BREEDING AND PROPAGATION OF MECONOPSIS

By

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ABSTRACT

Six species of Meconopsis were investigated for self-fertility and cross compatibility in order to incorporate desirable characters from both parents. The six species used were: M. cambrica, M. villosa, M. quintuplinervia, M. betonicifolia, M. horridula, M. aculeata. Chromosome counts were made: M. cambrica, n=14; M. villosa, n=16; M. quintuplinervia, n= c.42; M. betonicifolia, n=40; M. horridula, n=28 and M. aculeata, n=28. Interspecific compatibility was correlated with differences in ploidy level between parents. All crosses made except M. cambrica x M. quintuplinervia set seeds. M. cambrica, M. betonicifolia, M. horridula and M. aculeata are self-compatible.

Pollination mechanisms and the likelihood of apogamy were investigated in M. betonicifolia. No apogamy was found and insects are the likely pollinators for this species.

As some of the species do not flower at the same time, pollen staining, pollen germination and storage conditions for the six species were studied. Experimental alteration of flowering periods through controlling temperatures and day-lengths for M. betonicifolia was also carried out. This part of

the project shows: (1) pollen stainability (stained by lactophenol cotton blue) for the six species was 85% or more; (2) pollen of all six species except *M. quintuplinervia* germinated on an agar medium containing sucrose (5 g l^{-1}) and H_3BO_3 (0.1 mg l^{-1}); (3) pollen germination percentage decreased with storage time in a desiccator at 4-6°C; and (4) long daylength (16 h) was suitable for growth and flowering of *M. betonicifolia*. High temperature (17°C) induced earlier growth and flowering than low temperature (6°C) in *M. betonicifolia*.

Because of difficulties in vegetative reproduction and seed storage, *in vitro* establishment of *M. betonicifolia* was investigated. The unopened capsule sterilization method was used. The seeds in the capsule germinated on Murashige and Skoog medium. Derooted seedlings, hypocotyls and seedling roots from seedlings raised on sterile artificial medium were used as explants for the *in vitro* establishment. This experiment shows that half strength Murashige and Skoog medium is suitable for *in vitro* culture of this species. The cytokinin:auxin ratio and growth regulator concentration were found to control morphogenesis of *M. betonicifolia in vitro*. Derooted seedlings cultured first on nutrient medium containing N^6-benzyladenine

(BA) and kinetin (Kn) differentiated more multiple meristems than those originally cultured on medium containing N^6-(2-isopentenyl)-adenine (2ip) after being transferred into medium containing 2ip. Multiple meristems were divided and proliferated on medium containing 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 5 mg l^{-1} 2ip and 1 mg l^{-1} BA. Seedling roots and hypocotyls formed callus on media containing 1, 5 and 10 mg l^{-1} 2,4-D.

ABBREVIATIONS

MS	Murashige and Skoog medium (1962)
IAA	Indole-3-acetic acid
NAA	1-Naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
BA	N^6-Benzyladenine
Kn	Kinetin
2ip	N^6-(2-isopentenyl)-adenine
LD	Long day-length
SD	Short day-length
HT	High temperature
LT	Low temperature

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General Introduction

The genus Meconopsis, which belongs to the family Papaveraceae, was named by Viguier in 1814. It was based on the single species Papaver cambricum, and is differentiated from Papaver by the presence of a short style and the complete absence of a sessile stigmatic disc surmounting the ovary (Taylor, 1934). Meconopsis consists of 49 species. One of them is native to western Europe whilst the others are all distributed in the Sino-Himalayan Region of Asia (Wu and Chuang, 1980).

With the exception of M. cambrica, which inhabits damp, shady ravines at altitudes usually below 600 m, Meconopsis occurs in alpine woods, scrub, meadows, and scree slopes above 1,800 m. Several species grow at elevations above 5,000 m (Taylor, 1934). The genus, like other alpine plants, produces comparatively large, brilliantly coloured flowers. Flower colours are blue, yellow, white, red and purple. This genus is horticulturally useful (Taylor, 1934).

The taxonomy (Prain, 1906; Fedde, 1909; Taylor, 1934; Wu and Chuang, 1980), cytology (Maude, 1940; Ernst, 1965; Ratter, 1968),

pollen morphology (Henderson, 1965), hybrids (Taylor, 1934, 1951; Evans, 1959; Ramsden, 1962; Cobb, 1984), seed germination (Amsler, 1945; Thompson, 1968, 1969; Anonymous, 1984), cultivation and horticultural value (Harley, 1928; Cox, 1934; Harrow, 1930; Evans, 1974) of Meconopsis have been studied, but there are still some unresolved problems.

Most members of Meconopsis are monocarpic. Only a few species are polycarpic (Taylor, 1934; Evans, 1959). Within the genus, several controlled interspecific crosses have been reported between polycarpic species and monocarpic species (Taylor, 1934; Evans, 1959). The hybrids closely resembled their polycarpic parents. This shows that the possibility of controlled crossing between species exists. This fact stimulated interest in incorporating desirable floral habit from monocarpic species into polycarpic species to increase longevity of plants with desirable flower colour, number and size. The feasibility of such a breeding program is unknown because little is known about self-compatibility, interspecific cross compatibility, the pollination mechanisms or the possibility of apogamy in the genus. Thus, a hybridization and selfing test was designed to investigate self and cross pollination among the six species of this genus grown

in Vancouver. The hypotheses of this experiment were: (1) interspecific crosses are compatible among the six species; and (2) the species in this genus are self-compatible. If these hypotheses are true, it is possible to get hybrid seeds and seeds from selfed plants.

The six species available for study were: M. cambrica (L.) Vig., M. quintuplinervia Regel, M. betonicifolia Franch., M. horridula Hook f. & Thoms. and M. aculeata Royle (grown in the University of British Columbia Botanical Garden), and M. villosa (Hook. f.) G. Tayl. (grown in the VanDusen Botanical Garden, Vancouver). Possible pollination mechanisms and the possibility of apogamy were investigated in M. betonicifolia. M. betonicifolia was used because of its high horticultural usefulness, its pure blue flowers and availability of adequate plant material.

The morphological differences among these six species are:

M. cambrica: polycarpic, yellow to orange flower, tufted base of plant glabrous or sparsely pubescent, lamina of basal leaves pinnatisect towards the base and pinnatifid towards the apex.

M. villosa: polycarpic, yellow flower, tufted base of plant

beset with barbellate rufous hairs, lamina of basal leaves ovate to suborbicular, deeply bipinnately lobed.

M. quintuplinervia: polycarpic, flower pale lavender-blue, leaves all basal, pendulous flowers borne on basal scapes.

M. betonicifolia: monocarpic or polycarpic, flower colour ranging from rose-lavender to satiny-sky-blue, leaves basal and cauline.

M. horridula: monocarpic, flower light-blue to claret, leaf-lamina entire or rarely pinnately lobed at the margin.

M. aculeata: monocarpic, flower purplish-blue, leaf-lamina deeply pinnatifid or pinnatisect.

Among the six species investigated, M. quintuplinervia flowers earliest (in April) while M. villosa flowers in June in Vancouver. Their blooming times do not overlap. Consequently, an interspecific breeding program involving these species would require pollen storage or experimental alteration of flowering periods. Thus a pollen viability test was designed to investigate pollen staining, pollen germination and storage conditions for

the six species. The experiment to alter flowering time was designed to test the hypothesis that different temperatures and day-lengths can change flowering time of the species in Meconopsis in growth chambers. No such information was available from earlier studies. Such data would be helpful in improving breeding techniques for this genus by allowing controlled pollination between species that do not flower at the same period.

Meconopsis is usually raised from seeds. Vegetative reproduction is possible in only a few perennial species (Cox, 1934). Most Meconopsis are monocarpic so that regular successful seed set each year is essential for their cultivation. Under normal storage conditions the seed remains viable over only relatively short periods of a year or two so that continued cultivation of individual species is dependent first on their capacity to set seed under garden conditions, and then on the ability of the gardener to raise seedlings and bring them into flower. Adverse circumstances may occasionally prevent the raising of young plants and even a single generation lost in this way may lead to the loss of an entire stock. Very many attractive species which have flowered from seeds collected in the wild

have, after a few years, been lost to cultivation because of failures to maintain successful stocks of seedlings (Thompson, 1968). Hence, it is desirable to develop an alternative propagation method to ensure continued success in cultivation of particularly attractive clones.

Cox (1934) pointed out that summer heat, particularly when humidity is low, is the main reason for failure of Meconopsis in the south of England, in eastern and central Canada and the United States. Cultivars which could resist summer heat and low humidity would extend the range of cultivation of this genus.

Following the successful rapid multiplication of orchids by shoot meristem culture (Morel, 1965) there has been an increasing interest in recent years in the application of tissue culture techniques as an alternative means of asexual propagation of economically important plants. Potential advantages of the in vitro micropropagation are: (1) recovery of pathogen-free stock (Morel, 1960); (2) providing increased rates of propagation and facilitating asexual multiplication of plants that had previously proved difficult, or impossible, to propagate (Hussey, 1978); (3) potential benefits in long-term storage (Morel, 1975) and cryogenic preservation of germplasm (Seibert, 1976); (4)

international transport of genetic stocks (Kahn, 1976); and (5) proliferating the *in vitro* stocks at any time of the year (Boxus et al., 1977) after shipping or storage, while propagation with conventional practices is highly season-dependent. From the breeder's point of view, the tissue culture technique provides an effective tool to release new varieties rapidly (Hu et al, 1982). The production of haploid plants, specific desirable gene combination and regenerates with desirable characters from somaclonal variation can be very useful in plant breeding. If a successful method of tissue culture could be worked out for *Meconopsis*, it may help not only propagation, but also preservation of stock, and breeding of this genus.

No information about tissue culture of *Meconopsis* was available. *Meconopsis betonicifolia* was chosen to conduct the *in vitro* culture experiment because of its horticultural usefulness and plant material availability. The object of this experiment was to determine an appropriate explant and an optimal medium for its growth and multiplication *in vitro*.

The ultimate objectives of the project were: (1) investigation of some plant characters related to breeding of *Meconopsis*; and (2) development of a new propagation method for

M. betonicifolia. The thesis shows methods for pollen staining, pollen storage and germination testing, the feasibility of hybridization and selfing, and the relationship between interspecific compatibility and chromosome ploidy level of six species in this genus. Data regarding possible pollination mechanisms, apogamy, factors controlling flowering time, and the in vitro establishment of M. betonicifolia are also given.

Section A. Self-fertility and Cross Compatibility among Six Species of *Meconopsis*

Introduction

Within the genus *Meconopsis*, some hybrids were reported from previous studies. Taylor (1934) pointed out that with few exceptions the hybrids have arisen spontaneously and evidence of their parentage is therefore often hypothetical. The reported hybrids were:

(1) *Meconopsis* x *Harleyana*: *M. integrifolia* x *M. simplicifolia* (Taylor, 1934);

(2) *M.* x *Auriculata*: *M. betonicifolia* x *M. paniculata* (Taylor, 1934);

(3) *M.* x *Coxiana*: *M. betonicifolia* x *M. violacea* (Taylor, 1934);

(4) *M.* x *Musgravei*: *M. betonicifolia* x *M. superba* (Taylor, 1934);

(5) *M.* x *Sarsonsii*: *M. betonicifolia* x *M. integrifolia* (Taylor, 1934);

(6) M. x Beamishii: M. grandis x M. integrifolia (Taylor, 1934);

(7) M. x Hybrida: M. grandis x M. simplicifolia (Taylor, 1934);

(8) M. x Decora: M. latifolia x M. napaulensis (Taylor, 1934);

(9) M. x Cookei: M. punicea x M. quintuplinervia (Taylor, 1951);

(10) M. x Finlayorum: M. integrifolia x M. quintuplinervia (Taylor, 1951);

(11) M. x Sheldonii: M. grandis x M. betonicifolia (Evans, 1959);

(12) M. x Ramsdeniorum: M. dhwojii x M. napaulensis (Ramsden, 1962);

(13) Unnamed: M. quintuplinervia x M. cambrica (Cobb, 1984);

(14) Unnamed: M. simplicifolia x M. paniculata (Cobb, 1984);

(15) Unnamed: M. grandis x M. regia (Cobb, 1984).

Of the fifteen hybrids, five were controlled crosses. Two hybrids from controlled crosses: M. x Musgravei (M. betonicifolia x M. superba) and M. x Sarsonsii (M. betonicifolia x M.

integrifolia) were described as closely resembling the polycarpic parent (M. betonicifolia) and showing the flower colour influence of monocarpic parents (M. superba and M. integrifolia) (Taylor, 1934). This indicates the possibility of controlled crossing between species within the genus. Thus it is possible to incorporate desirable characters from both parents to create new varieties. The feasibility of self-compatibility, cross compatibility, pollination mechanisms or possibility of apogamy in the genus is unknown. The object of this experimental pollination study was to investigate self-compatibility and interspecific cross compatibility among the six species in this genus, which are cultivated in the University of British Columbia Botanical Garden and VanDusen Gardens, Vancouver.

No controlled crossing and selfing among the six species were reported from previous studies except one unnamed hybrid: M. quintuplinervia x M. cambrica (Cobb, 1984).

Meconopsis betonicifolia was chosen to study pollination mechanisms and the likelihood of apogamy for two reasons: (1) horticultural usefulness of its pure blue flower; and (2) plant material availablity.

Chromosome numbers have been determined for eighteen species in the genus. They vary from n=14 to n=60 within the genus (Ratter, 1968). Chromosome numbers reported for the six species were: M. cambrica, n=11 (Maude, 1940), n=14 (Sugiura, 1937, 1940; Ratter, 1968), n=28 (Ernst, 1962, 1965); M. villosa, n=16 (Ernst, 1962, 1965; Ratter, 1968); M. quintuplinervia, n=42 (Ratter, 1968); M. betonicifolia, n=41 (Ratter, 1968), n=40 (Ernst, 1965); M. horridula, n=28 (Ratter, 1968); and M. aculeata, n=28 (Ratter, 1968). Thus, this study was also designed to determine chromosome number for the six species cultivated in the University of British Columbia Botanical Garden and VanDusen Garden.

Previous studies about chromosome number for the six species show that they have different ploidy levels (Ratter, 1968). If this is true for the six species grown in Vancouver, then interspecific crosses can be tested between species with the same or different ploidy levels. The degree of interspecific cross compatibility can be compared with difference in ploidy level between parents. The hypotheses of this experiment were:

(1) the degree of compatibility between species differs with difference in ploidy level between parents;

(2) species of this genus are self-compatible;

(3) M. betonicifolia is not apogamous.

Materials and methods

Plants of *Meconopsis cambrica*, *M. villosa*, *M. quintuplinervia*, *M. betonicifolia*, *M. horridula* and *M. aculeata* cultivated in the two Botanical Gardens were used for selfing and crossing experiments. Three to eighteen replicates were used depending on plant availability. The availability of plant materials and difference in flowering time prevented all possible crosses from being made.

