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Sexual reproduction and crossing barriers in white pines: the case between *Pinus lambertiana* (sugar pine) and *P. monticola* (western white pine)

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Abstract The sexual reproductive process in *Pinus lambertiana* has not been completely described, and previous attempts to generate hybrids with *Pinus monticola* and other North American pines have not been successful. The nature of incompatibility barriers between *P. lambertiana* and *P. monticola* is unknown. This needs to be understood if strategies are to be developed to overcome the said barriers. In this paper, development on interspecific crosses is compared with that on intraspecific crosses on the same parent trees. Pollen grains of both species germinated on the nucellus of both species within a week after pollination. Seed cone receptivity in *P. lambertiana* came approximately 2 weeks after receptivity in *P. monticola*, and this delay was perpetuated throughout ovule development in the first year of the reproductive process. Development of the second-year seed cones proceeded more gradually in *P. lambertiana.* However, seed cones reached maturity only for *P. monticola* x *P. lambertiana*. In both crosses, the barriers to hybridization occurred during the second year of the reproductive process. With the *P. lambertiana* as the seed parent, it was manifested through the failure of the megaspores at the free-nuclear stage to resume development. When *P. monticola* was used as the seed parent, the male and female gametes failed to fuse. Our results clearly show that the barriers to hybridization in these species occur before or at fertilization. However, the exact mechanisms behind these are still unknown. Based on the results of this study, we present several strategies to bypass the developmental barriers and possibly produce hybrid progenies.

Keywords *Pinus lambertiana* · *Pinus monticola* · Crossing barriers

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Introduction

A considerable amount of information is available concerning interspecific hybridization in pines, and numerous examples of naturally occurring hybrids, as well as artificial hybrids, are available [2, 6, 22, 38]. In fact, interspecific hybridization has been explored more intensively in pines than in any other group of plants. Within the genus *Pinus*, the five-needle white pines (subgenus *strobus*) are highly desirable because of their fast growth rate, excellent wood qualities, extensive arboretum collections, and evident need for genetic improvement, especially for rust resistance. The five-needle white pines are also relatively easy to cross with each other with the exception of *Pinus lambertiana* Douglas (sugar pine). *P. lambertiana* is both an important component of ecosystems in California and Oregon and a valuable timber species. All efforts to cross this species with *P. monticola* Douglas ex D. Don (western white pine) and with other North American white pines, as well as with other North American pines have failed [10, 47]. On the other hand, *P. lambertiana* has been successfully crossed with two east Asian white pines, *Pinus armandii* Franchet and *Pinus koraiensis* Siebold & Zuccarini [7–11, 43].

All nine North American white pines are susceptible to blister rust, a disease caused by the introduced pathogen *Cronartium ribicola* J.C. Fischer. There are active resistance breeding programs for *P. lambertiana*, *P. monticola*, and *Pinus strobus* that concern all nine species [40]. Natural resistance in these species is rare, and the number of resistance mechanisms is unknown. For most breeding programs, current emphasis is on intraspecific variation, but options to hybridize white pines may be necessary if insufficient resistance exists within a species. In fact, due to

the low levels of resistance found in *P. strobus*, the merits of hybrid breeding involving *P. strobus* and other white pine species are currently being weighed in Canada (Pengxin Lu, personal communication). Because *P. lambertiana* is a source of a major gene for resistance [23–26] and the durability of resistance in this species to white pine blister rust is unknown, the study of its reproductive biology deserves our attention. In addition, the developmental basis of the incompatibility barriers in crosses involving this species is still unknown. This needs to be understood if strategies are to be developed to overcome the barriers and to take advantage of the genetic potentials locked in *P. lambertiana*.

