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N. Dumont-BéBoux · M. Weber · Y. Ma · P. von Aderkas

Intergeneric pollen – megagametophyte relationships of conifers in vitro

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Abstract Germinating pollen from larch (*Larix occidentalis*), Sitka spruce (*Picea sitchensis*) and white pine (*Pinus monticola*) were co-cultured with megagametophytes dissected from cones of other genera (*Pseudotsuga menziesii*, *Larix* × *eurolepis* and *Pinus monticola*). Pollen was presented to megagametophytes possessing archegonia which were either alive, degenerating or dead. In addition, pollen was presented to fertilized megagametophytes and to megagametophytes that had been cut in half. Megagametophyte penetration by pollen tubes and male gamete release into archegonia were verified by serial sections of glycomethacrylate-embedded specimens. Pollen tubes penetrated through any part of the apex of the megagametophyte. Division of the body cell into the two gametes was regularly observed. Delivery of gametes was confirmed between spruce and larch. Pollen tubes also penetrated fertilized megagametophytes, dead or degenerating archegonia as well as wounded and/or cut surfaces. This demonstrates the inability of the male gametophyte to optimize its mating efforts, since it is unable to differentiate between healthy and unhealthy archegonia. The megagametophyte cells are unable to optimize male selection. They may produce secretions of a generally attractive nature, as pollen is attracted to the apex of the megagametophyte, but archegonia themselves do not produce pollen-specific signals of either a promotive or inhibitory nature. These results open new avenues for the development of novel breeding strategies where natural breeding barriers may be bypassed.

Key words Conifers · In vitro fertilization · Intergeneric crosses · Prezygotic barriers · Gamete delivery

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N. Dumont-BéBoux (✉) · M. Weber · Y. Ma · P. von Aderkas
Centre for Forest Biology, Department of Biology, University of Victoria, P.O. Box 3020 STN CSC, Victoria, BC, V8W 3N5, Canada
Fax: +1-250-721-7120
e-mail: ndumont@uvic.ca

Introduction

In vitro fertilization (IVF) has recently been achieved in a gymnosperm, Douglas-fir (Fernando et al. 1998), consequently providing a tool for novel breeding strategies, the aims of which may be either to overcome prezygotic selection barriers against hybridization or to facilitate delivery of genetically engineered gametes. In this study we propose applying IVF methods to testing prezygotic reproductive barriers within the Pinaceae.

Prezygotic barriers show great variation within conifers. Examples of the relative weakness of prezygotic selection are the numerous hybrid swarms found in forests: in eastern Canada, *Picea rubens* × *mariana*; in western Canada, *Picea glauca* × *engelmannii*; and in southeastern United States, *Pinus* × *sondereggei*, to name but a few. Spontaneous hybridization, such as Dunkeld larch (*Larix decidua* × *leptolepis*) and Leyland's cypress (*Chamaecyparis nootkatensis* × *Cupressus macrocarpa*) are known to occur in arboreta (Mabberley 1990). Among the Pinaceae, a number of genera are more like species complexes or syngameons, in which species do not have well-defined reproductive boundaries (Otte and Endler 1989). However, examples of relative strength in prezygotic selection have been observed within *Picea* by Mikkola (1969) who found that alien pollen showed a pronounced inability to penetrate the nucellus. Whether prezygotic selection is strong or weak has never been the subject of experimental

reproductive biology in conifers, and no mechanisms have been elucidated.

Pollen germination in most genera of the Pinaceae depends on liquid, either in the form of a pollination drop as in *Picea*, *Abies* and *Pinus* (Owens and Blake 1985) or as a post-pollination prefertilization drop as in *Larix* and *Pseudotsuga* (Said et al. 1991; Takaso et al. 1996). Beyond promoting the germination of pollen from the same species or even genus, the drop may well inhibit the germination of pollen from other genera (von Aderkas, unpublished).

