EFFECTS OF HEAVY-METAL POLLUTED SOILS ON THE GENETIC STRUCTURE OF NORWAY SPRUCE SEEDLING POPULATIONS

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Abstract. Young seedlings raised from six different Norway spruce seed provenances were grown in heavy-metal contaminated soils at two severely polluted areas. After ten months, the rate of survival ranged from 30% – 42% and from 45% – 52% at the two polluted areas, respectively.

Comparisons of the genetic structure analysed at eight polymorphic enzyme loci showed that the heterozygote frequencies at four of these loci have substantially increased in the surviving seedling collections across all provenances as compared to the initial embryo samples. These results were discussed in relation to the metabolic role of the respective enzymes in response to environmental stress and the possibly greater reaction efficiency of the heterozygous enzyme phenotypes in order to recognize a probable overdominant selection regime.

Key words: heavy metal pollution, genetic structure, selection, isozymes, seedlings, provenances, Norway spruce

1. Introduction

European forest tree species have adapted to the particular environmental conditions of their ranges since the last glaciation, so that their gene pools reflect the spectrum of environments the species have experienced. Considerable alterations of environmental conditions resulting from air and soil pollution have affected many plant species during the past decades, but have caused a severe decline of several of the most important forest tree species in central and eastern Europe (Krause *et al.*, 1986). This observation indicates that the adaptability of these tree species is limited when sudden and drastic environmental changes are conceived as stress. On the other hand, it could repeatedly be demonstrated that stressful conditions were capable of changing the genetic structure of tree populations indicating the first steps of an evolutionary process in the course of adaptation to the polluted environment (see Gregorius *et al.*, 1985; Karnosky *et al.*, 1989, for review and literature compilation).

In particular, Norway spruce *(Picea abies)* was found to respond to severe air pollution by considerable genetic changes at several enzyme gene loci (Bergmann and Scholz, 1989), a few of which have important functions in metabolic (and resistance) processes that are demanded under very stressful environmental conditions (Rothe and Bergmann, 1995). Therefore, the question arises as to whether Norway spruce populations can also respond to severe soil pollution and in which way will their genetic structures be changed during the possible adaptation processes.

The objective of the present study was, therefore, to investigate the genetic changes in Norway spruce seedling populations growing on heavy-metal polluted soils for a limited time period. In order to start with different population structures, the seed samples used for seedling raising were chosen from different spruce provenances located in distant areas of the natural range. The genetic structures were analysed at those structural gene loci the enzymes of which have important functions in energy metabolism and some particular biosynthetic pathways.

2. Material and Methods

Two different severely polluted areas were chosen for these field studies. One (metal waste Oker) is located near an ore foundry on the northern foothills of the Harz mountains and contains several heavy-metals in high concentration (Zn: 1.2–5.1 g/kg soil and Pb: 4.2–7.9 g/kg soil). Because of the heterogeneity among soil samples from different sites of the area, only the range of heavy-metal concentration (determined by EDTA extraction) is presented. The control area which is located in an adjoining forest stand (about 4 km distance) contains less than 0.4 g Zn and 0.2 g Pb per kg soil. The second area, the so-called Silver Lake, is located near a big mining area in the Sauerland (western Germany) and contains also Zn: 1.4–12.5 g/kg soil and Pb: 1.4–6.1 g/kg soil. The control area is located at an edge of a spruce stand about 8 km away from the Silver Lake and contains less than 0.2 g Zn and less than 0,05 g Pb per kg soil. The relatively large distance between the polluted and the control area was required due to the widely polluted area around the study site at the Silver Lake.

Five seed lots for raising the seedling populations originated from Germany (Bärenfels/Ore mountains, Westerhof and Hilchenbach/Sauerland), Poland (Istebna/Upper Silesia) and Romania (Marginea/East Carpathians), whereas the sixth seed lot designed to exhibit high genetic variability was a provenance mixture (Mix) from stands in northern Finland, Sweden, Austria and Switzerland. Seeds from these six lots were germinated on a standard peat/vermiculite substrate in the greenhouse for about two months. Then the seedlings were transferred to the two study areas (Oker and Silver Lake) and 300 individuals per provenance (100 in each of three replicates) were planted in the polluted soil of each area, so that in total 1.800 seedlings were allowed to grow in each area. After ten months, at the end of the growing period, the survived seedlings were counted, harvested and homogenized for isozyme analysis. Additionally, 100 embryos of each of the six seed lots were homogenized and also surveyed for the same isozyme systems.