Prior research has shown that there may be differences between hard and soft pines in the stage where incompatibilities occur in interspecific hybrids. In hard pines [4, 5, 18, 19, 35], pollen grains from one species can usually germinate on the nucellus of another species but are unable to penetrate it. It has been reported that the nucellus wards off the germinating pollen and prevents it from growing any further. Only in the *Pinus elliottii* x *Pinus nigra* cross has it been observed that pollen grains are unable to germinate on the nucellus [35]. Therefore, in hard pines, irrespective of the stage when pollen growth is arrested on the nucellus, the type of incompatibility is still manifested prior to fertilization.

In 1944, Buchholz also introduced the idea that in some soft pines, the crossing barriers took place at or immediately following fertilization. [17] and [29–31] conducted various crosses and concluded that in soft pines, the inability to produce hybrids is due to incompatibility barriers that are manifested after fertilization, such as the failure of the proembryos to continue development or the inviability of the mature embryos.

Despite the apparent contrasting scenarios of hybrid failures in hard and soft pines, we believe that generalizations cannot be made because the developmental basis of the barriers have been examined in only a few crosses in these groups of pines. Unfortunately, no developmental study has been done recently to clarify our understanding of the nature of reproductive barriers in pines. The objectives of this study are to characterize the sexual reproductive process in *P. lambertiana* and to determine the nature of crossing barriers between *P. lambertiana* and *P. monticola* using histological analysis. The results of this study will further our understanding of the reproductive biology of *P. lambertiana* and expand our knowledge of the crossing barriers in soft or white pines.

Materials and methods

Study site, sources of pollen, and pollination

Pollinations were conducted in seed orchards at the Dorena Genetic Resource Center, Cottage Grove, OR from May to June of 2003 and 2004. *P. lambertiana* and *P. monticola* were used in this study. Developing seed cones of both species were bagged, before or just as they began receptivity. Thirty receptive *P. monticola* seed cones (from tree 3141) were pollinated with a mixed lot of *P. lambertiana* pollen grains. The reciprocal cross was made 2 weeks after because of the delay in the receptivity of the *P. lambertiana* seed cones. Thirty receptive *P. lambertiana* seed cones (from tree 4608) were pollinated with a mixed lot of *P. monticola* pollen grains. For the controls, intraspecific pollinations were conducted on 30 *P. monticola* and 24 *P. lambertiana* seed cones using the same mixed pollen lots used in the corresponding interspecific crosses. The pollination bags were removed after 3 weeks. In all the crosses, the species used as the seed parent was always written first and followed by the species used as the pollen donor; this applies to all discussions regarding this in the paper.

One-year-old *P. lambertiana* and *P. monticola* pollen grains that were stored in a freezer (at −20°C) were used in the pollinations. The mixed pollen lots contained pollen grains from three different individuals. The viabilities of the mixed pollen lots of *P. lambertiana* and *P. monticola* were about 95 and 94%, respectively. The pollen viability test used was the aqueous method, where a small amount of pollen (the size of a match head on a spatula) is put into a 5-mm test tube containing 1.5% sucrose solution (w/v). The sampling was placed on a shaker in a growth chamber with the following regimen: 12 h lights on at 22°C, then 12 h lights off at 20°C for 48 h. The samples were mounted on a slide, and the number of germinated and ungerminated pollen grains was determined from 100 counts. This was done five times, and the averaged percentage viability was determined.

Histological analysis

Collections of first-year *P. lambertiana* and *P. monticola* seed cones were done weekly for seven consecutive weeks starting from pollination. Collections of second-year seed cones were done biweekly for four consecutive times (from the first week of April through July 2003). Two seed cones were collected per cross. Immediately upon collection, the seed cones were packed in a Styrofoam box containing ice and sent through an overnight delivery service to the Department of Environmental and Forest Biology, State University of New York College of Environmental Science and Forestry, Syracuse, NY 13210. Immediately upon arrival, 25 randomly chosen cone scales per cross were dissected out from along the middle portion of the cones. This was done to avoid the high rates of abortion typically associated with the ovules in the apical and basal parts of the cone. The samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Pennsylvania). These were rinsed in cacodylate buffer, dehydrated by passing through a graded series of ethanol, transferred gradually into isopropanol, and infiltrated with melted paraffin. Thin sections (approximately 10 μm) were cut using a rotary microtome, mounted on slides and stained with Toluidine Blue O [39].

