# Multinucleate storage cells in Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco) and the effect of seed parasitism by the chalcid *Megastigmus spermotrophus* Wachtl

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Megagametophytes of Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco) accumulated storage products following fertilization. As megagametophytes matured, the number of nuclei per cell rose, resulting in syncytial storage cells. Studies carried out on trees in France and Canada confirmed that such previously unreported, free nuclear cells were a normal part of late megagametophyte development. Unfertilized megagametophytes showed that some binucleate cells before degeneration resulted in empty seed. Insect parasitism prevented megagametophyte abortion in unfertilized ovules. Oviposition by a torymid chalcid wasp (*Megastigmus spermotrophus* Wachtl) early in megagametophyte development resulted in normal megagametophyte development. Around the time of plant egg maturation, binucleate and trinucleate cells were observed. As megagametophytes matured, multinucleate mature storage cells rich in proteins, lipids and starch were formed. The insect was able to induce identical nuclear behaviour in infested, unfertilized megagametophytes, as that of uninfested, fertilized megagametophytes.

## Introduction

Early megagametophyte development of gymnosperms is analogous to endosperm development of angiosperms: both are characterized by an initial multinucleate phase. In gymnosperms, this occurs after meiosis; in angiosperms, it is after double fertilization. Although they differ in the initial events that bring about syncytial development (Raghavan, 2003), the outcome is similar: the initial cell increases in size while nuclei continue to divide in the absence of any cell wall formation. During this period, these cells can reach high ploidy levels. Cell wall formation then ensues, and a storage tissue is formed of uninucleate cells. Later in development, nuclei may exhibit other ploidy phenomena. Endosperms of some plant species have been shown to exhibit endomitosis, nuclear fusion, incomplete division, and other forms of nuclear behaviour that deviate from normal mitotic cycles (for an extensive review see Nagl, 1978). This diversity in nuclear behaviour is thought to reflect the diversity of angiosperms. In comparison, gymnosperms, a smaller and more ancient group, are thought to be more constrained. This may be due to the fewer studies of gymnosperm reproduction development;

recent studies in fertilization, for example, have shown more diversity than previously thought (Friedman and Carmichael, 1996).

Deviations in plant nuclear behaviour can also be insect-induced. The best known examples are galls that form in vegetative tissues of plants in response to insect parasitism. The insect's influence on host tissue may be a result of physical wounding – a consequence of mechanical wounding of the host during oviposition – or it may be biochemical, as is observed in many galls (Rohfritsch, 1992). In a survey of plant nuclear responses in insect-induced galls, Hesse (1971) noted that half of the infested species of plants exhibited changes in nuclear behaviour. Endomitosis accounted for 72% of such responses; the remaining 30% of responses were attributable to a variety of mitotic disruptions, including failure to form cell walls. In the latter case, it resulted in multinucleate cells that had more than a dozen nuclei per cell. Nothing is known about the effect of insects on the nuclear behaviour of the endosperms, or megagametophytes, of infested seeds. For gymnosperms, we were unable to find any cytological studies of the effects of oviposition and larval development on comportment of nuclei.

A group of insects that lends itself to such study is *Megastigmus*, a genus of seed chalcids that has many species-specific interactions with conifers throughout a wide range of families (Roques and Skrzypczynska, 2003). Hussey (1955) speculated that females of *Megastigmus spermotrophus* Wachtl are able to distinguish

fertilized ovules from unpollinated ones in Douglas-fir, but there is evidence that seed chalcids oviposit before plant fertilization takes place, and that larvae were even present in megagametophtyes that developed from unfertilized ovules (Niwa and Overhulser, 1992; Rappaport et al, 1993). They are therefore present in the developing ovule for a long period of time, and exercise an influence on megagametophyte development.

Douglas-fir megagametophyte development was histologically investigated. Unpollinated megagametophytes, which would normally abort, were compared with unpollinated but infested megagametophytes. These were also compared with pollinated megagametophytes, both uninfested and infested. M. spermotrophus induces storage tissue development in unfertilized megagametophytes, which would otherwise degenerate if uninfested. A novel finding was that most storage cells of infested and uninfested pollinated megagametophytes were multinucleate.

