The Impact of Phenological and Artificial Factors on Seed Quality in a Nematode-resistant *Pinus densiflora* Seed Orchard

By H. Ozawa[1)], J. Watanabe[1)], H. Chen[2)], K. Isoda[3)] and A. Watanabe[3),*)]

(Received 15th June 2007)

Abstract

To clarify the relationship between the impact of phenological and/or artificial factors on seed quality, we measured the numbers of strobili on nematode-resistant *Pinus densiflora* clones grown in an immature and relatively small scale (700 m^2) seed orchard. In addition, we established the clonal identities of all ramets, identified the paternal parent of the seeds, and assessed the resistance of seedlings to nematode infection. We also clarified the quantitative differences of strobili among clones; one clone produced 86.4% and 70.8% of all male strobili and female strobili, respectively. However, given that the total contamination ratio of the orchard was 82.0%, immigrant pollen had a larger impact on the success of actual crossing than phenology. Seedlings with a resistant maternal parent were resistant, even when their paternal parent was from outside the orchard. Two unselected clones were also planted in the seed orchard, one of which was not resistant and was associated with a maternal contribution of 34.7% of all seed stock. These findings suggest that, despite having a large impact on the crossing, immigrant pollen has a minor impact on seed resistance. Conversely, unselected and nonresistant clones have a marked impact on seed resistance. We concluded that artificial factors have larger impact on the seed quality than phenological factors in this orchard and the seeds will be of sufficient quality for supplying the market once nonresistant clones have been removed from the orchard.

Key words: nematode resistant, *Pinus densiflora*, pollen contamination, resistant ability, seed orchard, seed quality, SSR marker.

Introduction

One of the aims of clonal seed orchards is to facilitate the production of seeds with desirable genetic traits. Generally, crossing in seed orchards is primarily affected by phenological and/or artificial (human-inducible) factors, including quantitative variation in strobili between clones (Toda *et al.,* 1993; Matziris, 1997) and temporal asynchrony between maternal flowering and pollen shedding (Griffin, 1982; Erickson and Adams, 1989; Burczyk and Part, 1997; Paratainen and Pulkkinen, 2003; Owens *et al.,* 2005; Slavov *et al.,* 2005) causing differences in both the paternal (Goto *et al.,* 2002; Chaix *et al.,* 2003; Moriguchi *et al.,* 2004) and the maternal (Griffin, 1982; Matziris, 1992; Tang and Ide, 2001) contributions to the seed stock. Another phenological factor is the immigration of pollen from outside sources (Stoehr and Newton, 2002), which has resulted in contamination ratios of seed orchards ranging from 2.2% to 71.2% (El-Kassaby *et al.,* 1989; Adams *et al.,* 1997; Pakkanenn *et al.,* 2000; Goto *et al.,* 2002; Moriguchi *et al.,* 2004). Artificial factors contributing to contamination include mislabeling and misplanting of ramets (Harju and Muona, 1989; Wheeler and Jech, 1992; Kawauchi and Goto, 1999; Goto *et al.,* 2005). Taken together, these studies indicate that smaller scale and/or younger seed orchards are more susceptible to these factors.

While these studies have clarified the impacts of these negative factors on actual crossing, they have not explored how these factors affect seed quality (genetic traits). Orchard managers consider the effects of these negative factors to be minor if they have only a relatively small impact on seed quality, but the acceptable limits of such decreases in quality have not yet been ascertained. In addition, it is not yet known how soon after the establishment of a seed orchard can the seed be used to supply the market.

In the present study, we examined a *Pinus densiflora* seed orchard comprised of clones that are resistant to the pine wood nematode (*Bursaphelenchus xylophilus*). Pine species are one of the most important resources for environmental conservation in Japan because pine forests constitute approximately 10% of total forested area and also because the coastal vegetation in Japan is dominated pine species. However, over the last five decades, pine species such as *P. densiflora* and *P. thunbergii* have been seriously affected by pine wilt disease caused by the pine wood nematode (Fujimoto *et al.,* 1989). A breeding project employing nematode inoculation tests to select resistant trees from natural forests and their subsequent establishment in seed orchards was initiated in 1978 to improve the resistance of trees to the pine wood nematode (Fujimoto *et al.,* 1989). As a result of these efforts, resistant *P. densiflora* and *P. thunbergii* seed orchards have been established in Japan. These resistant seed orchards have generally been managed on a smaller scale than the seed orchards used for the timber industry and have been used to supply the market with seeds.

Nematode resistance in *Pinus* spp. and the associated genetic characteristics of this resistance are still unknown. Resistance has been observed to vary among pine species (Futai and Furuno, 1979); for example, resistance in *P. thunbergii* was found to be lower than in *P. densiflora*. In addition, the seed resistance of *P. thun-*

1) Fukushima Prefectural Forestry Research Centre, 1 Nishijimasaka, Narita, Asaka-machi, Koriyama 963-0112, Japan.

2) Hubei Provincial Forestry Bureau, 355 Xiongchudajie, Hongshan, Wuhan 430079, China.

3) Forest Tree Breeding Center, Independent Administrative Institution, 3809-1 Ishi, Juo, Hitachi, Ibaraki 319-1301, Japan.

*) The communicating author: A. Watanabe.
E-Mail: nabeatsu@affrc.go.jp

DOI:10.1515/sg-2009-0019
edited by Thünen Institute of Forest Genetics

bergii is reduced considerably by immigrant pollen (GOTO *et al.,* 2002). On the other hand, the effect of phenological and/or artificial factors on the seed resistance in *P. densiflora* is still unknown.

In order to assess the effect of these factors on seed quality (nematode resistance), we selected a relatively small and young resistant *P. densiflora* seed orchard that was susceptible to these factors and conducted the following field measurements and DNA analyses using SSR or microsatellite markers: 1) flowering characteristics of the clones; 2) clonal identity of all ramets; 3) crossing dynamics; 4) seed resistance.

