Screening Larch in Vitro for Resistance to *Mycosphaerella laricina*

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ABSTRACT

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Needle blight of larch caused by *Mycosphaerella laricina* seriously limits the productivity of susceptible trees in the north central and northeastern United States. Adventitious shoots, derived from cotyledon tissue culture, of selected European larch (*Larix decidua*) and a hybrid larch were inoculated in vitro with three isolates of *M. laricina*. Inoculation with any of the three isolates resulted in disease severity ratings similar to resistance rankings of seedlings in field trials. Tissue-cultured shoots showed evidence of infection within 3 wk after in vitro inoculation. Thus, in vitro screening offers a reliable system for determining the resistance of larch from various seed lots to infection by *M. laricina* within a few weeks rather than the years required in the field.

Mycosphaerella laricina (R. Hartig) Mig. in Thomé (anamorph *Cercoseptoria* sp.) causes a serious needle blight of European larch (*Larix decidua* Mill.) in the north central United States (11,13). Since the first reports of this disease in the United States (9,12), research has demonstrated that susceptibility to *M. laricina* varies by larch species and seed source within species (8).

Exotic larches have the potential for high productivity and are a valuable alternative to other conifers on many sites in the northeastern and north central United States (5). *M. laricina*, however, seriously threatens the success of plantings of susceptible larch by reducing their productivity or killing highly susceptible trees.

Before exotic larch species and selections can be recommended for large-scale planting, their resistance to the needle blight caused by *M. laricina* must be determined. Screening trees in the field is time-consuming and expensive, and results are confounded by many factors, including weather conditions and inoculum density.

Tissue culture and in vitro inoculation techniques are increasingly being used to select for disease resistance (4). Differential responses have been observed among European larch shoot cultures inoculated with *Gremmeniella abietina* (Lagerb.) Morelet (1). A rapid screening system for detecting resistance in vitro would have many advantages over the slower and more expensive traditional field screening techniques.

This paper reports the results of a study designed to compare in vitro screening with field screening of larch for resistance to *M. laricina*. A preliminary report was published (10).

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MATERIALS AND METHODS

Tissue culture. Seeds of selected European larch and *L.* × *eurolepis* Hort., a hybrid larch (*L. kaempferi* (Lamb.) Carrière × *L. decidua*), collected from six sources (8) were surface-sterilized and germinated according to the method of Abdul Rahman et al (1). Seedlings derived from these seed lots were previously evaluated over 4 yr for field resistance to *M. laricina* in Wisconsin and Iowa (8). For adventitious shoot production, cotyledons from germinated seed were cultured inverted in sterile, disposable petri plates (100 × 25 mm) containing half-strength LePoivre medium (2) supplemented with 250 mg/L of L-glutamine, 5 mg/L of 6-benzylaminopurine, 3% (w/v) sucrose, and 1% (w/v) Difco Bacto agar (pH 5.5) for 1 wk. The cotyledons were subcultured twice on fresh medium of the same composition at 3-wk intervals. Cotyledons with adventitious buds were cultured for 6 wk on half-strength Gresshoff and Doy medium (7) containing 1% (w/v) each of activated charcoal, sucrose, and Difco Bacto agar (pH 5.5). Individual elongated shoots from several cotyledons within a seed source were removed and placed on woody plant medium (6) supplemented with 2.58 g/L of Gelrite (Kelco) (pH 5.6). Shoots were transferred to fresh woody plant medium every 3 wk until used for the inoculation experiments. All cultures were incubated in a growth chamber with 16-hr photoperiod (cool-white fluorescent, 125–200 $\mu E \cdot m^{-2} \cdot s^{-1}$, 25 C).

Inoculum production. Three isolates of *M. laricina* were recovered from diseased larch needles collected from trees growing near La Crosse, Wisconsin. Isolate 532 was recovered in June and isolates 561 and 565 were recovered in September 1987. All cultures were maintained on Difco potato-dextrose agar in a continuously lighted incubator (cool-white fluorescent, 60–80 $\mu E \cdot m^{-2} \cdot s^{-1}$, 20 C). Cultures were grown for 7 wk before larch shoots were inoculated; at that time conidia were present in the aerial mycelium throughout the colonies.