## Materials and methods

### Plant material
Douglas-fir trees were sampled from two locations. Clones 3782 and 3788 were selected from the breeding orchard at the Institut National de la Recherche Agronomique (INRA) in Orléans, France. The same experiment was repeated at the British Columbia Ministry of Forests breeding orchard at Puckle Road Research Station (BCMF) in Saanichton. The clones utilized were 63-8L, 101-3L and 134-10L.

### Design
The following four treatments were used: (1) no pollination, no seed chalcid; (2) no pollination, introduction of M. spermotrophus seed chalcids; (3) manual pollination, no seed chalcids; and (4) manual pollination, introduction of M. spermotrophus seed chalcids. Anthesis in Douglas-fir precedes female receptivity, which necessitates emasculation of male cones on branches that have female cones. Branches bearing 3–20 female cones were then bagged to exclude pollen from all sources, wild M. spermotrophus, as well as any other seed predators. For the pollination treatments, a polymix of pollen was applied during the period of female cone receptivity either by brush (INRA) or by syringe injection (BCMF) on half of the bagged female cones. To ensure success, each female cone was pollinated twice. Paper exclusion bags were replaced 2 weeks later with woven bags to prevent overheating.

### Insects
Insects used in the INRA portion of the experiment were raised from infested seed collected from a seed orchard in Lavercantière (Lot, France). In the BCMF portion, the insects were from seed collected in natural population of Douglas-fir of Vancouver Island. The seeds were stored at ambient temperature, thereby synchronizing phenologies of parasite and host. Seed lots were placed at ambient temperature for adult emergence. Males began to emerge 9 days later and females 1 week after males. Adults were collected and transferred for 1 day to plastic boxes that contained a small amount of sugared water on a piece of foam. These boxes were then opened in the

woven bags. A total of 20 couples were introduced in bags with one to five cones and 40 couples when more cones were present in an exclusion bag. It was necessary to monitor female cone development in exclusion bags, as differences in developmental timing between cones were known to arise because of microclimate differences between branches, trees and sites.

### Histology
At INRA, two cones per treatment were harvested every week from late March to mid-July and dissected in the laboratory and selected ovules removed. The collection period included all developmental stages of the female gametophyte. At BCMF, the ovules were removed weekly for the period from cellularization (mid-May) to megagametophyte maturity in early July.

Ovules were processed and stained differently in the two studies. At INRA, material was isolated and fixed in FAA. Samples were washed under clear water overnight. These were dehydrated in an ethanol series, then infiltrated and embedded in paraffin. They were cut into 7 μm sections, affixed to gelatin-coated slides, then dried, dewaxed and stained with either Safranin-Fast Green or IKI. Slides were then dried and mounted.

Cones removed from trees at BCMF were dissected and selected ovules removed. The ovules were fixed in 2.5% gluteraldehyde in 0.075 M phosphate buffer at pH 7.2. The samples were stored at 4°C until they were processed for histochemical analysis. Samples were washed, dehydrated, and then infiltrated with glycol methacrylate (Technovit 7100) and embedded. Sections (5 μm) were cut using a Leica SM 2400 sledge microtome with a tungsten carbide knife. They were stained according to Gutmann's (1995) methods: Ponceau Red 2S-Azure Blue for proteins and cell walls, respectively, IKI for starch, or Toluidine Blue O as a general
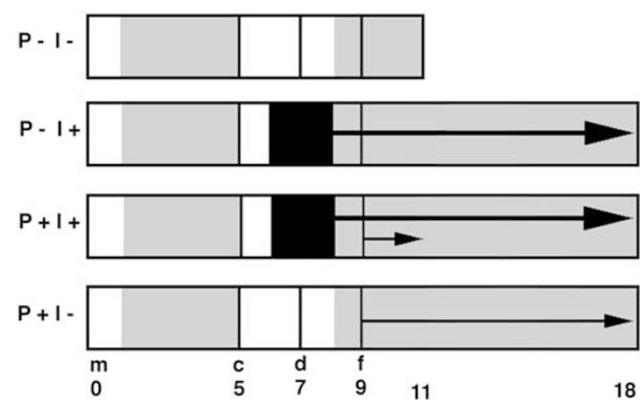