The sections were examined using a Leica DMLB compound microscope. Representative photomicrographs were taken using a digital camera (Optronics, California). Photomicrographs were taken mostly from the *P. lamberti-*

ana × *P. lambertiana* cross, and a few were taken from the *P. monticola* × *P. lambertiana* cross. The readers are referred to the papers of [3, 37], and [36] for illustrations on the reproductive biology of *P. monticola*.

Seed viability test

Eleven fully matured seed cones from the *P. monticola* × *P. lambertiana* cross yielded 1,596 seeds. These seeds were manually extracted and examined, and their viabilities were determined through x-ray analysis following the protocol of [1]. Seeds from controlled pollinations (*P. monticola* × *P. monticola* and *P. lambertiana* × *P. lambertiana*) were also collected and analyzed as above.

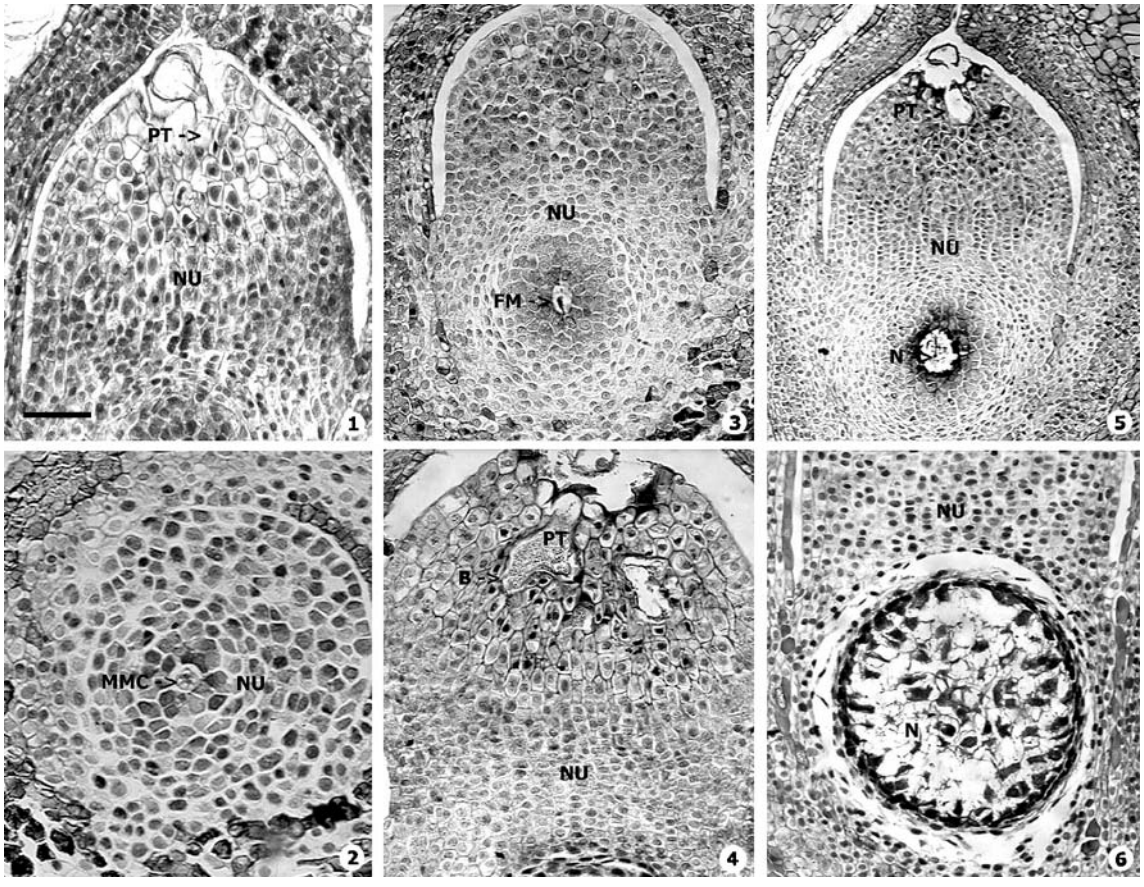
Results

First-year cones

Histological analysis of weekly samples showed that *P. monticola* and *P. lambertiana* pollen grains have germinated on the nucellus of *P. monticola* ovules 1 week after pollination. The receptivity of *P. monticola* seed cones occurred around the first week of June, which was 2 weeks earlier than that of *P. lambertiana* seed cones. In the *P. lambertiana* × *P. lambertiana* and *P. lambertiana* × *P. monticola* crosses, pollen grains from both species have also germinated on the nucellus 1 week after pollination (Fig. 1). At this stage, the ovules in all the four crosses were at the megaspore mother cell stage (Fig. 2), undergoing meiosis, or at the functional megaspore stage (Fig. 3).

Two weeks after pollination, the pollen tubes in all four crosses have elongated to about one third of the length of the nucellus. Analysis of the more advanced seed cones (representing stages 3 to 7 weeks from pollination) showed that the pollen tubes gradually enlarged but remained in this position for the rest of the year (Fig. 4). Branching