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Effects of Biotic Stress on Seed Quality and Antioxidant Content in Norway Spruce Trees from Approved Seed Stands in Slovenia

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Summary

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2003 was a seed year for Norway spruce. At the same time Norway spruce was heavily attacked by bark beetles. Because the nutritive tissue in conifers is derived entirely from the female parent the effect of biotic stress could influence the seed quality. Presence of biotic stress was detected through the visual signs caused by infestation with bark beetles and fungi and confirmed with higher antioxidant content in needles of non-vital trees. Seed quality of vital and non-vital subpopulations for three populations in Slovenia (Idrija, Pohorje, Bohor) was analysed. Attempt to find genetic differences between vital and non-vital trees was done. Statistically significant differences were observed in Idrija population for number of germinating seeds per cone, weight of 1000 seeds and weight of seeds per cone. No solid evidence against the use of seed derived from sanitary fellings due to biotic damages for production of forest reproductive material in the studied populations was found.

Introduction

Year 2003 was a seed year for Norway spruce (*Picea abies* (L.) Karst.) in Slovenia. In the second half of 2003 Slovenian State Forest Service reported high

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number of coniferous trees, mainly Norway spruce, attacked by bark beetles. This was caused by extremely high temperatures and lasting drought (ZGS 2004).

In conifers the health of the female parent could directly affect the seed quality because the nutritive tissue is derived entirely from the female parent. A healthy female parent will be able to contribute more resources to the nutritive tissue. Therefore seeds derived from non-vital trees could be smaller, lighter and have lower quality. Biotic stress could affect the seed quality in Norway spruce.

The antioxidants α-tocopherol (vitamin E) and ascorbic acid (vitamin C) have been identified as non-enzymatic protectors against peroxidation of lipids caused by different stress factors. A concentration ratio of ascorbic acid and α-tocopherol of about 10:1 to 15:1 is highly protective against damage (KUNERT & EDERER 1985). Normally the concentration of antioxidants increases with altitude. Normal level of ascorbic acid in Norway spruce needles is above 4 mg g^{-1} dry weight. Exposure of plants to different biotic and abiotic stresses changes the level of antioxidants and their ratio (RIBARIČ LASNIK & al. 1999).

The aim of the study was to investigate the seed quality and antioxidants content in the needles of Norway spruce trees with respect to the health status of mother trees. Another goal was to determine possible genetic differentiations between the subpopulations of vital and non-vital seed trees. The purpose of the obtained results was to see if any practical suggestions could be made for the use of forest reproductive material regarding the vitality of the mother trees.

Material and Methods

Plant material

10 cones per an adult tree from the upper part of the crown were collected from 10 vital and 10 non-vital Norway spruce reproductive trees felled in sanitary fellings in three seed stands in Slovenia (Idrija, Pohorje, Bohor) in November 2003. The origin of all 3 populations is probably of non-autochtonous source. Additionally, from every tree in the stands Idrija and Pohorje branches with needles were cut from the 7th whorl counted from the top of the crown. Current year and one-year-old needles were separated and lyophilised. Trees were assessed as vital or non-vital on the basis of visual signs caused by infestation with Scolytidae, *Heterobasidion annosum* (Fr.) Bref., *Armillaria mellea* (Vahl) P. Kumm., *Camponotus herculeanus* L., etc. relatively to the conditions on the plot. Non-vital trees from Pohorje population were assessed as such only on the basis of presence of *Heterobasidion annosum* because no attack by bark beetles was observed in this stand.

Table 1. Description of the site conditions of the three sampled populations.

Population	Idrija	Pohorje	Bohor
Average altitude [m]	630	900	650
Provenance region	Dinaric	Pohorje	Pre-dinaric
Average (min - max) temperature [°C]*	7.2 (2.8 - 12.0)	8.1 (3.6 - 13.3)	9.1 (4.5 - 14.2)
Precipitation [mm]*	1853	1252	1229
Soil type	distric cambisole	eutric cambisole, rendzic leptosole	distric cambisole

* Data from KUTNAR & al. 2002

Seed characterisation

Average length and width of cones, number and weight of seeds per cone, weight of 1000 seeds, moisture content, germination and vigour (Pohorje only) were measured according to ISTA 2003 in January 2004 (i.e. in optimal conditions).

Stress indicators

α-tocopherol and ascorbic acid were measured in current and one-year-old lyophilized needles from vital and non-vital trees from locations Pohorje and Idrija by ERICo Velenje using methods PM 4.03 and PM 4.32 (RIBARIČ LASNIK & al. 1999).