**Figure 1** Schematic time lines (weeks) of megagametophyte development in Pseudotsuga menziesii, Douglas-fir in different treatments. Treatments include unpollinated, uninfested (P−I−), unpollinated, infested (P−I+), pollinated, infested (P+I+), and pollinated, uninfested (P+I−). Stages are indicated in lower case letters: m, meiosis followed by multinuclear stage (gray shading); c, cellularization; d, differentiation of archegonia; f, fully mature, receptive egg. Multinuclear stages (grey) occur during two periods – syncytial megagametophyte development before week 5, and all stages following differentiation of archegonia. Insect oviposition is indicated by the black area; the period of larval development is indicated by the large arrow. The period of plant embryo development is indicated by a smaller black arrow.

metachromatic stain. Some sections were stained with Periodic Acid Schiff's (PAS) procedure for carbohydrates in combination with AnilineBlue Black for proteins (O'Brien and McCully, 1981). For black and white image (Figure 3f), sections were stained with Ponceau 2S-Azure Blue, photographed through a yellow filter to enhance the protein contrast with the background, and then processed through Adobe Photoshop 7 in which red hue and saturation was optimized before the image was converted to black and white.

## Results

Megagametophyte development was similar in all four treatments up until and including egg maturation (Figure 1). The first appearance of multinucleate cells was after cellularization and during archegonial differ-

entiation. Binucleate cells, though infrequent, were found near central cells at the micropylar end of the megagametophyte (Figure 2a). When central cells had further differentiated into eggs, both binucleate and trinucleate cells were found in the surrounding megagametophyte tissue (Figure 2b). After this point, megagametophyte development was treatment dependent.

Unpollinated megagametophytes degenerated approximately 2 weeks after their unfertilized eggs aborted (Figure 1). Degeneration occurred in three stages. At the time of egg death, binucleate cells were found in the surrounding cells at the apical end of the megagametophyte (Figure 2c). Afterwards starch accumulated in the central portion of the megagametophyte (not shown). No protein or lipid was stored. Within a few weeks, the megagametophyte had completely degenerated (Figure 2d).
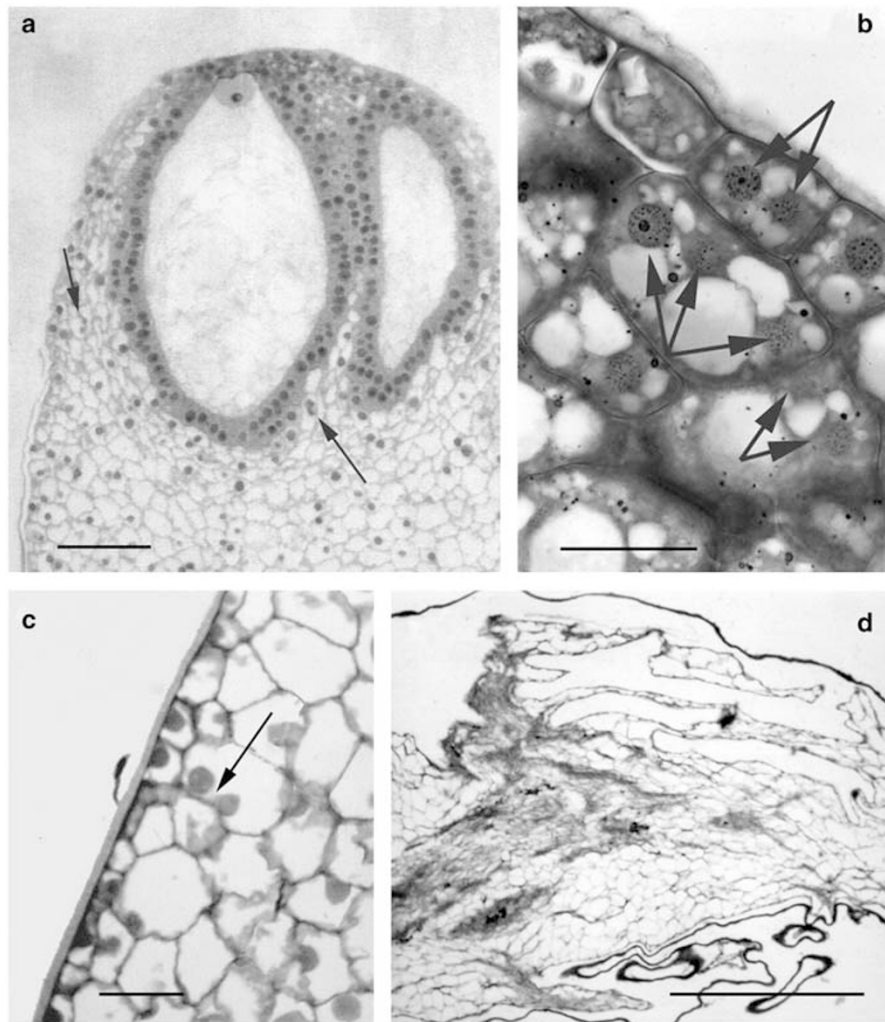