of the pollen tubes was observed from both crosses involving *P. lambertiana* pollen (Fig. 4). In addition, at this stage, the generative cells and vegetative nuclei have moved out of the pollen grains and occupied the pollen tubes. The ovules in all crosses were mostly at the functional megaspore stage.

Three weeks after pollination, the functional megaspores in all crosses have undergone initial free-nuclear divisions



Figs. 1-6 Reproductive stages in the *P. lambertiana* ovules crossed with *P. lambertiana* pollen grains on the first year of seed cone development. 1. Pollen tube (*PT*) penetrating the nucellus (*NU*) 1 week after pollination (*bar*=50 μm), 2. Megaspore mother cell (*MMC*) within the nucellus 1 week after pollination (*bar*=75 μm), 3. Functional megaspore (*FM*) within the nucellus 1 week after pollination (*bar*=100 μm), 4. Branched (*B*) and enlarged pollen tubes in the nucellus 2 weeks after pollination (*bar*=40 μm), 5. Pollen tube in the nucellus with the corresponding enlarging megaspore with free nuclei (*N*) 3 weeks after pollination (*bar*=100 μm), 6. Enlarged megaspore with free nuclei 7 weeks after pollination (*bar*=50 μm)

accompanied by gradual cell enlargement (Fig. 5). This continued at least up to August (Fig. 6). The seed cones remained dormant at the free-nuclear stage.

Other than the 2-week delay in the development of the seed cones in *P. lambertiana*, as compared with *P. monticola*, no difference was observed in the rates of growth of the pollen tubes on the nucellus of the recipient ovules in any of the interspecific and intraspecific crosses examined. There was also no difference in the development of the megaspores of the recipient ovules in the presence of pollen tubes from any of the four crosses. Pollen tubes were observed in all the ovules that were examined. Furthermore, no abnormality was observed on the development of the seed cones during the first year of the reproductive process.

