.

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