Improving the resistance of eastern white pine to white pine blister rust disease☆

Carolyn C. Pikea,⁎, Paul Berrangb, Scott Rogersb, Andy Davidc, Carrie Sweeneyb, Julie Hendricksonc

a USDA Forest Service, Northeastern Area, 715 W. State Street, West Lafayette, IN 47907, United States
b USDA Forest Service, Eastern Region, Milwaukee, WI, United States
c University of Minnesota, Department of Forest Resources, St Paul, MN, United States

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A B S T R A C T

Eastern white pine (EWP), Pinus strobus L., is an iconic forest tree in the north woods of eastern North America. White pine blister rust, caused by Cronartium ribicola, an invasive pathogen, entered North America in the early 20th century and infected all five-needled pines across the continent. Few genotypes of eastern white pine have demonstrated consistent, elevated resistance to the pathogen, so our objective was to identify additional genotypes with resistance. Since 1970, the USDA Forest Service has identified and grafted over 800 phenotypic plus tree selections from Michigan, Wisconsin and Minnesota. A protocol to artificially inoculate one-year old seedlings was used to screen 228 genotypes along with rust-resistant and susceptible standards across a four-year period, from 2010 to 2013. We identified 25 genotypes, based on survival assessed two years after inoculation, which exceeded the resistant standard. These genotypes will be grafted into new seed orchards to aid reforestation efforts aimed at restoring this keystone species to suitable habitats where regeneration is currently hindered by blister rust on privately-held land, state forests and national forests in the Lake States region.

1. Introduction

Eastern white pine, Pinus strobus, (EWP) is an iconic tree in North America, highly prized by European settlers for its use as ship masts and building material (Albion, 1926), and is recognized today as a keystone species in the eastern forest (Wendell and Smith, 1990). Large-scale exploitation of the species commenced in the late 1800s and early 1900s, and continued until wood resources were all but exhausted. Unfortunately, the white pine blister rust-causing pathogen Cronartium ribicola J.C. Fisch. entered North America on nursery stock shipped from Europe in the early 1900s, causing extensive mortality to regenerating white pine forests. The first known introduction on the east coast was noted in Geneva, NY in 1906 (Hummer, 2000), and later transport of infected nursery stock helped disperse blister rust across the region. In the span of a few decades, blister rust became a significant problem of all five-needled pines across North America (Maloy, 1997). In Minnesota, the combination of blister rust and herbivory, especially by white tailed deer (Odocoileus virginianus), resulted in decreased seedling survival, and a more simplified stand composition as the age-class distribution has skewed towards older, 100+ year trees (Vanderschaaf and Vongroven, 2013; White, 2012).

Blisters rust has a complex life cycle that requires an alternate host, most commonly currant and gooseberry belonging to the Ribes genus, although other plants function as alternate hosts as well (Kaitera and Hiltunen, 2012). In the fall, basidiospores disperse from Ribes leaves to pine needles, a process that is enhanced when temperatures are cool and relative humidity is high (15.5 °C, 100% Relative Humidity). In North America, blister rust rapidly spread on native and cultivated Ribes spp., which were relatively common on the landscape. Many of the northern forests, where EWP is a dominant or co-dominant species and native Ribes are a common understory plant, are replete with lakes and topographical features that enhance humidity levels and provide convective surfaces that favor the fungus’ propagation and spread. Cultivated Ribes nigrum is an epidemiologically important host in North America for blister rust (Van Arsdel et al., 2006), but has largely disappeared from the social and cultural ethos since it was connected with this pathogen. Extensive removal of native Ribes using mechanical, and later chemical methods (Martin and Spaulding, 1949) was attempted to...
reduce the inoculum across a large swath of the eastern forests in the early 20th century. The impact of *Ribes* removal is not known, but one study suggested that survival of EWP in the decade after the arrival of blister rust was likely enhanced by the removal of *Ribes* in combination with other strategies (Stewart and Ritter, 1962; Van Arsdel et al., 2006).