Materials and Methods

Seed orchard

The nematode-resistant *P. densiflora* seed orchard investigated was located in central Japan (37°51' N, 140°53' E, 68 m a.s.l.) (*Fig. 1*). According to GIERTYCH (1965), the 700-m^2 seed orchard was initially laid out as follows: the clonal ramets of ten resistant clones were planted at 3.5-m intervals throughout the orchard in 1998 (*Table 1*). Another six resistant clones were planted in 2002 to give a total of 422 ramets in 2006.

Phenological measurements

The number of male and female strobili on five ramets of each clone was counted in May 2003 (*Fig. 2a*). All of the cones from these ramets were collected in October 2004, and the weight of the seeds collected from these cones was measured.

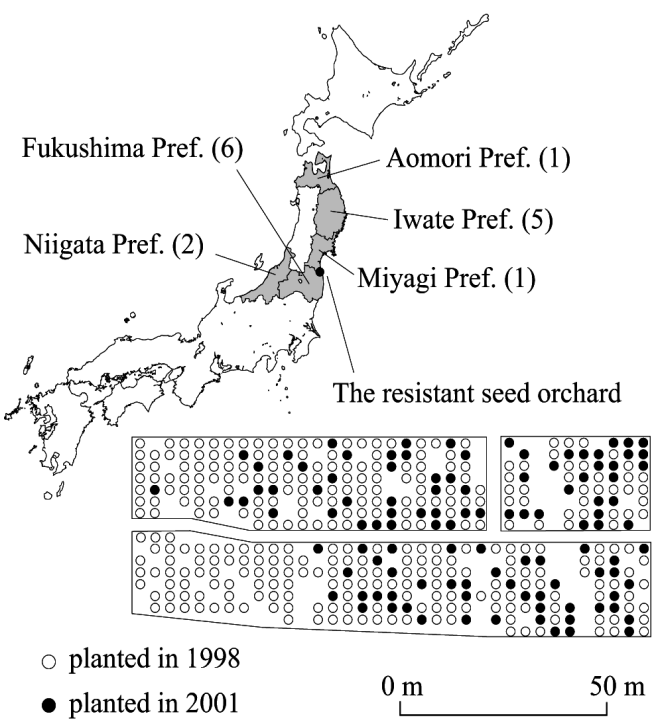

Figure 1. – Location and ramet allocation of the nematode-resistant *P. densiflora* seed orchard in Fukushima Prefecture. Gray areas indicate prefectures from which ortets were selected and figures in parenthesis show the number of ortets from which clones were derived for planting in the seed orchard.

Clonal identification

DNA was extracted from several needles collected from all ramets in July 2006 using a CTAB extraction protocol (SHIRAISHI and WATANABE, 1995). Microsatellite loci were amplified using the five primer pairs, pdms 009 (WATANABE *et al.,* 2006), pde14 (LIAN *et al.,* 2000), bcpd006 (GOTO *et al.,* 2005), bcpd502 and bcpd222 (ISODA and WATANABE, unpublished data). Genotypes were scored on the basis of PCR product length for each locus using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA) and GENESCAN (ver. 3.7) and GENOTYPER (ver. 2.0) genetic analysis software (Applied Biosystems).

Paternal analysis

Two to four ramets of six clones (Sanbongi-5, Kamiheii-101, Miyagi-101, Kariha-102, Iwaki-8 and Iwaki-26) were selected for paternal analysis (*Fig. 2b*). All of the cones from these ramets were collected in October 2004, and 100–200 seedlings per ramet were used for DNA analysis. DNA was extracted and genotyped using the methods described above. The paternal alleles of the seeds at every locus were inferred by subtracting the maternal alleles from the offspring alleles. A likelihood criterion allowing for pollen immigration was employed for the paternal parentage analysis (GERBER, 2000; CHAIX *et al.,* 2003) using the FaMoz software program (GERBER *et al.,* 2003).

Nematode inoculation test

The materials used for the nematode inoculation test consisted of 762 seedlings from five clones (Sanbongi-5, Ojika-102, Kariha-102, Kitakanbara-2 and Miyagi-101). Seeds were collected in 2002, and the seedlings were raised in the nursery of the Fukushima Prefectural Forest Research Centre (37°21' N, 140°20' E, 254 m a.s.l.) from 2003 to 2005 for use as the test seedlings. They were three years old at the time tests were conducted. The seedlings were transplanted into plastic pots (approx. 19 cm diam., 16 cm deep) in March 2005. The mean height of seedlings (incl. pots) was 49.1 ± 5.2 cm (± SD, n = 160) and the mean diameter at the ground surface was approximately 10 mm in July 2005.

The resistance controls were 100 three-year-old, open-pollinated seedlings from five clones (Sanbongi-3, Iwate-104, Ichinoseki-101, Iwaizumi-101, Kitakanbara-2). The resistance of these clones was comparable to that of *P. taeda*, which is considered to be the resistance standard in Japan (TERADA *et al.,* 1997). They were provided as one-year-old seedlings by the Forest Breeding Center in April 2004 and were raised in the same nursery as the resistance controls. The seedlings, transplanted into unglazed pots (approx. 24 cm diam., 21 cm deep) in March 2005, had a mean height (incl. pot) was 54.1 ± 9.1 cm (± SD, n = 100), and the mean diameter at the ground surface was approximately 12 mm in July 2005.

Paternal parentage and clonal identification analysis of the test seedlings were performed immediately before the test, and the methods used for DNA extraction, genotype scoring and paternal analysis were the same as those described above.

