

ARTICLES

Variation in Germination of Scots Pine (*Pinus sylvestris* L.) Pollen Exposed to Ozone

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Abraitienė, A., Kairiūkštis, L., Pliūra, A., Girgždienė, R., Abraitis, R. 2002. Variation in Germination of Scots Pine (*Pinus sylvestris* L.) Pollen Exposed to Ozone. *Baltic Forestry*, 8 (1): 2–7.

The pollen grains of 16 plus tree clones of Scots pine (*Pinus sylvestris* L.) collected at the clonal archive were screened for susceptibility to treatment of ozone concentrations 45 ppb and 90 ppb for 4 hours. The pollen sensitivity was characterized by reduction in pollen germinability. Differences in susceptibility among genotypes of *Pinus sylvestris* were analysed in addition to the effect of ozone on germinability of *Pinus sylvestris* pollen.

According to the results obtained ozone treatment caused significant reduction in pollen germinability. Great genetic effect on pollen viability and susceptibility to ozone was found. Also strong ($k=0.92-0.93$) and significant ($p<0.001$) correlations in pollen germinability between exposed and non-exposed pollen lots were obtained. The significant genetic variation in susceptibility of pollen to increased ozone concentration found in our study indicates good possibilities of long-term adaptation of *Pinus sylvestris*. Only small part of variation (5%) was caused by genotype x treatment interaction. It was concluded that some but not significant changes in the genetic structure in the next generation are possible because of pollen being affected by ozone peaks during flowering period.

Key words: Scots pine, *Pinus sylvestris*, pollen, ozone treatment, genetic variation.

Introduction

One of the most prominent hypothesis is that chronic exposure of forest trees to ozone is probably one of the primarily causes of forest decline (Schmieden & Wild 1995). For foliar yellowing and damage which may reach up to 77% for spruce, pine and beech forest area (Blank 1985), the ozone concentrations that were recorded over the last ten years may play the major role (Dalstein et al. 1997) and be the most important factor for reduction in tree growth (Bartholomay et al. 1997). Even relatively low concentrations of tropospheric ozone can significantly reduce the growth of mature forest trees and interactions between ambient ozone and climate are likely to be important modifiers of future forest growth and function (McLaughlin et al. 1994).

Ozone affects various spheres of plant life of *Pinus* species as well as the other ones. The studies

on ozone influence on growth response of *Pinus sylvestris* have received great attention (Kainulainen et al. 1994, Rantanen et al. 1994, Holland et al. 1995, Bortier et al. 1997). However there is still a lack of evidence concerning the influence of ozone on reproduction. According to the papers reviewed by Wolters & Martens (1987), *in vivo* and *in vitro* air pollutants affect pollen germination and pollen tube growth. These traits are even offered to be used as sensitive biological indicators. Stimulation and inhibition of pollen germination and pollen tube growth depend on the plant species that differ in their response to the air pollutants and other environmental factors.

The aim of this study was to assess the effect of ozone concentrations on germinability of *Pinus sylvestris* pollen grains. Evaluation of among-clone differences in susceptibility to ozone, i.e. their reaction norm, was the other aim.

Materials and methods

Pollen grains were collected on May 16, 2001 at the clone archive from 20 plus tree clones of *Pinus sylvestris*. After drying, pollen had been stored in the freezer at -12° C until ozone treatment. Pollen samples of 0.1-gram were germinated for 72 hours as non-treated controls or were ozonated at the concentrations of 45 ppb (7 clones) and 90 ppb (all 20 clones) for 4 hours at 23-25° C. These ozone concentrations were chosen as they were close to the natural concentrations in the atmosphere. The concentration of 45 ppb is observed frequently in Lithuania during the vegetation period while the concentration of 90 ppb occurs rarely and is observed under "ozone episode" (Girgzdiene & Girgzdys 2001). The concentrations were produced by use of the ozone generator. Further, 4 clones were excluded from the dispersal analysis because of low viability (germination of control pollen samples was below 20%).

To characterize the susceptibility of pollen from different clones, germinability of control and treated pollen samples was studied. Three 0.01±0.002 gram samples of pollen were taken from each pollen lot and each sample was placed in Petri dishes. The pollen was carefully spread over the bottom surface of each dish. 10 millilitres of distilled water were added to each Petri dish and the dishes were incubated at 23-25° C for 72 hours. The dishes were shaken lightly during incubation to keep pollen grains from clumping. On each occasion, approximately 450 grains per clone per treatment were examined (taking at random 3 samples from each of the 3 dishes, 50 pollen grains per sample within treatment). Pollen grains with tube lengths equal to or greater than the diameter of the grain were judged as germinated. The germination percentage was calculated and recorded for each pollen lot in every Petri dish.