After blister rust swept through North America, forests across the US and Canada were scoured in search of surviving trees to initiate a breeding program. These surviving trees were propagated by grafting into clone banks and seed orchards for federal, state and provincial governments in the United States and Canada. The Hartley plantation in Duluth, Minnesota was planted in 1914 with bare root nursery stock from unknown origin and continually monitored in a quest to locate trees with resistance. The first two infected pines were noted in 1927, and by the 1930s nearly 98% of the planting was infected, averaging over four infections per tree (Ritter, 1930). Two surviving trees were identified as having above-average resistance, bearing the namesake of Robert Patton (University of Wisconsin Madison): P-327 and P-312. Offspring from P-327 have been used in screening programs as a resistant standard demonstrating consistent, above-average levels of resistance in field and laboratory studies.

The largest known collection of seed sources selected for potential resistance to EWP was established in 1972–1974 near Tofte, Minnesota USA (47.57° N, 90.83° W). This site is located approximately six miles inland from Lake Superior in an area designated as being at high-hazard for blister rust (Van Arsdel, 1961; White et al., 2002). Over 40,000 open-pollinated tree seedlings were planted from over 800 mature mother trees from the forests in northern Minnesota (Ahlgren, 1979; Merrill et al., 1986). Each family was represented by five ten-tree row plots for a total of 50 progeny per mother tree at the site. Trees were planted at a roughly five foot (1.5 m) spacing, with a mixture of *Ribes* spp interplanted between rows to enhance exposure to the pathogen. By 1984, 97–99% of the seedlings were infected with rust. Differences in survival among families were significant, but rates of non-infection (number of trees that lacked any sign of the pathogen) were not significantly different among families (Merrill et al., 1986). In 1993, over 800 surviving trees selected as having no visible symptoms were field-tagged, and incorporated into a database. In 2015, fewer than 2000 live trees remained and no single family had more than five of its original 50 trees surviving.

Screening under controlled conditions is desirable to remove unwanted environmental variation that can mask gene expression for traits of interest. Artificial screening for resistance to blister rust entails cultivating and infecting *Ribes* leaves and infecting cultured white pine seedlings in an inoculation chamber or a nursery setting (Ahlgren, 1955; Riker et al., 1943; Riker and Patton, 1961). Needle lesions (spots), the first sign of infection, can be observed several months after inoculation. In side-by-side trials of EWP with western white pine (*Pinus monticola* Doug. ex D. Donn.) and sugar pine (*P. lambertiana* Douglas), EWP averaged 36 spots per tree compared to 34 and 25 for western white pine and sugar pine, respectively (Sniezko et al., 2008), >90% of seedlings for all three species exhibited needle lesions. Infection severity is usually assessed by observing stem infections as a binary trait (infected vs not), or expressed as the percent of trees per family with early stem symptoms (after one year) or later (3 years or beyond) (Sniezko and Kegley, 2003). Screening for resistance in EWP has proven challenging, producing either too much infection from over-inoculating seedlings (Cliff and Isabel Ahlgren, personal communication), or inconsistent infection from cultural practices that failed to synchronize the plants’ stomatal apertures with spore dispersal through inoculation (early efforts at USDA Forest Service). Programs that were successful in screening EWP found that no single genotype was fully immune, and that resistance occurred at a relatively low frequency in the population: in the aforementioned Tofte planting and at nursery experiments in Wisconsin fewer than 1% of one-year old seedlings expressed resistance (Merrill et al., 1986; Riker and Patton, 1961). In Ontario, scientists failed to identify alleles that conferred any meaningful resistance, leading them to establish a breeding program to hybridize EWP with Asian pine (*L. and Derbowka, 2009; Lu and Sinclair, 2006*). Efforts to improve rust resistance in EWP in the US have failed to identify additional genotypes emerged with phenotypic resistance equivalent to P-327.

Resistance to blister rust in EWP is assumed to be largely polygenic (Smith et al., 2006a, 2006b; Zambino and McDonald 2003), which means that resistance results from certain combinations of alleles at many gene loci, complicating efforts to improve resistance through traditional breeding methods. Polygenic resistance exists in other five-needled pines including sugar pine (*Kinloch et al., 2008*) and in populations western white pine (*Bingham et al., 1959*). Acquisition of resistance in a breeding program, for instances where traits are polygenic, may entail inheritance of resistance genes from both parents. In wind-pollinated conifers, this may be achieved through controlled pollinations, or by sequestering seed orchards from susceptible pollen donors.

