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# Isolation of reduced genotypes of *Hieracium pilosella* using anther culture

R. A. Bicknell & N. K. Borst
*New Zealand Institute for Crop & Food Research Ltd, Private Bag 4704, Christchurch, New Zealand*

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**Abstract**

Plants of reduced ploidy were derived from anther tissue of a pentaploid, apomictic biotype of *Hieracium pilosella* (mouse-ear hawkweed). Callusing was observed from both the somatic and generative tissue of the anther. Capitulum diameter was used as a correlative character to determine the best stage for harvesting the tissue. The highest degree of callusing coincided with the early uninucleate stage of microsporogenesis. Regenerants included tetraploids, triploids and two classes of aneuploids. Segregation for apomixis was observed among the tetraploid regenerants, an indication of the dominant inheritance of apomixis in this species.

*Abbreviations:* BA – 6-benzylaminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid

## Introduction

A close association between apomixis and polyploidy has been recorded in a wide range of plant taxa. Manton (1950) noted that polyploidy, in particular triploidy, is common among apomictic ferns, while diploids are predominantly sexual. Similarly, apomictic monocotyledons and dicotyledons are almost exclusively polyploid (Asker & Jerling 1992). It has been proposed that apomixis provides "an escape from sterility" (Darlington, 1939), rescuing newly formed polyploids with poor fertility. This hypothesis requires that apomixis spontaneously arises in these individuals before they perish, yet there is no evidence of polyploidy directly promoting the development of apomixis, or that apomixis is a common result of mutation.

Conversely, Nogler (1984) proposed that apomixis may encourage the establishment of polyploidy. Nogler studied the inheritance of apomixis in the alpine dicotyledon, *Ranunculus auricomus*. Apomixis in this species, which occurs through the mechanism of apospory, is conferred by a dominant allele at a single locus. The trait, however, can only be transmitted by a diploid or polyploid, heterozygotic gamete, as the dominant allele is gamete-lethal in a homozygotic state. This mechanism would restrict existing alleles to polyploid races, and, where the trait arose spontaneously in a diploid, it would favour the formation of polyploids through gamete selection.

*Hieracium pilosella* (mouse-ear hawkweed) is a useful model species for studying apomixis (Bicknell, 1994a). It is particularly amenable to experimental manipulation because of its small size, ease of culture, short generation time and autonomous development of an endosperm about the clonal embryo. Natural biotypes range from diploids to septaploids, including both sexual and aposporic forms (Tutin *et al.*, 1976). Diploid races of *Hieracium pilosella* are rare, and, in common with most species with apomictic biotypes, all the diploids studied have been recorded as sexual (Gadella, 1972). Apospory in *H. pilosella* also appears to be conferred by a single dominant allele (Gadella 1991).

Nogler's hypothesis predicts that diploid apomictic plants will not be recovered after crossing polyploids, but may be derived from unfertilized gametes. Nogler (1982) identified a diploid apomictic plant of *Ranunculus auricomus*, which appeared to have arisen from the spontaneous development of a reduced egg from a tetraploid parent (a dihaploid, according to Rutishauser (1948). Similarly, Skalinska (1971) reported the iso-

lation of a dihaploid plant of *Hieracium aurantiacum*, but did not record its reproductive state.

Despite perturbations in megasporogenesis in aposporic biotypes of *Hieracium* (Skalinska, 1971), meiosis during microsporogenesis is normal (Cristoff & Popoff, 1933), and therefore the recovery of reduced genotypes is possible through regeneration from post meiotic tissue. Anther culture, therefore, provides another potential avenue for recovering plants with reduced ploidy, while avoiding syngamy.

This paper outlines the development of an anther culture method for *Hieracium pilosella,* particularly in relation to the optimum time for harvesting anthers, and reports the inheritance of apomixis among the different ploidy classes recovered. Tissue culture methods for shoot regeneration from leaf tissue, micropropagation and acclimatization of a related species, *Hieracium aurantiacum,* have been reported separately (Bicknell, 1994b).

## Materials and methods

### *Plant material*

Plants of a pentaploid, aposporic race of *Hieracium pilosella* were obtained from an adventive population in Central Otago, South Island, New Zealand. The stock plants were maintained in a greenhouse and encouraged to flower by frequent propagation and exposure to a 16-h photoperiod. A pentaploid was chosen for this study to facilitate the differentiation of regenerants arising after the spontaneous doubling of a reduced genome, from maternal types, derived from somatic tissue. Apomixis was measured through the quantification of germinable seed, developed after decapitation of the capitulum (Richards, 1986), and confirmed by histological examination of megasporogenesis (data not shown). Sexuality of non-apomictic biotypes was determined by cross pollination.