Processing of the data was made by a two-way analysis of variance using MIXED procedure of the SAS, Type III sums of squares. There was no transformation done as the percentage of pollen germination followed normal distribution (stated with UNIVARIATE procedure). The following mixed ANOVA model was applied:

$$y_{ijk} = \mu + t_i + c_j + [t*c]_{ij} + e_{ijk} \quad (1)$$

where: μ - overall mean, t_i - fixed treatment effect, c_j - random clone effect, $[t*c]_{ij}$ - random effect of clone x treatment interaction, and e_{ijk} - random error term.

Among-clone differences within the treatment were compared using a Student t-test.

The evaluation of reaction norms (response functions) of each clone was done using intercepts (b_{0i}) and regression coefficients (b_{1i}) of linear regression of clone means. The following equation was used:

$$Y_{ij} = b_{0i} + b_{1i} X_j \quad (2)$$

where: Y_{ij} is the response of the clone i to the environment (e.g. ozone concentration) j , X_j is the mean response under the environment j .

Additionally to that, R-squared value of linear regression equation was calculated to fit the reaction norm to the linear model.

Ecovalence (W_i) of Wricke (1962) was calculated to evaluate the contribution of clones to the clone x treatment interaction. The following equation was used:

$$W_i = \sum_j (\bar{X}_{ij} - \bar{X}_i - \bar{X}_j + \bar{X}.)^2 \quad (3)$$

where: W_i - ecovalence estimate, ' X_{ij} ' - the mean of clone i under environment j , ' \bar{X}_i ' - the mean of the clone i , ' \bar{X}_j ' - the mean of environment j , ' $\bar{X}.$ ' - the grand mean.

Results

Elevated concentration of ozone (90 ppb, 4 hours) resulted in fairly significant among-clone differences in pollen germinability. Analysis of variance revealed significant effects of treatment and clone as well as treatment x clone interaction (Table 1).

The greatest part of variation in pollen germinability (84.5 %) was caused by clone effect. Clone x treatment interaction was responsible for just 5% of the total variation. The rest part of variation was attributed to the error term. However, it was not possible to compute the variation part caused by treatment effect properly due to too low number of the treatments applied.

The great amplitude of response in pollen viability among 16 clones is illustrated in Figure 1. It is evident that clones varied considerably in germinability of both treated and non-treated pollen.

Table 1. Variance components (%), estimate and standard error for random effects only and significance of effects among clones for pollen germinability based on mixed model of ANOVA. ** and *** show significance of effects at 1% level and 0.1% level, respectively, df means degrees of freedom, F is Fisher criteria

Source of variation	df	Random effect			F	Significance level
		Estimate	Standard error	Variance component, %		
Treatment	1				69.88	***
Clone	15	264.80	99.88	84.5	2.65	**
Treatment x clone	15	15.72	6.11	5.0	2.57	**

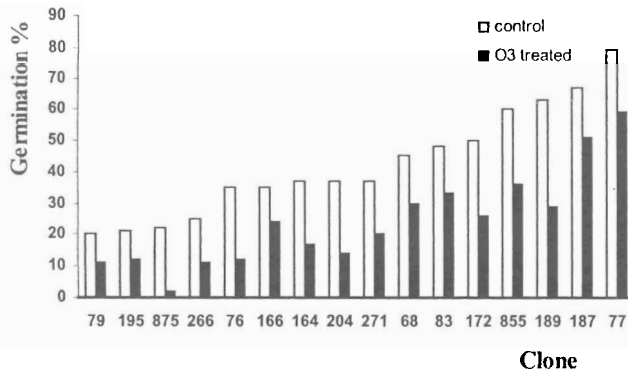


Figure 1. Among-clone differences in germination of ozone exposed (90 ppb, 4 hours) and non-exposed (control) pollen of *Pinus sylvestris*

The germinability of pollen grains of *Pinus sylvestris* plus trees was significantly reduced at both ozone concentrations (45 ppb and 90 ppb). Within-clone differences in germination between ozone-treated and non-treated pollen reached up to 35%, depending upon the clone screened. The degree of reduction in pollen germinability due to elevated ozone concentration varied among clones too.