Pollen penetration of the nucellus shows great variation among genera. In *Picea*, *Larix* and *Pseudotsuga*, the pollen pushes through this tissue in rapid succession (1 week), but in *Pinus* the pollen is arrested once within the nucellus and only resumes growth after nearly a year (Singh 1978).

When the pollen arrives at the megagametophyte, it moves into the archegonial chamber before penetrating the neck cells (Singh 1978). But other routes are also possible. Penetration through the wall of the archegonium has been reported for interior spruce (*Picea glauca* × *engelmannii*). This was attributed to abnormalities in ventral canal orientation, implying a role for this particular cell in directing pollen growth (Runions 1997). Some pollen may branch during penetration, either to provide anchorage (Willemse and Linskens 1969) or to optimize search strategies for archegonia.

Thus, barriers to foreign pollen may occur during pollen germination, pollen penetration of the nucellus and pollen penetration of the megagametophyte. These prefertilization processes have been well described in histological and ultrastructural studies, but no analysis of nucellar tissue or of any of the various interactions with the pollen has been carried out. This is largely due to the difficulties in working with intact cones, as well as the impossibility of mutation work with trees that have such long breeding cycles. Likewise, selection of male gametes within the egg has received no attention in gymnosperms.

The purpose of the work presented here was to look at the interactions between pollen and megagametophyte and to determine whether any barriers to foreign pollen exist during the events from pollen presentation to male gamete delivery within the egg. We tried a variety of combinations of genera within the Pinaceae. To do this we used the in vitro fertilization system successfully applied to Douglas-fir (Fernando et al. 1998) in which the pollen was germinated in vitro and presented to megagametophytes isolated from ovules and free of any nucellar tissue. We hypothesized that pollen of one genus could penetrate and deliver its gametes into the archegonium of another genus. We wished to know if recognition of neck cells was necessary for penetration by pollen and whether any barriers to pollen penetration existed within megagametophytes. Furthermore, we were interested in any possible changes in megagametophytes during culture which may affect pollen behaviour.

Materials and methods

Plant material

Male and female cones were collected from several sources. *Pinus monticola* male cones and *Pseudotsuga menzeisii* female cones came from British Columbia Ministry of Forests (BCMF) Glyn Road Station, Victoria. *Pinus monticola* female cones were collected at TimberWest seed orchard (Saanich), *Picea sitchensis* male cones came from Nootka orchard (Pacific Forestry), *Larix occidentalis* male cones came from BCMF Kalamalka Research Station. Seed cones of *Larix* × *eurolepis* and *P. menziesii* were collected on the campus of the University of Victoria. Male cones were collected in March and April of 1997, just prior to pollen shedding. Both bagged and unbagged female cones were collected from mid May to late June of the same year.

Pollen was collected from surface-sterilized cones prepared as described by Friedman (1987). The cones were washed for 15 s in 70% ethyl alcohol, followed by 30 s in 1% sodium hypochlorite. They were rinsed three times in sterile distilled water and blot-dried on sterile filter paper. The sterile cones were kept at 23°C in petri dishes covered with sterile filter paper until the shedding of pollen grains occurred. The dry pollen ($\leq$ 9% water content) was stored in airtight sterile glass vials over silica gel and kept at 4°C until required. Larch pollen needed to be hydrated prior to culture. This was done in 100% relative humidity according to Charpentier and Bonnet-Masimbert (1983). All manipulations were performed in a laminar flow hood.

The developmental stages of larch and Douglas-fir megagametophytes varied between central cell and late egg cell as determined by clearing in methyl salicylate (Fernando and Cass 1996). White pine megagametophytes were either at or beyond fertilization. In late June, some open-pollinated Douglas-fir cones were also collected.

Seed cones were surface-sterilized by flaming in 70% ethyl alcohol. Megagametophytes were dissected and the nucellus removed. When open-pollinated Douglas-fir cones were dissected, care was taken to keep only those megagametophytes in which pollen attached to the nucellus was found in the micropyle.