For each selfing and crossing, the stamens were removed using a pair of tweezers just before corolla opening. At this stage, anthers had not dehisced but stigmas were receptive. Fresh pollen was applied to the stigma of the female parent. Pollen of *M. quintuplinervia* stored in a refrigerator at 4-6°C was used because of differences in flowering time. The pollinated flower was covered with a waxed paper bag (Figure 1) and the bag was removed when the stigma withered. Capsules were collected when mature and open. Seeds from capsules were examined under a dissecting microscope. Seeds which looked flattened and non-viable were not counted while those which looked plump were recorded for each crossing and selfing. Where very large numbers

Fig. 1. Method of selfing and crossing.

(Meconopsis betonicifolia)

of seeds were set, they were recorded as greater than 300. Seeds from all successful crosses were sown in the pots by staff of the Botanical Garden Nursery at the University of British Columbia in the spring of 1985.

Pollination treatments used in M. betonicifolia were: (1) exposure to natural environment after emasculation; (2) bagging flowers without emasculation; (3) bagging flowers after emasculation; (4) pollination using different plants of the same species; and (5) pollination using the same flower.

For chromosome counts, whole flower buds were fixed in ethanol:chloroform:acetic acid (6:3:1) after pretreatment with p-dichlorobenzene. They were stored in a refrigerator and squashed in acetocarmine (4 g in 100 ml 45% acetic acid l^{-1}). The slides were observed using a Photomicroscope II (Carl Zeiss Oberkochen/West Germany).

In the following text, the combination: "A species x B species" means A as female and B as male parents; the combination: "A species x A species" means selfing.

Results

Percentage of capsules setting seeds (fruit set), seeds per pollination and seeds per capsule were calculated as measures of selfing and cross compatibility (Table 1). They were all linearly correlated: Seeds per pollination and fruit set, $r=0.74$, ($p<0.01$, Figure 2); seeds per capsule and fruit set, $r=0.58$ ($p<0.05$, Figure 3); seeds per capsule and seeds per pollination, $r=0.90$ ($p<0.01$, Figure 4).

The meiotic chromosome numbers found were: *M. cambrica*, n=14 (Figure 5) and *M. villosa*, n=16 (these represent tetraploidy with x=7 and x=8 respectively); *M. quintuplinervia*, n (approximate)=42 and *M. betonicifolia*, n=40 (Figure 6) (these are nearly duodecaploids); and *M. horridula*, n=28, and *M. aculeata*, n=28 (octoploids).

Since the number of seeds per pollination is highly correlated with both fruit set and seeds per capsule, it was used as the index to represent the degree of compatibility. Figure 7 illustrates all selfing and crossing results in Table 1. It can be seen clearly that: (1) of those species selfed, compatibility was highest in *M. cambrica*, *M. betonicifolia* and *M. horridula*,

Table 1.

Selfing and crossing among six species of Meconopsis

cross	rank (Fig.7)	no. of flowers pollinated	no. of capsules setting seeds	% of capsules setting seeds	total seed number	no. of seeds per polli-nation	no. of seeds per capsule
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
M.c.x M.c.	1	11	9	82	>300	>27.3	>33.3
M.c.x M.v.	1	15	5	33	128	8.5	25.6
M.c.x M.q.	3	14	0	0	0	0	0
M.c.x M.b.	3	11	2	18	6	0.6	3.0
M.c.x M.h.	2	16	4	25	28	1.8	7.0
M.c.x M.a.	3	15	3	20	7	0.5	2.3
M.v.x M.a.	1	4	4	100	>300	>75.0	>75.0
M.b.x M.c.	3	15	3	20	6	0.4	2.0
M.b.x M.v.	2	15	9	60	70	4.7	7.8
M.b.x M.q.	1	15	5	33	98	6.5	19.6
M.b.x M.b.	1	18	8	100	>300	>16.7	>16.7
M.b.x M.h.	2	15	6	40	42	2.8	7.0
M.b.x M.a.	1	12	10	83	180	15.0	18.0
M.h.x M.c.	2	6	3	50	15	2.5	5.0
M.h.x M.b.	3	3	1	33	2	0.7	2.0
M.h.x M.h.	1	15	3	20	135	9.0	45.0
M.h.x M.a.	1	10	8	80	>300	>30.0	>37.5
M.a.x M.a.	2	10	3	30	18	1.8	6.0

M.c.= *M. cambrica*; M.v.= *M. villosa*; M.q.= *M. quintuplinervia*
M.b.= *M. betonicifolia*; M.h.= *M. horridula*; M.a.= *M. aculeata*

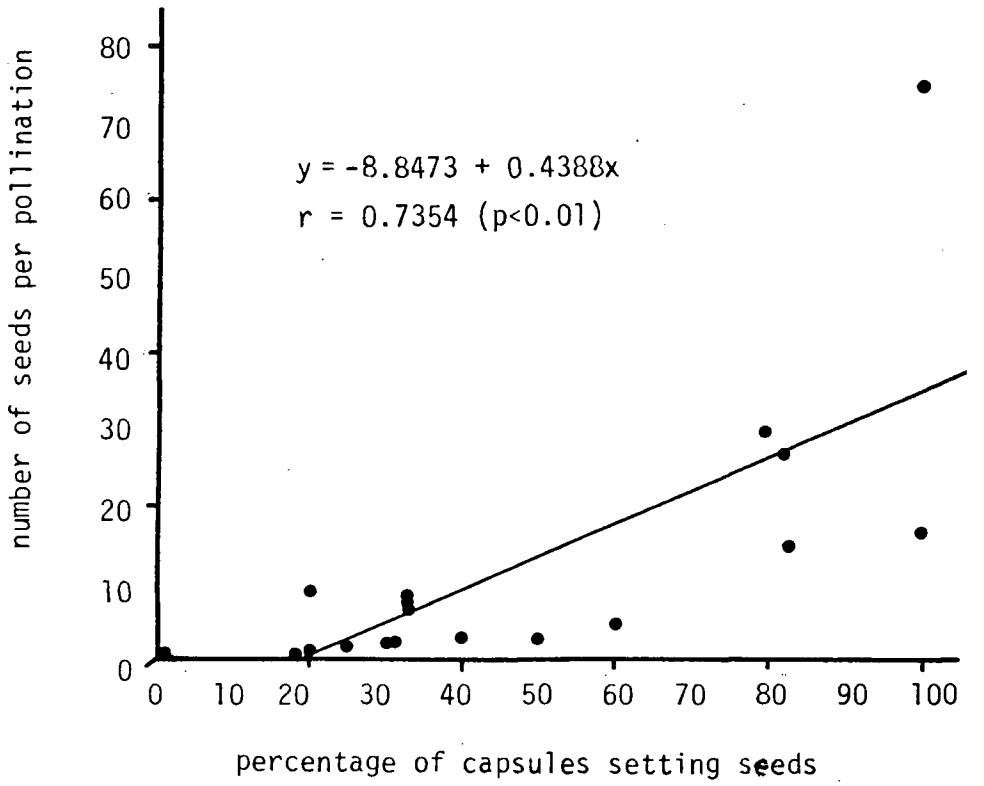


Fig. 2. Relationship between percentage of capsules setting seeds and seeds per pollination in Meconopsis (from Table 1 column 5 and 7).

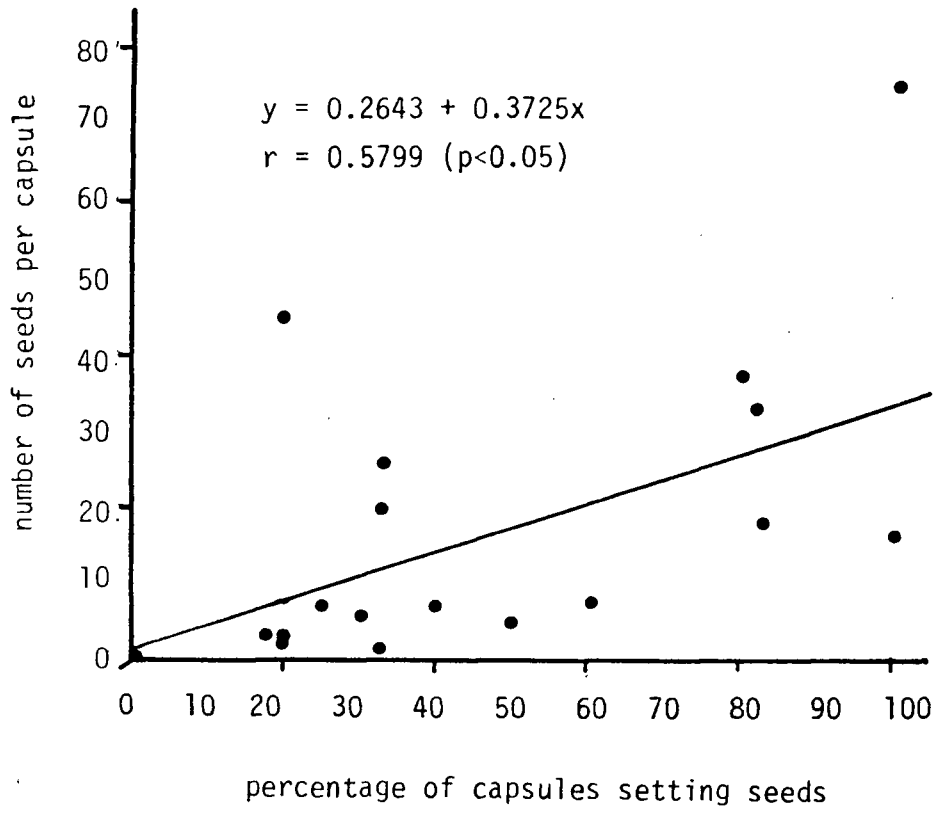


Fig. 3. Relationship between percentage of capsules setting seeds and seeds per capsule in Meconopsis (from Table 1 columns 5 and 8).

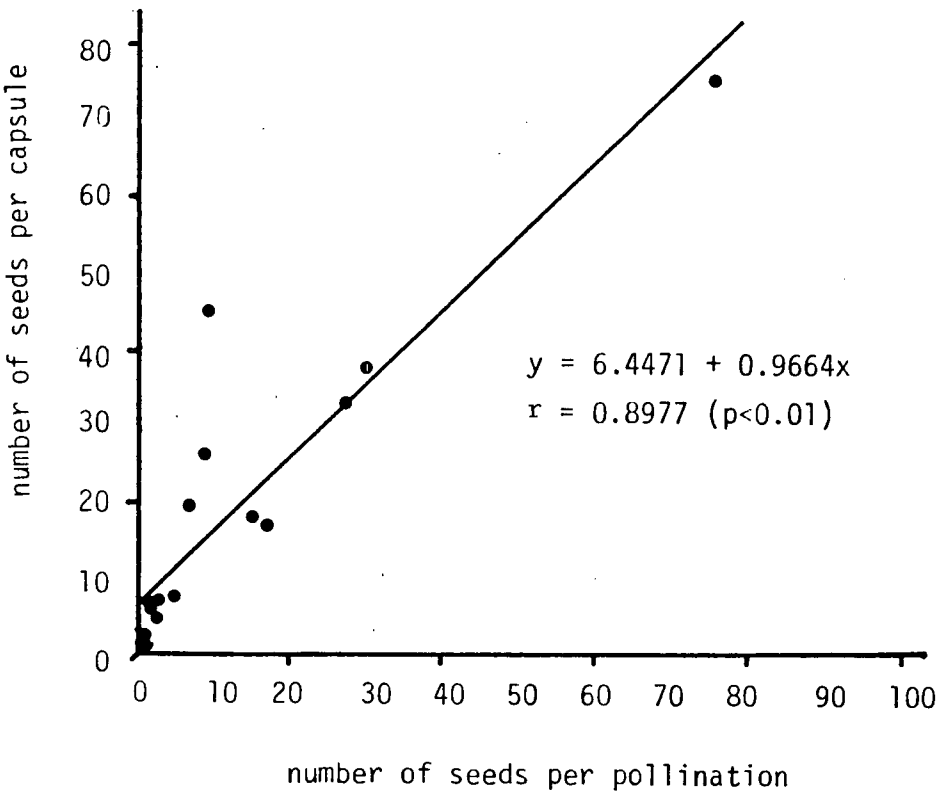


Fig. 4. Relationship between seeds per pollination and seeds per capsule in Meconopsis (from Table 1 columns 7 and 8).

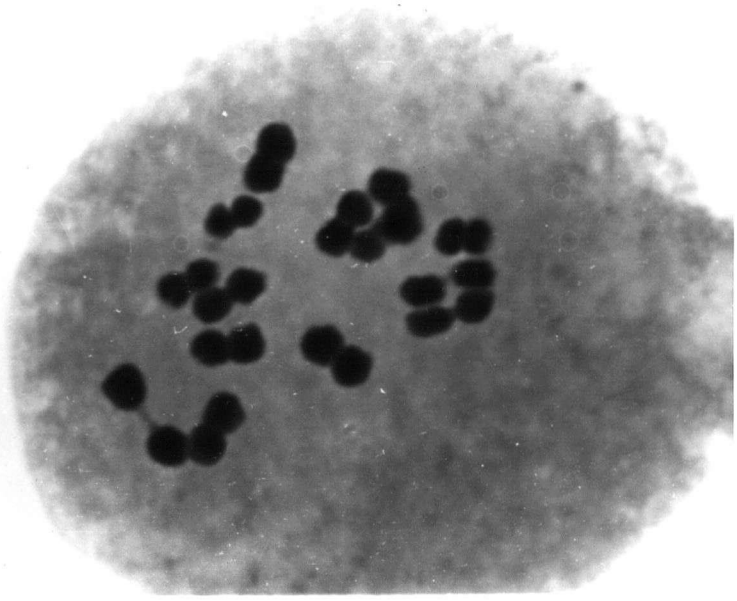


Fig. 5. Meiotic chromosomes of Meconopsis cambrica (x1000).

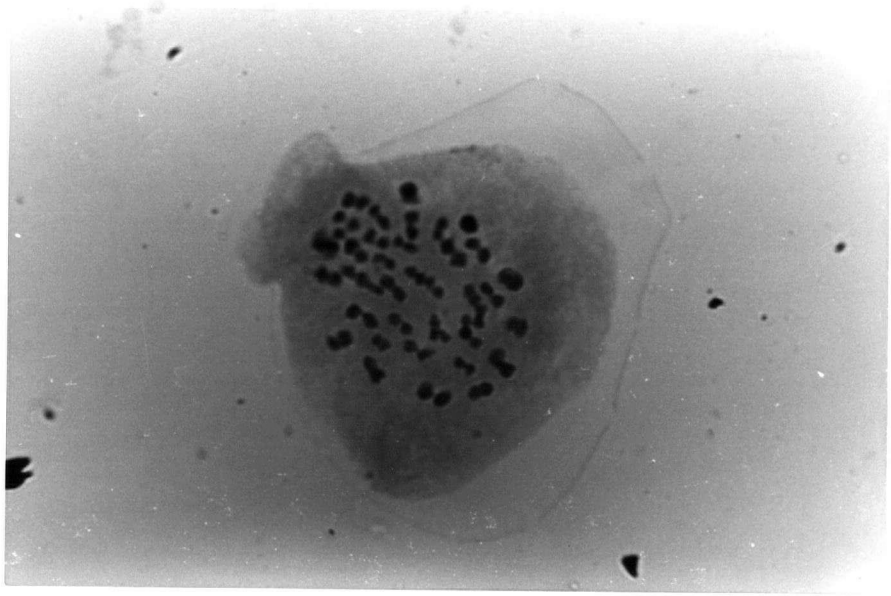


Fig. 6. Meiotic chromosomes of Meconopsis betonicifolia (x400).

