ON SOURCES OF VARIATION IN EXPRESSION OF PHOSPHOENOLPYRUVATE-CARBOXYLASE IN NORWAY SPRUCE (*PICEA ABIES* (L.) KARST.): PEPC GENOTYPE, GENETIC BACKGROUND AND GROWTH TEMPERATURE

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ABSTRACT

For gene-enzyme systems in forest trees it is unclear how much structural genetic diversity as compared to diversity of the genetic background contributes to phenotypic variability. The gene-enzyme-system of PEPC-A, phosphoenolpyruvate-carboxylase (PEPCase, EC 4.1.1.31), of Norway spruce (*Picea abies* (L.) Karst.) was chosen as an example to investigate the relative importance of the following sources of phenotypic variation in enzyme activity: (1) Variation at the structural PEPC-A-locus with three genotypes, (2) Variation in the genetic background, (3) Variation in growth temperature. The amount, specific activity and catalytic efficiency of PEPCase in crude needle extracts were assessed as quantitative traits. According to our ANOVA results, variation in the genetic background, *i.e.* epistasis in the general sense, is the most important source of variation compared to variation at the levels of both PEPC-A-genotype or growth temperature. Moreover, evaluation of the kinship partition of the genetic background revealed the individual level as most important. We compare to results of similar investigations for other species with different life histories and point to consequences for forest tree conservation genetics.

Key words: *Picea abies*, phosphoenolpyruvate-carboxylase (PEPC-A), isozyme, structural genetic variation, phenotypic variation, genetic background.

INTRODUCTION

Genetic diversity at the level of structural loci is often taken as a measure for the adaptability of populations (NEVO 1993; LANDE 1995; GREGORIUS 1989, 1996), assuming that variation in structural genes is the most important part of genetic variation, or at least representative for adaptability (NAMKOONG *et al.*, 1988). Much work has been done to investigate the physiology of the electrophoretic loci themselves. Allele frequencies have been shown to vary with environmental variables such as temperature, salinity or pollution (KOEHN *et al.* 1983; MEJNARTOWICZ 1983). There is also evidence for selection on the electrophoretic loci themselves (MITTON & GRANT 1984). Variation in regulatory genes, however, may be more important for phenotypic variation than variation in structural genes and should contribute to adaptability. Moreover, genetic variation in structural genes may follow different patterns than genetic variation in the regulatory domain.

Variation in regulatory genes is important in evolutionary processes (ZUCKERKANDL 1963; WALLACE 1963; SCHLICHTING & PIGLIUCCI 1995; DAMERVAL *et al.* 1994). Both experimental and theoretical studies demonstrate the importance of this variation (AYALA & MCDONALD 1980; LAURIE-AHLBERG *et al.* 1980; ALLENDORF *et al.* 1983; WU 1998). Even at the level of a single structural gene, the influence of regulatory genes on trait expression has been pointed out (WAGNER & ALTENBERG 1996; ASSELMEYER *et al.* 1996;

PAIGEN 1989).

To determine which portion of the genome is actually affected by natural selection, acting on phenotypes, POWERS *et al.* (1991) call for investigating more species for which a number of enzyme coding loci should be evaluated. They suggest studying model organisms with different life-history and adaptive strategies. So far, for *Drosophila melanogaster*, LAURIE-AHLBERG *et al.* already in 1980 demonstrated the importance of the genetic background of several structural loci for variation of enzymatic activity traits and quantitative traits associated with flight metabolism (LAURIE-AHLBERG *et al.* 1982; WILTON *et al.* 1982; BARNES & LAURIE-AHLBERG 1986).

Forest trees were not yet investigated for variation at structural loci and different genetic backgrounds as sources of variation of enzyme characteristics. Life-history and adaptive demands of trees differ substantially from that of *Drosophila* or other well-studied model organisms. Being long-lived and sessile they depend greatly on adaptability (HAMRICK *et al.* 1992; NEVO *et al.* 1983; KRUTOVSKII & NEALE 2001). Norway spruce as a phenotypical variable conifer species is adapted to a wide range of environments with temperature as a decisive factor (SCHMIDT-VOGT 1977; HAMRICK & GODT 1990).