Second-year cones

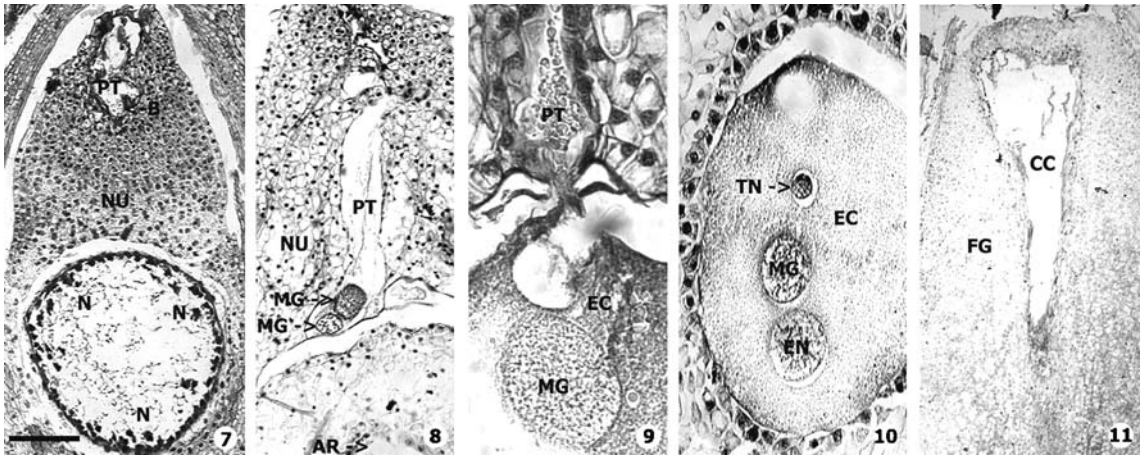
The cross between *P. lambertiana* seed cones and *P. monticola* pollen grains as well as the reciprocal cross resulted in the formation of second-year cones. However, only the *P. monticola* × *P. lambertiana* cross produced mature cones. Approximately 50% of the seed cones from this interspecific cross have aborted, and this figure is similar to that of the intraspecific crosses (data not shown). Therefore, the seed cone abortion observed in this study is likely due to external and internal factors other than an effect of the specific combination of species being examined. To confirm the quality of the pollen grains used in the study, we also determined the percentage of filled or viable seeds. The batch of *P. monticola* and *P. lambertiana* pollen grains used resulted in approximately 63 and 83% viable seeds, respectively.

Histological analysis showed that, in early spring (about the last week of March), the ovules in the *P. monticola* × *P. monticola* and *P. monticola* × *P. lambertiana* crosses were already at a more advanced developmental stage compared with those in the *P. lambertiana* × *P. lambertiana* and *P. lambertiana* × *P. monticola* crosses. In the *P. monticola* × *P. monticola* and *P. monticola* × *P. lambertiana* crosses, the ovules have already formed mature female gametophytes, i.e., cellularized and containing archegonia at the central cell stage. The pollen tubes in both crosses using *P. monticola* as the seed parent were still partway through the nucellus.

In the *P. lambertiana* × *P. lambertiana* and *P. lambertiana* × *P. monticola* crosses, the pollen tubes were also nearly in the same position as last year (Fig. 7). In the intraspecific cross, the ovules were still at the free-nuclear stage, although the megaspores have enlarged, as compared with their size at winter dormancy (Fig. 7). In the interspecific cross, however, all the ovules have collapsed at this stage. This was also already manifested in the seed cones, as these turned brown and shriveled.

By the middle of April, fertilization has passed in the *P. monticola* × *P. monticola* cross, and the ovules already contained four to eight nucleate proembryos. In the *P. monticola* × *P. lambertiana* cross, the ovules were still at the egg stage and most of the pollen tubes have reached the canal cells leading to the archegonia. At this stage, the generative cells have divided and formed two unequal-size male gametes. This usually occurred in the part of the pollen tube between the nucellus and the archegonia (Fig. 8). The bigger male gamete (60 μm long) usually precedes the smaller one (40 μm long). Branching of *P. lambertiana* pollen tubes was more prominent at this stage (Fig. 7).

By the first week of June, proembryos were growing in the corrosion cavities of ovules from the *P. monticola* × *P. monticola* cross. In the *P. monticola* × *P. lambertiana* cross, many of the egg cells have been inseminated (Fig. 9), and the male gametes were still clearly visible in the egg cytoplasm (Fig. 10). However, fertilization has not been observed in any of the samples examined. The unfertilized



