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In vitro fertilization from co-cultured pollen tubes and female gametophytes of Douglas fir (*Pseudotsuga menziesii*)

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Abstract Our previous attempt on in vitro fertilization (IVF) in conifers resulted in pollen tube penetration of female gametophytes, but because of the rapid decline in egg viability, no further interaction occurred. In this report, we describe for the first time that IVF has been achieved in conifers. Using Douglas fir (*Pseudotsuga menziesii*), we describe a two-step process which involved induction of pollen tubes in culture followed by introduction of isolated female gametophytes at the tips of growing pollen tubes. Pollen tubes penetrated the introduced isolated female gametophytes at various places, but a number of tubes entered the egg cell through the neck cells similar to the in vivo condition. Under our current culture conditions, longevity of pollen tubes and eggs has been improved resulting in the release of sperms, fusion of gametes, and initial formation of the proembryo. Continued plasmolysis of the egg limited the number of successful gametic interactions. IVF has been accomplished in flowering plants in several ways, but the gametophyte-gametophyte IVF system described in this paper is unique. IVF offers a novel breeding technology that takes advantage of the sexual reproductive route. When coupled with hybridization and genetic transformation, IVF could result in the development of stable novel genotypes of economically superior trees.

Key words Conifer · Female gametophyte · In vitro fertilization · Pollen tube · *Pseudotsuga menziesii*

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Introduction

In vitro fertilization (IVF) is a process whereby reproductive structures are isolated and introduced to each other enabling fusion of gametes to proceed under culture conditions. IVF offers the possibility of a novel breeding technology which has practical applications in breeding programs such as the introduction of transformed gametes and controlled selection of offspring (Tilton and Russell 1983). IVF also opens up new avenues of exploration including pollen tube and female gametophyte interactions and the study of cellular events surrounding fertilization. Another importance of IVF is its potential in the production of hybrids between plants that do not normally cross (Zenkteler 1990). In nature, interspecific and intergeneric crosses are possible to some extent, but biological incompatibility systems usually prevent the production of viable hybrids. Post-fertilization incompatibility barriers can be overcome by the rescue of immature hybrid embryos which are nurtured in vitro (Asano 1980; Stewart 1981; Ho 1987). However, many incompatibility reactions happen prior to fertilization (Steyn et al. 1996; Nettancourt 1997; Runions 1997). IVF offers a novel approach that could bypass pre-fertilization incompatibility barriers. IVF coupled with embryo rescue could avoid the problems of regeneration and somaclonal variation induced by somatic embryogenesis. Our IVF system allows pollen and female gametophytes to be maintained in culture so genetic transformations can be carried out on sperms or eggs. When transformed gametes are used during IVF, stable novel genotypes can be formed.

In flowering plants, IVF has been accomplished using multicellular reproductive structures such as isolated gynoecia (Tilton and Russell 1983), cob segments (Kranz and Lörz 1990), and ovules (Kanta et al. 1962; Kameya et al. 1966; Rangaswamy and Shivanna 1976; DeVerna et al. 1987). IVF has been most notably

accomplished using isolated male and female gametes of maize (Kranz et al. 1991; Kranz and Lörz 1993; Faure et al. 1994), wheat (Kovács et al. 1995), and tobacco (Tian and Russell 1997). At any level, IVF has not been done using conifers. Advances in IVF particularly in maize, have been very significant (Kranz et al. 1992; Faure et al. 1996; Rougier et al. 1996). In spite of the diversity of plant reproductive systems and current advances in IVF, only limited information can be transferred to conifers.

Our first attempt on IVF using Douglas fir (*Pseudotsuga menziesii*) showed that pollen tubes were able to penetrate archegonia in vitro but that further interaction was hindered by the rapid decline in egg viability (Fernando et al. 1997). This study presented here was aimed at optimizing culture conditions to extend egg viability and, at the same time, to allow further interaction of male and female reproductive structures in vitro. The success of IVF strongly depends on culture conditions (including media composition) that will support pollen tube formation, egg viability, pollen tube penetration, sperm release, and eventually the fusion of gametes.