For isozyme analysis, the seedling or embryo homogenates were subjected to horizontal starch gel electrophoresis using different enzyme specific buffer systems. Following electrophoretic separation, the gels were sliced into several replicate slabs and each slab was stained for one of the following enzyme systems. This procedure enables the recognition of multilocus genotypes of individual seedlings

TABLE I

Percentage of surviving seedlings at heavy-metal polluted and control areas

Study areas	Provenance name of seed lots						
	Ore Mts.	Sauerland	Westerhof	Carpathians	Silesia	Mix	Average
Silver Lake	33.3	30.4	33.7	42.2	29.6	8.9	29.7
Control area	97.4	93.6	94.5	90.2	92.8	70.6	89.9
Oker	50.5	45.6	44.8	47.2	51.5	41.0	46.8
Control area	97.9	91.4	97.1	97.9	92.9	97.9	95.8

and embryos. The enzyme systems and their gene loci scored (in brackets) were as follows: leucine aminopeptidase (LAP-B), glutamate oxalacetate transaminase (GOT-B), phosphoglucose isomerase (PGI-B), NADH dehydrogenase (NDH-A), glucose-6-phosphate dehydrogenase (G-6-PDH), 6-phosphogluconate dehydrogenase (6-PGDH-B, 6-PGDH-C) and phosphoenolpyruvate carboxylase (PEPCA-A). The specific procedure for seedling homogenization was described by Hosius (1994), the electrophoretic conditions and staining methods were compiled by Weeden and Wendel (1989). Studies on the genetic control and inheritance of these isozyme systems in Norway spruce have been reviewed by Geburek and von Wühlisch (1989) and Bergmann and Scholz (1989).

To determine any genetic change at these enzyme loci, the gene and genotype frequencies of the embryo samples (designated as initial genetic structures) were compared with the corresponding frequencies of the seedling populations (here designated as final genetic structures). The differences observed were evaluated for statistical significance by the maximum-likelihood test (G-Test). To assess one fitness component, the viability (final frequency of genotype divided through initial frequency of genotype) was estimated for most isozyme genotypes.

3. Results

3.1. MORTALITY OF SEEDLING POPULATIONS

A prerequisite for the detection of genetic selection is a high rate of mortality within the seedling populations. Although nothing is known about the inherent metal tolerance of these Norway spruce populations (the Ore Mts. sample was believed to be more tolerant than the other samples, since these spruce stands have been exposed to pollution of ore mines for several generations), it was presumed that the very toxic lead/zinc wastes at Oker and Silver Lake will represent harsh environmental conditions, capable of exerting strong selection forces on the seedlings.

The inspection of the study areas, eight months after planting, showed that many seedlings across all provenances became necrotic and several of them started dying. After the end of the growing period (ten months after planting) the proportion of seedlings which have survived on the heavy-metal polluted soils at Oker and Silver Lake was determined and the data compared among provenances (Table I). On the average, only 30% of all seedlings at the Silver Lake and 47% of all seedlings at Oker survived the toxic conditions of wastes. In the control areas located in the neighbourhood of the polluted areas, about 90 -98 % were still alive. The variation in the rate of survival among the five provenance collections was not very high, ranging from 30% to 42% at Silver Lake and from 45% to 52% at Oker, however, the artificial mixture of seedlings (Mix) clearly differed from these values since only 9% of its seedlings survived at Silver Lake and 41% of its seedlings at Oker (Table I)

3.2. DIFFERENCES IN GENETIC STRUCTURES BETWEEN EMBRYO AND SEEDLING POPULATIONS

The comparison of the gene and genotype frequencies between the embryo samples (initial structures) and the populations of seedlings survived at both polluted sites (final structures) revealed that remarkable and several statistically significant differences occurred at only four gene loci (LAP-B, PEPCA-A, 6-PGDH-B and 6-PGDH-C). The basic data were presented elsewhere (Hosius, 1994). Therefore, the data of only these gene loci were described in the following sections. Since for each provenance the genetic changes at the four loci were generally consistent for both polluted areas, the genetic data of the survived seedlings at Oker and Silver Lake were pooled to enlarge the respective sample sizes. The allele and genotype frequencies of these combined seedling collections together with their initial (embryo) frequency data were presented in Table II.