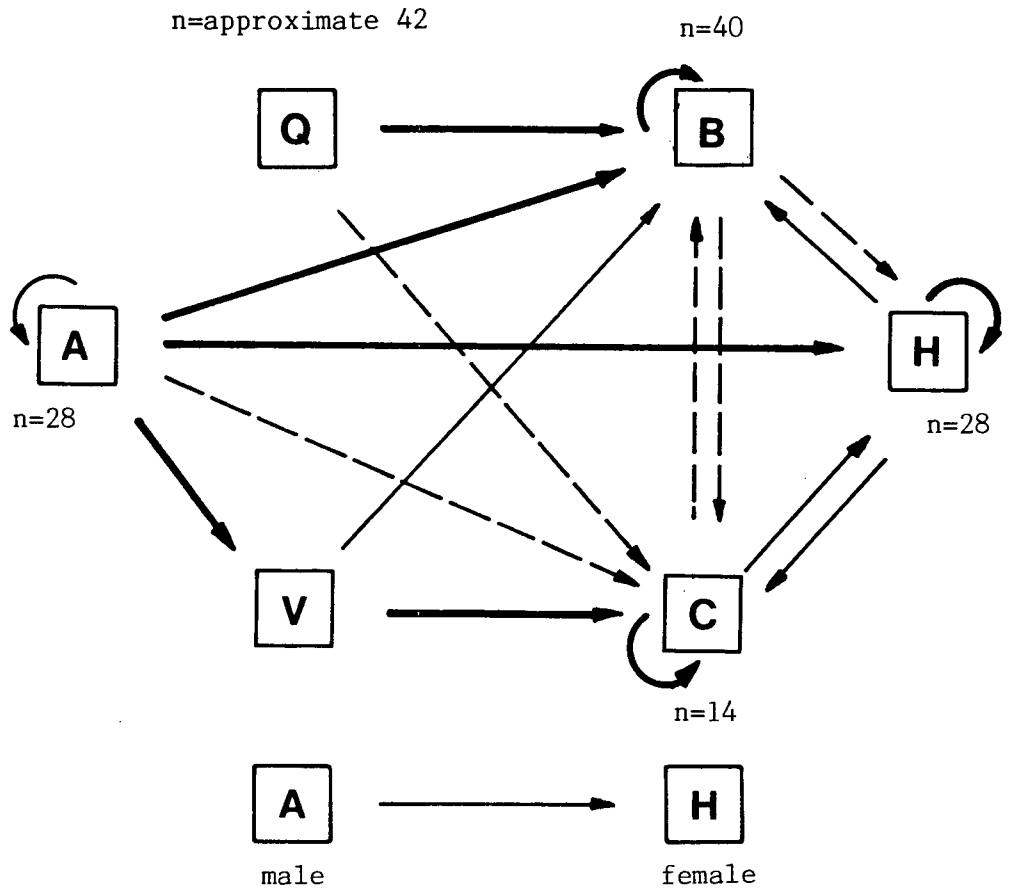


Fig. 7. Rank illustration of self-fertility and cross compatibility based on seeds per pollination (Species which are on the same horizontal line are at the same ploidy level).

Legend:

Rank	seeds per pollination	sign	compatibility
1	> 5	———	highest
2	1.8--5	———	
3	< 1.8	— — — —	lowest

Q=M. quintuplinervia; B=M. betonicifolia; A=M. aculeata;
H=M. horridula; V=M. villosa; C=M. cambrica.

and lowest in M. aculeata; and (2) the interspecific cross compatibility was highest between species at the same ploidy level and between M. betonicifolia x M. aculeata and M. villosa x M. aculeata. Seeds set from these crosses exceeded 5 seeds per pollination. Other crosses gave low seed set (less than 5 seeds per pollination).

Seeds sown in the Botanical Garden Nursery all germinated and grew into seedlings. The first hybrid (M. cambrica x M. horridula) flowered on 29 May, 1985. This hybrid showed the intermediate characters of both parents. For example, flower colour was purple at the base of the petals (M. horridula has purple flowers) and was orange at the top of the petals (M. cambrica has yellow to orange flowers)(information about seed germination and the hybrid produced were from the Botanical Garden Nursery of the University of British Columbia).

Pollination treatments in M. betonicifolia (Table 2) showed that: (1) exposure to the natural environment, after emasculation, gave 93% capsule set. (2) 18% of capsules set seeds after bagging flowers. (3) No seeds set when flowers were bagged after emasculation. (4) Selfing and intraspecific crossing gave 100% fruit set (treatments IV and V).

Table 2.

Pollination treatments for Meconopsis betonicifolia

no.	treatment	no. of flowers pollinated	no. of capsules setting seeds	total seeds number	% of capsules setting seeds	seeds per polli-nation	seeds per capsule
I.	emasculation + natural exposure	15	14	>300	93	>20.0	>21.4
II.	bagging flowers	11	2	17	18	1.6	8.5
III.	emasculation + bagging	10	0	0	0	0	0
IV.	pollen from different plant	9	9	>300	100	>33.3	>33.3
V.	pollen from the same flower	11	11	>300	100	>27.3	>27.3

Discussion

According to difference in ploidy level between parents, selfing and crossing carried out in the current experiment were divided into four groups (Table 3). In Groups I, II and III, the differences in chromosome ploidy level between parents are 8, 4 and 0 respectively. Group IV shows the selfing results. The correlation analysis showed that the greater the difference in ploidy level between parents, the less compatibility between species. The correlation between ploidy level differences and compatibility ranks was highly significant with $r=0.74$ ($p<0.01$).

The fact that compatibility (measured by seed set ability) was correlated with differences between parents in ploidy level may result from genetic or cytoplasmic incompatibilities that are expressed either in fertilization or at any developmental stage of the zygote between early cleavage divisions and maturity.

In interspecific crosses, pollen tubes sometimes burst in the styles of foreign species (Bucholz and Blakeslee, 1929). In addition, the thick pollen tubes of polyploid species sometimes have difficulty growing in the slender styles of diploids or lower polyploids (Allard, 1960). These may be reasons for lower

Table 3

Analysis of compatibility in Meconopsis based on chromosome ploidy level

No. of group	Cross (1)	Difference in ploidy level (2)	Rank in compatibility (3)
	M.c. x M.q.	8	3
I	M.c. x M.b.	8	3
	M.b. x M.c.	8	3
	M.b. x M.v.	8	2
	M.c. x M.h.	4	2
	M.c. x M.a.	4	3
	M.v. x M.a.	4	1
II	M.b. x M.h.	4	2
	M.b. x M.a.	4	1
	M.h. x M.c.	4	2
	M.h. x M.b.	4	3
	M.c. x M.v.	0	1
III	M.b. x M.q.	0	1
	M.h. x M.a.	0	1
	M.c. x M.c.	0	1
IV	M.b. x M.b.	0	1
	M.h. x M.h.	0	1
	M.a. x M.a.	0	2

(1) abbreviations are the same as Table 1.

(2) higher ploidy level of one parent minus lower ploidy level of another parent (see text for ploidy level).

(3) rank based on seeds per pollination (see Figure 7).

compatibility of the crosses: M. cambrica x M. quintuplinervia, M. cambrica x M. aculeata, M. cambrica x M. betonicifolia and M. horridula x M. betonicifolia (Figure 7). The female parents of these crosses all had lower ploidy level than the male parents. Overall seed set for these crosses was less than 1.8 seeds per pollination.

Meconopsis quintuplinervia has been grown in the Botanical Garden from seedlings received from Britain. It has not set seeds after several years even though hand pollination has been carried out (J. MacPhail, personal communication). Although a hybrid between M. quintuplinervia and M. cambrica has been reported (Cobb, 1984), there was no seed set from the cross of M. cambrica x M. quintuplinervia in the present experiment. Pollen grains of this species were viable after 10-day storage because they did successfully lead to seed set of M. betonicifolia. The hybrid reported between M. quintuplinervia and M. cambrica (Cobb, 1984) might be a reciprocal cross of M. cambrica (4x, female) x M. quintuplinervia (12x, male). The barrier to pollen tube growth of polyploid species in the styles of lower polyploids may be overcome by making the reciprocal cross (Allard, 1960). Although Cobb (1984) reported that this hybrid existed sixty years ago, he

failed to recreate it.

An early hypothesis suggested that a particular balance of chromosome sets between the major plant parts of the developing seed (maternal tissue:endosperm:embryo) was necessary for normal seed development (Peloquin, 1981). Muntzing (1933) suggested that a 2:3:2 ratio of the ploidy levels of the maternal tissue:endosperm:embryo was a requisite for normal seed development. Although it is not clear whether the endosperm of seeds in Meconopsis is triploid, the balance of the chromosome sets should be obtained more easily if both parents have the same ploidy level. In the present experiments, crosses between parents which have the same ploidy level have the highest compatibility (Figure 7).

The same level of compatibility was obtained for the reciprocal crosses: M. cambrica and M. betonicifolia, and M. cambrica and M. horridula, but not for the reciprocal crosses between M. betonicifolia and M. horridula. Differences between reciprocal crosses are often observed when the species that are hybridized do not have the same number of chromosomes (Allard, 1960). When the species with the larger number is female the cross is more successful than when it is male. This was

demonstrated in wheat and some dicotyledons (Thompson, 1930). This is also the case in the reciprocal crosses between M. betonicifolia and M. horridula. When M. betonicifolia (12x) was used as the female in the cross, higher seed set could be obtained than in the reciprocal one.

It is hard to explain the high compatibility of the crosses: M. betonicifolia x M. aculeata and M. villosa x M. aculeata based on information about ploidy level alone. This may be attributable to the fact that M. villosa and M. betonicifolia can readily set seeds in gardens (Evans, 1959, and personal observation).

The experiments carried out in M. betonicifolia show that this species had high self-fertility and intraspecific cross compatibility (treatments IV and V of Table 2). Exposed to the natural environment after emasculation, the flower could be pollinated from the pollen of other flowers with high success (treatment I of Table 2). Pollination agents would be necessary for cross-pollination in this case. The pollinator is unlikely to be wind, based on the result of treatment II (Table 2). Of 11 flowers treated in treatment II, two set seeds. These two flowers were inspected by opening the bags to see if there was pollen on the stigma. This may have caused self-pollination through

touching the flowers. Moreover, Meconopsis betonicifolia has large, brilliant blue flowers and its pollen grains are heavy and compact. Insects are assumed to be the pollinators in this situation. No apogamy in this species was observed (treatment III of Table 2).

Conclusions

(1) All interspecific crosses except *M. cambrica* x *M. quintuplinervia* in this study set seeds (Table 1) but the seed setting ability was highly correlated with the difference in ploidy level between parents. This supports the hypothesis that the degree of compatibility between species differs with difference in ploidy level between parents. The successful growth and flowering of hybrids shows that it may be possible to combine desirable characters from both parents.

(2) All selfed plants set seeds. The four species: *M. cambrica*, *M. betonicifolia*, *M. horridula* and *M. aculeata* are self-fertile. This fact supports the hypothesis that species of this genus are self-compatible.

(3) The hypothesis that no apogamy exists in *M. betonicifolia* is supported.

(4) Insects are the likely pollinators for *M. betonicifolia*.

Section B. Pollen Staining and Germination Test for Six Species in *Meconopsis*

Introduction

Henderson (1965) divided the pollen grains of *Meconopsis* into a number of morphological types based on shape and size of pollen grains, and thickness of exine and sexine. Of these six species, the pollen grains of *M. quintuplinervia*, *M. horridula* and *M. aculeata* belong to the horridula-type while those of the other three, i.e., *M. cambrica*, *M. villosa* and *M. betonicifolia* are allocated to cambrica-type, villosa-type and betonicifolia-type respectively.

Among the six species, *Meconopsis quintuplinervia* flowers in April while *M. villosa* flowers in June in Vancouver. Their blooming times do not overlap. An interspecific breeding program involving these species would require pollen storage to allow controlled pollinations between species that do not flower at the same period.

The object of the pollen viability test was to determine

some of factors affecting pollen staining, pollen germination and pollen storage of the six species.

Lactophenol cotton blue has been reported as an effective pollen staining agent (Radford et al., 1974).

Of the primary factors affecting pollen germination, carbohydrates in the form of sucrose or honey are most often added to germination media as an energy supply (Stanley, 1967). The optimal concentration varies with plant species (Darlington and La Cour, 1976). Boric acid usually has the most pronounced effect in stimulating pollen germination (Goddard and Matthews, 1981; Li, 1982). 0.5-2% agar was recommended for pollen germination (Goddard and Matthews, 1981; Li, 1982).

Matthews and Kraus (1981) recommended pollen storage in a desiccator with a drying agent at about 4°C.

The hypotheses of this experiment were:

(1) boric acid is necessary for pollen germination in Meconopsis;

(2) pollen grains of different types have different requirements for germination.

Materials and methods

Non dehisced anthers of *Meconopsis cambrica*, *M. villosa*, *M. quintuplinervia*, *M. betonicifolia*, *M. horridula* and *M. aculeata* were placed in small vials. The vials were covered with three layers of cheese cloth and placed in a desiccator which contained granular anhydrous calcium chloride. The desiccator was put in a refrigerator at 4-6°C for storage. Germination tests of the stored pollen were carried out every week. For staining and germination tests on fresh pollen, the vials were maintained at 23°C (± 2°C) until anther dehiscence.

For pollen staining tests, fresh pollen was put on a slide with a drop of lactophenol cotton blue. The percentage of pollen grains stained was recorded after 3 hours. Observations of pollen staining tests were carried out at x160 magnification using a Photomicroscope II (Carl Zeis Oberkochen/West Germany). For each species, two slides were made. The staining percentages on each slide were then estimated by counting a random sample of 100 pollen grains.

To test pollen germination, an agar medium was used. The medium consisted of 5 g sucrose l^{-1}, 0.5% or 1% Bacto agar

(Difco) with or without 0.1 mg H_3BO_3 l^{-1}. Pollen tubes of M. cambrica, M. villosa, M. horridula and M. aculeata burst on 0.5% agar medium. The susequent germination test was conducted on 1% agar medium for these species. For M. betonicifolia, only 0.5% agar was added because no germination was observed on 1% agar medium for this species. The medium was autoclaved at 121°C (15 psi) for 15 min and then poured into 100x15 mm petri dishes. After the medium solidified, 20x20 mm agar blocks were cut from the dishes and placed on slides. Each was then placed in a petri dish lined with filter paper that was saturated with distilled water. Pollen was placed on the agar blocks with a fine hair brush. The dishes were covered to prevent moisture loss and incubated at 23°C (± 2°C). Pollen germination was observed after 24 hours under the microscope using the same method as for pollen staining. Pollen grains with a tube length greater than their diameter were described as having germinated.