Materials and methods

Plant material

We used Douglas fir because normal ovule and female gametophyte development occurs in unpollinated cones. In the field, the collection of cones was restricted to the first week of June (Owens and Smith 1964). In trees at higher elevations, however, the developmental stage can be delayed up to a month (Allen and Owens 1972). This provided another collection period, which was in the first week of July. It has also been shown that the application of gibberellic acid induces cone bud development in Douglas fir (Ross et al. 1985). Potted ramets of Douglas fir previously treated with gibberellic acid were obtained from the British Columbia Ministry of Forests (BCMF) Research Branch at Glyn Road Research Station in Victoria. Accelerated cone development occurred in ramets that were transferred into the greenhouse of the Centre for Forest Biology, University of Victoria. Greenhouse incubation extended the availability of cones, and allowed collection periods during the last week of March and second week of April. A total of four staggered collection periods were therefore possible, two from the field and two from greenhouse trees. Seed cones were enclosed in isolation bags prior to pollen release. Methods for the collection of sterile pollen and isolation of unpollinated female gametophytes have been described previously (Fernando et al. 1997). To avoid the problem of self-incompatibility or inbreeding, we used two genetically different clones (clone 3202 as the source of pollen and clone 3265 as the source of female gametophytes). Stages of female gametophyte development were monitored using specimens cleared with methyl salicylate (Fernando and Cass 1997).

Media composition and culture conditions

We used a MS basal medium (Murashige and Skoog 1962) modified by the addition of some Brewbaker and Kwack salts (Fernando et al. 1997). This medium is referred to as MSBK. The stock was diluted 1:10 with distilled water and then supplemented with 150 m*M* or 300 m*M* lactose or sucrose, 30 m*M* polyethylene glycol 3350 (PEG), and 0.4% phytagel. The pH was adjusted to 5.8.

Approximately 1 mg of pollen was evenly dispensed per petri dish containing MSBK medium. Pollen cultures were wrapped with aluminum foil and incubated at 24°C. The length of pollen grains and tubes and percentage tube formation were determined after 7 days. Representative pollen tubes were marked and longevity monitored after another 5 and 7 days (12 and 14 days after pollen culture). The number of pollen tubes that developed swollen tips was determined after another 7 days (14 days from pollen culture).

Viability of the egg was based on whether it had undergone plasmolysis or not. Data on egg viability was obtained from 160 female gametophytes (40 per treatment). Since there are usually four eggs per female gametophyte, we referred to a female gametophyte as viable even if only one of the eggs was unplasmolyzed. A total of 656 female gametophytes were co-cultured by introducing newly isolated female gametophytes at the tips of pollen tubes. The co-cultures were incubated in the dark at 22°C. Co-cultured female gametophytes were classified as type 1 (all four eggs unplasmolyzed) or type 2 (one to three eggs unplasmolyzed). The types of female gametophytes were determined at the time of fixation, which ranged from 6 to 12 days after the initiation of co-culture.

Histological analysis

After the co-cultures were incubated for 6–12 days, viable female gametophytes in contact with unplasmolyzed pollen tubes were identified. Only viable female gametophytes which when lifted had firmly attached pollen tubes were fixed in 4% glutaraldehyde. Specimens were washed with distilled water and dehydrated through a graded series of alcohol up to 100% ethyl alcohol. Using a historesin embedding kit (Leica Instruments, Heidelberg), we gradually infiltrated specimens using mixtures (1:1, 1:2 and 0:1 ratio) of 100% ethyl alcohol and infiltration solution (made up of 100 ml hydroxyethyl methacrylate and 1 g dibenzolperoxide). Specimens were infiltrated in each mixture until the specimens sank. They were then left in pure infiltration solution for 3–4 days and embedded in fresh infiltration solution containing dimethyl sulfoxide (10:1 ratio) in molds made up of paraffin. The molds were sealed with melted paraffin and incubated under bright light for at least 2 h at room temperature. Sections (7–9 μm) were cut using glass knives on JB4 ultramicrotome. Slides were stained with toluidine blue (Trump et al. 1961).