A number of mechanisms are associated with resistance in EWP, P-327, including a hypersensitive reaction (Jurgens et al., 2003), proteins that may confer disease resistance (Smith et al., 2006b), and occlusions of the stomata and/or biochemical characteristics of wax that may inhibit or repel fungal hyphae (Smith et al., 2006a). Progeny from EWP families selected as having putative resistance did not match the elevated resistance of selected western white pine families (Sniezko et al., 2008), leading to questions about the effectiveness of artificial screening. This new protocol developed for EWP has many similarities to western screening programs (Zambino, personal communication), and could provide a new opportunity to test the hypothesis that EWP families possess resistance that is heritable using artificial inoculations. The objective of the screening described here is to identify additional genotypes, also referred to as families, whose progeny (young seedlings) express resistance to white pine blister rust that exceeds P-327. Our long-term goal is to select a genetically diverse population of superior genotypes to plant in seed orchards as a seed source to reforest areas where blister rust remains an impediment to regeneration of EWP.

2. Materials and methods

Material for inoculation originated from a clone bank based at the USDA Forest Service’s Oconto River Seed Orchard (ORSO), at 45°12’21.0”N 88°40’16.8”W in central Wisconsin, and from Tofte Minnesota (described earlier). The clone bank at ORSO includes a genetically diverse population of EWP of over 800 different genotypes from across the eastern US and Canada. Selections chosen for blister rust screening originated from the Lake States region only, and were selected if they were free of blister rust in a forest where blister rust infections were noted. Mother trees originated from two National Forests: the Chippewa National Forest (NF) in north central Minnesota (designated C-) and Superior NF in northeast Minnesota (S-). Selections were also acquired from the resistance program in Ontario (ON-), and from the state of Minnesota on land not affiliated with a national forest (ML-). Information on the locations of mother trees is maintained at the National Forest System but is not described further. Half-sibs for screening were derived from naturally open-pollinated collections at Tofte, open-pollinations that occurred in the wild of clones replicated at ORSO, or from mass-control pollination using a multi-genotype pollen mix (20–40 genotypes) of Minnesota-origin collected at ORSO. Each of four trials is described, herein referred to by the year that pine seedlings were germinated (2010, 2011, 2012, 2013). In the 2011 trial, twenty-two mother trees were each crossed with P-327, H-111, or were open-pollinated so that each family was represented by two full-sibs and one half-sib, resulting in 68 families. In 2010, 2012, and 2013, 26, 64, and 70 open-pollinated families were tested, respectively (Table 1) for each trial. Open-pollinated P-327 and H-111 were included as resistant and susceptible standards, respectively, in each trial.

3. Cultivation methods

Approximately 5000 EWP seedlings were germinated into individual Ray Leach Conetainer™ tubes, 10 cubic inches (164 cubic cm)
is the residual. (See Isik et al. (2008) for
estimated from agar plates placed randomly across the inoculation chamber.


lesions began to appear as seedlings broke dormancy in the spring, and

Ribes nigrum

seedlings were organized so that

–

4. Experimental design

Inoculated seedlings contained a combination of primary and secondary

EWP trees in Minnesota the same year. The infection was fostered

leaves were infected with fresh aeciospores in the spring, collected from

its leaves tolerate high levels of rust infection without senescing. The

were grown in individual one-gallon pots

5. Statistical analysis

We used survival (dead vs alive) at year two, from each trial as a

standardized measure of resistance. Since infection rates in EWP are so

high following artificial inoculations, we chose to analyze survival after

the second year, in spite of the fact that additional seedlings died after

the second year. The use of binary data is robust for datasets where the
data is highly skewed, provided that survival is within reasonable parameters (>20%, <80%), parameters that were met by all the trials. Survival was analyzed using a linear mixed model in Proc glimmix (SAS, v 9.4, SAS Institute Inc., Cary, NC) using the logit function (Eq. (1)). The model statement is shown in Eq. (2):

\[
y = \log \left[ \frac{p}{1-p} \right]
\]