Although a heavy-metal stress is believed to cause a more directional selection effect (Prus-Glowacki and Godzik, 1991), the genetic changes observed at the four enzyme loci in this study mainly concern the heterozygote frequencies, i.e. the frequency of many heterozygous genotypes increased from the initial to the final (seedling) structures (Table II). At LAP-B, the frequency of the genotype B_1B_2 was consistently, though not significantly, higher in spruce seedlings surviving the heavy-metal pollution than in the embryo samples. Since this frequency change occurred in all six provenances tested, the heterozygote advantage appeared to be related to the LAP-B locus itself and not to another adaptive gene locus linked to the LAP locus. Because of the heterozygote increase the initially infrequent allele B_2 has also increased in frequency in the seedling populations. Significant differences at a LAP locus between the seed and seedling stages could also be detected in beech growing under stressful conditions (Müller-Starck and Ziehe, 1991). Statistically significant differences could be observed at the gene locus PEPCA-A, where the heterozygote A_1A_2 appeared with higher frequency in the

survivors as compared to the embryo samples (Table II). Very similar results were found for young regenerations originating from severely air-polluted spruce stands in the Franconia Forest and Fichtel mountains (Bergmann and Scholz, 1989). A shift towards higher frequencies was also found for the heterozygotes at both 6-PGDH loci in most of the seedling populations, although the allele frequencies have generally not changed between the embryo and the seedling stages (Table II).

3.3. HETEROZYGOTE SUPERIORITY AND VIABILITY

Since the genetic changes between the initial (embryo) stage and the final (seedling) stage were found to tend to an increase in frequency of heterozygotes at expense of homozygotes (with a few exceptions) across all spruce provenances, the corresponding data of all provenance samples were pooled for each enzyme locus. If the direction of the genetic changes between the two stages is only arbitrary, the pooling of the provenance data will then provide no significant differences. If, on the other hand, there is a systematic shift in genotype frequencies, the pooling of data will make the general trend of genetic change more evident. Furthermore, the viability for each genotype could now be calculated with greater reliability. The change in genotype frequencies at the locus PEPCA-A between the two stages demonstrated the importance of the heterozygous genotype A_1A_2 for adaptation to polluted soils, which is supported by the highest viability among the three genotypes (Table III). Similar changes in heterozygote frequencies could be observed for the two 6-PGDH loci, at which the heterozygotes (B_1B_2 and C_1C_2) increased in frequency from the initial to the final stage and, accordingly, exhibited the highest viability values (Table III).

Since the two 6-PGDH loci function in the same metabolic pathway (oxidative pentose phosphate cycle), their genotypes might be associated in the adaptive response to pollution stress. Therefore, it was reasonable to compare their two-locus genotypes between the two stages. Not surprisingly, the double heterozygote $B_1B_2C_1C_2$ and the single heterozygote $B_2B_2C_1C_2$ showed the most pronounced increase in frequency from the initial to the survival stage and, accordingly, possessed the highest viability (Figure 1). In contrast, the double homozygotes $B_1B_1C_2C_2$ and $B_1B_1C_1C_1$ clearly decreased in frequency between the two stages and exhibited the lowest viability (Figure 1).