Statistical analysis

One-way ANOVA procedures were performed and differences detected with Tukey's honestly significant difference test using SPSS software (SPSS 1993). This statistical software was also used to calculate for the range, mean, and standard error.

Results

Pollen tubes and female gametophytes in culture

Pollen cultured on media with lower sugar concentrations had a faster rate of pollen elongation and longer pollen tubes (Table 1). Pollen tubes of Douglas fir formed on MSBK medium supplemented with either lactose or sucrose in combination with PEG. Percentage pollen tube formation was generally higher on medium supplemented with sucrose than with lactose

Table 1 Effects of sugars and sugar concentrations on growth of Douglas fir pollen tubes on MSBK medium ($n = 40$)

	Lactose		Sucrose	
	150 m*M*	300 m*M*	150 m*M*	300 m*M*
Length of pollen grains (μm)				
Mean	382a (±17)[a]	307b (±11)	370a (±13)	350c (±21)
Range	215–560	200–450	215–540	200–520
Length of pollen tubes (μm)				
Mean	652a (±71)	75b (±4)	763a (±102)	216c (±26)
Range	90–1170	55–90	90–1260	70–360
Pollen tube formation (%)				
Mean	4.9a (±2)	1.0b (±1)	5.7a (±2)	11.2c (±5)
Range	1.9–8.6	0.5–1.6	2.0–10.4	2.5–27.8
Pollen tube survival (%)				
5th day	65	–	42	36
7th day	49	–	25	30
Swollen pollen tubes (%)	52	98	77	85

[a] Mean values followed by the same letter do not differ significantly ($\alpha = 0.05$)

(Table 1). Although percentage pollen tube formation on 150 m*M* sucrose appeared to be higher than that of 150 m*M* lactose, no significant difference was observed between these treatments (Table 1). On medium containing 150 m*M* lactose, more pollen tubes survived after 5 and 7 days, and fewer pollen tubes swelled at the tips as compared to any concentrations of sucrose examined (Table 1). Survival of pollen tubes on 300 m*M* lactose was about 95%, but the data were not presented in the table since the tubes appeared stunted and did not elongate further; these tubes were not suitable for IVF. On all of the media tested, a number of pollen tubes plasmolyzed, branched or became swollen at the tips. These peculiarities occurred whether the pollen tubes were used in co-culture or not.

Isolated female gametophytes were cultured on MSBK medium supplemented with two concentrations of lactose and sucrose (Table 2). Egg viability appeared better on media with lower sugar concentrations. However, this effect occurred only during the first 8 days in culture (Table 2). After 10 days of culture, the effect of sugar concentrations was generally similar in all treatments (Table 2). A higher percentage and a more gradual decline in egg viability were observed on media supplemented with 150 m*M* lactose or sucrose. In a few female gametophytes all four eggs remained unplasmolyzed for up to 6 days in culture. Our results also showed that viability of eggs in culture can be extended for up to 10 days, although only at the 2–4% level (Table 2). Most female gametophytes at this stage have one unplasmolyzed egg. All eggs were plasmolyzed after 12 days in culture and, thus, the data were not included in the table. A total of 160 female gametophytes were used in this part of the experiment.

Based on the longevity of pollen tubes and eggs, co-cultures were done on MSBK medium supplemented with 150 m*M* lactose. Although pollen tubes at co-

Table 2 Effects of sugars and sugar concentrations on percentage female gametophyte (egg) viability ($n = 40$)

Days in culture	Lactose		Sucrose	
	150 m*M*	300 m*M*	150 m*M*	300 m*M*
1	95	78	89	80
6	44	11	31	12
8	25	10	18	7
10	3	4	2	4

culture ranged between 90 and 1170 μm long, female gametophytes were only introduced to tubes that were more than 500 μm long. During co-culture, some pollen tubes grew away from or continued elongating past the introduced female gametophytes. Other pollen tubes exhibited no peculiarities and may have penetrated some female gametophytes (Fig. 1). Of the 656