The structural gene locus PEPC-A which codes for Phosphoenolpyruvate-Carboxylase (EC 4.1.1.31, PEPCase) is a well studied enzyme in Norway spruce in terms of genotypic variation and variation in expressed biochemical traits. Segregation studies revealed only one expressed locus in Norway spruce and studies on biochemical enzyme characteristics showed variation in the expressed traits (BERGMANN & SCHOLZ 1989). For biochemical PEPCase traits we seek to quantify different sources of phenotypic variation: different genotypes of the structural gene locus PEPC-A, different genetic backgrounds in which these genotypes are acting, and different temperature regimes in which the investigated plants were cultivated. Temperature regimes were varied with respect to adaptability at the structural PEPC-A locus, as implied by the studies of BERGMANN & SCHOLZ (1989) as well as ROTHE & BERGMANN (1995). The influence of genetic background can be assessed by comparing given PEPC-A-genotypes operating in different Norway spruce clones. With appropriate choice of these clones an additional source of variation can be analysed when partitioning according to provenances, mother trees and half-sibs. The variation of temperature regime is aimed at assessing the influence of growth temperature on cloned plant material with given combinations of PEPC-A-genotype and genetic background.

MATERIALS AND METHODS

Plant material and growth temperature

In the plant material, *i.e.* clones of Norway spruce, *Picea abies* (L.) Karst., the variation of the structural gene locus was represented by the isozyme genotypes Pepc-A_1A_1, -A_1A_2 and -A_2A_2 (BERGMANN & SCHOLZ 1989). The variation of the genetic background for each of these PEPC-A-genotypes was obtained by choosing different spruce clones of the same PEPC-A-genotype. Among these clones a hierarchy of kinship was chosen. The clones belonged to a number of single tree progenies from several provenances in Rumania and former Czechoslovakia. Hence, the variation of the genetic background was partitioned according to provenance, mother trees in provenances, siblings in progenies of these mother trees and ramets of siblings (SCHOLZ & VENNE 1989). For incorporating growth temperature as source of variation and repetitions of the treatments, the siblings were propagated by cuttings, resulting in three to four ramets at each growth temperature. Age of plants in our setting was four years.

The plant material was incubated at 0 °C, 15 °C and 30 °C for two weeks using climate chambers. Relative humidity was maintained at 70 % and illumination was 200 $\mu \cdot E \cdot m^{-2} \cdot s^{-1}$ for 9 h per day.

Determination of PEPC-A-genotypes

PEPC-A-genotypes were determined using starch gel electrophoresis as described before (CHELIAK & PITEL 1984; SHAW & PRASAD 1970) with minor modifications, and interpreted according to SCHOLZ & BERGMANN (1994). Apical cones were dissected from buds and homogenized in 66 mM Tris-HCl pH 7.5, 2.3 mM polyvinylpyrrolidone, 5% (w/v) saccharose. Pieces of Whatman paper (5 x 5 mm) were soaked in the homogenate and briefly dried before electrophoresis at 190 mA for 5.5 hours. The gel was then cut into three layers and the middle layer stained for PEPCase as described by KONNERT (1992).

PEPCase amount, catalytic efficiency and specific activity

After incubation in the climatic chambers, amount, catalytic efficiency and specific activity of PEPCase were determined for each ramet. Needles were collected separately for each ramet in liquid nitrogen. Total protein concentration ([protein]), PEPCase enzyme protein concentrations ([PEPCase]) and enzyme activities ($V_{max} = [PEP]/t$) were measured in a crude protein extract from needles.