Figs. 7-11 Reproductive stages in *P. monticola* ovules crossed with *P. lambertiana* pollen grains on the second year of seed cone development. 7. *P. lambertiana* pollen tube (*PT*) growing in the nucellus (*NU*) of *P. monticola* with the corresponding enlarged megaspore containing free nuclei (*N*) at early spring (*bar*=200 μm), 8. Formation of two unequal-size male gametes (*MG*) in the part of the pollen tube that is at the base of the nucellus and near the archegonia (*AR*) (*bar*=110 μm), 9. Pollen tube that has inseminated the egg cell and showing one of the male gametes (*MG*) inside the egg cytoplasm (*EC*; *bar*=30 μm), 10. Egg cytoplasm containing a pollen tube nucleus (*TN*) and a male gamete (*MG*) near an egg nucleus (*EN*; *bar*=10 μm), and 11. A female gametophyte (*FG*) with a corrosion cavity (*CC*) from an unfertilized ovule (*bar*=250 μm)

egg cells eventually degenerated, but the female gametophyte tissues appeared to be unaffected. The seed cones still remained intact on the trees and did not show any manifestation of the degeneration that occurred in the ovules. Although there were no proembryos formed in the ovules of the *P. monticola* × *P. lambertiana* cross, the female gametophytes still formed corrosion cavities (Fig. 11).

In the *P. lambertiana* × *P. lambertiana* cross, the ovules by the first week of June have formed mature female gametophytes, the pollen tubes have produced male gametes of unequal size, and fertilization has taken place. The total length of time from pollination to fertilization is about 12 months.

Discussion

Sexual reproduction in *P. lambertiana*

The sexual reproductive process in *P. lambertiana* has not yet been completely described. The only histological analysis done on this species is that of [20], where descriptions of the formation of the egg from the central cell, the cytology of the pollen tube as it approaches the archegonia, and the immediate events surrounding fertilization are presented. However, information regarding the stages prior to the above is still lacking. In addition, knowledge of the stages involved during ovule development, corresponding to the stages that are occurring during pollen germination and tube growth on the nucellus from pollination to fertilization, is still not known. These are important in the advancement of our understanding of the reproductive behavior of *P. lambertiana*.

The developmental stages involved in the reproductive process in *P. lambertiana* are generally similar to that in *P. monticola* [3, 36, 37]. However, there are differences in the timing of these stages between the two species. Seed cone receptivity in *P. lambertiana* is 2 weeks delayed compared with that in *P. monticola*. This delay is perpetuated throughout ovule development on the first year of the reproductive process. There are also differences in the timing of development of the second-year seed cones. The ovules in early spring develop gradually in *P. lambertiana* unlike those in *P. monticola*; that is, by about the last week of March, free-nuclear divisions resume and continue for about four and two more weeks in these species, respectively. The timing of fertilization in *P. lambertiana* occurs about 5 weeks after the occurrence of fertilization in *P. monticola*. In total, the length of time involved from pollination to fertilization in *P. lambertiana* is approximately 12 months, which is about 2 months longer than that of *P. monticola*. Therefore, these two species are clearly incompatible based on phenology. There are also a few differences in the features of the reproductive structures between the two species. These include the formation of branched pollen tubes and male gametes of unequal size in *P. lambertiana* but not in *P. monticola*. Our observations for *P. lambertiana* and *P. monticola* are similar to those of [20] and [3], respectively.

Crossing barriers between *P. lambertiana* and *P. monticola*

In pines, lack of pollination or insufficient number of ovules containing germinating pollen results in ovule abortion. This is followed by the falling off of the developing seed cones in just a couple of months, which occurs on the first year of the reproductive process. If sufficient number of ovules contains germinating pollen, the ovules continue to develop and the seed cones remain attached on the tree. Therefore, the growth of pollen tubes in the nucellus is necessary for continued ovule development in pines [35, 41]. Therefore, the presence of seed cones on the tree, particularly on the second year of the reproductive process, has been used as a basis of pollination success.