Genetic characterization of populations

Extraction of DNA from grinded lyophilised needles (Idrija, Pohorje) was carried out with DNeasy Plant Mini Kit (Qiagen, Germany) according to slightly modified protocol. Primer pairs (annealing temperature in °C) Sb16 (55), Sb17 (57), Sb42 (56), Sb51 (55), Sb58 (57), Sb70 (57) (PERRY & BOUSQUET 1998), PA0002 (55) and PA0055 (55) (SCHUBERT & al. 2001) in combination with restriction enzymes AluI, AfaI, HaeIII, DraI were used. Products were separated by means of agarose gel electrophoresis and visualized by ethidium bromide staining. For each population allelic frequencies, degree of observed heterozygosity (H_o) and fixation indices (F) were calculated. The deviation from Hardy-Weinberg equilibrium was tested by χ^2 test. The degree of differentiation between subpopulations was quantified with the genetic distances proposed by NEI 1972. To test the homogeneity of allele frequencies between populations χ^2 tests were used. All computations were preformed with Popgene32 (YEH & al. 1997) software package.

Statistical analysis

Multiple comparisons of averages using ANOVA or multiple comparisons of medians with non-parametric Kruskal-Wallis test between populations and/or vital and non-vital individuals were carried out with the programme package Statgraphics® Plus 5.0 to establish possible correlations to vitality of the seed trees.

Results

Stress indicators ascorbic acid and α-tocopherol

Average content of ascorbic acid and α-tocopherol was slightly higher in non-vital trees from both populations. However the differences were not statistically significant. Highly significant differences were detected for α-tocopherol content between current year and one year old needles in both populations, the average values being higher in one year old needles. Average content of ascorbic acid and α-tocopherol in needles was higher in population from Idrija.

Table 2. Content of ascorbic acid and α-tocopherol in Norway spruce lyophilized needles in mg g^{-1} of dry weight.

		Mean (range) in mg g^{-1} dry weight			
		One year old needles		Current year needles	
Population / Vitality		Idrija	Pohorje	Idrija	Pohorje
ascorbic acid	Vital	2.65 (1.72-3.38)	2.01 (1.26-2.63)	2.58 (1.52-3.42)	2.08 (1.31-2.76)
	Non-vital	2.66 (1.77-3.45)	2.19 (1.75-2.72)	2.74 (1.71-3.16)	2.22 (1.51-2.88)
α- toco-pherol	Vital	0.23 (0.11-0.30)	0.11 (0.05-0.18)	0.15 (0.07-0.22)	0.07 (0.05-0.08)
	Non-vital	0.26 (0.17-0.38)	0.12 (0.07-0.16)	0.17 (0.10-0.28)	0.07 (0.05-0.11)

Table 3. Percentage of trees with highly protective ratio of ascorbic acid and α-tocopherol 10:1 to 15:1.

Percentage of trees with the ratio of ascorbic acid and α-tocopherol between 10:1 and 15:1						
Population	Idrija			Pohorje		
	All trees	Vital	Non-vital	All trees	Vital	Non-vital
One year old needles	63	70	50	46	44	50
Current year needles	37	50	20	0	0	0

In current year needles the highly protective ratio of ascorbic acid and α-tocopherol of about 10:1 to 15:1 was found only in 37% of trees for Idrija population. In other trees of this population the ratio was higher than 15:1. The ratio of ascorbic acid and α-tocopherol was also higher than 15:1 in all trees for current year needles for the Pohorje population (therefore the percentage is 0).

Seed characterisation

No statistical significant differences between vital and non-vital mother trees ($\alpha = 0.05$) were found for seed germination from any of the studied plots. However results indicate slightly higher germination rate for seeds derived from vital trees.

Table 4. Average values for seeds from vital and non-vital trees from 3 populations (* denotes statistically significant difference between subpopulations at $\alpha = 0.05$).

Location	Idrija		Pohorje		Bohor	
Vitality	Vital	Non-vital	Vital	Non-vital	Vital	Non-vital
Germination [%]	70.5	63.7	75.3	75.5	62.3	60.4
No. germinated seeds per cone	172	120*	248	228	113	108
No. seeds per cone	249	193	328	302	185	178
Weight of seeds per cone [g]	1.86	1.24*	2.29	2.42	1.64	1.22
Weight of 1000 seeds [g]	6.45	5.13*	5.81	6.56	6.94	6.37

The average values for germination vigour of seeds from the two subpopulations showed slightly faster germination in the first 6 days for seeds derived from vital mother trees. After the ninth day there was no noticeable difference in vigour (Fig. 1), the average vigour settling at 75%. Germination vigour for individual trees showed two patterns: one with highest germination in the first 6 days and one with the highest vigour between 6^{th} and 9^{th} day. The second pattern was found in 3 non-vital trees and 1 vital tree respectively. Trees with this pattern had the highest germination rate among non-vital trees.