The pollen lots that exhibited higher germinability under control conditions had also a superior germinability under the conditions of the highest ozone concentration (90 ppb) as the correlation coefficient between germinated pollen under both these conditions reached 0.92 and was significant at 0.1% level (Table 2). The correlation coefficient between germinability of non-treated pollen and reduction in germinability due to elevated ozone concentration was moderate (0.50) and significant at 5% level. However, the coefficient between germinability of treated pollen and reduction in germinability was poor and insignificant (Table 2).

Table 2. Pearson's rank correlation coefficients and their significances for relationships between pollen germinability expressed in per cent (above diagonal) and clone ranks (below diagonal) under the two conditions. Control and Treated refer to non-treated and ozone treated (90 ppb) pollen. Reduction means difference in pollen germination of the same clone between non-treated and ozone-treated pollen, which was obtained as follows: mean clone value of pollen germinability under control conditions (0 ppb) minus that at 90 ppb. * and *** show significance at 5% level, 1% level, and 0.1% level, respectively

	Treated	Reduction	Control
Treated		0.14	0.93***
Reduction	0.21		0.49*
Control	0.92***	0.50*	

Among-clone variation in pollen germinability showed a different pattern under control conditions and at 90 ppb ozone concentration (Figure 2). Elevated ozone concentration led to increased among-clone variation in pollen germinability than that under control conditions. There was detected some shifting in clone ranking between the germination conditions as well (Figure 2).

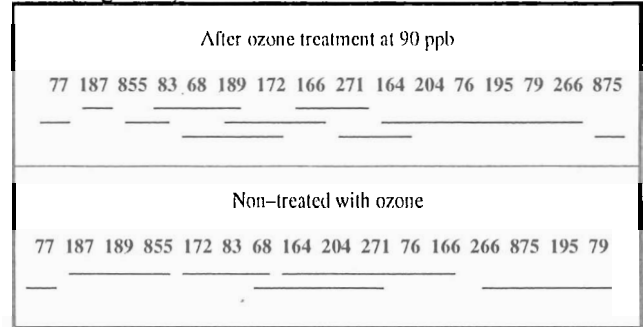


Figure 2. Grouping of clones by percentage of germinated pollen. Each line represents a separate group of clones within which the among-clone differences in pollen germination were insignificant

To reveal the among-clone differences in reaction norm, 7 randomly chosen clones only were tested under concentrations 45 ppb and 90 ppb. It was found that reduction in pollen germinability was directly related with the ozone concentration (Figure 3). There were almost no changes in clone ranking between the ozone treatments of 45 ppb and 90 ppb. The only exception was plus tree No. 189. Pollen of this clone showed high susceptibility to elevated ozone concentration.

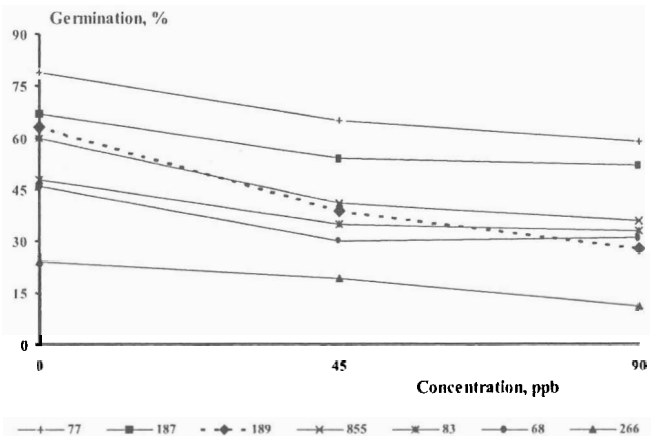


Figure 3. Reaction norm of *Pinus sylvestris* pollen germinability under different germination conditions. Ozone concentration of 0 ppb means control conditions

From Figure 3 it is evident that pollen germinability of the same clone tended to decrease more rapidly when the ozone concentration increased from 0 ppb to 45 ppb than that from 45 ppb to 90 ppb.

The reaction norms of *Pinus sylvestris* pollen viability in response to ozone concentration showed fairly large differences among the clones by a number of parameters used to estimate their environmental sensitivity (Table 3).

