Development of an In Vitro Technology for White Pine Blister Rust Resistance

Danilo D. Fernando
John N. Owens

Abstract—In spite of the progress made towards isolating blister rust resistant white pines, there is still a threat from the evolution of new pathogenic strains of blister rust in North America and/or introduction of new virulent strains from Asia. Through interspecific hybridization with the most resistant Eurasian white pines, resistance genes may be passed on to North American white pines, and the gene pool for rust resistance in North America diversified. An earlier approach using wide crosses with Eurasian white pines was abandoned because of failure to obtain viable seeds. We believe that wide hybridizations are possible through the removal of the nucellus which is a probable site of incompatibility reactions. This study aims to develop a novel approach to hybridization through in vitro fertilization (IVF). Our work involves co-culture of *Pinus aristata* female gametophytes with *P. monticola* pollen tubes (and vice versa). Female gametophytes were isolated and introduced to pollen tubes grown in culture for 2 to 3 days. Pollen tubes and female gametophytes were then co-cultured for 6 to 10 days. Histological analysis showed that in both types of interspecific crosses, pollen tubes did not only penetrate the female gametophytes through the neck cells of the archegonia, but also release their contents into the egg cytoplasm. The inability to maintain viability of female gametophytes in culture presently precludes successful IVF. Our results on the in vitro interspecific cross between *P. aristata* and *P. strobus* showed the same interaction as the reciprocal cross between *P. aristata* and *P. monticola*.

Key words: in vitro fertilization, *Pinus aristata*, *P. monticola*, *P. strobus*, pollen tube, female gametophyte, interspecific hybridization

Introduction

Blister rust (*Cronartium ribicola*) is one of the most destructive forest pathogens, and it affects all native North American white pines. Infection caused by this fungus results in the formation of large blister-like cankers on branches and the main stem leading to stunted growth and eventually death of trees. This disease has resulted in the significant loss of white pine timber values, but according to Kinloch (2000), the ecological damage may even be worse. Since the unwanted introduction of the fungus to North America in the early 1900s, white pine breeders have been concerned with isolating resistant trees through selection, screening and intraspecific breeding. As a result, blister rust resistant stocks are available (Kinloch and others 1970, Bingham 1983, Kinloch 1992, Blada 1994, Kinloch and others 1999). However, as new pathogenic strains develop and/or new races of wider virulence are reintroduced from Asia (Kinloch and Comstock 1981, MacDonald and others 1984, Kinloch and others 1996, Kinloch and Dupper 1999, Kinloch 2000), the rust problem remains a constant threat. Therefore, it is necessary to develop new strategies that can be incorporated into the current breeding programs to serve as insurance against new or different pathogenic races of blister rust. The need to widen the spectrum of rust resistance is imperative and one such strategy is in vitro fertilization (IVF) coupled with interspecific hybridization and/or genetic transformation (Fernando and others 1998).

The present "resistant" selection process that is underway in North America may not impart long-term resistance. Widening the spectrum of resistance in North American white pines entails interspecific hybridizations with the most resistant Eurasian white pines (Spaulding 1929, Bingham 1972). Interspecific hybridization may not only impart resistance genes, but may also diversify the gene pool for rust resistance. Of the species ranked by Bingham (1972), *Pinus armandii* is considered the most resistant, followed by *P. cembra* and *P. aristata*. These species constitute a repository of resistance genes that seem advisable to exploit in white pine breeding programs. In fact, hybrids have been formed between *P. armandii* and *P. lambertiana* (Stone and Duffield 1950, Heimburger 1972), and *P. cembra* and two of the most susceptible but economically important white pines, *P. monticola* and *P. strobus* (Blada 1994). Unfortunately, *P. armandii* or *P. aristata* crossed with *P. monticola* or *P. strobus* were all unsuccessful (Wright 1959, Patton 1964, Bingham 1972, Bingham 1983). The cross between *P. armandii* and *P. monticola* did not even produce any cone (Wright 1959), and while cones were produced between *P. armandii* and *P. strobus*, no filled seeds developed (Patton 1964).