DOI:10.1515/sg-2009-0019
edited by Thünen Institute of Forest Genetics

Table 1. – The profiles of resistant clones planted in the seed orchard.

Clone	abb.	No.	Planting		H (cm)		DBH (cm)		Location of ortets
			yr.	no.	mean	SD	mean	SD	pref.
Sanbongi-5	Sa	1	1998	35	354	20	5,5	0,5	Aomori
Ichinoseki-101	Ic	5	1998	33	289	28	3,8	0,8	Iwate
Iwaizumi-101	Ii	4	1998	30	309	31	3,4	1,1	Iwate
Iwate-104	It	2	1998	7	301	50	2,4	1,2	Iwate
Kamiheii-101	Km	6	1998	35	358	59	4,8	0,8	Iwate
Morioka-1	Mo	3	1998	14	246	45	2,0	0,7	Iwate
Miyagi-101	Mi	8	1998	39	315	49	4,4	1,2	Miyagi
Ojika-102	Oj	7	1998	35	254	41	3,2	0,9	Miyagi
Kariha-102	Ka	10	1998	43	313	22	4,9	0,8	Niigata
Kitakanbara-2	Ki	9	1998	43	283	53	4,2	1,2	Niigata
Iwaki-8	I8	14	2001	19	107	17	1,8	0,2	Fukushima
Iwaki-23	I23	11	2001	27	97	32	1,4	0,4	Fukushima
Iwaki-25	I25	13	2001	21	92	11	1,7	0,2	Fukushima
Iwaki-26	I26	12	2001	31	114	26	1,7	0,4	Fukushima
Iwaki-91	I91	16	2001	5	89	25	1,7	0,2	Fukushima
Iwaki-94	I94	15	2001	8	103	20	2,0	0,3	Fukushima

The tree height and DBH was measured in 2003. Measured ramets were showed in *Fig. 2 (a)*.

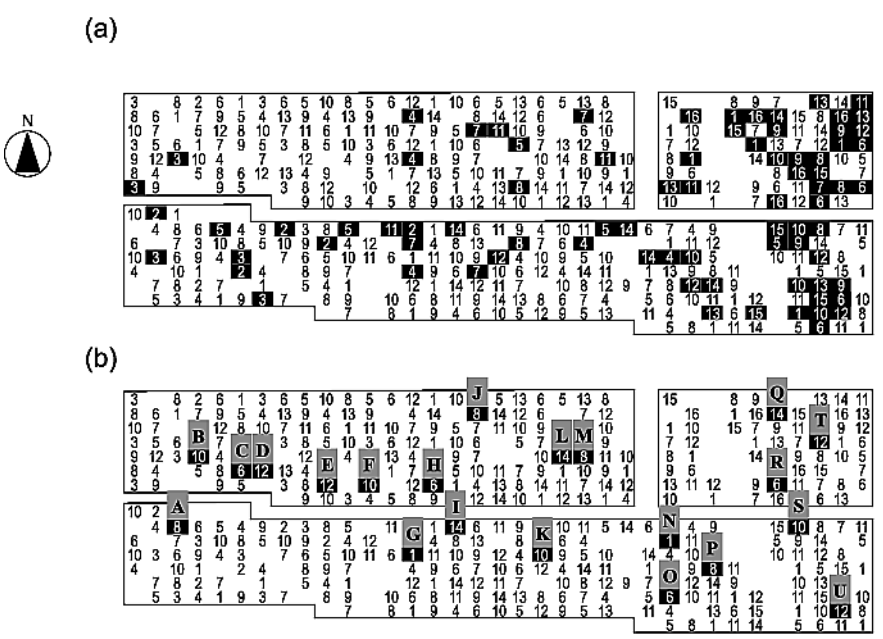

Figure 2. – Map of the resistant seed orchard with figures showing the clone names indicated in *Table 1.* a) Black boxes show the ramets used for strobili counts. b) Black boxes with A-U flags show ramets subjected to paternal parent analysis using seeds.

The inoculation tests were performed in a vinyl greenhouse (18.2 m long, 4.6 m wide, 2.7 m high). The test seedlings for each clone were divided into five groups using a randomized block design and a series of the resistant controls composed of four clones is positioned in the greenhouse. All of the seedlings were inoculated with 0.1 ml of water containing 10000 pine wood nematodes (isolate name: *Shimabara*) using a micropipette on July 5, 2005. After inoculation, all seedlings were watered with 200–450 ml of water via a sprinkler at 6–15 day intervals. The air temperature and humidity in the center of the greenhouse were monitored at hourly intervals at a location 35 cm above the floor surface. Survival was assessed ten weeks after inoculation, which is considered sufficient for the evaluation of resistant traits of the seedlings (FUJIMOTO *et al.,* 1989).

Results

Note that clone names are given in abbreviated form in the Results and Discussion sections (see *Table 1* for definitions).

DOI:10.1515/sg-2009-0019
edited by Thünen Institute of Forest Genetics

Quantities of strobili and seeds from clones

The number of male and female strobili differed markedly among clones (*Table 2*). The Sa clones produced the highest number of male and female strobili at 86.4% and 70.8%, respectively. Ic produced the second-highest number of male strobili (6.1%) and Ka produced the second-highest number of female strobili (17.8%). Other clones produced considerably fewer strobili than the Sa or Ic clones. The differences in the seed products reflected differences in the numbers of female strobili. Sa produced the greatest amount of seed, 344.3 g in total, representing 69.5% of all seed produced.

Clone identification and paternal analysis

Except for Sa, which consisted of three genotypes (n=35), each of the clones contained only one genotype, implying that the ramets were true ramets of each clone. One Sa genotype, SaA (n=18), was the same as

Table 2. – The amount of male and female flowers, and seed products of each clone.