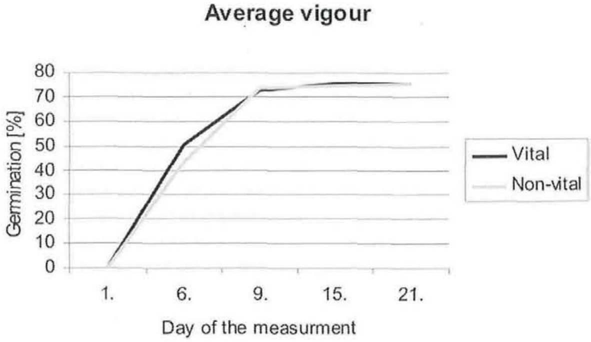

Fig. 1. Average vigour for vital and non-vital trees on Pohorje.

Population genetic analysis

Amplification was successful for all but primer pair PA0002 and primer pair Sb16 in Idrija population. Those two loci were therefore omitted from further analysis. Taking into consideration the six primer pairs with good amplification products 27 different multilocus genotypes were found in the 38 samples. In one sample from non-vital subpopulation from Idrija amplification was successful for only 2 loci and in one sample from non-vital subpopulation from Pohorje for 1 locus respectively. Those two samples were omitted from genotype designation. At every locus two alleles were identified. The frequencies of these alleles are given in Table 5.

Greater differences in frequency of alleles between subpopulations of vital and non-vital trees were noted for loci Sb17 and PA0055 in both populations. However statistical significance of these differences failed to be confirmed by χ^2 test ($\alpha = 0.05$).

Genetic distances between group of vital and non-vital trees are given in Table 7. The single-locus distance values as well as the gene pool distance (all loci) are quite low. The values of genetic distances are much lower within populations (1.16 % and 1.18 %) as between them (3.40 %).

Table 5. Allelic frequencies at 6 biallelic loci analysed in sampled vital and non-vital subpopulations of 2 spruce seed stands and results of homogeneity tests between subpopulations.

		Idrija			Pohorje		
Locus	Allele	Vital	Non-vital	Signif.	Vital	Non-vital	Signif.
Sb42	1	0.900	0.833	ns	0.800	0.833	ns
	2	0.100	0.167		0.200	0.167	
Sb17	1	0.389	0.278	ns	0.050	0.250	ns
	2	0.611	0.722		0.950	0.750	
Sb51	1	0.650	0.611	ns	0.900	0.889	ns
	2	0.350	0.389		0.100	0.111	
Sb58	1	0.400	0.333	ns	0.300	0.389	ns
	2	0.600	0.667		0.700	0.611	
Sb70	1	0.850	0.944	ns	0.800	0.778	ns
	2	0.150	0.056		0.200	0.222	
PA0055	1	0.800	0.625	ns	0.778	0.944	ns
	2	0.200	0.375		0.222	0.056	

ns not significant ($\alpha = 0.05$)

Table 6. Fixation index (F), significance of χ^2 test for HW proportions in vital and non-vital subpopulations of 2 spruce seed stands.

	Idrija				Pohorje			
Locus	Vital	Signif.	Non-vital	Signif.	Vital	Signif.	Non-vital	Signif.
Sb42	-0.111	ns	-0.200	ns	-0.250	ns	-0.200	ns
Sb17	-0.169	ns	0.169	ns	-0.053	ns	-0.333	ns
Sb51	-0.099	ns	-0.169	ns	-0.111	ns	-0.125	ns
Sb58	-0.667	ns	-0.500	ns	-0.429	ns	-0.636	ns
Sb70	0.608	5.65*	-0.059	ns	-0.250	ns	-0.286	ns
PA0055	1.000	12.80***	0.467	ns	-0.286	ns	-0.059	ns

* significant at the 5 % level, *** significant at 0.1% level, ns not significant ($\alpha = 0.05$)

Table 7. Nei's genetic distance.

	Sb42	Sb17	Sb51	Sb58	Sb70	PA0055	All loci
Idrija Vital : Non-vital	0.004	0.020	0.003	0.008	0.007	0.044	0.016
Pohorje Vital : Non-vital	0.001	0.037	0.000	0.013	0.001	0.024	0.018
Pohorje : Idrija	0.003	0.047	0.087	0.001	0.010	0.022	0.034

The average observed heterozygosity (H_o) was slightly higher in non-vital groups of trees (Idrija: $H_o = 0.375$, Pohorje: $H_o = 0.398$) then in vital (Idrija: $H_o = 0.359$, Pohorje: $H_o = 0.357$). The difference in observed heterozygosity between spruce subpopulations is relatively small and does not exceed 4.3 % in Idrija population and 10.3% in Pohorje population.