(1)

\[y = \mu + B_i + F_k + \epsilon_{ik}\]

where \(y\) = probability of survival, \(p\) = proportion of surviving trees, \(B_i\)

is the random effect for blocks, and \(F_k\) is the random effect for family, \(\mu\)
is the overall mean, and \(\epsilon_{ik}\) is the residual. (See Isik et al. (2008) for more detail on the assumptions of the binary model.) We obtained random effects for each family (also known as best linear unbiased predictors, or BLUPS), for survival, that estimate the log odds of survival. Breeding values (BV) are obtained by multiplying the BLUP by two for open-pollinated families, and by summing the BLUPs for both maternal and paternal parents for full-sib families. The breeding values are converted to percent probabilities of survival (%prob survival) using an inverse link function in Eq. (3):

\[
%\text{prob survival} = 1 - \left( \frac{1}{1 + \text{EXP}(\text{BV})} \right)
\]

(3)

where EXP(BV) is the inverse log of the breeding value. The resulting value, percent probability of survival, is herein referred to as the %prob survival. The %prob survival for each family was compared to the population mean survival (for each trial) to obtain a genetic gain. Genetic gains that exceed the population mean are positive; gains that are below the mean are negative. The percent gains for each family were ranked and compared with the standards, P-327 (resistant) and H-111 (susceptible) for each trial. Families were selected if their percent gain for survival exceeded P-327.

Individual heritabilities, \(h^2\), were calculated for half-sib families with Eq. (4), and calculate the percent of phenotypic variation that is additive (Falconer and Mackay, 1996):

\[
h^2 = \frac{4V_{ij}}{V_{ij} + V_b + V_e}
\]

(4)

where \(V_{ij}\) is the variance due to family, \(V_b\) is the variance due to the blocks, and \(V_e\) is error variance set to 3.29 (Gilmour, et al., 1985). For full-sib families, the numerator was calculated as \(2 \cdot V_{ij}\). Family heritabilities, that express the percent of phenotypic attributable to all genetic variation (including additive, epistatic and other gene interactions) (Falconer and Mackay, 1996) were calculated per Eq. (5):

\[
h^2_{\text{full}} = \frac{V_{ij}}{V_{ij} + \frac{V_{ij}}{b} + \frac{V_e}{n}}
\]

(5)

where \(V_{ij}\) is the family variance, \(V_b\) is the block variance, \(b\) is the number of blocks, \(n\) is the number of trees per family, and \(V_e\) is the residual error variance set to 3.29 (Gilmour et al., 1985). Standard errors for heritability estimates were obtained with the Delta method (Lynch and Walsh, 1998).

Table 1
Summary of experimental design for each of four trials. Average spores per cm² was estimated from agar plates placed randomly across the inoculation chamber.

<table>
<thead>
<tr>
<th>Year germinated</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. families</td>
<td>26</td>
<td>68</td>
<td>64</td>
<td>70</td>
</tr>
<tr>
<td>No. blocks</td>
<td>12</td>
<td>10</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>No. trees per family per block</td>
<td>7</td>
<td>7</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Average spores/cm²</td>
<td>&gt;10,000</td>
<td>&gt;9000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

6. Results

Over 90% of seedlings exhibited foliar spots in three trials; in 2013 the infection rate was lower, at 78% (Table 2). The uninoculated controls remained uninfected throughout the study and were removed from further analysis. After one year, more than half of all seedlings developed a visible stem canker indicating disease progression. Survival after the first year was >75%, but dropped to fewer than 50% in all trials by the end of the two-year observation period (Table 2). After two years,
individual heritabilities for survival ranged from 0.33 to 0.90, and family heritabilities ranged from 0.80 to 0.86 for all trials. Survival for open-pollinated P-327 ranged from 58 to 81%, and open-pollinated H-111 ranged from 12 to 24%.

In 2010, probabilities of survival for resistant standard, P-327 (67.6%), were highest while the ones for the susceptible standard, H-111, were the lowest (19.6%) among the 27 genotypes tested so no additional genotypes were selected. In 2012 and 2013, %prob survival for 15 and 8 families (Table 3), respectively, exceeded P-327. Nine of the families originated from the planting near Tofte (T-), eight from the Chippewa National Forest in 2012 (C-), and two from the Superior National Forest (S-). Two families in 2013 that exceeded P-327 originated from the Chippewa NF (C-), and two from Minnesota (M).