### *Anther culture*

Results from a preliminary experiment indicated that the anthers of *H. pilosella* could be induced to callus in a liquid medium containing 0.5 mg $l^{-1}$ 2,4-D and 200 mg $l^{-1}$ glutamine, and that the stage of anther development at harvest was an important determinant of success. One aim of the current study was to determine the optimum time for harvesting anthers.

In *H. pilosella,* the involucral bracts of the capitulum remain tightly closed throughout most of floral development, so that it is impractical to determine the optimum stage for harvesting anthers through direct observation of floret morphology. Capitulum diameter was therefore used as a correlative character to estimate the optimum stage for harvesting anthers. This was followed by an investigation of floret ontogeny to determine the developmental events associated with callus formation from isolated anther tissue.

Capitula were collected at a number of developmental stages, and the diameter of each recorded using a digital calliper. Surface sterilisation was conducted using a 1% sodium hypochlorite solution for 30 min at room temperature, followed by thorough rinsing in sterile distilled water. Using a dissecting microscope under sterile conditions, the involucral bracts were removed and the florets severed transversely through the developing anther tube. The upper portion of the bud, containing the developing anther tube, was transferred to a 32–mm diameter petri dish containing 1.2 ml of a modified liquid MS medium, comprising of MS salts (Murashige & Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1968), 200 mg $l^{-1}$ glutamine, 0.5 mg $l^{-1}$ 2,4-D and 3% sucrose at pH 5.7. The dishes were then sealed with plastic cling wrap and placed in the dark, within an incubator maintained at 27 °C.

After 10 days, calluses were clearly visible on some isolates. The anthers of individual *H. pilosella* florets, which are fused together into a tube, remained attached in the liquid medium after isolation. This permitted the identification of individual floret isolations from a bud. An inverted microscope was used to score each capitulum dissection for the number of floret isolations which had, or had not, callused after 10 days of culture. The results were expressed as a percentage of floret sections callused, and are presented as a scatter plot of percentage callused against capitulum diameter.

The liquid medium was replaced 10 days after tissue isolation, and again 10 days later. One month after isolation, calluses were separated from the degenerating floral tissue, and transferred to an agar-solidified, modified MS medium, containing MS salts, B5 vitamins, 1.0 mg $l^{-1}$ BA and 3% sucrose (Bicknell, 1994b), to stimulate shoot formation. Shoots were transferred three weeks later to a similar medium without growth regulators to encourage root growth. Rooted plantlets were acclimatized to potting medium in a high humidity mist tent, raised to flowering and assessed for apomixis.