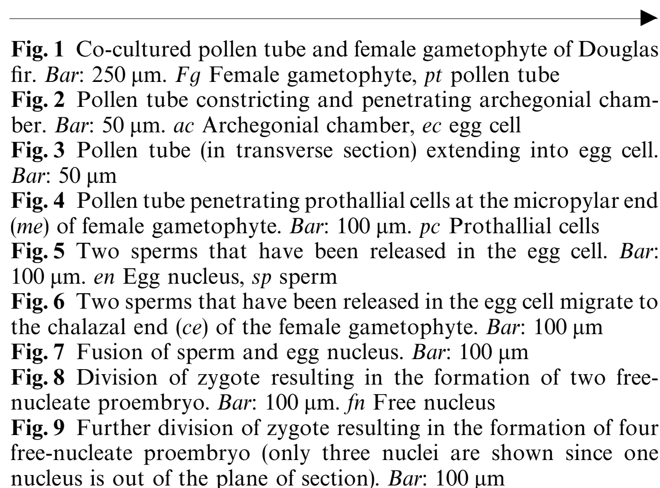
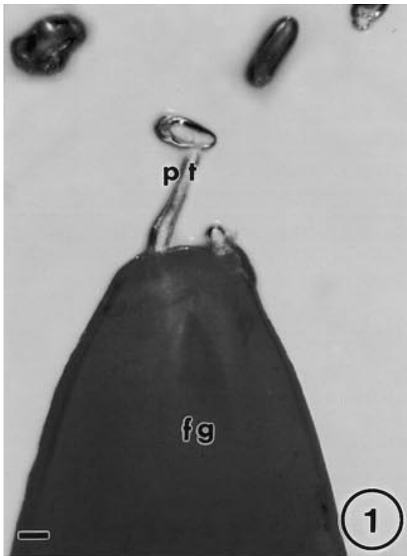