One of the important features of IVF is its capability to bypass prefertilization incompatibility barriers (Fernando and others 1998), and through IVF, species that do not normally hybridize in nature may be hybridized in culture. The ultimate aim of this project is to develop rust resistant white pines through interspecific hybridization in vitro. Because

In: Sniezko, Richard A.; Samman, Safiya; Schlarbaum, Scott E.; Kriebel, Howard B., eds. 2004. Breeding and genetic resources of five-needle pines: growth, adaptability and pest resistance; 2001 July 23–27; Medford, OR, USA. IUFRO Working Party 2.02.15. Proceedings RMRS-P-32. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station.

Danilo D. Fernando is with the Faculty of Environmental and Forest Biology, SUNY College of Environmental Science and Forestry, 461 Illick Hall, 1 Forestry Drive, Syracuse, NY 13210. E-mail: fernando@esf.edu. Phone: (315) 470-6746. Fax: (315) 470-6934. John N. Owens is with the Centre for Forest Biology, University of Victoria, Victoria, BC V8W 3N5, Canada.

there are no previous works on the culture of reproductive structures of any species of white pine that can be directly used, the current and immediate concerns of this research are basic. What are the nutrient and cultural requirements for growing pollen tubes and female gametophytes of pines in vitro? How long can pollen tubes and female gametophytes remain viable in culture? Will pollen tubes penetrate the archegonia of female gametophytes? Will in vitro fusion occur between two different pine species?

Materials and Methods

Plant Materials

Pollen and seed cones of *P. aristata* were obtained from the University of Victoria, British Columbia, while pollen and seed cones of *P. monticola* were obtained from Saanich Seed Orchard, Saanich, British Columbia. All crosses involving female gametophytes of *P. aristata* and *P. monticola* were done at the Centre for Forest Biology, University of Victoria, Victoria, British Columbia, Canada.

Pollen cones of *P. strobus* were collected from the SUNY-ESF Lafayette Experimental Station, Syracuse, New York, while seed cones were obtained from the SUNY-ESF Heiberg Memorial Forest, Tully, New York. Crosses involving female gametophytes of *P. strobus* were done at the Department of Environmental and Forest Biology, SUNY College of Environmental Science and Forestry, Syracuse, New York USA.

Surface Sterilization of Pollen and Seed Cones

Pollen cones of *Pinus aristata*, *P. monticola* and *P. strobus* were collected 2 to 3 days before dehiscence while seed cones were collected at central cell stage (Fernando and others 1997). Pollen and seed cones were surface-sterilized by washing in 70 percent ethanol, sterile distilled water, and 1 percent sodium hypochlorite for 30 seconds each step. They were rinsed three times with sterile distilled water for 10 seconds each time, blotted dry on sterile paper towels, and left in Petri dishes covered with sterile filter paper for 48 to 72 hours at 27°C. Dried sterile pollen grains were collected in sterile vials and stored at 4 °C for short-term or –20 °C for long-term storage.

After surface sterilization of seed cones, ovuliferous scales were separated individually using sterile forceps. Ovules were dissected under a stereomicroscope, and the female gametophytes were mechanically isolated and placed in culture. Representative female gametophytes from each seed cone used in culture were fixed in formalin-acetic-alcohol. These were used to monitor the initial stage of development and also serve as the control.

Media Composition and Co-Culture Conditions

The basal medium contained macro- and micronutrients and vitamins as described by Murashige and Skoog (1962), supplemented with boric acid and calcium nitrate following Brewbaker and Kwack (1963). The working solution was half-strength diluted with deionized distilled water, and supplemented with 15 percent sucrose and 0.4 percent phytagel. The pH was adjusted to 6.0 with KOH. This medium is referred to as MSBK.

Pollen grains were grown on MSBK and after 2 to 3 days, freshly isolated female gametophytes were introduced at the tips of growing pollen tubes. Viability of pollen tubes (table 1) was based on whether they had collapsed or not, while viability of female gametophytes (table 2) was based on whether the central cell had undergone plasmolysis or not (Fernando and others 1997). The co-cultures were incubated in the dark at 23 °C. Several intraspecific and interspecific crosses were done and a total of 1,200 female gametophytes were used (table 3).