Clone	no. of flowers and seed products / tree							
	Male			Female			Seed	
	mean	SD	%	mean	SD	%	total (g)	%
Sa	567,2	253,4	86,4	447,6	114,2	70,8	344,3	69,5
Ic	39,8	37,3	6,1	2,0	2,3	0,3	2,8	0,6
Ii	0,2	0,4	0,0	1,2	1,3	0,2	0,6	0,1
It	0		0	7,2	4,8	1,1	5,9	1,2
Km	0,2	0,4	0,0	3,4	2,6	0,5	2,0	0,4
Mo	3,2	3,6	0,5	7,4	2,8	1,2	6,3	1,3
Mi	6,0	6,4	0,9	26,0	11,4	4,1	70,3	14,2
Oj	12,6	9,5	1,9	1,2	2,2	0,2	1,0	0,2
Ka	0,2	0,4	0,0	112,6	51,8	17,8	45,0	9,1
Ki	7,0	2,8	1,1	12,4	2,2	2,0	7,8	1,6
I8	1,8	2,4	0,3	6,0	1,7	0,9	7,1	1,4
I23	3,4	5,0	0,5	0,4	0,9	0,1	0	0
I25	5,0	5,8	0,8	0,4	0,5	0,1	0,2	0,0
I26	3,4	7,6	0,5	2,2	2,3	0,3	1,9	0,4
I91	6,2	5,0	0,9	0,8	1,3	0,1	0	0
I94	0,6	1,3	0,1	1,2	2,7	0,2	0,2	0,0
Total							495,3	100

Measured ramets were showed in *Fig. 2 (a)*.

Table 3. – Results of the pollen parentage analysis of the seed.

Position	Clone	*n*		Candidate of pollen parent																													
				Outside		Inside																											
								Candidate of internal pollen parent																									
								SaA		SaB		SaC		Ic		Ii		Km		Mo		Mi		Oj		Ka		Ki		I23		I25		I26		I91	
		no.	%																														
A	Mi	29	(100)	26	(89,7)	3	(10,3)					1	(3,4)	2	(6,9)																		
B	Ka	96	(100)	89	(92,7)	7	(7,3)					1	(1,0)	3	(3,1)					2	(2,1)							1	(1,0)						
C	Km	86	(100)	81	(94,2)	5	(5,8)	2	(2,3)					1	(1,2)					1	(1,2)													1	(1,2)
D	I26	-	(100)																														
E	I26	14	(100)	12	(85,7)	2	(14,3)	2	(14,3)																								
F	Ka	120	(100)	113	(94,2)	7	(5,8)	2	(1,7)			1	(0,8)							2	(1,7)					1	(0,8)							1	(0,8)
G	SaA	113	(100)	101	(89,4)	12	(10,6)	6	(5,3)			4	(3,5)											2	(1,8)										
H	Km	80	(100)	73	(91,3)	7	(8,8)			2	(2,5)	2	(2,5)							1	(1,3)									1	(1,3)			1	(1,3)
I	I8	94	(100)	82	(87,2)	12	(12,8)	2	(2,1)	1	(1,1)	5	(5,3)					1	(1,1)					1	(1,1)					1	(1,1)			1	(1,1)
J	Mi	73	(100)	61	(83,6)	12	(16,4)			3	(4,1)	8	(11,0)	1	(1,4)																		
K	Ka	77	(100)	71	(92,2)	6	(7,8)	1	(1,3)	1	(1,3)	3	(3,9)											1	(1,3)										
L	I8	60	(100)	24	(40,0)	36	(60,0)	2	(3,3)	1	(1,7)	33	(55,0)																						
M	Mi	110	(100)	79	(71,8)	31	(28,2)	2	(1,8)	1	(0,9)	26	(23,6)											1	(0,9)									1	(0,9)
N	SaA	96	(100)	79	(82,3)	17	(17,7)	2	(2,1)	7	(7,3)	7	(7,3)											1	(1,0)										
O	Km	45	(100)	36	(80,0)	9	(20,0)			1	(2,2)	8	(17,8)																						
P	Mi	57	(100)	50	(87,7)	7	(12,3)	1	(1,8)			6	(10,5)																						
Q	I8	84	(100)	39	(46,4)	45	(53,6)			1	(1,2)	38	(45,2)	1	(1,2)	1	(1,2)					1	(1,2)	2	(2,4)									1	(1,2)
R	Km	95	(100)	84	(88,4)	11	(11,6)			2	(2,1)	6	(6,3)							1	(1,1)							1	(1,1)					1	(1,1)
S	Ka	116	(100)	98	(84,5)	18	(15,5)	2	(1,7)	7	(6,0)			1	(0,9)			1	(0,9)	1	(0,9)			1	(0,9)			1	(0,9)	2	(1,7)	1	(0,9)	1	(0,9)
T	I26	63	(100)	36	(57,1)	27	(42,9)			2	(3,2)	22	(34,9)	1	(1,6)							2	(3,2)												
U	I26	44	(100)	39	(88,6)	5	(11,4)					3	(6,8)	1	(2,3)																	1	(2,3)		
Total		1552	(100)	1273	(82,0)	279	(18,0)	24	(1,5)	29	(1,9)	174	(11,2)	11	(0,7)	1	(0,1)	2	(0,1)	8	(0,5)	3	(0,2)	9	(0,6)	1	(0,1)	3	(0,2)	2	(0,1)	3	(0,2)	1	(0,1)	8	(0,5)
							(100)		(8,6)		(10,4)		(62,4)		(3,9)		(0,4)		(0,7)		(2,9)		(1,1)		(3,2)		(0,4)		(1,1)		(0,7)		(1,1)		(0,4)		(2,9)

I26 (D) was unable to analyze because it did not have a cone. Position of the ramets were showed in *Fig. 2 (b)*.