Fig. 1 Co-cultured pollen tube and female gametophyte of Douglas fir. *Bar*: 250 μm. *Fg* Female gametophyte, *pt* pollen tube
Fig. 2 Pollen tube constricting and penetrating archegonial chamber. *Bar*: 50 μm. *ac* Archegonial chamber, *ec* egg cell
Fig. 3 Pollen tube (in transverse section) extending into egg cell. *Bar*: 50 μm
Fig. 4 Pollen tube penetrating prothallial cells at the micropylar end (*me*) of female gametophyte. *Bar*: 100 μm. *pc* Prothallial cells
Fig. 5 Two sperms that have been released in the egg cell. *Bar*: 100 μm. *en* Egg nucleus, *sp* sperm
Fig. 6 Two sperms that have been released in the egg cell migrate to the chalazal end (*ce*) of the female gametophyte. *Bar*: 100 μm
Fig. 7 Fusion of sperm and egg nucleus. *Bar*: 100 μm
Fig. 8 Division of zygote resulting in the formation of two free-nucleate proembryo. *Bar*: 100 μm. *fn* Free nucleus
Fig. 9 Further division of zygote resulting in the formation of four free-nucleate proembryo (only three nuclei are shown since one nucleus is out of the plane of section). *Bar*: 100 μm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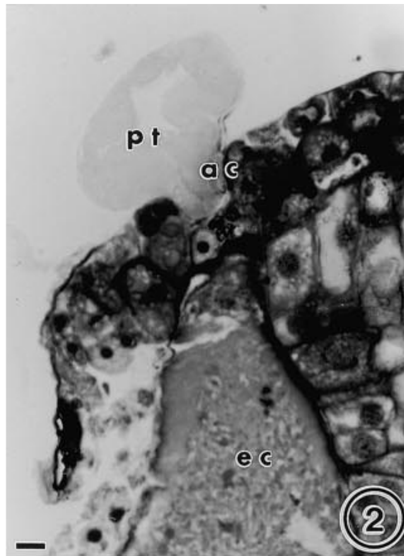
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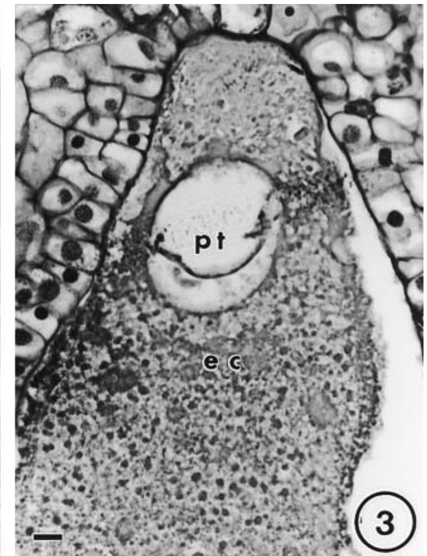
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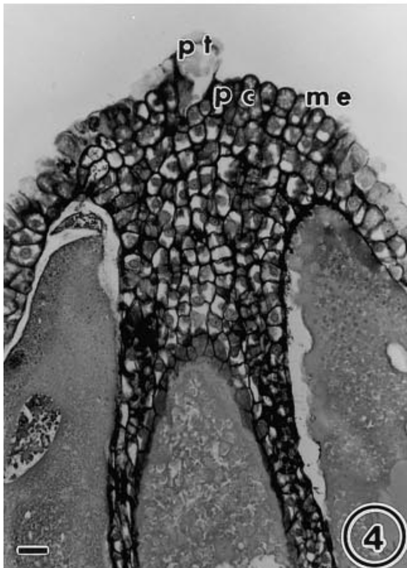
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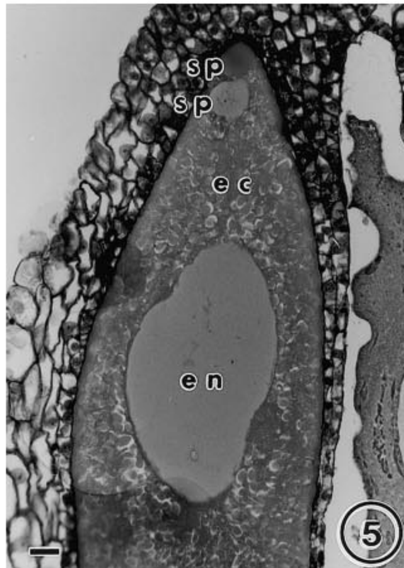
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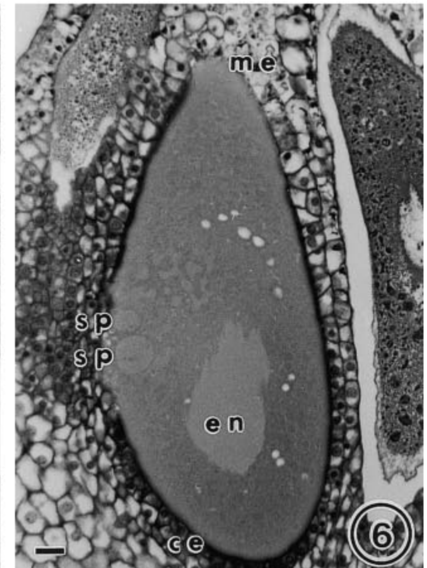
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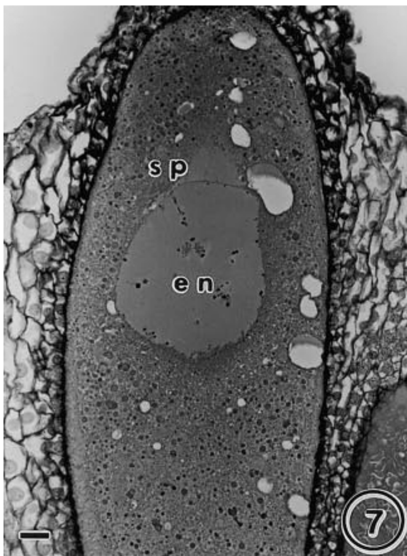
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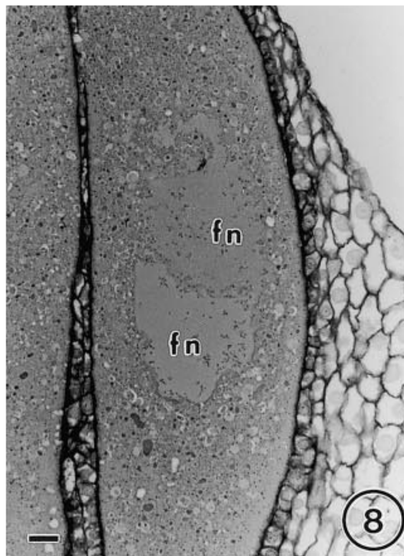
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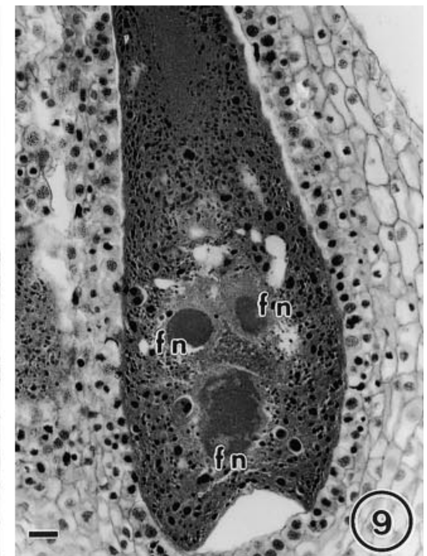
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Table 3 In vitro pollen tube penetration of viable female gametophytes (FG)

