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Delphinella Shoot Blight on *Abies lasiocarpa* Provenances in Norway

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Abstract: Delphinella shoot blight (*Delphinella abietis*) attacks true firs (*Abies* spp.) in Europe and North America. Especially subalpine fir (*A. lasiocarpa*), one of the main Christmas tree species in Norway, is prone to the disease. The fungus kills current year needles, and in severe cases entire shoots. Dead needles become covered with black fruiting bodies, both pycnidia and pseudothecia. Delphinella shoot blight has mainly been a problem in humid, coastal regions in the northwestern part of Southern Norway, but, probably due to higher precipitation in inland regions during recent years, heavy attacks were found in 2011 in a field trial with 76 provenances of subalpine fir in Southeastern Norway. However, the amount of precipitation seemed less important once the disease had established in the field. Significant differences in susceptibility between provenances were observed. In general, the more bluish the foliage was, the healthier the trees appeared. The analysis of provenance means indicated that, at least for the southern range, the disease ratings were correlated with foliage color. This study also includes isolation, identification, a pathogenicity test, a seed test and electron microscopy of the wax layer on the needles. The fungus was identified based on the morphology of spores and by sequencing the Internal Transcribed Spacer (ITS) regions of the ribosomal DNA. Koch's postulates were fulfilled. The fungus was found present on newly harvested seeds and may therefore spread via international seed trade. When comparing the wax layers on green and blue needles, those of the latter were significantly thicker, a factor that may be involved in disease resistance.

Keywords: subalpine fir; *Delphinella abietis*; foliar color; resistance

1. Introduction

Delphinella shoot blight, caused by the fungus *Delphinella abietis* (E. Rostrup) E. Müller, is a problem on true firs (*Abies* spp.) in Norway [1,2], especially in the Christmas tree and bough production, where quality demands are high. Subalpine fir [*A. lasiocarpa* (Hooker) Nuttall], a commercially important Christmas tree species in Norway, is unfortunately very susceptible to *D. abietis*. The current demand for Christmas trees in Norway consists of approximately 60% fir (hereof 50% subalpine fir) and 40% spruce (*Picea* spp.) [3]. No records exist concerning damage by *D. abietis* on Christmas trees in Norway, but fields have been abandoned due to the disease [4].

In Europe, the disease occurs mainly in humid regions in the northwestern parts, not the least in Scandinavia. The first description of the fungus was from Denmark under the name *Sphaerella abietis* [5], but, in recent years, the most severe damages have been reported from coastal regions in Southern Norway [1,2]. Already 80 years ago, Jørstad [6] reported that *D. abietis* was found along the coast in Southern Norway (Kristiansand to Trondheim) on European silver fir (*A. alba* Mill.), Greek fir (*A. cephalonica* Louden), and some other fir species. However, it was not detected on balsam fir [*A. balsamea* (L.) Mill.]. In 1999, Solheim [7] summarized that the disease thus far had been found on corkbark fir [*A. lasiocarpa* var. *arizonica* (Merriam) Lemmon], Greek fir, Noble fir (*A. procera* Rehder), Nordmann fir [*A. nordmanniana* (Steven) Spach], European silver fir, Spanish fir (*A. pinsapo* Boiss), subalpine fir, and Siberian fir (*A. sibirica* Ledeb.) in Norway. Later we also found it on Turkish fir (*A. bornmulleriana,* also known as *A. nordmanniana* subsp. *equi-trojani*) in a Christmas tree field. Interestingly, Korean fir (*A. koreana* E.H. Wilson) adjacent to subalpine fir that were severely damaged by *D. abietis* in a Christmas tree field in Western Norway were free from the disease [4]. Apart from that observation, no data are available on host resistance.

Limited attacks have been observed in regions further south in Europe. Minor symptoms of *D. abietis* were observed on European silver fir in Austria in 1990 and 1991 [8]. From Great Britain, *D. abietis* has been reported as being found in Devon, England and the highlands of Scotland [9], but, to our knowledge, it has not caused severe damage.

D. abietis also occurs in North America. Funk [10] reported infections on subalpine fir in Canada, but stated that the closely related species *D. balsameae* (A. M. Waterman) E. Müller is a more common pathogen on fir species there. The latter species was also reported in Vermont, USA on subalpine fir, white fir [*A. concolor* (Gordon & Glend.) Lindl. ex Hildebr.] and balsam fir [11]. In 2014, severe damage by *D. abietis* was also observed on subalpine fir in Idaho and Washington State, USA [12].

In severe cases of Delphinella shoot blight, where all of the needles on current year shoots turn chlorotic and die, the symptoms are often mistaken as spring frost damage. However, if only a few needles randomly distributed on a shoot die, it indicates a biotic causal agent. The life cycle for *D. abietis* has not been well described in the literature, but we often observed the small, black fruiting bodies (pseudothecia and pycnidia) reported by Rostrup [5] on infected needles during late summer and fall, especially on the upper needle surfaces. They are visible to the naked eye. We have found that the ascospores mature during spring, which make them ready for infecting soft tissue during budbreak and shoot elongation given the right conditions for the fungus (mild and humid). Only species within the genera *Abies* get infected by *D. abietis*.

In 2011, symptoms caused by *D. abietis* were found in a field trial with 76 provenances of subalpine fir at Jønsberg, Hedmark county in Southeastern Norway [13]. Provenance is a general term used throughout this paper for describing origin of seed sources. Many trees at Jønsberg were severely affected by *D. abietis*, but some trees were less damaged than others. Interestingly, trees with bluish foliage were seemingly more healthy than the ones with more green foliage. The provenance trial was of interest as a seed source and for future breeding material. If needle color could be used for selecting trees with resistance against *D. abietis,* it would be helpful for selecting the best material for progeny tests. Thus, the main objective of this study was to uncover whether variation in resistance to *D. abietis* correlated with needle color. We also studied the pathogen in detail and the damage it had caused.

2. Materials and Methods

2.1. Isolation and Identification of D. abietis

From earlier work with this fungus [14], it was known that isolation from needles, even when the surface was sterilized for 10 s in 70% ethanol (C_2H_6O) and 90 s in 0.5% sodium hypochlorite (NaOCl), often yields many secondary organisms covering *D. abietis*. In general, several so-called secondary pathogens may colonize tissue that have been dead for some time, as was the case here with respect

to the older needles with fruiting bodies. We have never found mature ascospores from *D. abietis* on samples collected and incubated during autumn or winter, except from the end of April onwards [14]. Thus, we collected and cold-stored infected material from Jønsberg on 10 May 2015, which was before budbreak. The sample consisted of twigs and branches with dead shoots due to a *D. abietis* attack from the previous season. On May 28, after mounting some pseudothecia on slides to look for ascospores in the microscope, we attached the infected shoots to the underside of the lid of a plastic container. We placed 8 open Petri dishes with acidified potato dextrose agar (PDAS—19 mL 10% tartaric acid per liter PDA) right below the branches (low pH in media supresses bacterial growth). To keep the relative humidity high inside the container, wet filter paper was placed at the bottom. The Petri dishes were standing on a metal grid to avoid contact with water. The container that served as a moist chamber was transparent and measured 59 × 40 × 38 cm. The experiment was carried out under room temperature (approximately 20 °C). The Petri dishes were left open for 48 h. An isolate was selected (No. 250510) to confirm our morphological identification via sequencing of the Internal Transcribed Spacer (ITS) region of the ribosomal DNA. Total genomic DNA was extracted from mycelium grown on a PDA plate using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). A DNA segment containing the 3′ end of the 18S rDNA, ITS1, 5.8S rDNA, ITS2 and the 5′ end of 28S rDNA was amplified by PCR using Taq polymerase and forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC) as described by White *et al.* [15]. DNA sequences were analyzed using the Lasergene Sequence Analysis Software (DNASTAR Inc., Madison, WI, USA).