DOI:10.1515/sg-2009-0019
edited by Thünen Institute of Forest Genetics

Table 4. – Results of pollen parentage analysis of the inoculated seedlings.

Clone		*n*	Candidate of pollen parent				UA	SU
			Total		outside	inside		
			no.	%				
	A		40	(100)	40 (100)	0 (0)		
Sa	B	161	29	(100)	29 (100)	0 (0)	2	6
	C		84	(100)	84 (100)	0 (0)		
Oj		117	116	(100)	112 (97,4)	4 (2,6)	1	
Ka		160	157	(100)	154 (98,1)	3 (1,9)	3	
Ki		161	158	(100)	157 (99,4)	1 (0,6)	3	
Mi		163	162	(100)	160 (98,8)	2 (1,2)	1	
Total		762	746	(100)	736 (98,7)	10 (1,3)		

Abbreviations are unable to analyze (UA) and seed parent unknown (SU).

Table 5. – Results of the nematode inoculation test.

	Clone	*n*		Dead		Survival	
				%	SD	%	SD
Seedling	Sa	161	(39)	58,5	4,1	41,5	4,1
	SaA	40	(14)	50,0		50,0	
	SaB	29	(5)	45,8		54,2	
	SaC	84	(18)	65,2		34,8	
	Oj	117	(22)	45,0	17,6	55,0	17,6
	Ka	160	(19)	47,0	17,8	53,0	17,8
	Ki	161	(39)	51,9	27,1	48,1	27,1
	Mi	163	(34)	58,2	7,3	41,8	7,3
Control		100		60,0		40,0	

% of the seedling was showed the mean of five groups. % of the control was the total of five families. There were not significant differences among groups and clones (Kruskal-Wallis test, $P < 0.01$). Figures in the brackets showed the number of uninoculated samples.

the Sanbongi-5 genotype maintained at the Forest Breeding Center. The other two genotypes, SaB (n=3) and SaC (n=14) did not correspond to any of the clones planted in the seed orchard or maintained by the Forest Breeding Center. SaA ramets were predominantly found on the western side of the orchard, while SaC ramets were mainly located on the eastern side. Ramets of SaB and SaC represented 4% of all the ramets planted.

Due to pollen immigration, almost all of the paternal parents of the seeds were from outside the orchard (*Table 3*). Km (C in *Fig. 2b*) and Ka (F) had the highest contamination ratios among all the ramets analyzed (94.2%) and I8 (L) had the lowest (40.0%). The total contamination ratio of the orchard was 82.0%, which meant that only 18.0% of all the analyzed seeds had a paternal parent that was a planted clone. Among the planted clones, the three Sa clones (SaA-C) contributed most to internal paternal parentage (81.4%), with the unselected SaC clone contributing 62.0% and SaA and SaB contributing 9.0% and 10.4%, respectively. The remaining 15 clones contributed 18.6% to the total internal paternal parentage (only 2.1% of all seeds analyzed).

Only 10 of the 1552 seeds analyzed were found to be self-fertilized, 8 from Sa (G and N), 1 from Ka (F), and 1 from I26 (U).

Inoculation tests

Immigrant pollen accounted for almost all of the paternal parents of the test seedlings (*Table 4*). Contamination ratios ranged from 97.4% (Oj family) to 100% (Sa family) and the total contamination ratio for the five families was 98.7%.

The air temperature in the greenhouse ranged from 16.7 to 52.2°C, with a mean temperature of less than 33.1°C. The relative humidity ranged between 68.8 and 97.7%, with a mean of above 68.8%.

Several of the surviving seedlings did not exhibit wilting at the site of inoculation, and some actually sprouted second shoots from this point (*Table 5*). These seedlings were assumed to have not been adequately inoculated and were excluded from the results and analysis.

The total survival ratio of the resistant controls was 40.0%. Test seedlings from the Oj, Ka, Ki and Mi were considered to be resistant because they had survival ratios of 41.8–55.0%, which were higher than those of the resistant controls (*Table 5*). SaA and SaB seedlings from had survival ratios exceeding 50% and were considered to be resistant, while SaC seedlings had a survival ratio of only 34.8% and were not considered to be resistant.

Discussion

In this nematode-resistant *P. densiflora* seed orchard, we found that despite the maternal contribution of clones being markedly unequal and the crossing dynamics being strongly influenced by immigrant pollen, resistance was maintained in seedlings from resistant maternal parents. Interestingly, unselected and nonresistant clones had a greater impact on resistance than immigrant pollen, and our data showed that resistance of *P. densiflora* in this orchard was more greatly influenced by artificial factors than by phenological factors.

DOI:10.1515/sg-2009-0019
edited by Thünen Institute of Forest Genetics

Differences in strobili and seed production among clones within a seed orchard have been reported previously (MATZIRIS, 1997) and quantitative differences among strobili from different *P. thunbergii* clones are known to be genetically fixed (TODA *et al.,* 1993). Similarly, in this study, the number of male and female strobili produced differed greatly among clones, and this tendency was also reflected in the different quantities of seeds produced by the different clones (*Table 2*); 86.4% of all male strobili and 70.8% of all female strobili were borne by Sa clones. In addition, Sa produced approximately 71% of all seeds. In the *P. taeda* seed orchard study of BYRAM *et al.* (1986), more than 15 years elapsed before 60% of the clones were involved in crosses as parents producing 90% of the seed. These findings suggest that, even though the number of planted clones involved in the production of seed is likely to increase over time, a quantitative imbalance with respect to flowering will be maintained in the orchard.

