

## Clonal Propagation of Meadowfoam (*Limnanthes* sp.) from Bisected Seeds

### Abstract

A method was developed to select and propagate meadowfoam lines that produce seeds with high oil content. Seeds of a meadowfoam hybrid (*Limnanthes* sp., ORL 85-765) were bisected so that a portion of the seed could be analyzed for oil content. The remainder of the seed was then germinated. Higher germination rates and more uniform plants were obtained from seeds cut transversely than from seeds cut longitudinally. Half seeds were stored frozen for 25 days without decrease in viability. Sterile plants were established by culture of shoot meristems taken from greenhouse-grown plants. Plants in sterile culture were cloned by micropropagation. This method of selection is more specific to genotype than sampling seeds from a population, and is easier than selection from tissue culture, because the half-seed method does not require sterile technique.

### Introduction

Meadowfoam (*Limnanthes* sp.) is a short-lived annual with potential as a new oil seed crop (Jolliff 1981, Reed 1991, Southworth and Kwiatkowski 1991). Agronomists are working to increase oil yield by improved cultivation methods and by selection of seeds with high oil content for use as parents (Jolliff 1981). Analysis of individual seeds for oil content in the search for superior oil producers requires sacrifice of a portion of the seed for analysis. What is needed is the ability to identify oil-rich seeds and then to germinate them. If a portion of the seed could be analyzed and the remainder stored and later cloned, selection of high oil-producing parents would be greatly simplified. A half-seed technique has been used for analysis and subsequent germination in such genera as *Cucurbita* (Gathman and Bemis 1981) and *Brassica* (Pleins and Friedt 1989). This investigation addresses the feasibility of bisected seed storage, germination and clonal propagation of meadowfoam.

### Methods and Materials

Seeds of *Limnanthes* sp. seed line ORL 85-765 selected from hybrids of *L. floccosa* ssp. *grandiflora* Arroyo X *L. alba* Hartw. in Benth. were obtained from Dr. Gary Jolliff, Department of Crop Science, Oregon State University. A plant variety protection application is pending.

#### Bisection of Seeds

Establishment of meadowfoam plants was compared for the following treatments: (a) whole seeds; (b) longitudinally bisected dry seeds; (c) longitu-

dinally bisected seeds that had been pre-imbibed at 10°C before bisection; (d) transversely bisected dry seeds; and (e) transversely bisected dry seeds which were stored at -14°C for 25 d following bisection. For treatments (a)-(d), 30 seeds were cut and divided into three Petri dishes for germination. For frozen storage, 32 seeds were cut, stored frozen, and divided into four dishes for germination.

Results were analyzed by a chi-square contingency table to determine the significance of deviations from the pooled estimate of germination (Samuels 1989).

"Seed coats" in *Limnanthes* consist of a testa-pericarp with the fruit wall adhering to seed coat and embryo in both dry and imbibed conditions. In preliminary experiments, the testa-pericarp was chipped off with a scalpel, but this process was laborious and potentially damaging to the embryo. We proceeded without removing the testa-pericarp. Seeds were sliced longitudinally or transversely through the center by holding them with curved forceps on a hard surface, and slicing with a number 10 blade fitted scalpel. Longitudinal cuts, (b) and (c), were made through the long axis of the seed. The position of the cotyledons in relation to the longitudinal cut was not determined, as no obvious external marks on the seed indicated cotyledon position. Some longitudinal cuts separated the cotyledons; others cut both cotyledons lengthwise. Frequently the embryonic axis (radicle-hypocotyl-epicotyl) was cut. Transverse cuts, (d) and (e), were made across the seed, halfway between the basal attachment scar and the seed tip. This cut off the distal portion of both cotyledons, but did not cut the embryonic axis.

For the pre-imbibition treatment, seeds were hydrated on filter paper moistened with distilled water at 10°C in the dark for 40 h prior to bisection. For frozen storage at -14°C, seeds were transversely bisected, placed in a 96-well polystyrene microtitre plate and sealed with parafilm.

### Seed Germination

Seeds from all treatments were imbibed on moist filter paper in sealed Petri dishes at 10°C in darkness for 9 d, after which they were planted in flats of soil by placing the emerging radicle, when present, in the downward position, and barely covering the seeds with potting soil. Sterile technique was not used as a test of the utility of the method to growers. Flats were placed in a greenhouse under a 10-11 h photoperiod in October and November in Ashland, OR, with temperatures of 22°C day and 15°C night, and watered daily. No fertilizer was applied.

After 12 d in the greenhouse, plants were counted as established if one or more true leaves had formed. Results were analyzed by a chi-square contingency table with the expected rate of plant establishment determined from the pooled estimate.