Type of FG	Number of FG with pollen tubes at fixation	Number of FG Penetrated (sectioned specimens)
1	16	6
2	54	15
Total	70	21

female gametophytes that were co-cultured, 70 had one or more viable eggs with firmly attached pollen tubes at fixation. A total of 586 female gametophytes were discarded because all eggs had plasmolyzed. Examination of sectioned specimens showed that female gametophytes isolated at the late-central cell stage proceeded to the egg stage in culture.

In vitro penetration, release of sperm, and fertilization

Examination of sectioned specimens revealed that only 21 female gametophytes were actually penetrated by pollen tubes (Table 3). Most pollen tubes were either only clinging to the female gametophytes or became detached during tissue processing and, therefore, were difficult to distinguish from sectioned specimens. Ten pollen tubes penetrated the archegonia similar to the in vivo situation where part of the pollen tube constricts and penetrates between neck cells (Fig. 2). Three of these pollen tubes elongated beyond the neck cells, resulting in part of the tube extending into the egg cytoplasm (Fig. 3). Pollen tube penetration was not always through the neck cells; 11 pollen tubes penetrated the prothallial cells at various parts of the female gametophyte (Fig. 4).

Rupture of the pollen tubes to release sperm was not observed from any of the tubes that extended into the egg cell or penetrated the prothallial cells. Pollen tubes that passed through the neck cells released sperm that were similar in size (Fig. 5). Peculiarities were observed in the activities of some of the sperm that were released in vitro, such as migration of both toward the chalazal end instead of only one or migration not to the micropylar end of the egg nucleus but to its side (Fig. 6). Fusion of gametes was observed in one occasion from specimens fixed at 8 days in culture (Fig. 7). Initial free-nuclear divisions of the zygote were observed on two occasions from specimens fixed at 10 days in culture (Figs. 8 and 9). Sperm release, gamete fusion, and proembryo formation were only observed from female gametophytes with fewer than four unplasmolyzed eggs. Most pollen tubes that penetrated prothallial cells were observed from female gametophytes with all four unplasmolyzed eggs.