2.2. Inoculation Test

Although pathogenicity for *D. abietis* is well known—Ellis and Ellis [16] among others describe the fungus as parasitic on living needles of firs—we wanted to try out an inoculation method that potentially could be used for screening provenances and progenies for susceptibility to *D. abietis.*

In order to carry out the inoculation as naturally as possible under laboratory conditions, we used the same principles as for the isolation described above. Nineteen one-year-old and six two-years-old plants from subalpine fir (seed sources from Chuwhels Mountain and White River in British Columbia, respectively) that had just started breaking buds were placed at the bottom of a 70-litre plastic drum (26 June 2015). Cold-stored branches from the same material used in the isolation trial were attached to the lid of the drum. No wounding of the new needles took place. The plants were kept inside the drum for five days, before they were exposed to shaded, outdoor conditions like before the treatment. Control plants got no treatment. The experiment was terminated on 7 October 2015, and reisolations were carried out. Since the needles were thin and small, they received a less tough surface sterilization than normal before reisolation (only 5 s in 70% C_2H_6O, 5 s in 0.5% NaOCl + rinsed in distilled, autoclaved water). From each provenance, ten needles were selected for reisolation and plated out on PDAS.

2.3. Seed Tests

In 2015, some trees produced cones in the provenance trial at Jønsberg, and some promising trees for future Christmas tree production were selected as parents for a control-pollination. Since a number of diseases are known to be seed-borne on *Abies* spp. [17], a bulk sample of cones from seven trees (mix of provenances) were collected in the second week of September and analyzed for *D. abietis*. The trees where the cones were harvested had various degrees of Delphinella shoot blight symptoms. The sample was dried over night at room temperature before the seeds for the test were cleaned for debris and the wings removed. From each sample, 100 non-surface-sterilized seeds and 100 surface-sterilized seeds (1% NaOCl for 10 min, dried on sterile blotters) were plated on PDA, 5 seeds per 9-cm Petri dish. The PDA plates were incubated for seven days at 20 ± 2 °C under alternating 12 h NUV-light and 12 h darkness. After incubation, the plates were scanned under a dissecting microscope, and sporulating cultures were further examined in a light microscope

for morphological identification. Young *D. abietis* cultures are light brown and produce abundant aseptate conidia in a slimy, beige-colored spore mass.

2.4. Provenance Trial

2.4.1. Study Sites, Experimental Design and Provenaces

The field trial at Jønsberg was established in 1999 with four (4/0) and three (3/0) year old bareroot seedlings produced from seed collections of populations from Colorado, Utah, New Mexico and Arizona in 1994, and in Idaho, Oregon, Washington, Wyoming, Montana, Alberta and British Columbia in 1995, respectively. In addition, five commercial seed sources were included. All seedlings were grown in Norway. The provenance trial at Jønsberg is the last remaining trial from a series of eight around the country, all with the same seed sources. The aim of provenance testing was to identify suitable seed sources for Christmas tree production in different climatic zones [18,19].

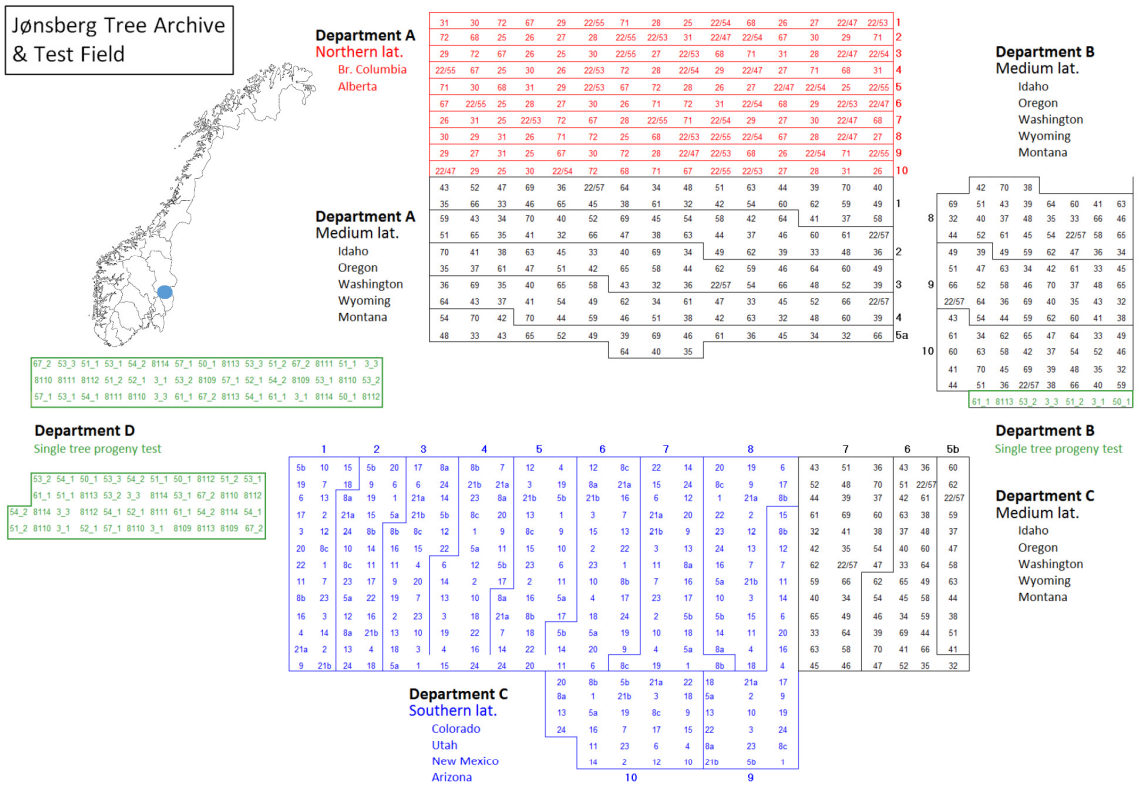

Figure 1. Outline of the provenance trial at Jønsberg. The three regions Northern latitude, Medium latitude, and Southern latitude are marked in red, black, and blue, respectively. The progeny tests in departments D and B (marked in green) were not included in this study. An inserted, small map of Norway indicates the area where Jønsberg is located (blue dot).