4. Discussion

Heavy-metal polluted soils have detrimental effects on the survivorship of Norway spruce seedlings as demonstrated with experimental plots in two severely polluted areas near to heavy-metal emitents. The mortality of seedlings from six different spruce provenances was expected to also reveal selection effects on particular enzyme gene loci, since natural spruce regeneration growing for more than ten

TABLE II

Allele and genotype frequencies of embryos and surviving seedlings from six Norway spruce seed lots. These seedling frequencies represent pooled data from both polluted areas. Asterix specify statistical significance (G-Test)

Enzyme locus / Genotype	Ore Mountains Bärenfels		Sauerland Hilchenbach		Westerhof		Carpathians Marginea		Silesia Poland Istebna		Mix	
	embryo	seedling	embryo	seedling	embryo	seedling	embryo	seedling	embryo	seedling	embryo	seedling
LAP–B												
B_1B_1	0.44	0.31	0.68	0.69	0.79	0.73	0.69	0.53	0.69	0.65	0.64	0.56
B_1B_2	0.34	0.38	0.12	0.22	0.10	0.20	0.16	0.32	0.18	0.20	0.17	0.33
B_1B_3	0.06	0.14	0.04	0.03	0.01	–	–	–	0.04	0.05	–	0.02
B_1B_4	0.06	0.05	0.10	0.05	0.09	0.03	0.10	0.09	0.02	0.02	0.09	0.03
B_2B_2	0.08	0.08	0.05	0.01	0.01	0.03	0.05	0.06	0.06	0.08	0.05	0.06
B_4B_4	0.02	0.04	0.02	–	0.01	0.01	–	–	0.01	–	0.05	–
Allele						*						*
B_1	0.67	0.59	0.80	0.83	0.88	0.84	0.82	0.73	0.81	0.79	0.76	0.75
B_2	0.25	0.28	0.11	0.12	0.05	0.14	0.13	0.22	0.15	0.18	0.14	0.23
B_3	0.03	0.08	0.02	0.03	0.01	0.01	–	–	0.02	0.02	0.01	0.01
B_4	0.05	0.05	0.07	0.03	0.05	0.01	0.05	0.04	0.02	0.01	0.07	0.02
PEPCA–A	*			*				*		*		
A_1A_1	0.76	0.63	0.64	0.55	0.78	0.81	0.82	0.66	0.74	0.56	0.75	0.75
A_1A_2	0.22	0.26	0.21	0.37	0.15	0.13	0.15	0.25	0.21	0.36	0.17	0.20
A_2A_2	0.02	0.11	0.15	0.08	0.07	0.06	0.03	0.09	0.05	0.08	0.08	0.05

Table II (Continued)

| Enzyme locus Genotype	Ore Mountains Bärenfels embryo	seedling	Sauerland Hilchenbach embryo	seedling	Westerhof embryo	seedling	Carpathians Marginea embryo	seedling	Silesia Poland Istebna embryo	seedling	Mix embryo	seedling
Allele		**						*		**		**
A_1	0.87	0.75	0.75	0.74	0.86	0.87	0.89	0.78	0.85	0.74	0.84	0.85
A_2	0.13	0.25	0.25	0.26	0.14	0.13	0.11	0.22	0.15	0.26	0.16	0.15
6-PGDH-B				*						***		**
B_1B_1	0.17	0.15	0.18	0.13	0.18	0.16	0.35	0.24	0.32	0.10	0.36	0.14
B_1B_2	0.33	0.39	0.47	0.37	0.24	0.28	0.28	0.41	0.39	0.43	0.35	0.51
B_2B_2	0.49	0.45	0.34	0.50	0.58	0.55	0.36	0.35	0.29	0.47	0.30	0.35
Allele						*				***		*
B_1	0.34	0.34	0.42	0.31	0.30	0.31	0.50	0.45	0.49	0.31	0.53	0.39
B_2	0.66	0.64	0.58	0.68	0.70	0.69	0.50	0.54	0.50	0.69	0.47	0.61
6-PGDH-C												
C_1C_1	0.14	0.14	0.22	0.20	0.22	0.16	0.22	0.11	0.11	0.14	0.16	0.14
C_1C_2	0.53	0.49	0.39	0.53	0.40	0.59	0.38	0.49	0.51	0.56	0.35	0.40
C_2C_2	0.33	0.37	0.39	0.26	0.38	0.24	0.39	0.40	0.38	0.30	0.49	0.45
Allele												
C_1	0.40	0.38	0.42	0.48	0.42	0.46	0.42	0.36	0.38	0.42	0.34	0.35
C_2	0.60	0.62	0.57	0.52	0.57	0.53	0.58	0.63	0.61	0.57	0.66	0.65