**Figure 2** Megagametophyte development in uninfested ovules. (**a**) Light micrograph of glycol methacrylate-embedded Douglas-fir megagametophyte (7 weeks) from a pollinated, uninfested ovule. The left archegonium of the two prominent archegonia has a large central cell with a very large nucleus within which a dark nucleolus can be seen. Arrows point to binucleate cells. Stained with Aniline Blue Black for proteins and Periodic Acid Schiff's reagent for insoluble carbohydrates. Scale bar = 50 μm. (**b**) Light micrograph of glycol methacrylate-embedded Douglas-fir megagametophyte (8 weeks) from a pollinated, uninfested ovule. Arrows point to nuclei in multinucleate cells at the archegonial end of the megagametophyte. Stained with Toluidine Blue O. Scale bar = 20 μm. (**c**) Light micrograph of glycol methacrylate-embedded Douglas-fir megagametophyte (9-week-old) from an unpollinated, uninfested ovule. A binucleate cell is visible in the centre (arrow). Most cells are uninucleate Stained with Toluidine Blue O. Scale bar = 20 μm. (**d**) Paraffin section of dead, degenerated megagametophyte (16-week-old ovule). Stained with Fast Green. Scale bar = 100 μm.

Following oviposition by *M. spermotrophus* in unpollinated megagametophytes (Figure 3a), larvae hatched and began consuming the central zone (Figure 3b). Eggs were laid in megagametophytes that were differentiating archegonia which would eventually house the plant eggs (Figure 1). Binucleate and, later, trinucleate cells were observed near the micropylar end of the megagametophyte (not shown); this was similar to what was observed in uninfested seed. Infested megagametophytes of unpollinated ovules did not degenerate as would have been expected, but continued to develop. The early instar larva ate cells of the central zone in which starch had accumulated (not shown). These cells were uniformly uninucleate. Those not consumed by the larva broke down not long after filling with starch, forming a space continuous with the space created by the aborted eggs, collectively known as the corrosion cavity (Figure 3c). By this stage, prothallial cells were generally multinucleate (Figures 3d,e). These cells accumulated protein (Figure 3f) and lipid storage reserves (not shown). The nuclei were not spaced around the cell margins, as was the case in the earlier synctial stage, but were either randomly distributed or clustered close to one another in various locations.

Pollinated but infested megagametophytes were fertilized, producing plant embryos that only reached an early stage of development (Figure 4a) before being eaten by the insect. Megagametophytes began to store protein, lipid and starch after week 11. This coincided with an increase in multinucleate cells throughout the megagametophyte (Figure 4b). Nuclei were distributed randomly within such cells. These cells, as well as the developing plant embryo (not shown), were consumed by the insect larva (Figures 1, 4c).

Megagametophytes of pollinated uninfested ovules exhibited bi- and trinucleate cells at the same stage as in other treatments, that is, as archegonia were maturing (Figure 1). The embryo (Figure 4d) was nourished by storage tissue composed of multinucleate cells (Figure 4e).