Except for Jønsberg, all trials were situated at Christmas tree farms. The trial at Jønsberg was planted on highly productive former farmland. The trial consists of 76 provenances that, prior to planting, were divided into three regions based on their latitudinal origin: South (Southern latitude) with 28 provenances from Colorado, Utah, New Mexico, and Arizona in USA), Middle (Medium latitude with 33 provenances from Idaho, Oregon, Washington, Wyoming, and Montana in USA), and North (Northern latitude) with 15 provenances from British Columbia and Alberta in Canada. The regions were then planted in separate departments of the field, A, B and C (Figure 1). Within each department, the provenances from respective regions were planted in square 9-tree-plots (3 × 3 trees) randomly distributed in 10 replicates. Hence, the provenance trial analyzed here originally consisted

of a total of 6840 trees. Although trees from different regions were planted adjacent to each other, there was no overlap in the provenances planted within departments, and the site must therefore be considered as three separate trials in formal statistical analyses. The trials consisting of provenances from the Middle and North regions were thinned in 2009, and on average 3–4 trees were still present within each plot when registrations took place in 2013 (with a variation from 1 to 7). Trees from the South region were not thinned, and the number of remaining trees there were on average 5–6 (with a variation from 1 to 9).

2.4.2. Rating of Needle Color and Disease Symptoms in the Provenance Trial at Jønsberg

In the first week of July 2013, current year shoots on all remaining trees were scored for symptoms caused by *D. abietis* on a scale from 0 to 4 according to the proportion of damage (d) (0 = 0% d, 1 = 1–20% d, 2 = 21–60% d, 3 = 61–100% d. Furthermore, a forth category was used; 4 = 61–100% d of current year shoots in addition to d on previous years shoots (2011–2012) (Figure 2). The latter was possible since dead needles stick to the trees for a long period.

Additionally, needle color was scored on a scale from 1 to 4 (1 = blue, 2 = greenish blue, 3 = bluish green and 4 = green). Two teams of two people carried out the ratings in the field, and the data were directly recorded on a computer.

Figure 2. In 2013, ratings of disease severity caused by *Delphinella abietis* (scale from 0 to 4) on subalpine fir (*Abies lasiocarpa*) were carried out in the provenance trial at Jønsberg (**a**) 0% damage; (**b**) 1%–20% damage, (**c**) 21%–60% damage; (**d**) 61%–100% damage; and (**e**) 61%–100% damage of current year shoots in addition to damage in previous years. Photos: Odd Ragnar Johnskås.

2.4.3. Statistics Used to Analyse Field Data

Provenances from the three regions South, Middle and North were planted in separate parts of the site (Figure 1). Even though the regions were planted in close vicinity, there is no overlap allowing comparison of provenances across regions. Hence, the effect of region is confounded with the environment at the site and cannot be estimated properly. The formal statistical analysis from the field data were therefore performed for the three regions as separate trials. The analysis of variance and covariance were made in SAS PROC MIXED [20] using two variants of the analysis of variance/co-variance model:

$$Y_{ijn} = u + bX_1 + R_i + P_j + RP_{ij} + E_{ijn}$$

Here, Y is the value for foliar color or damage percentage by *D. abietis* registered on individual trees. The other letter codes represent: i = replicate; j = provenance; n = single tree in a 9-plot square; u = the grand mean; bX_1 = the continuous normal score transformed value for color with an estimated

b (beta); R = the effect of replicate; P = the effect of provenance; RP = the interaction between replicate and provenance (plot-effect); E = the residual error. R, RP and E are assumed to be normally and independently distributed with a mean of 0 and respective variances. The model used for analysing needle color was a pure analysis of variance not containing the regressor X_1. The model used for analysing percentage of needle damage contained needle color as the covariate X_1. Provenances were considered to be fixed effects since they were sampled as candidate provenances for seed supply and breeding (they were not a random sample made across the landscape). The significance of the model effects were evaluated on the basis of the *p* value obtained from the F and Z test of fixed and random effects, respectively.

To fulfill the assumption of normally distributed residuals in the formal analysis, scores for needle color were transformed to "normal scores" within replicates using the BLOM-transformation in SAS PROC RANK [20], which equalized the differences between replicate means. Percentages of needle damage were transformed using the ARCSIN(SQRT(Y)) transformation in order to stabilize variance.

In addition to the formal analyses within regions, a less formal visual analysis (map) was made across regions. Least square means presented in Appendix Table A1 and in the map were estimated from untransformed values based on a simpler version of the analysis of variance model only containing the effects R, P and E.

2.5. Studies of Cuticular Thickness of Needles

Since susceptibility to *D. abietis* seemed to be correlated with needle color, we examined the structure of the cuticle of the needles by scanning electron microscopy (SEM). A blue and a green shoot were chosen at Jønsberg in March 2015. The SEM used was a Quanta 250 (FEI Worldwide Corporation Headquarters), and the software used for making the measurements was Scandium by Olympus (Olympus Soft Imaging Solutions, Münster, Germany). Pictures were taken in low vacuum mode. Needles were frozen at −70 °C before cross-sections were cut with a razorblade. No coating or staining took place. Twelve measurements of cuticular thickness were taken from seven green and seven blue needles (totally 168 measurements). To find possible significant differences between blue and green needles, these data were analyzed by analysis of variance using Minitab 16 Statistical Software (State College, PA, USA: Minitab, Inc.).

3. Results and Discussion

3.1. Disease Symptoms Observed in 2008–2015

Yellowing of current year needles was observed in the provenance trial at Jønsberg, Hedmark county in Southeastern Norway for the first time in 2008, but at that time it was assumed that the damage was caused by frost. In 2011, the damaging agent was identified as *D. abietis*. As explained in Section 2.4.2., damage by *D. abietis* was rated on all trees in July 2013. However, characteristic symptoms and signs caused by *D. abietis* were also observed in other growing seasons between 2011–2015. Infected needles turned yellow and subsequently died (Figure 3). In severe cases, the new buds and shoots were killed, but, more commonly, the buds survived and produced new shoots the following spring. The edges of the dead needles curled towards the stomatal bands on the underside of the needles. Dead needles were firmly attached to the shoots, thus, the majority did not fall off during the winter, nor even during the following growing season. Sometimes three generations of dead needles were found. Numerous, small fruiting bodies (pseudothecia and pycnidia) formed on dead needles and were visible to the naked eye, especially as they matured and became swollen in the spring, nearly a year after the needles got infected. Blue provenances were observed during all five years as generally healthier (few to no dead needles) than those with green foliage (Figure 4).

Figure 3. *Delphinella abietis* on subalpine fir (*Abies lasiocarpa*) in the provenance trial at at Jønsberg in 2015. (**a**) Dead needles on current year shoots in September; (**b**) small, black fruiting bodies on needles in March. Photos: Venche Talgø.

Figure 4. The provenance trial of subalpine fir (*Abies lasiocarpa*) at Jønsberg. (**a**) Blue varieties were generally healthier looking than green varieties; (**b**) typical symptoms of *Delphinella abietis* on the tree with the green foliage to the right. Photos: Odd Ragnar Johnskås.