Discussion

Pollen tubes and female gametophytes in culture

Pollen tubes of conifers are generally easy to induce in culture with a few exceptions, such as *Larix* and Douglas fir. We used Douglas fir because of its economic importance, the wealth of basic information available, and an accessibility to specimens. The induction of Douglas fir pollen tubes using Brewbaker and Kwack medium has been successful (Takaso et al. 1996; Dumont-BéBoux and von Aderkas 1997), but the effect of this medium on egg viability has not been established. For IVF to be successful, the culture medium should be able to support the growth of both pollen tubes and female gametophytes. Therefore, in our previous work (see Fernando et al. 1997) we used a more complete medium, such as that of Murashige and Skoog (1962). After a series of modifications, which involved several changes in the concentrations of boric acid and calcium nitrate and several types of osmoticum, we achieved 10–20% pollen tube formation (Fernando et al. 1997). This medium, referred to as MSBK, supported the growth of eggs only up to 5 days (Fernando et al. 1997).

The effects of several types of sugars on pollen germination and tube growth have been examined for Douglas fir (Dumont-BéBoux and von Aderkas 1997). We believed that finding the optimum conditions entailed exploring the effects of more types of sugars. Furthermore, the effects of sugars and sugar concentrations on egg viability had not been established on Douglas fir or any other conifer. We tried lactose because it has been shown to be superior to sucrose for stimulating pollen germination and tube growth in angiosperm species (Bamberg and Hanneman 1991; Rihova et al. 1996). Polyethylene glycol has also been shown to promote pollen tube growth (see Shivanna and Sawhney 1995) and has become a common constituent of many culture media (see Bonga and von Aderkas 1992). Our results showed that MSBK supplemented with lactose improved the survival of pollen tubes and reduced swelling of tube tips. Both these features help in increasing the chances of penetration and release of gametes.

Female gametophytes isolated at the late-central cell and early egg stages were cultured to find the optimum conditions in which they would survive. Using media with lower sugar concentrations improved longevity of the eggs. Our results also showed that female gametophytes isolated at the late-central cell stage proceeded to form eggs. The eggs exhibited tremendous variability under culture conditions. Female gametophytes with four unplasmolyzed eggs only lasted 6 days in culture, after this stage, all female gametophytes had fewer than four unplasmolyzed eggs. Longevity was mostly due to female gametophytes with only one unplasmolyzed egg.

When pollen tubes and female gametophytes were co-cultured, some pollen tubes grew away from the introduced female gametophytes while other tubes continued to elongate without penetrating the female gametophyte. In spite of pollen tube peculiarities such as branching, swelling, and plasmolysis, approximately 7% of the female gametophytes examined by Fernando et al. (1997) had been penetrated. Under our improved conditions, the percentage of penetration has been increased to approximately 30%.

In vitro penetration, release of sperms and fertilization

The longevity of pollen tubes and eggs has been sustained to increase the chances of fertilization and to allow sperm release and fertilization to proceed under culture conditions. IVF in conifers has been achieved from co-cultured pollen tubes and female gametophytes of Douglas fir. Initial proembryo formation also occurred in culture. Our results represent a significant contribution to increase the longevity of unfertilized eggs in culture. In vivo, the eggs of Douglas fir have been reported to degenerate within a week if unfertilized (Owens et al. 1991). Early deterioration of unfertilized female gametophytes has also been noted in *Picea* (Runions 1997).

Our previous report showed that pollen tubes were able to penetrate archegonia in vitro but that successful gametic interaction did not proceed because of a rapid decline in egg viability (Fernando et al. 1997). Pollen-tube penetration of female gametophytes in vitro is variable, ranging from a condition similar to that in vivo to the penetration of prothallial cells. The latter has not been reported in vivo. Pollen tubes that extend into the egg cell are not unique to in vitro conditions since this phenomenon also been observed in vivo (Runions 1997).

Since our previous work represents the only literature on IVF in conifers, many details need to be refined in order to attain an ideal IVF system for this group of plants. The longevity of the pollen tubes and eggs of Douglas fir has been improved, but this remains to be a problem limiting the number of successful IVF events. We also believe that sustaining growth of proembryos in culture is difficult and, consequently, no mature embryo has yet been produced. Our culture conditions have allowed IVF to occur, but these have to be improved to lessen the number of peculiarities, increase the number of successful IVF events, and sustain embryo formation to at least, the stage when embryo rescue can be done. With minor modifications, using this IVF protocol on other conifer species might achieve a better result.

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