Inoculation and evaluation. Larch shoots approximately 1 cm long were selected for inoculation. The upper portion of each shoot was inoculated by applying a 2-mm^2 segment of a *M. laricina* colony to the unwounded adaxial surface of a needle. For each isolate of *M. laricina*, 50 shoots (five petri plates with 10 shoots each) from each larch seed source were inoculated. Uninoculated shoots served as controls. All shoot cultures were incubated in a growth chamber with 18-hr photoperiod (cool-white fluorescent, 125–200 $\mu E \cdot m^{-2} \cdot s^{-1}$, 25 C). Shoots were rated for disease severity 3 and 6 wk after inoculation according to: 0 = no symptoms; 1 = slight mycelial growth, 1–25% of the needles with browning; 2 = moderate mycelial growth, 26–50% of the needles with browning; 3 = extensive mycelial growth, 51–75% of the needles with browning; and 4 = severe, shoot overgrown by mycelium, 76–100% of the needles with browning. The presence of conidiomata (acervuli) of *M. laricina* on shoots was also recorded. Isolations were made from necrotic needles to confirm the presence of *M. laricina*. In addition, 6 wk after inoculation symptomatic inoculated needles and several uninocu-

lated needles were fixed in Formalin–acetic acid–ethanol (FAA) for 24 hr. The needles were dehydrated in a graded ethanol series and embedded in Paraplast. Sections (10-μm) were stained with safranin/fast green and examined under a microscope for presence of mycelial growth and conidiomata. At 5 wk after inoculation, all asymptomatic shoots were reinoculated with the original isolate and incubated as before. Disease development was recorded again after 5 wk.

The various inocula were compared with respect to mean disease severity ratings for the larch sources using the Quade test (3), a nonparametric analysis of ranks. Larch sources were compared with respect to overall distributions of plants among disease severity categories using chi-square tests. Chi-square tests were also used to compare individual *M. laricina* isolates on each larch source with respect to the number of shoots that developed conidiomata. Finally, chi-square tests were used to compare larch sources with respect to the total number of shoots that developed conidomata regardless of *M. laricina* isolate.

RESULTS

Larch shoots inoculated in vitro with any of the three isolates of *M. laricina* resulted in disease severity ratings that were not significantly different from ratings obtained in the field (Table 1). However, the disease severity ratings for all larch sources except XLD-1-84 and 9953 differed significantly from each other (Table 2).

Dense mycelial growth, needle necrosis, and conidiomata were evident within 3 wk of inoculation on many shoots from the most susceptible larch source. The incidence and severity of disease were maximum at 6 wk; data from this reading are reported here. Disease severity of inoculated shoots was uniform within a given seed source. The majority of reinoculated shoots of the most resistant larch sources remained disease-free. The few shoots that did become diseased had ratings similar to the mean rating given to the larch source in the first inoculation. Mature conidia were recovered from all conidiomata sampled.

Symptomatic needles that were detached and incubated on potato-dextrose agar yielded colonies of *M. laricina*. Light microscopy of necrotic needles showed extensive destruction of host mesophyll and the presence of conidiomata with conidia. No uninoculated shoots became diseased, and needle sections appeared normal.

Disease severity rating was consistently highest on shoots of all larch sources inoculated with isolate 561 (Table 1). Isolate 565 caused the least disease on all larch sources except XLL-LD-12-84, and isolate 532 was intermediate. The different isolates did not significantly change the relative ranking of the most resistant larch sources. Larch source 9953, however, was highly susceptible in the field but was ranked moderately resistant when tested in vitro. Inoculation of all larch sources with isolate 532 resulted in a ranking similar to that obtained in the field. All three of the most resistant sources, based on the field trials, were identified when isolate 532 was used, and two of the three were identified when isolate 561 was used.

European larch from seed source XLD-14-84 and hybrid larch from seed source XLL-LD-12-84 were generally the most resistant. European larches from seed sources XLD-5-84 and 9950 were the most susceptible.

Conidiomata developed significantly more often on shoots from larch sources XLD-5-84 and 9950 than on shoots from the other larch sources (Table 3). In general, conidiomata developed more frequently in the necrotic portions of

Table 1. Mean needle blight disease severity ratings of European (*Larix decidua*) and hybrid (*L.* × *eurolepis*) larch 6 wk after inoculation with three *Mycosphaerella laricina* isolates and under natural inoculum in the field in Iowa and Wisconsin

Larch source[w]	Field inoculum[x]	In vitro isolates[y]			Combined means[z]
		532	565	561	
XLD-14-84	0.75	0.94	0.70	1.06	0.88
XLL-LD-12-84	0.84	1.54	1.68	1.80	1.59
XLD-1-84	2.03	1.75	1.30	2.10	1.75
9953	2.41	1.87	1.42	1.88	1.80
9950	2.25	2.30	1.84	2.68	2.27
XLD-5-84	2.06	2.37	2.33	2.51	2.37

[w] All were European larch except XLL-LD-12-84.

[x] Field ratings (combined for Iowa and Wisconsin for 1986 and 1987): 0 = no disease; 1 = slight, 1–25% foliage affected; 2 = moderate, 26–50% foliage affected; 3 = extensive, 51–75% foliage affected; 4 = severe, 76–100% foliage affected.