Male and female gametes were co-cultured on Murashige and Skoog (1962) medium (M1) modified by Fernando et al. (1997) and supplemented with 150 m*M* sucrose and 10% polyethylene glycol 4000 (Sigma). An exception to this was larch pollen, which was raised on a different medium (M2) containing Brewbaker and Kwack (1963) minerals diluted 1:10, 7.5% sucrose and 16% polyethylene glycol 4000. All media were solidified with 0.4% phytagel, and the pH was adjusted to 5.6 $\pm$ 0.1.

Pollen/megagametophyte interactions

Pollen was sown onto M1 and cultured at 23°C in the dark. Sitka spruce and white pine pollen were introduced to megagametophytes after 48 h on M1. Larch pollen was cultured on M2 for 5–7 days before being presented on a small block of M2 medium to megagametophytes cultured on M1.

Megagametophytes were placed in close proximity (approx. 100 μm) to growing pollen tubes. Megagametophytes in a variety of developmental states were used: unfertilized, fertilized, degenerating and dead. Megagametophytes were also bisected into the chalazal and micropylar halves. Both intact and cut ends were presented to pollen tubes. The male and female gametes were co-cultured for about 10 days or until penetration by pollen tubes had been observed.

Histological analysis

Megagametophytes with attached pollen tubes were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2). Five specimens of each kind of attempted cross were dehydrated using a graded series of ethyl alcohol and embedded in Technovit 7100. About 225 archegonia were sectioned. Serial sections (2–8 μm) were cut using glass knives mounted on a Sorvall JB4 ultramicrotome. Sections were stained 1 min with 0.5% Toluidine blue.

For electron microscopy, megagametophytes were embedded in Spurr's resin and cut into semi-thin (0.9–1 μm) and ultra-thin (50–90 nm) sections on a Leica ultramicrotome. The sections were examined and photographed with a Hitachi H-7000 electron microscope.

Results and discussion

Pollen and megagametophytes are not difficult to co-culture. Pollen reached the micropylar end of the female gametophyte (Fig. 1A), penetrating the archegonium (Fig. 1B, inset) and delivering male gametes to the egg (Fig. 1B). No barriers to gamete delivery existed within the members of the Pinaceae tested in vitro. Penetration of isolated megagametophytes by pollen tubes was verified histologically in all of the crosses attempted (Table 1). Prezygotic events in vitro differ from those in situ in a number of ways: pollen behaviour, penetration sites, megagametophyte development, and male gamete development and release.

Pollen behaviour in vitro

In situ, pollen must penetrate a nucellus, which may direct or aid in penetration of the megagametophyte (Mikkola 1969). In our in vitro experiment, pollen developed normally, growing a tube and penetrating megagametophytes in the absence of nucellar tissue. Body cells migrated down the tubes and divided to generate two male gametes. Pine or spruce pollen germinated quickly, growing long, branching tubes. It was not uncommon to see several tubes anchored in megagametophyte apices. On some occasions, both branches of a pollen tube entered the megagametophyte (not shown). This branching phenomenon is common in vivo, and the extra branches are thought to anchor the tubes more firmly in the nucellus (Willemse and Linskens 1969) or else to correspond to haustoria-like structures (Dawkins and Owens 1993). Larch pollen, which is difficult to germinate in vitro, produced unbranched pollen tubes after 5–7 days in culture and successfully penetrated megagametophytes of *Pinus monticola* and *Pseudotsuga menzeisii*.