Pollen staining and germination percentages were transformed to arcsin square root before analysis of variance. The values shown in the results are values transformed back.

Results

Pollen stainability for all six species tested equalled or exceeded 85% (Table 4). No significant differences were found among the six species. Figures 8 and 9 show stained pollen grains of *M. betonicifolia* and *M. aculeata*. They belong to different pollen types: betonicifolia pollen type and horridula pollen type respectively.

Pollen of *Meconopsis cambrica*, *M. villosa*, *M. betonicifolia*, *M. horridula* and *M. aculeata* germinated on the artificial medium tested (Table 5). Figure 10 shows the pollen tube growth of *M. aculeata*. 1% agar medium was suitable for pollen germination of *M. cambrica*, *M. villosa*, *M. horridula* and *M. aculeata* while 0.5% agar medium was suitable for pollen germination of *M. betonicifolia*.

Pollen germination percentage decreased with storage time (Table 5). Pollen of *M. cambrica*, *M. horridula* and *M. aculeata* germinated after one or two-week storage while that of *M. villosa* and *M. betonicifolia* did not germinate even after only one-week storage.

No pollen germinated on sucrose medium in the absence of boric acid.

Table 4.

Pollen staining percentage in six species of Meconopsis

species	percentage	test result
M. cambrica	85	no significant
M. villosa	92	differences
M. quintuplinervia	93	exist among
M. betonicifolia	91	the six means.
M. horridula	94	
M. aculeata	91	

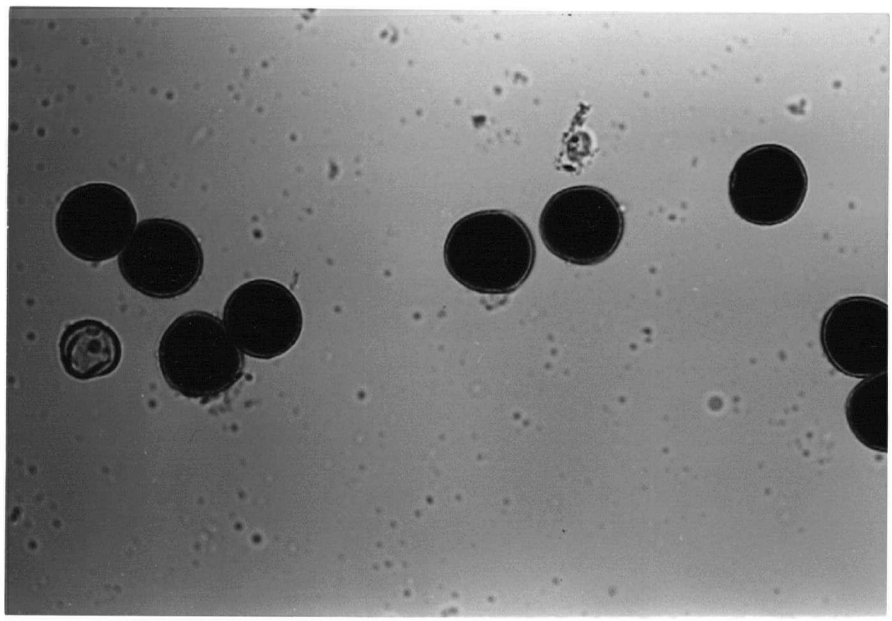


Fig. 8. Stained pollen grains of Meconopsis aculeata (with one unstained pollen grain), (x160).

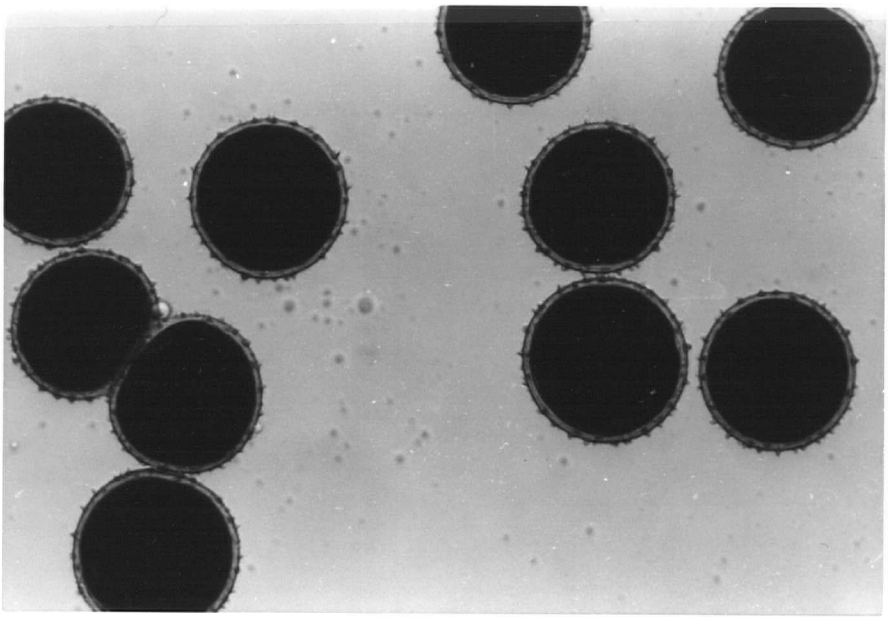


Fig. 9. Stained pollen grains of Meconopsis betonicifolia (x160).

Table 5

Pollen germination percentage in six species of Meconopsis

storage time (week)	0	1	2	3
species				
M. cambrica	29.5 a*	7.7 b	2.5 c	0 d
M. villosa	7.9 a	0 b	___	___
M. quintuplinervia	___	___	0	___
M. betonicifolia	20.0 a	0 b	___	___
M. horridula	18.5 a	13.0 b	2.9 c	0 d
M. aculeata	24.0 a	11.0 b	2.9 c	0 d

___=germination tests were not carried out where no value is presented.

* values not labeled by the same letter in the same row differ significantly at 5% level based on Duncan's Multiple Range Test.

Note: pollen of M. betonicifolia germinated on 0.5% agar medium. Pollen of other species germinated on 1% agar medium.

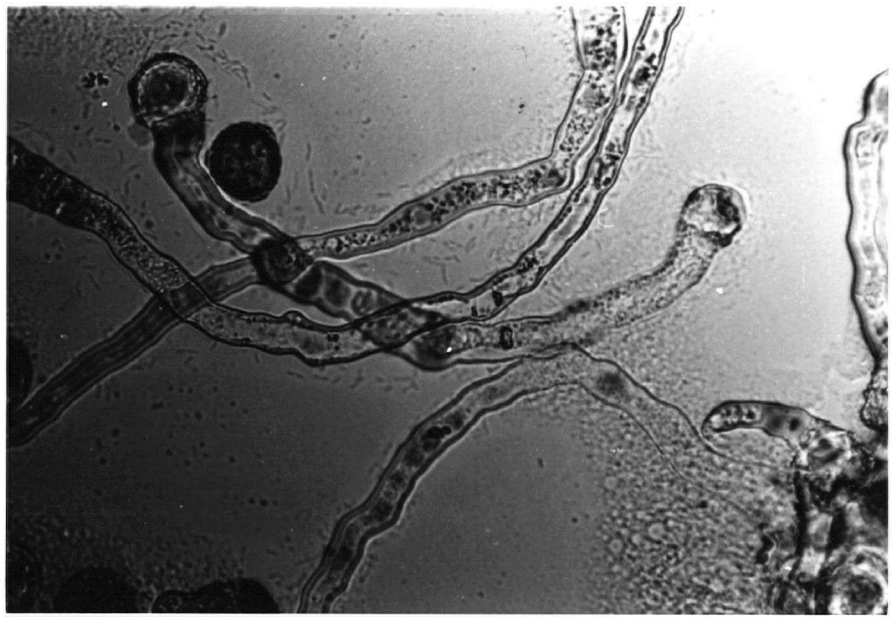


Fig. 10. Pollen tube growth of Meconopsis aculeata (x160).

Discussion

Lactophenol cotton blue is effective in pollen staining of the six species of Meconopsis. Pollen staining effectiveness was similar for different pollen types.

Pollen of Meconopsis quintuplinervia, M. horridula and M. aculeata belongs to the same horridula pollen type. Pollen of M. horridula and M. aculeata germinated after two week storage but that of M. quintuplinervia did not. When pollen germination tests were carried out, no fresh pollen of this species was available because this species flowered earliest of the six species. It is not known whether or not fresh pollen of M. quintuplinervia can germinate on the medium tested. However, this test shows that pollen of the same pollen type from different species behaves differently.

Pollen from different pollen types has different requirement for germination. Pollen of M. betonicifolia (betonicifolia pollen type) germinated on the 0.5% agar medium but not on the 1% agar medium (Figures 11 and 12). One reason for this may be due to the water potential difference between 1% agar medium and the pollen grains. Pollen grains of M. betonicifolia may have a higher

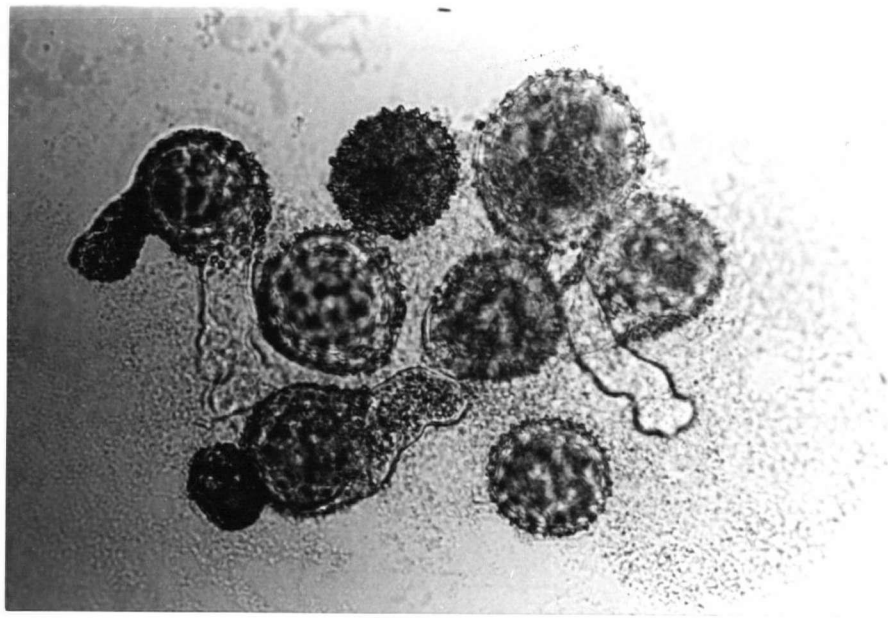


Fig. 11. Fresh pollen germination of Meconopsis betonicifolia on 0.5% agar medium (x160, 3 hour incubation).

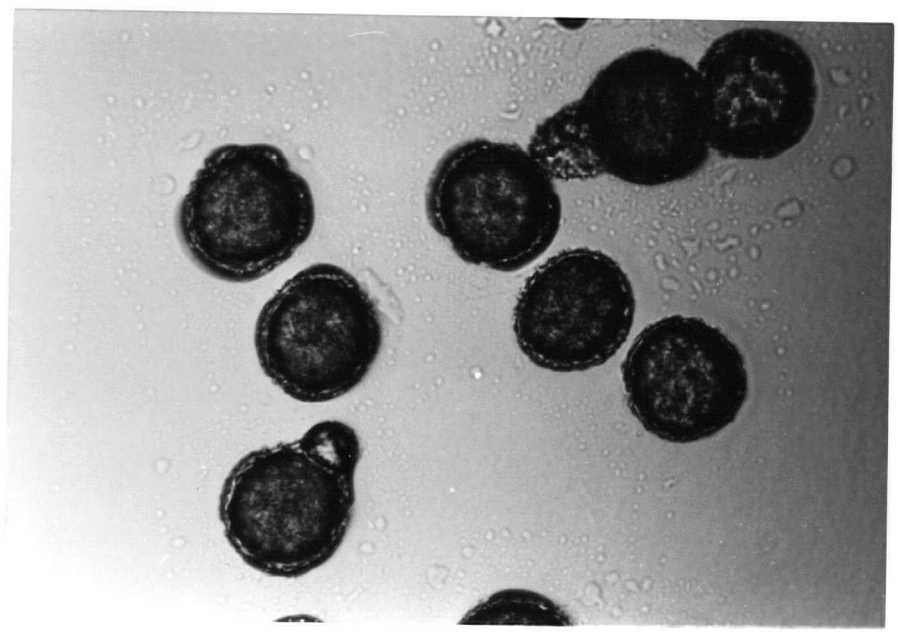


Fig. 12. Fresh pollen of Meconopsis betonicifolia on 1% agar medium (x160, 24 hour incubation).

water potential than those of the other species from the other pollen types tested, and thus the speed of hydration, exudation and germination of pollen is reduced. Another reason may be the absence of apertures on its pollen grains (Figure 9). Henderson (1965) described the absence of apertures as one of the most striking features of betonicifolia pollen type.

The sequence recorded by Watanabe (1955) as typifying a successful pollination includes hydration, exudation, and germination phases. Watanabe (1955, 1961) established that exudation is an essential prelude to germination in the grasses; grains that do not exude, do not germinate. Liquid is exuded both from the aperture and the non-apertural exine (Heslop-Harrison, 1980). More exudation was observed in 0.5% agar medium (Figure 11) than in 1% agar medium (Figure 12). It is possible that non-aperture pollen grains of M. betonicifolia cannot exude and thus germinate as quickly as that of the other species tested on 1% agar medium. Pollen can hydrate, exude and germinate more readily on the semi-solidified agar medium (0.5%) than on 1% agar medium because of more rapid hydration and exudation. The more fully hydrated the pollen grains, the shorter the interval before germination because less time is needed for water uptake (Heslop-

Harrison, 1980). Figure 12 shows that some pollen tube tips emerged but did not grow on 1% agar medium. It was assumed during the experiment that after longer incubation, these grains might produce germ tubes. Although the germination test lasted for one week for this test, no germination was observed because of bacterial and fungal contamination. This phenomenon was also observed for pollen germination of the other species tested after two-day incubation on the artificial media. Contaminants can reduce germination (Goddard and Matthews, 1981). Thus, 0.5% agar medium is more suitable than 1% agar medium for pollen germination of M. betonicifolia.

Pollen germination often needs boron as a stimulus (Li, 1982). It is also necessary for pollen germination in Meconopsis. In sucrose medium in the absence of boric acid, no pollen germination was observed.

The data in Tables 4 and 5 indicate that pollen germination percentage on the artificial medium was consistently lower than percentage pollen grains effectively stained by lactophenol cotton blue. This is also the case in avocado pollen (Sahar and Spiegel-Roy, 1984) and rose pollen (Pearson, 1984). Visser et al. (1977) called stainable pollen grains "normal pollen", but

considered them as having only the potential to germinate. Erlanson (1931) reported that many apparently morphologically perfect grains were unable to effect fertilization. Pollen germination alone is not a complete measure of fertility since there are other contributing factors. In addition, germination of pollen in artificial culture is not the same as on the stigma. However, *in vitro* pollen germination capacity can estimate an important aspect of fertility (Goldy and Lyrene, 1983).