Histological Analysis

Pollen grains and tubes were examined at various stages of development by fixing in 4 percent paraformaldehyde in saline phosphate buffer and staining with DAPI (4',6-diamidino-2-phenylindole). The specimens were examined using a Leica DMLB fluorescence microscope. A total of 1,200 female gametophytes were co-cultured with pollen tubes. After the co-cultures were incubated for 6 to 10 days, female gametophytes which when lifted, had firmly attached pollen tubes were fixed in 4 percent glutaraldehyde in phosphate buffer. Specimens were rinsed with phosphate buffer and dehydrated through a graded series of ethanol.

Table 1—Length and longevity of pollen tubes in culture (n = 50).

Species	Days in culture	Length of pollen tubes (μm)	Viability (%)
		Mean (range)	
P. aristata	15	650 (530-850)	100
	20	750 (690-980)	100
	30	920 (840-1090)	98
P. monticola	15	600 (460-800)	100
	20	760 (540-920)	98
	30	800 (630-990)	95
P. strobus	15	580 (340-700)	95
	20	620 (440-880)	88
	30	770 (650-910)	85

Table 2—Viability of female gametophytes in culture (n = 100).

Number of days in culture	Number of viable female gametophytes		
	P. aristata	*P. monticola*	*P. strobus*
2	92	85	70
4	70	61	54
6	58	49	37
8	34	23	12
10	19	12	05

Table 3—Intraspecific and interspecific crosses in vitro (n = 200).

Pollen tubes	Female gametophytes		
	P. aristata	*P. monticola*	*P. strobus*
P. aristata	x	x	x
P. monticola	x	x	-
P. strobus	-	-	x

x indicates intraspecific or interspecific cross; - indicates no cross was made

Specimens were gradually infiltrated with a solution containing hydroxyethyl methacrylate (Technovit 7100 embedding kit, Energy Beam Sciences Inc., MA). Sections (8 to 10 µm) were cut using a JB4 ultramicrotome, mounted on glass slides and stained with Toluidine Blue O. Specimens were examined under a brightfield microscope and images captured using a digital video camera (Optronics, CA).

Results and Discussion

Viability and Longevity of Pollen Tubes and Female Gametophytes

Percentage pollen viability in *P. aristata*, *P. monticola* and *P. strobus* were very high (table 1). Growth of pollen tubes was maintained in vitro for 30 days with at least 85 percent viability (table 1). Of the three species of pine examined, *P. aristata* appears to be the most vigorous because of relatively greater longevity and length of pollen tubes. Our results also show that pollen tubes of all three species continue to elongate under in vitro conditions. This shows that in vitro, there is no stage that corresponds to the resting stage that occurs in vivo (Gifford and Foster 1989). Under our in vitro conditions, the average length of pollen tubes (table 1) attained by all three species of white pines (that is, 830 mm) is much longer than the average length of the nucellus (that is, 700 mm) that the pollen tubes traverse prior to reaching the archegonia in vivo.

The longevity of pollen tubes in culture makes them suitable as targets for genetic transformation. In fact, pollen grains are natural vectors for delivering foreign DNA since they are involved in the normal fertilization process. Transformed pollen grains are being used to artificially pollinate flowers in several plants and these have resulted in the formation and recovery of transgenic progenies (Häggman and others 1997, Aronen and others 1998). This technique is very promising because it avoids the use of elaborate and time-consuming tissue culture steps. In white pines, the protocols for the transformation of pollen grains and tubes have already been optimized for *P. aristata* and *P. monticola* (Fernando and others 2000). There are several broad-spectrum pathogenesis related genes that are available for flowering plants (Shewry and Lucas 1997, Osusky and others 2000, Powell and others 2000), and these need to be tested in white pines.