In vivo, pollen-tube growth towards the archegonia may take a few days (*Larix*, *Pseudotsuga*), a few weeks (*Picea*) or a year (*Pinus*) (Dawkins and Owens 1993). In vivo, pollen tubes grow through the nucellus with which they are in close contact and which may possess mechanisms to control, regulate or prevent pollen-tube growth (Mikkola 1969). In contrast, cultured pollen tubes appeared within a few days of sowing, and no apparent mechanism regulating their growth was evident. Although they differ greatly amongst one another in their in vivo behaviour (Willemse and Linskens 1969; Allen and Owens 1972; Singh 1978; Owens and Molder 1979; Owens and Molder 1986), pine, Douglas-fir, larch and spruce pollen behaved similarly in vitro. In only a matter of days, all species were equally able to penetrate megagametophytes from different genera.

Pollen penetration

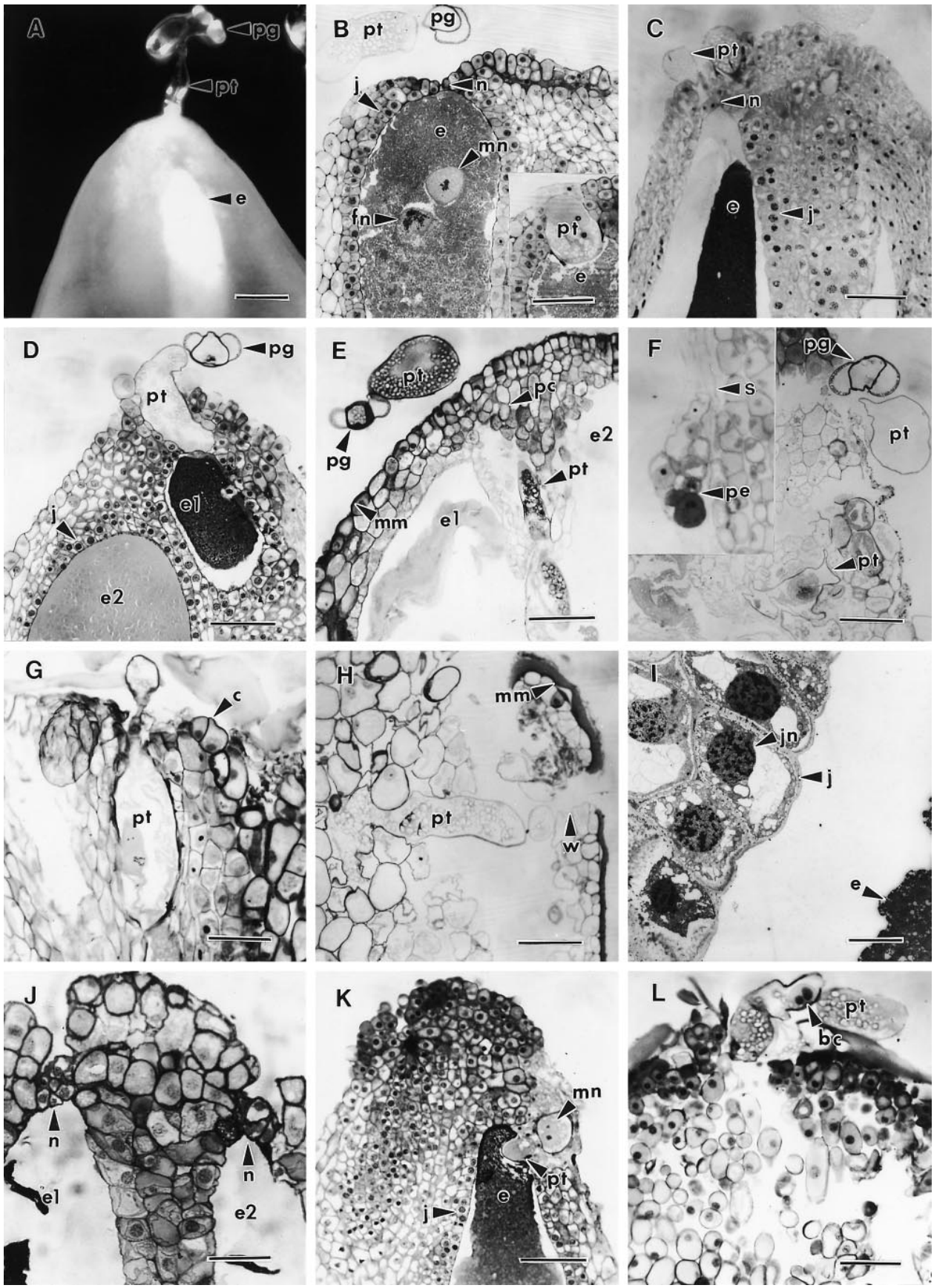
Although pollen tubes may enter the archegonia through the neck cells, this was seldom observed (Fig. 1C). More commonly, pollen was seen growing into the megagametophyte through its apex. Pollen entrance was from cells beside the neck (Fig. 1D) as well as through prothallial and jacket cells at the sides of the micropylar end of the megagametophytes (Fig. 1E). Pollen tubes generally grew towards the archegonia (Fig. 1E). A few pollen tubes missed the archegonium altogether, growing past it and through the prothallial cells which separated the archegonia from each other (not shown). The pollen point of entry had nothing to do with the internal organization of the archegonium, as many different points of entry were recorded for archegonia of normal structure and appearance.