Conclusions

(1) Fresh pollen grains of the six species in *Meconopsis* can be stained using lactophenol cotton blue. The stainability for the six species was 85% or more.

(2) Pollen of *Meconopsis cambrica*, *M. villosa*, *M. horridula* and *M. aculeata* germinated on the 1% agar medium with sucrose (5 g 1^{-1}) and boric acid (0.1 mg 1^{-1}). Pollen of *M. betonicifolia* germinated on the same medium with only 0.5% agar. The hypothesis that pollen from different pollen types have different requirements for pollen germination is supported.

(3) The hypothesis that boric acid is necessary for pollen germination of the species in *Meconopsis* is supported. No pollen germinated on sucrose medium in the absence of boric acid.

(4) Pollen germination percent decreased with storage time.

Section C. Influence of Temperature and Day-length on Flowering of *Meconopsis betonicifolia*

Introduction

No previous information concerning control of flowering of *Meconopsis* was available. Experimental change of flowering time could be an alternative method to pollen storage for species which do not flower simultaneously. *Meconopsis betonicifolia* was chosen to study flowering time alteration in the growth chambers because of the availability of plant materials and their horticultural usefulness.

The growth and flowering requirements for *M. betonicifolia* were investigated. The outdoor daily mean temperature in 1983 was approximately 11°C (data from the Climatology Station of the University of British Columbia). The flowering period of *M. betonicifolia* in the Botanical Garden is from early May to the end of June. The photoperiod outdoors is approximately 15-16 hours during this period in Vancouver. It is therefore possible that long daylength is needed for its flowering.

The geographical range of M. betonicifolia is in South-eastern Tibet, north-western Yunnan and northern Upper Burma at altitudes from 3,000-4,000 m (Taylor, 1934). Thus it was assumed that plants of M. betonicifolia could grow and flower at low temperatures.

The hypothesis for this experiment was that flowering of M. betonicifolia is determined by day-degree accumulation.

Materials and methods

One-year-old potted seedlings of *M. betonicifolia* from the Botanical Garden Nursery of the University of British Columbia were studied for their flowering response in 4 daylength/temperature regimes: short days (SD, 8 hour) and long days (LD, 16 hour) combined with low temperature (LT, $6^{o}\pm2^{o}C$) and high temperature (HT, $17^{o}\pm2^{o}C$). Temperatures were constant day and night. The experiments were initiated on December 20, 1983 in growth chambers (Conviron Model EF7 with eight F48T12/CW/HO fluorescent tubes and four 40 w incandescent lamps) at an energy fluence rate of 126 micro Einsteins m^{-2} s^{-1}.

After 70 days, seedlings in SD, LT were transferred to LD, LT conditions because no growth was observed in SD, LT after 70 days.

There were 5 seedlings for each treatment. Plants were examined every other day, and the following dates were recorded: when noticeable growth commenced (new leaves visible), when the terminal flower bud could be seen macroscopically, when the first flower unfolded and when the last flower wilted.

Selfing was carried out at both low and high temperatures.

Results

At low temperature, seedlings began to grow and the apical flower bud became visible later (Table 6). Plants needed longer from the beginning of growth to flowering at low temperature (Table 6).

No difference was observed in the number of days needed to begin flowering at high temperature between LD and SD (Table 6).

Table 6

Daylengh and temperature change in relation to growth and flowering of M. betonicifolia

(days from the begining of experiment)

treatment	days to begin growth	days to flower bud	days to first flower	days to last flower	days from growth to first flower	flowering period: days	flowering period: dates (1984)
	(1)	(2)	(3)	(4)	(5) (3)-(1)	(6) (4)-(3)	(7)
6°C,LD	77.0 a*	157.3 a	200.3 a	238.5 a	123.3	38.2	July 8-Aug.15
17°C,LD	6.0 b	51.3 b	82.0 b	118.5 b	76.0	36.5	Mar.12-Apr.18
17°C,SD	6.0 b	64.0 b	93.0 b	110.0 b	87.0	17.0	Mar.23-Apr.9
probability	p<0.01	p<0.01	p<0.01	p<0.05			

* values labeled by different letter in the same column differ significantly (based on Duncan's Multiple Range Test).

Discussion

Potted seedlings of *M. betonicifolia* grew and flowered at both low temperature (6°C) and high temperature (17°C) (Figures 13 and 14). Temperature affected growth and flowering of *M. betonicifolia*. High temperature induced earlier growth and flowering. The number of days needed to begin growth (new leaves visible) at low temperature is nearly thirteen times as many as that needed at high temperature (Table 6). The number of days needed to reach flowering doubles at low temperature (Table 6).

Welander (1984) reported that time until the first flower opened, decreased with increasing temperature in *Aeschynanthus speciosus*. Low temperature also delays induction of flowering in *Chrysanthemum morifolium* (Van Ruiten and De Jing, 1984). Fitter and Hay (1981) concluded that the length of time required for completion of the annual growth cycle increases as the climate becomes cooler. The primary effect of low temperature is the reduction of rate of growth and metabolic processes.

Under the same day-length (16 hour photoperiod), flowering could be induced under both low and high temperature but low temperature induced later flowering (Table 6). Accumulated

Fig. 13. Flowering of Meconopsis betonicifolia at low temperature (6°C, LD).

Fig. 14. Flowering of Meconopsis betonicifolia at high temperature (17°C) (comparison of flowering plants of M. betonicifolia with SD plant on the left and LD plant on the right).

temperature plays a role on flower initiation within a certain range of temperature. Table 6 shows that under LD conditions, days needed to first flower at 6°C and 17°C are 200 and 82 respectively. The heat units (0°C base) were 1,200 and 1,394 Celsius degree-days respectively. The outdoor heat units (data from the University of British Columbia Climatology Station) from January to May, 1984 (flowering time of this species in the University of British Columbia Botanical Garden) were 1,220 Celsius degree-days. These values are similar numerically. It is possible that a specific number of heat units are required to initiate flowering of this species. Li (1983) also reported that 450-570 Celsius degree-days were required for initiation of plum fruit buds.

However, the data collected from the Climatology Station (the University of British Columbia) may not be the same as those in the University of British Columbia Botanical Garden because of microclimate differences. Moreover, variation between growth chambers existed. Sometimes accidental temperature fluctuation within the growth chamber during the experiment occurred. These factors prevented a precise experiment from being conducted. The relationship of flower induction to accumulated heat units could

be tested by further experimentation.

Day-length was also found to affect the growth and flowering of this species. After 70 days of low temperature treatment, some seedlings began to grow under LD but not under SD. When re-potting seedlings, it was found that new roots had grown from seedlings subjected to LD while no new roots grew in SD (see Figures 15 and 16 for comparison). The longer day-length and greater irradiation energy stimulated growth at low temperature.

Light provides energy for the synthesis of carbon compounds. Carbohydrate accumulation is associated with light. Therefore many growth processes such as flowering, fruiting and seed production are indirectly affected by light. Root growth is also affected, and occurs after the photosynthate requirements of the top portions of the plant have been met (Janick et al., 1981). Net photosynthesis is measured by both gross photosynthesis in the light and respiration in the dark. In the present experiment, long day-lengths, and thus more irradiation energy and carbohydrate accumulation, fulfil the photosynthate requirements of the top portions of the plant and promote both shoot and root growth. Carbohydrates cannot accumulate sufficiently to satisfy plant requirements in short days, or at least cannot accumulate

Fig. 15. Potted seedlings of Meconopsis betonicifolia after 70-day SD treatment at 6°C (show no growth of new roots).

Fig. 16. Potted seedlings of Meconopsis betonicifolia after 70-day LD treatment at 6°C (show growth of new roots).

as rapidly as in the long day-lengths.

Day-length does not seem to have an effect on growth and flowering at high temperature as stated in the hypothesis because both LD and SD could induce growth, flower bud formation and flowering in approximately the same time (Table 6). But qualitative differences existed (Figure 14). Under SD, flowers were much smaller (5 cm in diameter). Some flower buds wilted before unfolding. The leaf area of the largest leaf was only 24 cm^2. Under LD, flower size doubled. The leaf area of the largest leaf was 78 cm^2. It can be seen from column 6 of Table 6 that the number of days from first flower to last flower (flowering period) under LD conditions at high temperature is double that under SD conditions.

Selfing in the growth chamber set seeds at both low and high temperature but the seed number (7-15 seeds per capsule) was only half as many as selfing of M. betonicifolia grown outdoors.

The information provided by this experiment could be useful in a breeding program related to this species. The flowering time was from March 12 to April 18 at high temperature and LD in the growth chamber, was from early May to the end of June outdoors in the University of British Columbia Botanical Garden, and was from

July 8 to August 15 at low temperature combined with LD in the growth chamber.

Pollen of M. betonicifolia can be used to pollinate the species which flower during these five months without pollen storage. Pollen from other species might be transferred to M. betonicifolia to set hybrid seeds during this period of time.

Conclusions

(1) Higher temperature (17°C) induced earlier growth and flowering than lower temperature (6°C). Temperature can change flowering time of *M. betonicifolia* under the growth chamber conditions. This is supportive of the hypothesis that flowering of *M. betonicifolia* is determined by day-degree accumulation.

(2) Long daylength (16 hour photoperiod) produced more vigorous plants with larger flowers than short daylength.

Section D. *In Vitro* Establishment of *Meconopsis betonicifolia*

Introduction

In vitro propagation of genera from many plant families has been studied but no previous information about tissue culture of *Meconopsis* has been reported. *Papaver*, which is closely related to *Meconopsis*, has been grown in tissue culture systems. The information from this genus was used for reference in the *in vitro* culture of *Meconopsis* based on the assumption that plants with close phylogenetic relationships may have similar requirements for growth and development.

To establish *in vitro* cultures, explants, media, plant growth regulators and incubation conditions should be considered.

To obtain explants, seed surface sterilization and capsule sterilization methods have been used in cotyledon and ovule culture of *Papaver somniferum* (Maheshwari, 1958; Ilahi, 1982). Seedlings were raised on sterilized artificial medium from mature seeds and young ovules. Callus was induced from cotyledons

(Ilahi, 1982), hypocotyls (Schuchmann and Wellmann, 1983; Yoshikawa and Furuya, 1983), and seedling roots (Furuya et al., 1972). Plants of Papaver somniferum were regenerated from callus culture and flowerd in vitro (Yoshikawa and Furuya, 1983).

Murashige and Skoog medium (1962) (MS medium), with some modifications, is the one used most frequently and with greatest success (Hu and Wang, 1983). Full strength and half strength MS media were used for cotyledon culture (Ilahi, 1982), seedling root culture (Furuya et al., 1972) and hypocotyl culture (Yoshikawa and Furuya, 1983) of Papaver somniferum. One third strength MS also supported meristem cultures of Solanum tuberosum (Wang, 1977; Wang and Hu, 1982) and nodal segment cultures of Brassica campestris (Kuo and Tsay, 1977). The preliminary seed germination test conducted in the present study showed that one third strength MS is suitable for seed germination of M. betonicifolia.

Plant growth regulator concentrations in the culture medium are critical to the control of growth and morphogenesis (Skoog and Miller, 1957). Three cytokinins are frequently used: kinetin (Kn), N^6-benzyladenine (BA) and N^6-(2-isopentenyl)-adenine (2ip). Although a particular cytokinin may not be effective for one

species, it may be effective in others (Hu and Wang, 1983). 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most potent auxin in tissue culture (Hu and Wang, 1983).

In tissue culture of Papaver somniferum, different concentrations of different growth regulators were used. MS medium with 2,4-D (1 mg l^{-1}), Kn (0.1 mg l^{-1}) and 7% coconut milk induced callus from hypocotyl (Yoshikawa and Furuya, 1983). Regeneration occurred in the medium supplemented with 0.1-1.0 mg l^{-1} Kn (Yoshikawa and Furuya, 1983). Indole-3-acetic acid (IAA), 2,4-D, Kn and BA were used in cotyledon culture. Combination of Kn and 2,4-D at concentrations (mg l^{-1}): 0.1, 0.5, 1.0 and 2.0 proved to be beneficial for callus induction and its further proliferation (Ilahi, 1982). Kn (0.4 and 0.5 mg l^{-1}), IAA (5 mg l^{-1}) and 1-naphthaleneacetic acid (NAA, 0.1 mg l^{-1}) were used in ovule culture (Maheshwari et al., 1961; Pontovich et al., 1966). These growth regulators enhanced proliferation of the embryonal cells and induced formation of embryos. 2,4-D (0.1 mg l^{-1}), IAA (1 mg l^{-1}) and Kn (0.1 mg l^{-1}) were used for callus formation in seedling root culture (Furuya et al., 1972).

Solanaceous species have been used as model systems of in vitro studies. Among solanaceous species, if the cytokinin:auxin

ratio is <1, callus formation is enhanced; if >1, shoot formation is obtained (Flick et al., 1983). In bud culture of Chinese cabbage, Kuo and Tsay (1977) demonstrated that an exogenous cytokinin:auxin ratio >1 was necessary for good shoot growth and <1 for root differentiation.

The incubation conditions were illumination of about 4,000 lux for 16 hour photoperiod at 16-18°C for shoot formation and regeneration of Papaver somniferum (Yoshikawa and Furuya, 1983). These conditions also support plant growth of M. betonicifolia in the growth chambers (Section C).

The aim of the present work was to determine an optimal explant and medium which supported the in vitro growth and differentiation of M. betonicifolia. Growth and differentiation responses were investigated by using different explants cultured in various media. These media contained different strengths of MS salts and different concentrations of plant growth regulators. It is generally agreed that the lowest concentration of growth regulators which will give desired morphogenetic effect should be used (Murashige, 1974).

The hypothesis of this experiment was that if cytokinin:auxin ratio is greater than 1, then shoot

proliferation in M. betonicifolia would occur.

Materials and Methods

In this experiment, Murashige and Skoog (1962) medium was used as the basic medium. Commercial MS Plant Salt Mix (Flow laboratories, cat. no. 26-330-22, lot 26330016) was supplemented with sucrose (30 g l^{-1}), myo-inositol (100 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), pyridoxine HCl (0.5 mg l^{-1}), thiamine HCl (0.1 mg l^{-1}), glycine (2 mg l^{-1}) and casein hydrolysate (1 g l^{-1}). The medium was adjusted to pH 5.7±0.1 with 1 N HCl or 1 N NaOH before agar addition. The medium was dispensed into 25x150 mm culture tubes (20 ml/tube) and then autoclaved at 121°C (15 psi) for 15 minutes.

All cultures and subcultures were incubated in growth chambers (either Sherer Model Cel 255-6 or Conviron Model EF7 with eight F48T12/CW/HO fluorescent tubes and four 25 w or 40 w incandescent lamps) at 16-18°C, relative humidity of 65-70% and light energy of 126 micro Einsteins m^{-2} s^{-1} for 16 hour photoperiod.

Explant preparation

Unopened capsules of *M. betonicifolia* collected in the

University of British Columbia Botanical Garden were washed with distilled water and then rinsed in 70% ethanol for 10 sec. They were shaken in 20% "Sunbrite" bleach (1.05% sodium hypochlorite) for 5 minutes and then rinsed several times with sterilized distilled water before being split.