In culture, the viability of female gametophytes declined very rapidly reaching very low numbers after 10 days in culture (table 2). The decline in the viability of female gametophytes in *P. aristata* appears less drastic when compared to those of *P. monticola* or *P. strobus* (table 2). It has long been known that unlike some other conifers, unpollinated ovules in pine do not develop into maturity. In vivo, development of pine ovules proceeds only in the presence of germinated pollen (McWilliam 1959). This suggests that the presence of developing pollen tubes on the nucellus provides some stimulatory factors that are required for the maturity of the female gametophytes. Apparently, the co-culture of pollen tubes and female gametophytes does not have the same effect. It will be interesting to find out if pollen tube extracts added to the culture medium will improve the response of female gametophytes in culture.

Interactions Between Pollen Tubes and Female Gametophytes

When freshly isolated female gametophytes of *P. monticola* were co-cultured with 2 to 3 day old *P. aristata* pollen tubes (and vice versa), the pollen tubes continued to elongate resulting in the penetration of the female gametophytes. In several instances, pollen tubes of *P. aristata* entered the canal leading to the neck cells of the archegonia in *P. monticola* (fig. 1). This is similar to what has been reported to happen in nature under intraspecific crosses (Owens and Morris 1990). This type of penetration was also observed between *P. monticola* pollen tubes and *P. aristata* female gametophytes. In both types of interspecific crosses, some pollen tubes also penetrated the female gametophytes through the prothallial cells far from the neck cells of the archegonia (fig. 2). Only in one instance was a pollen tube observed to reach the neck cells of viable female gametophytes (fig. 3).

It is interesting to note that in vitro, pollen tubes formed minute projections to penetrate between neck cells (fig. 4), as

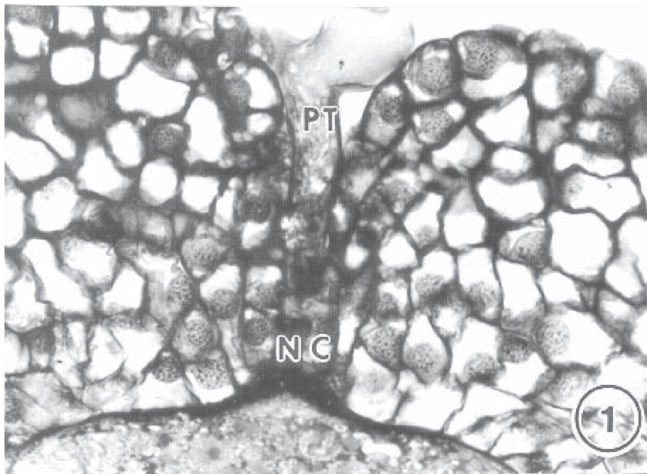

Figure 1—Pollen tube penetrating female gametophyte through neck cells. Figures 1-5 – Acronyms are: EC egg cell, EN egg nucleus, FG female gametophyte, NC neck cells, PC prothallial cells, PT pollen tube, SC starch grains.

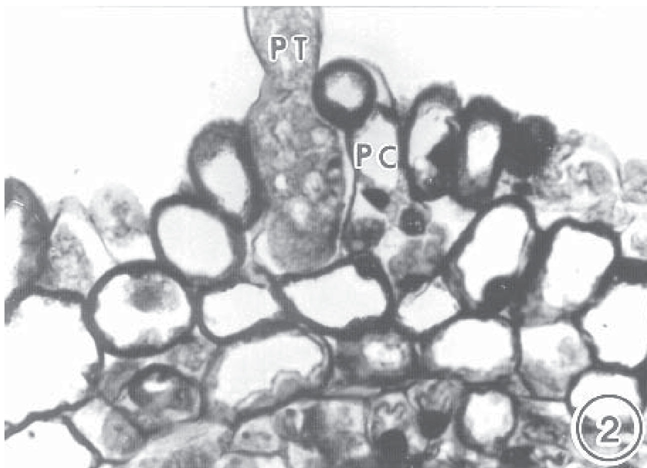

Figure 2—Pollen tube penetrating prothallial cells of female gametophyte.

happens when pollen tubes make contact with the neck cells in vivo (Owens and Morris 1990). In culture, however, formation of minute projections appeared to form not only from the tips of pollen tubes but also from the lateral walls as seen in *P. aristata*.