3.2. Damage by D. abietis at Jønsberg May Be Related to Increased Precipitation

Since Jønsberg is situated to the eastern side of the Scandes, a mountain range running from the south to the north of Norway, the climate is normally much dryer there than on the western and coastal side. However, since the test field was established in 1999, there have been several years with abnormally high precipitation (Figure 5). Before the wet period 2009–2012, symptoms that were most likely caused by *D. abietis*, as mentioned in Section 3.1., had been observed at Jønsberg. Thus, inoculum was probably present before this last wet period. Wetter climate, coinciding with the plantation becoming denser, create in principle an ideal microclimate for fungal growth and subsequent build-up of inoculum. During budbreak and shoot elongation under such conditions, the new needles will generally be under heavy disease pressure from spores released from the still-attached dead needles from the previous season. A high inoculum level and moisture content (even dew dry up slowly in dense plantations) must have been the case in the spring of 2013 at Jønsberg in the year we report from here, although the growing season as a whole was dryer than normal. The same must have been the case for the spring of 2015 because, even after several seasons with precipitation below normal (2013–2015), extensive damage on current year shoots were observed. Thus, the amount of precipitation seemed less important once the disease had established in the field.

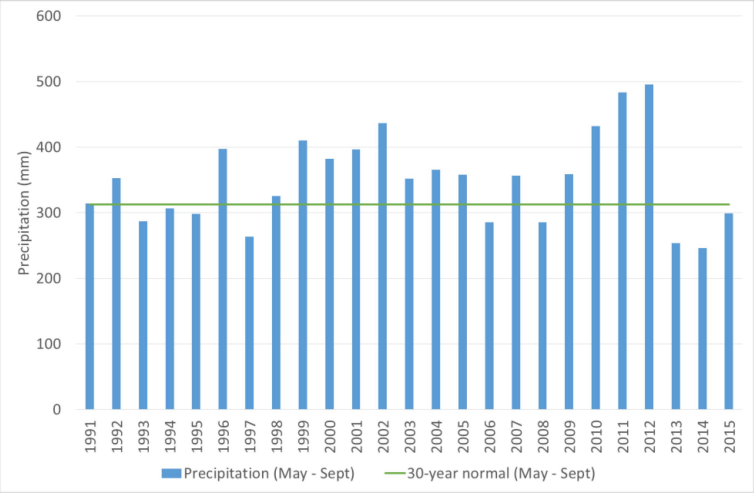

Figure 5. Precipitation during the growing season (1 May until 31 October) for the location Ilseng (near Jønsberg) in Eastern Norway, compared to the 30-year normal (green line) for precipitation (1960–1990) from the Norwegian Meteorological Institute.

3.3. D. abietis Was Readily Isolated from Ascospore Dispersal

Ascospores were present on the microscope slides prepared from the material collected at Jønsberg in May 2015. Morphology of the spores matched these earlier observations reported for *D. abietis* in Norway; 16 ascospores per ascus, hyaline, one septum, and measuring 10.9 − (13.4) − 15.9 × 3.4 − (4.2) − 5.7 µm (n = 50) [14]. The ascospores can potentially be transported by air over larger distances and may give raise to epidemics, but the fungus also produces conidia that get dispersed by water splash to needles within the same tree or to neighbouring trees in dense plantations.

Numerous colonies of *D. abietis* occurred on the eight Petri dishes that were placed in the moist chamber below the infected material from Jønsberg (Figure 6). Apart from two colonies of [*Sydowia polyspora* (Bref. & Trv.) E. Müller], some *Penicillium* and some bacterial growth, *D. abietis* dominated, and pure cultures were easily obtained.

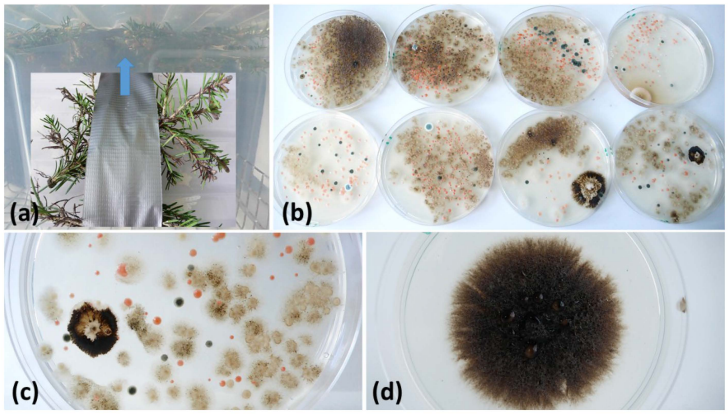

Figure 6. Isolation of *Delphinella abietis* from ascospores. (**a**) Shoots from subalpine fir (*Abies lasiocarpa*) with dead needles due to attack by *D. abietis* were attached to the underside of the lid (indicated by blue arrow) of a moist chamber to disperse ascospores; (**b**) numerous, brown colonies of the fungus appeared on 8 Petri dishes (containing acidified PDA) located below the branches inside the moist chamber for 48 h; (**c**) the largest, black colony is *Sydowis polyspora*; (**d**) a pure, 17 day old culture of *D. abietis*. Photos: Venche Talgø.

The internal transcribed spacer (ITS) sequence of isolate No. 250510 was identical to an isolate of *D. abietis* obtained from a Christmas tree plantation of subalpine fir at the northwestern coast of Southern Norway in 2009 (GQ412731.1). *D. abietis* is only 5 bp different from *S. polyspora* in the ITS region (99.1% similarity), another troublesome disease-causing organism on fir species [21].

3.4. Fulfilment of Koch's Postulates for D. abietis

The two provenances we had available for the inoculation test were not represented in the trial at Jønsberg; thus, we had no data available concerning susceptibility to *D. abietis*. When the experiment ended in October 2015 (Figure 7), 14 out of the 17 plants from the provenance Chuwhels Mnt. had died, three had some dead shoots, and two looked healthy. The fact that two plants were healthy may have to do with budbreak. Generally, firs have very irregular budbreak, even within the same plant; thus, the stage the plants were at during the inoculation period may have caused escape of infection. From the White River provenance, four plants had dead top and/or side shoots, and two plants were dead. Control plants were healthy. Under field condition, we did not see subalpine fir dying from *D. abietis*, but younger plants may have less tolerance towards the pathogen. In addition, the plants may have been exposed to some drought stress during the summer. *D. abietis* was reisolated from two needles from the bigger plants (White River). Reisolation did not succeed from needles from the small dead plants. Grey mould (*Botrytis cinerea*) quickly covered the agar plates and may have supressed the growth of *D. abietis*. More needles should have been used for reisolation. One plant from the White River provenance will be kept until spring 2016 to look for development of fruiting bodies.

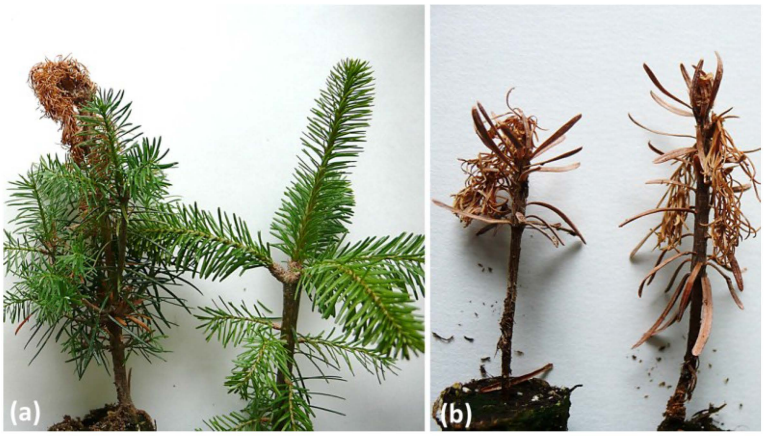

Figure 7. Plants from subalpine fir (*Abies lasiocarpa*) that were exposed to ascospores of *Delphinella abietis* dispersed from infected material collected in the provenance trial at Jønsberg in 2015. (**a**) Dead needles on a two-year-old plant from the provenance White River compared to a healthy control plant; (**b**) dead plants of the provenance Chuwhels Mnt. (clearly the current year shoots died at an early stage). Photos: Venche Talgø.