There were no differences between French and Canadian material at equivalent stages in any of the treatments.
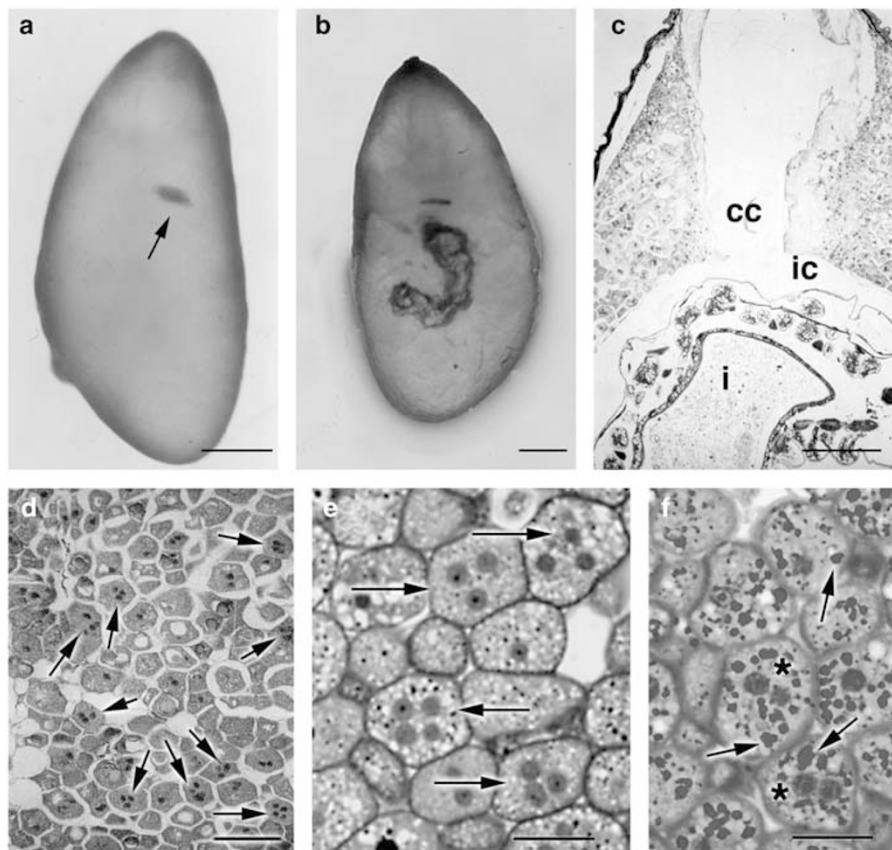