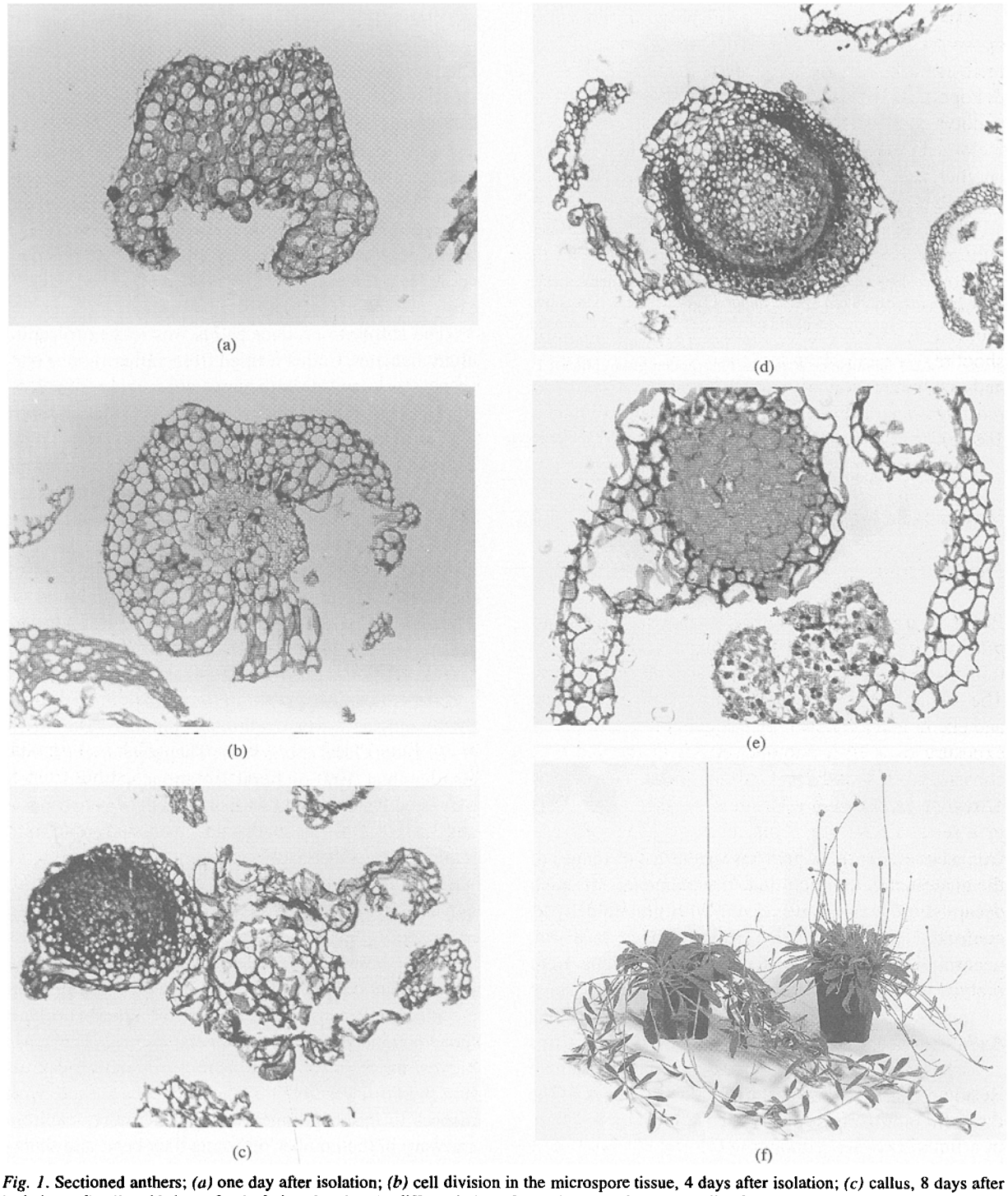


*Fig. 1.* Sectioned anthers; *(a)* one day after isolation; *(b)* cell division in the microspore tissue, 4 days after isolation; *(c)* callus, 8 days after isolation; *(d)* callus, 12 days after isolation showing the differentiation of a meristematic layer; *(e)* callus formation from the anther wall tissue; *(f)* progenitor pentaploid variety (left) and a triploid derivative (right).

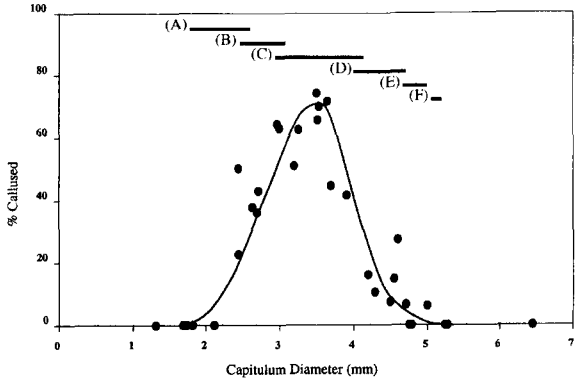


*Fig. 2.* Percentage of floret isolations producing callus after isolation from immature capitula at a range of floral bud diameters. Bars above the graph represent the developmental stages observed in sectioned florets. The stages are; A. Meiosis; B. Tetrads; C. Early uninucleate stage; D. Late uninucleate stage; E. First pollen grain mitosis; F. Second pollen grain mitosis.

*Table 1.* Chromosome counts from a random sample of 50 anther regenerants.

| Chromosome count | Ploidy | Number of regenerants | | | |
|---|---|---|---|---|---|
| | | Total | Apomictic | Sexual | Sterile |
| 45 | 5x | 19 | 19 | - | - |
| 36 | 4x | 23 | 18 | 4 | 1 |
| 29 | 3x+2 | 2 | - | - | 2 |
| 27 | 3x | 5 | 5 | - | - |
| 26 | 3x-2 | 1 | - | - | 1 |
| 18 | 2x | 0 | - | - | - |

*Cytology and histology*

Immature capitula were harvested at a range of developmental stages, and the diameter of each recorded. The tissue was fixed with glutaraldehyde, embedded in paraffin wax, and sectioned at 6 $\mu$m, according to established techniques. Sections were stained with Toluidine Blue, examined, and the stage of microsporogenesis recorded. Chromosome counts were conducted by aceto-orcein staining of root tips squashes. Counts were conducted on a random sample of 50 regenerants, to determine the frequencies of the different ploidy classes recovered