Table 3

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>% with foliar spot</td>
<td>94%</td>
<td>99%</td>
<td>87.6%</td>
<td>78%</td>
</tr>
<tr>
<td>1st year survival</td>
<td>95%</td>
<td>72%</td>
<td>88%</td>
<td>89%</td>
</tr>
<tr>
<td>2nd year survival</td>
<td>39%</td>
<td>34%</td>
<td>29%</td>
<td>42%</td>
</tr>
<tr>
<td>2nd year survival (SE)</td>
<td>0.08</td>
<td>0.10</td>
<td>0.17</td>
<td>0.45</td>
</tr>
<tr>
<td>%prob survival</td>
<td>81%</td>
<td>72%</td>
<td>58%</td>
<td>50%</td>
</tr>
<tr>
<td>H-111: % survival</td>
<td>5.60%</td>
<td>24%</td>
<td>12%</td>
<td>21%</td>
</tr>
</tbody>
</table>

Table 2

Infection success, survival (No. alive/total No. seedlings), and heritabilities for four trials. The percent of trees (across families) with foliar spots indicating infection (%foliar spot) were assessed 7 months after inoculation. P-327 and H-111 are the resistant and susceptible standards, respectively. See Table 1 for experimental design, and the text for additional descriptions of the trials.

Table 4

Percent probability of survival (% Prob survival), derived from the sum of maternal and paternal BLUPS with a log-link function, and gain (relative to the test population) for best maternal * paternal combinations in the 2011 trial (those that exceeded open-pollinated P-327). Maternal parents MI-004, S-151, and S-130 are selected (underlined) because they exceeded P-327 when open-pollinated or crossed with H-111.

For decades, with the exception of P-327, no genotype of EWP produced progeny that consistently demonstrated resistance to white pine blister rust. We identified 25 new families, out of 228 screened (11%), whose progeny exceeded the resistant standard (open-pollinated progeny of P-327), herein referred to as top-tier selections. These top-tier selections originated from a variety of locales including the planting near Tofte, Chippewa and Superior National Forests. The fact that heritabilities for resistance were relatively high supports the notion that families can be distinguished by resistance levels, although major-gene resistance appears to be largely absent in our selected population. Heritabilities were lower than those reported for a binary assessment of resistance (diseased vs not-diseased) of fusiform rust in loblolly pine (Işık et al., 2008) which is not surprising given the polygenic nature of EWP.
resistance in EWP. In spite of the challenges in controlling infection rates across different years of the trials, heritabilities were similar for the different trials indicating that inoculation protocol achieved reasonable control over unwanted environmental variation. Furthermore, the resistant and susceptible standards differentiated in all trials, following expectations.

Progeny from P-327 had higher survival than the trial mean for all four years, and was exceeded by 25 other genotypes across three trials (2011, 2012 and 2013). Two of Patton’s selections, P-312 and P-343, included in the 2010 trial, had been screened previously at USDA Forest Service’s Dorena Tree Improvement Center in Oregon (Sniezko et al., 2008) and failed to surpass resistance of P-327 in the 2010 trial. At Dorena, survival of P-312 and P-343 was relatively low (6.9 and 5.1%) compared to western white pine families with confirmed resistance (survival 64% and 34%). Genotypes from other provenances (Tofte, Chippewa NF, and Superior NF) were represented within the top tiers (9, 10, and 3 families, respectively). Taken together, these findings confirm that the protocol to artificially screen EWP seedlings is effective at identifying resistance, and suggest that genes for resistance do not originate from a single provenance, but instead are distributed across the landscape.

Our goal to identify a suite of genotypes with resistance is only achievable with controlled environmental conditions and consistent genetic expression across the different trial years. Each trial contains inherent error, and our selection criteria is relatively coarse, limited to families that exceed P-327 even though breeding values that are below P-327 may be within the margin of error. Replication of all genotypes at multiple trials was not feasible because of the need to maximize the number of genotypes screened each year. Only three families, besides the two standards, were tested in different years: MI-004, T-104, and C-101. MI-004 appears to have strong resistance that was expressed even when crossed with the susceptible standard in the 2011 trial, and as an open pollinated family in both 2011 and 2012. Additional investigations are warranted to explore its potential resistance as a pollen parent. Open-pollinated T-104 was tested in 2012 and 2013, and was not selected because it performed below the mean in both trials. Genotype C-101 was selected in 2012 as a top-tier family, but not in 2011 (full-sib trial), where its breeding values were below the mean for all crosses. We recognize that genotypes that fall at or near P-327 may warrant additional testing in the future as maternal or pollen parents.