Protein extracts were prepared from needles according to ROTHE and BERGMANN (1995) in Tris-HCl (100 mM), MgSO-7H_2O (0.5 mM), EDTA-Na (0.5 mM), 10 % (w/v) polyvinyl-pyrrolidone (PVP, PolyclarAT-pract.), 20 % (w/v) glycerine, 1 % (w/v) Triton-X-100, pH 8.5 and centrifuged for 5 min at 20300 g at 4 °C. The supernatant was aliquoted for subsequent activity assays. The amount of total protein, [protein], in the supernatant was determined by the method of POPOV *et al.* (1975). PEPCase enzyme protein concentrations, [PEPCase], were determined in an immunoassay as ACP-ELISA, slightly modified according to KOENIG and PAUL (1982). Supernatant was diluted to between 1 : 3000 and 1 : 24000 and incubated over night at 4 °C in the well of an ELISA-plate. After washing with 0.05 % (w/v) Tween in PBS (137 mM NaCl, 2.7 mM KCl, 4 mM Na HPO, 1.5mM KH PO) the wells were saturated with 1 % BSA-protein at 25 C for 24 hours. The first antibody (rabbit-anti-Flaveria-PEPCase) and the second antibody (anti-rabbit-PO-conjugate) were applied for 20 min each, separated by four PBS washing steps. Tetramethylene-benzidine was the staining reagent. The PEPCase enzyme protein concentrations were determined as absorption at 450 nm in relation to a standard made from a mixture of 80 pooled supernatants, collected in this study.

Enzyme activities were measured according to ROTHE and BERGMANN (1995). The reaction mixture contained 20 µl of the supernatant and 40 mM Tris-HCl pH 8.0, 0.1 mM NADH, 8 mM HCO_3, 4 mM MgCl, 0.83 mM DTE. The components were mixed and incubated at 10 °C, 20 °C, 30 °C or 40 °C for 4 min. Afterwards the enzymatic reaction was started by adding 145 µl aqueous PEP-solution to a final concentration of 4 mM and the reaction was monitored for 5 min.

Based on these measurements three phenotypic traits were calculated for each supernatant: Specific PEPCase activity $A_{[protein]}$ (= V_{max}/[protein], V_{max} = $[PEP]/t$), amount of PEPCase P (= $[PEPCase]/[protein]$) and catalytic efficiency k_{cat} (= $A_{[protein]}/P$).

Experimental Design

Two experimental designs were used:

Design 1 focused on comparison of the influence of PEPC-A-genotypes, genetic background, and growth temperature on variation of the biochemical traits under investigation. The plant material was chosen, such that the three PEPC-A-genotypes were available in a number of different spruce clones representing different genetic backgrounds. Choice of the clones was governed by the available clone material. PEPC-A-genotype A_1A_1 operated in 4 different genetic backgrounds, genotype A_1A_2 in 8 different backgrounds and genotype A_2A_2 in 11 backgrounds. Hence, for this design the genetic background is nested within the respective PEPC-A-genotype.

Design 2 focused on the partitioning of genetic background according to sources of its variation, *i.e.* provenance, mother tree and offspring clone (= half sib). Here, because of the limited material, only clones carrying the most frequent PEPC-A-genotype (A_2A_2) were used, allowing a three level hierarchical design in the genetic background.

In both designs, for each combination of effects, three to four ramets were used as repetitions. The positions of the ramets in the climate chambers were randomized and randomly changed during the experiment.

Statistical analysis

PEPC-A-genotype (G), genetic background ($R(G)$), the provenance (P), mother tree ($M(P)$), half-sibs of the progeny ($H(M,P)$), and the growth temperature (T) were modelled as sources of variation of the biochemical traits $A_{[protein]}$, k_{cat} and P. For both designs log-transformed phenotypic values gave better fits of the total model than untransformed values (data not shown).

For design 1, an additive model with fixed effects was used. Design 1:

$$\log(y_1) = T + G + R(G) + G{\times}T + CT + e$$

where y_1 gives the measured value for the traits $A_{[protein]}$, k_{cat} and P in design 1.