A better option for screening material for resistance may be inoculation by conidial spore suspension on new shoots, which was successfully carried out for *S. polyspora* [22].

3.5. D. abietis May Be Seedborne

D. abietis was found on 4% of the treated and 2% of the untreated seeds, the former meaning that the fungus had entered inside the seeds. It may seem strange that more *D. abietis* was found on surface-sterilized seeds, but we have experienced the same with other seed-borne pathogens [23]. On untreated seeds, there are often many secondary organisms that may supress the pathogen or simply overgrow it.

The fact that we found the fungus on fresh seeds does not necessarily mean that this would occur on dry, stored seeds. On newly harvested seeds from Nordmann fir, we discovered a very high number (64%) of seeds infected by the canker fungus *Neonectria neomacrospora* (C. Booth & Samuels) Mantiri & Samuels, but, on dried seeds from the same seed orchards and year, we found no seed-borne inoculum [24]. Nevertheless, since trade with subalpine fir seeds is common between countries and continents, seed contamination should be further investigated.

3.6. Some Provenances of Subalpine Fir Show Stronger Resistance against D. abietis

There were highly significant provenance differences in both needle color and infection by *D. abietis* in all three regions ($p < 0.001$, Table 1), although the provenance by replicate interactions were strong ($p < 0.001$).

Table 1. Parameters from the analysis of variance (and covariance) for needle color and damage caused by *D. abietis* in the provenance trial at Jønsberg. Parameters given are: Estimate, the variance (with standard error) for random effects and the *b* (beta) for the covariate X_1; the degrees of freedom (df) of the numerator (Num) and denominator (Den) in the test of the fixed provenance (Prov.) effect; F or Z values for fixed or random effects, respectively; and *p* values for the test of significance. As the values for needle color were normal score transformed within replicates, parameters for replicate (R) were not possible to estimate. RP = interaction between provenance and replicate.

Color						
Region	**Effect**	**Estimate**	**Num df**	**Den df**	**F or Z Value**	***p* Value**
South	Prov.		27	235	20.61	<0.0001
	RP	0.147(0.019)			7.74	<0.0001
	Error	0.289(0.012)			25.11	<0.0001
Middle	Prov.		32	287	7.21	<0.0001
	RP	0.150(0.024)			6.34	<0.0001
	Error	0.463(0.022)			21.38	<0.0001
North	Prov.		14	126	11.78	<0.0001
	RP	0.099(0.031)			3.14	0.0008
	Error	0.413(0.034)			12.12	<0.0001
Needle damage						
Region	Effect	Estimate	Num df	Den df	F or Z value	*p* value
South	X_1 (Color)	0.06(0.01)	1	1261	24.16	<0.0001
	Prov.		27	235	6.48	<0.0001
	Repl.	0.003(0.002)			1.59	0.056
	RP	0.011(0.002)			4.29	<0.0001
	Error	0.082(0.003)			25.08	<0.0001
Middle	X_1 (Color)	−0.038(0.01)	1	917	11.46	0.0007
	Prov.		32	287	4.49	<0.0001
	Repl.	0.006(0.003)			1.85	0.032
	RP	0.02(0.004)			5.97	<0.0001
	Error	0.072(0.003)			21.4	<0.0001
North	X_1 (Color)	0.057(0.02)	1	293	8.30	0.0043
	Prov.		14	126	3.07	0.0004
	Repl.	0.001(0.002)			0.61	0.27
	RP	0.022(0.006)			3.76	<0.0001
	Error	0.066(0.005)			12.16	<0.0001

Provenance means for needle color and susceptibility towards *D. abietis* (including standard errors) are given in Table A1 in the Appendix, including data of origin of the seed sources.

The map in Figure 8 shows the geographic variation in needle color and resistance against *D. abietis*. The blue-colored provenances mainly originate from the southern part of the natural distribution areas, and some coastal regions in the Pacific North West (PNW-Oregon and Washington in USA and British Columbia in Canada). In the southern region, resistance against *D. abietis* seems to follow a general pattern connected with foliar color, in which blue provenances show higher resistance. This is confirmed with the positive *beta* value for needle color in the analysis of needle damage (0.057, Table 1), indicating the general trend that greener provenances are more damaged by *D. abietis*. Similarly, the provenance No. 71, Grassie Mnt. from Vancouver Island is bluer and at the same time less damaged than the other provenances from the same northern region (Figure 8). This provenance is frequently used by Norwegian Christmas tree growers, as it is doing especially well in coastal regions in Western and Southwestern Norway, where disease pressure by *D. abietis* is known to be high.

In the Middle region, the relationship between needle color and needle damage is, in fact, opposite of that of the one observed in South and North, as the *beta* for the covariate needle color is negative (−0.038, Table 1). Regardless, in this region, there are also highly significant provenance differences both for needle color and damage.

In a study of *D. abietis* on subalpine fir in a provenance trial in Idaho, USA [12], it was also evident that provenances from higher altitudes in the southern distribution area, displayed high resistant against *D. abietis*, especially cork bark fir.

Scoring of the disease on 2012 needles correlated well with the data presented for 2013 needles here, but, since some of the 2012 needles had fallen of when the scoring was carried out in 2013, the data were slightly unreliable and are therefore not presented further here.