Table 3. Mean values of pollen germination (\pm standard error SE), difference of variation in germination means (at 0 ppb minus at 90 ppb), Wrinkle covalence (W_i), and parameters (intercept b_0 , slope b_1 , and regression coefficient R^2) that fit the linear effect model of the clones studied

Clone No.	Germination \pm SE, %			Difference 90 ppb-0 ppb	W_i	Parameters		
	90 ppb	45 ppb	0 ppb			b_0	b_1	R^2
77	59 \pm 0.59	65 \pm 0.74	79 \pm 0.35	20	0.087	87.7	10.0	0.95
187	52 \pm 0.51	54 \pm 0.25	67 \pm 0.31	15	2.622	72.7	7.5	0.85
189	28 \pm 0.38	39 \pm 0.31	63 \pm 0.31	35	2.986	78.3	17.5	0.96
855	36 \pm 0.42	41 \pm 0.37	60 \pm 0.29	24	2.658	69.7	12.0	0.90
83	33 \pm 0.41	35 \pm 0.37	48 \pm 0.35	15	2.622	53.7	7.5	0.85
68	31 \pm 0.70	30 \pm 0.50	46 \pm 0.35	15	3.265	50.7	7.5	0.70
266	11 \pm 0.33	19 \pm 0.27	24 \pm 0.44	13	7.797	31.0	6.5	0.98

Discussion and conclusions

Pollen, the reproductive material, was found to be very sensitive to unfavourable environmental conditions, including ozone stress (Feder 1968, Feder & Sullivan 1969). In the present study, considerable among-clone differences in pollen germinability were present already under control (0 ppb) germination conditions (Fig. 1). Genotypic features or susceptibility to environmental conditions (air temperature, humidity, etc.) before pollen collection is done might be responsible for this, as germination of *Pinus sylvestris* pollen is known to decrease when exposed to open air conditions (Lindgren & Lindgren 1996). Usually, more than half of the pollen remained germinable after few days outdoors but further more than four days outdoors resulted in very low pollen germination.

Significant ozone effect detected in *Pinus sylvestris* pollen germinability was in accordance with other studies on pine pollen sensitivity to ozone. *Pinus strobus* germination was found significantly reduced after exposure at ozone concentration of 0.15 ppm under wet conditions (Benoit et al. 1984). The greater reduction in germinability of pollen of sensitive clones can appear because of some irreversible changes within pollen cell structure. Since some ultrastructural changes has been referred in the case of study two *Petunia* cultivars. Over 50% of the ozonated (at 0.50 ppm for 3 hours) pollen of the ozone sensitive culti-

var had a peripheral band of cytoplasm, which was free of all organelles except ribosomes (Harrison & Feder 1974). Fewer grains of the ozone-tolerant pollen showed the cytoplasmic change (Harrison & Feder 1974). Ozone stress also is referred as altering the free amino acid and peptide pools (Mumford et al. 1972).

The results obtained in this study have revealed fairly high among-clone variation in *Pinus sylvestris* pollen susceptibility to ozone concentrations already at 45 ppb as well as at 90 ppb (Figure 2). Genetic variation in sensitivity of the species makes some buffer to survive the increased concentration of ozone during the flowering period. Significant differences in pollen tube growth were found among plus trees, the pollen donors, which indicated genetic potential for male gametophytic competition. The variation in pollen tube growth rate at the population level was considered to have an impact on the genetic composition of seed produced at the seed orchards (Venäläinen et al. 1999). Some parameters used to evaluate the environmental sensitivity revealed clear differences among clones (Table 3).

The highest slope range as well as range between mean clone values of germinated pollen (0 ppb minus 90 ppb) caused by the highest susceptibility to the ozone concentration were shown by clones No. 189 ($b_1=17.5$, range 35) and No. 855 ($b_1=12.0$, range 24). The most stable clone was No. 266 ($b_1=6.5$, range 13), which showed more than twice less lower slope and range in pollen germinability while comparing with the most sensitive one. However, when comparing pollen germinability of this clone with the overall mean pollen germinability, this clone was the worst ($W_i=7.8$). The best adaptation was shown by clone No. 77 ($W_i=0.1$). The most sensitive (189) and the most stable (266) clones originated in the southern (Marcinkonys) and western (Viesvile) parts of Lithuania, respectively. Fairly large range of slope (b_1 varied from 7 to 17) but not the intercept (b_0 from 73 to 78) was demonstrated by the clones of plus trees originating in the southern part of the country. The vegetative progenies of the most productive stand of *Pinus sylvestris* in Lithuania (clones 77, 68, and 83, the latter being from the tallest *Pinus sylvestris* tree in Lithuania) located at Punia forestry in Prienai forest enterprise did not show such high variation in sensitivity pattern (b_1 from 7 to 10). However, a higher number of clones to be studied parallelly with their flowering phenology are needed to draw conclusion on the effect of original mother tree growth place on pollen susceptibility to ozone.