The term CT (cuvette temperature) was added to correct for different temperatures in the cuvettes of the enzymatic reaction assay of four experimental series (for the traits $A_{[protein]}$ and k_{cat}). $R(G)$ gives a factor coding for the genetic background in order to guarantee a different code for each combination of P, M, and H. $G{\times}T$ is the interaction of PEPC-A-genotype and growth temperature. The other possible interactions were never significant. $e = y_{estimated} - y_{measured}$ gives the error.

Analyses of variance were performed using the GLM-procedure of SAS (SAS-INSTITUTE 1992). The standard deviations of the estimated factor effect values were calculated and served as measure to compare the importance of the different sources of variation.

For design 2, a mixed-effects approach was used. The two factors M and H were modelled as random factors, to account for the random choice of the belonging plant material on these partition levels:

Design 2:

$$\log(y_2) = T + P + M(P) + H(M,P) + P{\times}T + CT + e$$

where $M(P)$ and $H(M,P)$ were modelled as random variables. Their significance was tested using a Log-likelihood test (PINHEIRO & BATES 2000). $P \times T$ gives the interaction of provenance and growth temperature. The other possible interactions were never significant. $e = y_{estimated} - y_{measured}$ gives the error. For CT see design 1.

As we wanted to compare the importance of the different factors also in design 2, we needed a measure to compare fixed and random factors. We have chosen to compute the standard deviations of the estimated factor effect values for the fixed effect factors and the estimated standard deviation for the random effect factors. Note that such a comparison has to be taken with care, because of the different estimation methods for fixed and random effects. The analysis was carried out using the R-language (IHAKA & GENTLEMAN 1996).

RESULTS

PEPC-A genotypes and growth temperature

The biochemical traits examined are dependent on both variation of the PEPC-A-genotype as well as on growth temperature with significant interactions. Figure 1 shows this for the specific activity, $A_{[protein]}$, as mean values over the genetic background. Interactions, as expected for the adaptive isozyme locus, are evident.

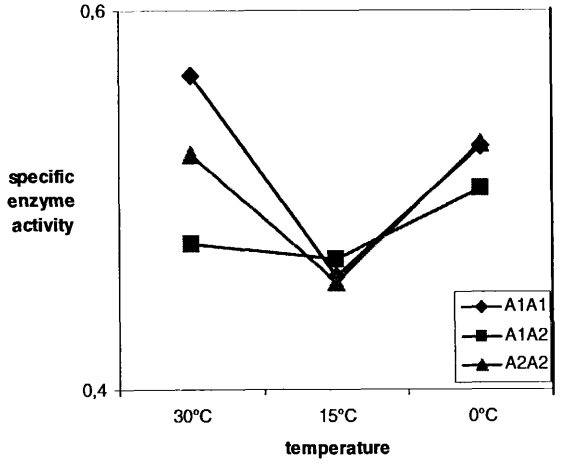

Figure 1. Dependence of specific enzyme activity ($A_{[protein]}$- mean values over clones) on PEPC-A genotype and growth temperature.

PEPC-A genotype, genetic background and growth temperature (design 1)

ANOVA of our results identified genetic background as the main source of variation of the phenotypic traits. The contributions of different sources vary only slightly among the three traits. Figure 2 shows the quantitative influence of the different sources of phenotypic variation for design 1. Table 1 give the detailed results of ANOVA analyses for this model, and table 3 the values for the standard deviations for the factor estimates. Summarizing, the influence of PEPC-A-genotype is less than a third of that of genetic background, and less than that of growth temperature. Interactions between PEPC-A-genotype and growth temperature are significant, and their contribution is similar to the single effects of growth temperature and PEPC-A-genotype.