Compared to the flowering bias of the clones, with a total contamination ratio of 82.0%, pollen contamination had a greater impact on the seed harvested in 2004 (*Table 3*). This suggests that the amount of pollen around female strobili from male strobili within the seed orchard was markedly lower than the amount of immigrant pollen which has been observed to be maintained at constant levels for a few years in a Picea abies seed orchard (PAKKANEN *et al.,* 2000). The findings suggest that if the total amount of internal pollen does not increase significantly, and if the homogeneous distribution of the surrounding forest does not change markedly, high levels of pollen contamination can be expected.

In this orchard, because the number of strobili of the six clones planted in 2002 has increased markedly over time (OZAWA *et al.,* 2005)., it is possible that the order of seed production by each clone may change even if a quantitative flowering imbalance is maintained However, given the small size of the seed orchard and absolute internal pollen deficiency, it appears likely that a high level of contamination will be maintained in the future.

Immigrant pollen was also observed to have a considerable impact on the amount of seed harvested two years previously in 2002 (*Table 4*). Nevertheless, test seedlings from Oj, Ka, Ki and Mi were found to be more resistant than the resistant controls (*Table 5*), suggesting that the resistance of the seeds harvested in 2004 would be maintained, even though 82.0% of the paternal parentage was attributed to immigrant pollen.

One reason why immigrant pollen is thought to have only a slight impact on resistance is because maternally conferred resistance is more important than paternally conferred resistance. In nematode inoculation tests, the survival ratio of *P. densiflora* seedlings from parents that were both resistant was 94.0%, but this ratio declined to 69.5% when the paternal parent was not resistant (HANDA *et al.,* 1995). The 69.5% decline in the survival ratio observed by Handa et al. is greater than that observed in the resistance standard, *P. taeda*, because the general survival ratio of *P. taeda* is 30–50% (OKADA and TSUDA, 1989; TAKEUCHI *et al.,* 1989; TODA *et al.,* 1989). In other resistant *P. densiflora* orchards, the survival ratio for many seedlings after natural crossing was generally higher than that observed in *P. taeda* (OKADA and TSUDA, 1989; TAKEUCHI *et al.,* 1989; TODA *et al.,* 1989; ENDO and NAKAGAWA, 2005).

Another reason why immigrant pollen is thought to have only a slight impact on resistance is because some of the pollen in wild *P. densiflora* forest is shed by resistant individuals. There are numerous *P. densiflora* forests surrounding the orchard and not all have been damaged by the pine wood nematode. However, because resistant individuals represent only 0.8% of the total population in wild P. densiflora forests, it is unlikely that all of the immigrant pollen originates from males with high resistance (FUJIMOTO *et al.,* 1989).

Artificial factors also influence crossing. For example, unselected clones (SaB and SaC) were detected in this orchard, and although they represented only 4% of all planted ramets, they accounted for 72.4% of the total internal paternal parentage and 13.0% of the total paternal parentage. However, as the maternal parent, SaB only had a minor impact on resistance because the test seedlings of this clone had a higher survival ratio (54.2%) than the controls.

But, considering that the survival ratio of widely distributed unselected *P. densiflora* seedlings is less than 30%, this level of resistance is unusually high for the seedlings from unselected clones (OKADA and TSUDA, 1989; TODA, 2004). One of the reasons why the test seedlings from SaB exhibited higher resistance than controls or unselected individuals is that the ramets of SaB that were mistakenly introduced at the time of the clonal proliferation originally had higher resistance. The ten clones planted in 1998 in this orchard were selected from among populations considered to have the most desirable characteristics for the timber industry (*Table 1*). Specifically, these "elite" trees (and their open-pollinated seedlings) generally exhibited higher levels of resistance than unselected trees (TODA *et al.,* 1993; TODA, 2004).

Another unselected clone, SaC, was observed to have a marked impact as a maternal parent and most of the test seedlings obtained from SaC had a lower survival ratio (34.8%) than the controls (*Table 5*). Given that 52.2% of the Sa seeds produced were SaC (84/161) (*Table 2*), which represented 34.7% of all seed produced (344.3*0.52/495.3), SaC was considered to have largest maternal contribution among all clones (*Table 4*). In other words, almost 35% of the total seed produced in 2004 was not resistant, which would have a serious impact on seed orchard management.

We concluded that after removing the unselected and nonresistant clones from this orchard, the seeds obtained from this young, small-scale seed orchard could be used to supply the market. But, to resolve these crossing problems it is necessary to improve the resistance of the seed as soon as possible. It is also necessary for orchard managers to implement additional measures, increasing the contribution of planted clones to seed production by employing methods such as supplemental mass pollination or artificial crossing to suppress pollen contamination (EL-KASSABY and RITLAND, 1986; ERIKSSON *et al.,* 1995; STOEHR *et al.,* 1998; STOEHR *et al.,* 2006).

DOI:10.1515/sg-2009-0019
edited by Thünen Institute of Forest Genetics

Literature cited

ADAMS, W. T., V. D. HIPKINS, J. BURCZYK and W. K. RANDALL (1997): Pollen contamination trends in a maturing Douglas-fir seed orchard. Can J For Res **27**: 131–134.

BURCZYK, J. and D. PART (1997): Male reproductive success in *Pseudotsuga menziesii* (Mirb.) Franco: the effects of spatial structure and flowering characteristics. Heredity **79**: 638–647.

BYRAM, T. D., W. J. LOWE and J. A. MCGRIFF (1986): Clonal and annual variation in cone production in loblolly pine seed orchards. Forest Science **32**: 1067–1073.

CHAIX, G., S. GERBER, V. RAZAFIMAHARO, P. VIGNERON, D. VERHAEGEN and S. HAMON (2003): Gene flow estimation with microsatellites in a malagasy seed orchard of *Eucalyptus grandis*. Theor Appl Genet **107**: 705–712.

DI-GIOVANNI, KEVAN P. G. (1991): Factors affecting pollen dynamics and its importance to pollen contamination: a review. Can J For Res **21**: 1155–1170.