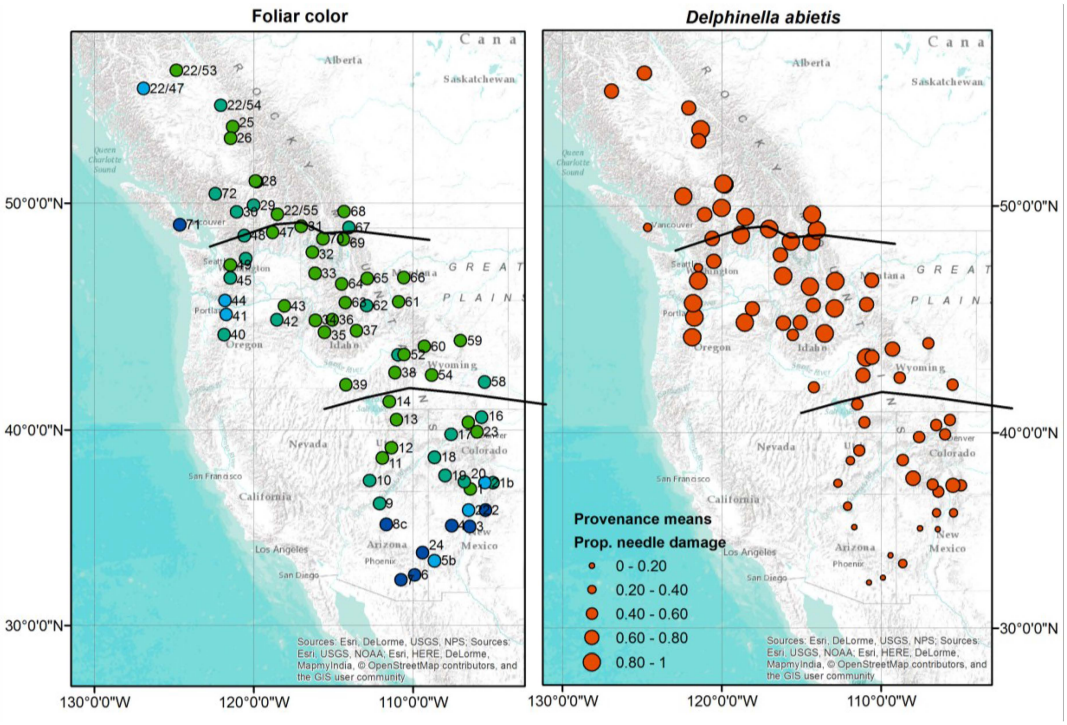

Figure 8. The 76 provenances of subalpine fir (*Abies lasiocarpa*) at Jønsberg projected on a map covering their origin in Canada and USA. Colored dots on the left map indicate the gradient of foliar color on each provenance (from blue to green). In addition, provenance numbers are given next to the dots (see Table A1 for further information about the provenances). Red dots on the map to the right show the level of infection by *Delphinella abietis* on each provenance at Jønsberg in 2013. The three regions (Northern-, Medium- and Southern latitude) are divided by two black crosslines.

3.7. Blue Needles on Subalpine Fir Has a Thicker Cuticle than Green Needles

Images from the SEM analyses are displayed in Figure 9. Generally, cuticular thickness varied a lot, even within the same needle, but was seemingly larger on the blue needles. This was confirmed by statistical analysis of the measurements taken. The cuticle of the blue needles was on average 1.04 µm thicker (17%) than on the green needles, and the difference was significant (p = 0.002) (Figure 10). In general, wax is a major component of the cuticle layers on a leaf/needle surface. From outside-in these wax layers are: epicuticular wax crystals; epicuticular wax; and intracuticular wax. Other components include cutin and polysaccarides [25].

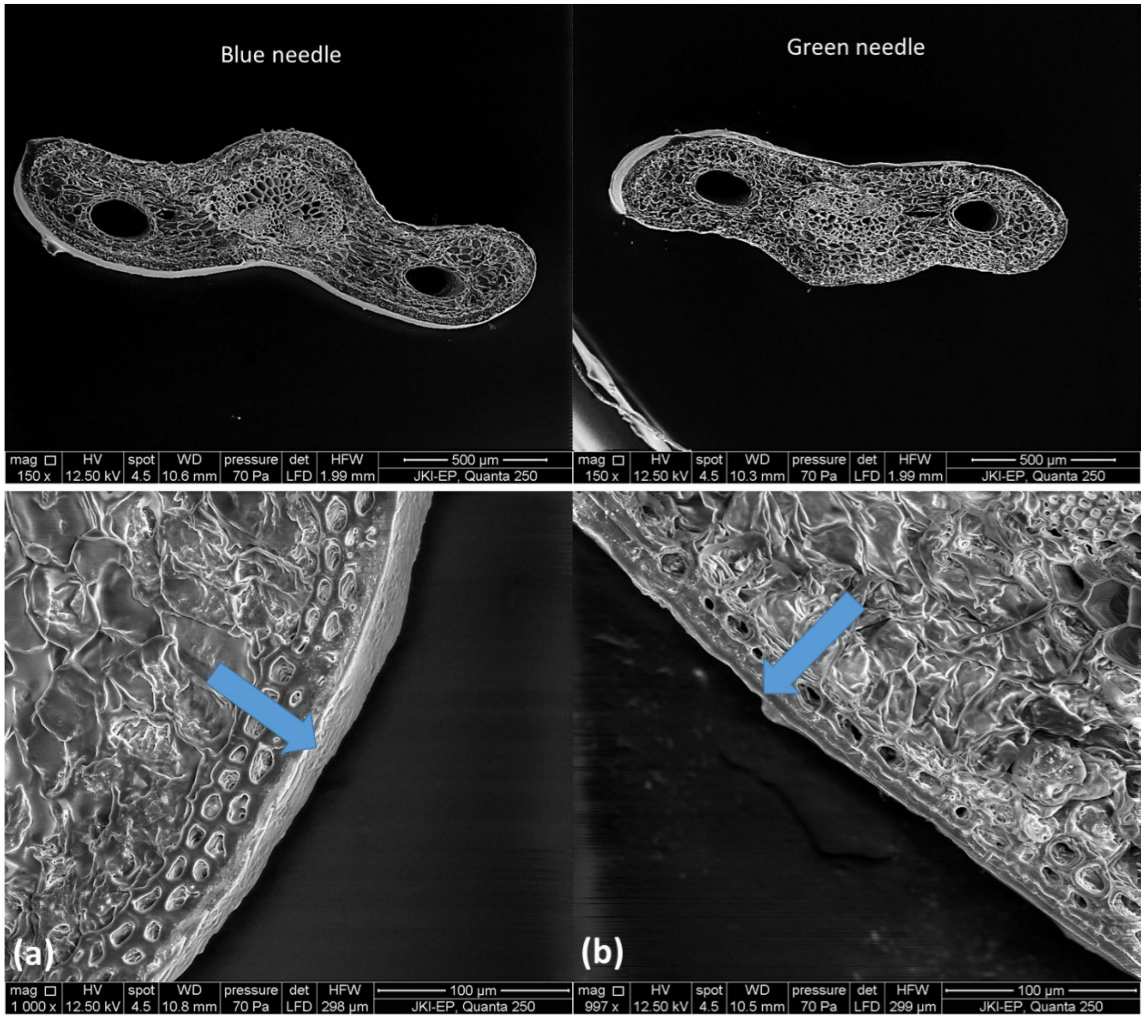

Figure 9. Cuticle (blue arrows) on two needles from subalpine fir (*Abies lasiocarpa*) from the provenance trial at Jønsberg. The cell layers beneath the cuticle are epidermis, hypodermis and mesophyll, respectively. Scale bar and other parameters (mag = magnitude, HV = high voltage, spot, WD = working distance, pressure, det, LFD = large field detector and HFW = horizontal field with) are given below each image. (**a**) Blue needle; (**b**) green needle. Photos: Corina Junker.

The fact that the blue needles on the subalpine fir had a thicker wax layer than the green is interesting, but we cannot conclude that that is the reason for the observed differences in resistance towards *D. abietis*. Although it may play a role as a physical barrier, we do not know anything about the thickness of the cuticle layer on newly emerging needles at the stage when they are prone to infection. Furthermore, this study was only done on needles from two individual trees from one field.

Further studies are necessary to reveal the mechanism of resistance. In addition to thicker wax layer, the cell layer beneath the wax seems thicker on the blue needles (Figure 9), which may play a role concerning resistance. We also need to study how *D. abietis* colonizes the needles, whether it penetrates the epidermis or enters via stomata. Being genetically close to *S. polyspora*, it may similar to that fungus produce components dissolving the wax [26].

If the thickness of the wax layer turn out to play a role concerning resistance against *D. abietis*, a method for protecting the wax, or even possibly adding wax to new, vulnerable shoots, should be investigated in the future.