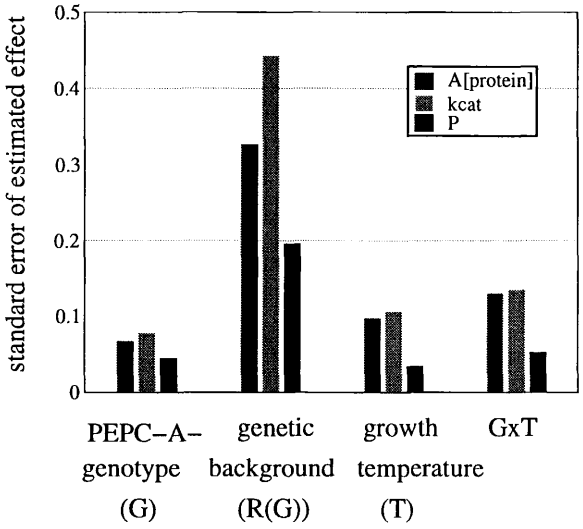

Figure 2. Relative influences of PEPC-A genotype, genetic background, growth temperature, and interactions, on variation of the biochemical traits, measured as standard errors for the estimated effects of ANOVA for evaluating design 1.

Partitioning of genetic background (design 2)

For the most frequent PEPC-A-genotype, A_2A_2, the genetic background was partitioned into levels of different degree of kinship, *i.e.* provenance, mother tree and half-sib. Figure 3 gives the contributions for the sources of variation for this design (see table 2, and also table 3 for the detailed values): The main contribution of the genetic background is at the half-sib (clonal) level, $H(M,P)$. Note that the low number of siblings for each mother tree and the comparatively large variance of the error term make it impossible to accurately estimate the standard deviation for the effects of mother trees ($M(P)$). This results in very large confidence intervals for the standard deviation of $M(P)$. However, the corresponding intervals for halfsibs $H(M,P)$ are of reasonable size.

The influence of different provenances is not significant (p-values: $p(A_{[protein]}) = 0.63$, $p(k_{cat}) = 0.13$,

Table 1. ANOVA table for design 1, trait $A_{[protein]}$, $R^2 = 0.77$.

Source	$A_{[protein]}$, $R^2 = 0.77$					k_{cat}, $R^2 = 0.76$					PEPCase amount, $R^2 = 0.24$				
	DF	SS	MS	F	P	DF	SS	MS	F	P	DF	SS	MS	F	P
Model	32	655	20.5	149	0.0001	32	655	20.5	149	0.0001	29	35	1.2	6.21	0.0001
PEPC-A genotype (G)	2	2.9	1.4	11	0.0001	2	2.9	1.4	11	0.0001	2	1.4	0.7	3.6	0.0278
Genetic background	21	112	5.3	39	0.0001	21	112	5.3	39	0.0001	21	27	1.3	6.8	0.0001
Growth Temperature (E)	2	7	3.3	24	0.0001	2	7	3.3	24	0.0001	2	2.1	1	5.4	0.0045
Cuvette Temperature	3	523	174	1271	0.0001	3	523	174	1271	0.0001	–	–	–	–	–
G × E	4	7	1.8	12	0.0001	4	7	1.8	13	0.0001	4	0.2	0.04	0.23	0.9211
Error	1411	194	0.14	–	–	1411	194	0.14	–	–	582	112	0.19	–	–
Sum	1443	849	–	–	–	1443	849	–	–	–	611	147	–	–	–

DF: degrees of freedom, SS; sum of squares, MS: mean sum of squares, F: F-test statistics, P: p-values.

Table 2a. Log-Likelihood-Tests for random factors (L. Ratio / p-value) for design 2.

Trait	Source	L. - Ratio	*p*
P	Mother tree (M)	86.6	<0.0001
	Half sib (H)	102.1	<0.0001
$A_{[protein]}$	Mother tree (M)	86.	<0.0001
	Half sib (H)	55.6	<0.0001
k_{cat}	Mother tree (M)	70.2	<0.0001
	Half sib (H)	70.9	<0.0001