**Figure 3** Megagametophyte development in ovules. (**a**) Light micrograph of megagametophyte (7 weeks). The insect egg (arrow) is located in the central zone. Scale bar = 100 μm. (**b**) Light micrograph of megagametophyte (8 weeks) after pollination. The larva has begun to eat, forming a cavity. Scale bar = 100 μm. (**c**) Paraffin section of insect (i) in unpollinated megagametophyte (13 weeks). Insect cavity (ic) and corrosion cavity (cc) are continuous. Stained with Safranin-Fast Green. Scale bar = 50 μm. (**d**) Paraffin section of storage tissue in infested, unpollinated megagametophyte (13 weeks). Arrows indicate multinucleate cells. Stained with Safranin-Fast Green. Scale bar = 30 μm. (**e**) Light micrograph of glycol methacrylate-embedded Douglas-fir megagametophyte (11 weeks) from an unpollinated, infested ovule. Multinucleate cells are common (arrows). Stained with Ponceau Red 2S-Azure Blue. Scale bar = 20 μm. (**f**) Light micrograph of glycol methacrylate-embedded Douglas-fir megagametophyte (11 weeks) from an unpollinated, infested ovule. Protein bodies (pb) are abundant; three are indicated with arrows. Two binucleate cells (asterisks) are present. Stained with Ponceau Red 2S-Azure Blue: image processed as described in Material and methods. Scale bar = 20 μm.
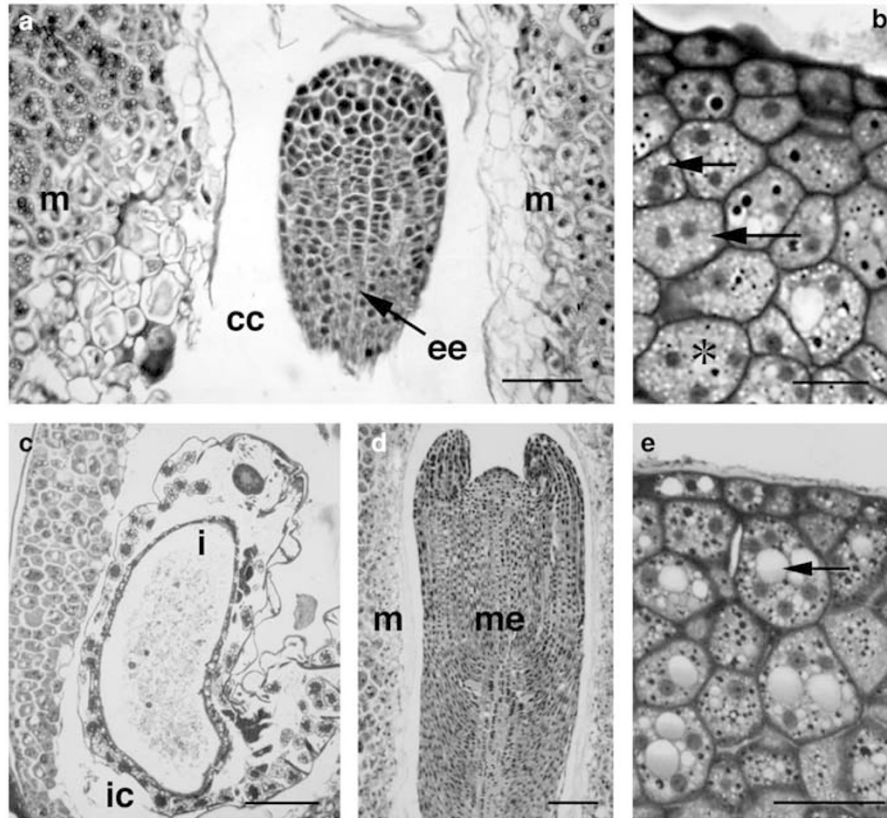
**Figure 4** (**a**) Paraffin section of early stage plant embryo in corrosion cavity (cc) of infested megagametophyte. Stained with Safranin-Fast Green. Scale bar = 50 μm. (**b**) Light micrograph of glycol methacrylate-embedded Douglas-fir megagametophyte (13 weeks) from a pollinated, infested ovule with binucleate and trinucleate cells. Stained with Toluidine Blue O. Scale bar = 20 μm. (**c**) Paraffin section of insect (i) in its cavity (ic) of pollinated infested megagametophyte (15 weeks). Stained with Safranin-Fast Green. Scale bar = 50 μm. (**d**) Paraffin section of plant embryo from a pollinated uninfested megagametophyte (17 weeks). Stained with Safranin-Fast Green. Scale bar = 50 μm. (**e**) Light micrograph of glycol methacrylate-embedded Douglas-fir megagametophyte (11 weeks) from a pollinated, uninfested ovule. Stained with Ponceau Red 2S-Azure Blue or Toluidine Blue O. Arrow points to a cell with five visible nuclei. Below it is a cell with three nuclei. Scale bar = 30 μm.

## Discussion

The results presented here differ in many respects from previously reported incidents of multiple nuclei during embryogeny. Multinucleate cellular development is characteristic of early megagametophyte growth in gymnosperms, such as *Ginkgo*, cycads and conifers. Megagametophyte development first goes through a prolonged syncytial state during which both cell volume and number of nuclei increase. This stage ends with alveolation and cellularization, resulting in megagametophytes uniformly composed of evenly distributed uninucleate cells. In *Ginkgo*, binucleate and multinucleate cells were occasionally found after cellularization, but cells with multiple nuclei were not found in megagametophytes at later stages (Carothers, 1907). Developing megagametophytes of *Cephalotaxus* also exhibited occasional multinucleate cells. Some of these had as many as seven nuclei; these eventually fused to form polyploid masses (Fujita, 1961). As in *Ginkgo*, mature storage tissue was uniformly composed of uninucleate cells, implying that the multinucleate cells were ephemeral. The only conifer megagametophytes that have multinucleate cells at maturity are found in the genus *Podocarpus* (Konar and Oberoi, 1969). An inner

zone of cells with multiple nuclei extends from the micropylar end to the centre.