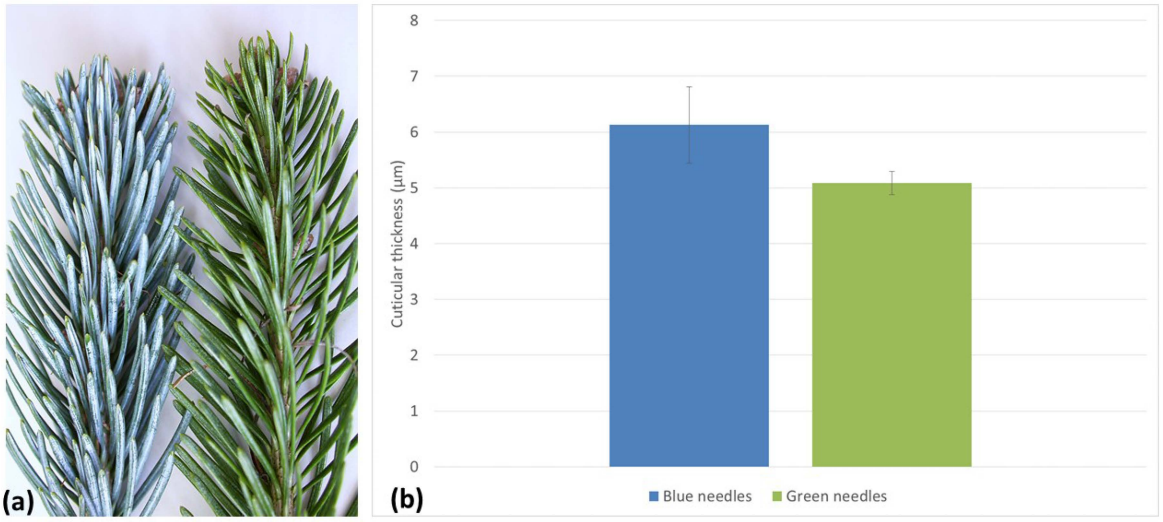

Figure 10. Results from measurements of cuticle thickness of needles from subalpine fir (*Abies lasiocarpa*) at Jønsberg. (**a**) The blue and green needles that were examined by electron microscopy; (**b**) mean cuticular thickness (µm) based on twelve measurements from each of seven green and seven blue needles. Photo: Venche Talgø.

4. Conclusions

Susceptibility towards *D. abietis* on subalpine fir is clearly dependent upon origin of seed sources in the native stands in North America. Data suggest that provenances with blue foliar color are generally more resistant to *D. abietis* than the green ones, although this is more evident in the southern part of the natural distribution area than in the PNW. Further studies of resistance mechanisms are necessary.

A selection of provenances with thick wax layer may possibly be used in the breeding of Christmas trees in the future. The variation observed within and among provenances indicates a presence of genetic variation that can be utilized to (i), in the short term, select provenances with a desired color and stronger resistance towards *D. abietis*; and (ii), in the long term, alter the needle color and improve resistance towards *D. abietis* in a breeding program.

A number of management strategies probably need to be implemented at Jønsberg to control the disease, ranging from mechanical (e.g., removal of the most susceptible trees, thinning, and good weed control) to chemical treatments. Use of fungicides have proven effective against *D. abietis* in Norway [2].On the contrary, by not controlling the disease, the most resistant material can be chosen in the future. For the Christmas tree industry, a selection of resistant provenances against *D. abietis* is a much more feasible approach for managing this disease than using fungicides, both from an economic and an environmental perspective.

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Author Contributions: Venche Talgø identified *D. abietis* in the provenance trial at Jønsberg in 2011, carried out morphological studies of the fungus, and wrote the paper in collaboration with the co-authors; Jan Ole Skage has been in charge of the provenance trial at Jønsberg since it was established in 1999, and, prior to that, he was involved in the planning and organizing of the project; Arne Steffenrem performed the statistical analyses of the field data; Corina Junker carried out the electron microscopy studies at JKI, Germany; Håvard Eikemo analyzed precipitation and electron microscopy data; May Bente Brurberg was responsible for the molecular identification of the fungus; Odd Ragnar Johnskås initiated, and was responsible for carrying out, the disease and color ratings at Jønsberg in 2013. He also participated in the seed collection in USA and Canada in 1994 and 1995.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix

Table A1. Provenance (P) means and standard errors (in brackets) for foliage color and infection level by *Delphinella abietis* registered in the subalpine fir (*Abies lasiocarpa*) provenance trial at Jønsberg in July 2013. Origin of seed sources in each state is given by longitude (Long.), latitude (Lat.) and altitude (Alt.–given in meters above mean sea level). Regions (R) reflect the three regions given in Figures 1 and 8 (1 = South, 2 = Middle, 3 = North).

R	P (No. and Name)	State	Long.	Lat.	Alt.	Color *	*D. abietis* **
1	1 Spruce Hole	Colorado	−106.417	37.1	3150	3.02(0.13)	0.54(0.05)
1	10 Duck Lake	Utah	−112.733	37.53333	2770	2.38(0.12)	0.3(0.04)
1	11 Big Lake	Utah	−111.95	38.65	2870	3.14(0.13)	0.33(0.05)
1	12 Willow Lake	Utah	−111.367	39.13333	2900	3.62(0.13)	0.5(0.04)
1	13 South Fork	Utah	−111.05	40.5	2750	3.63(0.13)	0.46(0.05)
1	14 Monte Cristo	Utah	−111.5	41.35	2700	3.37(0.14)	0.49(0.05)
1	15 Rabbit Ears Pass	Colorado	−106.55	40.36667	2900	3.41(0.13)	0.57(0.04)
1	16 Crown Point (Fort Collins)	Colorado	−105.7	40.61667	3150	2.82(0.13)	0.52(0.05)
1	17 Seaman Park	Colorado	−107.633	39.78333	2750	2.75(0.12)	0.42(0.04)
1	18 Divide Fork	Colorado	−108.667	38.68333	2700	2.57(0.13)	0.51(0.04)
1	19 The Meadows	Colorado	−108.017	37.78333	3100	2.92(0.14)	0.72(0.05)
1	2 Alamitos Creek (Gravel Pit)	New Mexico	−105.467	36.05	2900	1.41(0.13)	0.21(0.04)
1	20 Wolf Creek Pass	Colorado	−106.783	37.46667	2900	2.66(0.12)	0.49(0.04)
1	21a Apishapa River (Cordova Pass)	Colorado	−105	37.41667	3000	2.49(0.14)	0.52(0.05)
1	21b Apishapa River (Cordova Pass)	Colorado	−105.5	37.41667	3250	2.21(0.15)	0.65(0.05)
1	22 Cerro Pavo	New Mexico	−106.533	36.05	2900	1.94(0.12)	0.39(0.04)
1	23 Church Park	Colorado	−106	39.91667	2925	3.25(0.15)	0.5(0.06)
1	24 Big Lake	Arizona	−109.417	33.86667	2850	1.59(0.14)	0.14(0.05)
1	3 Sandia Crest	New Mexico	−106.45	35.21667	3100	1.67(0.12)	0.19(0.04)
1	4 La Mosca (Mount Taylor)	New Mexico	−107.583	35.26667	3200	1.08(0.12)	0.08(0.04)
1	5a Bearwallow Mtn.	New Mexico	−108.667	33.45	2950	1.79(0.13)	0.12(0.05)
1	5b Sør for Bearwallow Mtn.	New Mexico	−108.667	33.45	2950	1.74(0.13)	0.21(0.05)
1	6 Old Columbine/ Mt. Graham	Arizona	−109.9	32.7	3000	1.34(0.13)	0.15(0.05)