It would appear unlikely that the ventral canal cell plays any role in pollen entry as has been suggested in the case of interior spruce (Runions 1997). Additionally, pollen tubes were not strongly attracted to the neck cells, indicating that the neck was not involved in directing pollen-tube growth. Furthermore, the neck cells did not act as species-specific recognition sites as pollen from one genus was able to pass through the neck cells of megagametophytes from another genus (Fig. 1C).

In vivo, it has been observed that Douglas-fir pollen would often reach the apex of a megagametophyte and then grow along the apical edge until an archegonial chamber was encountered, at which point the pollen would penetrate the megagametophyte (Owens and Morris 1990, 1991). Said et al. (1986) suggested that in *Larix leptolepis* the megaspore wall could provide some

Table 1 Intergeneric pollen – megagametophyte co-cultures

Male gametes	Female gametes	Penetration
L. occidentalis	*P. monticola*	+
P. monticola	*P. menzeisii*	+
P. sitchensis	*P. monticola*	+
P. sitchensis	*L.* x *eurolepis*	+
P. sitchensis	*P. menzeisii*	+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recognition mechanism at the apex of the megagametophyte. Our results clearly do not indicate the presence of any recognition mechanism, as the apices of all megagametophytes tested were unable to discriminate foreign pollen tubes. It may be possible that the apex is generally attracting the pollen, providing a signal of a very non-specific nature.

Megagametophyte development

The physiological state of the archegonia had no influence on pollen behaviour. Pollen penetrated living as well as dying eggs with equal facility (Table 2). Pollen were unable to distinguish living from dead eggs, as pollen were frequently seen entering dead archegonia immediately adjacent to live ones (Fig. 1D). Pollen was observed to penetrate non-functional archegonia which had been put into culture past the point of fertilization. In one example, pollen penetrated a megagametophyte in which an embryo was developing in the corrosion cavity (Fig. 1F).

Such behaviour would suggest that no inductive signal exists in vitro promoting pollen tubes to invade a healthy archegonium, nor is there an inhibitory signal to prevent pollen from entering a degenerating or fertilized one. In Douglas-fir, Owens and Morris (1991) reported that only one pollen tube could enter the archegonial chamber in situ and, consequently, the egg cell. In angiosperms, several studies have shown that ovules are penetrated by only one pollen tube and that pollen tubes are not attracted by fertilized ovules (Russell 1992). To our knowledge, no penetration of dead archegonia has been reported in vivo. In intraspecific IVF trials of Douglas-fir, a similar lack of pollen discrimination of archegonia has been noted (Dumont-BéBoux and von Aderkas, unpublished). The inability of the pollen to discriminate viable archegonia from the non-viable has implications for male selection during reproduction in conifers. Pollen compete during prezygotic stages, such that the fastest germinating, fastest growing pollen may be the first to successfully reach the archegonia (Willson and Burley 1983), but any initial advantage is lessened by the inability of the pollen to distinguish which archegonia are viable. Events in situ must be coordinated sufficiently well to prevent delays in pollen penetration which would lead to significant degeneration of archegonia. However, archegonial abortion has been recorded in *Picea* (Mikkola 1969), and in such cases pollen may also be unable to distinguish the living from the dead.