A sterilized scalpel and tweezers were used to split the capsules and the seeds inside were sown on the 1/3 strength MS medium solidified with 6 g l^{-1} Bacto agar (Difco).

Seeds were incubated in the same conditions as for cultures except that some were incubated in the dark to obtain suitable hypocotyls as explants (seedlings from seeds germinated under light have no hypocotyls). In the following text, seedlings from seeds germinated in the dark are referred to as dark-raised seedlings and those from seeds germinated under light are called light-raised seedlings.

After seed germination, derooted seedlings, hypocotyls and seedling roots were used as explants for *in vitro* establishment.

To test storage conditions for seedlings, some tubes with germinated seeds were put in a cold room (4-6°C) for three months.

Experiment I

To develop a medium that would support the growth of derooted seedlings of M. betonicifolia, several media were tested using different strengths of MS salts, cytokinins and auxin concentrations. In the first culture, the treatments were: full strength MS or 1/3 strength MS solidified with 6 g l^{-1} Bacto agar. The growth regulators used were: 0 or 0.2 mg l^{-1} 2,4-D, 1 mg l^{-1} BA, Kn or 2ip. The experiment was a 2x2x3 factorial with 45 seedlings per treatment. Shoots which survived were counted after four and eight weeks of incubation.

Surviving shoots from the first culture were subcultured (subculture 1) into 1/2 strength MS medium solidified with 8 g l^{-1} agar which was used for all subsequent cultures because 1/3 strength MS medium gave higher survival percentage than full strength MS medium but yellowish leaves in 1/3 strength MS medium suggested insufficient mineral nutrient supply. In the subcultures, the aim was to develop a medium that would stimulate multiple shoots and multiple meristems to increase propagation rate. 2 or 5 mg l^{-1} BA, Kn or 2ip were used with 0.2 mg l^{-1} 2,4-D. The differentiated multiple meristems and multiple shoots (Figures 17-21) were counted after four and eight weeks of subculture.

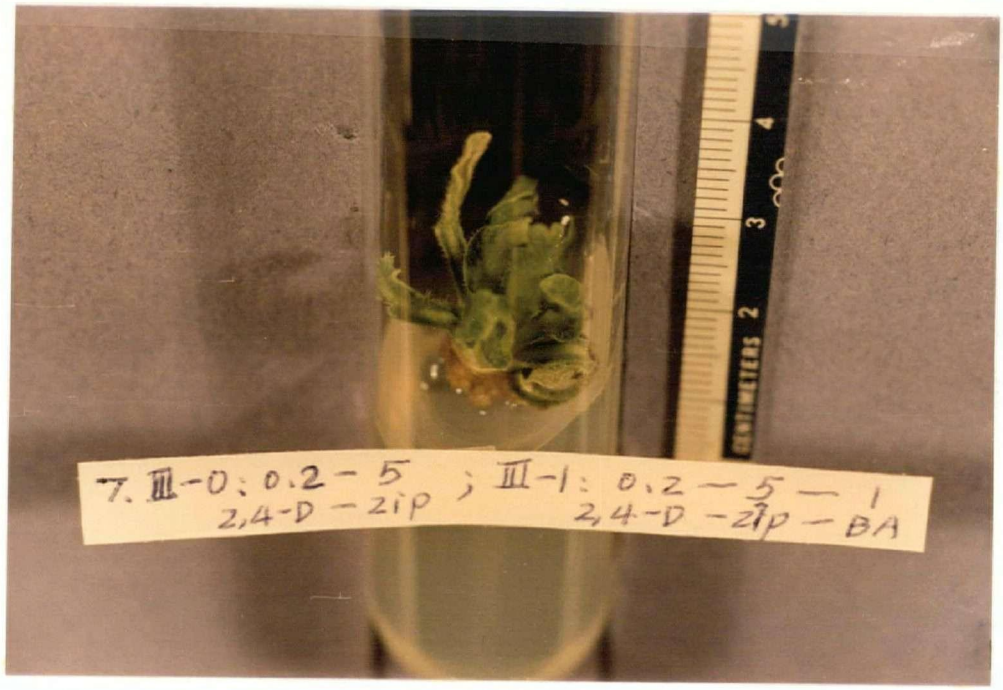


Fig. 17. In vitro shoots from derooted seedlings of Meconopsis betonicifolia.

Fig. 18. In vitro shoots from derooted seedlings of Meconopsis betonicifolia (showing callus at base of shoot)

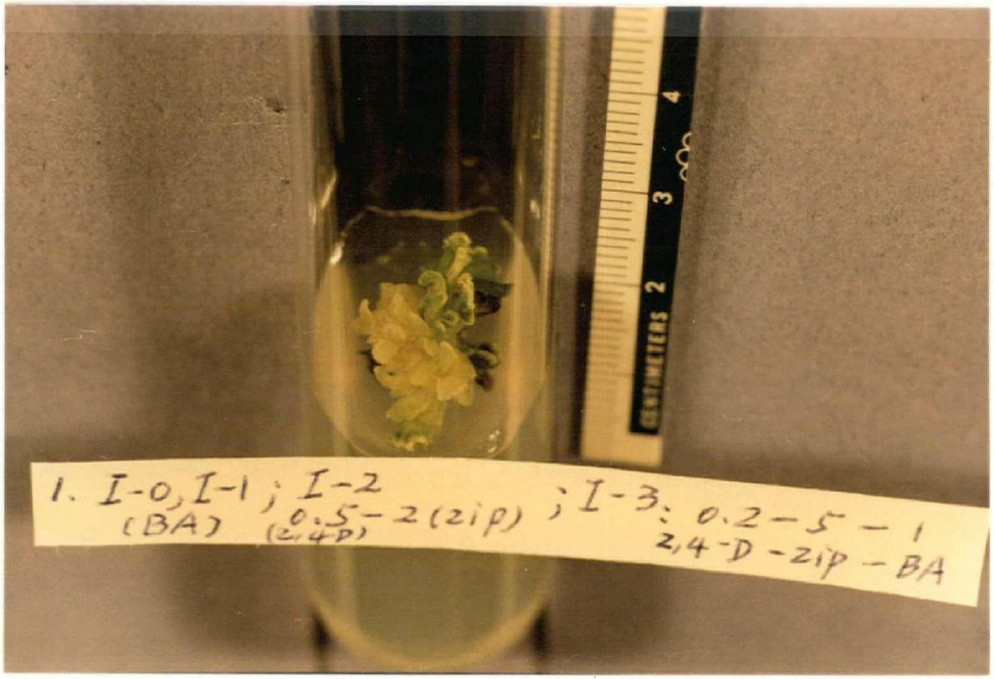


Fig. 19. Multiple meristems differentiated from derooted seedling of Meconopsis betonicifolia.

Fig. 20. Multiple meristems differentiated from callus induced from derooted seedlings of Meconopsis betonicifolia.

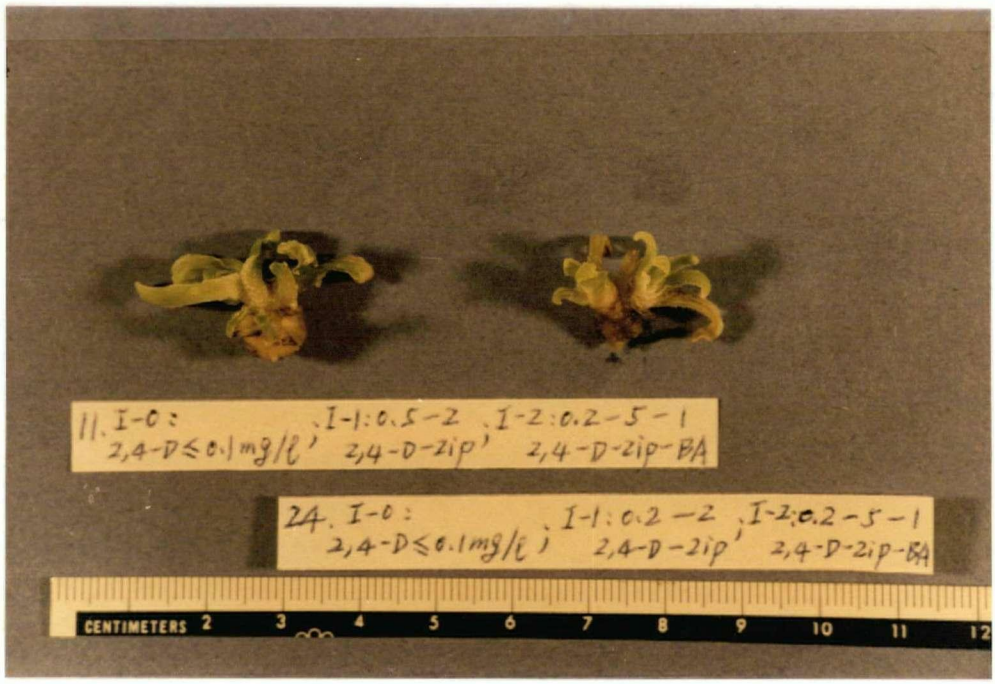


Fig. 21. Multiple shoots differentiated from derooted seedlings of Meconopsis betonicifolia.

The second subculture: multiple meristems and multiple shoots from the first subculture were divided and subcultured on the same medium as for the first subculture. The medium was supplemented with 0.2 or 0.5 mg 1^{-1} 2,4-D combined with 2 or 5 mg 1^{-1} 2ip. After eight weeks, the surviving explants were subcultured on a medium with 0.2 mg 1^{-1} 2,4-D, 5 mg 1^{-1} 2ip and 1 mg 1^{-1} BA to allow shoot proliferation.

Experiment II

The aim of this experiment was to define the ranges of concentrations of 2,4-D and 2ip that were effective in controlling morphogenetic responses of explants. Three morphological types of explants were used in this experiment: (1) hypocotyls and seedling roots from dark-raised seedlings; (2) derooted seedlings and seedling roots from light-raised seedlings; (3) petioles from light-raised seedlings.

Four levels of 2ip (0, 0.1, 1.0, 10.0 mg 1^{-1}) and five levels of 2,4-D (0, 0.1, 1.0, 5.0, 10.0 mg 1^{-1}) were used in a factorial experiment. There were 8 replicates for each treatment. After eight weeks, the morphological changes of different explants from each treatment were recorded.

Statistical analysis

The data for this experiment are of binomial distribution because an observation can be called either a success or a failure. A chi-square test may be used for binomial populations while the analysis of variance deals with normal populations. However, a chi-square test requires large samples to make the hypothetical frequencies equal to or larger than 5 in each cell. This was not always the case in the current experiments due to differences in survival between treatments. Li (1964) demonstrated that the chi-square test is equivalent to the analysis of variance with the total mean square being used as the error term in the binomial population, with the F value being designated as F'. The analysis of variance of the current data is only an approximate test in some cases because of disproportional and unequal sample sizes in the factorial experiment.

In the text, the results are those of eight-week-old cultures or subcultures. Survival percent means percentage of surviving explants out of explants cultured. Differentiation percent means percentage of explants which differentiated into multiple meristems and multiple shoots out of surviving explants in cultures and subcultures.

Results

Explant preparation

Seedlings from seeds germinated in the dark have long hypocotyls and yellowish, smaller cotyledons while those from seeds germinated under light have no hypocotyls and have green, large cotyledons.

Germinated seedlings stored at 4-6°C for three months grew well after being transferred into culture conditions.

Experiment I

In the first culture, no significant differences existed in explant survival between presence or absence of 2,4-D, or among three different cytokinins (Tables 7-9). Survival was higher in 1/3 MS than that in full MS (Table 9). Of 540 derooted seedlings tested, 11 differentiated multiple shoots and multiple meristems.

In the first subculture, BA, Kn or 2ip and their concentrations had no effects on differentiation percent (Tables 10 and 11).

In the second subculture, concentration of 2ip had no effect on the differentiation percent but concentration of 2,4-D changed

differentiation percentage (Tables 12-14). The cytokinin contained in the first culture and in subculture 1 exhibited an effect on the differentiation percentage of subculture 2 (Table 14). Explants originally cultured on media containing BA and Kn developed more multiple meristems and multiple shoots than those originally cultured in 2ip containing media (this is the same cytokinin used in the second subculture).

Proliferation was obtained on the medium which contained 0.2 mg l^{-1} 2,4-D, 5 mg l^{-1} 2ip and 1 mg l^{-1} BA.

Experiment II

Where petiole explants were used, no growth or differentiation was observed. After eight weeks all explants died.

The responses of derooted seedlings and seedling roots from light raised seedlings to exogenous auxin and cytokinin are shown in Figure 22. Figure 22 shows that when 2,4-D was 0.1 mg l^{-1} or less, shoots grew well and rooted if 2ip was 1 mg l^{-1} or less (treatments 1-3 and 5-7 of Figure 22). When 2ip concentration increased to 10 mg l^{-1} while 2,4-D concentration remained 0.1 mg l^{-1} or less, less than half of the shoots grew, and no rooting

Table 7

Survival percentage of derooted seedlings of M. betonicifolia after treatment with 2,4-D, cytokinin and strength of MS medium (the first culture)

A 2,4-D (mg l^{-1})	B strength of MS	C (cytokinins, 1 mg l^{-1}) BA	Kn	2ip
0	full	20.0	20.0	28.9
	1/3	44.4	48.9	51.1
0.2	full	28.9	17.8	22.2
	1/3	44.4	57.8	53.3

Note: 45 replicates were used for each treatment.

Table 8

Analysis of variance of Table 7

(the first culture)

source	degrees of freedom	sum of squares	mean squares	F'	probability
(treatment)	(11)	(10.9981)	(0.9998)	(4.3067)	($p<0.01$)
2,4-D	1	0.0463	0.0463	0.1994	$p>0.05$
cytokinin	2	0.1815	0.0907	0.3909	$p>0.05$
strength of MS	1	9.8685	9.8685	42.5083	$p<0.01$
interactions	7	0.9019	0.1288	0.5550	$p>0.05$
error	528	144.1333	0.2162		
total	539	125.1315	0.2322		

Table 9

Summary of test results from Tables 7 and 8

(the first culture)

	factors	levels and test results		
	2,4-D (mg l^{-1})	0	0.2	
A	survival (%)	35.6 a*	37.4 a	
	strength of MS	full	1/3	
B	survival (%)	23.0 b	50.0 c	
	cytokinin (mg l^{-1})	BA	Kn	2ip
C	survival (%)	34.4 d	36.1 d	38.9 d

* values not labeled by the same letter in the same row differ significantly at 1% level (based on Table 8).

Table 10

Differentiation percentage

with different concentrations of BA, Kn or 2ip

(subculture 1)

concentration of cytokinins ($mg\ l^{-1}$)	cytokinins			
	BA	Kn	2ip	mean
2	27.8	11.1	23.3	20.0 b*
5	16.7	27.6	25.9	23.8 b
mean	21.4 a	19.6 a	24.6 a	

* values labeled by the same letter in the same row or the same column do not differ (based on Table 11).

Note: 18 to 30 replicates were used for each treatment.