Several mechanisms are considered by which both pollution and climate change are likely to affect pollination processes, altering the genetic structure of forest tree populations. Stand density, distance of

pollen dispersal, asynchrony and sexual asymmetry in reproductive efficiency among different genotypes could all be altered, resulting in rearranged mating systems (Giannini & Magnani 1994). Patterns of resource allocation, which are related to paternal reproductive success, could be modified. The antropogenic impact could interact with male gametophytic selection as well as with early zygotic selection, either as a direct stress or by reducing pollen load (Giannini & Magnani 1994). Mechanisms of self-incompatibility could also be modified. This could strongly affect both adaptedness and adaptability of forest tree populations to the further changes. However, the strong ($k=0.92-0.93$) and very significant ($p<0.001$) correlation between ozone-exposed and non-exposed to ozone pollen germinability (evaluated in percent and clone ranks) was found in our study (Table 2). It shows that *Pinus sylvestris* exposition to 45-90 ppb of ozone probably cannot make drastic changes in genetic structure of the next generation through the changes in pollen viability if permanently elevated ozone concentration occurs during the flowering time. A small part of variation, which was caused by genotype \times treatment interaction, also supports this conclusion. Some changes in genetic structure can be expected due to enhanced differentiation in pollen germinability among genotypes (Figure 2) when short peaks of ozone act on different initial pollen germinability caused by different flowering time or other peculiarities of genotypes.

The present study has revealed the possibility of tropospheric ozone to make impact on *Pinus sylvestris* pollen viability. The high genetic variation in pollen susceptibility indicates good possibility for *Pinus sylvestris* to get adapted to increased ozone concentrations during the flowering period in the future. However, further investigations are needed to get a better evidence of ozone effect on changes in genetic structure under natural conditions and susceptibility of material selected/bred for forest regeneration.

In conclusion, the exposition of *Pinus sylvestris* pollen grains to ozone at 45 and 90 ppb for 4 hours significantly reduced their germinability. The great variability in pollen germinability as well as susceptibility to ozone appeared due to genotype while genotype \times environment interaction explained only a minor part (5%) of the total variation. Considerable influence of genotype on pollen susceptibility to ozone may be caused by the differences in flowering time. Significant phenotypic correlations between ozone-exposed and non-exposed pollen germinability ($k=0.92$ and $k=0.93$ for percentage of germinated pollen and clone ranks, respectively, $p>0.999\%$) as well as between pollen germinability reduction because of ozone treat-

ment and initial germinability ($k=0.49-0.50$, $p>95\%$) were obtained. Therefore, it does not look like exposition of *Pinus sylvestris* pollen to 45-90 ppb of ozone is able to make drastic changes in the genetic structure of the next generation through the changes of pollen viability if permanently elevated ozone concentration occurs during the flowering time. However, some alteration in genetic structure might be possible when *Pinus sylvestris* genotypes with different flowering time are exposed to short peaks of elevated ozone concentration. The significant genetic variation in susceptibility to increased ozone concentration that has been found in our study indicates good possibilities of long-term adaptation of *Pinus sylvestris* populations.

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Received 26 February 2002

ВЛИЯНИЕ ПОВЫШЕННЫХ КОНЦЕНТРАЦИИ ОЗОНА НА ВСХОЖЕСТЬ ПЫЛЬЦЫ СОСНЫ ОБЫКНОВЕННОЙ (*PINUS SYLVESTRIS* L.)

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Резюме

Исследовалась чувствительность пыльцы сосны обыкновенной к повышенным концентрациям озона (45 и 90 ppb) при 4-часовой экспозиции. Чувствительность к воздействию озона оценивалась по уменьшению ее всхожести. Пыльца была собрана в кленовом архиве с 16 плюсовых деревьев.

Воздействие пыльцы озонем привело к значительному уменьшению всхожести. Разница по всхожести пыльцы подверженной и неподверженной к воздействию озонем статистически достоверна ($p < 0.001$). Влияние повышенных концентраций озона на всхожесть пыльцы, собранной с отдельных клонов, была неодинакова. Воздействие окружающей среды на генотипы оказало только незначительное влияние на изменчивость полученных данных. Разница в чувствительности отдельных клонов к повышенным концентрациям озона свидетельствует о хороших возможностях к длительной адаптации сосны. Влияние озона на всхожесть пыльцы может привести к некоторым изменениям в генетической структуре следующего поколения.

Ключевые слова: сосна обыкновенная, *Pinus sylvestris*, пыльца, воздействие озонем, генетическая вариация.