The fixation index showed that loci Sb70 and PA0055 for vital trees in Idrija population had statistical significant deviations of genotype frequencies from the HW equilibrium (Table 2). F-value of 1.000 on locus PA0055 is explained with the fact that eight homozygotes 11 and two homozygotes 22 were found in this subpopulation. For both mentioned loci a deficit of heterozygotes was detected, the deficiency on locus PA0055 also being present in non-vital trees. Locus Sb17 showed excess of heterozygotes in vital trees and a heterozygote deficiency in non-vital trees. For Pohorje population all loci showed slight excess of heterozygotes but never statistically significant.

Discussion

Nurseries and seed banks are in the need of high quality seed for their operation. Therefore it is important to find out how much biotic stress could diminish seed quality in seed stands during a seed year. Additionally we have aimed at describing the effects of biotic stress on antioxidant content and tried to see, if collectives with different damage degrees differ in their genetic structure. The importance of this study is in integration of different methods and parameters: seed testing, analysis of stress components and genetic aspects. This gives us a preliminary insight into the possible influences of damage caused by biotic stress on the quality of seed.

Due to the origin of the studied populations from different provenance regions and slightly different criteria for evaluation of vitality in each of the populations owing to the situation on the studied plots combining of vital subpopulations and non-vital subpopulations amongst the three populations was not possible.

Average content of ascorbic acid and α-tocopherol in needles was higher in population from Idrija despite its lower height above the sea level. When compared to population from Pohorje this could indicate more stressful conditions at the Idrija sampling site. Slightly higher average values of ascorbic acid and α-tocopherol in non-vital trees could indicate more stressful conditions for non-vital subpopulations of Idrija and Pohorje populations. The observed differences confirmed our determination of trees as vital or non-vital on the basis of visual symptoms of infestation. Higher average values of α-tocopherol found in one year old needles in both sampled populations are consistent with the results published by RIBARIČ LASNIK & al. 1999. The ratio between ascorbic acid and α-tocopherol over 15 is characteristic for locations from higher elevations (RIBARIČ LASNIK & al. 1999) and demonstrates higher production of ascorbic acid and less α-tocopherol as a result of oxidative stress.

Statistically significant differences between Idrija subpopulations for weight of seeds per cone and weight of 1000 seeds might indicate that there is less nutritive tissue in seeds belonging to maternal trees of the non-vital subpopulation and consequently mean lower competitiveness in comparison to the vital subpopulation. This might also be confirmed if statistically significant higher germination for vital trees would have been observed. In our case germination was higher in vital trees by 10.7% but this was not statistically significant.

In populations Pohorje and Bohor no statistically significant differences were found between the subpopulations indicating that in these two populations the seed quality was not substantially affected by biotic stress. However higher values for vital trees in Bohor population were observed.

Under field conditions rapid germination is an advantage for seedling establishment (SCHMIDT 2000). In Pohorje population the difference in vigour was 9.3% in favour of the vital subpopulation in the first six days of germination period. After the 15^{th} day of measurements the differences were not observed anymore.

The genetic variability within subpopulations was similar. The level of observed heterozygosity (H_o) in both populations was lower in the vital subpopulations in contrast to the study made with izoenzyme markers by PRUS-GLOWACKI & GODZIK 1995 where higher level of heterozygosity indicated higher survival ability of Norway spruce trees exposed to industrial pollution. Partial cDNA clone PA0055 was putatively identified as ATP synthase β chain precursor (SCHUBERT & al. 2001). In addition to their role as building blocks of nucleic acids they are the carriers of the energy to power the numerous chemical reactions occurring within cells (RAVEN & al. 1999).

Deviations of genotype frequencies from Hardy-Weinberg equilibrium might be caused by drift effects because the number of analysed trees was very low.

The genetic differences between the populations of spruce and their vital and non-vital subsets are relatively small for the gene pool studied.

Based on the results of our study we are not able to verify the existence of genetic differentiation between the vital and non-vital subpopulations of seed trees.

Owing to the limited number of studied trees and therefore small samples of seed trees the inference of general rules for the 3 studied populations is not feasible. However we have found no solid evidence against the use of seed derived from sanitary fellings due to bark beetles and other biotic damages for production of forest reproductive material in the studied populations. A study with higher sample sizes under more uniform conditions (same provenance region, altitude, cause of biotic stress, etc.) would be advisable to verify the trends observed in the presented study. If the trends turned out to be significant the impact of diminished seed quality on the economical result of nurseries should be revised. Additional suggestions for the collection of forest reproductive material should be considered.

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