Table 11

Analysis of variance of Table 10

(subculture 1)

source	degrees of freedom	sum of squares	mean squares	F'	probability
(treatment)	(5)	(0.5859)	(0.1172)	(0.6798)	(p>0.05)
concentration	1	0.0544	0.0544	0.3155	p>0.05
cytokinin	2	0.0698	0.0349	0.2024	p>0.05
interaction	2	0.4616	0.2308	1.3387	p>0.05
error	149	25.9561	0.1742		
total	154	26.5419	0.1724		

Table 12

Differentiation percentage with different 2ip and 2,4-D concentration after subculture from BA, Kn or 2ip media

(subculture 2)

A	B	C (original cytokinins)		
2ip (mg l^{-1})	2,4-D (mg l^{-1})	BA	Kn	2ip
2	0.2	66.7	85.7	41.7
	0.5	100.0	37.5	9.1
5	0.2	100.0	77.8	25.0
	0.5	50.0	85.7	20.0

Note: 3 to 12 replicates were used for each treatment.

Table 13

Analysis of variance of Table 12

(subculture 2)

source	degrees of freedom	sum of squares	mean squares	F'	probability
(treatment)	(11)	(8.0098)	(0.7282)	(2.8794)	(p<0.01)
2ip concentration	1	0.2334	0.2334	0.9229	p>0.05
2,4-D concentration	1	1.0362	1.0362	4.0979	p<0.05
original cytokinin	(2)	(5.3991)	(2.6995)	(10.6754)	(p<0.01)
BA, Kn/2ip	1	5.2755	5.2755	20.8600	p<0.01
BA/Kn	1	0.1236	0.1236	0.4887	p>0.05
interaction	7	1.3412	0.1916	0.7576	p>0.05
error	75	13.7373	0.1832		
total	86	21.7471	0.2529		

Table 14

Summary of test results from Tables 12 and 13

(subculture 2)

	factors	levels of factors and test results		
	2ip (mg l^{-1})	2		5
A	differentiation (%)	45.5 a*		55.8 a
	2,4-D (mg l^{-1})	0.2		0.5
B	differentiation (%)	61.4 b		39.5 c
	original cytokinin	BA	Kn	2ip
C	differentiation (%)	80.0 d	71.0 d	24.4 e

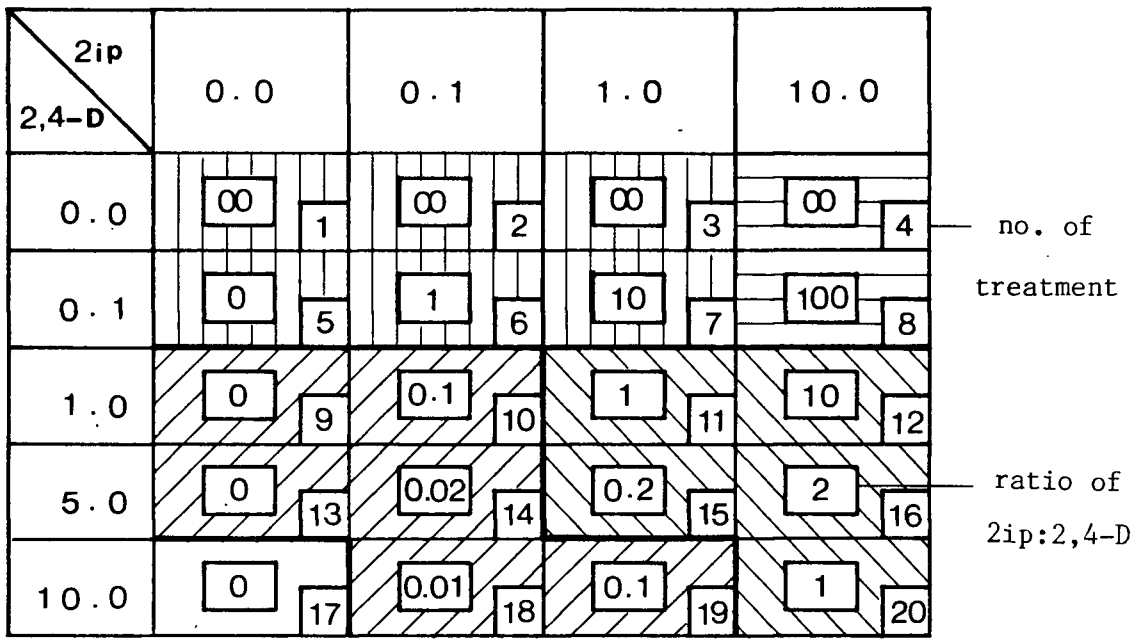
* values not labeled by the same letter in the same row differ significantly at 5% level (based on Table 13).

or callus growth occurred (treatments 4 and 8). With increased concentration of 2,4-D (1 mg l^{-1} or above), shoot growth was inhibited while callus growth increased (treatments 9-20). Shoots died and seedling roots produced callus in treatments 9, 10, 13, 14, 18 and 19. Less than one quarter of the shoots grew, but some root and shoot base callus formed in treatments 11, 12, 15, 16 and 20. Shoots died and root callus browned in 10 mg l^{-1} of 2,4-D (treatment 17).

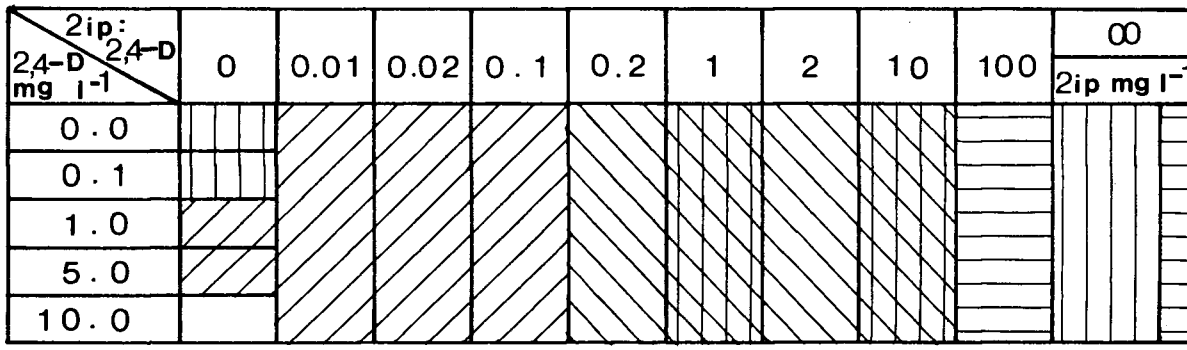
Hypocotyl and seedling root responses from dark raised seedlings to exogenous auxin and cytokinin are shown in Figure 23. This shows that callus was not induced in the absence of 2,4-D (treatments 1-4). Nor was callus induced with the absence of 2ip or 10 mg l^{-1} 2ip, combined with 0.1 mg l^{-1} 2,4-D (treatments 5 and 8). 2ip is not critical to the formation of callus when the concentrations of 2,4-D exceeded 0.1 mg l^{-1}. Callus obtained in treatments 10, 11, 15 and 16 showed no browning after eight weeks. When the callus was subcultured in media containing: 1.0 mg l^{-1} 2,4-D with 0.1 or 1.0 mg l^{-1} 2ip, or 5.0 mg l^{-1} 2,4-D with 1.0 or 10.0 mg l^{-1} 2ip, callus proliferation (callus multiplication) was obtained (Figures 24 and 25).

Fig. 22. Responses of derooted seedling and seedling root cultures from light-raised seedlings to 2ip and 2,4-D concentrations and the ratio of 2ip to 2,4-D by weight

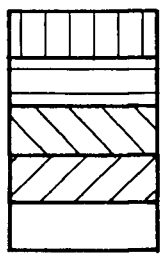
Concentration Response (mg l^{-1}):



Ratio Response:



Legend:



>1/2 shoots grew, root regeneration occurred

<1/2 shoots grew, no root regeneration or callus

<1/4 shoots grew, some root and some shoot base callused

shoots died, root callus formed

shoots died, root callus turned brown

Fig. 23. Responses of hypocotyl and seedling root cultures from dark-raised seedlings to 2ip and 2,4-D concentration and the ratio of 2ip to 2,4-D by weight

Concentration Response (mg l^{-1}):

2ip / 2,4-D	0.0	0.1	1.0	10.0
0.0	∞ 1	∞ 2	∞ 3	∞ 4
0.1	0 5	1 6	10 7	100 8
1.0	0 9	0.1 10	1 11	10 12
5.0	0 13	0.02 14	0.2 15	2 16
10.0	0 17	0.01 18	0.1 19	1 20

no. of treatment

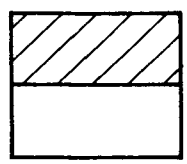
ratio of 2ip:2,4-D

Ratio Response:

2ip: 2,4-D / 2,4-D mg l^{-1}	0	0.01	0.02	0.1	0.2	1	2	10	100	∞
0.0										
0.1										
1.0										
5.0										
10.0										

← better results →

Legend:



no callus, explants died

75-100% callused

Fig. 24. Callus induced from hypocotyls of Meconopsis betonicifolia.

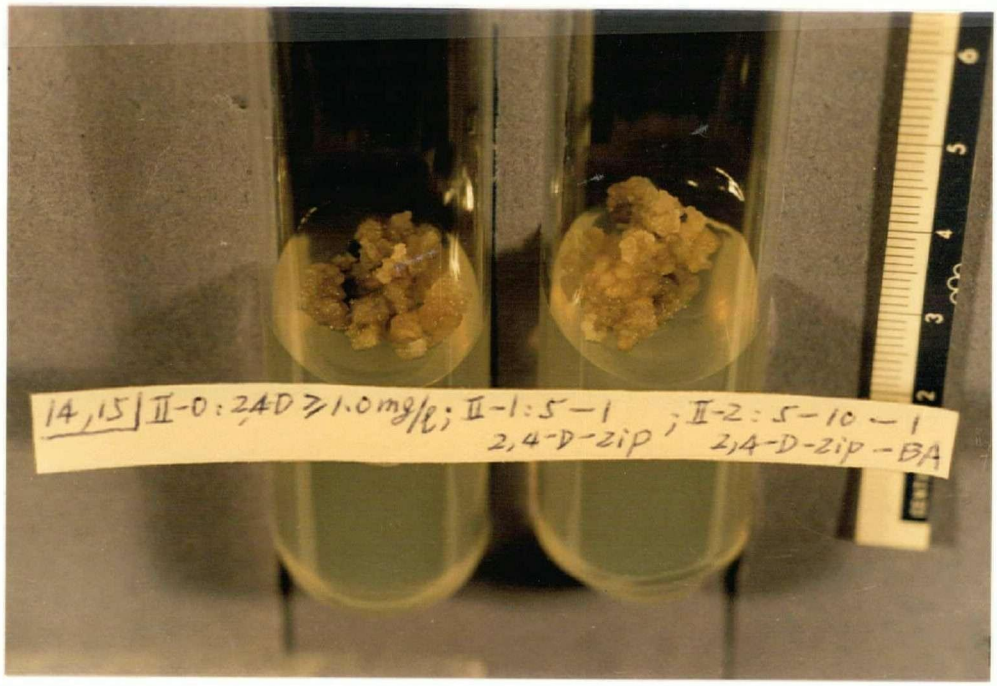


Fig. 25. Callus induced from seedling roots of Meconopsis betonicifolia

Discussion

Experiment I

Strength of MS medium affects the survival of derooted seedlings in culture (Table 9). MS medium is a high N, P, and K salt medium. When the salt concentration in the medium is lowered to one-half or one-third of the standard strength, improved cultures resulted in seedling nodal segment cultures of *Grevillea rosmarinifolia* (Ben-Jaacov and Dax, 1981), in shoot tip cultures of *Malus sylvestris* (M. 7) (Werner and Boe, 1980), and in tuber sprout tip meristem cultures of *Solanum tuberosum* (Wang, 1977; Wang and Hu, 1982).

Ma and Wang (1977) reported that azalea explants turned brown and died in full MS strength medium, while with reduced salt concentrations, the shoot tips turned green and made substantial growth.

Of the media tested, half strength MS supported excellent growth in the cotyledon cultures of opium poppy, *Papaver somniferum* (Ilahi, 1982). *Papaver* is a genus closely related to *Meconopsis*.

The current study shows that survival of explants on one-

third strength MS medium is twice as high as survival on full strength MS medium. Salt concentration thus plays an important role in the explant survival of M. betonicifolia.

Leaves produced on one-third MS medium were yellowish but survival percentage was higher than on full strength MS medium. This suggested insufficient mineral nutrient because too low a salt concentration in the medium sometimes results in poor top growth in in vitro culture (Wang, 1978). Therefore, the subcultures were all conducted on half strength MS medium.

In the first culture, only 2% of the derooted seedlings differentiated. The majority were from media which contained 0.2 mg 1^{-1} 2,4-D. Cytokinin treatment was at 1 mg 1^{-1} in the first culture for all three cytokinins. When cytokinin concentration increased to 2 or 5 mg 1^{-1} in subculture 1, the differentiation percentage increased to 19-24% (Table 10).

An interesting phenomenon was observed in the second subculture. When subcultured on the medium containing 2ip in subculture 2, the explants which had originally been cultured on media lacking 2ip (i.e., BA and Kn) produced more multiple meristems and multiple shoots than those from 2ip-containing medium (Table 14). This indicates that changing cytokinin during

the subculture process may improve growth and differentiation. This was also the case in apple culture. Werner and Boe (1980) reported that periodic substitution of 2ip for BA after about 4 months on BA-containing multiplication medium reduced or eliminated BA toxicity in cultures. This suggests that continual culture on the same cytokinin-containing medium might cause toxicity in cultures.

The medium containing 0.2 mg l^{-1} 2,4-D induced more differentiation than the medium containing 0.5 mg l^{-1} 2,4-D in derooted seedling cultures (Table 14). The reasons for this could be:

(1) the young shoot apex contains enough auxin for growth. This was demonstrated in the first culture and in Experiment II (Figure 22). Shoots can grow well without exogenous auxin;

(2) cytokinins do not act alone. The interaction of cytokinin with other plant growth factors has been demonstrated in many ways (Hall, 1973). Jordan and Skoog (1971), in a study with coleoptile tips, showed that BA stimulated the synthesis of auxin. They also reported that, in tobacco tissue cultures which normally require exogenous cytokinins and auxin for growth, sufficient auxin for growth was synthesized in the presence of

high levels of cytokinin. This might be the case in the current experiment;

(3) too high an auxin level causes dedifferentiation, not redifferentiation. This was demonstrated in Experiment II (Figure 22).

Experiment II

The qualitative response of different explants of M. betonicifolia to 20 combinations of cytokinin 2ip and auxin 2,4-D was tested.

Auxin:cytokinin interactions have been documented since Skoog and Miller's work (1957) with tobacco callus cultures. Their work showed the importance of auxin:cytokinin ratios in controlling shoot and root initiation in vitro.

The current experiments show that not only the ratio itself but also the amounts of cytokinin and auxin play important roles in the morphogenic response.