## Results and discussion

Plants with altered ploidy were successfully regenerated from tissue cultured *Hieracium pilosella* anther tissue (Fig. 1; f). In contrast with most published methods of anther culture (Dunwell, 1985; Prasad *et al.*, 1990, Thengane et al 1994), terminal sections of immature florets were used rather than individual isolated anthers. This greatly simplified harvesting anther tissue from immature capitula, and reduced difficulties associated with desiccation in the laminar flow hood. Terminal sections included tissues from the immature corolla, pistil and anther tube. Callus, however, was only observed to develop from cut surfaces of the anthers. Most callus appeared to originate from the microspore mass (Fig. 1 a-d), although some regeneration from the anther wall was also observed (Fig. 1 e).

The ability to produce callus was related to capitulum diameter. Callus formed from anther tissues harvested from capitula with diameters ranging from 2.4–5.0 mm (Fig. 2). Anthers isolated from smaller capitula failed to develop at all, while those from larger capitula continued normal development, often forming apparently mature pollen grains. The bud stage found to yield the highest percentage of anther calluses, 3.5 mm in diameter, corresponded to the early uninucleate stage of microsporogenesis in most of the developing florets. The early-to-mid uninucleate stage is reported as being an appropriate time for harvesting anthers of cereals (Dunwell, 1985), *Brassica* (Wenzel & Foroughi-Wehr, 1984) and the Asteraceous species safflower (*Carthamus tinctorius*) (Prasad *et al.*, 1990) and sunflower (*Helianthus annuus*) (Bohorova *et al.*, 1985; Gurel *et al.*, 1991; Thengane *et al.*, 1994). Greater than 50% of floret isolations callused when harvested from capitula with diameters of between 2.9 and 3.8 mm. Only the regenerants from this group were retained for further analysis.

Capitulum diameter proved to be a useful, non-invasive correlative character for estimating microspore development. The limitations of this approach, however, are reflected in the spread of experimental data obtained (Fig. 2). Floret development on a capitulum is centripetal, buds in the outer whorl are more advanced than those in the centre. The mass harvesting of floret tissue from an immature capitulum therefore results in a heterogeneous isolate, with respect to microsporogenesis. Furthermore, capitula can vary in the number of florets they bear, and therefore in the association between size and stage of development. To minimise the influence of these difficulties during the routine use of this technique, isolates are only taken from capitula with the optimum diameter for harvest, and a study of the association between capit-

ulum size and anther callusing is conducted whenever the technique is applied to different species or varieties of *Hieracium*.

Over 200 independent regenerants were isolated from capitula with diameters between 2.9 and 3.8 mm. Chromosome numbers from a random sample of 50 of these plants are summarised in Table 1. Of the plants scored, 19 (38%) were found to have the parental chromosome number of 45, indicating that they arose from the somatic tissue of the anther. All of these plants retained the apomictic phenotype of the parent. A further 23 (46%) of the regenerants were tetraploids. Tetraploids may have formed either as the result of a highly imbalanced division of the pentaploid parent, through microspore fusion, or by the spontaneous doubling of diploid derivatives. It is not possible to determine conclusively the mechanism(s) involved from the results available. The absence of diploids, prevalence of tetraploids, and the occurrence of two triploid tetrasomics among the regenerants, however, strongly suggests that spontaneous doubling occurred in some cases. It is interesting to note that the tetraploid regenerants segregated with respect to the expression of apomixis, an indication of the dominant inheritance of the trait (Gadella 1991). Only 5 (10%) of the regenerants studied were triploids, all were apomictic. Hexaploids, which would be expected from the spontaneous doubling of a triploid were not detected. This result is in contrast to the high number of tetraploids detected. The reason for this disparity is unclear.

A total of three aneuploids were identified among the 50 plants studied; one triploid monosomic, and two triploid tetrasomics. Gadella (1991) reported that aneuploids were not recovered when a pentaploid was crossed, as the pollen parent, to a sexual tetraploid. The recovery of aneuploids through anther culture of similar material indicates that gamete selection acts against this pollen class either during microgametogenesis or fertilization. The aneuploids isolated in this study grew very poorly and were unable to complete floral development. They have been recorded as sterile because premature capitulum abortion precluded seed formation.

In summary, the technique described has provided a mechanism for generating a range of ploidy derivatives of *H. pilosella*. It is intended to use this to study the inheritance of apomixis in this species, through the isolation and use of apomictic diploids and aneuploids.

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