TABLE III

Genotype frequencies of the embryo sample and sur-
viving seedlings and their viabilities at the loci PEP-
CA-A, 6-PGDH-B and 6-PGDH-C. The differences
at the gene loci are statistically significant

Enzyme loci Genotypes	Embryo sample	Surviving seedlings	Viability
PEPCA-A			
A_1A_1	0.75	0.64	0.852
A_1A_2	0.18	0.28	1.516
A_2A_2	0.07	0.08	1.212
6-PGDH-B			
B_1B_1	0.25	0.14	0.547
B_1B_2	0.35	0.40	1.141
B_2B_2	0.40	0.46	1.158
6-PGDH-C			
C_1C_1	0.18	0.15	0.834
C_1C_2	0.42	0.52	1.222
C_2C_2	0.40	0.33	0.815
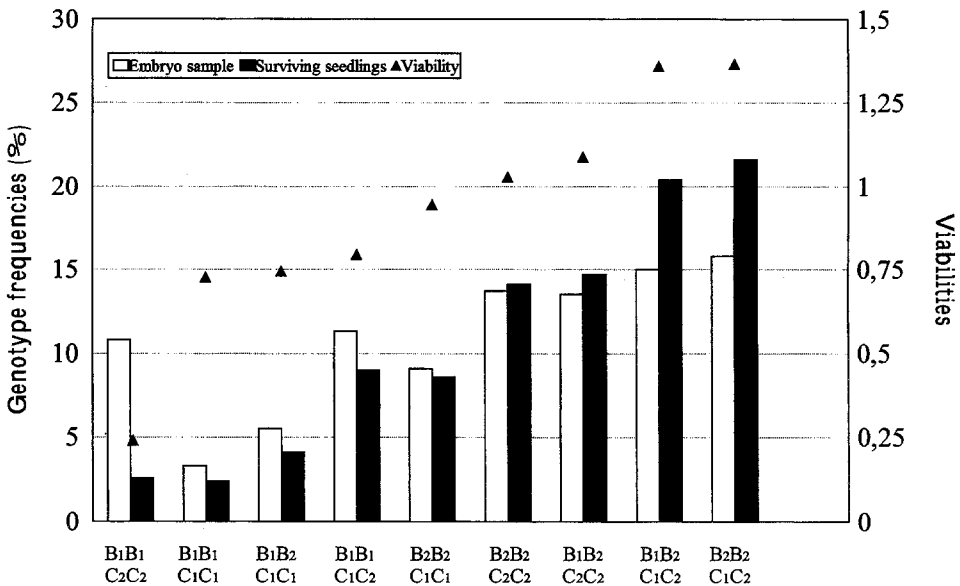
Fig. 1.   Two-locus genotype frequencies of the embryo samples and surviving seedlings and their
viabilities at the loci 6-PGDH-B and 6-PGDH-C.

years at the most polluted site of Silver Lake exhibited altered genetic structures as compared to the old parent stand from an adjacent unpolluted area (Hosius and Bergmann, 1993). Surprisingly, the genetic changes observed at four of the eight enzyme gene loci scored mainly concerned the heterozygous genotypes, whose frequencies have increased in the collection of surviving seedlings (the data were pooled over both polluted areas) as compared to the initial (embryo) genetic structures. Genetic changes at particular enzyme loci have also been observed during early life stages of Scotspine (Mejnartowics, 1983) and beech (Müller-Starck and Ziehe, 1991).