In Figure 22, treatments 1-3 and 5-7 have a ratio (2ip: 2,4-D) which ranges from 0 to infinity. Explants from these treatments all display similar morphological responses, i.e., more than half of the shoots grew and root regeneration occurred.

In these treatments, exogenous auxin and cytokinin in the media were either absent or at a very low concentration.

Auxin is essential for shoot tip culture and root initiation. Since the young shoot apex is an active site for auxin biosynthesis, exogenous auxin is not always needed in shoot tip culture (Kuo and Tsay, 1977; Ancora et al., 1981; Yang et al., 1981; Nemeth, 1981) and in root regeneration (Hasegawa, 1980; Meredith, 1979; Papachatzi et al., 1981).

Although a small quantity of cytokinin may be synthesized by shoots grown in vitro (Koda and Okazawa, 1980), roots are the principal sites of cytokinin biosynthesis. It is likely that endogenous cytokinin present in the explants supports shoot growth initially. The regenerated roots may act as a new source of cytokinin before the residual hormone is exhausted. In vitro establishment can be achieved without supplemental cytokinin in Solanum (Wang, 1977; Wang and Hu, 1982), Phaseolus (Kartha et al., 1981), and Fragaria (Kartha et al., 1980).

With increased concentration of 2ip (10 mg l^{-1}) combined with 0 or 0.1 mg l^{-1} 2,4-D (treatments 4 and 8 of Figure 22), neither rooting, nor callus formation occurred. More than half of the shoots died. The ratio of 2ip:2,4-D in these two treatments

is 100 or more. It seems that this 2ip:2,4-D ratio is too high for the initiation of shoot, root or callus growth.

It is possible that callus induction depends on the presence of auxin. 0 or 0.1 mg l^{-1} 2,4-D cannot induce callus in derooted seedlings and root cultures in this species, but 1, 5 or 10 mg l^{-1} 2,4-D induced callus (Figure 22).

When the 2,4-D concentration was 1 mg l^{-1} or more, the ratio of 2ip:2,4-D affected morphological response (Figure 22, ratio responses). Ratios from 0.01 to 0.1 induced seedling root callus growth. Shoot growth did not occur at these low ratios. Ratios from 0.2 to 10 induced some root and shoot base callus, and also kept some shoots alive. The fact that a high exogenous cytokinin:auxin ratio favoured shoot growth has also been observed by other workers (Kuo and Tsay, 1977).

The present experiment showed that the same ratio can give rise to different morphological responses. Ratios 1 and 10 can either support good shoot growth (treatments 6 and 7) or induce root callus (treatments 11, 12 and 20) depending on the 2,4-D level. The critical concentration in this case is between 0.1 and 1.0 mg l^{-1}. With a 2,4-D concentration of 0 or 0.1 mg l^{-1}, either good shoot growth and rooting can occur (treatments 1-3 and 5-

7), or shoot growth can be poorer with no rooting and no callus formation (treatments 4 and 8). The critical concentration of 2ip in this case was between 1 and 10 mg l^{-1}. Media without 2ip (the ratio of 2ip:2,4-D is 0) induced three kinds of morphological responses (treatments 5, 9, 13 and 17 of Figure 22) depending on the concentration of 2,4-D used: 0.1 mg l^{-1} 2,4-D stimulated shoot growth; 1 or 5 mg l^{-1} 2,4-D favoured root callus; and 10 mg l^{-1} 2,4-D induced little growth. James and Newton (1977) carried out a similar experiment using strawberry plants. Their results showed that cytokinin ranging from 0.06-0.6 mg l^{-1}, coupled with auxin concentrations ranging from 0.05-0.2 mg l^{-1}, were most suitable for bud proliferation. Higher concentration of cytokinin inhibited bud proliferation. Higher concentration of auxin (2.0 mg l^{-1}) caused extensive callus formation.

It can be seen from Figure 22:

(1) when cytokinin:auxin ratio is <1, four kinds of morphological responses occurred:

a. more than half of the shoots grew and roots regenerated (treatment 5);

b. less than a quarter of the shoots grew, some root and shoot base callus formed (treament 15);

c. shoots died, root callus formed (treatments 9, 10, 13, 14, 18, 19);

d. shoots died, root callus browned (treatment 17).

Of 9 treatments mentioned above, 7 treatments (b and c) favoured callus formation. The general rule that if cytokinin:auxin ratio is <1, callus formation is enhanced applies to M. betonicifolia. This rule also applies to most solanaceous species (Flick et al., 1983). In general, this ratio (<1) is not suitable for root differentiation in M. betonicifolia because only treatment 5 favours shoot growth and root regeneration.

(2) when cytokinin:auxin ratio is =1 or >1, three kinds of morphological responses occurred:

a. more than half of the shoots grew and root regenerated (treatments 1, 2, 3, 6 and 7);

b. less than half of the shoots grew, roots did not regenerate nor was callus induced (treatments 4 and 8);

c. less than a quarter of the shoots grew, some root and shoot base callus formed (treatments 11, 12, 16 and 20).

Although callus formed in some of treatments described above, all 11 treatments supported some shoot growth. The general rule that if cytokinin:auxin ratio is >1, shoot formation is

obtained applies to M. betonicifolia. This rule also applies to both solanaceous species and Chinese cabbage (Flick et al., 1983; Kuo and Tsay, 1977).

This experiment shows: concentrations of 2ip ranging from 0-1 mg l^{-1}, coupled with 2,4-D concentrations ranging from 0-0.1 mg l^{-1}, are suitable for shoot growth. Higher concentrations of 2,4-D (1 mg l^{-1} or above) favours callus formation. Higher concentrations of 2ip (10 mg l^{-1}) are not suitable for shoot growth and root regeneration.

The responses of hypocotyls and seedling roots from dark-raised seedlings to exogenous cytokinin and auxin are shown in Figure 23. It is clear that auxin is essential to induce callus. No callus formed without exogenous auxin. 0.1 mg l^{-1} 2,4-D only or its combination with 10 mg l^{-1} 2ip could not induce callus (treatments 5 and 8). When 2,4-D concentration was equal to or greater than 1 mg l^{-1}, no matter what concentrations of 2ip was used , callus formed in the culture. It was noted that treatments 10, 11, 15 and 16 induced white, fragile callus which showed no browning after eight weeks. The ratios of 2ip:2,4-D for these four treatments are from 0.1 to 2 (Figure 23). Therefore, concentrations of 2ip ranging from 0.1-10.0 mg l^{-1}, coupled with

2,4-D concentrations ranging from 1-5 mg l^{-1}, are suitable for callus growth.

Conclusions

(1) General culture conditions, i.e., explants, basic medium, and incubation conditions, used in the tissue culture system of *Papaver* are suitable for the *in vitro* culture of *M. betonicifolia*. It seems that plants with close phylogenetic relationships have similar requirements for growth and development *in vitro*.

(2) Lower strength of MS (half or one third strength) is better for the growth of *M. betonicifolia* than full strength of MS.

(3) Germinated seeds can be stored at 4-6°C for at least three months.

(4) In general, if the ratio is <1, callus formation is enhanced; if >1, shoot formation is obtained. This supports the hypothesis that if cytokinin:auxin ratio was >1, shoot proliferation in *M. betonicifolia* would occure. Concentrations of 2ip ranging from 0-1 mg l^{-1}, coupled with 2,4-D concentrations ranging from 0-0.1 mg l^{-1}, are suitable for shoot growth. Higher concentrations of 2ip (10 mg l^{-1}) are not suitable for shoot growth and root regeneration. Higher concentration of 2,4-D (1 mg

1^{-1} or above) favours callus formation in hypocotyl and seedling root cultures. 0.1-10 mg 1^{-1} 2ip combined with 1-5 mg 1^{-1} 2,4-D are suitable for callus growth.

(5) 2 or 5 mg 1^{-1} BA, Kn or 2ip combined with 0.2 mg 1^{-1} 2,4-D induced multiple meristems and multiple shoots in the derooted seedling cultures of M. betonicifolia.

(6) Derooted seedlings cultured first on media containing BA or Kn differentiated more multiple meristems and multiple shoots after being transferred into media containing 2ip. Changing cytokinin during the subculture process may improve growth and development in vitro.

Overall Discussion

Little research has been conducted on Meconopsis although the genus has been known for nearly 200 years. Records of hybrids exist in the genus but some of the original plants are no longer in cultivation. Rarely have specimens been preserved, and thus no description, or only a very incomplete one, can be found (Taylor, 1934). Most hybrids have arisen spontaneously and their parentage is therefore often hypothetical. Difficulty in propagation and limited capacity for producing seed prevent the establishment of hybrids under cultivation (Taylor, 1934).

Some difficulties exist in vegetative reproduction and seed storage in Meconopsis (Cox, 1934; Thompson, 1968). An entire stock may be lost if adverse circumstances prevent the raising of young plants in one generation. Failures to maintain successful stocks of seedlings have led to the loss of many attractive species (Thompson, 1968). Therefore, it is very important to establish species of this genus under cultivation. Taylor (1934) pointed out that with the increase in the number of species under cultivation it may be expected that attempts will be made to

produce hybrids. Unfortunately, little scientific work has been conducted on this aspect for fifty years. This genus has not been widely used as a garden plant.

The present studies provide information on the breeding and propagation of Meconopsis. They revealed:

(1) interspecific crossing may be used to combine desirable characters from both parents. Most crosses conducted set hybrid seeds. This will improve the horticultural usefulness of this genus and extend its range of cultivation;

(2) pollen storage, pollen viability tests, and experimental alteration of flowering time of the species in Meconopsis are practical. This will facilitate desirable crosses between species which do not flower simultaneously;

(3) in vitro propagation is possible for M. betonicifolia. This may provide an alternative propagation method to ensure continued cultivation of this species. The same methods used in the tissue culture of M. betonicifolia could be tested on other species and hybrids which are not easily cultivated under garden conditions.

Although most interspecific crosses conducted in the present study set seeds and viable F_1 hybrid plants were obtained, some

hybrids died in the later seedling stage before flowering. The hybrid (M. cambrica x M. horridula) which flowered in 1985 was sterile. The anthers were shrivelled and petals did not open normally. No seeds set after flowering. Hybrid inviability and hybrid sterility can be caused by single genes, by general incompatibility of the genotypes of the parents, or by disharmonies of gene recombination and deficiencies, duplications, and other alterations of chromosomal segments (Allard, 1960). Great diversity in chromosome number, morphology and flower colour of Meconopsis may explain this kind of genetic unbalance.

Evolutionary development of Meconopsis has corresponded to changes of environmental conditions by changing morphologically (Chuang, 1981). Geographic isolation may lead to ecological isolation and increase differences in gene pool of species groups. Speciation may take place after mutation, chromosome aberration and selection. The fact that some species in this genus are polyploids probably indicates that their evolutionary diversification has taken place from an ancestral stock of high polyploid level. Because of reproductive isolation, the interspecific hybridization usually either cannot set seeds or

leads to hybrid inviability and hybrid sterility. Therefore, the hybrids produced in this study may be lost, like those hybrids reported before, if they cannot be reproduced vegetatively or be made into allopolyploids.

The present study may solve this problem. The tissue culture system established for M. betonicifolia could be used for propagation of Meconopsis hybrids. Hybrid seeds could be sown on artificial medium and derooted seedlings could be used as explants for micropropagation. Germinated seedlings could be stored at low temperature if necessary. Multiple shoots may be separated and allowed to proliferate in vitro. Hybrids could be preserved in this way for further study.

A medium which can induce somatic embryogenesis from callus culture of M. betonicifolia may be developed. The maintenance of chromosomal and genetic integrity is essential if the goal of somatic embryogenesis is clonal reproduction. The embryogenic cultures can produce large number of embryos per culture flask, many more than the multiple shoots generated via organogenesis (Ammirato, 1983). For breeding programs, new varieties could be developed quickly via haploid culture, somaclonal variation and somatic hybridization. This project on Meconopsis could improve

the horticultural usefulness of the genus.

Overall Conclusions and Summary

(1) All interspecific crosses among the six species: *Meconopsis cambrica, M. villosa, M. quintuplinervia, M. betonicifolia, M. horridula* and *M. aculeata*, except *M. cambrica* x *M. quintuplinervia*, are compatible but the seed setting ability differs with the difference in ploidy level between parents. The successful growth and flowering of hybrids show the possibility of combining desirable characters from both parents.

(2) *Meconopsis cambrica, M. betonicifolia, M. horridula* and *M. aculeata* are self-fertile. *Meconopsis betonicifolia* has high intraspecific cross compatibility.

(3) Insects are the likely pollinators for *M. betonicifolia* and no apogamy exists in this species.

(4) Fresh pollen of the six species can be stained using lactophenol cotton blue. Percent pollen stainability was 85% or more.

(5) Pollen of Meconopsis cambrica, M. villosa, M. horridula and M. aculeata germinated on the 1% agar medium with sucrose (5 g l^{-1}) and boric acid (0.1 mg l^{-1}). Pollen of M. betonicifolia germinated on the same medium with only 0.5% agar. Pollen from different pollen types has different requirement for germination.

(6) Pollen stored in a desiccator at about 4-6°C germinated in vitro but the germination percent decreased with storage time. Pollen of M. cambrica, M. horridula and M. aculeata germinated after two-week or less storage while that of M. villosa and M. betonicifolia did not germinate even after only one-week storage.

(7) Boric acid is necessary for pollen germination of the six species of Meconopsis investigated.

(8) Experimental alteration of flowering time of M. betonicifolia can be obtained by controlling temperature and day-length in growth chambers. High temperature (17°C) induced earlier growth and flowering than low temperature (6°C). Long day-length (16 hour photoperiod) is suitable to flowering of this

species.

(9) Plants with close phylogenetic relationships have similar requirements for growth and development *in vitro*. General culture conditions used in the tissue culture system of *Papaver*, which is closely related to *Meconopsis*, are suitables for the *in vitro* culture of *M. betonicifolia*. These culture conditions are: explant type, basic medium and incubation conditions.

(10) Germinated seedlings can be stored at 4-6°C for at least three months. Stored seedlings grew well after being transferred to culture condition.

(11) Changing cytokinin during subculture process may improve growth and development. Derooted seedlings cultured first on the media containing BA and Kn differentiated more multiple meristems and multiple shoots after being transferred into media containing 2ip than those initially cultured on 2ip-containing medium.

(12) Cytokinin:auxin ratio and their concentrations control

morphogenesis of M. betonicifolia in in vitro culture. In general, if the ratio was <1, callus formation was enhanced; if >1, shoot formation was obtained. 2 or 5 mg l^{-1} BA, Kn or 2ip combined with 0.2 mg l^{-1} 2,4-D induced multiple meristems and multiple shoots in derooted seedling culture of M. betonicifolia. When 2,4-D concentration was 1 mg l^{-1} or more, callus formed from both hypocotyls and seedling roots of this species. 0-1 mg l^{-1} 2ip with 0-0.1 mg l^{-1} 2,4-D were suitable for shoot growth in derooted seedling cultures. 0.1-10.0 mg l^{-1} 2ip with 1-5 mg l^{-1} 2,4-D were suitable for callus growth in hypocotyl and seedling root cultures.

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