During co-culture, the elongating pollen tubes could have all passed under or over the female gametophytes, but instead many penetrated the archegonia through the neck cells. This suggests that some sort of cellular recognition do exists under in vitro conditions. Furthermore, some pollen tubes that penetrated the archegonia released their contents into the egg cytoplasm (fig. 5). This suggests not only that in vitro pollen tubes recognize their target destination,

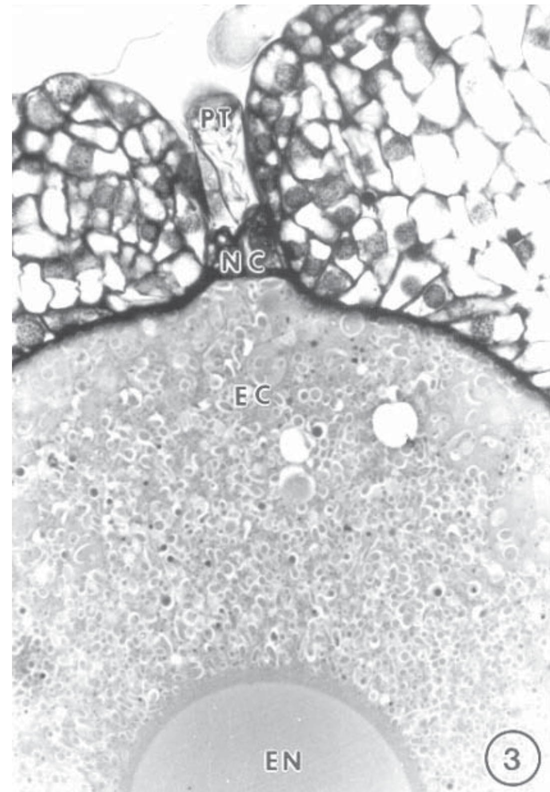

Figure 3—Female gametophyte with pollen tube and unplasmolyzed egg.

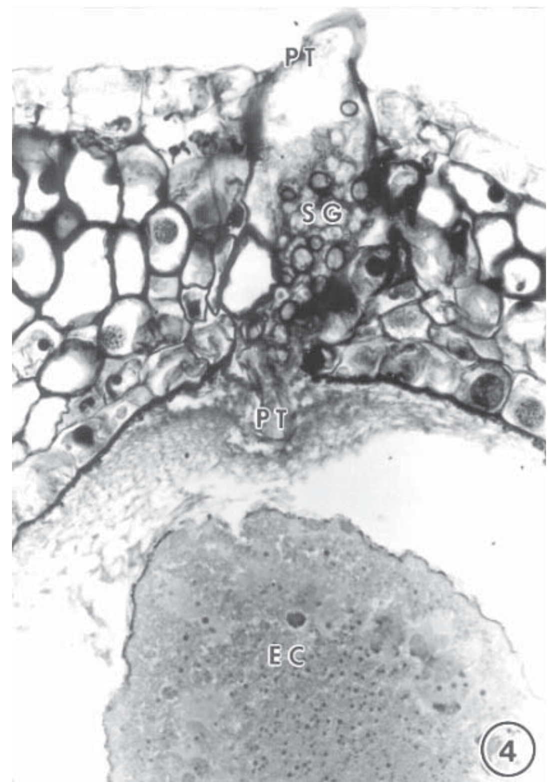

Figure 4—Pollen tube tip inside plasmolyzed egg cell; pollen tube containing starch grains.