EL-KASSABY, Y. A. and K. RITLAND (1986): The relation of outcrossing and contamination to reproductive phenology and supplemental mass pollination in a Douglas-fir orchard. Silvae Genetica **35**: 240–244.

EL-KASSABY, Y. A., D. RUDIN and R. YAZDANI (1989): Levels of outcrossing and contamination in two *Pinus sylvestris* L. seed orchards in northern Sweden. Scand J For Res **4**: 41–49.

ENDO, R. and S. NAKAGAWA (2005): Evaluation of families produced in a pinewood seed orchard selected through the 'Breeding Project on Resistance to Pine-wood Nematodes' (Chiba Pref.) For Tree Breed **215**: 7–10 (in Japanese with English summary).

ERICKSON, V. J. and W. T. ADAMS (1989): Mating success in a coastal Douglas-fir seed orchard as affected by distance and floral phenology. Can J For Res **19**: 1248–1255.

ERIKSSON, U., G. JANSSON, R. YAZDANI and L. WILHELMSSON (1995): Effects of supplemental mass pollination (SMP) in a young and a mature seed orchard of *Pinus sylvestris*. Tree Physiology **15**: 519–526.

FUJIMOTO, Y., T. TODA, K. NISHIMURA, H. YAMATE and S. FUYUNO (1989): Breeding project on resistance to the pine-wood nematode. An outline of the research and the achievement of the project for ten years. Bull For Tree Breed Inst **7**: 1–84 (in Japanese with English summary).

FUTAI, K. and T. FURUNO (1979): The variety of resistances among pine-species to pine wood nematode, *Bursaphelenchus lignicolus*. Bulletin of the Kyoto university forests **51**: 23–36.

GERBER, S., S. MARIETTE, R. STREIFF, C. BODENES and A. KREMER (2000): Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. Molecular Ecology **9**: 1073–1048.

GERBER, S., P. CHABRIER and A. KREMER (2003): FaMoz: a software for parentage analysis using dominant, codominant and unparentally inherited markers. Molecular Ecology Notes **3**: 479–481.

GIERTYCH, M. M. (1965): Systematic lay-outs for seed orchards. Silvae Genetica **14**: 91–94.

GOTO, S., F. MIYAHARA and Y. IDE (2002): Monitoring male reproductive success in a Japanese black pine clonal seed orchard with RAPD markers Can J For Res **32**: 983–988.

GOTO, S., A. WATANABE, F. MIYAHARA and Y. MORI (2005): Reproductive success of pollen derived from selected and non-selected sources and its impact on the performance of crops in a nematode-resistant Japanese black pine seed orchard. Silvae genetica **54**: 69–76.

GOTO, S., F. MIYAHARA and Y. IDE (2002): Contribution of pollen parents to the nematode-resistance in seedlings of Japanese black pine. J Jap For Soc **84**: 45–49 (in Japanese with English summary).

GRIFFIN, A. R. (1982): Clonal variations in radiata pine seed orchard. I. Some flowering, cone and seed production traits. Aust J For Res **12**: 295–302.

HANDA, T., K. KATOH, T. UEKI and K. KAWAMURA (1995): The decline of the resistant ability of the resistant clone crossing with non-resistant pollen. Trans. 106rd Mtg. Jpn. For. Soc.: 295–296 (in Japanese).*)

HARJU, A. and O. MUONA (1989): Background pollination in *Pinus sylvestris* seed orchards. Scand J For Res **4**: 513–520.

KAWAUCHI, H. and S. GOTO (1999): Monitoring clonal management in nematode-resistant Japanese black pine seed orchard in Kagoshima Prefecture. J Jpn For Soc **81**: 338–340 (in Japanese with English summary).

LIAN, C., M. MIWA and T. HOGETSU (2000): Isolation and characterization of microsatellite loci from the Japanese red pine, *Pinus densiflora*. Mol Ecol **9**: 1186–1187.

MATZIRIS, D. (1992): Variation in cone production in a clonal seed orchard of black pine. Silvae Genetica **42**: 136–141.

MATZIRIS, D. (1997): Variation in growth, flowering and cone production in a clonal seed orchard of Aleppo pine grown in Greece. Silvae Genetica **46**: 224–228.

MORIGUCHI, Y., H. TAIRA, N. TANI and Y. TSUMURA (2004): Variation of paternal contribution in a seed orchard of *Cryptomeria japonica* determined using microsatellite markers. Can J For Res **34**: 1683–1690.

OKADA, S. and T. TSUDA (1989): Selection of resistant pine trees to the pine-wood nematode in Kinki and Setonaikai. Bull For Tree Breed Inst **7**: 85–118 (in Japanese with English summary).

OWENS, J. N., J. BENNETT and S. L'HIRONDELLE (2005): Pollination and cone morphology affect cone and seed production in lodepole pine seed orchards. Can J For Res **35**: 383–400.

OZAWA, H., H. L. CHEN, J. WATANABE and A. WATANABE (2005): The vision of breeding project of pine-wood resistant to nematode in Fukushima prefecture. For Tree Breed **217**: 1–5 (in Japanese with English summary).

PAKKANEN, A., T. NIKKANEN and P. PULKKINEN (2000): Annual variation in pollen contamination and outcrossing in a *Picea abies* seed orchard. Scand J For Res 15:399-404.

PARATAINEN, A. and P. PULKKINEN (2003): Flowering and Airborne Pollen Occurrence in a *Pinus sylvestris* Seed Orchard Consisting of Northern Clones. Scand J For Res **18**: 111–117.