When megagametophytes were bisected into micropylar and chalazal halves prior to culture, pollen tubes penetrated both cut ends, growing either towards the archegonia (not shown) or towards the chalazal tip of the megagametophyte (Fig. 1G). Pollen tubes also penetrated through the uncut portion of the chalazal tip or sides of megagametophytes and grew towards the micropylar end; however, in such incidents pollen penetration was always associated with a wound in the megagametophyte membrane (Fig. 1H). At the chalazal end, pollen was never seen to penetrate through an intact megagametophyte membrane.

The megagametophyte is surrounded by a megaspore membrane similar in composition to the exine of pollen (Sedgley and Griffin 1989). Contrary to a report from Owens and Morris (1990), it was found that the megaspore wall was thinner at the micropylar end than at the chalazal end (compare Fig. 1E and H). As in primitive gymnosperms (Pettitt 1977), conifer pollen is known to possess hydrolytic enzymes such as pectinase and cellulase in *Pinus sylvestris* (Willemse and Linskens 1969) and acid phosphatase and esterase in *Pine*, *Picea*,

Table 2 : Co-culture of *P. sitchensis* pollen and various *P. menzeisii* megagametophytes

Megagametophytes	Penetration
Unpollinated	+
Pollinated/fertilized	+
Micropylar end (intact)	+
Chalazal end (intact)	−
Wounds, cut surfaces	+
Dead archegonia	+
Degenerating archegonia	+

Fig. 1 **A** Light micrograph of unpollinated Douglas-fir megagametophyte with spruce pollen grain (*pg*) anchored in its apex. One dead egg (*e*) appears as an opaque structure. *pt* pollen tube. *Bar*:100 μm. **B** Light micrograph of spruce pollen tube delivering gametes in an unpollinated larch megagametophyte (inset: serial section showing tube entering the archegonium). *fn* Female nucleus, *j* jacket, *mn* male nucleus, *n* neck cells. *Bar*:25 μm. **C** Light micrograph of spruce pollen tube penetrating into the neck of an unpollinated Douglas-fir megagametophyte and growing towards a dead archegonium. *Bar*:25 μm. **D** Light micrograph of spruce pollen tube penetrating beside the neck cells of an unpollinated Douglas-fir megagametophyte. The pollen tube grows towards a dead archegonium (*e*1) while a live archegonium (*e*2) lies adjacent to it. *Bar*:50 μm. **E** Light micrograph of spruce pollen growing towards a dead archegonium (*e*2) after penetrating through the side of an unpollinated Douglas-fir megagametophyte cut into two halves. *pc* Prothallial cells, mm megaspore membrane. *Bar*:50 μm. **F** Light micrograph of spruce pollen invading a fertilized Douglas-fir megagametophyte (inset: developing embryo). *s* Suspensor, *pe* proembryo. *Bar*:25 μm. **G** Light micrograph of the chalazal end cut surface (*c*) of a Douglas-fir megagametophyte with spruce pollen penetrating and growing towards the chalazal tip. *Bar*:25 μm. **H** Light micrograph of a pollen tube penetrating through a side wound (*w*) of the chalazal half of a Douglas-fir megagametophyte. *Bar*:25 μm. **I** Electron micrograph of jacket cells of unpollinated Douglas-fir megagametophyte, showing nuclei at prophase and degenerating egg. *jn* Jacket nucleus. *Bar*:10 μm. **J** Light micrograph of neck cells of an unpollinated Douglas-fir megagametophyte showing clusters of neck cells at the megagametophyte apex. *Bar*:25 μm. **K** Spruce pollen tube with two male gametes from body cell division within an unpollinated Douglas-fir megagametophyte. *Bar*:50 μm. **L** White pine pollen tube with body cell (*bc*) dividing at a position outside of an open-pollinated Douglas-fir megagametophyte. *Bar*:25 μm