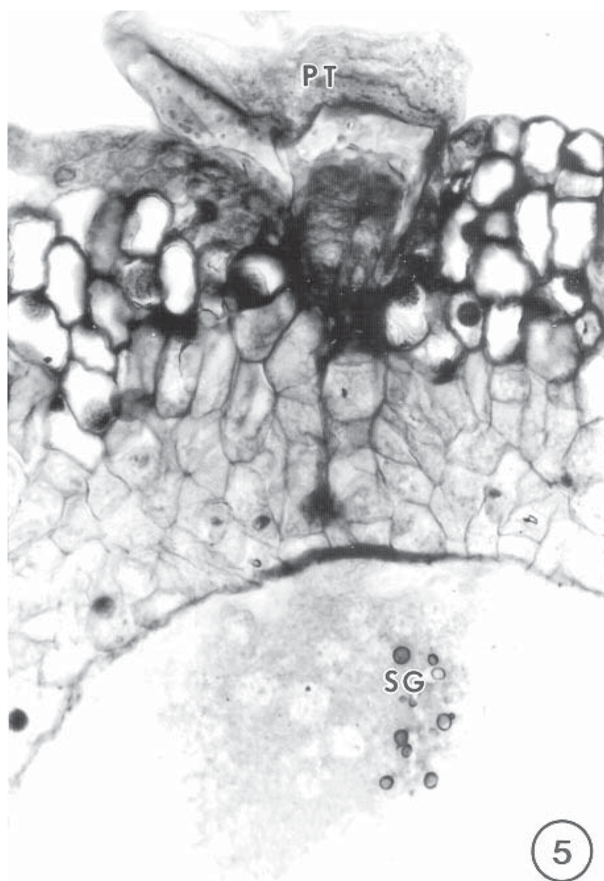

Figure 5—Contents of pollen tube such as starch grains are in the egg cytoplasm.

but they also react in the same way as in vivo. None of the pollen tubes that penetrated the prothallial cells of the female gametophytes released their contents.

Summary

Our results show that our current culture conditions are optimum for growth and development of *P. aristata*, *P. monticola*, and *P. strobus* pollen tubes. The activities of pollen tubes as they penetrate the archegonia of the female gametophytes in culture resemble those that have been reported to occur in vivo. Our results on histological analysis show similar pattern for intraspecific and interspecific crosses (table 3). It is also important to note that the activities of pollen tubes are not hindered by the source of the co-cultured female gametophytes, suggesting that in vitro, no incompatibility reaction is manifested.

Sustaining growth and development of female gametophytes in culture is extremely difficult. Although we have tried different media and supplements without success (unpublished data), there are still countless options to try. Because the culture medium is not optimized for female gametophyte development, no interaction occurred after pollen tube penetration and release of gametes into the egg cytoplasm. Therefore, there is a need to develop a culture medium that is suitable to sustain growth and development of female gametophytes, and at the same time allow the sperm cells that are released in the egg cytoplasm to fuse with the egg nucleus and develop into embryo.

Although in vitro fertilization was not achieved, our results are promising. If we succeed in sustaining the growth of female gametophytes in culture, this IVF technology can offer several novel alternative approaches such as interspecific hybridization and imparting resistance genes into pollen tubes or archegonia followed by IVF. Another benefit of this work applies to all pines and their breeding system. The normal life cycle from pollination to mature embryos takes about 15 months. Through IVF, the time from "pollination" to development of mature embryos could be shortened to 3 to 4 months.

References

Aronen, T.S., Nikkanen, T.O., Häggman, H.M. 1998. Compatibility of different pollination techniques with microprojectile bombardment of Norway spruce and Scots pine pollen. Can J. For. Res. 28:79-86.

Bingham, R.T. 1972. Taxonomy, crossability and relative blister rust resistance of 5-needled white pines, pages 271-280. *In*: Biology of Rust Resistance in Forest Trees, Bingham, R.T., Hoff, R.J. and McDonald, G.I. Miscellaneous Publication No 1221. USDA, Washington DC.

Bingham, R.T. 1983. Blister rust resistant western white pine for the inland empire: the story of the first 25 years of the research and development program. USDA General Tech Rep INT-146.

Blada, I. 1994. Interspecific hybridization of Swiss stone pine (*Pinus cembra* L.). Silvae Genet. 43:14-20.

Fernando, D.D., Owens, J.N. and Misra S. 2000. Transient gene expression in pine pollen tubes following particle bombardment. Plant Cell Rep. 19:224-228.