Our findings of improved resistance among other families besides P-327 are promising, but several caveats are worth discussing. Firstly, field resistance in natural conditions is untested. Seedling structure and morphology, especially those pertaining to stomatal apertures, may be different in greenhouses than in more natural settings which could impact the receptivity of seedlings to infection. Secondly, we don’t know if the resistance traits would be expressed, or be as effective, in older seedlings or saplings. Lastly, by imposing artificially high rates of infection, our selection may be more stringent than biologically necessary, creating an artificial bottleneck that eliminates, by chance, resistance traits that are only expressed on older plants. In the planting near Tofte, many of the mature trees contain large, elliptical cankers with well-defined edges and have been characterized as being “slow to infect,” or slow-rusters. This slow-resistance may be important in mature trees but is unlikely to be expressed in seedlings. Additional studies at Tofte, or new field trials may provide opportunities to compare performance of seedlings with mature trees that have survived natural levels of inoculum.

The addition of full-sib families in the 2011 experiment provided some insight into the resistance conferred by the susceptible and resistant standards, H-111 and P-327, across the 26 families tested. The fact that survival with P-327 as a male parent was nearly twice that of open-pollinated trees, or those crossed with H-111, suggests that P-327 has a genetic component of resistance that exceeds wild type pollen, and that the resistance of the paternal parent may be an important consideration. Scientists at the University of Minnesota who conducted artificial inoculations also noted low resistance for open-pollinated EWP (Smith et al., 2006a). Jurgens et al. (2003) noted the exceptionally poor performance of bulked seed sources commonly used for seedling production. Artificial inoculations of full or partial diaries would provide estimates of specific combining ability, or SCA, to ascertain if specific maternal or paternal combinations are optimal for achieving resistance. If SCA, or paternal resistance, is important then seed orchards would either require isolation from outside pollen to generate seedlings with maximum resistance for planting in areas with a high risk for blister rust.

Fungi are capable of adapting rapidly to environmental change due to their fast generation times and high levels of genetic recombination. Ultimately, an improved population of EWP with a multitude of resistance mechanisms should possess greater long-term resistance to blister rust than a population that is dependent on one or a few resistant genes. Additional investigations into the mechanisms that underlie resistance in these 25 families is warranted so we can develop seed orchards with genotypes that possess a variety of mechanisms. Trees that survived inoculation after three years are being conserved, propagated, and planted out for long-term observation. The addition of forward selection, to identify and conserve best individuals within best families, should produce new genotypes with heightened levels of resistance. Three additional screening trials have since been installed, and may reveal new genotypes with improved resistance to bolster diversity in our population. Grafted seed orchards are being developed using the 25 genotypes identified from this study, and are expected to provide seed with improved resistance for regeneration and restoration projects for planting on public land (state and National Forests), as well as on privately-held land from seedlings sold at the Minnesota State nursery, and potentially other public or private nurseries as well.

8. Conclusions

Regeneration of eastern white pine has decreased in the north woods of the Lake States and Ontario since blister rust arrived in North America over 100 years ago. Resistance in P-327 remains a benchmark, but genetic diversity is essential for regeneration to succeed across heterogeneous environments for long-lived species like EWP. We identified 25 additional genotypes, out of 228 that were screened, with resistance that exceeded the standard through artificial inoculations. Resistance was governed by both maternal and paternal parents so new orchards may require isolation, or mass controlled pollinations to maximize resistance to white pine blister rust. Additional work is warranted to characterize the diversity of resistance mechanisms to ensure the stability resistance into the future. Additional screening trials are in progress and that may yield new genotypes to bolster this improved population. Seed orchards, constructed from grafts of the 25 top-tier genotypes, are in development to provide a seed source for restoration or reforestation where regeneration is hindered by blister rust in the Lake States region.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foreco.2018.03.001.

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