### Meristem Culture

After an additional 28 d in the greenhouse, when plants were maturing, but not flowering, apical meristems were aseptically excised and cultured on a liquid medium consisting of MS salts, vitamins (Nitsch and Nitsch 1969), indoleacetic acid (IAA, 0.1 mg/l), gibberellic acid (GA, 0.2 mg/l), kinetin (0.04 mg/l), and sucrose (30 g/l). The pH was adjusted to 5.6 with NaOH. Medium was dispensed, 10 ml per tube, into 20 x 150 mm culture tubes with polypropylene caps and autoclaved for 12 min at 110°C (Brown and Kwiatkowski 1988). After 10 d culture on liquid media, meristems were subcultured to semi-solid media containing carbenicillin (250 mg/l) for control of a slow-growing bacterial contaminant.

Cytokinins were tested to determine which best promoted shoot multiplication. The test material consisted of shoot clumps of *L. alba* var *Foamore* derived from somatic embryos grown on the above MS medium without plant growth regulators, except trans-zeatin (0.2 mg/l) and with agar (6 g/l) (Southworth and Kwiatkowski 1991). Under a laminar flow hood, clumps were dissected to single

shoots and transferred, three shoots per 250-ml flask, to the same medium containing N<sup>6</sup>-benzyladenine (BA), trans-zeatin, kinetin, or N<sup>6</sup>-( $\Delta^2$ -isopentenyl)-adenine (2iP), all at concentrations of 1 mg/l. Flasks were placed in the growth chamber under 8-h d (19  $\mu\text{Em}^{-2}\text{s}^{-1}$ ) and 18°C/12°C day/night temperature regime. After 43 d when shoot clumps were mature and senescence was beginning, clumps were dissected to count the number of shoots.

Data were subjected to one-way analysis of variance (ANOVA) to test whether sample means were drawn from populations with the same mean (Samuels 1989).

Plant material from one flask of each treatment was subcultured to semi-solid (6 g/l agar) modified MS media containing IAA, GA, and kinetin, as above (Brown and Kwiatkowski 1988), for rooting and then transplanted to soil in the greenhouse.

For shoot multiplication, plants derived from meristem culture of *Limnanthes* sp. (ORL 85-765) were placed on 50 ml semi-solid MS media containing trans-zeatin (1 mg/l) and carbenicillin in 250 ml flasks plugged with cotton. Flasks were placed in the growth chamber as above.

### Results

All treatments yielded high germination percentages (Table 1). After incubation of seeds for 8 d, radicle emergence was least for longitudinally bisected dry seeds in which 73 percent of seeds produced radicles. In all other treatments, at least 97 percent of seeds produced radicles. Based on chi-square analysis, germination percentages differed significantly from the pooled estimate of germination which was 0.93.

The manner of cutting affected plant growth. The number of plants established in the greenhouse after 8 d incubation at 10°C and 12 d in the greenhouse was greatest for transversely bisected dry seeds of which 100 percent gave rise to plants. Plant establishment was least for pre-imbibed, longitudinally bisected seeds in which 77 percent of the seeds yielded plants (Table 1). Of transversely bisected frozen seeds, 97 percent formed plants. In some dry seeds, cotyledons shattered or cracked when bisected while pre-imbibed seeds did not shatter or crack, but cut smoothly. However, a larger fraction of seeds that were cut dry gave rise to plants (Table 1). Some seeds with emergent radicles after the cold treatment failed

TABLE 1. Effect of cuts on radicle emergence and establishment of plants.

Treatment	Emergence of Radicles <sup>1</sup>		Establishment of Plants <sup>2</sup>	
	Germ.	Ungerm.	Growth	No Growth
Whole seed	29	1	28	2
Longitudinal bisect	22	8	26	4
Imbibe, longi-bisect	30	0	23	7
Transverse bisect	29	1	30	0
Trans-bisect, freeze	31	1	31	1
Chi-square value	21.39		12.48	

<sup>1</sup>Radicle emergence 8 d after imbibition;  $p < 0.0001$ .

<sup>2</sup>Determined 12 d after planting;  $0.001 < p < 0.01$ .

to develop into plants, and some seeds lacking emergent radicles eventually grew.

After 12 d in the greenhouse, seedlings from whole seeds showed the greatest vigor, being larger than those from all other treatments. Seedlings from pre-imbibed seeds were the smallest. Seedlings from the dry, transversely bisected seeds, whether germinated immediately or stored, and the dry longitudinally bisected seeds had similar intermediate vigor, but the longitudinally bisected seeds showed more variability. They had both larger and smaller seedlings than the transversely bisected seeds in which seedlings were more uniform. These differences in seedling size continued. After 43 d in the greenhouse, plants from whole seeds were larger than those from all other treatments, and had many flower buds. Plants from bisected seeds were smaller, and had fewer flower buds.

Meristems from ORL 85-765 grew rapidly, although 25 percent were lost due to contamination. Meristems grew from an excised length of 0.3-1.0 mm to lengths of 3-15 mm in 9 d. Subculture to semi-solid media from liquid media was necessary after 10 d. After an additional 55 d on media with zeatin (1 mg/l) and carbenicillin, seven out of ten shoots survived and produced an average of  $7 \pm 3.1$  shoots per clump.