Abies and *Cedrus* (Pettitt 1985). These enzymes are implicated in the penetration of the nucellus (Pettitt 1985) and possibly the megaspore membrane. In *Pinus contorta*, enzyme activity is higher in the presence of compatible pollination than in the presence of incompatible pollination (see Pettitt 1985). No penetration of the megaspore membrane, other than just above the archegonial chamber, has ever been reported in vivo. Our study did not reveal pollen penetration of the thick megaspore membrane of the megagametophyte chalazal end. The megaspore membrane presents a physical barrier, and possibly a chemical one, over the greater part of the megagametophyte that prevents the pollen tube from entering.

Megagametophytes continued to develop in vitro in unforeseen fashion. The cells of the apex continued to divide and expand, and so did various cell types of the archegonium. Even when the egg and ventral canal cell had died, jacket cells of the archegonium remained alive (Fig. 1I), and neck cells continued to divide (Fig. 1J). In spite of the increased number of actively secreting neck cells, pollen tubes were still not strongly attracted to them (Fig. 1J). It would appear that the secretion is not used in attracting pollen but may have some other, as yet undetermined, function.

Male gamete development

Division of the body cell to produce two male gametes occurred in a variety of locations and did not depend on pollen-tube entry of archegonia. Division was observed near the tip of pollen tubes that had penetrated either a megagametophyte (not shown) or an archegonium (Fig. 1K) or away from the tip, in parts of the pollen tube that were still outside of the megagametophyte (Fig. 1L). It took place under all circumstances, whether or not the archegonia were alive.

In white spruce pollen germinating in vivo, body cell division occurs early, when the tube is just entering the nucellus and the body cell is still at the proximal part of the tube (Dawkins and Owens 1993). In the same study, when pollen was germinated in culture, no division was observed and it was speculated that division was triggered by the nucellus (Dawkins and Owens 1993). This is in contrast to our results for spruce. Migration of the body cell and its division have already been reported for in vitro-raised Douglas-fir pollen (Dumont-BéBoux and von Aderkas 1997; Fernando et al. 1997). Gamete formation appears to be internally controlled as it occurs independently of the megagametophyte and in the absence of the nucellus.

Male gamete delivery

Male gamete delivery to the egg was confirmed between Sitka spruce pollen and a larch megagametophyte (Fig. 1B). Serial sections revealed that the pollen tube bypassed the neck of the archegonium and entered through the cells of the apex of the megagametophyte. Upon penetration of the archegonium, the tip of the pollen tube ruptured and released its male gametes. The ventral canal/egg nucleus axis does not appear to be necessary for successful delivery of gametes in vitro, although this is the only path portrayed in the histological literature (Singh 1978). The mechanism(s) which delivers male gametes to the egg nucleus appears to function irrespective of generic differences.

Conclusion

This is the first report of intergeneric crosses attempted in vitro for conifers. Male gametes can be delivered between different genera. We have shown that no recognition mechanism affects alien pollen growth in vitro. Furthermore, pollen can develop and grow towards and into megagametophytes belonging to different genera. The body cell is able to divide before and/or during penetration. Neck cells neither guide nor attract pollen. Penetration can occur through any undamaged part of the apex of the megagametophyte and through wounds. However, pollen will not penetrate unwounded chalazal ends and, therefore, cannot make its way through the thicker part of the megagametophyte membrane.

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