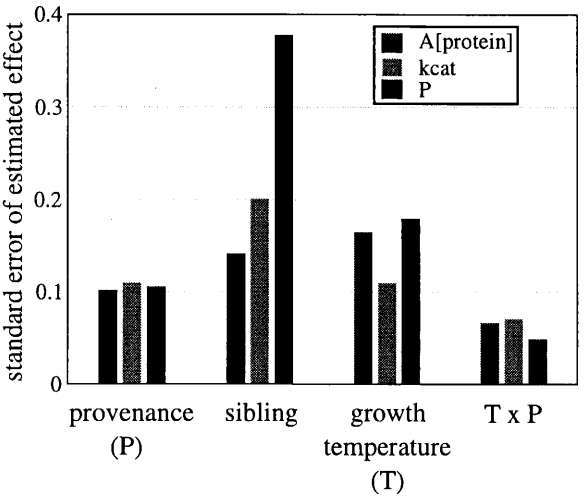

Figure 3. Relative influences of provenance, sibling, growth temperature, and interactions, on variation of the biochemical traits, measured as standard errors for the estimated effects of ANOVA for evaluating design 2.

$p(P) = 0.26$). Growth temperature accounted for about half of the phenotypic variation. The interaction between provenance and growth temperature was significant for the specific activity, $A_{[protein]}$, and the catalytic efficiency, k_{cat}, and similar important as the growth temperature influence. Table 2 shows the detailed results for both ANOVA of the fixed effects and the Log-likelihood tests for the random effects. The variation in the three levels of genetic background remain the major source of phenotypical variation if compared to growth temperature or interaction effects.

DISCUSSION

Genotype variation at a structural gene locus and variation of the genetic background of this locus were compared as sources of variation in belonging quantitative enzymatic traits in a forest tree species. Genotype variation was realised by choosing the three isozyme

Table 2b. ANOVA tables for fixed effects, design 2.

Trait	Source	Num. DF	Den. DF	F	p
P	Growth-Temperature (T)	2	1458	22.7	<0.0001
	Provenance (P)	2	9	0.5	0.63
	T × P	4	1458	1.5	0.2
$A_{[protein]}$	Growth-Temperature (T)	2	1454	77	<0.0001
	Provenance (P)	2	9	2.6	0.13
	T × P	4	1454	9.2	<0.0001
	Cuv.-Temperature	3	1454	1220	<0.0001
k_{cat}	Growth-Temperature (T)	2	1454	17.1	<0.0001
	Provenance (P)	2	9	1.6	0.26
	T × P	4	1454	4.7	0
	Cuv.-Temperature	3	1454	629	<0.0001

Table 3. Standard errors of estimated factor effect values for designs 1 and 2.

Trait	Design 1				Design 2					
	G	R(G)	T	G×T	M	H	T	P	P×T	CT
P	0.043	0.195	0.033	0.051	0.025	0.376	0.178	0.104	0.047	–
$A_{[protein]}$	0.066	0.325	0.096	0.128	0.010	0.147	0.163	0.100	0.065	0.701
k_{cat}	0.077	0.442	0.106	0.134	0.014	0.200	0.109	0.109	0.070	0.702

electrophoretic genotypes of PEPCase in Norway spruce. Variation of genetic background was achieved by investigating different spruce clones for each PEPC-A genotype. As these clones were cuttings from different half sibs from mother trees from different progenies, we could partition the variation of the influence of the genetic background according to the given kinship structure. Assessed quantitative traits were amount, specific activity and catalytic efficiency of the enzyme encoded by the structural locus PEPC-A.

Our results show that also for an adaptive locus in a forest tree species variation of quantitative enzyme traits can be governed mainly by the non-structural genetic background, while the genotype of the structural locus plays a minor role. Moreover, in this example partitioning the observed phenotypic variation according to the kinship structure of the genetic background revealed the individual (= clonal) level within progenies, as most important.