[y] Laboratory ratings: 0 = no symptoms; 1 = slight mycelial growth, 1–25% of needles with browning; 2 = moderate mycelial growth, 26–50% of needles with browning; 3 = extensive mycelial growth, 51–75% of needles with browning; 4 = severe, shoot overgrown by mycelium, 76–100% of needles with browning.

[z] Because the relative ranking of seed sources was not significantly different (Quade nonparametric rank test, $P = 0.05$) after 4 yr in the field or after in vitro tests, field and laboratory ratings were combined.

Table 2. Number of larch plants in each disease rating category[z]

Rating	Larch source					
	XLD-14-84	XLL-LD-12-84	XLD-1-84	9953	9950	XLD-5-84
0	50	18	20	14	8	3
1	91	63	27	20	28	8
2	16	63	64	85	64	96
3	2	15	17	11	40	39
4	4	7	8	5	25	17

[z] Larch sources are ranked left to right according to resistance (0 = high, 4 = low). The distributions among rating categories differ significantly from each other except for XLD-1-84 and 9953 ($P = 0.05$, chi-square test). Because no significant differences were found among the inocula (Quade nonparametric rank test), the numbers of plants in each rating category were pooled from field and in vitro inoculations.

Table 3. Larch shoots inoculated in vitro with *Mycosphaerella laricina* on which conidiomata developed after 6 wk

Larch source[x]	Percent/sample size of isolates			
	565	561	532	Total[y]
XLL-LD-12-84	0/50 a[z]	0/50 a	0/50 a	0/150 a
XLD-14-84 a	0/50 a	0/48 a	20/49 b	7/147 b
9953 b	45/40 a	0/40 b	0/39 b	15/119 c
XLD-1-84 a	0/40 a	0/40 a	75/40 b	25/120 c
XLD-5-84 c	0/49 a	63/49 b	90/49 c	51/147 d
9950 d	6/50 a	84/50 b	92/50 b	61/150 d

[x] Sources within column not followed by the same letter are significantly different in respect to production of conidiomata by individual *M. laricina* isolates ($P = 0.05$). Larch source XLL-LD-12-84 could not be included in analysis because of 0 values.

[y] Values within column not followed by the same letter indicate significant differences among larch sources in respect to production of conidiomata across all *M. laricina* isolates ($P = 0.05$).

[z] Values within rows not followed by the same letter are significantly different ($P = 0.05$).

needles from susceptible sources inoculated with isolates 532 and 561. Conidiomata rarely developed on shoots inoculated with isolate 565. Conidia were produced only sparsely in culture plates of isolate 565 as well. Conidiomata did not develop on the hybrid larch source XLL-LD-12-84 and developed infrequently on larch source XLD-14-84; both sources are highly resistant in the field.

DISCUSSION

Generally, larches with a rating of 2.0 or lower in the field and laboratory trials were assumed to have an acceptable level of resistance. The two most resistant larch sources in the field trials, XLD-14-84 and XLL-LD-12-84, were also identified as resistant in the in vitro test. Inoculation with isolate 532 resulted in a ranking of the three most resistant larch sources in the same order as in the field trial.

Because there were no significant differences in aggressiveness on the larch sources among the three *M. laricina* isolates compared with the field inoculum, the in vitro ranking of the most resistant larch sources was similar to the field ranking. However, larch source 9953, the most susceptible in the field, was ranked more resistant in the in vitro test and was within the acceptable standard. This may indicate that genotype × environment interactions may influence disease susceptibility of larch from some sources more than others.

Production of conidiomata on inoculated shoots varied by isolate and larch source, even though the three *M. laricina* isolates were recovered from infected larch within the same geographic area. It is not known if the latent period of *M. laricina* could influence disease severity on different larch sources by reducing inoculum potential in the field. This in vitro inoculation technique could be useful in the study of the differences in aggressiveness of *M. laricina* throughout its range and its ability to sporulate on various larch selections.

Vegetative hyphae were used as inoculum in this study because of the difficulty in obtaining a quantified spore suspension. In culture, conidia of *M. laricina* develop slowly, are borne on simple conidophores within the aerial mycelium, and are often sparse. In contrast, conidiomata developed within 3 wk on inoculated shoots of susceptible larch grown in vitro, providing an opportunity for obtaining a large number of conidia for further inoculation tests.

Screening larch in vitro for resistance to *M. laricina* should be considered a preliminary step to field screening. The ability to screen larch from several seed lots in vitro under highly controlled conditions for resistance to *M. laricina* offers the potential for faster determination of the suitability of larch selections for inclusion in a tree improvement program. In vitro screening allowed us to determine the relative susceptibility of larch from different seed sources within 6 wk of inoculation, rather than the 4 yr required in the field. However, additional research is needed to determine if variability in the aggressiveness of *M. laricina* exists within the north central United States before test isolates are selected.

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