In hard pines, many interspecific crosses result in the failure of the pollen tubes to penetrate the nucellus, and in a few cases, some pollen tubes penetrate the nucellus, but since these are only few, the impact is not sufficient to induce development of the seed cones [4, 35, 41]. Irrespective of the stage of pollen tube arrest, incompatibility in hard pines is regarded as a prefertilization phenomenon and has been the case in all of the hard pines examined thus far. In soft or white pines, interspecific crosses result in pollen germination and penetration of the nucellus of the recipient ovules. The development of the seed cones and ovules during the first year of the reproductive process is normal regardless of species combination. This has also been observed in several studies using different white pine species combinations ([9, 17, 32, 44]; this paper). Incompatibility in white pines occurs during the development of second-year seed cones. It has been reported that it is manifested through the failure of the proembryos to continue development or through the inviability of the mature embryos [17, 29–31]. However, there are also reports suggesting that this is not entirely the case in other white pines. In the cross between *P. armandii* and *P. monticola*, no seed cone or hybrid seed was produced [2, 47]. In the cross involving *P. monticola* × *P. lambertiana*, consistently higher early seed cone and ovule abortion occurred compared with that in the intraspecific cross; based on this, [9] concluded that the incompatibility between these two species occurs before fertilization. On the other hand, no hybrid seed was produced whether *P. lambertiana* or *P. monticola* was used as the female parent [9]. Our pollination experiments showed that the *P. monticola* × *P. lambertiana* cross resulted in many fully matured seed cones, while no seed cones reached maturity from the reciprocal cross. In both types of pollinations, no hybrid seed was obtained, therefore confirming the findings of [9]. However, our histological analysis showed that in the cross between *P. lambertiana* seed cones and *P. monticola* pollen grains, the barrier to hybridization occurs earlier compared with that in the reciprocal cross. The breakdown is manifested by the failure of the megaspores at the free-nuclear stage to resume development. We believe that this is related to the presence of the incompatible pollen tube on the nucellus. However, the mechanism behind this incompatibility reaction is unknown.

In pines, previous reports on the prefertilization incompatibility reactions from interspecific crosses have centered on the nucellus; either the incompatible pollen grains are unable to germinate on it or it prevents the pollen tubes from growing further. Our report clearly shows that the nucellus allows the incompatible pollen tube to penetrate it. In fact, seed cone development in many interspecific crosses involving various white pine species is normal on the first year of the reproductive process ([9, 17, 32, 44]; this paper). Our report is the first to clearly describe a prefertilization incompatibility reaction that is manifested in the female gametophytic tissues.

Our results also showed that when *P. monticola* seed cones were pollinated with *P. lambertiana* pollen grains, germination occurred and the pollen tube developed partly through the nucellus. There was no difference in the development of the ovules in the *P. monticola* × *P. lambertiana* cross from the intraspecific cross on the first year of the reproductive process. The failure to form hybrids between *P. monticola* seed cones and *P. lambertiana* pollen grains occurs in the spring of the second year. This is due to the inability of the male and female gametes to fuse with each other. This observation supports the report of [4] that incompatibility in some white pines occurs at fertilization. Unfortunately, the mechanism behind this phenomenon is unknown.

Despite the absence of fertilization in the *P. monticola* × *P. lambertiana* cross, the female gametophytes still formed corrosion cavities. Therefore, the presence of corrosion cavities is not necessarily correlated with the occurrence of fertilization or the presence of developing proembryos, contrary to the reports of [17] and [29–31]. The presence of corrosion cavities in the absence of fertilization has also been documented in several studies involving in vitro culture of isolated female gametophytes [13–15].

Our study clearly shows that hybridization barriers between *P. lambertiana* and *P. monticola* occur before or at fertilization. It also validates previous observations of the occurrence of prefertilization incompatibility barriers in white pines [2, 9, 47]. On the other hand, if the presence of corrosion cavity is not used as a basis for the occurrence of fertilization, reports on postfertilization incompatibility barriers in white pines will probably be not as common as previously reported [17, 29–31]. However, since the manifestations of incompatibility reactions depend on the extent of genetic difference between the species being crossed [35], it is very likely that postfertilization barriers do occur in white pines. Therefore, the current generalization of the nature of incompatibility reactions in white pines is no longer appropriate.