Variation of most quantitative traits is usually only to a small extent associated with allozyme genotype variation (BUSH & SMOUSE 1992; KARHU *et al.* 1996). Already WILSON (1976), and later SCHEINER (1993), suggested that polymorphisms at other than structural loci could be more important for adaptability. Instead, variation of regulatory genes might be of major importance for phenotypic variability. Our results are an example for their notion that, often genetic polymorphisms other than those of the regarded structural loci play a more important role for phenotypic variability. In this sense, PEPC-A could be either a part of a complex gene regulatory system, where the structural genetic variation at the PEPC-A-locus is combined with that of other regulatory loci. Or it may be a marker locus, only marginally linked to the genetic variation which mainly governs the adaptive response. It may be linked by any form of genetic linkage phenomena, like hitchhiking, to the main genetic basis of adaptive variability. Another possibility is genetic variation at the PEPC-A-locus other than the electrophoretic genotypes so far known and investigated here (see IPSEN *et al.* 1998). On the other hand, the effect of structural genetic variation remains measurable as average effect over a lot of different genetic backgrounds, as already assumed as a representative model for tree breeding by NAMKOONG *et al.* 1988. These mean PEPC-A-genotype effects clearly interact with the growth temperature as a determining factor for the quantitative traits under investigation (see for example figure 1), possibly also reflecting the adaptive nature of the isozyme locus PEPC-A.

For the choice of organism, we followed POWERS *et al.* (1991), studying a species with a life history different to well-studied organisms like *Drosophila,*

Mus, and *Caenorhabditis*. Forest trees are assumed to need a comparatively high amount of adaptability because they are long-lived and sessile. These life-history and ecological characteristics may cause a different level and organisation of genetic diversity (HAMRICK *et al.* 1992). Comparison of our results on partitioning sources of variation "structural locus versus genetic background" (design 1) to findings in similar experiments for *Drosophila*, however, reveals similar proportions. LAURIE-AHLBERG (1980) was the first to investigate isozyme loci regarding these aspects. Our results are in accordance with her findings, yet with respect to a forest tree species. Also, similar to our results, MIYASHITA *et al.* (1986) found for an isozyme locus in *Drosophila* a contribution of about 10 % to the observed variation. Moreover, our results from partitioning sources of variation "individual versus mother tree versus provenance" (design 2) show that variation on the individual level of genetic background governs phenotypic variation. For mother trees and provenances no significant influence was detected, which again is in accordance with the results of LAURIE-AHLBERG *et al.* (1982).

Interactions were important sources of phenotypical variation (figures 1, 2 and 3) as expected for an isozyme locus for which adaptive variation according to temperature regime was shown before (BERGMANN & SCHOLZ 1989; ROTHE & BERGMANN 1995). Figure 2 reveals the G × T interaction in the experiment on partitioning "structural locus and genetic background" (design 1) experiment to be more important for phenotypical variation than both the single contribution of PEPC-A-genotype or growth temperature, but lower than the genetic background. This is in accordance with the results for a variety of other species as reviewed by SCHEINER (1993).

Other examples for the regulatory importance of variation in the genetic background are found when investigating genetic disruption of transgenic organisms. Here, trait expression depends on the genetic background as shown for example by LATHE (1996), PHILLIPS *et al.* (1999) for mice and SCOTT *et al.* (1998) for clover. For *Drosophila* LEIPS AND MACKAY (2000) showed that the effects of lifespan QTLs are significantly dependent on genetic background.

Genetic variation is regarded as prerequisite for adaptability of populations (KRUTOVSKII & NEALE 2001; NEVO 1993; WATT 1994; GREGORIUS 1989; GREGORIUS 1996) as supported by results on variation of allozyme genotypes in relation to phenotypic or even adaptive variation (KOEHN *et al.* 1983; reviewed for example by HAMRICK & GODT 1990). Hence, genetic variation at structural gene loci, such as adaptive isozyme-loci, is thought to quantify the adaptability of populations (NEVO 1993; LANDE 1995). Here, as the results of our work exemplify, it is of great importance, for which sample of the genome genetic diversity is assessed. As far as genetic diversity is assessed only by gene markers based on structural loci, conclusions and hypotheses do not include the impact of genetic diversity at the level of non-structural genes. If this decisive level can be included, the basis for the hypothesis that genetic diversity is an important precondition for adaptability of biological populations will be broadened.

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