Shoot multiplication of meadowfoam also occurred on media containing zeatin, kinetin, or 2iP, but not on media containing BA. Multiplication was best on zeatin (Table 2), but kinetin and 2iP gave satisfactory results. Differences in treatments were significant ( $F = 29.11$ , d.f. 3, 32,  $P < 0.0001$ ). Based on the Newman-Keuls procedure, the means of numbers of shoots developed on zeatin and on

TABLE 2. Shoot multiplication in response to cytokinins in *L. alba* var. Foamore.

Cytokinin (1 mg/l)	Shoots per Clump <sup>1</sup>	
	$\bar{X}$	SE
Zeatin	9.8	0.9
Kinetin	6.7	0.4
N <sup>6</sup> -( $\Delta^2$ -isopentenyl)-adenine	6.7	0.9
N <sup>6</sup> -benzyladenine	1.0	0

<sup>1</sup>43 d in culture;  $n = 9$ .

BA differed from those of all other cytokinins at the 1% level (Samuels 1989). Meristems treated with kinetin did not differ significantly from those treated with 2iP.

## Discussion

Transversely bisected seed was easier to work with than longitudinally bisected seed. Plants grown from transversely bisected seeds were more uniform, and germination and growth were more reliable. With longitudinally bisected seeds, it was difficult to determine which half would be viable, as both halves may have contained some apical tissues. We observed that only one half formed a plant. Longitudinally bisected seed consisted of proportional amounts of testa-pericarp and cotyledonary tissues, and in addition, apical tissues in varying amounts. Transversely bisected seed provided a more uniform tissue for analyses, because the decapitated portion consisted only of proportional amounts of testa-pericarp and cotyledonary tissues.

These results show that a rather crude cut under non-sterile conditions yields a half seed that can be stored frozen while the other half is analyzed for oil.

While *Limnanthes* sp. grows well in tissue culture, this method requires relatively high technology with sterile conditions (Reed 1991, Southworth and Kwiatkowski 1991). Establishment of *Limnanthes* sp. embryos in culture occurred with the loss of a high percentage of embryos due to handling damage or contamination (Southworth and Kwiatkowski 1991). The half-seed method applies appropriate technology to develop a useful selection procedure.

The rapid growth of plants from meristems indicates that meadowfoam is well suited to meristem

culture. Refinements in meristem excision should overcome contamination, as the meristem itself appeared to be free of microorganisms. When excising apical meristems, it was noted that most leaf petiole bases were contaminated with soil particles. Only the innermost six to eight leaf bases surrounding the meristem were reliably free of soil particles. This level of surface contamination so deep within the rosette indicated difficulty in surface sterilization for shoot culture, as noted by Reed (1991). Meristem culture should overcome leaf axil contamination, as the innermost primordia seemed to be clean.

Failure to obtain shoot multiplication on BA differed from the results of Reed (1991) who used

BA as the sole cytokinin and obtained 2.4 shoots per clump. The differing outcomes may be due to genetic differences in selected lines, to developmental differences induced by previous culture conditions such as growth on zeatin, or to environmental differences such as warmer days.

The ability to store bisected seeds and later to regenerate and clonally increase plants from those half seeds should enhance the effort to breed a superior oil-producing meadowfoam.

### Acknowledgements

This research was supported by NSF grant DCB 8801902 through Research at Undergraduate Institutions.

### Literature Cited

- Brown, C. R., and S. Kwiatkowski. 1988. Eradication of PVS from potato clones through excision of meristems from in vitro, heat-treated shoot tips. *Amer. Potato J.* 65:633-638.
- Gathman, A. C., and W. P. Bemis. 1981. Non-destructive fatty acid analysis of Cucurbit seed. *Cucurbit. Gen. Coop.* 4:36.
- Jolliff, G. D. 1981. Development and production of meadowfoam (*Limnanthes alba*). In E. J. Pryde, L. H. Princen, and K. D. Mukherjee (eds.), *New Sources of Fats and Oils*. Monogr. 9, Amer. Oil Chem. Soc., Champaign, IL. Pp. 269-285.
- Nitsch, J. P., and C. Nitsch. 1969. Haploid plants from pollen grains. *Science* 163:269-285.
- Pleins, S., and W. Friedt. 1989. Genetic control of linolenic acid concentration in seed oil of rapeseed (*Brassica napus* L.). *Theor. Appl. Genet.* 78:793-797.
- Reed, B. 1991. Micropropagation of meadowfoam (*Limnanthes* spp.). *Plant Cell Repts.* In press.
- Samuels, M. L. 1989. *Statistics for the life sciences*. Dellen, San Francisco.
- Southworth, D., and S. Kwiatkowski. 1991. Somatic embryogenesis from immature embryos in meadowfoam (*Limnanthes alba*). *Plant Cell, Tissue, Organ Culture* 24:193-198.

Received 25 January 1991

Accepted for publication 18 September 1991.