Strategies to bypass crossing barriers between *P. lambertiana* and *P. monticola*

The ability to reforest using genetically improved seeds depends on tree improvement programs. These programs rely on breeding technologies devised to capture desirable traits and transmit them to the offspring. Strategies that will allow improvement of genetic stocks beyond the capability of traditional breeding need to be developed if long-term solution is desired. In vitro fertilization (IVF) offers a novel alternative technology which has practical applications in breeding programs such as overcoming prefertilization incompatibility barriers, controlling the production of offspring, and reducing the time required for the development of embryos [14]. This technology also offers the development of new genetic pools for future forest development, provides new genetic combinations for new niche growing areas that are likely to be more prevalent with future climate change, and buffers the forestry industry against diseases.

In many pines, it appears that the most critical period in the development of hybrid seeds occurs before fertilization ([4, 16, 33–35, 42, 46] this paper). It is in cases like these where the reproductive barrier involved in hybridization is a prefertilization event where IVF becomes most applicable [13–15]. IVF in conifers involves the culture of male and female reproductive structures to facilitate the penetration of the egg and eventually the fusion of gametes [14]. Through IVF, species that do not normally hybridize in nature may be hybridized in culture. Therefore, to be able to introduce resistance genes into susceptible white pines, one needs to develop an IVF strategy based on the specific incompatibility barriers involved.

In the *P. lambertiana* × *P. monticola* cross, the failure to produce hybrid seed is due to the abortion of the developing female gametophytes in the early spring of the second year of the reproductive process. Since fully matured female gametophytes of *P. lambertiana* can be isolated from ovules produced through intraspecific pollination, these can be cocultured with *P. monticola* pollen tubes in vitro. Therefore, this IVF strategy allows the reproductive structures of the two species at the ideal developmental stages to come together and possibly yield a hybrid progeny.

In the *P. monticola* × *P. lambertiana* cross, the situation appears to be more complicated since the actual reason for the failure of the male and female gametes to fuse is unknown. However, based on our study, we propose two possible reasons why the egg and sperm of *P. monticola* and *P. lambertiana*, respectively, do not fuse; these include the size of the male gamete fusing with the egg and the timing of fertilization.

In intraspecific crosses, fertilization occurs in about 10 and 12 months in *P. monticola* and *P. lambertiana*, respectively. In the interspecific cross involving *P. monticola* as the seed parent, the male gametes from *P. lambertiana* were delivered into the egg cytoplasm in about 12 months. This means that there was a delay in the receipt of the male gametes in the eggs of *P. monticola*. We believe that this may have affected the physiological condition of the eggs, making them not amenable for fertilization. Therefore, if the barrier between such species combination is due to the asynchrony in the availability of the male and female gametes, then IVF will be a helpful strategy to bring together these two reproductive structures at the stage when both gametes are just initiated and probably most receptive. This can be done by isolating female gametophytes of *P. monticola* obtained from intraspecific pollinated ovules

and introduced to *P. lambertiana* pollen tubes bearing male gametes under culture conditions.

If the reason behind the failure of fertilization is related to the size of the male gamete fusing with the egg nucleus, then this may involve some kind of species-specific recognition mechanisms similar to those reported in animals and lower plants [21, 45]. Therefore, the IVF strategy in this case involves isolating the female gametophyte containing the egg cell with male gametes in it, and then subjecting it to electroporation. Subjecting cells to intense but very short electrical impulses has been shown to cause membranes to fuse, and this was successfully demonstrated in maize [12, 27, 28].

It is possible that gametic incompatibility exists in pines and is due to reasons other than those speculated here. However, the lengthy reproductive process in pines complicates the examination of a possible genetic mechanism that is involved in this process. Therefore, as compared with other possible scenarios, we believe that our suggestions to overcome the crossing barriers in white pines are noteworthy and relatively easy to address experimentally.

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