Megagametophytes are apoptotic in nature, as has been shown in white spruce (He and Kermode, 2003a, b). Whether multiple nuclei formation is part of the process in this or other species has yet to be established. Postfertilization development in many conifers results in starch accumulation in the centre of the megagametophyte, but this breaks down to form a corrosion cavity. In Douglas-fir, these cells are generally uninucleate. It is only as the corrosion cavity expands, as it does during normal seed development, that it begins to incorporate multinucleate cells. This is different in *Podocarpus*, in which the central zone is already multinucleate (Konar and Oberoi, 1969). It would appear that there are evident differences in the only two conifer systems reported to have multinucleate storage cells. This phenomenon of multinucleate cells is likely to be underreported, as past studies of embryo and megagametophytes naturally employed stains that enhanced the abundant storage products, thus masking unstained nuclei.

Gymnosperm megagametophytes are analogous with angiosperm endosperm. The latter exhibit syncytial growth during the earliest stages. Once cellularized, the cells are uninucleate, but are often endopolyploid,

sometimes to a high degree. Restitution mitoses occur rarely; they result in multinucleate cells, the nuclei of which may also be endopolyploid (Nagl 1978). Among gymnosperms, *Ginkgo* (Avanzi and Cionini, 1971), *Cupressus* (Pichot and El Maâtaoui, 1997) and *Sequoia* (Ball, 1978) are known to show varying levels of endopolyploidy. The occurrence of endomitosis has been shown by flow cytometric study of *Cupressus sempervirens* L. While cellular nuclear content of embryos remained undeviatingly diploid, megagametophyte cells showed a range of ploidy levels. These cells had single nuclei with high C values, which was assumed to be the outcome of endopolyploidy (Pichot *et al*, 1998). Other pinaceaeous genera (*Abies*, *Cedrus* and *Pinus*) that have been tested show no endopolyploidy (Pichot and El Maâtaoui, 1997). Experimentally, multiple nuclei can be induced, as Douglas-fir megagametophtyes cultured *in vitro* have neck cells that are binucleate (Ma *et al*, 1998).

According to Brown and Lemmon (2001), syncytial cells characteristically have evenly distributed microtubular networks. In early stage megagametophytes of *Ginkgo biloba*, this results in free nuclei being evenly spaced (Brown *et al*, 2002). This needs to be studied in our system, in which nuclei are not evenly spaced. It may be that syncytial cells formed at maturity and destined to degenerate while releasing their nutrients during embryo growth and germination are different than syncytial cells formed during early gymnosperm megagametophyte development, early embryogenesis of conifers or early angiosperm endosperm development.

Douglas-fir and larch megagametophytes are unusual among conifers as they are able to form mature receptive eggs even in the absence of pollination. Megagametophytes only abort if fertilization, an event 6–10 weeks after pollination, fails to occur, at which point the megagametophyte collapses and its contents are resorbed by the surrounding ovular tissues. It is conceivable that fertilization provides one or more signals that permit further development and/or prevent abortion. In the absence of fertilization further development is impossible, syncytial cells do not occur and the female gametophyte aborts.

The presence of the insect parasite does not appear to interfere with normal megagametophyte development, nor does it induce a gall-like response, in which tissue structure is altered to produce nutrient-rich cells. While the larva develops, the megagametophyte continues to accumulate storage products in spite of its continuous reduction due to insect feeding. The plant responds as if it was feeding its own embryo. The histological study shows that the processes are identical in treated and untreated megagametophtyes. The presence of the insect does not induce a visible wound reaction, which suggests a reduced defense response by the host. However, the insect parasite does prevent normal abortion in unfertilized female gametophytes, co-opting the female conifer reproductive tissue. Discovering the nature of the insect's signal(s) to the unfertilized megagametophyte will be the subject of future work.

## Acknowledgements

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