Table A1. *Cont.*

R	P (No. and Name)	State	Long.	Lat.	Alt.	Color *	*D. abietis* **
1	7 Mount Lemon	Arizona	−110.767	32.43333	2750	1.68(0.13)	0.19(0.05)
1	8a Agassiz Peak	Arizona	−111.7	35.33333	3000	1.45(0.12)	0.19(0.04)
1	8b Agassiz Peak	Arizona	−111.7	35.33333	3000	1.49(0.13)	0.18(0.04)
1	8c Agassiz Peak	Arizona	−111.7	35.33333	3000	1.34(0.12)	0.15(0.04)
1	9 De Motte	Arizona	−112.117	36.38333	2650	2.4(0.13)	0.24(0.05)
2	22/57 Biri Frøplantasje	Norge	10.6	60.95	190	2.51(0.13)	0.85(0.05)
2	32 Spruce Mtn.	Idaho	−116.333	47.98333	1500	3.61(0.14)	0.65(0.06)
2	33 Davies Pass	Idaho	−116.167	47.1	1250	3.13(0.13)	0.8(0.05)
2	34 Goose Lake	Idaho	−116.15	45.05	1950	3.16(0.14)	0.77(0.06)
2	35 Deadwood Summit	Idaho	−115.567	44.53333	2050	3.16(0.13)	0.56(0.05)
2	36 Williams Creek Summit	Idaho	−115.083	45.08333	2050	3.12(0.14)	0.68(0.06)
2	37 Big Eightmile	Idaho	−113.567	44.6	2250	3.13(0.14)	0.82(0.05)
2	38 Webster Ridge	Idaho	−111.15	42.7	2400	3.47(0.14)	0.74(0.06)
2	39 Monument Peak	Idaho	−114.233	42.13333	2400	3.29(0.14)	0.49(0.06)
2	40 Santiam Pass	Oregon	−121.867	44.41667	1500	2.45(0.14)	0.87(0.06)
2	41 Still Creek	Oregon	−121.733	45.31667	1450	2.33(0.14)	0.86(0.06)
2	42 Tower Mt.	Oregon	−118.567	45.08333	1650	2.98(0.14)	0.91(0.06)
2	43 Horseshoe Prairie.	Oregon	−118.083	45.68333	1450	3.37(0.14)	0.79(0.06)
2	44 Red Mtn.	Washington	−121.817	45.91667	1450	2.2(0.13)	0.95(0.05)
2	45 Chinook Pass	Washington	−121.483	46.9	1500	2.44(0.13)	0.8(0.05)
2	46 Sugarloaf Peak	Washington	−120.517	47.71667	1550	2.88(0.14)	0.69(0.06)
2	47 Handsqrabble Mtn.	Washington	−118.817	48.81667	1500	3.35(0.14)	0.87(0.05)
2	48 Rattlesnake Creek	Washington	−120.617	48.68333	1550	2.79(0.14)	0.8(0.06)
2	49 Snoqualmie Pass	Washington	−121.483	47.45	1200	3.04(0.13)	0.37(0.05)
2	51 Teton Pass	Wyoming	−110.95	43.5	2600	2.52(0.14)	0.84(0.06)
2	52 Sheep Creek	Wyoming	−110.583	43.51667	2580	3.36(0.14)	0.62(0.06)
2	54 Louis Lake	Wyoming	−108.85	42.58333	2650	3.4(0.14)	0.51(0.06)
2	58 Eagle Peak	Wyoming	−105.517	42.26667	2350	2.77(0.14)	0.5(0.06)
2	59 Munkres Pass	Wyoming	−107.033	44.15	2850	3.53(0.15)	0.58(0.06)
2	60 Wood River	Wyoming	−109.3	43.88333	2500	3.21(0.14)	0.61(0.06)
2	61 Middle Fork, Bridger	Montana	−110.917	45.86667	2000	3.36(0.14)	0.75(0.06)
2	62 Quartz Hill	Montana	−112.933	45.7	2550	2.95(0.13)	0.86(0.05)
2	63 N.Trapper Peak	Montana	−114.267	45.83333	2000	3.21(0.13)	0.63(0.05)
2	64 Lost Park	Montana	−114.483	46.65	1750	3.33(0.14)	0.91(0.06)
2	65 Marcum Mtn.	Montana	−112.9	46.88333	1500	3.5(0.14)	0.8(0.06)
2	66 Jeffersons Creek	Montana	−110.6	46.91667	2200	3.06(0.13)	0.71(0.05)
2	69 Whitefish Range	Montana	−114.383	48.53333	1800	3.35(0.13)	0.84(0.05)
2	70 Quartz Mtn.	Montana	−115.667	48.55	1750	3.38(0.14)	0.8(0.06)
3	22/47 Grizzly Lake	Br.Columbia	−126.917	54.4	1000	2.3(0.14)	0.74(0.06)
3	22/53 Inzana Lake	Br.Columbia	−124.867	55.05	1100	3(0.14)	0.8(0.06)
3	22/54 Spring Mt.	Br.Columbia	−122.083	53.78333	1220	2.97(0.14)	0.67(0.06)
3	22/55 Blue Joint	Br.Columbia	−118.517	49.55	1850	3.12(0.14)	0.93(0.06)
3	25 Cunningham Creek	Br.Columbia	−121.333	52.98333	1300	3.23(0.14)	0.9(0.06)
3	26 Cedar Creek	Br.Columbia	−121.467	52.55	1150	3.12(0.14)	0.69(0.06)
3	27 McGillvray Lake	Br.Columbia	−119.833	50.86667	1400	3.29(0.14)	0.84(0.06)
3	28 Sun Peaks	Br.Columbia	−119.917	50.88333	1200	3.37(0.14)	0.9(0.06)
3	29 Pennask Mtn.	Br.Columbia	−120.017	49.91667	1700	2.85(0.14)	0.81(0.06)
3	30 Upper Coldwater	Br.Columbia	−121.083	49.65	1200	2.79(0.14)	0.78(0.06)
3	31 Kootenai Pass	Br.Columbia	−117.033	49.06667	1650	3.19(0.14)	0.92(0.06)
3	67 Summit Lake	Alberta	−114.033	49.01667	1900	2.97(0.14)	0.81(0.06)
3	68 N. Racehorse Creek	Alberta	−114.333	49.66667	1700	3.09(0.14)	0.86(0.06)
3	71 Grassie Mtn.	Br.Columbia	−124.667	49.13333	1150	1.53(0.14)	0.34(0.06)
3	72 Duffy Lake	Br.Columbia	−122.417	50.38333	1500	2.42(0.14)	0.86(0.06)

* = scale from 1 to 4 (1 = blue, 2 = greenish blue, 3 = bluish green and 4 = green); ** = percentage of needles damaged.

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