Fernando, D.D., Owens, J.N. and von Aderkas, P. 1998. In vitro fertilization from co-cultured pollen tubes and female gametophytes of Douglas fir (*Pseudotsuga menziesii*). Theor. Appl. Genet. 96:1057-1063.

Fernando, D.D., Owens, J.N., von Aderkas, P. and Takaso, T. 1997. In vitro pollen tube growth and penetration of female gametophyte in Douglas fir (*Pseudotsuga menziesii*). Sex. Plant Reprod. 10:209-216.

Gifford, E.M. and Foster, A.S. 1989. Morphology and Evolution of Vascular Plants. W.H. Freeman Company, NY.

Häggman, H.M., Aronen, T.S. and Nikkanen, T.O. 1997. Gene transfer by particle bombardment to Norway spruce and Scots pine pollen. Can. J. For. Res. 27:928-935.

Heimburger C. 1972. Breeding of white pine for resistance to blister rust at the interspecies level, pages 541-549. *In*: Biology of Rust Resistance in Forest Trees, Bingham, R.T., Hoff, R.J. and McDonald, G.I. Miscellaneous Publication No 1221. USDA, Washington DC.

Kinloch, B.B. Jr. 1992. Distribution and frequency of a gene for resistance to white pine blister rust in natural populations of sugar pine. Can. J. Bot. 70:1319-1323.

Kinloch, B.B. Jr. and Comstock, M. 1981. Race of *Cronartium ribicola* virulent to major gene resistance in sugar pine. Plant Dis. Rep. 65:604-605.

Kinloch, B.B. Jr. and Dupper, D.E. 1999. Evidence of cytoplasmic inheritance of virulence in *Cronartium ribicola* to major gene resistance in sugar pine. Phytopathology 89:192-196.

Kinloch, B.B. Jr., Davis, D. and Dupper, G.E. 1996. Variation in virulence in *Cronartiumribicola*: What is the threat? pages 133-136. In: Kinloch, B.B. Jr., Marosy, M. and Huddleston, M.E. Proceedings of a Symposium presented by the California Sugar Pine Management Committee: Sugar pine: status, values and roles in ecosystems. Publication No. 3362, University of California, Division of Agriculture and Natural Resources, Davis, California.

Kinloch, B.B. Jr., Parks, G.K. and Fowler, C.W. 1970. White pine blister rust: simply inherited resistance in sugar pine. Science 167:193-195.

Kinloch, B.B. Jr., Sniezko, R.A., Barnes, G.D. and Greathouse, T.E. 1999. A major gene for resistance to white pine blister rust in western white pine from the western cascade range. Phytopathology 89:861-867.

McDonald, G.I., Hansen, E.M., Osterhaus, C.A. and Samman, S. 1984. Initial characterization of a new strain of *Cronartium ribicola* from the Cascade Mountains of Oregon. Plant Dis. 68:800-804.

Osusky, M., Zhou, G.Q., Hancock, R.E.W., Kay, W.W. and Misra, S. 2000. Transgenic potatoes expressing cationic peptides confers broad-spectrum resistance to phytopathogens. Nature Biotech. 18:1162-1166.

Owens, J.N. and Morris, S.J. 1990. Cytoplasmic basis for cytoplasmic inheritance in *Pseudotsuga menziesii*. II. Pollen tube and archegonial development. Amer. J. Bot. 78:1515-1527.

Patton, R.F. 1964. Interspecific hybridization in breeding for white pine blister rust resistance. 367-376.

Powell, W.A., Catranis, C.M. and Maynard, C.A. 2000. Design of self-processing antimicrobial peptides for plant protection. Let. Appl. Microbio. 31:163-168.

Shewry, P.R. and Lucas, J.A. 1997. Plant proteins that confer resistance to pests and pathogens. Adv. Bot. Res. 26:135-192.

Spaulding, P. 1929. White pine blister rust: a comparison of European with North American conditions: USDA Technical Bulletin 87.

Stone, E.C. and Duffield, J.W. 1950. Hybrids of sugar pine by embryo culture. J. Forestry 48:200-201.

Wright, J.W. 1959. Species hybridization in the white pines. Forest Science 5:210-222.