SHIRAISHI, S. and A. WATANABE (1995): Identification of chloroplast genome between *Pinus densiflora* Sieb. et Zucc and *P. thunbergii* Parl. based on the polymorphism in *rbc*L gene. J Jap For Soc **77**: 429–436 (in Japanese with English summary).

SLAVOV, G. T., G. T. HOWE and W. T. ADAMS (2005): Pollen contamination and mating patterns in a Douglas-fir seed orchard as measured by simple sequence repeat markers. Can J For Res **35**: 1592–1603.

STOEHR, M., H. MEHL, G. NICHOLSON, G. PIEPER and C. NEWTON (2006): Evaluating supplemental mass pollination efficacy in a lodgepole pine orchard in British Columbia using chloroplast DNA markers. New Forests **31**: 83–90.

DOI:10.1515/sg-2009-0019
edited by Thünen Institute of Forest Genetics

STOEHR, M. U., B. L. ORVAR, T. M. VO, J. R. GAWLEY, J. E. WEBBER and C. H. NEWTON (1998): Application of a chloroplast DNA marker in seed orchard management evaluations of Douglas-fir. Can J For Res **28**: 187–195.

STOEHR, M. U. and C. H. NEWTON (2002): Evaluation of mating dynamics in a lodgepole pine seed orchard using chloroplast DNA markers. Can J For Res **32**: 469–476.

STOEHR, M. U., J. E. WEBBER, C. C. A. HOLLEFREUND and R. A. PAINTER (2004): Potential pollen contamination effects on progeny from an off-side Douglas -fir seed orchard: 9-year field results. Can J For Res **34**: 981–984.

TAKEUCHI, H., H. HANDA, T. OHGURO and M. OKAMURA (1989): Selection of resistant pine trees to the pine-wood nematode in Shikoku. Bull For Tree Breed Inst **7**: 119–143 (in Japanese with English summary).

TANG, D. Q. and Y. IDE (2001): Genetic variation in fruitfulness in a Hinoki (*Chamaecyparis obtusa* Endl.) seed orchard and its impact on the maintenance of genetic diversity in seedlots. Journal of Forest Research **6**: 67–72.

TERADA, M., T. TODA and T. NOGUCHI (1997): The selection of the nematode-resistant candidates from the damaged forest in the cold district. The control trees of the inoculation test in the breeding project on resistance to pine-wood nematodes in Tohoku. Bull Tohoku For Tree Breed Inst **27**: 57–58 (in Japanese).*)

TODA, T., Y. FUJIMOTO, K. NISHIMURA, H. YAMATE and S. FUYUNO (1989): Selection of resistant pine trees to the pine-wood nematode in Kyushu. Bull For Tree Breed Inst **7**: 145–178 (in Japanese with English summary).

TODA, T. (2004): Studies on the breeding for resistance to the pine wilt disease in *Pinus densiflora* and *P. thunbergii* Bull For Tree Breed Inst **20**: 83–217 (in Japanese with English summary).

TODA, T., M. TAJIME, K. NISHIMURA and H. TAKEUCHI (1993): Resistance breeding to the pine wood nematode in Kyushu district. – Progress of study after selection of the resistance clones – Bull For Tree Breed Inst **11**: 37–88 (in Japanese with English summary).

WATANABE, A., M. G. IWAIZUMI, M. UBUKATA, T. KONDO, C. LIAN and T. HOGETU (2006): Isolation of microsatellite markers from *Pinus densiflora* Sieb. et Zucc. using a dual PCR techniques. Mol Ecol Notes **6**: 80–82.

WHEELER, N. C. and K. S. JECH (1992): The use of electrophoretic markers in seed orchard research. New Forests **6**: 311–328.

*) The titles are approximate translations of the original Japanese title by the authors of this paper.

Genetic Variation in Seed Size and Germination Patterns and their Effect on White Spruce Seedling Characteristics

By S. CARLES[1),*)], M. S. LAMHAMEDI[2)], J. BEAULIEU[3)], D. C. STOWE[1)], F. COLAS[2)] and H. A. MARGOLIS[1)]

(Received 4th December 2007)

Abstract

We determined the degree to which families differ in seed and germination characteristics and examined the extent to which these characteristics influence the early growth of 75 open-pollinated white spruce families. Seed characteristics (1000-seed weight, length, width, area, volume) were measured for 400 seeds per family. Germination variables (germination capacity, peak value, germination value) were determined for each of the 75 families under controlled conditions and germination patterns were modelled using the Weibull function. Seedling characteristics (height, diameter, shoot and root dry weights) were measured at the end of the first and second growing seasons under standard nursery cultural practices. Statistically significant family variation ($p < 0.0001$) was found for all seed characteristics and germination variables measured. The between-family variance explained 23% to 98% of the total variance of morphological and physiological seed characteristics. Family differences at the seed stage explained up to 33% (root dry weight) and 12% (shoot dry weight) of the family differences observed at the one-year and two-year seedling stages, respectively. Since, in this study based

1) Centre d'étude de la forêt (CEF), Faculté de foresterie, de géographie et de géomatique, Pavillon Abitibi Price, Université Laval, 2405 rue de la Terrasse, Québec, QC, G1V 0A6, Canada.

2) Direction de la recherche forestière, Forêt Québec, ministère des Ressources naturelles et de la Faune, 2700 rue Einstein, Québec, QC, G1P 3W8, Canada.

3) Natural Ressources Canada, Canadian Forest Service, Canadian Wood Fibre Centre, 1055 rue du P.E.P.S., P.O. Box 10380, Sainte Foy, Québec, QC, G1V 4C7, Canada.

*) Corresponding Author: SYLVIE CARLES. Tel: 418-656-2629, Fax: 418-656-5262. E-Mail: sylvie.carles@sbf.ulaval.ca

DOI:10.1515/sg-2009-0020
edited by Thünen Institute of Forest Genetics