Based on a presumed mixed mating system in the parent populations, the respective seed progenies used for seedling raising may have consisted of associated homozygous (through inbreeding) and heterozygous (through outbreeding) genotypes. After elimination of homozygotes carrying deleterious recessive alleles during the exposure to stressful conditions, the remaining samples will be composed of higher proportions of heterozygotes as compared to the initial structures (for review and literature compilation, see Mitton and Jeffers, 1989). Under these assumptions, the significantly higher heterozygote frequencies in the surviving seedlings should be the result of selection against inbred genotypes, which occurred particularly under stressful conditions. However, the finding that these genetic changes occurred at only four of the eight enzyme gene loci scored and that two of the unaffected loci (PGI-B, GOT-B) exhibit typical major polymorphism better suited to reveal some inbreeding including selfing effects, does not support the inbreeding hypothesis. Rather, an explanation based on overdominant relationships may be more appropriate for the data interpretation in this study.

In order to evaluate the functional basis of probable heterozygote superiority, it is primarily necessary to discuss the metabolic role of the enzymes encoded by the four gene loci, in connection with possible response mechanisms. The greatest alterations occurred at the locus for PEPCA, which plays an important role in anaplerotic and CO_2 fixation pathways (Latzko and Kelly, 1983). In particular, increased biosynthetic processes required for response to environmental stress are closely related to anaplerotic replenishments. Specifically, the heavy-metal induced biosynthesis of phytochelatin, an oligopeptide capable of binding heavy-metals in plant cells (Grill et al., 1985), must use different branches of plant anabolism necessarily connected to the anaplerotic ability of PEPCA. Probably, aminopeptidases like LAP are also involved in these metabolic pathways, since the phytochelatins are composed of three amino acids (Grill et al., 1985), which will be released from polypeptides. The involvement of enzymes like PEPCA and LAP in the response metabolism of plants does not always predict the advantage of heterozygous genotypes. However, it was established in a biochemical study that the heterozygous PEPCA compound functions with greater efficiency under stressful conditions than both homozygous enzyme types (Rothe and Bergmann, 1995).

A higher viability of 6-PGDH heterozygotes in heavy-metal polluted soils does not appear to be an arbitrary result, since the natural spruce regeneration grown at Silver Lake was found to exhibit higher proportions of 6-PGDH-C heterozygotes as compared to the parent population (Hosius and Bergmann, 1993). Thus, it is concluded that seedlings heterozygous at one or both 6-PGDH loci are generally better adapted to soil pollution than the respective homozygotes. A possible explanation for the differential effects of homozygotes and heterozygotes on the viability of seedlings should take into account the by far broader reaction range of three or five allozymes produced by heterozygotes as compared to one allozyme of the homozygotes. Changes of the intracellular milieu, therefore, will be better tolerated by the heterozygous enzyme complement, thus providing a greater metabolic efficiency.

Although enzymes like 6-PGDH are not directly involved in stress response, the significance of their heterozygous state must be considered in a new context. Based on extensive studies with different animal species, it was concluded that heterozygosity at particular enzyme loci provides an increase in physiological efficiency, especially under stressful conditions. This greater physiological efficiency of heterozygous genotypes enables energy otherwise used for the basic metabolism to be saved and then to be allocated to stress-specific response processes (for review, see Koehn and Bayne, 1989, Clark and Koehn, 1992, Mitton, 1993). The advantage of heterozygous 6-PGDH genotypes should, therefore, be regarded as an indication of greater metabolic efficiency of particular enzyme compounds also in plant species. This relationship is supported by results from earlier studies with spruce where heterozygosity at these gene loci was found to be associated with greater vitality and better performance (Bergmann and Ruetz, 1991), greater reproductive success (Wittland, 1992) and effective vegetative regeneration (Stimm and Bergmann, 1994).

In contrast to our results, Prus-Glowacki and Godzik (1991) found a general decrease in heterozygosity among Scots pine seedlings grown in heavy-metal polluted soils. One explanation for this discrepancy is in the choice of different categories of enzyme systems used for population surveys, as they have chosen mostly enzyme loci with minor polymorphisms (see also Hosius and Bergmann 1993). Another reason for the opposing data may be the diversity of seed material, since we investigated material originating from six different provenances whereas their pine seedlings resulted from seeds of only one population.

Yet, the results presented in this study must be supported by further experiments at other heavy-metal polluted areas.

Acknowledgments

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